Characterization of a Second Estrogen Receptor Species in Chick Oviduct†

Reid W. McNaught‡ and Roy G. Smith*,‡,§

Roy and Lillie Cullen Department of Urologic Research, Department of Urology, and Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

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ABSTRACT: Scatchard analysis of estradiol binding to chick oviduct cytosol is consistent with the existence of two high-affinity estrogen receptors which bind this ligand with equilibrium dissociation constants (K_d) of 0.06 and 0.8 nM. While the higher affinity receptor has been characterized and purified [Smith, R. G., & Schwartz, R. J. (1979) *Biochem. J. 184*, 331–343], the properties of the lower affinity receptor have not previously been defined; thus, its existence has been questioned. We now report the separation of the two receptors by low-capacity affinity chromatography, by sucrose density gradient centrifugation, and the partial separation by diethylaminoethyl ion-exchange chromatography. The former utilizes differences in estrogen binding kinetics associated with each receptor; the rate of dissociation (k_{-1}) of [^{3}H]estradiol from each receptor is identical ($k_{-1} = 7.7 \times 10^{-5}$ s^{-1}) while rates of association (k_1) reflect the 10-fold differences in K_d. The lower affinity receptor has a $k_1 = 1.63 \times 10^5$ M^{-1} s^{-1}, and the higher affinity receptor has a $k_1 = 1.33 \times 10^6$ M^{-1} s^{-1}. The lower affinity receptor sediments at 3.5 S on sucrose density gradients compared to 4.2 S for the high-affinity form; it has high specificity for estrogen and is tissue specific, and its nuclear occupancy is associated with increases in ovalbumin gene transcription; thus, this macromolecule meets the criteria of an estrogen receptor. Augmentation of this receptor is possible by treatment of the cytosol with ATP/Mg^{2+}, illustrating the existence of a non-steroid binding species. Moreover, we have demonstrated that ATP/Mg^{2+} induces a quantitative interconversion of the high-affinity to the lower affinity receptor. These data suggest that the estrogen receptors are different molecules containing a common protein in which the steroid binding moiety exists in two different conformations and that the intracellular ratio of the conformers can be regulated by ATP.

The mechanism of estrogen receptor action in the chick and hen oviduct has become the basis for numerous studies focused on the molecular biology of steroid action. The hormonally responsive avian oviduct is an excellent model to investigate the estrogenic modulation of gene expression (Harris et al., 1975; Mulvihill & Palmiter, 1977; Woo et al., 1978). Identified as mediators in such processes are two estrogen-specific receptor species, conveniently labeled as X and Y (Smith et al., 1979) to avoid confusion with type I and type II estrogen binding sites described in the rat uterus (Clark et al., 1978; Taylor & Smith, 1982). Possessing a 10-fold difference in their equilibrium dissociation constants of 0.1 and 1.0 nM, respectively, both binding site proteins appear to have specific and discrete functions in eliciting an estrogenic response in oviduct epithelial tubular gland cells (Smith & Taylor, 1981).

Although the physiological significance of receptor polymorphism is not fully understood, present evidence suggests that the discrete chick oviduct binding species mediate specific cellular responses. The high-affinity X receptor directly modulates RNA polymerase II activity (Smith & Schwartz, 1979; Taylor & Smith, 1979, 1982, 1985) whereas the lower affinity Y receptor appears to be linked to induction of estrogen-dependent gene transcription, such as ovalbumin synthesis (Taylor et al., 1980; Smith & Taylor, 1981). For example, selective down regulation in vivo of the Y receptor, by administration of the drug danazol, results in inhibition of ovalbumin mRNA synthesis, although RNA polymerase II activity is unaffected. Furthermore, the estrogen regulation of faithful transcription of the ovalbumin gene is associated with the nuclear accumulation of both receptor species (Smith & Taylor, 1981).

The existence of a second lower affinity chick oviduct estrogen binding site has already been recognized and indeed partially defined (Smith et al., 1979). This report is the first to describe specific characteristics that illustrate both the similarities and the differences from the previously documented high-affinity receptor (Smith & Schwartz, 1979). Additionally, the relationship of the two receptors is considered in a framework of multiple binding site heterogeneity and how their individual form and function may be related.

Experimental Procedures

Chemicals. All reagents were of analytical grade. Tris(hydroxymethyl)aminomethane (Tris)[1] base was purchased from Boehringer Mannheim (Indianapolis, IN) while sucrose came from Schwarz/Mann (Orangeburg, NY). Ammonium sulfate, dioxane, EDAC, EDTA, glycerol, hydrochloric acid, monothioglycerol, Norit-A charcoal, potassium chloride, and sodium chloride were obtained from Fisher Scientific (Pittsburgh, PA). DES, sodium molybdate, and triamcinolone acetonide came from Sigma (St. Louis, MO). Dextran T70, DEAE-Sephadex A-25, CNBr-activated Sepharose were supplied by Pharmacia (Piscataway, NJ). Diaminodipropylamine was supplied by Eastman Kodak (Rochester, NY). Bio-Gel A-0.5M and DEAE-Bio-Gel A were from Bio-Rad (Richmond, CA). 17β-Estradiol, estriol, 17β-estradiol hemisuccinate, cortisol, progesterone, testosterone, and 5α-dihydrotestosterone were purchased from Steraloids (Wilton,

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* Address correspondence to this author at the Department of Urology, Baylor College of Medicine.

‡ Department of Urology.

§ Department of Cell Biology.

[1] Abbreviations: DEAE, diethylaminoethyl; DES, diethylstilbestrol; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; DCC, dextran-coated charcoal.

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NH), while 17β-[2,4,6,7-^{3}H]estradiol was supplied by Amersham (Arlington Heights, IL).

Hormone Treatment. White Leghorn chicks (Texas Animal Specialties, Humble, TX) aged 10–12 days were treated with daily subcutaneous injections of 2 mg of DES in sesame seed oil over a 14-day period. The chicks were then withdrawn from the hormone for 2–10 days prior to sacrifice, with the actual time of withdrawal depending on the specific needs of the experiment being undertaken. Short-term withdrawn animals (typically ≤2 days) contained only the Y-soluble receptor, while longer periods of clearance from the hormone favored primarily the X receptor.

Preparation of Cytosol. All procedures were performed at 4 °C unless otherwise stated. Chick oviducts, rinsed in ice-cold saline, were weighed, minced, and homogenized with 5 volumes (w/v) of TESH buffer (10 mM Tris-HCl, pH 7.4 at 24 °C, 1.5 mM EDTA, and 12 mM monothioglycerol) with 5 × 5 s pulses by means of a Polytron tissue homogenizer (Brinkmann, Westbury, NY). The homogenate was centrifuged at 5000*g* for 20 min, and the resulting fat plug on the surface was removed by filtering through two layers of organza cloth. The supernatant was then centrifuged at 105000*g* for 90 min with an intermediate stop to aspirate off the floating fatty material. A final aspiration at the end of the second ultracentrifugation spin resulted in a clear supernatant suitable for quantitation of the estrogen receptor or further purification as circumstances dictated.

Ammonium Sulfate Fractionation. A saturated solution of ammonium sulfate prepared in TESH buffer (pH 7.4) was slowly added to cytosol over a 25–30-min period with gentle stirring until a final concentration of 30% ammonium sulfate was attained. the mixture was stirred for an additional 30 min, and the resultant suspension was centrifuged for 20 min at 5000*g* to collect the sediment. Following this step, the pellet was carefully rinsed with TESH buffer and redissolved in 1.66–2.0 volumes of TESH buffer (viz. 1.66–2.0 mL/g of oviduct tissue). A sustained gentle swirling action ensured that the pellet redissolved within a 10–15-min time period. The resulting solution, containing the partially purified estrogen receptor, was centrifuged at 15000*g* for 30 min to sediment remaining insoluble material.

Preparation of Affinity Resin. An adaptation of the protocol of Sica et al. (1973) as employed by Smith and Schwartz (1979) was used. Details are as follows: 15 g of 1 mM HCl washed and activated CNBr–Sepharose suspended in 50 mL of 0.2 M $NaHCO_3$, pH 9.0, was coupled with 2.0 mM, 3,3′-diaminodipropylamine by gentle end over end rotational mixing at 4 °C overnight. The following day the resin was washed with 1 L of 1 M NaCl and 1 L of distilled water. Then remaining CNBr-activated sites on the gel beads were inactivated by additional gentle mixing of the washed gel cake with 50 mL of 5 M ethanolamine dissolved in 0.2 M $NaHCO_3$ at pH 9.0 for an additional 2 h at 25 °C. Following this step, the gel was extensively washed with 3 × 1 L aliquots of 1 M NaOH and 2 × 1 L of distilled water. Ten milligrams of 17β-estradiol hemisuccinate was then coupled to the diaminodipropylamine side-chain spacer arms by reacting 15 mg of EDAC dissolved in 15 mL of distilled water with the Sepharose resin suspended in 35 mL of dioxane. The mixture was gently shaken at 25 °C for 5 h at which point an additional 15 mg of EDAC similarly dissolved in water (1 mL) was added. The mixing was continued for an additional 9 h. The freshly prepared affinity resin was thoroughly washed with 1 L of dioxane, 1 L of distilled water, and finally with 8 L of 80% methanol. The resin, stored at 4 °C in 80% methanol, was stable for 2–3 months and contained 200 pmol of estradiol hemisuccinate/mL of resin.

Affinity Resin Purification. Sufficient estradiol hemisuccinate–Sepharose 4B affinity resin was washed at 4 °C in 50 mL of 80% methanol and 100 mL of distilled water. The gel was then added to a 5-fold excess of 30% ammonium sulfate fractionated cytosol and gently mixed overnight at 4 °C by end over end rotation. Affinity resin beads were separated from the suspension by centrifugation at 2000*g*. The clear supernatant, designated as the purified binding protein source, contained only the low-affinity Y estrogen receptor species while the high-affinity X receptor remained selectively bound to the sedimented affinity resin pellet. If required, the immobilized X estrogen receptor could be eluted as described previously (Smith & Schwartz, 1979).

Hormone Binding Assay. Saturation analysis of both the affinity resin purified and crude cytosol estrogen receptor was performed in the presence of [^{3}H]estradiol at final concentrations ranging from 50 pM to 10 nM. Nonspecific binding was determined in the presence of a 100-fold excess of DES. Incubations were carried out mainly at 4 °C for 18 h, although in specific applications exchange conditions of 3 h at 30 °C were used. Conventional charcoal adsorption assays were used (Smith et al., 1979) to measure total and nonspecific bound [^{3}H]estradiol. Specific binding was quantitated by Scatchard analysis (Scatchard, 1949), with the Rosenthal correction protocol (Rosenthal, 1967) being used to resolve complex two-component Scatchard curves.

Ion-Exchange Chromatography. Bio-Rad DEAE-Bio-Gel A and Pharmacia DEAE A-25 resins were used with similar protocols. Both materials were extensively washed with eluting buffer, prior to use in minicolumns of dimension 1.2 × 5.5 cm containing 5 bed volumes of gel per milliliter of receptor source. Soluble proteins were eluted from the column at 4 °C either with linear gradients of 0–400 mM KCl in TESH buffer or on a semipreparative scale with stepwise KCl elutions at varying salt concentrations (typically 0, 50, 100, 150, 200, 250, and 300 mM). Prelabeled receptor specimens assayed on a continuous gradient were run in duplicate with a 100-fold excess of cold DES to quantitate for nonspecific binding and were counted as individual fractions. Stepwise elutions using nonlabeled cytosol proteins were assayed by Scatchard analysis to determine the affinity of any specific estradiol binding.

Molecular Sieve Chromatography. All experiments, carried out at 4 °C, were performed with a Bio-Gel agarose A-0.5M gel packed in a 1.6 × 70 cm glass column fitted with flow adapters. The column was thoroughly washed with TESH buffer containing 0.4 M KCl and 10 mM sodium molybdate in preparation for use. Receptor samples prelabeled with [^{3}H]estradiol were introduced onto the column and eluted with additional buffer, ensuring that a flow rate of 12–15 mL/h was maintained. One-milliliter fractions were collected and assayed for specific [^{3}H]estradiol incorporation. Standardization of the column was achieved with the use of the following ^{14}C-tagged protein standards: β-amylase, bovine serum albumin, ovalbumin, and myoglobin. The void volume was delineated with blue dextran, while the total volume of the column was determined with vitamin B_{12}.

Sucrose Density Gradient Centrifugations. Linear gradients of 5–20% sucrose (w/v) in TESH buffer containing 400 mM KCl were prepared in a Buchler gradient former (Buchler Instruments, Fort Lee, NJ) at room temperature and then allowed to equilibrate at 4 °C for 6 h. Three hundred microliter aliquots of cytosol or 30% ammonium sulfate fractionated cytosol prelabeled with 5 or 10 nM [^{3}H]estradiol at

3 °C for 3 h and treated with an equivalent volume pellet of DCC to absorb unbound hormone were carefully layered onto each prepared gradient immediately before commencement of the centrifugation process.

The gradients were developed for 9 h at 45 000 rpm in a Beckman L5-50B ultracentrifuge fitted with an SW50.1 rotor (Beckman Instruments, Palo Alto, CA). At the termination of the spin period, the gradients were fractionated from below with a Buchler gradient tapper, and 36 fractions (approximately 130 μL each) were duly collected. Duplicated gradients containing receptor sample prelabeled in the presence of a 100-fold excess of unlabeled DES were additionally run for the determination of nonspecific binding. The sedimentation coefficient of the resolved [^{3}H]estradiol binding protein was estimated by comparing its relative gradient location with [^{14}C]ovalbumin (3.7 S) and β-[^{14}C]amylase (8.2 S).

Association/Dissociation Kinetic Studies. So that the respective experiments could be satisfactorily carried out, it was necessary to isolate only the Y estrogen receptor from X- and Y-containing preparations by affinity chromatography. The receptor sample was divided into two fractions—one was assayed to quantitate the concentration of receptor binding site at ligand saturation by Scatchard analysis, and the other was incubated with 1 nM [^{3}H]estradiol at 4 °C with aliquots being removed at various intervals. At each time point, aliquots in duplicate were transferred to tubes containing dextran charcoal to adsorb unbound [^{3}H]estradiol.

Assuming that a single molecule of [^{3}H]estradiol is bound to each receptor molecule, and over short reaction times the dissociation phenomenon is negligible, the binding of hormone to protein can be interpreted as a second-order reaction such that

$$d[ReS]/dt = k[S\text{–}ReS][Re\text{–}ReS]$$

where [S] is the concentration of [^{3}H]estradiol added to initiate the association reaction (1 nM), [Re] is the receptor concentration, and [ReS] is the concentration of labeled receptor–hormone complex at time t. The association rate constant (k) is defined in terms of reciprocal concentration times reciprocal time (viz., M^{-1} s^{-1}) and can be derived from this equation as the resultant slope of a plot of

$$\frac{1}{[S\text{–}Re]} \ln \left[\frac{[Re]}{[S]} \left(\frac{[S\text{–}ReS]}{[R\text{–}ReS]} \right) \right]$$

vs. time, where the intercept is ln ([S]/[Re])/[S–Re].

Chick oviduct estrogen receptor dissociation rate constants were determined by a standard protocol as described previously (Smith & Schwartz, 1979). Receptor was labeled with 10 nM [^{3}H]estradiol for 18 h at 4 °C. The experiment was then commenced with the addition of 1 μM unlabeled DES. Duplicate samples were immediately removed to ascertain the time zero point and processed by treatment with dextran-coated charcoal in the conventional manner to separate bound from free [^{3}H]estradiol. Additional samples were taken at 10-min intervals for the first hour, at 30 min thereafter for the following 3 h, and finally at 18 and 24 h. Nonspecific binding was ascertained with a parallel receptor sample prelabeled with [^{3}H]estradiol in the presence of a 100-fold excess of cold estrogen.

The addition of an excess of unlabeled DES effectively precluded the reassociation of estradiol with the receptor, such that the dissociation can be defined by first-order kinetic parameters. Thus, a plot of ln [ReS] against time, where [ReS] is the concentration of the hormone–receptor complex at time t, will yield a straight line from which the dissociation constant may be calculated from its slope.

Results

Identification of X and Y Species. The existence of two estrogen receptors was initially identified by analyzing biphasic Scatchard plots (Smith et al., 1979; Taylor & Smith, 1982). Interpretation of these curvilinear plots by the graphical method of Rosenthal (1967) gave equilibrium dissociation constants (K_d's) of 0.1 nM (±0.028 nM, n = 10) and 1.2 nM (±0.31 nM, n = 10). The high- and low-affinity estrogen receptors have been labeled X and Y, respectively.

Following these findings, a series of experiments were undertaken to conclusively demonstrate that the lower affinity binding component was not due to an artifactual aberration, be it exacerbated by endogenous ligand present in the crude cytosol, or the possibility of occupied activated high-affinity receptor sites displaying altered binding parameters, such that they could be mistaken for the lower affinity Y species. To exclude both options, the receptor source was initially labeled with 10 nM [^{3}H]estradiol at elevated temperatures (30 °C) to saturate and heat-activate the receptor molecules. The labeled receptors were then treated with charcoal at elevated temperatures to strip away both bound and free hormone before an equilibrium binding assay was performed. By these means, any [^{3}H]estradiol remaining bound to the receptor could be estimated by scintillation counting. Optimal conditions were a 10-min exposure to dextran-coated charcoal at 38 °C, resulting in a net removal of 92% of the introduced labeled estradiol with retention of >90% of the receptor binding activity. The remaining bound [^{3}H]estradiol was accounted for when the subsequent equilibrium binding assays were performed under exchange conditions. Exchange conditions were used so that this residual labeled ligand could enter the pool of [^{3}H]estradiol to allow a new equilibrium to be established. By use of various concentrations of [^{3}H]estradiol (50 pM–15 nM), a single saturation curve representing specific estrogen binding was constructed. Figure 1A demonstrates that binding is saturable, apparently consisting of a single class of binding sites with a concentration of 1.6 nM. The concentration of [^{3}H]estradiol required to saturate 50% of the receptors, i.e., the K_d obtained from this saturation curve, is 0.6 nM. However, when these data were replotted by using Scatchard analysis (Figure 1B), biphasic plots were obtained, consistent with those found under more conventional assay conditions. Following a Rosenthal correction, and allowing for all endogenous ligand by exchange with [^{3}H]estradiol, the K_d's for the X and Y receptors are 0.06 and 0.8 nM, respectively. If the temperature of the charcoal treatment for the removal of endogenous ligand is increased from 38 to 42 °C, and the period of treatment is extended to 20 min, it is possible to selectively destroy the Y estrogen receptor so that only the 0.06 nM K_d high-affinity component is apparent by Scatchard plot. These observations together illustrate that biphasic curves are not the product of residual endogenous hormone and nonequilibrium conditions or of negative cooperativity but are the presence of two discrete receptor species.

To further define the significance of our findings, Scatchard plot analyses were conducted on cytosol subjected to a variety of manipulations. Ammonium sulfate fractionation, which removes >80% of any remaining ovalbumin in cytosol, did not alter the ratio of X and Y receptors. Freshly prepared cytosol was filtered through phosphocellulose prior to analyses to remove receptor-specific protease from chick oviduct cytosol (Birnbaumer et al., 1983). The ratios of the receptors X and Y pre- and post-phosphocellulose chromatography, however, remained constant (data not shown), indicating that the

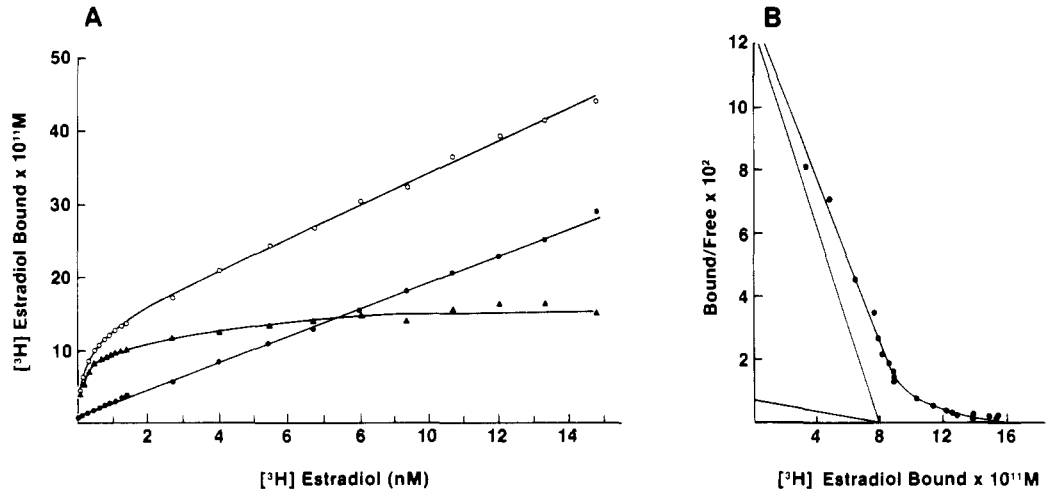

FIGURE 1: Exchange assay analysis of estradiol binding by chick oviduct estrogen receptors X and Y. Animals were stimulated with DES for 14 days and then withdrawn from hormonal stimulation for 4 days before being sacrificed. (A) Binding of [^{3}H]estradiol following elevated-temperature charcoal stripping of prelabeled cytosol. Samples were treated with 10 nM [^{3}H]estradiol for 3 h at 30 °C to ensure suitable conditions of exchange with any remaining endogenous hormone. Ligand–receptor complexes were then stripped of bound estradiol by exposure to dextran-coated charcoal at 38 °C for 10 min. Finally, varying concentrations of labeled estradiol (50 pM–15 nM) were incubated with the stripped receptor sample for 3 h at 30 °C to ascertain total binding (○). Parallel experiments with a 100-fold molar excess of cold DES were used to measure the level of nonspecific binding (●). Specific [^{3}H]estradiol binding is similarly shown (▲). (B) Scatchard analysis of the same specific bound data (●). The two components of the biphasic curve were determined by the method of Rosenthal.

component was not associated with receptor-specific proteolysis. Cytosol assayed in the presence or absence of 10 mM molybdate demonstrated no relative differences in the concentrations of X and Y (data not shown), indicating that the presence of Y was not associated with molybdate-inhibitable phosphatase activity. Filtration of freshly prepared cytosol or chromatography of cytosol activated by ammonium sulfate precipitation (30%) through DNA–cellulose indicated that no preferential affinity of either X or Y receptors for DNA was evident.

Isolation of the Lower Affinity Estrogen Receptor. While the preceding experiments have established the apparent presence of two estrogen receptors in chick oviduct, it might nevertheless be argued that at relatively high concentrations of ligand (>10 nM) nonspecific binding to nonreceptor proteins could lead to a subjective demonstration of two high-affinity binding components as sometimes identified with the Rosenthal (1967) protocol of resolving complex binding curves. Unequivocal proof of the existence of two discrete receptors would thus require their isolation and subsequent characterization. Such a separation has been achieved with the application of a low-capacity affinity resin.

Irrespective of the level of available receptor sites in a typical oviduct cytosol preparation, a useful 3–4-fold level of purification could be achieved with the addition of saturated ammonium sulfate to a final concentration of 30%. This procedure was necessary as a prerequisite for affinity resin separation of X and Y since it removes cytosol esterase activity (Kuhn et al., 1975) that, if present, would hydrolyze and free the estrogen-tagged linkage groups of the Sepharose gel.

The outcome following exposure of ammonium sulfate fractionated cytosol with the affinity resin (4 °C for 18 h) was dependent upon the specific capacity of the gels synthesized. A low-capacity 17β-estradiol 17-hemisuccinyl-diaminodipropylamino-Sepharose 4B resin, prepared such that it contained 100–200 pmol of estradiol/mL of packed beads, when incubated with solutions containing both X and Y receptors resulted in preferential adsorption of the X species, with 80–90% of the Y form remaining in solution. These results are illustrated by analysis of the receptor solution before and after exposure to the affinity resin (Figure 2). Following this

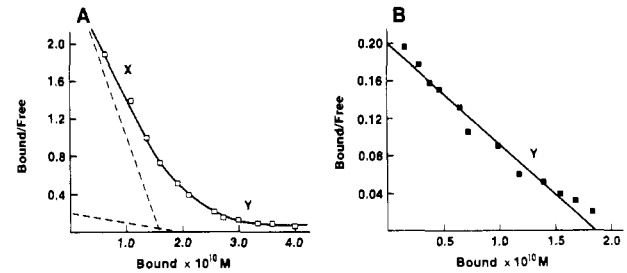

FIGURE 2: Separation of the chick oviduct cytosol X and Y receptors by affinity chromatography. Scatchard analysis of specific [^{3}H]estradiol binding was performed with increasing concentrations of labeled estradiol ranging from 50 pM to 10 nM for 18 h at 4 °C to quantitate for the X and Y and for the Y components, respectively. Saturation analysis in the presence of a 100-fold molar excess of unlabeled DES was used to determine nonspecific binding. Curvilinear plots were corrected by the Rosenthal method to determine K_d's and binding site concentrations. (A) 30% ammonium sulfate fractionated receptor preparation prior to incubation with 17β-estradiol 17-hemisuccinyl-diaminodipropylamino-Sepharose 4B resin. (B) Supernatant fraction postaffinity resin treatment.

treatment, Scatchard analysis showed just a single binding component with a K_d of 1 nM, demonstrating that only Y remained in the sample aliquot. This figure also demonstrates the accuracy of the Rosenthal method for quantitation of the receptors. Higher capacity resins, containing 800–1000 pmol of estradiol/mL of gel, had sufficient affinity to adsorb both the X and Y receptors, with just minor amounts ($\leq$15%) of the low-affinity species remaining free in solution. Experiments designed to show the selective adsorption and release of bound high-affinity receptor X from the affinity resin have previously been utilized to purify this binding protein to homogeneity (Smith & Schwartz, 1979; Taylor & Smith, 1979, 1982).

Estrogen Receptor Kinetic Studies. The association (k_1) and dissociation (k_{-1}) rates were determined for the high- and low-affinity species. As described under Experimental Procedures, the basis for calculating the rate constants of both receptors was based on the assumption that such kinetics could be appropriately defined by second-order and first-order kinetics, respectively. The rate of association can be approximated to a second-order reaction for short reaction times where dissociation and subsequent establishment of equilibrium are

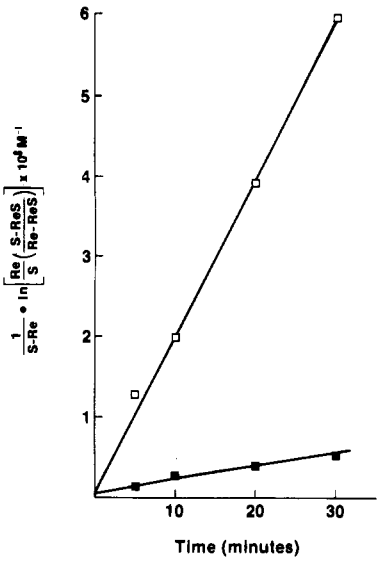

FIGURE 3: Association kinetics of chick oviduct 30% ammonium sulfate fractionated X and Y receptors. The on rates for X and Y and for isolated Y-containing receptor sources were measured in the presence of 1 nM [^{3}H]estradiol at varying times in duplicate. A 100-fold molar excess of unlabeled DES was used to quantitate for specific binding. Association rate constants of 1.33×10^6 and 1.63×10^5 M^{-1} s^{-1} for X and Y, respectively, at 4 °C were calculated from the means of the slopes from three experiments. (□) X + Y; (■) Y. Intercepts were calculated from the integration constant, ln ([S]/[Re])/[S–Re], and were <0.005.

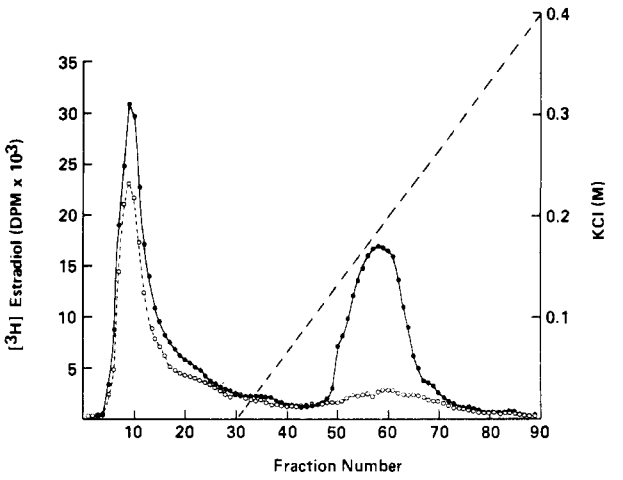

FIGURE 4: Analysis of chick oviduct X and Y estrogen receptors by ion-exchange chromatography. Cytosol labeled with 5 nM [^{3}H]estradiol for 18 h at 4 °C was charcoal treated to remove free ligand immediately before application to the column of DEAE-Bio-Gel A (1.2 × 5.5 cm) which had been preequilibrated in TESH buffer. Elution was achieved with a linear gradient of 0–400 mM KCl in a total volume of 90 mL. The flow rate was approximately 1 mL/min. KCl concentration in the effluent (---) was determined conductimetrically. Total binding of cytosol sample (●). Nonspecific binding prelabeled in the presence of a 100-fold molar excess of cold DES (○). Comparable void volume and salt-eluted peaks were obtained with Y only preparations.

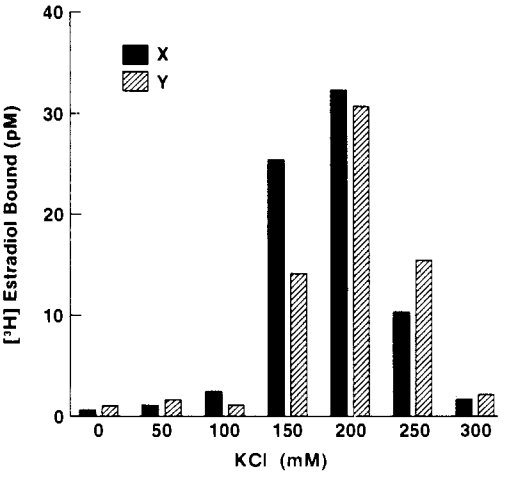

FIGURE 5: Stepwise elution of chick oviduct X and Y estrogen receptors by ion-exchange chromatography. Cytosol or 30% ammonium sulfate fractionated samples were applied to a column of DEAE-Bio-Gel A (6 × 1.6 cm) equilibrated in TESH buffer and then eluted with 0, 50, 100, 150, 200, 250, and 300 mM KCl discrete step gradient increments. The flow rate was approximately 2 mL/min, and increases in the effluent KCl concentration were measured conductimetrically. Fractions representing the eluted respective salt gradient zone were pooled and standard binding assays conducted at 4 °C for 18 h using increasing concentrations of labeled estradiol (80 pM–10 nM). Nonspecific binding was quantitated in the presence of a 100-fold molar excess of cold DES. Scatchard analysis was performed to determine the K_d's and binding site concentrations of each eluted sample. The results are summarized with the X receptor form being designated by the shaded bars and the Y species by the hatched bars.

avoided. Both sets of resultant data illustrate the nature of the variance in binding kinetics that characterize the 10-fold difference in the equilibrium dissociation constant (K_d) between the X and Y chick oviduct estrogen receptors (Figure 1B). Figure 3 illustrates the association of [^{3}H]estradiol to cytosol containing both receptor species. The rate constant of 1.33×10^6 M^{-1} s^{-1} is virtually identical with that previously measured for the purified X receptor ($k_1 = 1.4 \times 10^6$ M^{-1} s^{-1}; Smith & Schwartz, 1979). However, when the X receptor is removed by affinity chromatography, the rate of association of [^{3}H]estradiol to the Y receptor can be measured and was found to be 1.63×10^5 M^{-1} s^{-1} (Figure 3). The rates of dissociation were identical where $k_{-1} = 7.7 \times 10^{-5}$ s^{-1}, similar to our previously reported value for the X receptor (Smith & Schwartz, 1979).

Physical Properties of the Cytosolic Y Receptor. Subtle differences in the relative heat stabilities of the chick oviduct cytosolic X and Y estrogen receptors illustrate one of the apparent major differences in the two species. Incubating the receptor solutions at elevated temperatures in the presence of dextran-coated charcoal resulted in the selective destruction of the low-affinity receptor species. The procedure, optimized at 42 °C for 20 min, additionally resulted in a 30–40% degradation of the more heat-stable X component.

Attempts to effect a useful separation of the two estrogen receptors by ion-exchange chromatography on DEAE-Bio-Gel A and DEAE-Sephadex A-25 were at best only partially successful. A variety of receptor sources were tried, including crude cytosol, ammonium sulfate fractions, and affinity resin purified preparations. However, in all cases where a continuous salt gradient (0–400 mM KCl) was employed, just one clearly resolved peak displaying predominantly specific binding for estradiol was observed, eluting at 180–185 mM KCl (Figure 4). However, if stepwise elution was used, the results demonstrated that there was a small but preferential elution of the higher affinity species with lower salt concentrations. Commencing at 150 mM KCl and increasing in either 25 or 50 mM increments, the ratio of X to Y was observed to follow the progression of X > Y, X = Y, to X < Y (Figure 5). Since the cytosol, prior to chromatography, contained equal concentrations of X and Y, these results are indicative of subtle differences in the relative charge ratios of the two receptors such that the lower affinity species displayed more acidic characteristics. Considering the small change of ratios observed, and the ability to selectively saturate the complete ion-exchange bed volume at each designated stepwise salt

concentration, it is not too surprising that a continuous gradient was unable to detect such small changes. When the receptors X and Y were separated by affinity chromatography and chromatographed separately on DEAE-Bio-Gel A, they eluted at 150 and 200 mM KCl, respectively.

Naturally, the above experiments serve to reinforce the status of just how similar the two receptor species are, as other related studies have previously demonstrated (Taylor, 1981). Emphasizing this situation, it was interesting to be able to compare the findings of parallel experiments carried out with Y only cytosol preparations. Irrespective of whether or not the high-affinity species was present, the resolved specific binding peak still eluted at 180 mM KCl via continuous gradient analysis and predominantly at 200 mM KCl with a stepwise elution. Parallel experiments employing phosphocellulose chromatography were similarly ineffectual in our endeavor to separate the chick oviduct estrogen binders. Thus, both receptors were predominantly eluted at 200 mM KCl, although the lower affinity Y receptor was preferentially retained, confirming the subtle differences in receptor acidity observed with DEAE chromatography.

Other protocols involving separation on the basis of molecular size resulted in coincident elution of the two receptor species. A technique developed in this laboratory to effect a quantitative conversion of the higher affinity X receptor to the lower affinity Y form was employed to further study this size relationship. Similar to the parallel nucleotide-mediated augmentation of the active lower affinity receptor species from a non-steroid binding form of Y (Y_{nb}; Raymoure et al., 1985), this X and Y transformation has a specific requirement for magnesium ions and elevated temperature (30 °C). The interconversion does not occur at 4 °C even in the presence of ATP and Mg^{2+} (unpublished results). Aliquots of cytosol prelabeled with 5 nM [^{3}H]estradiol at 30 °C and, where appropriate, treated with ATP/Mg^{2+} at this temperature to effect the transformation of the high-affinity to the lower affinity form were chromatographed on a column packed with Bio-Gel A-0.5M as described under Experimental Procedures. Elution profiles for both labeled X and converted X to Y soluble receptor sources showed concurrent binding peaks trailing a few fractions behind the void volume marker (data not shown). No other specific [^{3}H]estradiol binding peaks were observed, however, effectively ruling out the possibility of receptor-derived proteolytic fragments as reported with gel filtration studies on the chick oviduct progesterone receptor (Sherman & Diaz, 1977; Vedeckis et al., 1980).

The use of sucrose density gradient analysis under conventional conditions met with varying success. Traditional methodology specifying prolonged centrifugation (~18 h) was concurrent with poor resolution of estradiol binding peak(s), exacerbated by excessive dissociation of the estrogen receptor complex during the course of the lengthy centrifugation period. Additionally, low-salt conditions were frequently observed to favor conditions leading to the formation of aggregates with specific [^{3}H]estradiol binding being localized in gradient zones of 10 S or greater. Such operational problems appear to be relatively widespread with sucrose density gradient analysis of estrogen receptor complexes (Harrison & Toft, 1975). However, a simple amendment to the protocol, whereby the spin time was halved and the centrifugation speed increased, gave consistent results with good resolution.

A typical sedimentation profile for the Y estrogen receptor in high-salt conditions is illustrated in the top panel of Figure 6, with specific estradiol binding being localized at 3.5 S. The advantages of the reduced centrifugation time are apparent

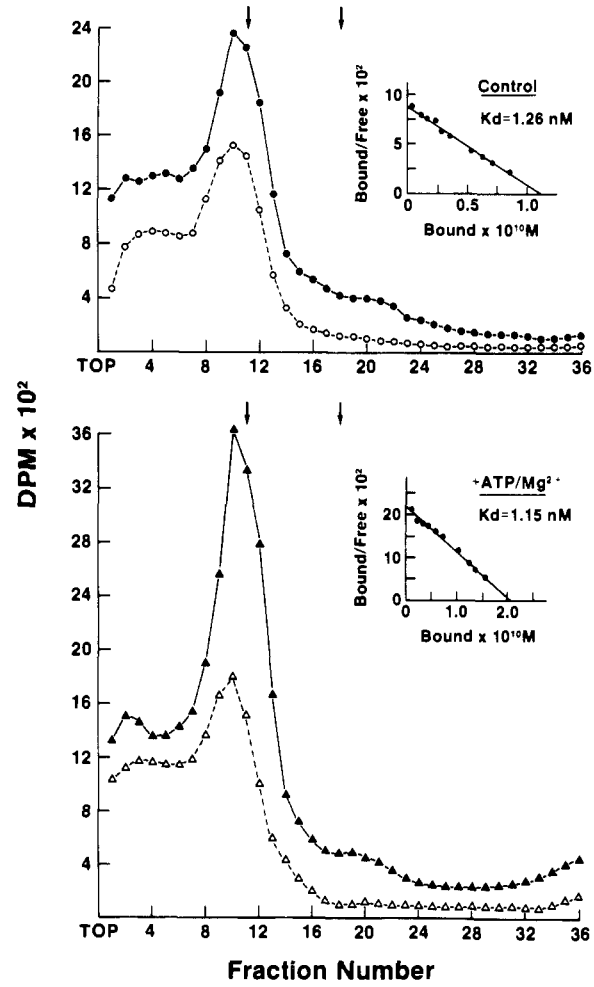

FIGURE 6: Prelabeled sucrose density gradient analysis of receptor Y in cytosol from 2-day-withdrawn estrogen-stimulated chick oviducts. Linear gradients containing 5–20% (w/v) sucrose in the presence of 400 mM KCl were prepared, centrifuged, and fractionated as described under Experimental Procedures. Cytosol labeled with 5 nM [^{3}H]-estradiol for 3 h at 30 °C and charcoal-treated immediately before application to the gradient to remove free ligand was used to determine total binding (●). Nonspecific binding was measured in the presence of a 100-fold molar excess of unlabeled DES (○). Receptor Y was augmented with 2.5 mM ATP/2.5 mM Mg^{2+} for 3 h at 30 °C. Otherwise, conditions are applicable as for the controls. Total binding (▲) and nonspecific binding (△). No augmentation occurred at 30 °C for 3 h in the absence of Mg^{2+}/ATP. Internal standards: ovalbumin, 3.7 S; β-amylase, 8.2 S. Inserts depict Scatchard analysis of the control and nucleotide-augmented Y receptor binding assays.

with significantly reduced levels of dissociation and a correspondingly greater resolved [^{3}H]estradiol binding peak. Although the level of specific binding in this particular example is relatively low, the phenomenon of receptor augmentation by treatment with ATP/Mg^{2+} at 30 °C (Aurrichio, 1982; Raymoure et al., 1985) results in a substantial amplification of the 3.5S peak, with a doubling in specific binding (Figure 6, lower panel). The integrity of the Y receptor species after nucleotide-mediated augmentation by activation of the non-steroid binding receptor (Y_{nb}) was defined by maintenance of its equilibrium dissociation constant (inserts in Figure 6) and specificity of hormone binding (Smith & Taylor, 1981; Raymoure et al., 1985). This augmentation could not be induced by ATP/Mg^{2+} at 4 °C or in the absence of ATP/Mg^{2+} at 30 °C.

To establish that the peak at 3.5 S was not an artifact of [^{3}H]estradiol dissociating from a higher sedimenting form and then binding nonspecifically to a 3.5S protein, similar gradient analyses were conducted, but the fractions were labeled with

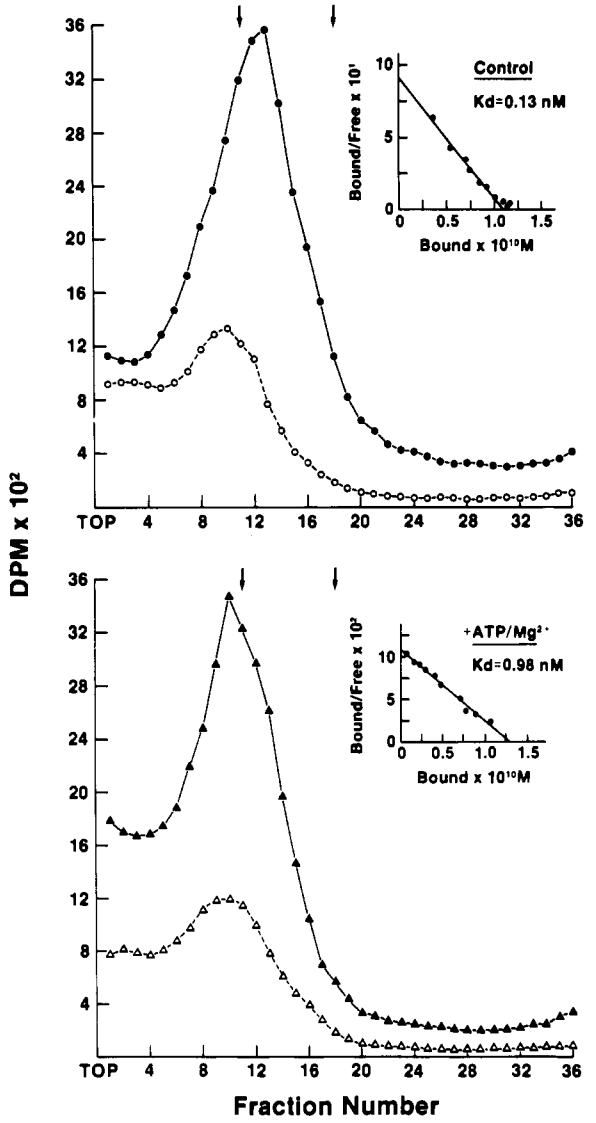

FIGURE 7: Sucrose density gradient of receptor X and its conversion to receptor Y. Linear gradients containing 5–20% w/v sucrose in the presence of 400 mM KCl were prepared, centrifuged, and fractionated as described in the text. X only receptor preparations from 10-day DES-withdrawn chick oviduct cytosol were labeled either with 5 nM [^{3}H]estradiol for 3 h at 30 °C or with 2.5 mM ATP/2.5 mM Mg^{2+} at 30 °C for 3 h. Nonspecific binding was measured in the presence of a 100-fold molar excess of [^{1}H]DES. Total binding control (●); nonspecific binding (○). Total binding nucleotide treated (▲); nonspecific binding (△). Internal standards: ovalbumin, 3.7 S; β-amylase, 8.2 S. Inserts depict Scatchard analysis of the control (R_X) and treated (R_Y) cytosols.

5 nM [^{3}H]estradiol after collection. Following incubation (18 h at 4 °C), free steroid was separated from bound by using dextran-coated charcoal (Smith et al., 1979). By use of this postlabeling technique, the sedimentation coefficient of the Y receptor was confirmed to be 3.5 S.

For comparison purposes, the high-affinity receptor X (K_d = 0.1 nM) was subjected to similar sucrose density analysis. These studies, as illustrated in the top panel of Figure 7, were consistent with our previous findings that the higher affinity species sedimented at approximately 4.3 S (Smith & Schwartz, 1979; Taylor & Smith, 1979). When this receptor preparation was treated with ATP/Mg^{2+}, no receptor augmentation occurred, indicating the absence of Y_{nb}. However, the peak was shifted to 3.5 S, and Scatchard plot analysis demonstrated a change in K_d from 0.1 to 1 nM in a quantitative manner, consistent with a conversion of X to Y (Figure 7, lower panel). The absence of Y_{nb} in these X only preparations is typical since Y_{nb} is not found in cytosol obtained from oviducts withdrawn from DES in excess of 48 h (unpublished results). While the observed change in sedimentation coefficient accompanying the X to Y conversion is not large, it is, however, consistently reproducible and was always measured by including internal ^{14}C standards in the gradients. The sensitivity of measuring the change is extremely high since the X and Y forms sediment at either side of the [^{14}C]ovalbumin standard ($p < 0.001$).

Discussion

Since the initial demonstration of estrogen binding heterogeneity in the rat uterus (Clark et al., 1978) and the chick oviduct (Smith et al., 1979), a number of reports have followed describing polymorphic estrogen binding sites. However, an evaluation of this work has been complicated by an apparent major difference in the primary characteristics of the respective lower affinity species. While the chick oviduct Y receptor, identified with a K_d of 1 nM, can be readily translocated to the internuclear compartment and maintained there for extended periods of time following estrogen administration and is assayable in the presence of sulfhydryl reagents, the low-affinity form in the rat, conveniently tagged as "type II" and possessing a measured K_d of 40 nM, seemingly remains inert to such hormonal stimulation and can only be assayed in the absence of sulfhydryl reducing agents. More recently, a third estrogen binding protein in the chick oviduct has been identified (Taylor & Smith, 1982) and additionally characterized (Ruh & Toft, 1984). Similar to the type II rodent binding protein, this new component, designated Z, is nontranslocatable to the nucleus and has a equilibrium dissociation constant of 30 nM but has liberal steroid binding specificity.

Heterogeneity of estrogen binding sites has subsequently been confirmed in a number of diverse species including mouse mammary tumor (Watson & Clark, 1980), normal and malignant human breast tissue, and myometrial tissue (Panko et al., 1981; Syne et al., 1982). Nevertheless, the status of the lower affinity species in these reports remains unclear. Syne and her colleagues (Syne et al., 1982), in their quantitation of breast tumor nuclear estradiol binding sites, have calculated apparent equilibrium dissociation constants of 0.17 and 5.0 nM for the high- and low-affinity components, respectively. The latter represents a virtual 10-fold decrease from the initially reported values for the equivalent uterine type II binding sites. Interestingly, these resolved K_d's are similar in magnitude to those reported by our laboratory in a study of human uterine estrogen receptors (Smith et al., 1979; Gibbons et al., 1982). In premenopausal tissue, there are two binding site species, with respective K_d's of 0.11 and 2.9 nM, that are very close to our reported values in the chick oviduct.

Insofar that many experiments were undertaken to demonstrate differences in the two receptor forms, the results have essentially demonstrated the inverse. It is now apparent that the X and Y binding components, on the basis of a variety of criteria, are remarkably similar. Conventional protocols designed to resolve discrete protein species such as molecular sieve and ion-exchange chromatography have proven ineffective in efficiently separating the X and Y forms. Not surprisingly, one of the immediate conclusions from such observations is that we may be dealing with a homogeneous receptor such that a mixture of nascent (unoccupied) and transformed (occupied) forms could readily be perceived by Scatchard analysis as an apparent two-component system of high- and low-affinity sites. To conclusively rule out this possibility, a series of experiments were undertaken employing charcoal stripping of receptor solutions, prelabeled with [^{3}H]estradiol at elevated temperature. Following quantitation

for residual bound [^{3}H]estradiol, 20-point binding assays were performed under exchange conditions (3 h at 30 °C), with the net result that biphasic Scatchard plots were still readily evident. Graphical correction analysis by the method of Rosenthal (1976) demonstrated the existence of two receptors displaying calculated K_d's of 0.06 and 0.8 nM for the X and Y species, respectively. These values are comparable to the control (4 °C) assays carried out concurrently with the stripping experiments, with resolved K_d's of 0.08 and 1.0 nM, as well as with the previously published values of 0.05 and 0.8 nM when the data from 20-point binding assays were subject to the LIGAND computer program of Munson and Rodbard (Taylor & Smith, 1982). Naturally these experiments also rule out any possible hint of an artifactual element contributed by unbound endogenous estrogen or other ligands to the biphasic binding kinetics.

The use of traditional methods of protein isolation and purification met with limited success in the separation of the two estrogen receptor species. For example, cytosol samples containing just the Y receptor, as well as affinity resin purified Y samples, upon DEAE ion-exchange salt gradient chromatography or stepwise elution essentially matched the polymorphic receptor elution profiles. Stepwise elution followed by Scatchard analysis demonstrated that varying mixtures of X and Y were eluted by 0.15, 0.20, and 0.25 M KCl. These subtle differences in acidities were supported by similar results obtained with phosphocellulose chromatography. Continuous salt gradient ion-exchange chromatography, however, was unable to demonstrate such small changes. A recent study by Ruh and Toft (1984) investigating the ion-exchange chromatography of an avian estrogen receptor preparation reported very similar continuous gradient results with a single broad peak of specific estradiol being eluted at approximately 200 mM KCl.

Undoubtedly the best evidence for the existence of the two receptor forms is their physical separation which was achieved by capitalizing on the different binding kinetics, with the use of a low-capacity 17β-estradiol 17-hemisuccinyl-diaminodipropylamino-Sepharose 4B affinity resin. Preferential adsorption of the high-affinity receptor to the Sepharose gel gave essentially quantitative recovery of the Y species. The optimum conditions specified a resin possessing 100–200 pmol of bound estradion hemisuccinate/mL. The results cannot be explained by a gradual leaching of estradiol from the affinity resin as the posttreatment supernatant was routinely treated with charcoal prior to commencement of the binding assays. Scatchard analysis of the data from these experiments showed no significant variance in the K_d's and binding site concentrations irrespective of whether the assays were carried out at 4 °C overnight or under exchange conditions (30 °C for 3 h), thus confirming the absence of endogenous ligand. To demonstrate the selective property of these laboratory-synthesized estrogen affinity resins, an increase in the immobilized estradiol concentration to 800–1000 pmol/mL of resin resulted in both receptors being adsorbed to the gel matrix. This separation by an affinity resin based protocol further strengthens the already strong case for the existence of two distinct estrogen receptors.

Experiments investigating the preferential binding of the X receptor to the estrogen-linked affinity resin gave an initial indication of the probable explanation for the recognized 10-fold difference in equilibrium dissociation constants for the two chick oviduct binding sites. In a bimolecular reaction, the concentration of ligand has a direct impact on the kinetics of association between ligand and protein. It was thus rationalized that the selective uptake of the high-affinity receptor species to the lower capacity Sepharose-based affinity resin was a direct result of a more rapid association rate. Conversely, the use of an affinity resin containing high concentrations of immobilized ligand would effectively mask the differences in association rates such that it would not now be possible to separate the two receptor forms. Subsequently, it was possible to experimentally confirm this relationship with a study of the receptor association kinetics. Indeed, since the rates of dissociation for X and Y were essentially identical, the 10-fold difference in their resolved K_d's was readily accounted for with the measured association rate constants of 1.33×10^6 and 1.63×10^5 M^{-1} s^{-1} for X and Y, respectively.

These observations, illustrating the 10-fold slower rate of association of the lower affinity Y receptor, raise an important issue relating to the analysis of equilibrium binding studies. While equilibrium appears to be established after 18 h at 4 °C in the presence of 1 nM estradiol, it must be stressed that the association reaction is bimolecular; thus, at constant receptor concentration, the concentration of estradiol becomes rate determining. Binding and hence equilibrium will occur more slowly at low concentrations of ligand. When binding studies are conducted at various concentrations of ligand for a fixed incubation time, if equilibrium has not been established for the lower concentrations of ligand and the results are plotted according to Scatchard, a hooked curve will result. This could lead one to incorrectly assume that the receptor–ligand interaction is cooperative in nature.

The possibility that the lower affinity estrogen binder may be a degradation product of receptor X was effectively ruled out with a series of gel filtration experiments. Analysis of the Y receptor on agarose 0.5M showed a major peak of specific estradiol binding immediately following the void volume; this was essentially identical with the peak resolved by chromatography of a high-affinity X receptor preparation. With the discovery of nucleotide-dependent processes to augment lower affinity Y receptor from either the high-affinity X or a nascent inactive form of the receptor (Y_{nb}), it has furthermore been possible to additionally demonstrate the integrity of the Y estrogen binder already partially characterized with regard to the equilibrium dissociation constant, and the binding specificity that is maintained throughout these procedures (Smith et al., 1979; Smith & Taylor, 1981; Raymoure et al., 1985). Thus, concurrent with an ATP/Mg^{2+}-dependent conversion of X to Y, no change was observed in the sizing column resolved specific binding peak, although the transformation was verified with shifts in the respective K_d's from 0.1 to 1.0 nM and sedimentation coefficients from 4.2 to 3.5 S. Conversely, augmentation of receptor Y from its nonbinding analogue (Raymoure et al., 1985), similarly identified by gradient analysis and binding kinetics as well as steroid specificity, gave parallel findings. Pretreatment of cytosol samples with filtration through phosphocellulose to remove receptor-specific proteases (Birnbaumer et al., 1983) gave no indication of an adjustment in the relative ratio of X and Y. It can therefore be concluded that the increase in concentration of the lower affinity receptor resulting either from the conversion of X to Y or from its augmentation from Y_{nb} in the absence of receptor X is not explained by proteolysis. Although both receptors apparently elute from gel filtration columns as tetramers, differences in receptor size cannot be ruled out. These findings suggest that X and Y are the same protein unit(s) capable of existing in different conformations.

Previous reports dealing with estrogen receptor conformational forms have primarily involved various mechanisms of

receptor activation, irrespective of whether it be induced with elevated temperature, salt concentrations, or exposure to ligand. The resultant 4S to 5S transformations of rat and human uterine cytoplasmic estrogen binding proteins have been well documented (Notides, 1978; Thampan & Clark, 1981) and are thought to be indicative of a generalized model based on dimerization of 4S monomer units. However, the X and Y receptors of the chick oviduct are not representative of a simple activation phenomenon, since the two forms bind to DNA and nuclei; they can coexist and can be differentiated according to the rate of association of [^{3}H]estradiol and not the rate of dissociation. From molecular weight estimates of the denatured X receptor (Smith & Schwartz, 1979), the gel filtration data suggest that both native X and Y receptors exist as tetramers which under the stress of sucrose density gradient centrifugation induced hydrostatic pressure (Auricchio et al., 1978) dissociate into their monomer units. This speculation is supported by the observation that there is no evidence of aggregation following sucrose gradient analysis. Since the monomers differ in their sedimentation coefficients, it is possible that receptor X in addition to its alternative steroid binding site conformation is a covalent dimer of Y.

The data presented here strongly promote the concept of two intrinsically related receptor forms that are very similar in a number of physical and biochemical properties, while exhibiting subtle differences in their interaction with estrogen. Studies in progress are currently investigating a possible physiological relationship between the X and Y species. Preliminary findings indicate that for these interconversions to take place there is a prerequisite for ATP and Mg^{2+}. The implications of these findings are relevant to a number of aspects of hormone-directed mechanisms and their study. The avian oviduct lower affinity Y estrogen receptor appears to be required for integrity of transcription for the ovalbumin gene (Smith & Taylor, 1981); thus, its in vivo regulation may be an important aspect of egg white protein synthesis. Certainly a nucleotide-mediated process that could efficiently control the relative concentrations of the X and Y receptors, as well as the nonbinding form, would have a profound impact on our understanding of estrogen receptor action and its contribution to ovalbumin gene transcription of DNA replication. The ATP-mediated conversion of the X to the Y receptor may additionally help explain the mechanism of anti-estrogen action as subsequent studies have demonstrated that efficient conversion requires the continued presence of estradiol.

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