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Analysis of the Molecular Species of the Chick Oviduct Progesterone Receptor Using Isoelectric Focusing†

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ABSTRACT: Conditions are described for the preparative isoelectric focusing in flat beds of Sephadex of the progesterone receptor from the chick oviduct. The method allows the fractionation of the receptor into two molecular species, one focusing at p*I* 6 and the other at p*I* 7 with good purification and recovery. The p*I* 6 and p*I* 7 receptor species were purified 2- and 26-fold, respectively. The assaying of the focused fractions with the charcoal binding method provides an accurate identification and quantitation of the [^{3}H]progesterone receptor. The method is reproducible in recovery, quantitation, and resolution of the two receptor species. The receptor with an apparent p*I* of 6 sediments at ~4 S on linear sucrose gradients, while the receptor with an apparent p*I* of 7 sediments at ~3.5 S. On the basis of the sedimentation values and elution patterns from diethylaminoethyl (DEAE) chromatography, the p*I* 6 component is equivalent to the "B" receptor species and the p*I* 7 component is equivalent to the "A" receptor species described previously [Schrader, W. T., & O'Malley, B. W. (1972) *J. Biol. Chem. 241*, 51–59].

Isoelectric focusing has proven to be a useful technique for the separation of proteins and other amphoteric substances which possess different isoelectric points. Many earlier studies on the isoelectric focusing of steroid receptor proteins have utilized predominantly vertical columns containing gradients of sucrose as the media to support the pH gradient and the zones of focused protein (Schrader & O'Malley, 1972; Sherman et al., 1974). However, IEF[1] in sucrose gradients has several inherent disadvantages which include (1) limitations in the amount of sample which can be successfully focused, (2) failure of the gradient to support proteins which precipitate at their p*I*, (3) extended times required for focusing proteins to equilibrium (24–72 h), and (4) diffusion and mixing of focused zones during the elution procedure (Radola, 1973a, 1975; Sherman, 1975). These technical difficulties have been essentially circumvented by the use of either polyacrylamide gel or granulated gel as the support media (Radola, 1973a,b, 1975). The granulated gel has advantages over polyacrylamide with respect to improved recovery of proteins.

This paper describes an IEF method for fractionating the [^{3}H]progesterone–receptor complex into two molecular species which correspond to the "A" and "B" receptor species described previously (Schrader & O'Malley, 1972; Schrader et al., 1972). Partially purified [^{3}H]P–R isolated from the oviducts of estrogen-pretreated immature chicks was focused on flat beds of Sephadex G-75 superfine resin by using select conditions of temperature, buffers, and periods of focusing. This method separates the [^{3}H]P–R into two molecular species in a relatively short period (8 h) with good recovery, reproducibility, resolution, and partial purification.

Materials and Methods

Steroids. [1,2-$^3H_2$]Progesterone (40–60 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Unlabeled progesterone was obtained from Sigma Chemical Co. (St. Louis, MO). The degree of chemical purity of these steroids was checked by high-pressure liquid chromatography using a linear gradient of 30–65% acetonitrile in water over a 1-h period in a reverse phase C_{18} μBondapak column (Waters Associates, Milford, MA). The radioactivity eluting from the column was collected by a fraction collector and the fractions were counted. Greater than 90% purity was found for all preparations used. The stock [^{3}H]progesterone solution in benzene–ethanol (9:1) was frozen, freeze-dried, and redissolved to the original volume with ethanol. When needed, this stock was diluted 1:5 with water to give a 4 μM [^{3}H]progesterone solution in 80% water–20% ethanol (v/v). This diluted stock solution of [^{3}H]P was added directly to the cytosol preparations to obtain labeled progesterone–receptor complexes as described below.

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[1] Abbreviations used: [^{3}H]P, [^{3}H]progesterone; [^{3}H]P–R, [^{3}H]-progesterone–receptor; IEF, isoelectric focusing; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; $(NH_4)_2SO_4$, ammonium sulfate.

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Preparation of Progesterone–Receptor Complex. The [^{3}H]P–R complex was prepared by the method of Schrader & O'Malley (1972) with minor modifications. Immature chicks were injected subcutaneously (5 times/week) with 5 mg of diethylstilbestrol for 20–25 days to obtain fully developed oviducts (O'Malley et al., 1969). The oviducts were excised, stripped of fat, and weighed. Subsequent steps were performed at 4 °C unless stated otherwise. The tissue was briefly homogenized in 3 volumes (v/w) of buffer A (0.05 M Tris-HCl, 0.001 M EDTA, 0.012 M thioglycerol, pH 7.4) for 30 s at 2000 rpm in a Waring blender to fragment the tissue. The homogenate was then homogenized in a glass Teflon homogenizer to break cells. The suspension was centrifuged at 20000*g* for 10 min and the resulting supernatant recentrifuged for 1 h at 100000*g* in a Beckman L3-50 centrifuge. The supernatant (cytosol) was diluted to 20 mg of protein/mL, quantitated by using absorbance at 280 nm with reference to a standard curve of absorbance vs. cytosol protein as determined by the Lowry method (Lowry et al., 1951). This cytosol preparation was incubated for 2 h with 5 μL of the stock [^{3}H]P solution (described above) to give a final concentration of ~20 nM progesterone. The labeled cytosol was then precipitated by addition of a saturated ammonium sulfate solution in buffer B (0.01 M Tris-HCl, 0.001 M EDTA, 0.012 M thioglycerol, pH 7.4) to a final concentration of 35% saturation. The [^{3}H]P–R was sedimented by centrifugation at 20000*g* for 10 min by using a Sorvall HB-4 swinging bucket rotor. These pellets of partially purified [^{3}H]P–R were frozen and stored at −80 °C. When needed, the partially purified receptor pellets were resuspended in buffer B containing 10% (v/v) glycerol and 0.3 M KCl at half the volume of the original cytosol solution and dialyzed against 20 volumes of buffer B containing 10% (v/v) glycerol for 1 h and the solution was clarified by centrifugation at 20000*g* for 10 min.

Isoelectric Focusing. A slurry of prewashed Sephadex G-75 superfine resin was prepared by mixing 95 mL of buffer B containing 10% (v/v) glycerol with 5 mL of LKB (pH 3.5–10) ampholine solution (40% w/v) and 5 g of the resin. The final concentration of ampholine was 2% (w/v). All focusing buffers contained glycerol unless indicated otherwise. The suspension was poured onto a silicone rubber-rimmed glass plate (23 × 11 cm) and dried with a light stream of cool air until the weight (i.e., water content) decreased by 25%. The plate was placed on the cooling unit (0–4 °C) of an LKB 2117 Multiphor. Electrode strips (10.7 cm) were soaked in either 1 M phosphoric acid (anode) or 1 M sodium hydroxide (cathode) and placed at the ends of the IEF plate. In early experiments, the plate was then prefocused at 4 W/plate for 2–4 h at 0–4 °C to establish the pH gradient. Subsequent studies indicated that the prefocusing was unnecessary. The partially purified [^{3}H]P–R sample was applied in the pH 7–8 region of the gel and focused for 8 h at 8 W/plate at 0–4 °C, unless stated otherwise. Following focusing, the pH gradient was determined either with a flat membrane electrode (Ingold, Lexington, MA) on the surface of the gel or by extracting a small segment of each gel filtration with preboiled water and measuring the pH directly. The resin was then sectioned into 30 fractions and each section eluted with 9 mL of buffer B containing 10% (v/v) glycerol at 4 °C by using plastic columns. These eluants either were quantitated immediately by using the dextran charcoal assay described below or were precipitated with 50% $(NH_4)_2SO_4$ in buffer B and stored.

Estimation of the Specifically Bound Progesterone. The amount of specifically bound progesterone in the receptor preparations and IEF eluants was quantitated by using a modification of the dextran-coated charcoal procedure described by Korenman (1968). Tubes containing 0.1–0.2 mL of labeled receptor were incubated either alone at 4 °C to assess the total bound radioactivity or in the presence of a 100–200-fold excess of unlabeled steroid at 37 °C for 1 h to denature and/or exchange the [^{3}H]P–R complex to measure nonspecific binding. These conditions have been shown to eliminate specifically bound [^{3}H]P by irreversible denaturation of the receptor and exchange of labeled steroid for unlabeled progesterone (Buller et al., 1975; Schrader et al., 1978). Following incubation, the heated tubes were recooled to 4 °C. A 1.0-mL aliquot of charcoal suspension [1% (w/w) Norit A, 0.05% (w/w) T-70 dextran] in buffer C (0.01 M Tris-HCl, 0.001 M EDTA, 0.25 M sucrose, pH 7.4) was then added to each tube for 5 min. The tubes were centrifuged at 2500 rpm for 10 min by using an IEC PR-6000 centrifuge. An aliquot of each supernatant was counted in 5 mL of a toluene-based scintillation solution containing 6.4 g of PPO (2,5-diphenyloxazole) and 0.08 g of POPOP [1,4-bis[2-(5-phenyloxazolyl)benzene]] per L. The counting efficiency was 40–50%. Specific binding was calculated as the difference between total and nonspecific binding.

Sedimentation Analysis of the Progesterone Receptor. All steps are carried out at 4 °C. Partially purified [^{3}H]P–R preparations were focused, and the individual peaks of activity were pooled and precipitated with 50% saturation of $(NH_4)_2SO_4$. The pellets were resuspended in buffer B containing 0.3 M KCl, dialyzed against buffer B, and centrifuged at 20000*g* for 10 min to remove insoluble material. Portions (0.2 mL) of the resulting supernatant were layered onto linear gradients of 5–20% sucrose in buffer B containing 0.3 M KCl which were prepared in 5-mL polyallomer tubes (Beckman Instruments). The gradients were centrifuged at 45 000 rpm for 16 h at 0–4 °C in a Spinco SW 50.1 rotor. Ovalbumin (3.7 S) was run as a marker protein. Gradient fractions (10 drops) were collected from the bottom and immediately incubated with 0.5 mL of dextran-coated charcoal suspension for 5 min. The tubes were then centrifuged at 2500 rpm for 10 min by using the IEC PR-6000 centrifuge. An aliquot of each supernatant was counted as described above. The gradient containing the ovalbumin marker was fractionated as described above except that each fraction was diluted with 0.9 mL of H_2O and the absorbance measured at 280 nm.

DEAE Chromatography. Anion-exchange chromatography was performed as previously described by Schrader & O'Malley (1972). Briefly, Whatman DE-52 ion-exchange resin was washed and equilibrated with TESH buffer (0.10 mM Tris-HCl, 0.01 mM EDTA, 0.12 mM monothioglycerol, pH 7.4). The slurry was poured into a 2.0 × 15 cm column and allowed to settle until the bed height was equivalent to the diameter of the column. The column was then washed thoroughly with the same buffer before the application of the receptor preparation. Following sample application, the column was eluted with TESH buffer until the unbound radioactivity (free [^{3}H]P) was removed. A stepwise elution procedure was then conducted by using TESH buffer containing 0.15 M KCl followed by 0.3 M KCl, which eluted two peaks of [^{3}H]P–R activity designated "A" and "B", respectively (Schrader & O'Malley, 1972). The fractions in each peak of receptor were pooled, and the receptor was precipitated with 50% saturation of $(NH_4)_2SO_4$.

Results

Initial studies were directed at establishing the optimal conditions for focusing the progesterone receptor. The prefocused preparations of bound progesterone in the chick

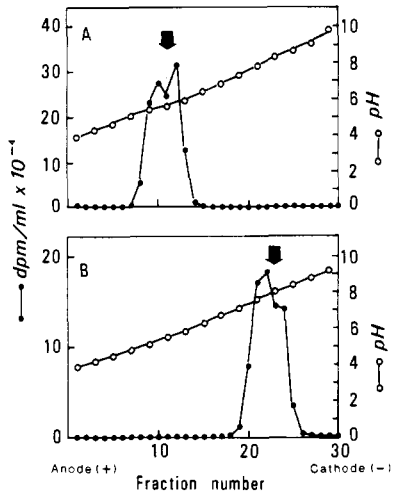

FIGURE 1: Isoelectric focusing of free [^{3}H]progesterone. Free [^{3}H]progesterone was applied either in the anode region (A) or the cathode region (B) of an isoelectric focusing plate. The plate was prepared as described under Materials and Methods. Following 8 h of focusing at 0–4 °C using a constant wattage at 8 W/plate, the gel was fractionated into 30 sections. Each section was placed in small columns and the protein eluted. A 100-μL aliquot of each fraction was counted for radioactivity. The pH gradient (open circles) was determined as described under Materials and Methods. The arrow indicates the position where the receptor sample was applied.

oviduct were previously shown to be the progesterone receptor via its affinity and specificity of binding steroids, tissue specificity, and ability to translocate to the oviduct nuclei (Schrader & O'Malley, 1972; Schrader et al., 1972; Spelsberg, et al., 1971, 1972). Since [^{3}H]P–R preparations consist of steroid–receptor complexes in equilibrium with free steroid, it was important to analyze the isoelectric focusing pattern of free progesterone. Figure 1 illustrates the profiles obtained when [^{3}H]P was applied either in the anode region (Figure 1A) or in the cathode region (Figure 1B) of the focusing gel. Following 8 h of focusing (required to focus standard proteins such as hemoglobin or ovalbumin), the free ligand demonstrated no migration in either case. These results indicated the necessity for measuring the specifically bound radioactivity of the receptor samples after focusing; otherwise, peaks of free steroid could be mistaken for focused steroid receptor species. Therefore, all subsequent focusing experiments included the dextran-coated charcoal assay on each of the IEF fractions (see Materials and Methods) to estimate specifically bound [^{3}H]P.

A time course of isoelectric focusing of partially purified [^{3}H]P–R complexes was also conducted in order to determine the optimal time for complete focusing of the receptor by using the resolution of the two molecular species of the receptor and the recovery of specifically bound [^{3}H]P as indicators. Figure 2 shows the patterns and recoveries of samples which were focused for various times at 0–4 °C in the presence of 10% (v/v) glycerol. In these experiments, the IEF plate was prefocused for 2 h and the sample was applied in the pH 7–8 region of the gel. After 4 h of focusing (Figure 2A), the recovery of specifically bound receptor complexes was 27%; however, the peak of activity showed only slight migration from the sample application site. Following an 8-h focusing time (Figure 2B), the [^{3}H]P–R complexes were distributed into two peaks of activity which focused with apparent pI values of 6 and 7. The recovery was 19% under these conditions. Parts C and D of Figure 2 illustrate the focusing patterns obtained with 16 and 20 h of focusing, respectively. These longer focusing times gave slightly better resolution of the two peaks of radioactivity, but resulted in marked decreases in the recoveries of the bound [^{3}H]P–R compared to 8 h of focusing. Therefore, subsequent receptor preparations were focused for 8 h. The short period of prefocusing (2 h) of the gel before applying the sample was found to have no effect on the focusing pattern or recovery of receptor complexes (data not presented). Thus, this step was later omitted from the procedure.

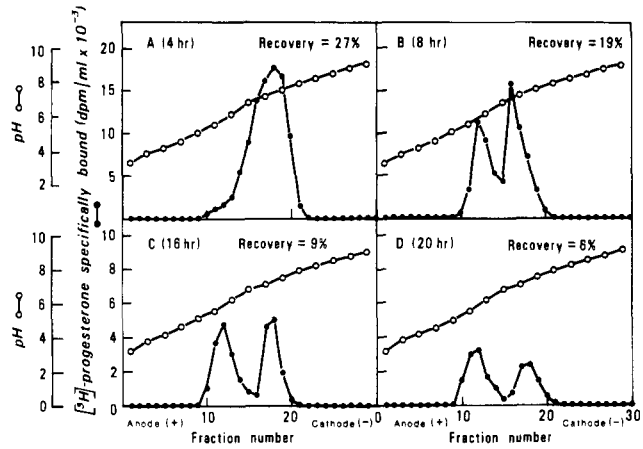

FIGURE 2: Time course of the isoelectric focusing of [^{3}H]P receptors. Partially purified preparations of the [^{3}H]progesterone receptor (2.5 mL) were applied to the pH 7–8 region of prefocused gel plates. The plates were focused at 8 Watts/plate for either 4 (A), 8 (B), 16 (C), or 20 h (D). Following the focusing, the gel was fractionated and the receptor eluted. The open circles represent the pH of the fractions. Aliquots of each of the eluates were then assayed by using the dextran-coated charcoal procedure to determine the specifically bound [^{3}H]progesterone, represented by the closed circles. Recoveries were calculated based upon the specific binding originally present in the unfocused sample.

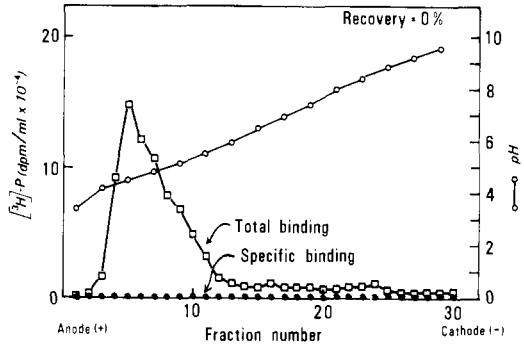

FIGURE 3: Isoelectric focusing of [^{3}H]P receptors which were applied near the anode. A partially purified [^{3}H]P–R preparation (2.5 mL) was applied in the pH 5 region of a prefocused gel. Following focusing for 8 h at 8 W, a dextran-coated charcoal assay was performed on the focused fractions. (□) Total [^{3}H]P binding; (●) specifically bound [^{3}H]P; (○) pH of the fractions.

To reaffirm that this method was monitoring receptor-bound steroid and not steroid adsorbed to other macromolecules, some receptor preparations were heat denatured prior to the IEF. Steroid receptor proteins are known to denature irreversibly at elevated temperatures (Puca et al., 1971; Schrader et al., 1978). Receptor samples which were incubated at 50 °C for 30 min prior to focusing demonstrated insignificant amounts of specifically bound progesterone by the charcoal assay of eluants from the IEF resin. A similar loss of specifically bound [^{3}H]P was observed when [^{3}H]P was layered in the acid region of prefocused gels followed by the standard focusing procedure as shown in Figure 3. Thus, the method described distinguishes between specific and nonspecific binding, providing the charcoal binding assay is performed on the eluted fractions. Also, the instability of the [^{3}H]P–R with high heat or low pH conditions in this IEF method is similar to that reported for

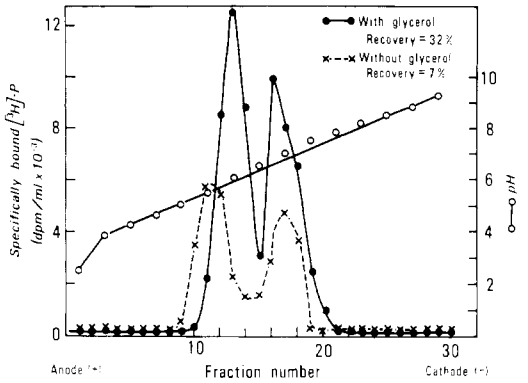

FIGURE 4: Effect of glycerol on the recoveries of the [^{3}H]P-R from the IEF resin. The same preparation of [^{3}H]P receptor was focused either with or without 10% (v/v) glycerol (represented by ● and ×, respectively) in both the isoelectric focusing gel buffer and the sample buffer. After 8 h of focusing at 8 W/plate, the fractions were eluted and assayed for specific binding by using the dextran-coated charcoal procedure.

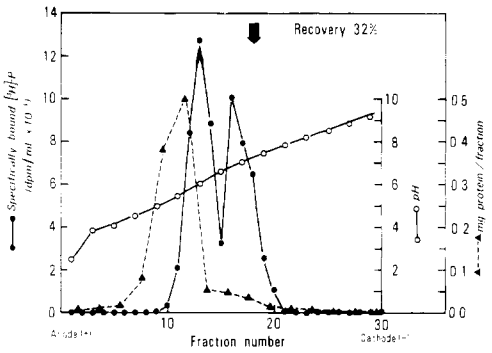

FIGURE 5: A typical isoelectric focusing pattern used to isolate the two molecular species of receptor; distribution of [^{3}H]P and protein. Partially purified [^{3}H]P-R complexes were focused and assayed as described under Materials and Methods. (●) Specifically bound [^{3}H]P; (○) pH of the fractions; (▲) milligrams of protein in the eluted fractions as determined by the Coomassie Blue method (Bromhall et al., 1969).

this receptor in crude or partially purified cytosol preparations (Schrader et al., 1972, 1978; Sherman et al., 1970).

In the course of developing the IEF method, a variety of conditions were studied in an effort to achieve optimal recoveries and resolution of the two species of [^{3}H]P-R. For example, the addition of glycerol (10% v/v) to all buffers, including the resins, increased the recovery of both species of receptor. As shown in Figure 4, the recovery of specifically bound [^{3}H]P was 32% for this preparation in the presence of glycerol, whereas only 7% recovery was obtained in its absence. Also, maximal recoveries of [^{3}H]P-R complexes were obtained when the samples were applied in the pH 7–8 region of the gel and the gel temperature was maintained at 0–4 °C during the focusing. The resolution of the two peaks of activity was greatly improved when the samples contained less than 3×10^6 dpm of specifically bound [^{3}H]progesterone since a broadening of the peaks occurred with greater amounts of receptor. Finally, the [^{3}H]P-R preparations partially purified by ammonium sulfate precipitation demonstrated several-fold increases in the recoveries of specifically bound [^{3}H]P compared to crude cytosol samples. Although the latter preparations also focused as two peaks of activity with p*I* values of 6 and 7, the amount of activity in the peak which focused at p*I* 7 was reduced (data not shown).

Figure 5 illustrates a typical IEF pattern of a [^{3}H]P-R preparation and the corresponding protein profile. The bulk of the protein focused in the region of the p*I* 6 receptor, while

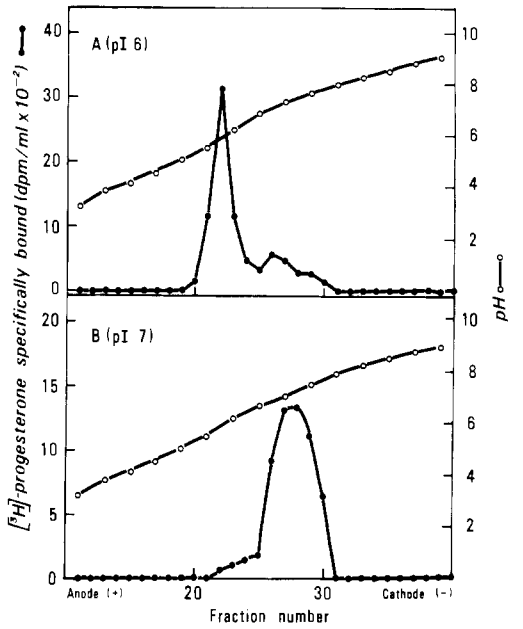

FIGURE 6: Refocusing of the isolated [^{3}H]progesterone receptor species. A partially purified [^{3}H]P-R sample (2.5 mL) was focused, and specifically bound [^{3}H]P was determined as shown in Figure 5. The fractions of each peak of [^{3}H]P-R activity (i.e., fractions 10–14 and 15–20) were then pooled and concentrated by using $(NH_4)_2SO_4$ precipitation. The [^{3}H]P-R pellets were resuspended, dialyzed, and refocused as above. The closed circles denote specifically bound [^{3}H]P, and the open circles show the pH value of the focused fractions. The top panel (A) illustrates the refocused pattern of the p*I* 6 [^{3}H]P-R species, and the bottom panel (B) represents the refocused p*I* 7 [^{3}H]P-R species.

very little protein focused in the region of the p*I* 7 species.

A series of experiments were conducted to determine whether the IEF method was reproducible with respect to the distribution of [^{3}H]P between the two species and total recovery of the receptor. The IEF patterns from a series of separate experiments performed on different days by using the same partially purified receptor preparation demonstrated only a few percent (~3%) difference in the total recovery of specifically bound [^{3}H]P and in the distribution of [^{3}H]P-R complexes between the two peaks. However, the recoveries of [^{3}H]P-R from different receptor preparations varied between 17 and 35% with the majority giving recoveries between 20–25%. The reason for the variability among receptor preparations is unknown currently. Attempts to improve recoveries by postlabeling the focused fractions with [^{3}H]P were unsuccessful. Therefore, the loss of [^{3}H]P-R during the focusing analysis appears to be irreversible.

Studies were then conducted to estimate the degree of resolution of the two receptor species and to determine whether these species would focus consistently at the same pH without interconversion. The two receptor species were separated by the focusing procedure (see Figure 5), and the individual peaks of activity were pooled and precipitated with ammonium sulfate. The p*I* 6 (fractions 10–14) and p*I* 7 (fractions 15–20) receptor species were then refocused as shown in parts A and B of Figure 6, respectively. The [^{3}H]P-R complexes which initially focused with an apparent p*I* of 6 also refocused at the same value in the pH gradient. Likewise, the [^{3}H]P-R complexes which originally focused with a p*I* value of 7 refocused at the same pH. Therefore, there does not appear to be interconversion of the two [^{3}H]P-R entities during the focusing procedure. Other studies on the isolation, $(NH_4)_2SO_4$ precipitation, and dialysis of the receptor also failed to show any interconversion of the p*I* 6 receptor and the p*I* 7 receptor

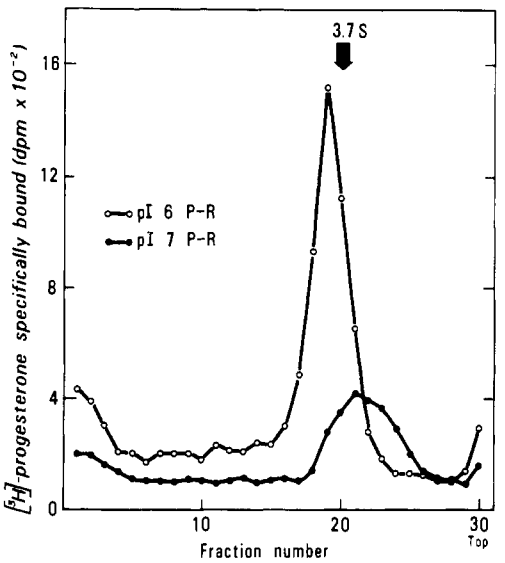

FIGURE 7: Sucrose gradient centrifugation of focused [^{3}H]P receptor species. Samples (2.5 mL) of partially purified [^{3}H]P–R were focused and assayed as shown in Figure 5. The peaks of [^{3}H]P–R activity were pooled and concentrated by using $(NH_4)_2SO_4$ precipitation. These focused [^{3}H]P–R species were layered onto linear gradients of 5–20% sucrose containing 0.3 M KCl and centrifuged at 45 000 rpm for 16 h at 0–4 °C. Ovalbumin (3.7 S) was sedimented as an internal standard. The gradient fractions were collected and incubated with dextran-coated charcoal to adsorb unbound [^{3}H]P. The open circles indicate the sedimentation profile of the p*I* 6 [^{3}H]P–R species, and the closed circles represent the p*I* 7 [^{3}H]P–R species.

species. Thus, the two species of receptor appear to be stable entities which are separated with 90% resolution by the isoelectric focusing method.

Sedimentation analysis of the two species was then performed to reaffirm the macromolecular nature of the focused species and to compare it with the native receptor. Figure 7 illustrates the profiles obtained when the two IEF peaks were pooled as described above and subsequently analyzed on linear 5–20% sucrose gradients containing 0.3 M KCl. Ovalbumin (3.7 S) was used as the marker protein. The two species of receptor displayed slightly different sedimentation values. The p*I* 6 species of [^{3}H]P–R sedimented slightly ahead of the marker (i.e., ~4 S), while the p*I* 7 species sedimented slightly behind the protein standard (i.e., ~3.5 S). Interestingly, these sedimentation values are very similar to those described by Kuhn et al. (1977) for the chick oviduct progesterone receptor "A" subunit (3.6 S) and "B" subunit (4.2 S).

The two components of the chick oviduct progesterone receptor, first detected by Sherman et al. (1970) using molecular sieve chromatography, were isolated by DEAE chromatography and purified and characterized by Schrader and co-workers (Schrader et al., 1972, 1977; Kuhn et al., 1977; Coty et al., 1979). To determine the relationship between the two species resolved by the IEF method and those by DEAE chromatography, we isolated the "A" and "B" species of the [^{3}H]P–R by DEAE chromatography and reanalyzed them by the IEF method described in this paper. Figure 8A depicts an elution profile of a [^{3}H]P–R preparation by using DEAE chromatography. After the elution of free steroid, the peak of "A" receptor was obtained following stepwise elution with TESH buffer containing 0.15 M KCl. Subsequently, the "B" receptor (plus any A–B dimers) was eluted with 0.3 M KCl. When the "A" (0.15 M KCl) and the "B" (0.3 M KCl) peaks from the DEAE chromatography were pooled and analyzed individually by the IEF method, as much as 30% of the bound [^{3}H]P was contaminated with the other species (parts B and

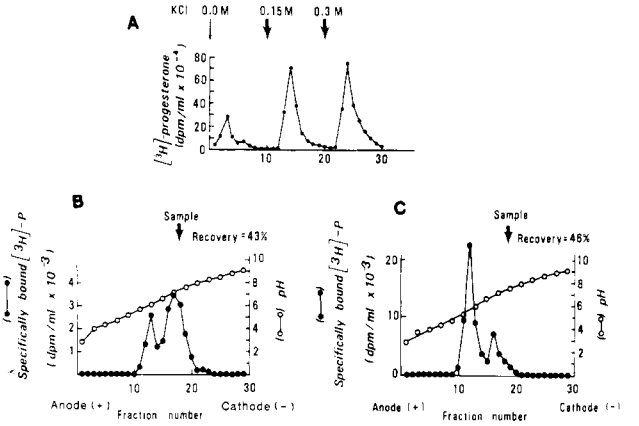

FIGURE 8: Isoelectric focusing of the two receptor species isolated by DEAE ion-exchange chromatography. (A) The [^{3}H]P–R preparation was applied to a DEAE anion-exchange column and eluted with a stepwise gradient of KCl as described under Materials and Methods. The fractions under each of the "A" and "B" peaks of bound [^{3}H]P, representing elution with 0.15 and 0.3 M KCl, respectively, were separately pooled and concentrated by ammonium sulfate precipitation. The "A" and "B" peaks were then separately focused for 8 h at 8 W/plate as described under Materials and Methods. The IEF of the "A" and "B" species from the DEAE chromatography are illustrated in parts B and C, respectively. (●) Specifically bound [^{3}H]P determined by the charcoal assay; (○) pH gradient.

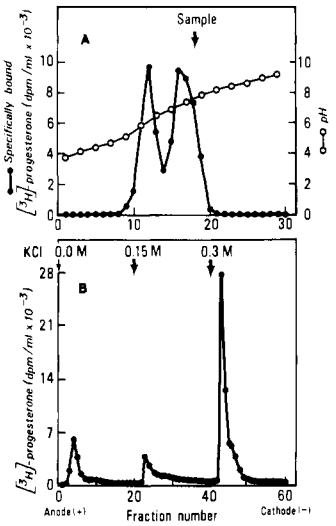

FIGURE 9: DEAE ion-exchange chromatography of the p*I* 6 receptor species isolated by isoelectric focusing. (A) A partially purified [^{3}H]P–R preparation (2.5 mL) was focused and assayed as described under Materials and Methods. The closed circles represent specifically bound [^{3}H]P, and the open circles represent the pH gradient. The p*I* 6 peak of activity was pooled and concentrated as described in the legend to Figure 6. The precipitated pellet was washed 3 times with a saturated solution of $(NH_4)_2SO_4$–buffer B to remove the ampholines, which interfere with ion-exchange chromatography. The pellet was then resuspended in 3 mL of TESH buffer, dialyzed, and applied to a DEAE column as described under Materials and Methods and the legend to Figure 8. The bottom panel (B) shows the elution profile of the p*I* 6 [^{3}H]P–R species from the DEAE resin.

C of Figure 8). Rechromatography of the "A" and "B" components on DEAE columns followed by focusing resulted in a greater enrichment of the individual entities focusing at p*I* values of 7 and 6, respectively. Reciprocal analysis involving DEAE chromatography of the p*I* 6 receptor isolated by the IEF method showed that 90% of the bound [^{3}H]P was eluted with 0.3 M KCl (Figure 9). Unfortunately, DEAE chromatography of the p*I* 7 species was not successful due to significant dissociation and/or denaturation of this [^{3}H]P–R component. Multiple washings of the ammonium sulfate

Table I: Purification of the Two Species of the Chick Oviduct Progesterone Receptor

sample	total protein[b] (mg)	total sp binding[c] (dpm × 10^{-6})	sp act. (dpm × 10^{-5}/mg of protein)	purifcn
cytosol[a]	100.00	5.32	0.55	1
0–35% $(NH_4)_2SO_4$ precipitation	6.12	3.38	5.55	10
IEF–p*I* 6 ("B" receptor)	0.90	1.03	11.40	20.5
IEF–p*I* 7 ("A" receptor)	0.13	1.90	146.00	265.0

[a] Prepared as described under Materials and Methods using 1.25 g of oviduct tissue. [b] Cytosol determined by Lowry et al. (1951); other fractions were determined by the Coomassie Blue method (Bromhall et al., 1969). [c] Determined by the dextran-coated charcoal assay described under Materials and Methods.

precipitated IEF fractions were necessary when going from focusing experiments to DEAE chromatography to remove ampholines, which interfere with ion-exchange chromatography. Since the "A" receptor is less stable than the "B" receptor (Coty et al., 1979), the p*I* 7 receptor species probably was not able to withstand these washing steps, whereas the p*I* 6 species survived. In any case, the results on the correlation between p*I* values of the two species with their elution from the DEAE column and the sedimentation values indicate that the p*I* 7 and p*I* 6 species isolated by IEF represent the "A" and "B" receptors, respectively.

Figure 5 suggested that the p*I* 7 species is significantly purified by the IEF. Table I outlines the purification of the p*I* 6 ("B") and p*I* 7 ("A") receptor species separated by the IEF method with respect to crude cytosol. The focusing step provided only a slight purification (~2-fold) of the p*I* 6 ("B") receptor, whereas the p*I* 7 ("A") receptor was markedly purified (~26-fold). This is in contrast to the DEAE method, wherein the "B" component is purified 10-fold and the "A" species is purified only 2-fold (Schrader & O'Malley, 1972).

Discussion

This report describes a flat-bed isoelectric focusing method for quantitating and partially purifying two species of [^{3}H]P–R which focus at p*I* values of 6 and 7. These receptor entities were found to correspond to the "B" and "A" receptors, respectively, identified by Schrader & O'Malley (1972) by using DEAE chromatography. The procedure combines the advantages of rapid focusing times with stabilized focused regions obtained when using polyacrylamide gel as the support medium, with the simple elution of focused proteins obtained when using sucrose gradient columns. However, the flat bed of Sephadex gel eliminates the possible errors in estimating the apparent p*I* of focused proteins which precipitate at their p*I* and sediment by gravity to the lower portions of the column of sucrose. It also gives a much higher recovery of protein than does the IEF in polyacrylamide.

Previous reports on the isoelectric focusing of the [^{3}H]-progesterone receptor of the chick oviduct using sucrose gradient columns have indicated p*I* values between 4 and 5 (Schrader & O'Malley, 1972; Sherman et al., 1974). However, it has been shown that the [^{3}H]P–R complex begins to destabilize irreversibly below pH 6, with a complete loss of binding activity at pH values below 5 (Schrader et al., 1978). Our results indicating the denaturation of the [^{3}H]P–R when it was placed in the region of the prefocused gel below pH 5 substantiate this instability. However, Sherman et al. (1974) demonstrated that the [^{3}H]P focusing at pH 4–5 was bound to receptor protein. The differences in the support medium itself may explain the differences in the estimated p*I* for [^{3}H]P–R between the Sephadex system and the sucrose column method. The p*I* values of 6 and 7 for the [^{3}H]P–R complexes of chick oviduct appear to be in good agreement with the known physicochemical properties of this steroid receptor and are similar to those reported for other steroid receptors in other systems (DeSombre et al., 1969; Puca et al., 1971; Mainwaring & Irving, 1973; Katsumata & Goldman, 1974; Kalimi et al., 1975; Coffer & King, 1976).

Although more time consuming and expensive than DEAE chromatography, the IEF method gives more reproducible quantitation and better resolution of the "A" (p*I* 7) and "B" (p*I* 6) receptor species compared to the anion-exchange method. Furthermore, the IEF procedure provides a greater purification of the "A" (p*I* 7) component compared to the DEAE method. The following paper describes the application of this IEF procedure for the separation and quantitation of the two molecular species of the progesterone receptor in the chick oviduct during different periods of the year.

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Seasonal Changes in the Molecular Species and Nuclear Binding of the Chick Oviduct Progesterone Receptor†

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ABSTRACT: A seasonal variation in the capacity of the progesterone–receptor complex to bind to isolated chromatin and partially purified acceptor protein bound to deoxyribonucleic acid (DNA) from the developed oviducts of estrogen-treated immature chicks has been described. Receptors isolated during the late winter fail to bind to these sites, whereas receptors isolated during the rest of the year show a marked binding. The level of binding to DNA remains unchanged throughout the year. A similar seasonal variation in the nuclear translocation in vivo of [^{3}H]progesterone is demonstrated. Further, the effect of progesterone on the endogenous ribonucleic acid polymerase II (B) activity in vivo also displays a seasonal variation. The variations in the in vivo binding and transcriptional effects show a similar periodicity and timing as the in vitro binding. The level of one of the two molecular species of the progesterone–receptor complex is markedly reduced during the winter, thus explaining the overall decrease in receptor amounts during this same period. The receptor preparations isolated during the late winter–early spring show greatly reduced amounts of the "A" species as compared to those isolated during the other periods of the year. The ability of a particular receptor preparation to bind to the chromatin in vivo and in vitro or to the nuclear protein acceptor sites in vitro correlates with the level of the "A" receptor species. These data support the role of certain nonhistone protein–DNA complexes as acceptor sites for the progesterone receptor in the chick oviduct and that either the "A" receptor species or a combination of the "A" and "B" receptor species is required for nuclear binding of the progesterone–receptor complex.

This laboratory has been investigating the interaction of steroid–receptor complexes with chromatin and its components. The binding of steroid–receptor complexes to target cell nuclei is required for the steroid-induced alteration of gene expression (Jensen & DeSombre, 1972). Various methods have been studied for analyzing nuclear binding of progesterone in the chick oviduct in order to chemically identify the nuclear acceptor site which binds the progesterone receptor. Details of these studies have been published in a recent review (Thrall et al., 1978). During these investigations, seasonal variations in oviduct weights, cytosol receptor levels, and the capacity of the receptor to bind to nuclear acceptor sites were observed (Spelsberg et al., 1979a,b; T. C. Spelsberg and F. Halberg, unpublished experiments). A circannual rhythm in the binding of [^{3}H]P–R[1] to nuclear acceptor sites was observed by using receptor preparations isolated throughout the year. The binding of [^{3}H]P–R to pure DNA was essentially constant throughout the year (Spelsberg et al., 1979a,b; T. C. Spelsberg and F. Halberg, unpublished experiments). The period of the isolation of the receptor, and not the nuclear preparation, was found to be the cause of this rhythm. Thus, the variability is a function of the receptor. Maximal [^{3}H]P–R binding to the nuclear acceptor sites was observed with receptor preparations isolated in the summer and fall, while minimal binding of [^{3}H]P–R was observed with receptor preparations isolated in the winter and early spring. The seasonal variation in the receptor binding to the NAP, but not to the pure DNA, was verified as a circannual rhythm by using computer-determined cosine-fitted curves (T. C. Spelsberg and F. Halberg, unpublished experiments). Although circannual rhythms were confirmed, the cycles did not repeat on an exact year basis (Spelsberg et al., 1979a,b; T. C. Spelsberg and F. Halberg, unpublished experiments).

Interestingly, as the [^{3}H]P–R lost its capacity to bind the acceptor sites, the level of receptor in the cytosol decreased ~50% (Spelsberg et al., 1979a,b; T. C. Spelsberg and F. Halberg, unpublished experiments). This report further describes the biological relevance of these rhythms and the cause of the overall decrease in receptor levels. First, evidence is presented that the seasonal variations in nuclear binding of the progesterone–receptor complex occur in vivo. Second, this loss of nuclear translocation of [^{3}H]P in vivo during the winter is accompanied by a marked decrease in the effect of the hormone treatment on RNA polymerase II activity. Finally, a correlation is shown between the capacity of [^{3}H]P–R to bind nuclear acceptor sites and its effect on transcription and the

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[1] Abbreviations used: [^{3}H]P, [^{3}H]progesterone; [^{3}H]P–R, [^{3}H]-progesterone–receptor; IEF, isoelectric focusing; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; NAP, nucleoacidic protein.

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