Hinkle, P. M., & Kirsch, J. F. (1971) *Biochemistry 10*, 2717.
Holloway, M., & Hardman, M. (1973) *Eur. J. Biochem. 33*, 1537.
Hui Bon Hoa, G., & Douzou, P. (1973) *J. Biol. Chem. 248*, 4649.
Hunkapillar, M. W., Forgac, M. D., & Richards, J. H. (1976) *Biochemistry 15*, 5581.
Ingraham, L. L. (1972) *Biochim. Biophys. Acta 279*, 8.
Ingraham, L. L. (1973) *Biochim. Biophys. Acta 304*, 891.
Jentoft, J. E., Neet, K. E., & Stuehr, J. E. (1977) *Biochemistry 16*, 117.
Johnson, S. L. (1967), *Adv. Phys. Org. Chem. 5*, 237.
Knowles, J. R. (1976) *CRC Crit. Rev. Biochem.*, 165.
Lewis, C. A., & Wolfenden, R. (1977) *Biochemistry 16*, 4890.
Lowe, G., & Yuthavong, Y. (1971) *Biochem. J. 124*, 117.
O'Leary, M. H., Urber, M., & Young, A. P. (1974) *Biochemistry 13*, 2077.
Reid, T. W., & Wilson, I. B. (1971) *Biochemistry 10*, 380.
Robertus, T. P., Kraut, J., Alden, R. A., & Birktoft, J. J. (1972) *Biochemistry 11*, 4293.
Robinson, D. W. (1970) *J. Am. Chem. Soc. 92*, 3138.
Schultz, R. M., Konovessi-Panayotatos, A., & Peters, J. (1977) *Biochemistry 16*, 2194.
Smith, D. J., Maggio, E. T., & Kenyon, G. (1975) *Biochemistry 14*, 766.
Wang, J. (1968) *Science 161*, 328.
Wang, J. (1970) *Acc. Chem. Res. 3*, 90.
Wang, J., & Parker, L. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 2451.
Westerik, J. O., & Wolfenden, R. (1972) *J. Biol. Chem. 247*, 8195.
Whitaker, J. R., & Bender, M. L. (1965) *J. Am. Chem. Soc. 87*, 2728.
Whitaker, J. R., & Perez-Villaseñor, J. (1968) *Arch. Biochem. Biophys. 124*, 70.
Zannis, V., & Kirsch, J. F. (1978) *Biochemistry 17*, 2669.

Estrogen-Binding Proteins of Calf Uterus. Purification to Homogeneity of Receptor from Cytosol by Affinity Chromatography†

Vincenzo Sica and Francesco Bresciani*

ABSTRACT: The estrogen receptor has been purified to homogeneity from calf uterus cytosol by sequential affinity chromatography by using heparin–Sepharose 4B and 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose 4B. The procedure yields about 1.2 mg of receptor protein from 1 kg of calf uteri, with a recovery of 53%. The receptor protein, as a complex with 17β-[^{3}H]estradiol, is purified more than 99%. A single band is seen on polyacrylamide gel electrophoresis under nondenaturing conditions. 17β-[^{3}H]Estradiol comigrates with the protein band. As computed from the specific activity of radioactive hormone, 64 450 g of purified receptor protein binds 1 mol of 17β-estradiol. 17β-[^{3}H]Estradiol bound to the proten is displaced by estrogenic steroids but not by progesterone, testosterone, or cortisone. As judged by chromatography on calibrated Sephadex G-200 columns, the purified receptor is identical with native receptor in crude cytosol: both show a Stokes radius of 6.4 nm. On sucrose gradient in low-salt buffer, the purified receptor sediments at 8 S. On electrophoresis in $NaDodSO_4$ gels, the purified receptor migrates as a single protein band with an apparent molecular weight of 70 000. The sedimentation coefficient measured on sucrose gradients in the presence of chaotropic salts [1 M NaBr or NaSCN (0.1 M)] is 4.2 S. We conclude that the estrogen receptor of cytosol consists of a single subunit weighing about 70 000 daltons and endowed with one estrogen binding site. Under native conditions in cytosol, several subunits associate to form a quaternary structure with a Stokes radius of 6.4 nm.

As soon as the estrogen receptor was identified as a main molecular link in the mechanism of estrogen action, attempts to achieve its purification began in several laboratories (Jungblut et al., 1965, 1967; De Sombre & Gorell, 1975) including ours (Puca et al., 1970, 1971a,b, 1972, 1975; Sica et al., 1973a,b; Molinari et al., 1977; Bresciani et al., 1978). The final goal was to make available purified receptor in tangible amounts, suitable to definitive physical, chemical, and biochemical characterization of this important regulatory molecule.

In a series of earlier publications we have described the results of purification work performed using both classical protein separation methods (Puca et al., 1970, 1971a,b, 1972, 1975) as well as affinity chromatography with solid-state adsorbents prepared linking 17β-estradiol 17-hemisuccinate to agarose via a polypeptide arm (Sica et al., 1973a,b). With classical protein separation methods we achieved partial purification of receptor. With affinity chromatography the complete purification was obtained but preparation of purified receptor on a large scale proved to be difficult, mainly because of considerable "bleeding" of hormone from the solid-phase adsorbent during incubation with uterine cytosol.

The above problem has now been solved by including in the purification procedure an initial step consisting of chromatography using heparin–Sepharose. Heparin binds the receptor protein but not most of the other cytosol components, including

† From the Department of Pathology, University of Toronto, and Instituto di Patologia generale, Università di Napoli, 2 Napoli, 80138, Italy. *Received November 17, 1978.* Research was supported by the National Cancer Institute of Canada and, in part, by Progetto Finalizzato "Controllo della Crescita Neoplastica" del Consiglio Nazionale delle Ricerche, Rome, Italy. This is the fourth in a series of articles concerning purification and characterization of estrogen-binding proteins from calf uterus. Sica et al. (1976) is the preceding article of this series.

* Address correspondence to this author at Istituto di Patologia generale, Università di Napoli, S. Andrea delle Dame, 2 Napoli, 80138, Italy.

0006-2960/79/0418-2369$01.00/0

those responsible for attacking the many susceptible bonds in 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose, with following release of hormone from the solid–state adsorbent. This and other improvements, including a new method of elution from the affinity adsorbent by using a chaotropic salt, have now made it possible to prepare milligram amounts of homogeneously pure receptor protein. The method of purification as well as the results of a number of characterization studies performed on the purified receptor protein is described in this paper.

Materials and Methods

Uteri from immature calves were collected at a local slaughterhouse into plastic bags. The bags were sealed and buried in crushed ice. The uteri under ice were rapidly brought to the laboratory cold room where they were cleaned of connective tissues, sliced open, and frozen in liquid nitrogen. They were kept in liquid nitrogen or in a refrigerator at −70 °C until further use.

Chemicals. All reagents were of analytical grade. Tris was from Sigma (Trizma Base, reagent grade). EDTA, disodium salt, was Sigma grade. Dithiothreitol was from Calbiochem. Sucrose was "ultra-pure" grade from Schwarz/Mann. Dimethyl suberimidate dihydrochloride was from Aldrich. Heparin used for preparation of the Sepharose–heparin complex (see later) was stage 14 crude sodium heparin from Inolex Corporation, Chicago. Heparin used for elution of receptors adsorbed on Sepharose–heparin was grade I (sp act. 170 USP units/mg) from Sigma. Reference proteins used for calibration of Sephadex G-200 columns for Stokes radius determination of receptor or determination of the Svedberg constant on sucrose gradients were as follows: γ-globulin (human) and cytochrome *c* (equine heart) A grade from Calbiochem, San Diego, CA, and bovine serum albumin crystallized and lyophilized and myoglobin (horse heart) type III lyophilized from Sigma Chemical Co., St. Louis, MO.

Sepharose 4B, Dextran T 70, Sephadex G-200 and G-50, and Blue Dextran 2000 were purchased from Pharmacia. Ovalbumin for preparation of 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose 4B was grade V crystallized and lyophilized from Sigma. 17β-Estradiol 17-hemisuccinate was from Steraloids, Pawling, NY. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was from Pierce Chemical Co., Rockford, IL. Acrylamide, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine, Coomassie brilliant blue, ammonium persulfate, and sodium dodecyl sulfate were from Bio-Rad. Charcoal Norit A was from Matheson Coleman and Bell, Norwood, OH. Radioactive estradiol (17β-[6,7-^{3}H]estradiol; sp act. 60 Ci/mmol) was from New England Nuclear. 17β-Estradiol, estriol, estrone, testosterone, progesterone, and cortisone were from Sigma.

Buffers. The following buffers were used: TED (pH 7.4), Tris-HCl (10^{-2} M), EDTA (10^{-3} M), and dithiothreitol (10^{-3} M); TD (pH 7.4), Tris-HCl (10^{-2} M) and dithiothreitol (10^{-3} M). When necessary, heparin grade I from Sigma was added to the buffers, at concentrations specified in the text.

Protein Assay. Protein determinations were performed by the Bio-Rad protein assay based on the work of Bradford (1976). Thiol groups, Tris, and EDTA do not interfer with the Bio-Rad assay. Eluates from chromatography columns were monitored at 280 nm, except when a precise determination of protein was required. In this case, the Bio-Rad assay method was used.

Radioactivity Measurement. Aqueous samples up to 1 mL were counted in 10 mL of Aquasol II (New England Nuclear) in scintillation vials by using an Intertechnique scintillation spectrometer. Gel slices were counted in 10 mL of Aquasol after overnight incubation at room temperature in the dark. Counting efficiency was determined by the external standard method.

Gel Filtration. Gel filtration was performed by using Sephadex gels and Pharmacia columns equipped with upward-flow adaptors. The upward flow was generated by Pharmacia peristaltic pumps. Effluents were monitored at 280 nm by Uvicord II. Fractions were collected by LKB Ultrorac fraction collectors equipped with drop counters. Sephadex G-200 columns were calibrated under standard working conditions by using Blue Dextran 2000 (Pharmacia) to assess V_0, [^{3}H]leucine to assess V_i, and proteins of known Stokes radius for calibration: γ-globulin (human), 5.3 nm; bovine serum albumin, 3.55 nm; myoglobin (horse heart), 2.07 nm; and cytochrome *c* (equine heart), 1.64 nm.

Sucrose Gradient Centrifugation. Linear gradients of 5–20% sucrose in TD buffer, or TD buffer containing 1 M NaBr, were prepared in Beckman cellulose nitrate tubes (5 mL) and stored for several hours at 4 °C before use. Bovine plasma albumin, 1 mg (0.1 mL of a 1% solution on the same buffer of sample), was used as internal standard in every tube. After layering cold sample and internal standard on top of the gradients, the centrifugation was carried out in a Beckman-Spinco SW 50.1 rotor, at 2–4 °C for 16 h at 45 000 rpm in a Beckman-Spinco L5-75 ultracentrifuge.

After collecting 8-drop fractions from the punctured bottom of the tube, 1 mL of H_2O per fraction was added, and albumin was determined by measuring the adsorbance of the fractions at 280 nm. Thereafter, radioactivity was assessed in each fraction in order to detect the receptor–[^{3}H]estradiol complex.

Concentration. Concentration of both the eluate from the second Sepharose–heparin column and the fraction "excluded" from the final Sephadex G-50 column chromatography was carried out by filtration on Amicon UM-20 Diaflo membranes by using the Amicon Model 402 on the Amicon Model 12 stirred cells, respectively. Filtration was performed in a room at 2–4 °C, by using N_2 at 60 psi.

Preparation of Heparin–Sepharose 4B. Heparin–Sepharose 4B was prepared as follows. One kilogram of freshly CNBr-activated Sepharose 4B was added to a solution of 75 g of stage 14 crude sodium heparin (Inolex Corporation, Chicago) in 2 L of 50 mM borate buffer, pH 9.0. The mixture was magnetically stirred for 15 h at 4 °C and, thereafter, it was filtered through a Buchner funnel; the resulting cake of gel was first washed with 5 L of KCl (1 M) and then with water and finally incubated with 1 L of glycine 2 M in water for 2 h at room temperature, in order to eliminate residual activated agarose sites. The heparin–Sepharose derivative was washed again on a Buchner funnel with 5 L of KCl (1 M), followed by 5 L of water. As judged by the amount of unreacted heparin in the washing solutions, about 25 mg of heparin is covalently coupled per mL of packed sepharose gel by the above procedure.

Heparin Determination. Heparin was assayed by the carbazole method of Bitter & Muir (1962).

Preparation of 17-Hemisuccinyl-17β-estradiol–Ovalbumin–Sepharose 4B. This specific affinity adsorbent was prepared according to a previously described method (Sica et al., 1973b) except that, because of economic reasons, ovalbumin instead of poly(L-lysyl-DL-alanine) was used as a macromolecular spacer.

Polyacrylamide Gel Electrophoresis under Nondenaturing Conditions. Gel electrophoresis was carried out at 5 °C by using the Weber and Osborn Tris system (1975) with one

single modification: heparin, final concentration 0.02%, substituted for $NaDodSO_4$. Glass tubes were 0.55 cm in diameter and 12 cm long. The concentration of the stacking gel was 3% and that of the lower gel was 5%. After gelification, the tubes were inserted through watertight rubber into a transparent plastic drum in which water at 5 °C was circulated. Upper and lower chambers were both filled with precooled (5 °C) reservoir buffer, pH 8.3, containing, in 1000 mL of H_2O, 6 g of Tris base, 28.8 g of glycine, and 0.1 g of heparin grade I (Sigma). After a 30-min initial cooling period, the samples were carefully layered on top of the stacking gel by using a cold pipette, and electrophoresis was started. Sucrose (final concentration 20%) and Bromophenol blue (5 μL of a 0.02% solution in water) were added to each sample before layering. Electrophoresis was carried out for the initial 2 h at 1 mA/gel and for another 4 h at 2 mA/gel. At the end of the run, the dye front was marked by inserting a small piece of fine copper wire, and the gel was kept for 2 h in 10% perchloric acid before being either stained with Coomassie blue as described below or sliced by using a Bio-Rad gel slicer into 2 nm-thick slices, which were thereafter collected into scintillation vials containing 10 mL of Aquasol II. After overnight extraction, the 17β-[^{3}H]estradiol content of slices was determined in a β counter. Staining was achieved by overnight incubation of the gel into a 0.04% solution of Coomassie blue G-250 in 3.5% perchloric acid, followed by developing in 7.5% acetic acid.

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis. The $NaDodSO_4$–phosphate system of Weber & Osborn (1975) was used. The final acylamide concentration in the gel was 5%. In general, all operations were performed according to Weber & Osborn (1975). Specifically, the receptor preparation or the reference proteins were dialyzed for several hours against the sample buffer (0.01 M sodium phosphate, pH 7.2, 1% $NaDodSO_4$, and 1% 2-mercaptoethanol) and heated for 1 min in a boiling water bath. In comparative tests, mercaptoethanol concentration was increased up to 3% and heated up to 3 min.

After cooling to room temperature, sucrose (final concentration 20%) and Bromophenol blue (5 μL of a 0.02% solution in water) were added to the sample. The upper and lower chambers were filled with reservoir buffer, pH 7.2 (3.9 g of $NaH_2PO_4 \cdot H_2O$, 1.93 g of $Na_2HPO_4 \cdot 7H_2O$, 1 g of $NaDodSO_4$, and water to 1000 mL), and, after 30-min preelectrophoresis at 8 mA/gel, the samples were carefully layered on top of the gels and electrophoresis carried out at 8 mA/gel for 4 h. All operations were performed at room temperature. At the end of electrophoresis, gels were cut at the dye front, fixed, and stained with Coomassie blue G-250 as described above. Standards for molecular weight determination were ovalbumin and bovine plasma albumin polymers, prepared by cross-linking with dimethyl suberimidate as described by Carpenter & Harrington (1972). Standards were run in parallel, under experimental conditions identical with those of the samples.

Determination of Hormone Binding Specificity. Hormone binding specificity of pure receptor was tested by competition studies at 4 °C, under conditions of increased ligand turnover brought about by chemical perturbation with NaSCN (0.5 M). We have found (unpublished experiments) that NaSCN (0.5 M) at 4 °C increases the rate of association (K_1) of the receptor–17β-estradiol complex (calf uterus) from 7.4×10^{-6} to 12.7×10^{-6} M^{-1} min^{-1} and the dissociation rate (K_{-1}) from 1.1×10^3 to 11.4×10^3 min^{-1}. As a consequence, the K_d increases from 1.4×10^{10} to 9.0×10^{10} M. The net result of these changes is a considerable increase of the ligand turnover rate.

Direct exchange experiments in which preformed 17β-estradiol–receptor complex of calf uterus is incubated in TD buffer with an excess of 17β-[^{3}H]estradiol (17β-[^{3}H]E_2/17β-E_2 = 25) show that after 16 h at 4 °C more than 98% of the cold estradiol has been exchanged for radioactive hormone, with no measurable loss of binding sites.

The experimental conditions of the binding specificity tests were as follows. TED buffer (0.5 mL) containing NaSCN (0.5 M) and 50 ng of 17β-[^{3}H]estradiol without or with a 1000-fold excess of the nonradioactive steroid to be tested (for ability to compete for the estrogen binding sites) was added to 0.5 ng of pure receptor in 30 μL of TED buffer. The hormones tested were as follows: 17β-estradiol, progesterone, testosterone, cortisone, estriol, and estrone. After incubation overnight (at least 16 h), free from bound hormones were separated by gel filtration on standard Sephadex G-25 columns as previously described (Puca et al., 1971a). Radioactivity was assayed in the "excluded" fraction as described above.

Adsorption Spectra. The adsorption spectrum of the pure receptor protein was obtained between 240 and 340 nm by using an ACTA III double-beam spectrophotometer. Despite maximum adsorbance of 17β-estradiol at 280 nm, contribution of bound 17β-estradiol to optical density in the 240–340 nm band is negligible because of the low level of hormone in the preparation, i.e., 27×10^{-6} mg/mL. The E_{280}^{1mg} for 17β-estradiol is 1.12.

Assay of Specific 17β-[^{3}H]Estradiol Binding Capacity (Receptor Assay). The samples (0.1–0.3 mL) to be tested were brought to 0.6 mL with TED buffer containing 1 ng of 17β-[6,7-^{3}H]estradiol (60 Ci/mmol) and incubated for 2 h. After incubation, separation of free from macromolecule-bound estradiol was accomplished by adding 0.6 mL of Dextran-coated charcoal (0.05% Dextran T 70; 0.1% charcoal Norit A), followed by incubation for 15 min at 4 °C. After centrifugation at 5000*g* for 5 min, 0.6 mL of supernatant was added to 10 mL of Aquasol II, and the radioactivity was measured as described above. The nonspecific binding, i.e., the relatively low-affinity binding of estradiol by macromolecules other than receptors, was measured by incubating parallel samples in the presence of 1000-fold excess of cold estradiol.

Results

Preparation of Cytosol. In the typical preparation to be described here, 1 kg of calf uteri frozen in liquid nitrogen was pulverized in a mortar. Four liters of ice-cold TED buffer was added to the powder, and the mixture was briefly treated in a blender in order to obtain a homogeneous slurry. About 150 mL of slurry was poured into plastic bottles kept immersed in crushed ice and homogenized with a Polytron machine (PT 100-00). Homogenization was performed with four 15-s bursts (speed setting 2.5) intercalated by 45-s periods of cooling during which the bottles were rotated in the ice.

The homogenate was transferred into appropriate bottles and centrifuged for 40 min at 2 °C at 35 000 rpm in Spinco L5-65 ultracentrifuges by using type 35 heads (g_{max} 142 800). Two ultracentrifuges were used in parallel to speed up the centrifugation step. After centrifugation, the supernatant fractions were pooled together. A total of 4.35 L of supernatant, or cytosol, was obtained and further treated as described below. Protein content and specific 17β-estradiol binding capacity of cytosol (active receptor content) were assessed on a small aliquot set aside for this purpose. The results of these determinations for the typical preparations

Table I: Purification of Estrogen Receptor

purification step	total vol (mL)	protein (total mg)	bound 17β-estradiol (total mol)[a]	sp act. (mol of 17β-E_2/mg of protein)	recovery %	purification factor (times)
(1) cytosol	4350	35 670	3.58×10^{-8}	1×10^{-12}	100	
(2) first heparin-Sepharose	1800	5760	2.54×10^{-8}	4.4×10^{-12}	71	4.4
(3) 17-hemisuccinyl-17β-estradiol-ovalbumin-Sepharose 4B	1100	n.m.[b]	n.m.	n.m.	n.m.	n.m.
(4) second heparin-Sepharose	414	n.m.	2.22×10^{-8}	n.m.	57	n.m.
(5) Sephadex G-50	49	1.225	1.9×10^{-8}	1.55×10^{-8}	53	15 585

[a] Computed from the specific activity of 17β-[^{3}H]estradiol employed. [b] n.m. = not measurable.

being described here are shown in Table I.

First Purification Step. Affinity Chromatography Using Heparin–Sepharose. Initially, Shyamala (1971) showed that heparin is able to inhibit aggregation of receptor in cytosol. This result was later confirmed and expanded by others (Chamness & McGuire, 1972; Auricchio et al., 1978). Recently, Molinari et al. (1977) have shown that heparin–agarose interacts with considerable affinity with the estrogen–receptor complex and that this property can be exploited to achieve about 100-fold purification of 17β-estradiol–receptor complex from cytosol. Affinity chromatography with heparin–Sepharose was used as the initial step of our purification procedure because (data not shown) the cytosol components eliminated by this procedure include those which have a destabilizing effect on 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose, the specific affinity adsorbent to be used in the next purification step. This aspect will be further commented upon under Discussion. Furthermore, this first step achieves a fourfold purification of ligand-free estrogen receptor (vs. about a 100-fold purification when the receptor is bound to ligand) and, at the same time, a decrease of volume to be treated to less than half the volume of original cytosol; these results are of considerable advantage for both the procedures involved and the quality of the outcome of the next purification step.

Pilot experiments were set up in order to find out optimal conditions for affinity chromatography of receptor on heparin–Sepharose prepared as described under Materials and Methods. All operations were carried out at 4 °C, and all buffers and other materials were precooled to 4 °C before use. The results of these experiments are graphically shown in Figure 1 and may be summarized as follows: (1) about 0.15 g of heparin–Sepharose binds more than 90% of receptor in 1 mL of cytosol (Figure 1A); (2) the binding reaction is completed after 60 min of batchwise incubation at 4 °C with gentle agitation (Figure 1B); (3) after washing the heparin–Sepharose with adsorbed receptors by using TED buffer, we found that elution is best carried out columnwise, with TED buffer containing 4 mg/mL of purified heparin (Sigma, grade 1) as the eluting medium (Figure 1C). Four milligrams per milliliter of heparin is the minimal concentration of the aminoglycan which achieves maximal release of receptor from the column (65–80%). All elutable receptor is recovered after passage through the column of a volume of eluting buffer equivalent to 2.5-fold the volume of the gel.

Based on the above results, 600 g of heparin–Sepharose, washed with 4 L of TED buffer just before use, was added to the 4.35 L of uterine cytosol from the previous purification step; incubation lasted 1 h at 4 °C, with slow stirring with a magnetic bar.

At the end of incubation, the liquid phase was separated from the gel by filtration on a Buchner filter (coarse), and the gel was washed on the same filter with TED buffer until the OD at 280 nm was below 0.05. About 6 L of TED buffer is

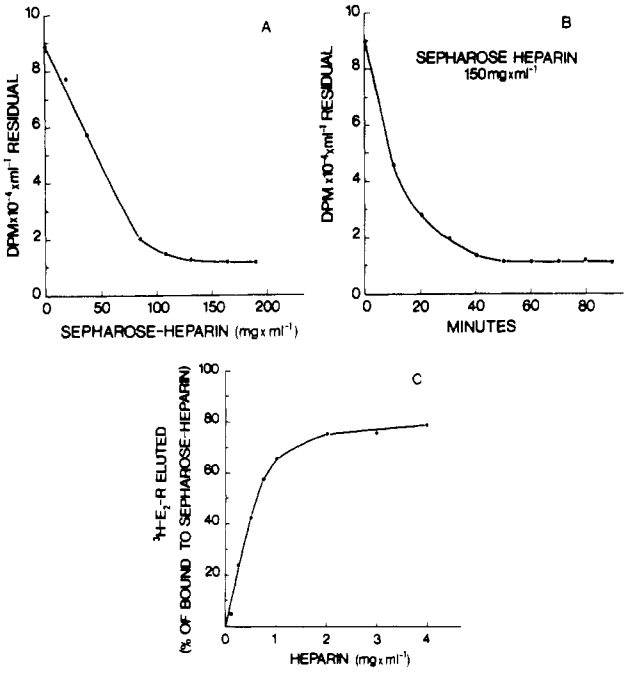

FIGURE 1: Interaction of estradiol–receptor with heparin–Sepharose. The binding of receptor is a function of Sepharose–heparine concentration (A) and of time of incubation (B). Elution of estradiol–receptor was with buffer containing different heparin concentrations (C).

required to achieve this goal. Finally, to the washed cake of heparin–Sepharose with adsorbed receptor, sufficient TED buffer was added to produce a slurry which could be easily decanted into a large, cylindrical plexiglass column (20 cm i.d.) with a nylon filter at one end. When excess buffer had just filtered away and the gel was neatly packed, a second nylon filter was set on top of the gel column, in order to avoid disturbing the packed gel, and 1.5 L of TED buffer containing 4 mg/mL of purified sodium heparin (Sigma grade I, sp act. 170 USP units/mg) was filtered through the gel by gravity. Filtration took about 45 min. Protein and receptor content of eluate were measured as described under Material and Methods, and the results are shown in Table I. One can see that 71% of receptor was recovered in 1.8 L of buffer and that, based on specific activity, a 4.4-fold purification of receptor was achieved.

Second Purification Step. Affinity Chromatography Using 17-Hemisuccinyl-17β-estradiol–Ovalbumin–Sepharose 4B. Pilot experiments were set up to find out the optimal conditions for affinity chromatography with this specific adsorbent. All operations were carried out at 4 °C, as well as buffers and other reagents which were precooled at 4 °C before use. The results are graphically shown in Figure 2 and may be summarized as follows. (1) One-tenth gram of adsorbent binds more than 90% of receptor in 1 mL of eluate from the first purification step, within 60 min of batchwise incubation at 4

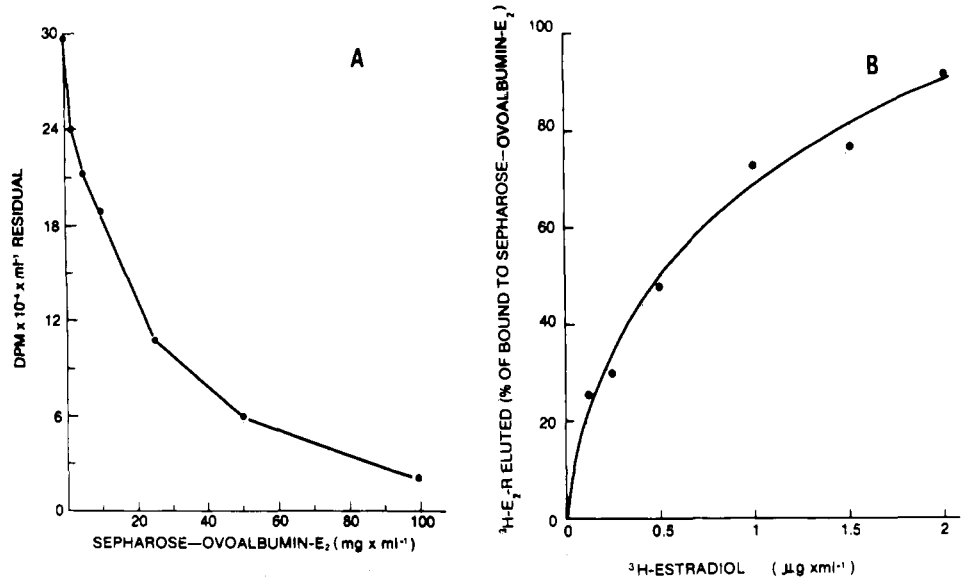

FIGURE 2: Interaction of estradiol–receptor with 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose. Eluate from heparin–Sepharose was incubated with increasing concentrations of 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose for 60 min at 4 °C. After incubation, residual estradiol–receptor concentration is measured in the supernatants (5000*g*, 5 min) (A). Elution of estradiol–receptor from 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose as a function of different hormone concentrations was in the buffer containing 0.5 M NaSCN at 4 °C (B).

°C with gentle stirring (Figure 2A). (2) After the adsorbent was washed with bound receptor, elution is best carried out batchwise, at 4 °C, by using TED buffer containing NaSCN (0.5 M) and an amount of 17β-[^{3}H]estradiol corresponding to 100-fold, molarwise, the 17β-estradiol residues in the solid-state adsorbent (Figure 2B). The eluting buffer/gel volume ratio is 4:1. The exchange is completed within 16 h, without loss of 17β-estradiol binding activity. This method of elution at 4 °C in the presence of NaSCN was found to be far superior to heating at 30 °C for 20 min, the method previously used (Sica et al., 1973a,b), both for recovery and for lack of receptor aggregation.

Based on the above results, the large-scale purification was performed as follows. About 230 g of 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose 4B, prepared as described under Material and Methods and stored at 4 °C, was washed with 1 L of TED buffer on a Buchner filter (coarse) and added to the 1.8 L eluate from the first purification step. The mixture was magnetically stirred for 1 h at 4 °C, filtered through a Buchner filter (coarse), and washed with 4 L of TED buffer containing KCl (0.5 M). The resulting cake was suspended in 1.1 L of TED buffer containing NaSCN (0.5 M) and 10 mg of 17β-[^{3}H]estradiol (sp act. 0.6 Ci/mmol) and incubated overnight (16 h) at 4 °C. After incubation, the suspension was filtered through a Buchner filter and the 1.1-L filtrate, containing the receptor–17β-[^{3}H]estradiol complex, was added to 4 L of TED buffer in order to decrease NaSCN concentration to about 0.1 M, a level of chaotropic salt which does not interfere with receptor binding to heparin–Sepharose, as required by the next purification step.

As a consequence of the very high concentration of 17β-[^{3}H]estradiol in the eluate on one side and the extreme protein dilution on the other, assessment of binding activity and protein content at this stage of purification proved to be unfeasible.

Third Purification Step. Second Chromatography Using Heparin–Sepharose. All operations were performed at 4 °C, and buffer and other reagents were precooled at 4 °C before use. Pilot experiments (data not shown) furnished the following information. (1) The estradiol–receptor complex is quantitatively bound by a column of heparin–Sepharose in the presence of a NaSCN concentration not exceeding 0.1 M; binding by heparin–Sepharose *columnwise* is about twice as efficient compared to *batchwise* and is virtually independent of receptor concentration in the medium; when receptor concentration in the medium is very low, these advantages more than offset the considerable longer time required by column operation. (2) After binding of the estradiol–receptor complex to heparin–Sepharose, the column can be washed with TED buffer containing KCl up to about 0.17 M, a more efficient washing procedure compared to use of TED buffer alone. Washing with high-salt buffers can be applied *only* when receptor is complexed with 17β-estradiol; when receptor is ligand-free, the KCl-containing buffer produces receptor inactivation for reasons which still elude us (data not shown).

Based on the above pilot experiments, the large-scale purification was continued as follows. Three hundred grams of heparin–Sepharose 4B was packed in a cylindrical plexiglass tube (10 cm i.d.) with a nylon filter on one end and washed with 1 L of TED buffer at the rate of about 300 mL/h. Thereafter, the diluted eluate from the previous purification step, containing the 17β-[^{3}H]estradiol–receptor complex, was filtered through the column at the same rate, immediately followed first by 1.5 L of TED buffer and then by 0.5 L of TED buffer containing KCl (0.17 M). Radioactivity and adsorbance at 280 nm of eluate were continuously monitored during these operations (Figure 3). When radioactivity in the eluate fell to a low level following washing with KCl-containing buffer, specific elution began by using TED buffer containing 4 mg/mL of purified heparin (Sigma grade I, sp act. 170 USP units/mg). The result was a peak of radioactivity emerging in about 400 mL of eluate. Protein concentration in the eluate was below the sensitivity of the assay method. On the basis of radioactivity, recovery of receptor is 57% at this stage (Table I). Thus, with small loss of receptor, about 14%, this purification step achieves (1) elimination of 17β-[^{3}H]estradiol and NaSCN used for the specific elution in the previous step and (2) concentration of receptor into a manageable volume of medium.

Fourth Purification Step. Concentration and Gel Filtration on Sephadex G-50. All operations were carried out at 0–4 °C. The eluate from the previous purification step was rapidly concentrated to about 40 mL by filtration under pressure by

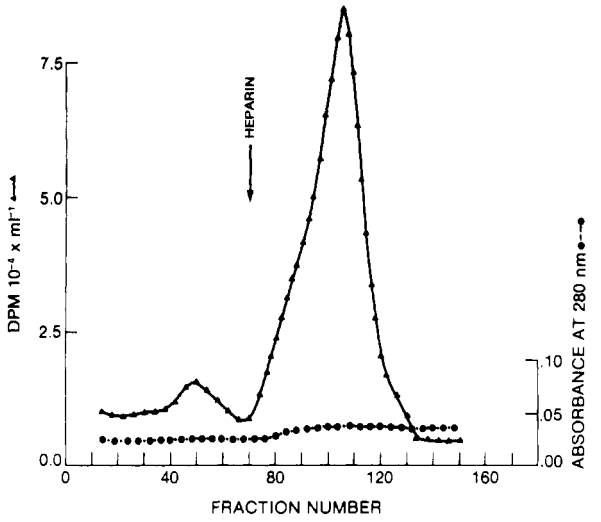

FIGURE 3: Third purification step. The diluted eluate from 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose was filtered through a column of heparin–Sepharose (300 g). The column was then washed with 1.5 L of TED buffer to eliminate most of free 17β-[^{3}H]estradiol. Figure 3 is the recording of radioactivity and adsorbance of radioactivity and adsorbance at 280 nm from the moment in which a final wash was started by using 0.17 M KCl in TED buffer, followed by the specific elution with heparin, 4 mg/mL in TED buffer (arrow). The large radioactive peak eluted by heparin is the 17β-[^{3}H]estradiol–receptor complex.

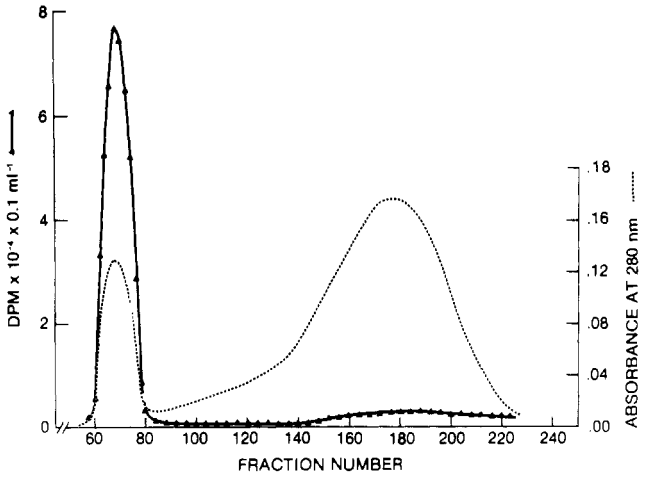

FIGURE 4: Fourth purification step. Sephadex G-50 chromatography of eluate from preceding step after concentration to about 40 mL by filtration on a UM 20 Diaflo membrane. Virtually all 17β-[^{3}H]-estradiol is excluded from the gel matrix, together with a peak of adsorbance at 280 nm. The broad included peak of adsorbance at 280 nm is oxidized dithiothreitol. Heparin, too, is included. Heparin is transparent at 280 nm. Temperature was 2–4 °C. This step achieves elimination of both (1) heparin used for elution in the preceding step and (2) other low molecular weight compounds (see text for further specifications).

using an Amicon Model 402 stirred cell with a UM 20 Diaflo membrane. No radioactivity passed through the filter or was bound to the membrane.

The concentrate was applied to a Pharmacia K26/100 column containing 400 mL of Sephadex G-50 (coarse) in TED buffer to which 0.01% (w/v) of heparin was added. Heparin was Sigma grade I (sp act. 170 USP units/mg) and was previously filtered on Sephadex G-50; only the included fractions were used for preparation of the TED–heparin in buffer. Elution was performed with the same TED–heparin buffer, and the elution pattern is shown in Figure 4. One can see that virtually all radioactivity is excluded by the gel matrix, together with a peak of adsorbance at 280 nm. There is, too,

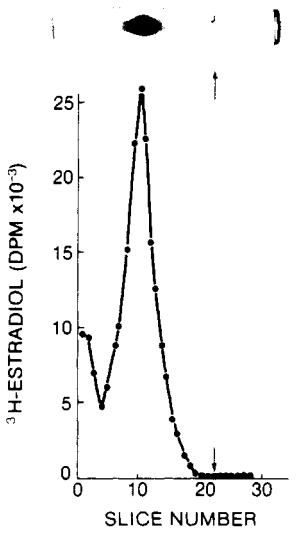

FIGURE 5: Disc gel electrophoresis at 5 °C under denaturing conditions of the 17β-[^{3}H]estradiol–receptor complex. Stacking gel was 3% and lower gel was 5%. Of a duplicate gel, one was used for measurement of radioactivity and the other stained was used with Coomassie blue. The single protein band is somewhat smeared because of overloading of the gel (35 μg of protein) for the purpose of detecting contaminants. The faint band at the front accounts for not more than 1% of total adsorbance of gel at 590 nm. 17β-[^{3}H]Estradiol comigrates with the protein. This electrophoresis is evidence that estrogen receptor has been purified to at least 99% homogeneity (see text for further details).

a broad included peak of optical density at 280 nm due to oxidized dithiothreitol. Heparin is perfectly transparent at 280 nm.

With this fourth purification step, one achieves (1) concentration to a small volume (40–50 mL) of the purified receptor; (2) elimination of heparin used for elution in the third purification step; and (3) elimination of low molecular weight compounds. Heparin at very low concentration (0.01%) was kept in the buffer used for the Sephadex G-50 chromatography in order to avoid aggregation of receptor; indeed, preliminary trials had shown that receptor, even at this final stage of purification, is still aggregation prone when concentrated in the absence of heparin.

As shown in Table I, the total protein content in the final Sephadex G-50 excluded peak is 1.225 mg and the total 17β-[^{3}H]estradiol is 2.12×10^7 dpm (sp act. 0.6 Ci/mmol). One may thus easily compute that in the final preparation the ratio grams of protein per mole of 17β-estradiol is 64450. The purification factor is about 16000-fold, with 53% recovery of original receptor activity.

Disc Gel Electrophoresis of Purified Receptor under Nondenaturing Conditions. All operations were carried out at 0–4 °C. The Sephadex G-50 excluded peak from the previous purification step was further concentrated to about 7 mL by filtration under pressure as described before except that the Amicon cell was the smaller, Model 12 type. The protein content of this final concentrate thus increased to 175 μg/mL. Samples from this concentrate were submitted to disc gel electrophoresis at 5 °C under nondenaturing conditions (see Materials and Methods).

Both the radioactivity pattern and the Coomassie blue stained gel of a typical duplicate electrophoresis run are shown in Figure 5. One can see that there is a single large protein band and that 17β-[^{3}H]estradiol comigrates with this protein. When heparin was not included in the gel and reservoir buffer, there was not penetration of protein into the gel. The gel was overloaded with protein (35 μg), and this explains the quite large and somewhat smeared protein band. Overloading was

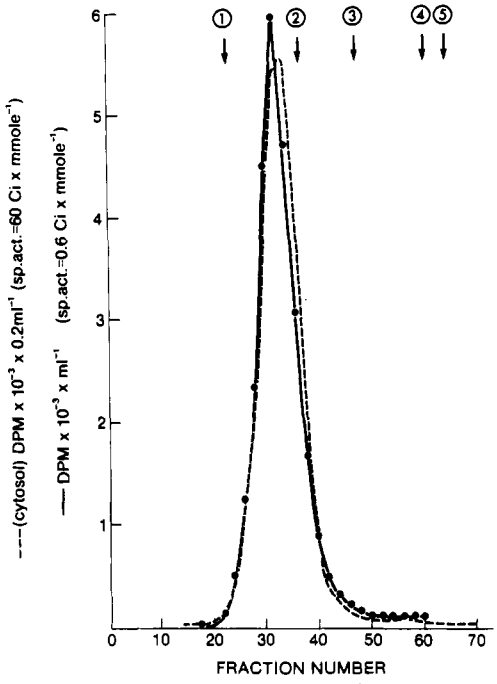

FIGURE 6: Chromatography on a calibrated column of Sephadex G-200 of pure receptor (solid line), as compared to uterine cytosol to which 4 mg/mL of heparin was added (broken line). Arrows indicate elution peaks of (1) Blue Dextran; (2) IgG; (3) bovine plasma albumin; (4) myoglobin; and (5) cytochrome *c*. Stokes radius of receptor computed according to Porath (1963) was 6.4 nm. Molecular weight computed according to Andrews (1964) was 43 000. Temperature was 2–4 °C. Note coincidence of the elution volume of pure receptor with that of the heparin-stabilized cytosol form (see text for further specifications).

necessary in order to detect possible contaminants. The results show that, with the exception of a very faint band migrating with the dye front, contaminants are absent. The faint band at the front must be low molecular weight material. As shown by scanning the gel at 590 nm, this faint band accounts for less than 1% of total adsorbance.

In conclusion, as judged by disc gel electrophoresis under nondenaturing conditions, estrogen-binding receptor has been purified to at least 99% purity. Further proof of purity is given by the results of gel electrophoresis in $NaDodSO_4$, which will be described later.

Determination of Stokes Radius of Purified Receptor by Gel Filtration. The pure receptor was submitted to gel filtration on a calibrated column of Sephadex G-200 in TD buffer. The elution pattern of bound 17β-[^{3}H]estradiol, together with the elution volume of reference proteins, is shown in Figure 6. There is a single sharp peak, for which a Stokes radius of 6.4 nm was computed on the calibration curve plotted according to Porath (1963). When the elution data are plotted according to Andrews (1964), a molecular weight of about 430 000 is derived for the purified receptor. The broken line in Figure 6 describes the elution pattern of *crude* calf uterus cytosol, to which 4 mg/mL of heparin was added previous to gel filtration on the same calibrated column used for the purified receptor. It is noteworthy that the elution patterns virtually coincide. This coincidence strongly suggests that we have purified, without modifications, the heparin-stabilized form originally present in the uterine cytosol. Because we know that heparin interacts with receptor, as well as with other proteins of cytosol (Waldman et al., 1975), this stabilizing action of the aminoglycan may be envisaged as formation of a negative electric refractory to protein–protein interaction involving the receptor in cytosol.

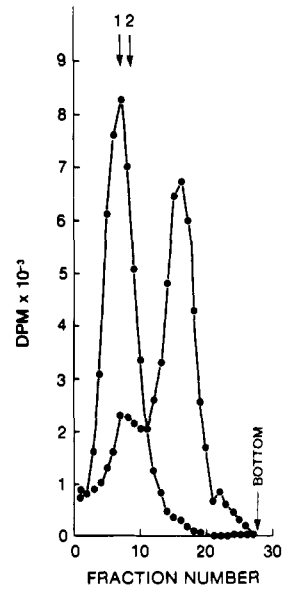

FIGURE 7: Patterns of sedimentation of pure receptor on 5–20% sucrose gradients in TD buffer (double-peak pattern) or in TD buffer containing 1 M NaBr (single-peak pattern). Temperature was 2–4 °C. Arrows indicate peaks of BPA, used as internal standard in the TD buffer tube (1) and in the TD buffer + NaBr tube (2). The major receptor peak in TD buffer is at 8 S, and the peak in TD buffer + NaBr is at 4.2 S (see text for further details).

Finally, it should be pointed out that addition of heparin (0.02%), NaSCN (0.1 M), or 1 M NaBr to the TD buffer did not modify the elution volume of purified receptor (data not shown). For NaSCN, this is apparently in contrast with previous results in which the chaotropic salt was added to *crude* or *partially* purified receptor preparations (Sica et al., 1976).

Sedimentation of Purified Receptor on Sucrose Gradients. As shown in Figure 7, when pure receptor is centrifuged on a 5–20% sucrose gradient in TD buffer, most of it sediments at 8 S (bovine plasma albumin was the internal reference), while some sediments as a shoulder at about 4 S. If 1 M NaBr is present in the sucrose gradient, all of the receptor sediments at 4.2 S. The effect of NaBr thus shows that the 8 S can quantitatively change into a 4.2 S. It is noteworthy that while NaBr (1 M) produces a dramatic change in the sedimentation rate of receptor, it does not, however, influence, as shown in section 2, its elution pattern from Sephadex G-200. Possibly, hydrostatic pressure may play a role in such difference. Evidence that pressure-induced, or favored, dissociation may play a role during high-speed centrifugation of receptor has been recently produced (Auricchio et al. 1978). This problem will be discussed later in this communication.

UV Adsorption Spectrum. The UV spectrum of pure receptor was analyzed in an ACTA III double-beam spectrophotometer between 240 and 340 nm. The spectrum shows a peak of adsorbance at 278 nm. The E_{280}^{1mg} is 2.1. The contribution of bound estradiol to adsorbance in the 240–340-nm region is negligible: although peak adsorbance of 17β-estradiol is at 280 nm, there is only 27 × 10^{-6} mg/mL of hormone in the pure preparation analyzed and the E_{280}^{1mg} for 17β-estradiol is 1.12.

Hormone Binding Specificity. The results of the tests of hormone binding specificity, performed as described under Materials and Methods, are shown in Table II. Only 17β-estradiol and, to a lesser degree, estriol compete for the binding sites. Thus, the binding site is estrogen specific.

$NaDodSO_4$ Disc Gel Electrophoresis. The pure receptor was submitted to gel electrophoresis in the $NaDodSO_4$–phosphate system of Weber & Osborne (1975).

Table II: Specificity of Binding Site of Pure Estrogen Receptor[a]

	residually bound 17β-[^{3}H]-estradiol (5)
50 ng of 17β-[^{3}H]estradiol	100
+ 50 000 ng of 17β-estradiol	3
+ 50 000 ng of progesterone	115
+ 50 000 ng of testosterone	110
+ 50 000 ng of cortisone	99
+ 50 000 ng of estriol	64
+ 50 000 ng of estrone	94

[a] Residually bound radioactive 17β-estradiol after addition of 1000-fold excess of cold steroid, followed by overnight incubation at 4 °C in the presence of NaSCN (0.5 M). Buffer: TED. Total volume of incubation mixture: 0.5 mL. Receptor: 5 μg.

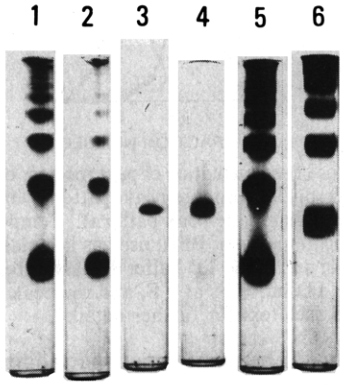

FIGURE 8: Parallel NaDodSO$_4$ disc gel electrophoresis (5% gel) in the NaDodSO$_4$–phosphate system of Weber & Osborne (1975). Ovalbumin and polymers: 1, 2, and 5. Pure receptor: 3 and 4. Plasma albumin and polymers: 6. Polymers were prepared by cross-linking with dimethyl suberimidate. The amount of loaded proteins was different in different gels. A single receptor protein band is observed, positioned between the plasma albumin monomer and the ovalbumin dimer. Room temperature. Results were consistently reproducible (see text for details).

Typical electrophoresis patterns of receptor and of reference proteins run in parallel are shown in Figure 8. Both receptor and reference proteins were run at two different concentrations, with the same results. A single band of receptor protein is observed, which is positioned between the plasma albumin monomer and the ovalbumin dimer. By interpolation in the semilogarithmic plot of molecular weights of reference proteins against their R_f values, an apparent molecular weight of 70000 is found for the denatured estrogen receptor. Assessment of the molecular weight of denatured receptor was repeated many times, always with precisely the same result.

Discussion

Clearly, the qualifying step in the above described procedure of purification of estrogen receptor is the specific affinity chromatography step using 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose 4B. Aside from inherent advantages like high recovery and rapidity, affinity chromatography guarantees, compared to classical separation methods, that the purified receptor is strictly *estrogen* receptor. Such a guarantee is important considering (1) that all steroid hormone receptors are present in the uterus and (2) that they all have very similar physical–chemical properties and are, therefore, amenable to parallel purification with classical methods which separate proteins according to these properties. Nor it is sufficient proof of purification of a specific steroid hormone receptor the finding that only *one* steroid hormone is bound by the purified protein. Other steroid hormone receptors may in fact be present as protein with inactive binding sites. This could be the consequence of the binding sites of the sought-for receptor being protected during the purification procedure, because the specific hormone has been added and is present in saturating amounts during purification. In contrast, the binding sites of other steroid receptors are ligand free and thus are unprotected and inactivated during purification.

A corollary advantage of affinity chromatography is that in the final preparation of receptor all binding sites are filled with 17β-[^{3}H]estradiol used for exchange elution from the specific affinity adsorbent. Therefore, knowing the specific activity of the 17β-[^{3}H]estradiol used, one can compute the protein/bound hormone ratio and thus, based on the molecular weight of the receptor molecule, derive in a direct manner the number of binding sites per receptor molecule. For the estrogen receptor purified here, this ratio was found to be 64450 g of protein per mol of bound hormone.

The chromatography steps using heparin–Sepharose are a considerable step forward compared to our previous affinity chromatography procedure in which uterine cytosol was directly incubated with the solid-state affinity adsorbent (Sica et al., 1973b). Incubation with cytosol induces the release of 17β-estradiol from the affinity adsorbent, as result of the action of chemical (reducing substances, nucleophiles) and biochemical components of cytosol (proteases, esterases) attacking the many susceptible bonds between the 17β-estradiol end and the agarose matrix (data not shown). However, when cytosol is first chromatographed on heparin–Sepharose, as in the novel procedure described in this paper, release of 17β-estradiol from 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose 4B no longer occurs, conceivably because the cytosol components producing this effect have been eliminated. Additional pluses of this initial heparin–Sepharose purification step are (1) about fourfold purification; (2) decrease of cytosol volume to less than half; and (3) inhibition of aggregation, without inactivation, of estrogen-free receptor in cytosol by heparin. It should be specified that the difference between the only fourfold purification achieved here and the about 100-fold purification of Molinari et al. (1977) finds its explanation mainly in the fact that Molinari et al. (1977) wash their estrogen receptor–heparin–Sepharose complex with salt-containing buffers, while we cannot apply this efficient washing procedure because in our case the heparin-bound receptor is estrogen free and is rapidly inactivated, by a mechanism which still eludes us, when such washing procedure is applied.

Also, the heparin–Sepharose chromatography step 3 was found to be the best manner for rapidly and efficiently eliminating both the free 17β-[^{3}H]estradiol and the NaSCN used in the preceding step, eluting the receptor from the specific affinity adsorbent.

The purification described in this paper is the typical procedure used by us to prepare about 1.2 mg of estrogen receptor of cytosol from 1 kg of calf uteri. The whole procedure takes about four working days. 17β-[^{3}H]Estradiol is used for eluting receptor from the specific affinity adsorbent. However, now that the optimal eluting conditions have been established, the same procedure may be performed by using cold 17β-estradiol instead. Under our working conditions, purification starting with 1 kg of uteri was optimal. It is obvious, however, that the operation can be scaled up or down, according to requirement.

As judged from the results of polyacylamide gel electrophoresis under nondenaturing as well as denaturing conditions (NaDodSO$_4$ + 2-mercaptoethanol), the purified receptor is more than 99% pure. The purified protein comigrates with bound radioactive hormone on nondenaturing polyacrylamide

Table III: Properties of Estrogen Receptor Purified to Homogeneity

purity of receptor protein:[a] hormone specificity	99% estrogen	
	native	dissociated
Stokes radius (a)	6.4 nm	3.9 nm[b]
sedimentation coefficients (s) (sucrose gradient)	8 S[c]	4.2 S[d]
M_r (based on gel filtration only)	430 000[e]	
M_r (based on s and a)	220 000[f]	
M_r ($NaDodSO_4$ gel electrophoresis)		70 000
frictional ratio		1.2[b]
axial ratio (prolate)		3[b]
λ_{max}	278 nm	
E_{280}^{1mg}	2.1	
no. of hormone binding sites per 70 000-dalton subunit		1

[a] Purity expressed as percent of receptor protein per total protein in preparation. [b] Computed from sedimentation coefficient (4.2 S) and molecular weight (70 000). [c] In view of possible pressure-induced dissociation during ultracentrifugation, it is uncertain that this coefficient corresponds to the undissociated protein with 6.4 nm of Stokes radius. [d] In the presence of 1 M NaBr. [e] Measured according to Andrews (1964). [f] Measured according to Siegel & Monty (1966).

gel electrophoresis. Consistent with earlier observations with less purified preparations, the pure receptor has a strong tendency to aggregate irreversibly. Aggregation can be counteracted by keeping the isolated protein in buffers containing trace amounts of heparin. If necessary, heparin can be eliminated by chromatography on Sephadex G-50, best in high-salt buffers (KCl, 0.25 M): the receptor protein is "excluded" by the gel matrix, while the aminoglycan is "included". As a precautionary measure, we use heparin (Sigma, grade I) previously included by Sephadex G-50.

The purified receptor protein is relatively stable when stored at 4 °C in TED buffer in the presence of trace amounts of heparin (≤1 mg/dL). Freezing in liquid nitrogen, followed by thawing at 5–10 °C or lyophilization, produces partial or complete denaturation of the protein, with loss of bound hormone. Conceivably, with further experience storage conditions can be improved.

The method of purification described here furnishes a homogeneous receptor protein with all binding sites filled with 17β-[^{3}H]estradiol of known specific activity. It is then easy to compute that 1 mol of hormone is bound per 64 450 g of pure receptor protein. Because, as assessed by acrylamide gel electrophoresis in $NaDodSO_4$, the molecular weight of the single polypeptide chain is 70 000, there can only be one binding site per polypeptide chain. The small discrepancy between the molecular weight as derived directly from $NaDodSO_4$ electrophoresis (70 000) and indirectly from bound ligand (64 450) cannot be considered significant.

As judged by chromatography on calibrated columns of Sephadex G-200, the final, purified, native nondenatured receptor is the same as that originally present in crude cytosol. In fact, both show a Stokes radius of 6.4 nm. Also, this value of Stokes radius coincides with the value found in earlier work (Puca et al., 1971a,b) for partially purified estrogen receptor in low-salt buffers. Thus, the available evidence is that during the purification procedure described in this paper there is no loss of receptor components. This conclusion is further supported by the 8S sedimentation constant found by centrifugation on sucrose gradients in low-salt buffers of the 99% purified receptor, as well as of receptor in crude cytosol and, in earlier work (Puca et al., 1971a,b), of partially purified receptor.

For a protein with a usual axial ratio, a Stokes radius of 6.4 nm corresponds to a molecular wieght of about 430 000 (Porath, 1963). However, the molecular weight of the protein is considerably lower if the axial ratio is unusually large. An unbiased estimate of molecular weight and of frictional ratio can be achieved based on the sedimentation coefficient and the Stokes raidus of a protein (Siegel & Monty, 1966). This method, already applied in earlier work (Puca et al., 1971a,b, 1972), gives a frictional ratio of 1.65 and a molecular weight of about 240 000, instead of 430 000, for the nondenatured receptor in low-salt buffer.

Based on the above calculation, the nondenatured receptor of cytosol appears thus to be a protein of unusually large axial ratio. However, recent evidence suggests that the quaternary structure of some protein (Erickson, 1974; Olmsted et al., 1974), including receptor (Auricchio et al., 1978), is destabilized by pressures the same as those developing during ultracentrifugation. If subunit interaction falls apart, partially or completely, during ultracentrifugation, then we may be dealing with a different protein according to the method being used, i.e., ultracentrifugation or gel filtration. Under these circumstances, application of the method of Siegel and Monty would lead to erroneous conclusions.

While there is uncertainty concerning the molecular weight of the native cytosol receptor, we are on solid ground with regard to the physical parameters of the receptor subunit. $NaDodSO_4$ gel electrophoresis of purified cytosol receptor shows a single protein band with an apparent molecular weight of 70 000, and sucrose gradient centrifugation under dissociation conditions [1 M NaBr or NaSCN (0.1–0.5 M)] shows a single peak with a sedimentation coefficient of about 4 S. One can indirectly compute a Stokes radius of 3.9 nm and a frictional ratio of 1.2. These results are in good agreement with earlier estimates of physical parameters of the smaller, about 4S receptor form (Puca et al., 1971a,b, 1972) in partially purified preparations, as well as with indirect estimates in this paper of receptor subunit weight (64 500) based on the weight of protein corresponding to 1 mol of bound 17β-estradiol.

Thus, our results indicate that the calf uterus estrogen receptor subunit is *one* and carries a *single* hormone binding site. It appears that in the native state in cytosol, several subunits are associated to form a larger quaternary structure, but the number of interacting subunits cannot be specified pending uncertainty about the molecular weight of the native receptor state. Known properties of purified receptor are summarized in Table III.

In conclusion, a method is now available which allows purification of estrogen receptor of cytoplasm in tangible amounts. Availability of pure receptor opens the way to its definitive biochemical characterization and to further in-depth understanding of the role of receptor in the control of gene expression.

References

Andrews, P. (1964) *Biochem. J. 91*, 222.

Auricchio, F., Rotondi, A., Sampaolo, P., & Schiavone, E. (1978) *Biochem. J. 171*, 137.

Bitter, T., & Muir, H. M. (1962) *Anal. Biochem. 4*, 330.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Bresciani, F., Sica, V., & Weisz, A. (1978) *Biochem. Actions Horm. 6*, 461.

Carpenter, F. H., & Harrington, K. T. (1972) *J. Biol. Chem. 247*, 5580.

Chamness, G. C., & McGuire, W. L. (1972) *Biochemistry 11*, 2466.

De Sombre, E. R., & Gorell, T. A. (1975) *Methods Enzymol. 36A*, 349.

Erickson, H. P. (1974) *J. Supramol. Struct. 2*, 393.
Jungblut, P. W., Morrow, R. I., Reeder, G. L., & Jensen, E. V. (1965) *Abstracts, 47th Meeting of the Endocrine Society*, 56.
Jungblut, P. W., Hatzel, I., De Sombre, E. R., & Jensen, E. V. (1967) *Colloq. Ges. Physiol. Chem. 18*, 58.
Molinari, A. M., Medici, N., Moncharmont, B., & Puca, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4886.
Olmsted, J. B., Marcum, J. M., Johnson, K. A., Allen, L., & Borisy, G. G. (1974) *J. Supramol. Struct. 2*, 429.
Porath, G. (1963) *Pure Appl. Chem. 6*, 233.
Puca, G. A., Nola, E., & Bresciani, F. (1970) *Res. Steroids 4*, 319.
Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1971a) *Adv. Biosci. 7*, 97–118.
Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1971b) *Biochemistry 10*, 3769.
Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1972) *Biochemistry 11*, 4157.
Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1975) *Methods Enzymol. A36*, 331.
Shyamala, G. (1971) *Nature (London), New Biol. 231*, 246.
Sica, V., Nola, E., Parikh, I., Puca, G. A., & Cuatrecasas, P. (1973a) *Nature (London), New Biol. 244*, 36.
Sica, V., Parikh, I., Nola, E., Puca, G. A., & Cuatrecasas, P. (1973b) *J. Biol. Chem. 248*, 6543.
Sica, V., Nola, E., Puca, G. A., & Bresciani, F. (1976) *Biochemistry 15*, 1915.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346.
Waldman, A. A., Marx, G., & Goldstein, J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 2352.
Weber, K., & Osborne, M. (1975) *Proteins 1*, 180.

Pyridoxal Phosphate Induced Alterations in Glucocorticoid Receptor Conformation†

John A. Cidlowski* and John W. Thanassi

ABSTRACT: The molecular properties of dexamethasone–receptor complexes from rat thymocytes are markedly affected by pyridoxal phosphate as shown by both sucrose density gradient centrifugation and molecular exclusion gel chromatography. In the absence of added pyridoxal phosphate, unactivated cytoplasmic receptor complexes sediment as 7–8S and 2.9S species, whereas activated cytoplasmic receptor complexes sediment as 4–5S and 2.9S species. When unactivated cytoplasmic receptor complexes are exposed to pyridoxal phosphate either before or during sucrose density gradient centrifugation, the sedimentation profile is converted to one that is identical with that found for activated receptor; i.e., the 7–8S form is apparently converted to a 4–5S form. Dexamethasone receptors extracted from rat thymocyte nuclei with pyridoxal phosphate sediment as a single 3.5S peak. Findings consistent with the above are made by using gel filtration techniques. After treatment with pyridoxal phosphate followed by reduction with sodium borohydride, all cytoplasmic and nuclear dexamethasone receptors are converted to a single 2.9S form. The 2.9S form is insensitive to the presence of 0.4 M KCl during centrifugation. These observations suggest that there is an irreversible, covalent modification of dexamethasone–receptor complexes by pyridoxal phosphate and sodium borohydride. Presumably this modification occurs at a lysine residue(s) on the various forms of the receptor and causes either conformational changes in the receptors or the expression of a subunit that is common to and characteristic of all of the forms of the nuclear and cytoplasmic dexamethasone receptors found in rat thymocytes. The 2.9S forms of the dexamethasone receptors produced by treatment with pyridoxal phosphate and sodium borohydride appear to be similar in size with those produced by treatment of thymocyte cytosols with Ca^{2+}.

Current thinking on the mechanism of steroid hormone action is that the hormone enters a target cell and associates with cytoplasmic receptors. Subsequent translocation of the cytoplasmic steroid–receptor complex to the nucleus requires activation which is assumed to be accompanied by a conformational change. In vitro, the conversion of unactivated cytosolic steroid–receptor complexes can be accomplished by warming cytosolic extracts to physiological temperatures (Munck & Leung, 1977; Wira & Munck, 1974). Turnell et al. (1974) have shown by sucrose density gradient centrifugation in low-salt buffer that there are two forms of glucocorticoid receptors in cytosol from rat thymocytes which have sedimentation coefficients of approximately 7 S and 3.5 S. Studies on the molecular properties of glucocorticoid receptors from other tissues, e.g., fibroblasts (Middlebrook & Aronow, 1977) and liver (Koblinsky et al., 1972; Beato & Feigelson, 1972; Wrange & Gustafsson, 1978), indicate single ~7S receptor forms which can be converted to smaller ~4S forms by 0.4 M KCl. Even smaller forms (~3 S) of glucocorticoid receptors have been produced by limited trypsin digestion (Wrange & Gustafsson, 1978) and treatment with calcium chloride (Sherman et al., 1978). The relation between receptor size and nuclear binding is not clear at the present time.

Vitamin B_6 is an essential cofactor for a large variety of enzymes that are concerned with the intermediary metabolism of amino acids (Snell, 1958; Braunstein, 1960). Very recently, evidence has appeared suggesting that pyridoxal phosphate,

† From the Department of Biochemistry, University of Vermont, College of Medicine, Burlington, Vermont 05405. *Received August 29, 1978.* This research was supported by U.S. Public Health Service Grants AM20892-01, 5429-16-9, and 5429-16-18.

0006-2960/79/0418-2378$01.00/0