Characterization of Trypsin-Treated Forms of the Estrogen Receptor from Rat and Lamb Uterus†

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ABSTRACT: The molecular properties of uterine estrogen receptors, following limited proteolysis, have been characterized. Mild trypsinization of cytoplasmic estrogen receptor preparations from lamb and rat uterus causes a marked disaggregation of the receptor, giving lower molecular weight forms that retain unimpaired their ligand binding properties. These trypsinized receptors have little tendency to reaggregate and thus are amenable to further purification and to detailed characterization. Sedimentation coefficients obtained by sucrose gradient centrifugation are 3.04 ± 0.04 S for the lamb and 4.01 ± 0.09 S for the rat trypsinized receptors; Stokes radii, determined by gel partition chromatography, are 2.76 ± 0.23 nm for lamb and 2.94 ± 0.25 nm for rat uterine receptor. Discontinuous polyacrylamide gel electrophoresis in two systems (pH 7.8 and 10.2) shows the trypsinized lamb receptor as three distinct components (two major and one minor); the rat receptor is somewhat more disperse, but appears as mainly one component. Molecular weight estimates based on combined sedimentation coefficients and Stokes radii are 35 300 for the lamb components and 49 600 for the rat. Molecular weights estimated by electrophoretic mobility (Ferguson plots) show the rat receptor to be somewhat lighter (41 800 ± 10 000) than the two principal lamb components (46 800 ± 11 000 and 46 400 ± 11 000). Isoelectric focusing in polyacrylamide gels gives apparent p*I* values of 5.5 and 5.8 (major components), and 6.2 (minor) for the lamb and 6.4 for the rat receptor. These readily characterizable, trypsinized forms of the estrogen receptor may be related to other smaller molecular weight forms of receptors for estrogens and other steroids. Thus, the availability of these receptor preparations should be of major assistance to studies that are aimed at understanding the interaction of estrogenic compounds with the receptor binding site and the relationship between various forms of steroid receptors.

Until recently, estrogen receptors have been characterized mainly by their ability to bind estrogens with high affinity and specificity and by their characteristic sedimentation velocities at different salt concentrations. While these techniques have contributed greatly to elucidating many features of steroid hormone action, we have encountered situations that demanded higher resolution and less ambiguous characterization methods. Therefore, we sought to investigate forms of the estrogen receptor that might be amenable to analysis by such high resolution techniques as discontinuous polyacrylamide gel electrophoresis and isoelectric focusing.

The proclivity of the estrogen receptor toward aggregation and the interference by nonreceptor binding proteins have been major obstacles to achieving high resolution electrophoretic separations. However, Rat et al. (1974) have reported that mild trypsinization of the calf endometrial estrogen receptor produces a form of receptor with lowered molecular weight that appears to be both homogeneous and relatively resistant to further aggregation. We have found that this trypsinization procedure can be applied to lamb and rat uterine estrogen receptors to give forms that behave well upon electrophoresis. In this report we describe the characterization of these complexes, and we note differences in the physical properties of trypsin-disaggregated estrogen receptors prepared from lamb and rat uteri.

Experimental Section

Materials were obtained from the following sources: Photo-Flo 200, acrylamide, and *N,N'*-diallyltartardiamide (DATD)[1] (Eastman Kodak); 17β-estradiol (Searle); [6,7-^{3}H]17β-estradiol, 48 Ci/mmol (New England Nuclear); charcoal Norit A, tris(hydroxymethyl)aminomethane (Tris, dextran grade C), and L-[^{14}C]leucine, 312 mCi/mmol (Schwarz/Mann); Triton X-114 (Central Solvents & Chemical Co.); bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bistris), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), dimethylarsinic acid (cacodylic acid), trypsin, and soybean trypsin inhibitor (Sigma); (ethylenedinitrilo)tetraacetic acid (EDTA) (Baker); Fast Green (Matheson, Coleman and Bell); periodic acid (G. F. Smith); ampholytes (LKB); Sephadex G-200 and Blue Dextran 2000 (Pharmacia); *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Aldrich).

Methods. All cytosol preparations, storage, and experiments were done in TEA buffer (0.01 M Tris-HCl–0.0015 M EDTA–0.02% sodium azide, pH 7.4 at 25 °C). The charcoal–dextran slurry used to remove unbound ligand was prepared as previously reported (Katzenellenbogen et al., 1973) and was generally used at 1 part to 10 parts of cytosol solution.

Cytosol. Rat cytosol was prepared from immature Holtzman rats (21–25 day females) (see Katzenellenbogen et al., 1973) and was stored in liquid nitrogen or at −20 °C in 25%

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[1] Common names (and abbreviations) used in this paper are: estradiol, 1,3,5(10)-estratriene-3,17β-diol; TEA buffer, 0.01 M Tris–0.0015 M EDTA–0.02% sodium azide (pH 7.4 at 25 °C); BSA, $(BSA)_2$, $(BSA)_3$, $(BSA)_4$, bovine serum albumin monomer, dimer, trimer, tetramer; STI, soybean trypsin inhibitor (Kunitz); CA, beef erythrocyte carbonic anhydrase B; OV, hen ovalbumin; AM, *B. subtilis* α-amylase; TF, human transferrin; CTG, bovine pancreatic α-chymotrypsinogen A; MYO, sperm whale myoglobin; Tris, tris(hydroxymethyl)aminomethane; DATD, *N,N'*-diallyltartardiamide; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

v/v glycerol (Katzenellenbogen et al., 1974). Lamb cytosol was prepared and stored as previously described (Katzenellenbogen et al., 1977c).

Serum. Blood collected from immature lambs and from 9- and 21-day old female rats was allowed to clot at 0 °C for 1 h and was centrifuged at 800*g* for 10 min.

Ammonium Sulfate Fractionation. All procedures were at 0–4 °C. The lamb cytosol was brought to 30% saturation by addition of a saturated solution of aqueous ammonium sulfate (pH adjusted to 7.2 with NaOH). After 15 min, the resulting precipitate was collected by centrifugation at 20 000*g* for 20 min. Rat cytosol receptor was routinely precipitated by ammonium sulfate brought to 40% saturation.

Trypsinization. A trypsin solution was prepared immediately before use at 10 mg/mL in 0.001 M HCl. For lamb receptor preparations, 20 μg of trypsin was added for each mg of protein, and the proteolysis maintained at 0 °C for 1 h. Rat receptor preparations required 40 μg of trypsin per mg of protein for 1 h. Trypsinization was stopped by the addition of 2.5 μg of soybean trypsin inhibitor for each μg of trypsin. While trypsin undergoes rapid autolysis in the absence of Ca^{2+} (TEA buffer), these conditions appear optimal for effecting receptor disaggregation while minimizing loss of binding activity (Rat et al., 1974).

Gel Filtration. A 1.5 × 50 cm column was packed with Sephadex G-200. The column was eluted at 4 °C with TEA buffer by inverted flow at a rate of 5–6 mL/h and 2.8-mL (100 drop) fractions were collected. The distribution coefficient, K_{av}, was calculated from the relationship, $K_{av} = (V_e - V_0)/(V_t - V_0)$ (Laurent and Killander, 1964), where V_e is the elution volume of the protein, V_0 is the void volume measured with Blue Dextran 2000, and V_t is the total bed volume measured with L-[^{14}C]leucine.

Protein Determination. Proteins were precipitated with 10% trichloroacetic acid, redissolved, and measured using the procedure for insoluble proteins of Lowry et al. (1951).

Scintillation Counting. Samples were counted in 5 mL of a xylene–Triton X-114 based scintillation fluid as described in Katzenellenbogen et al. (1974). Counting efficiency for single-labeled samples was 50% (tritium) and for double-labeled samples was 40% (tritium) and 57% (carbon-14).

Electrophoresis. Gels were cast in 12 cm long × 6 mm i.d. glass tubes. The tubes had been cleaned in a 6% KOH–methanol bath for ~30 min, rinsed, coated with a 0.5% aqueous solution of Kodak Photo-Flo 200, and oven dried. The catalyst solution (0.28% ammonium persulfate) was prepared fresh daily.

The high pH electrophoresis system was modified from Ornstein (1964) and Davis (1964), substituting *N,N'*-diallyltartardiamide (DATD) for methylenebisacrylamide to cross-link the separation gel (Anker, 1970). The gel composition is designated as % T [total gel concentration; (g of acrylamide + g of DATD)/100 mL solution] and % C [cross-linker: 100 × g of DATD/(g of acrylamide + g of DATD)]. The separation gel was prepared at 6.48% T and 7.35% C. The neutral pH system described by Rodbard and Chrambach (1971) (system D) was modified as above, to be cross-linked with DATD at 7.35% C; gels were cast at 8.1% T. The running pHs of the separation gel for these two systems are 10.2 and 7.8 (Chrambach and Rodbard, 1971).

A Searle Analytic-Buchler electrophoresis apparatus (Polyanalyst) was used. The lower buffer reservoir is jacketed, and the lower buffer is magnetically stirred and cooled to 0–2 °C by circulation of chilled methanol from a Lauda Kryomat. The gel tubes are immersed in the lower buffer so that the entire gel is surrounded by the cooled solution.

In the high pH system only, gels were pre-run at room temperature with an upper reservoir buffer which was a 1:10 dilution of the buffer in the stacking gel (A. Chrambach, personal communication). Pre-running was done at 2 mA/gel until the ion front (marked by bromophenol blue) reached the interface of the stacking gel and separation gel. The upper reservoir pre-run buffer was replaced with the running buffer, and the gels and lower buffer bath were cooled prior to application of the samples and initiation of the normal run also at 2 mA/gel.

Following electrophoresis, gels for radioactivity determinations were frozen on dry ice and sliced into 2.15-mm slices. Each slice was dissolved in 0.5 mL of 2% periodic acid at room temperature and counted in 5 mL of scintillation fluid. When free [^{3}H]estradiol is removed from the receptor preparation prior to electrophoresis, the average recoveries of radioactivity in the separation gels are, 70% in the pH 7.8 system and 74% in the pH 10.2 system. For optical scanning, the position of the dye band was marked by India ink, and the gels were stained in fast green using the procedure of Gorovsky et al. (1970) and were scanned on a Varian Techtron 635D spectrophotometer with a gel scanning adapter.

Isoelectric Focusing. Isoelectric focusing was done in polyacrylamide gels containing 5% T–15% C (Baumann and Chrambach, 1976) and 2% w/v pH 5–8 range ampholytes (Ampholine, LKB). The experiment was performed in the same apparatus used for electrophoresis (upper bath 0.4% ethanolamine; lower bath 0.5 M acetic acid). Focusing was initiated at 0.5 mA/gel until a voltage of 200 V was reached; the voltage was then maintained at 200 V for 8–10 h. Focusing of the receptor proteins was complete within 8 h and the p*I* values were stable up to 20 h. After focusing, the gels were sliced into 2.15-mm sections. The pH profile was determined by suspending each slice from a gel in 1.0 mL of 0.02 M KCl for 2 h at 4 °C. The gel slice and the KCl eluate were counted in 10 mL of scintillation fluid. Profiles of radioactivity and pH were determined on the same gels.

Density Gradient Centrifugation. Linear 5–20% sucrose gradients (3–4 mL) were prepared in TEA buffer. The gradients were centrifuged at 270 000*g* for 16 h (SW Ti 60 rotor in a Beckman L5-65 ultracentrifuge at 50 000 rpm). All other operations were carried out as described previously (Katzenellenbogen et al., 1973).

Standard Proteins. The physical properties of the proteins used as standards to estimate sedimentation velocities and Stokes radii are listed in Table III (see paragraph concerning supplementary material at the end of this paper). ^{14}C-labeled proteins were prepared according to the procedure of Stancel and Gorski (1975). Dansylated bovine serum albumin was prepared by the method of Weber (1952) and was detected on a Hitachi MPF-2A fluorimeter.

Results

Disaggregation and Partial Purification of the Estrogen Receptor. To obtain high resolution electrophoretic profiles of the uterine estrogen receptor, it is necessary to disperse the receptor aggregates that form in cytosol preparations; it is also advantageous to effect a partial purification in order to remove the bulk of the nonreceptor binding proteins. Ammonium sulfate precipitation is a convenient first step to use in the purification; it removes many of the nonreceptor binding proteins (mainly serum albumin) and gives a two- to fivefold purification in 50–60% yield (Table I in supplementary material). With lamb receptor, the greatest purification is obtained by precipitation with 20 to 30% saturated ammonium sulfate; 50% of receptor activity is recovered. The rat receptor, which is

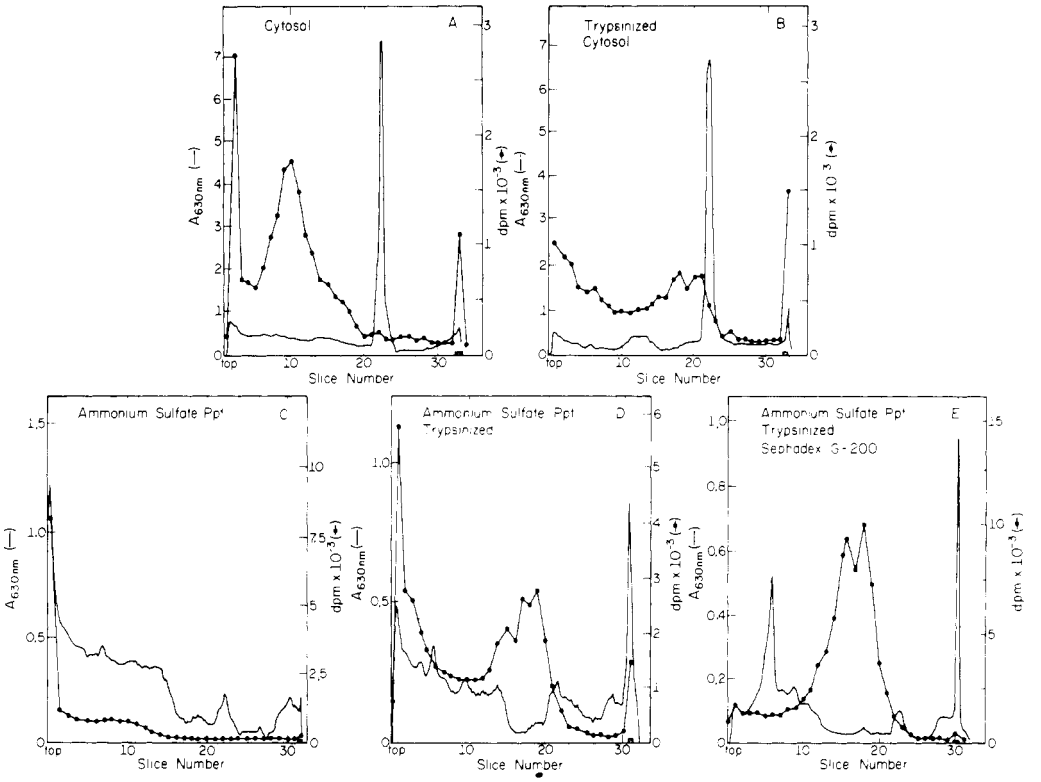

FIGURE 1: Electrophoretic analysis of different preparations of lamb uterine estrogen receptor. All samples were run on duplicate 8.1% T-7.35% C gels at pH 7.8. One gel was sliced for radioactivity determination (●—●) and the other was stained with Fast Green and scanned at 630 nm for total protein (—). The absorbance and radioactivity scales are adjusted to obtain full scale peaks. Only the separation gel is shown. The relative purity of each preparation, determined by comparison of the area of the radioactivity profile with the area of the absorbance profile, is given below in parentheses; the purification factors correspond closely to those determined on the total sample (cf. Table I). (A) Whole cytosol (relative purity 1); (B) trypsinized cytosol (0.74); (C) ammonium sulfate precipitated cytosol (4.98); (D) preparation C after trypsinization (7.00); (E) preparation D after Sephadex G-200 chromatography (21.6).

routinely stored in 25% glycerol, requires a higher percentage of ammonium sulfate to precipitate. In all experiments discussed below, we have used 30% ammonium sulfate for lamb receptor preparations and 40% for rat receptor preparations.

While the ammonium sulfate precipitation is effective in removing nonreceptor binding proteins, it produces a highly aggregated form of the receptor (see Figure 1C). This aggregate elutes in the void volume of a Sepharose 4B column (mol wt $>10^7$; data not shown) and is awkward to purify further.

Many procedures for disaggregation of the estrogen receptor have been reported in the literature; however, most of these proved unsatisfactory for our purposes either because of their use of high salt conditions (Stancel et al., 1973; Notides and Nielsen, 1974; Stancel and Gorski, 1975; Chamness and McGuire, 1975; Sica et al., 1976, 1977; Puca et al., 1976) or their requirement for fresh cytosol preparations (Puca et al., 1971, 1975, 1976; Sica et al., 1976, 1977). Trypsin has been utilized to partially cleave the calf uterine estrogen receptor (Rat et al., 1974) and to extract an insoluble estrogen receptor from chicken liver nuclei (Lebeau et al., 1973, 1974). These latter reports suggested a disaggregation procedure that would avoid the restrictions of the former methods.

We have found that trypsin converts both the lamb and rat uterine receptor to smaller forms which can be further purified by chromatography, electrophoresis, and isoelectric focusing. The maximum conversion of the lamb receptor to the smaller form (75%) can be achieved without loss of binding activity by treatment with between 2 and 50 μg of trypsin per mg of protein for 1 h at 0 °C; routinely 20 μg of trypsin is used. The rat receptor appears somewhat less sensitive to trypsin treatment than the lamb, and routinely we use 40 μg of trypsin per mg of protein for 1 h at 0 °C to effect maximum disaggregation (75%). We have found it advantageous to terminate all trypsinization reactions by the addition of soybean trypsin inhibitor (Vallet-Strouve et al., 1974). Failure to do this often results in loss of binding activity within a few hours. After trypsinization, chromatographic fractionation of receptor on a Sephadex G-200 column provides an additional twofold purification (Table I, supplementary material) and removes small amounts of uncleaved aggregates.

Electrophoresis. The major difficulty in developing an electrophoretic system suitable for the analysis of the estrogen receptor appears to have been a combination of the tendency of the receptor to form large, heterogeneous aggregates and the dissociation of the steroid from the receptor complex. The partial purification–trypsinization sequence, which produces a small form of the receptor that is no longer prone to aggregation, overcomes the first of these problems. However, an investigation of a number of parameters was still required in order to develop a satisfactory electrophoretic system for these preparations.

We have found it essential to have rigorous temperature control during the electrophoresis. The cooled electrophoresis apparatus described in Methods proved more satisfactory than attempts to run the electrophoresis in a cold room with air cooling of the gels.

In the high pH system, an important modification is prerunning the gel; failure to do this results in a receptor profile that is a series of poorly defined peaks, representing only a fraction of the expected binding capacity. (Underlaying the sample with thioglycolate, a procedure that has been used to

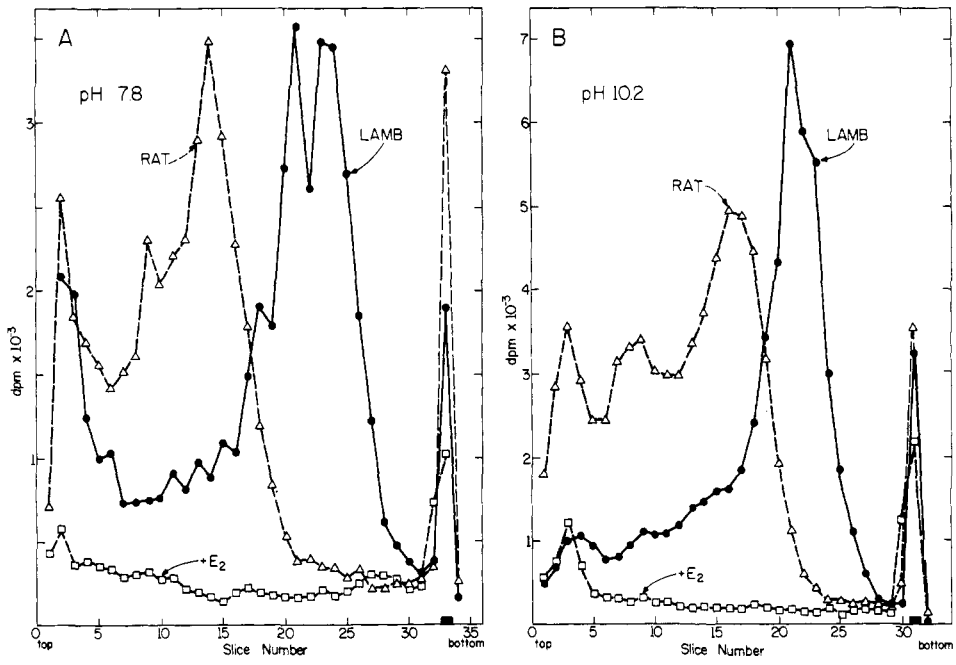

FIGURE 2: Profiles of rat and lamb uterine estrogen binding activity electrophoresed at pH 7.8 (panel A) and pH 10.2 (panel B). Cytosol preparations were ammonium sulfate precipitated, trypsinized, and then labeled by incubation with 30 nM [^{3}H]estradiol for 1 h at 0 °C. Free steroid was removed from the samples by charcoal adsorption prior to electrophoresis on 6.5% T-7.35% C gels. Only the separation gel is shown. The featureless profiles of samples labeled in the presence of a 100-fold excess of unlabeled estradiol (□—□) indicate the low level of nonspecific binding in these preparations. (Only data from rat are shown, but lamb data are essentially identical.)

remedy oxidative degradation (Dirksen and Chrambach, 1972), failed to improve the receptor pattern on a gel that has not been pre-run.) While pre-running of a gel with the normal upper reservoir buffer (Tris-glycine, pH 8.3) destroys the pH and ion discontinuity necessary to stack the proteins, the stacking phenomenon can be preserved by pre-running with the same pH and ion constituents present in the stacking gel, as described in the Methods section. Pre-running is less important in the neutral pH system, where similar receptor patterns are obtained whether the gel is pre-run or not. Inclusion of 10% glycerol in both the gel and the buffer, as has been used for the progesterone receptor (Miller et al., 1975), did not improve the resolution of the estrogen receptor in either gel system.

Electrophoretic profiles of estrogen receptor in cytosol preparations (not ammonium sulfate precipitated) can only be observed after charcoal adsorption. If this is not done, a large amount of estradiol migrates with the albumin band, and the tail of this peak obscures the high affinity estrogen binding.

The purification procedure can be followed nicely by electrophoresis on 8.1% T neutral pH polyacrylamide gels (Figures 1A-E). Duplicate gels were run at each stage of the purification, one being sliced to obtain the radioactive profile of estrogen binding activity and the other being stained to indicate the profile of total proteins. The uptake of the Fast Green stain is linear with protein concentration (Gorovsky et al., 1970); therefore, the optical scan is a quantitative representation of the protein content of the gels. In this gel system, free estradiol bearly enters the separation gel.

High affinity, estrogen specific binding activity in whole lamb uterine cytosol migrates as a fairly broad peak with a R_f of 0.24 (Figure 1A). Treatment of this preparation with trypsin has little apparent effect on the total protein profile (optical trace, Figure 1B); bound estradiol, however, now migrates as three distinct components having R_f values of approximately 0.44, 0.54, and 0.62, and having a total binding capacity (integrated area under the three peaks) very close to that of the untrypsinized cytosol.

Ammonium sulfate precipitation removes most of the albumin from the uterine cytosol preparation (optical trace, Figure 1C), but estrogen binding activity becomes highly aggregated and fails to penetrate the gel. Trypsinization of the precipitated cytosol produces only minor alterations in the pattern of total proteins (Figure 1D), but the estrogen binding activity is disaggregated and reappears as three distinct components with R_f's comparable to those seen in trypsinized whole cytosol (Figure 1B). Sephadex G-200 column chromatography removes many of the protein contaminants and some of the receptor aggregates that remain (Figure 1E). The binding activity still appears in three components, but the amount of the slowest moving one is reduced. The altered ratio of the peaks may be the result of an inadvertent fractionation since only the peak fraction from the Sephadex G-200 column effluent was used.

A comparison of the mobilities of the trypsinized estrogen receptor from rat and lamb uterus in the two electrophoretic systems is shown in Figure 2. In the pH 7.8 system, the lamb receptor shows the three peak pattern previously described. The rat receptor, which migrates less far in the gel, also seems to contain at least two components. The specificity of the estrogen binding activity in these preparations is evident by the low level of nonspecific binding (bottom curve, Figure 2A). On the high pH gel system, the components of the lamb receptor are only marginally separated. The rat receptor binding, which is less stable to electrophoretic fractionation, shows three peaks. The least mobile one corresponds to free estradiol, which in this high pH system migrates just beyond the stacking gel (note similar peak in lowest curve).

In order to ascertain that the peaks of radioactivity observed in the gels actually correspond to the profile of estrogen binding activity (and do not merely represent ligand that has dissociated from receptor during the electrophoretic run), receptor binding activity was measured directly after electrophoresis.

TABLE II: Physical Data of Estrogen Receptors from Lamb and Rat Uterus.

	Lamb			Rat
	Whole cytosol	Trypsinized		
		Peak I[a]	Peak II	Trypsinized
$s_{20,w}$[b]	8.6		3.04 ± 0.04 (3)	4.01 ± 0.09 (3)
R_S (nm) G-200[c]	d		2.76 ± 0.23 (4)	2.94 ± 0.25 (2)
M_1 (s, R_S)[e]			35300	49600
f/f_0 (M_1, R_S)[f]			1.15	1.11
a/b[g]			3.7	3.2
$\bar{R}$ (nm) PAGE[h]	4.78 ± 0.23	2.39 ± 0.19	2.38 ± 0.19	2.30 ± 0.19
M_2[i] PAGE	377000 ± 55000[j]	46800 ± 11000	46400 ± 11000	41800 ± 10000
f/f_0 (M_2, R_S)[f]		1.07	1.06	1.18
a/b[g]		2.4	2.4	4
pI[k]		5.5	5.8	6.4

[a] Peak I is the most mobile peak in Figures 1B, D, and E, and Figure 2A. [b] Sedimentation coefficients ($s_{20,w}$) for receptors were determined relative to two protein standards by density gradient sedimentation (Figure 3). Error estimates represent the standard deviation from three determinations (noted in parentheses). [c] Stokes radii (R_S) for receptors were calculated from the unweighted linear regression of log R_S on K_{av} for four protein standards (Figure 4, insert), using the mean K_{av} from 2–4 determinations of each standard and unknown (noted in brackets). The error estimates represent 95% confidence limits for R_S and are derived from the uncertainty in the determination of K_{av} and the uncertainty in the standard curve (Rodbard and Chrambach, 1971). [d] Excluded from Sephadex G-200. [e] Calculated from eq 2 (Siegel and Monty, 1966). [f] Calculated from R_S and M_1 or M_2 as indicated in parentheses using eq 3, and assuming $\bar{v}$ = 0.73 cm^3 g^{-1} and a degree of hydration of 0.2 g per g of protein (Tanford, 1961). [g] Axial ratios for a prolate ellipsoid model were determined using the contour plots of Oncley (1941). [h] Molecular radii ($\bar{R}$) for receptors were calculated from the unweighted linear regression of $\bar{R}$ on $(K_R)^{1/2}$ for nine protein standards (Figure 5B). The error estimates represent 95% confidence limits for $\bar{R}$ and are derived from the uncertainty in a single determination of $(K_R)^{1/2}$ and the uncertainty in the standard curve (Rodbard and Chrambach, 1971). [i] Calculated from $\bar{R}$ according to eq 4, assuming $\bar{v}$ = 0.73 cm^3 g^{-1}. Confidence limits (95%) for M_2 are propagated from the error estimates of $\bar{R}$. [j] Lamb albumin, visible as a band stained with bromophenol blue, has $\bar{R}$ = 2.61 ± 0.18 nm, M_2 = 61 300 ± 13 000. [k] The third (minor) receptor component of the lamb receptor has a pI of 6.2.

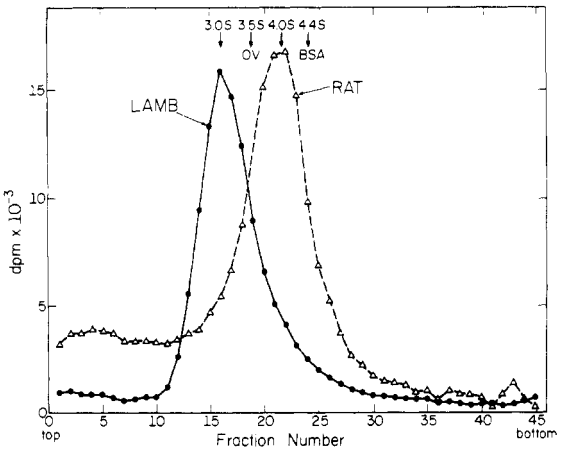

FIGURE 3: Estimation of the sedimentation coefficient of estrogen receptor species by sucrose gradient centrifugation. Receptor preparations from lamb (●—●) and rat (▲- -▲) uterus were purified by ammonium sulfate precipitation and disaggregated by trypsinization, and were then labeled by incubation with 30 nM of [^{3}H]estradiol for 1 h at 4 °C. Free steroid was removed by treatment with charcoal-dextran, and 0.2-mL samples were layered onto 3.4-mL 5–20% sucrose gradients prepared in TEA buffer and centrifuged at 270 000g for 16 h. The carbon-14-labeled marker proteins were ovalbumin (OV) and bovine serum albumin (BSA). The exact $s_{20,w}$ values assumed for the protein standards are given in the supplement and those calculated for the receptors in Table II.

Receptor preparations (either unfilled, or after incubation with 30 nM [^{3}H]estradiol for 1 h at 0 °C and charcoal treatment) were electrophoresed. The gels were sliced (without freezing), and the individual gel slices (2–4 mm) were incubated with a small volume of 12 nM [^{3}H]estradiol in TEA buffer for 2.5 h at 0 °C and then 1 h at 25 °C, to extract the receptor. The gel slice was removed, and the solution remaining was divided into two parts that were incubated further for 18 h at 25 °C, either in the same solution or after the addition of a 200-fold excess of unlabeled estradiol. Estrogen binding activity was determined by charcoal dextran adsorption; 60–70% of the activity is recovered by this exchange procedure (Katzenellenbogen et al., 1974). Whether filled or unfilled receptor was used, high affinity estrogen binding activity was found coincident with all the peaks in Figure 2 except for the rat preparation in the pH 10.2 system. In this case, the least mobile peak corresponds to free estradiol, and the most mobile one is receptor. The middle peak probably represents ligand that has dissociated during the run. Similar results were seen by Secco-Millet et al. (1977) with the trypsinized calf uterine receptor on the high pH system.

The estrogen-binding serum protein, α-fetoprotein, may potentially interfere with measurements of uterine receptor in immature rats (cf. Uriel et al., 1976, and Radanyi et al., 1977). We have been careful to determine that none of the binding we see by gel electrophoresis is due to serum proteins. The levels of high affinity estrogen binding activity in serum of 21-day old rats or immature lambs are much lower than the binding we observe in uterine cytosol preparations. Furthermore, the estrogen-binding activity in 9-day old female rat serum (presumed to be α-fetoprotein; Nunez et al., 1974) is not precipitated by 30–40% ammonium sulfate; it is unaffected by trypsinization, and, in the pH 7.8 electrophoretic system, the serum estrogen binder migrates at a R_f of 0.74, well ahead of all the uterine estrogen binding activities.

Physical Properties of the Trypsinized Estrogen Receptors from Lamb and Rat Uterus. We have utilized sucrose gradient sedimentation, Sephadex G-200 column chromatography, electrophoretic mobility at different acrylamide concentrations, and isoelectric focusing to determine the size, the molecular weight, and the isoelectric point of the trypsin-disaggregated forms of the estrogen receptor from rat and lamb uterus (Table II).

Figure 3 indicates that the trypsinized lamb and rat receptors have considerably different sedimentation coefficients, the lamb being ca. 3.0 S, and the rat ca. 4.0 S. As the widths of the peaks for both receptors are comparable to the peak widths for the marker proteins, there is no evidence of heterogeneity by this technique. Furthermore, these trypsinized preparations are free from large aggregates, such as normally are found after freezing, storage, or ammonium sulfate precipitation of receptor.

A Sephadex G-200 column was calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen, and myoglobin. On this column, the trypsinized rat receptor elutes just ahead of ovalbumin, while the lamb receptor comes just behind (Figure 4). According to Determann and Michel (1966), there is a linear relationship between the distribution coefficient of a protein on a Sephadex column and the logarithm of its Stokes radius (R_S). Stokes radii of 2.76 nm and 2.94 nm for the trypsinized forms of the lamb and rat receptors, respectively,

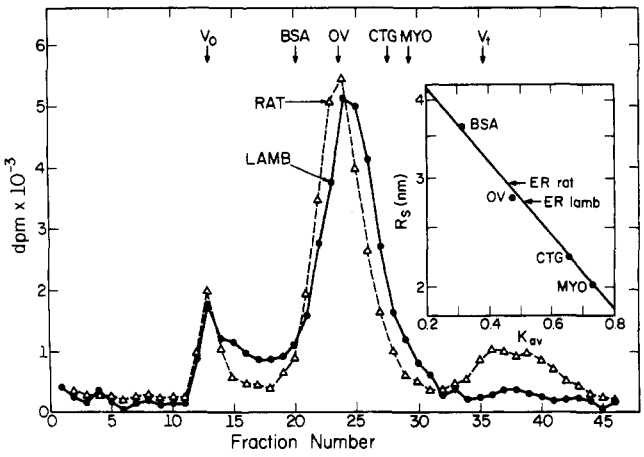

FIGURE 4: Estimation of Stokes radii (R_S) of estrogen receptor species by molecular exclusion chromatography on Sephadex G-200. Estrogen receptor preparations from lamb (●—●) and rat (Δ- -Δ) uterus, purified by ammonium sulfate precipitation and disaggregated by trypsinization, were labeled by incubation with 30 nM [^{3}H]estradiol for 1 h at 4 °C and freed of excess unbound steroid by treatment with charcoal-dextran. Samples (1.0 mL) were applied to a 1.5 × 50 cm column of Sephadex G-200, and 2.8-mL fractions were collected at an upward flow of 5–6 mL/h. Arrows indicate the elution position of the following standards: Blue Dextran 2000 (V_0 determined by A_{640}), dansyl-bovine serum albumin (BSA; determined by fluorescence λ_{ex} = 350 nm, λ_{em} = 500 nm), [^{14}C]ovalbumin (OV), α-chymotrypsinogen A (CTG, determined by A_{280}), myoglobin (MYO, determined by A_{420}), and L-[^{14}C]leucine (V_t). The inset shows the standard curve relating the distribution coefficient (K_{av}) of four protein standards with the logarithm of their Stokes radii (R_S) (see Table III in supplementary material for the R_S values of the protein standards). The horizontal arrows indicate the elution positions of the receptor species; the precise values are given in Table II. This line was determined by an unweighted linear regression of K_{av} upon log R_S.

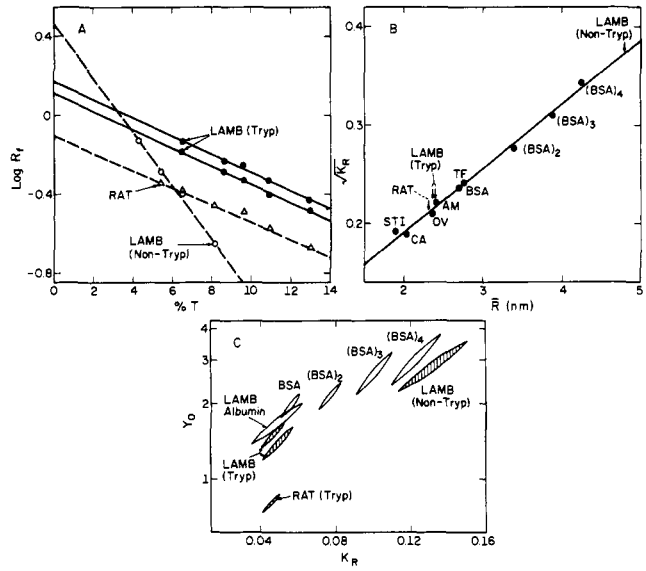

FIGURE 5: Estimation of molecular radii ($\bar{R}$) of the receptor species by electrophoretic mobility. (A) Ferguson plots of the two major trypsinized forms of the lamb receptor (●—●), trypsinized rat receptor (Δ- -Δ), and whole lamb receptor (O- -O). These lines were determined from a weighted linear regression of log R_f on % T, as reported by Rodbard and Chrambach (1971). The lines for the receptor species (shown) are based upon 8–12 observations; multiple data points at a single % T have been averaged on this figure but were used individually in the regression analyses. Lines for the standard proteins (not shown) are based on 4–12 observations. Minus the slope of each line is designated as the retardation coefficient K_R. (B) Standard curve relating $(K_R)^{1/2}$ of several standard proteins to their molecular radii (see Table III in the supplementary material for the molecular radii of the protein standards). The vertical arrows indicate the position of the receptor species on the standard curve. This line was determined from an unweighted linear regression of $(K_R)^{1/2}$ on $\bar{R}$. (C) Comparison of receptor species and selected protein standards. The ellipses represent the joint 95% confidence limits of the values of log Y_0 and K_P (Rodbard and Chrambach, 1974). Protein standards were included only in those cases where data were based on more than 8 observations.

can be determined from such a plot (Figure 4, insert; Table II, R_S G-200).

By combining sedimentation data and Stokes radii, one can calculate the anhydrous molecular weight for the trypsinized forms of the rat and lamb uterine receptors, according to the relationship of Siegel and Monty (1966)

$$M_1 = \frac{6\pi\eta N}{1 - \bar{v}\rho} sR_S = 4205sR_S \qquad (1)$$

where η (solvent viscosity) is 0.01 P, ρ (density) is 1.00 g cm^{-3}, $\bar{v}$ (partial specific volume) is assumed to be 0.73 cm^3 g^{-1}, N is Avogadro's number, R_S is in nanometers, and s, in Svedberg units (10^{-13} s^{-1}), refers to 20 °C and water ($s_{20,w}$). The values obtained in this manner are shown in Table II (M_1).

The asymmetry of the receptor forms can be evaluated in terms of $(f/f_0)_{shape}$, the frictional ratio due to shape. This frictional ratio is defined as

$$(f/f_0)_{shape} = R_S\left[\frac{4\pi N}{3M(\bar{v} + \delta/\rho)}\right]^{1/3} = 13.95\frac{R_S}{M^{1/3}}$$

$$\text{or } \frac{R_S}{\bar{R}}\left[\frac{\bar{v}}{\bar{v} + \delta/\rho}\right]^{1/3} = 0.9225\frac{R_S}{\bar{R}} \qquad (2)$$

where $\bar{v}$ is assumed to be 0.73 cm^3 g^{-1}, δ (the degree of solvation) is considered to be 0.2 g per g of protein, and R_S and $\bar{R}$ are in nanometers. The frictional ratios based on M_1 and the corresponding axial ratios for a prolate ellipsoid model, estimated from the contour plots of Oncley (1941), are given in Table II. All the species have relatively low asymmetry.

The molecular radius of a protein can be estimated by determining to what extent its electrophoretic mobility is retarded by increasing acrylamide gel concentrations (Ferguson, 1964; Rodbard and Chrambach, 1971). Plots of the log R_f of individual proteins vs. % T, at constant % C (Ferguson plot) appear as lines. Minus the slope of each line (called the retardation coefficient, K_R) is related to the molecular radius of the protein, and the ordinate intercept (log Y_0) depends on its charge-to-surface-area ratio and is a measure of its free electrophoretic mobility. Ferguson plots of the trypsinized rat receptor, the untrypsinized lamb receptor (whole cytosol), and the two major components in the trypsinized lamb receptor on 4–13% acrylamide gels are shown in Figure 5A. The slope and intercept of the lines shown in this figure have been calculated using the weighted linear regression program of Rodbard and Chrambach (1971).

A linear plot relating the square root of the retardation coefficient $[(K_R)^{1/2}]$ of standard proteins to their molecular radii ($\bar{R}$), shown in Figure 5B, can be used as a calibration curve to give an estimate of the molecular radii of the different receptor forms: these radii and molecular weights (M_2), calculated according to eq 3,

$$M_2 = \frac{4\pi N\bar{R}^3}{3\bar{v}} = 3456\bar{R}^3 \qquad (3)$$

where N and $\bar{v}$ are defined as for eq 1 and $\bar{R}$ is in nanometers, are given in Table II. The two trypsinized lamb components are essentially identical in mass; the rat receptor appears slightly lighter, and the whole untrypsinized lamb receptor is considerably larger. Compared with the frictional ratios and axial ratios calculated using M_1, those calculated using M_2 or $\bar{R}$ (see eq 3) are smaller for the trypsinized lamb receptor, but

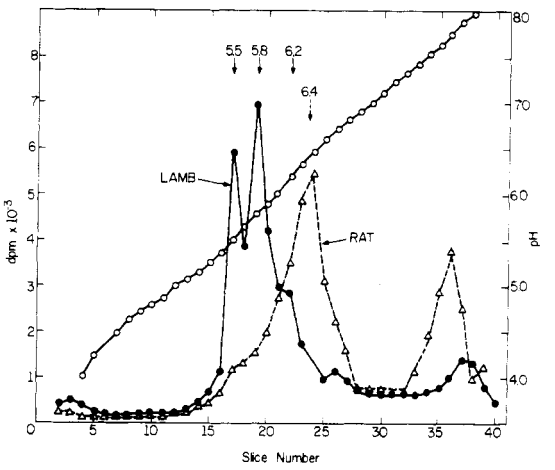

FIGURE 6: Gel isoelectric focusing patterns of partially purified lamb (●—●) and rat (△- -△) estrogen receptors. Receptor preparations, purified by ammonium sulfate precipitation and disaggregated by trypsinization, were labeled by incubation with 30 nM [^{3}H]estradiol for 1 h at 4 °C and freed of unbound steroid by charcoal adsorption. Samples (50 μL) were layered onto 6 × 85 mm polyacrylamide gels (5% T–15% C) containing 2% of the pH 5–8 range ampholines. After focusing for 10 h, gels were processed as described in Methods. The radioactivity and pH measurements were done on the same gel. Essentially identical pH profiles were obtained from the two gels used to construct this figure.

slightly larger for the rat receptor (Table II). Again, the asymmetry of the trypsinized receptors appears to be relatively low.

The relatively large estimated errors in these molecular weight determinations for the receptors reflect stringent statistical analysis of the data: The error bounds are 95% confidence limits that represent error both in the measurement of K_R (the slope of the Ferguson plots, Figure 5A) and uncertainty in the standard curve (Figure 5B) (Rodbard and Chrambach, 1971).

When comparing similarities or differences between various proteins on the basis of their electrophoretic behavior, it is more appropriate to compare their Y_0 and K_R values determined directly from the Ferguson plots, rather than their estimated molecular weights, which contain additional uncertainty contributed by the calibration curve based on the protein standards. As Rodbard and Chrambach (1974) have pointed out, errors in Y_0 and K_R are highly correlated. For this reason, comparisons are best made on a plot of log Y_0 against K_R, where the Y_0 and K_R values for individual proteins are shown together with their joint 95% confidence limits (Figure 5C). Individual proteins appear as ellipses: The size of each ellipse represents the uncertainty in the determination of the slope and intercept from the Ferguson plot and, generally, decreases with replicate analyses; vertical displacements between ellipses represent differences in net charge per surface area, and horizontal displacements represent differences in size. Lack of overlap between two ellipses indicates nonidentity of the two proteins with $p < 0.0025$.

The four receptor species, together with lamb albumin and the bovine serum albumin oligomers, are shown in Figure 5C. The trypsinized lamb and rat receptor species have clearly distinct charge-to-surface-area ratios, but very similar sizes. The lamb receptors are also distinct from lamb albumin, which itself is quite similar to bovine serum albumin (partial overlap of ellipses).

It is clear from analysis of Figures 5A and 5C that the electrophoretic separation seen between the trypsinized forms of the rat and lamb receptor and the two components of the trypsinized lamb receptor is due primarily to differences in charge-to-surface-area ratios. This suggests that these components should be cleanly separable by isoelectric focusing.

Isoelectric focusing profiles of the trypsinized rat and lamb receptor in highly cross-linked (nonretarding) polyacrylamide gels (5% T, 15% C) are shown in Figure 6. The lamb receptor focuses as two major and one minor band with apparent p*I* values of 5.5, 5.8, and 6.2. The rat receptor appears more disperse, with most of the activity focusing with an apparent p*I* of 6.4; this behavior is also consistent with its electrophoretic mobility.

Extraction of the gel slices from isoelectric focusing, as described previously for electrophoresis, confirmed that the distribution of radioactivity corresponded to the profile for estrogen binding activity.

Discussion

In this report we have described that controlled trypsinization of estrogen receptor preparations from rat and lamb uteri produces distinct forms of receptor that are relatively low in molecular weight and are amenable to detailed physical-chemical characterization, including electrophoresis and isoelectric focusing on polyacrylamide gels.

Electrophoresis of androgen receptors has been performed in a routine fashion on acrylamide (Ritzen et al., 1973) or acrylamide–agar composite gels (Hansson et al., 1974), and detailed electrophoretic studies by Sherman have characterized several forms of the chick oviduct progesterone receptor (Sherman et al., 1976; Miller et al., 1975). Attempts to electrophorese the estrogen receptor have been less successful. Unfractionated cytosol preparations generally yield low resolution patterns on acrylamide (Smethurst et al., 1974) or agar gels (Wagner, 1972; Davies et al., 1975; Tuelings et al., 1975). Electrophoretic profiles of calf uterine estrogen receptor, after several purification steps, have been published by DeSombre and Gorell (1975). More recent efforts by Secco-Millet et al. (Secco-Millet, 1974; Secco-Millet et al., 1977) and by Vallet-Strouve et al. (1976) have yielded electrophoretic results with purified-trypsinized calf receptor similar to ours with lamb and rat receptor.

The sedimentation velocity we have observed for the trypsinized rat receptor is substantially greater than that for the lamb receptor (Table II), and the rat receptor appears somewhat larger than the lamb by gel exclusion. Thus, when molecular weights are estimated by combining size and sedimentation data (Table II, M_1), the rat receptor appears heavier than the lamb. However, the rat receptor appears somewhat lighter than the lamb, when molecular weights are estimated by electrophoretic mobility (Table II, M_2). It is neither surprising nor uncommon to find discrepancies of this magnitude between molecular weights estimated by alternative techniques, as the techniques may not respond in the same manner to differences between the asymmetries of the unknown proteins and the protein standards (Miller et al., 1975; Rodbard, 1976a).

Studies by Soulignac (1974) and Vallet-Strouve et al. (1974, 1976) have indicated that trypsinization of the calf uterine estrogen receptor has little effect on its steroid binding properties. Although we have not done an exhaustive study, the steroid binding properties of the rat and lamb receptor also seem unaltered by trypsinization. However, after trypsinization, heat-activated receptor loses its ability to bind to nuclear constituents (Vallet-Strouve et al., 1974; Sala-Trepat and Vallet-Strouve, 1974); we have found this to be true for the lamb uterine receptor as well (K. E. Carlson, and J. A. Katzenellenbogen, unpublished data).

It is not clear at this point whether the three components of the trypsinized lamb receptor derive from physiologically distinct estrogen binders or whether they are merely different cleavage products generated from a common precursor. It is of note, however, that proteolysis of steroid receptors resulting in small molecular weight forms may, in fact, be an important aspect of steroid hormone action in vivo. For example, the calcium-activated receptor transforming factor from calf uterus described by Puca et al. (1972, 1977) appears to be a protease that converts the estrogen receptor to a 60 000 molecular weight form with a low axial ratio. Small molecular weight forms of the estrogen receptor from human breast tumor tissue (3 S, Miller and Sherman, 1977), human myometrium (3 S, Notides et al., 1976), and adult rat uterus (4 S, Steggles and King, 1970) have been described; these forms may also represent proteolytic fragments of larger forms of the receptors (Steggles and King, 1970; Notides et al., 1973). Sherman and co-workers have described similar low molecular weight forms of other steroid receptors ("mero-receptors"): progesterone receptor from chick oviduct (after calcium treatment; Sherman et al., 1974, 1976) and glucocorticoid and mineralocorticoid receptors from rat kidney (Sherman and Edelman, 1977). Thus, it is possible that the trypsinized forms of the lamb and rat receptor that we have studied may be related to forms of the estrogen receptor that are significant physiologically.

From the results of these studies, it is evident that mild trypsinization of the estrogen receptors from lamb and rat uterus produces lower molecular weight forms of the receptor that can be readily characterized by a variety of biochemical techniques. While the limited proteolysis does alter the biological activity (nuclear affinity) of the receptor, it has little effect on ligand binding. Thus, the availability of these well-characterized forms of the estrogen receptor should be of major assistance to studies aimed at elucidating the nature of the interaction of estrogens with the receptor binding site and the relationship between various forms of the estrogen receptor proteins (cf. Katzenellenbogen et al., 1977c).

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Supplementary Material Available

Table I giving partial purification and recovery data of uterine estrogen receptor and Table III listing the physical properties (with literature references) of the proteins used as standards to estimate sedimentation velocities and Stokes radii (3 pages). Ordering information is given on any current masthead page.

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