Purification of a Human Progesterone Receptor†

Roy G. Smith,* Michela d'Istria,‡ and Nguyen T. Van§

ABSTRACT: The cytoplasmic progesterone receptor from human uterus has been purified to apparent homogeneity by a combination of ammonium sulfate fractionation and affinity chromatography. Affinity resins prepared by conventional means were compared to those prepared by a modified method. The latter gave more reproducible results. A consistent finding was that low capacity resins gave the highest fold purification of the receptor. The pure receptor sedimented at 3.6 S on sucrose density gradient centrifugation, was eluted as a single band by 0.2 M KCl from DEAE-cellulose, and migrated as a single band of molecular weight 42000 on $NaDodSO_4$–polyacrylamide gel electrophoresis. Molecular weight determinations, obtained from Stokes' radii and sucrose gradient centrifugation, the receptors' behavior on ion exchange resins, and hormone binding specificity were all similar to those of the receptor found in crude cytosol. When the crude cytosol receptor was photoaffinity labeled by using ^3H-labeled 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione followed by $NaDodSO_4$–polyacrylamide gel electrophoresis, only protein of M_r 42000 was labeled. This is consistent with our previous findings that alkylation of the pure receptor using 11-deoxycorticosterone bromo[^3H]acetate showed labeling of a single protein of M_r 42000. These properties confirm that the identity and integrity of the receptor have been maintained throughout its purification.

It has become generally accepted that prior to a steroid hormone manifesting a biological response in its target tissues it must first bind to its intracellular cytoplasmic receptor (Jensen & DeSombre, 1973; O'Malley & Means, 1974; Taylor et al., 1980). It is our long-term goal to intefere with progesterone action by designing progesterone antagonists.

To design such agents in a methodical manner, we must first purify the receptor to homogeneity and then determine the topography of the hormone binding site. The most abundant source of the human progesterone receptor is uterine cytosol, which contains only 0.002% of the receptor when expressed as a percentage of total protein. Any attempts to use crude uterine cytosol for progesterone binding site structure determinations, using progesterone derivatives containing alkylating moieties, would be complicated by nonspecific reactions with other proteins. Thus purification of the receptor is a prerequisite for the determination of the structure of the hormone binding site by alkylation of specific amino acid residues.

The receptor is a very labile molecule and is present in very low concentrations in uterine cytosol; therefore, a very efficient purification procedure is required. Fortunately, the receptor has the unique feature of having a high affinity for the progesterone molecule. By immobilizing progesterone on an inert matrix, we can exploit this property and use affinity chromatography as the main method of purification. We previously reported the purification of this particular receptor by affinity chromatography (Smith et al., 1975), but we encountered many problems regarding the reproducibility of this technique. For this reason, it was imperative to refine our methodology, and we now describe a more reproducible method for preparing affinity resins and discuss some of the pitfalls of the conventional methodology. We also compare the properties of the pure receptor with the crude receptor to show the integrity of the pure molecule.

Experimental Procedures

Chemicals. All reagents were of analytical grade. Tris[1] base, ammonium sulfate, urea, and sucrose were purchased from Schwarz/Mann (Orangeburg, NY). Norit A charcoal, dioxane, glycerol, hydrochloric acid, sodium hydroxide, sodium chloride, and potassium chloride were from Fisher Scientific (Pittsburgh, PA). Sepharose 4B, CNBr-activated Sepharose, Sephadex G-75, and Dextran T70 were from Pharmacia (Piscataway, NJ). Acrylamide and Bio-Rad protein assay reagent were from Bio-Rad (Richmond, CA). 11-Deoxycorticosterone hemisuccinate, 11-deoxycorticosterone (DOC) progesterone, estradiol, and cortisol were from Steraloids (Wilton, NH). [^3H]Progesterone, [^3H]R5020, and [^{14}C]-deoxycorticosterone were from New England Nuclear (Boston, MA).

Preparation of Cytosol. All procedures were performed at 4 °C unless otherwise stated. Weighed and rinsed, ice-cold fresh uterine specimens were introduced into a food processor (Waring) with 4 volumes (v/w) of buffer A [10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 12 mM thioglycerol, and 10% (v/v) glycerol]. The tissue was fragmented by 5 × 1 s chopping pulses and allowed to cool for 1 min, and the process was repeated 10 times to yield finely minced tissue suitable for subsequent homogenization with a Tissuemizer (Tekmar, Cincinnati, OH). The Tissuemizer was used at an energy setting of 40 with 5 × 10 s bursts, with a cooling time of 1 min between bursts. The resulting homogenate was centrifuged at 10000*g* for 10 min. Fat was then removed from the surface of the supernatant by aspiration, and the supernatant was centrifuged at 105000*g* for 1 h to furnish cytosol. Progesterone receptors were quantitated in the crude cytosol by Scatchard analysis (1949) with a concentration of 1–40 nM [^3H]progesterone ± 100-fold excess of radioinert progesterone,

† From the Department of Surgery (Urology) and Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, Texas 77030. *Received October 17, 1980.* Supported by the World Health Organization (Grant No. 77005), a postdoctoral grant from the CNR, Italy, to M.I., and a grant from the Southampton Medical Research Fund.

‡ Present address: Department of Zoology, University of Naples, Naples, Italy.

§ Present address: Department of Laboratory Medicine, M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, TX 77030.

[1] Abbreviations used: R5020, 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; DOC, 11-deoxycorticosterone; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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in the presence of 0.1 μM cortisol; a dextran–charcoal assay (Smith et al., 1974) was used, and protein was determined according to Bradford (1976).

Ammonium Sulfate Fractionation. A saturated solution of ammonium sulfate in buffer A was added to cytosol over 30–40 min with gentle stirring until a final concentration of 30% ammonium sulfate was attained. After the mixture was allowed to stir gently for a further 30 min, the resulting suspension was centrifuged at 10000*g* for 15 min. The supernatant was discarded, and the pellet and the insides of the centrifuge tube were washed with buffer A to remove any adhering ammonium sulfate solution. The pellet was then redissolved in 1 volume of buffer A (1 mL/g of uterine tissue) with the aid of a rubber policeman and Teflon/glass homogenizer (Glenco, Houston, TX). The resulting solution containing the partially purified receptor was centrifuged at 200000*g* for 30 min to sediment insoluble material. Progesterone receptors were again quantitated by Scatchard analysis, and protein was measured.

Preparation of Affinity Resin: Procedure I. This procedure was modified from that of Sica et al. (1973). Briefly, 50 mL of a solution containing 20 mg of BSA in 0.2 M $NaHCO_3$ and 8 M urea at pH 9 was mixed with 15 g of CNBr–Sepharose 4B freshly activated with 1 L of 1 mM HCl. The entire mixture was shaken overnight at 4 °C. The gel was recovered by filtration, and the filtrate was assayed spectrophotometrically at 280 nm for protein concentration. The amount of BSA bound was obtained directly from the difference of the input protein and of the protein in the filtrate. In general, each milliliter of activated Sepharose bound approximately 0.3 mg of BSA. The resulting gel was washed with 1 L of 1 M NaCl and 1 L of distilled water. The remaining active CNBr sites on the gel beads were inactivated by shaking the washed gel cake with 50 mL of 1 M ethanolamine in 0.2 M $NaHCO_3$ buffer at pH 9 for 2 h. Following this step, the gel was thoroughly washed with 3 L of 1 M NaCl and 1 L of distilled water. The BSA covalently attached to the Sepharose could be revealed by immersing a small amount of the gel in the Bio-Rad protein reagent. At this stage, the beads show an intense blue coloration where the supernatant should be light brown. A blue coloration in the supernatant was indicative of insufficient washing. The next step was to couple 11-deoxycorticosterone (DOC) hemisuccinate to the BSA–Sepharose. BSA–Sepharose was added to 5 mg of 11-deoxycorticosterone hemisuccinate in 35 mL of dioxane. Then 10 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC) in 15 mL of water was added, and the mixture was shaken at room temperature for 5 h. An additional 10 mg of EDAC in 1 mL of water was added, and the shaking was continued for a further 6 h. The resulting resin was washed thoroughly with 1 L of dioxane, 1 L of water, and 10 L of 80% methanol. The resin was stable for several months in 80% methanol at 4 °C. The amount of DOC bound to 1 mL of resin was determined by base hydrolysis followed by ether extraction of the DOC and subsequent radioreceptor assay using uterine cytosol as a receptor source to quantitate the DOC.

Procedure II. In this procedure, the 11-deoxycorticosterone hemisuccinate was first coupled to BSA by using the technique modified from that described by Erlanger et al. (1957) for preparation of the BSA conjugate of testosterone. In this procedure, 11-deoxycorticosterone hemisuccinate (10 mg, 26.2 μM), tri-*n*-butylamine (0.4 mL, 1.5 nM), and dioxane (15 mL) were introduced in a 50-mL round bottom flask containing a small magnetic stirrer bar. The mixture was stirred in the hood at room temperature, in an atmosphere of nitrogen, for 5 min at which time freshly distilled isobutyl chloroformate (0.2 mL, 1.8 mM) was added. The stirring was continued for a further 30 min, and then the mixture was cooled to 15 °C. A solution of BSA was prepared by adding slowly, with stirring, 1 g of BSA to 55 mL of 0.035 M NaOH. When dissolution was complete, 55 mL of dioxane was slowly added, and the mixture was cooled in the ice bath. The precooled DOC solution was added slowly to the ice-cold alkaline solution of BSA under stirring. Gas evolution was clearly visible at this stage. After 1 h, 1 mL of 1 M NaOH was added and the stirring continued for a further 3 h. At this time, the pH was checked and was between 9 and 9.8. The DOC–BSA conjugate was precipitated by bringing the pH of the solution to 3.5 with HCl to remove nonreactive reagents as well as DOC. The resultant precipitate was gathered by centrifugation (10000*g* × 20 min) and then redissolved in 500 mL of water at pH 7–8. The precipitation step was repeated at least 3–4 times to yield a product free of reagents and DOC. The concentration of DOC covalently bound to BSA was determined spectrophotometrically by using the molar absorbancy of DOC at 250 nM (A_{250nm} = 15 600 M^{-1} L^{-1}) and by using the difference spectrum of the BSA–DOC conjugate and that of BSA alone at an equal protein concentration. The coupling of the DOC–BSA conjugate to CNBr-activated Sepharose 4B was performed in the same manner as described previously for BSA alone. The binding of the DOC–BSA conjugate to CNBr–Sepharose proceeded at almost the same rate as with BSA alone.

Radioreceptor Assay of DOC Bound to Sepharose 4B. One milliliter of DOC–BSA–Sepharose 4B was hydrolyzed with 1 M NaOH at room temperature for 24 h. The hydrolyzed 11-deoxycorticosterone (DOC) was extracted with 3 × 10 mL of ether. The ether extracts were pooled, dried with $MgSO_4$, and then evaporated under nitrogen. The DOC was redissolved in 100 μL of ethanol and 4.9 mL of buffer A. A series of DOC solutions containing 0.1, 0.3, 3, 10, 30, 100, 300, and 1000 ng/mL was prepared. In the calibration curve, each assay tube contained 0.2 mL of the above standard solutions, together with 0.2 ng of [^{3}H]progesterone (55 Ci/mmol) in 0.2 mL buffer A and 0.2 mL of freshly prepared uterine cytosol. The assay tubes were incubated for 18 h at 4 °C. The amount of [^{3}H]progesterone bound to the receptor was measured by using charcoal absorption (Smith et al., 1974) and was plotted vs. the dose of DOC. The amount of DOC in the resin hydrolysate was obtained by inspection of the standard curve.

Binding of Receptor to Affinity Resin (All Steps at 4 °C). One milliliter of affinity gel was washed just prior to use with 3 × 30 mL of 80% methanol and 3 × 30 mL of distilled water. To each milliliter of resin, 30 mL of ammonium sulfate purified receptor was added to a 50-mL sterile disposable centrifuge tube. Binding of the receptor to the resin was achieved by rotating the tube at 1 rpm for 18 h. A control incubation was performed by rotating in the same device an identical tube containing 1 mL of BSA–Sepharose with a similar volume of receptor. After incubation, the gel was separated by centrifugation. Aliquots of supernatants from the control and the affinity resin incubations were quantitated by Scatchard analyses using [^{3}H]progesterone (55 Ci/mmol; 1–40 nM). The amount of receptor bound to the affinity resin was then determined by difference. The resin containing the receptor was washed with 4 × 50 mL of 0.4 M KCl in buffer A, to remove loosely associated proteins, followed by 50 mL of buffer A alone. The receptor was eluted by incubation of the resin for 30 min at 22 °C with 5 mL of buffer A containing 3 μM

[^{3}H]progesterone (5 Ci/mmol). The amount of receptors eluted was quantitated by molecular sieving.

Molecular Sieving Chromatography for Quantitation of Receptor. Sephadex G-75 was stored overnight in distilled water, boiled for 1 h, and then filtered under vacuum. The gel cake was resuspended in 10 volumes of distilled water, then rotated gently for 10 min, and then filtered again. This last process was repeated at least 4 or 5 times until the wash contained no visible residue upon subsequent lyophilization. This thorough washing of the gel is extremely important because Sephadex G-75 is packed with adjuvants such as Dextran, which bind to the [^{3}H]progesterone, which occasionally yield small excluded peaks of radioactivity upon gel filtration of solutions containing ^{3}H-labeled steroids.

Two techniques were generally used to quantitate radioactivity in the excluded peak. The first, termed analytical, used a 10-mL column with a bed volume of 6–10 mL to chromatograph small samples (0.2–0.5 mL). When 0.5-mL fractions were collected, the excluded peaks were usually found in fraction 7 or 8. The second technique, termed preparative, was used to obtain all the excluded radioactivity from the whole sample. A 25-mL glass column was used, and a maximum 5-mL sample was applied to the column. The time necessary to run a larger column results in apparently low yields of receptor. This is due, however, to the dissociation of [^{3}H]progesterone during chromatography of the receptor. We, therefore, always used an analytical column in addition to the preparative column for quantitating the yield of receptor.

DEAE-Cellulose Chromatography. Whatman DE 52 DEAE-cellulose was washed extensively with buffer A until the conductivity and pH of the washes were the same as those of the buffer. The ion-exchange resin was used in a column of dimension 1.3 × 7 cm. The bed height used was 3–5 cm for the crude receptor and 0.5 cm whenever the pure receptor was chromatographed. Receptors were eluted at 4 °C with a linear gradient of 0–0.4 M KCl in buffer A.

Phosphocellulose Chromatography. Whatman P-11 phosphocellulose was equilibrated in buffer A. Column sizes and dilution conditions were similar to those described previously for DEAE-cellulose.

Agarose Gel Filtration. All procedures were performed at 4 °C. Agarose (1.5 M) (Bio-Rad, Richmond, CA) beads in buffer A containing 0.3 M KCl were packed upon a thin layer of sea sand in a 1.6 × 40 cm glass column fitted with a porous glass disk. The column was washed extensively with the above buffer, and a Whatman No. 1 filter was placed on the top of the gel. Receptor samples labeled with [^{3}H]progesterone were placed on the column and eluted with buffer A containing 0.3 M KCl. Fractions (2 mL) were collected and monitored for ^{3}H incorporation. The flow rate was maintained at 10–12 mL/h. Stokes' radii of the receptor were obtained after standardizing the column with the protein standards, human γ-globulin, bovine serum albumin, and ovalbumin according to Siegel & Monty (1966).

Protein Concentration Determination. Protein concentrations were measured directly by the use of the Bio-Rad protein assay reagent with BSA as standard. When the protein concentration was extremely low (less than 1 μg/mL), such as in the eluent from the affinity column, the receptor solution was desalted by using Sephadex G-75 and then lyophilized. $NaDodSO_4$–polyacrylamide gel electrophoresis was then performed on the concentrated receptor in parallel with protein standards in the concentration range 0.2–2 μg. The intensity of the stained bands was compared by densitometry (Quick Scan, Helana Laboratories).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Ten percent acrylamide–$NaDodSO_4$–gel electrophoresis was performed as described by Laemmli (1970). Since the sample eluted from the affinity resin contains very little protein, it was found necessary to concentrate the eluate by lyophilization to achieve protein concentration suitable for $NaDodSO_4$–gel electrophoresis. The lyophilysate was dissolved in 20 μL of a buffer containing 20% glycerol, 5% mercaptoethanol, and 2% $NaDodSO_4$ and then heated for 20 min at 85 °C in a water bath. Electrophoresis was performed at constant current (~2 mA/gel) for 400 min. Gels were fixed overnight in 12% Cl_3CCOOH, stained in 0.05% Coomassie blue R250 for 24 h, and then destained by diffusion for several hours. In experiments in which radioactivity of labeled protein bands was determined, the gels were sliced with clean razor blades into 4-mm slices. The latter were dissolved in hydrogen peroxide (0.5 mL/slice) at 70 °C for 6 h and then counted in Aquasol (4.5 mL) by using a Beckman scintillation counter.

Photoaffinity Labeling of the Progesterone Receptor. Uterine cytosol was incubated with 1, 2, and 10 nM [^{3}H]-R5020 (100 Ci/mmol) overnight at 4 °C in the absence or presence of a 100-fold excess of radioinert R5020 or progesterone. The specific binding was determined by subtracting the latter from the former incorporation of [^{3}H]R5020. Prior to irradiation, excess free R5020 was removed by treating the incubated solution with an equal volume of Dextran-coated charcoal suspension (0.5% charcoal, 0.05% dextran) for 5 min at 0 °C. The sample was irradiated with a UV light (Hanovia, 100-Watt Model 8A36) after the sample was first placed in a glass tube immersed in a 4 °C water bath. The light source was placed 1 in. from the sample tube, and the UV light was filtered through two layers of borosilicate glass to remove all radiation below 300 nm. For prevention of local heating, the 4 °C water bath was stirred with a magnetic stirrer, and condensation was minimized by blowing air between the light source and the glass surface of the water bath. Irradiation was performed for 0, 15, 30, and 60 min.

Results

Comparison of Affinity Resins Prepared by Procedures I and II. In procedure I, BSA was bound to the resin first, followed by coupling of DOC using EDAC. In using the quantitites described under Experimental Procedures, the amount of DOC bound as measured by radioreceptor assay in a useful affinity resin was found to be 0.20–0.75 nmol of DOC/mL of gel. This preparation is of sufficient capacity to bind progesterone receptor and to limit the binding of other proteins which have a much lower affinity for the progesterone molecule. Scatchard plots of the binding data obtained by using this resin of capacity 0.20 nmol of DOC/mL are shown in Figure 1. It can be seen from Figure 1 that 1 mL of this particular resin was not sufficient to bind all of the receptors present. However, when the volume of resin was increased to 4 mL, more than 60% of the receptors were bound. Thus, the receptor can be titrated by using various concentrations of affinity resin. The resins obtained by using preparative procedure I were not as reproducible as we would have liked. The reason for the poor reproducibility appeared to be in the varying quality of the EDAC reagent. The activity of a given batch of EDAC could be maintained by storing the reagent desiccated at −5 °C.

In procedure II, where 11-deoxycorticosterone hemisuccinate was conjugated to BSA, the UV spectrum of the resulting conjugate was changed compared to that of the starting BSA solution (Figure 2). From the molar extinction coefficient of BSA at 280 nm and that of 11-deoxycorticosterone hem-

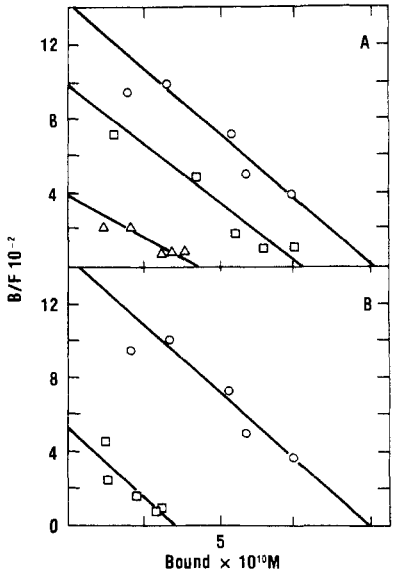

FIGURE 1: Examples of the receptor binding capacities of affinity resins prepared by (A) procedure I with 0.20 nmol/mL of resin or (B) procedure II with 15 nmol/mL of resin. The capacities are compared by performing Scatchard analysis on the breakthrough fraction after incubation for 18 h at 4 °C as described under Experimental Procedures. (○) Receptor solution incubation with 1 mL of Sepharose–BSA as control; (□) a similar solution incubated with 1 mL of Sepharose–BSA–DOC; (▲) incubation with 4 mL of affinity resin.

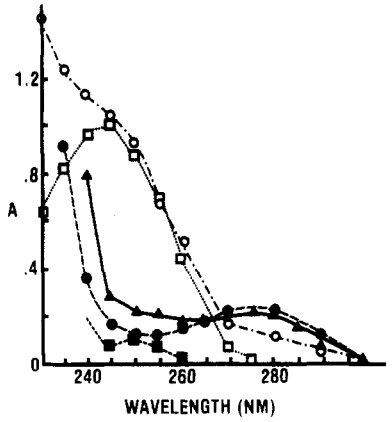

FIGURE 2: Spectroscopic assay for DOC bound to BSA. (●) BSA (0.2 mg/mL) in distilled water; (▲) BSA–DOC conjugate in distilled water (0.2 mg/mL); (■) difference spectrum; (○) a highly conjugated BSA–DOC adduct, (□) absorption spectrum of DOC in distilled water, λ_{max} 248 nM.

isuccinate at 250 nm, it could be calculated that each BSA molecule with 60 potential sites for coupling with 11-deoxycorticosterone hemisuccinate contained two to three steroid molecules. In a different preparation in which the 11-deoxycorticosterone hemisuccinate concentration was increased to 3 mM, 50% of the lysine residues were coupled to DOC (Figure 2). The BSA–DOC conjugates containing up to 30 DOC residues per molecule of BSA coupled readily to CNBr-activated Sepharose.

We have used the resins prepared by procedure II for purification of receptor. This method was preferred since it avoids the use of EDAC, which eliminated certain problems regarding reproducibility. It was found that a conjugation level of one to three steroids per BSA molecule is sufficient for receptor purification when the protein concentration per milliliter of resin is 0.5–1 mg. Figure 1 shows a comparison of receptor binding to this resin compared to that prepared according to procedure I. A lower protein concentration consistently yields resin with too low a capacity for receptor

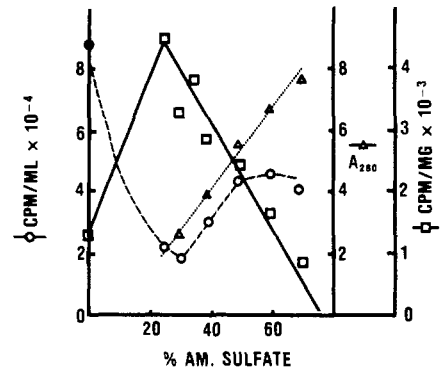

FIGURE 3: Purification of the progesterone receptor by ammonium sulfate fractionation. The ammonium sulfate concentration shown on the abscissa was achieved by adding saturated ammonium sulfate solution to 10 mL of cytosol. The precipitates were collected by centrifugation and the pellets were redissolved in 10 mL of buffer A. Specific progesterone binding was determined by incubating aliquots of the redissolved pellets with 10 nM [^{3}H]progesterone overnight in the presence or absence of 1 μM radioinert progesterone or 1 μM radioinert cortisol to determine progesterone specific binding. (○) cpm/mL of original cytosol; (▲) absorbance at 280 nm which is linearly related to the protein concentration; (□) cpm/mg of protein.

binding in spite of the fact that the steroid concentration is still theoretically high enough to bind receptors. This apparent low functional capacity might be explained by the fact that when BSA was conjugated to 11-deoxycorticosterone hemisuccinate under alkaline conditions, the protein is completely denatured, and conjugation might take place randomly with respect to the 60 sites on the BSA molecule. When the BSA–DOC conjugate is coupled to CNBr-activated Sepharose (pH 9), the coupling might proceed in such a way that many of the lysine residues bound to DOC are unexposed or buried inside the BSA molecule. In this way, the effective steroid concentration of the beads is much less than suggested by spectroscopic analysis. The concentration of DOC per milliliter of Sepharose affinity gel in a functional affinity resin was usually 15 nmol/mL when the gel was prepared by procedure II and only 0.75 nmol/mL with procedure I. These concentrations are orders of magnitude lower than those described by Sica & Bresciani (1979) for the purification of the estrogen receptor (1–2 μmol/mL of resin). In our experience, only the low-capacity affinity resins gave good yields of receptor and high levels of purification. Whenever we used high-capacity resins (20 nmol–2 μmol of DOC/mL of resin), either the receptor could not be eluted or if it was eluted it was eluted together with contaminating proteins such that the purification was generally <200-fold.

Stability of the Affinity Resin. The susceptibility of the ester linkage, which couples the steroid molecule to the inert matrix, toward hydrolysis was investigated during incubations of the resin with crude cytosol receptors or receptors which had been partially purified by using ammonium sulfate fractionation. The affinity resin used in these studies was prepared by using [^{14}C]deoxycorticosterone hemisuccinate. The latter was prepared by esterification of [^{14}C]DOC with acetic anhydride. Aliquots of this resin were incubated overnight at 4 °C with either cytosol or ammonium sulfate purified receptors. After incubation, the receptor solutions were separated from the resin by centrifugation, and the supernatants were extracted with chloroform. The extracts were dried, transferred to scintillation vials, evaporated, and then assayed for measurement of ^{14}C. A comparison of the cytosol with the ammonium sulfate fractionated receptors showed that the former had released 60% of the ^{14}C-labeled deoxycorticosterone from the affinity matrix whereas the latter had released

Table I: Affinity Chromatography Purification of the Human Progesterone Receptor[a]

purification step	total protein[b] (mg)	total receptor sites[c] ($\times 10^{-7}$ dpm)	sp act.[d] (dpm/mg)	yield (%)	purification (fold)
cytosol	2082	7.495	3.6×10^4	100	1
ammonium sulfate	69.2	1.522	2.2×10^5	20	6
affinity eluate	~0.0001–0.0015	0.295	2.4×10^9	4	~67 000

[a] Receptors were quantitated in cytosol, ammonium sulfate (30%) precipitations, and the breakthrough function from the affinity resin by Scatchard (1949) analyses using [^{3}H]progesterone (55 Ci/mmol). Protein was determined according to Bradford (1976). The theoretical specific activity of the pure receptor is 3.05×10^9 dpm/mg of protein. [b] Protein was determined by the Bradford procedure (1976) except on the affinity eluate where total protein was estimated visually from the relative staining of 0.2–2 μg of protein standards on $NaDodSO_4$–polyacrylamide gels as described under Experimental Procedures. [c] Receptor sites were determined by using a Scatchard plot, or by total disintegrations per minute of macromolecular progesterone binding in the affinity eluate. [d] Theoretical maximum specific activity is estimated as 3.05×10^9 dpm on the basis of a molecular weight of 42 000.

only 2% of the [^{14}C]deoxycorticosterone.

Purification of the Receptor. The receptor was purified by using a combination of ammonium sulfate fractionation and affinity chromatography, approximately 67 000-fold. Figure 3 shows a profile of the ammonium sulfate purification, indicating that a 6-fold purification of the receptor can be achieved by precipitation at a concentration of 25–30% ammonium sulfate. The yield of the receptor was generally 20–25%. Subsequent affinity chromatography resulted in a further 11 000-fold purification in 19% yield (Table I). When this particular resin was used, only 25% of the receptor was retained for purification; thus the corrected yield is 76%. The cytosol containing nonadsorbed receptor can be immediately recycled over fresh resin for a subsequent purification cycle. No significant additional purification of the affinity-chromatographed receptor could be achieved by ion-exchange chromatography on DEAE-cellulose; rather this step resulted in a loss of 90% of the receptor–progesterone binding activity. The affinity column eluate was homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis, showing a single band of M_r 42 000 (Figure 4). On the basis of this molecular weight and a specific activity of 55 Ci/mmol for [^{3}H]progesterone, the specific activity of the pure protein would be 3.05×10^9 dpm/mg of protein. On this basis, the receptor preparation is 79% pure; however, this does not account for any receptor denatured during elution from the affinity column which no longer has the capacity to bind progesterone. The limited accuracy of measuring the protein concentration of the affinity column eluent may also result in the underestimation of purity.

Hormone Specificity and Affinity of the Pure Receptor. The specificity of the protein eluted from the affinity column was shown in two ways. First, after binding the receptor to the resin followed by copious washing, elution using 2 μM concentrations of [^{3}H]progesterone, [^{3}H]R5020, [^{3}H]dihydrotestosterone, or [^{3}H]estradiol at room temperature for 30 min was attempted on aliquots of the resin. Only the aliquots eluted with [^{3}H]progesterone or [^{3}H]R5020 gave a peak of macromolecular bound radioactivity on subsequent gel filtration. In the second method, the receptor was eluted from the affinity gel with 3 μM [^{3}H]progesterone (specific activity 5 Ci/mmol). Radioinert progesterone at a concentration of 3 and 6 μM, estradiol at 7 μM, and cortisol at 6 μM were added to aliquots of the progesterone-labeled receptor, and these aliquots were incubated at room temperature for 20 min. Gel filtration was then performed on each aliquot to quantitate the amount of [^{3}H]progesterone bound to the receptor. Figure 5 shows that only radioinert progesterone displaces [^{3}H]progesterone from the receptors.

To further characterize the affinity column eluent, it was necessary to determine whether the specific progesterone binding was of high affinity and limited capacity. The eluant was chromatographed on Sephadex G-75 to remove free

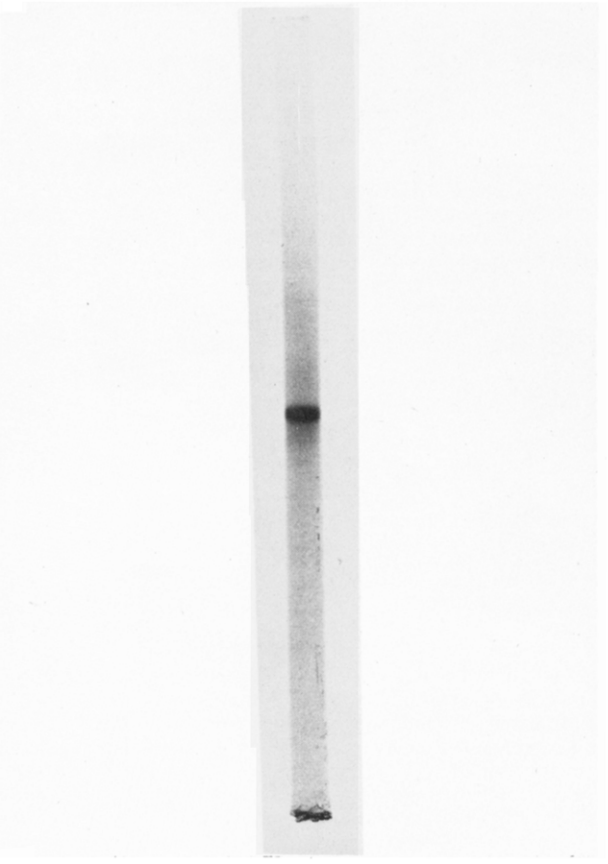

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of the pure receptor (~1.5 μg). The pure receptor in the affinity column eluate was concentrated and submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis as described under Experimental Procedures. An accompanying gel was run at the same time containing the following molecular weight standards: bovine serum albumin (M_r 68 000), ovalbumin (45 000), chymotrypsinogen (25 000), and cytochrome *c* (17 500). The log of the molecular weight of the standards was plotted vs. their relative mobility in the gel. The resulting straight line through these points was used to estimate the molecular weight of the stained band from its relative mobility. The band shown migrated at a molecular weight of 42 000.

[^{3}H]progesterone (5 Ci/mmol). The excluded peak containing 2 nM [^{3}H]progesterone was incubated overnight at 5 °C in the absence or presence of 2, 3, 5, 25, and 60 nM radioinert progesterone. The [^{3}H]progesterone bound was then determined by using a modified dextran-coated charcoal assay (Smith et al., 1974) with an incubation time of 1 min. Although this type of assay underestimates receptor binding at low protein concentrations and therefore cannot be used for accurate quantitations, it was chosen as the most efficient method for assaying the six duplicate assay points. The results represented in Figure 6 show that 50% of [^{3}H]progesterone

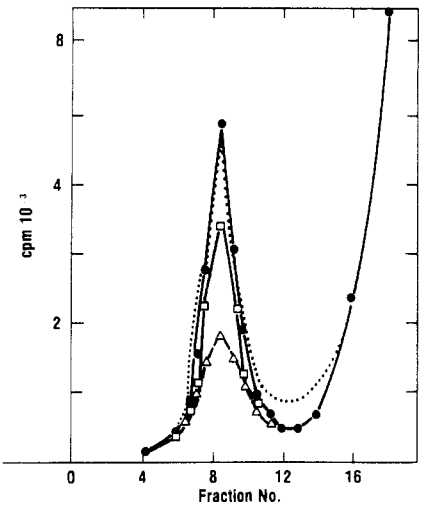

FIGURE 5: Exchangeability of [^{3}H]progesterone bound to the pure receptor determined by gel filtration. (●) Excluded radioactivity of receptor eluted from affinity resin with 3 μM progesterone (5 Ci/mmol); (□) exchange reaction using (22 °C/20 min) a further 3 μM unlabeled progesterone; (△) as before but with 6 μM progesterone added; (---) elution profile obtained after exchange of [^{3}H]progesterone with either 6 μM cortisol or 7 μM estradiol.

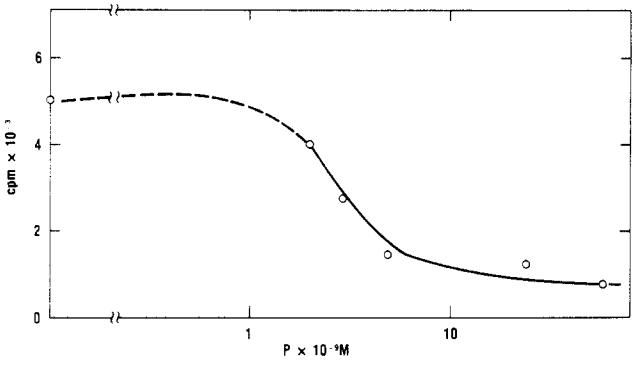

FIGURE 6: Displacement of [^{3}H]progesterone by various concentrations of unlabeled progesterone (P). After elution from the affinity resin, all excess progesterone was removed by gel filtration, the excluded peaks were pooled, and various aliquots were incubated with either 0, 2, 5, 25, or 65 nM unlabeled progesterone overnight at 4 °C in duplicate. Free steroid was then removed by a 1-min incubation with DCC as described in the text. Macromolecular bound [^{3}H]progesterone is plotted vs. log concentration of competitor.

binding is displaced by as little as 3 nM radioinert progesterone, showing that the affinity column eluent contains high-affinity saturable binding.

For comparison of [^{3}H]progesterone binding in the eluent with binding to a nonspecific binding protein, BSA was incubated with 3 μM [^{3}H]progesterone for 30 min at 22 °C. The specific activity of the binding to the affinity column eluent was 2.4×10^9 dpm/mg of protein whereas with BSA the specific activity was 1.8×10^4 dpm/mg of protein. The ratio of these specific activities is consistent with the relative equilibrium dissociation constant of the progesterone receptor (3 nM; Smith et al., 1975) and BSA (10 μM; Westphall, 1971).

Physical Properties of the Progesterone Receptor. The pure receptor was applied to a phosphocellulose column. Only 10% of the receptor was retained by the column. When subjected to salt gradient elution (0–0.4 M KCl), this proportion was eluted at 0.2 M KCl. The receptor–[^{3}H]progesterone complex which was not retained by phosphocellulose was applied to a DEAE-cellulose column and subjected to elution using a salt gradient (0–0.4 M KCl). The receptor–[^{3}H]progesterone complex was eluted at 0.2 M KCl. The receptor prior to

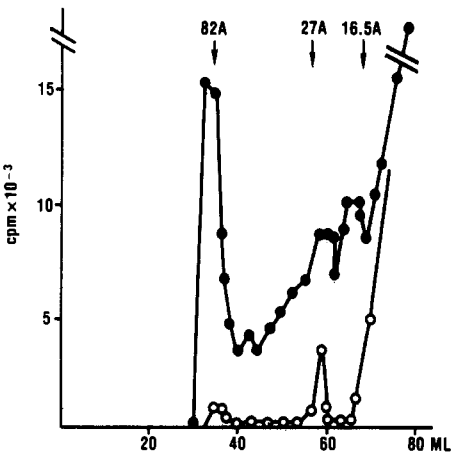

FIGURE 7: Gel filtration of progesterone receptors on 1.5 M agarose. (●) Crude progesterone receptors showing proteins having Stokes radii of 82, 27, and 16.5 Å. The percentage of [^{3}H]progesterone displaced from each of these peaks by a 200-fold excess of radioinert progesterone was 68%, 55%, and 40%, respectively. (○) Affinity column eluent containing the pure receptor with a Stokes radius of 28 Å. The crude receptors were labeled by using 16 nM [^{3}H]progesterone (100 Ci/mmol) overnight at 4 °C. The affinity column eluent contained 3 μM [^{3}H]progesterone (5 Ci/mmol). The Stokes radii were obtained by using the following protein standards: human γ-globulin, bovine serum albumin, and ovalbumin as described by Siegel & Monty (1966). The peak of Stokes' radius 16.5 Å was not observed in all specimens of uterine cytosol.

purification shows a similar elution profile on DEAE-cellulose chromatography. NaDodSO$_4$–polyacrylamide gel electrophoresis on the various fractions eluted from the ion-exchange columns showed that the major band at a molecular weight of 42 000 was not retained by phosphocellulose but was retained by DEAE-cellulose.

Crude uterine cytosol and the pure receptor were chromatographed on a 1.5 × 50 cm 1.5 M agarose column (Figure 7). Stokes' radii, frictional ratios, and molecular weights were determined on the specific progesterone binding proteins according to Siegel & Monty (1966). The crude receptor preparations showed peaks of macromolecular bound [^{3}H]-progesterone of Stokes' radii 16.5, 27, and 82 Å, which corresponded to molecular weights of 25 500, 42 000, and 247 000, respectively. The latter value was obtained based on a sedimentation coefficient of 7 S whereas the calculation for the smaller molecules was based on a more abundant 3.6S peak (Janne et al., 1975; Smith et al., 1975). All three peaks could be displaced by radioinert progesterone; however, the smaller molecule of Stokes' radius 16.5 Å could not be detected in all preparations of uterine cytosol. The pure receptor chromatographed with a Stokes radius of 28 Å, corresponding to a molecular weight of 43 000. The frictional ratios in crude cytosol were 2.0, 1.2, and 0.9. The pure receptor gave a calculated frictional ratio of 1.2, corresponding to a major component of progesterone binding in crude cytosol. It was not possible to confirm the molecular weight estimates by polyacrylamide gel electrophoresis under nondenaturing conditions in the presence of [^{3}H]progesterone since both crude receptor and the pure receptor aggregated at the top of the gel.

Photoaffinity Labeling of the Progesterone Receptor with [^{3}H]R5020. Since we were unable to confirm that progesterone was bound to the pure protein of M_r 42 000 by NaDodSO$_4$–polyacrylamide gel electrophoresis under nondenaturing conditions, we attempted to affinity label the receptor in a specific manner and then determine the molecular weight of the progesterone binding macromolecule under denaturing

Table II: Photoaffinity Labeling of the Crude Cytosol Receptor after Cytosol Was Incubated Overnight at 4 °C with the Appropriate Concentration of [^{3}H]R5020[a]

	time of irradiation (min)	cpm at [^{3}H]R5020 concentration of								
		1 nM			2 nM			10 nM		
		T	NS	S	T	NS	S	T	NS	S
(a)	0	5719	1632	4085	7050	1498	5552	15505	3601	11904
	15	752	395	357	116	763	373	5019	3819	1207
	30	564	537	27	1156	1085	71	5304	5014	290
	60	904	1018	−114	2021	1908	113	9584	9087	497
(b)	0	5710	1632	4085	7050	1498	5552	15505	3601	11904
	15	528	159	369	1205	206	1001	2569	663	1906
	30	510	166	399	966	202	764	2299	540	1754
	60	507	148	359	1003	210	793	2321	687	1734

[a] T = total binding; NS = nonspecific binding which was determined by incubating the [^{3}H]R5020 in the presence of a 100-fold excess of radioinert R5020; S = specific binding (T − NS); all were determined by Dextran-charcoal (DCC) with an incubation time of 5 min at 4 °C. Experiments in (a) were performed by UV irradiation of the incubation mixture prior to treatment with DCC; in (b), samples were treated with DCC prior to UV irradiation.

conditions. The progestin R5020 has a high affinity for the progesterone receptor, and its λ_{max} of 320 nm, which is substantially displaced from the λ_{max} of proteins, makes it an ideal substrate for the photoaffinity labeling of the receptor. When the 100000*g* uterine cytosol was labeled with [^{3}H]R5020 at 1, 2, and 10 nM concentrations, overnight at 4 °C, and then irradiated with UV light as described under Experimental Procedures, results such as shown in Table IIa were obtained. Irradiation was found to decrease both total binding and nonspecific binding, with the total binding decreasing at a more rapid rate than the nonspecific binding. After 30 min of irradiation, specific binding approached zero in all cases. Further irradiation increased both total and nonspecific binding at a similar rate.

We have ruled out heat produced by irradiation as a cause of the loss in specific binding because the temperature of the irradiated samples was maintained close to 0 °C with an ice bath. A light-shielded receptor–R5020 sample, irradiated under the same condition, suffered no loss in specific binding. We also found that an irradiated R5020 sample (30 min) can no longer bind to the progesterone receptor. For this reason, it is very likely that the decrease in both specific and nonspecific binding at the initial stage of the UV irradiation was due to photodecomposition of bound R5020 (both specific and nonspecific). The products of irradiation of R5020 are free radicals which react covalently with molecules found in their vicinity. Thus, when R5020 is in excess, these free radicals can interact with many proteins in cytosol, hence increasing the amount of nonspecific labeling upon prolonged irradiation (Table IIa). When excess R5020 (in the R5020 receptor complex) was removed with charcoal prior to irradiation, we observed a similar initial drop in both the total and nonspecific binding as had been seen earlier. However, following this, both types of binding remained stable for at least 60 min (Table IIb). The specific binding did not decrease appreciably in the 15–60-min irradiation interval. Under the condition of irradiation in which excess R5020 was removed, it is very likely that a majority of the reactive photoproducts of R5020 were formed in the vicinity of a progesterone receptor molecule because both low temperature and short irradiation times would ensure that most R5020 and receptor complexes were not dissociated. The generated free radicals will form covalent bonds with the receptor molecule. Figure 8 shows a radioactivity profile of $NaDodSO_4$–polyacrylamide gel electrophoresis of uterine cytosol labeled with R5020 and UV irradiated in the absence of excess free R5020. It can be seen that the R_f of the radioactivity indicated that a macromolecule of molecular weight close to 42 000 has been labeled by R5020.

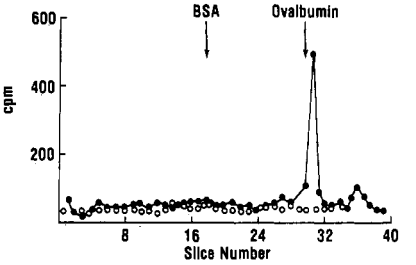

FIGURE 8: $NaDodSO_4$–polyacrylamide gel electrophoresis of crude cytosol after incubation with [^{3}H]R5020 followed by UV irradiation. Performed as described under Experimental Procedures, Specimens (100 μL) obtained from duplicate incubations using 100 nM [^{3}H]-R5020 in the absence or presence of 1 μM radioinert R5020 were applied to the gel. The covalently attached R5020 were 2900 cpm for the former (●) and 1200 cpm for the latter (○).

A duplicate irradiation, also shown in Figure 8, in the presence of a 100-fold excess of radioinert R5020 shows that the binding is specific. Similar molecular weight estimates were obtained when the pure receptor was run on $NaDodSO_4$–polyacrylamide gels, which suggested that the integrity of the human progesterone receptor had been retained throughout purification.

Discussion

Purification of the human progesterone receptor has been achieved by using a combination of ammonium sulfate fractionation and affinity chromatography on low-capacity resins (0.20–15 nmol/mL) prepared by two different methods. The second method, where the steroid ligand is linked to BSA prior to immobilizing the BSA to the Sepharose matrix, gave similar purifications to the first method but proved to be more reproducible. The reasons for the better reproducibility are 2-fold. First, the use of the reagent EDAC, which appears to vary in its reactivity in this system, is avoided. Second, since relatively large amounts of BSA–DOC adduct can be prepared at one time and then stored, we could maintain the quality of subsequently prepared resins. This is most important since not only will the ratio of BSA–DOC in the adduct be constant but so will the amount of steroid accessible to the receptor. The latter is particularly important since this parameter can vary, and in many resins, only approximately 1% of the steroid is sufficiently exposed to allow binding to receptors (Smith & Schwartz, 1979).

The major reasons for our success in attaining high levels of purification with affinity chromatography were in the use of low levels of steroid ligand immobilized on the inert matrix and in the use of small volumes of resin. The former only permitted proteins with high affinity for progesterone to bind

Table III: Comparison of the Physical Properties of the Receptor in Crude Cytosol with Those of the Pure Receptor[a]

physical property	crude cytosol	pure receptor
sedimentation coefficient[b] (S)	7, 3.6	3.6
equilibrium dissociation constant (nM)	3[b]	
steroid binding specificity	R5020, P > DHT, E_2, F[c]	R5020, P > DHT, E_2, F
nuclear translocation in vitro	yes[b]	yes[b]
DEAE-cellulose binding	eluted 0.2 M KCl	eluted 0.2 M KCl
Stokes' radius, R_s (Å)	82, 27, 16.5	28
mol wt from R_s and s value	247 000, 42 000, 25 000	43 000
mol wt by $NaDodSO_4$–polyacrylamide gel electrophoresis	42 000[d]	42 000[d]
frictional ratio	1.98, 1.17	1.21

[a] P = progesterone; DHT = 5α-dihydrotestosterone; E_2 = 17β-estradiol; F = cortisol. [b] Properties from Janne et al. (1975) and Smith et al. (1975). [c] From Smith et al. (1974). [d] Molecular weights determined by affinity labeling followed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

to the immobilized steroid, and the latter allowed rapid and efficient washing to remove proteins loosely associated with the resin matrix. To show the generality of the method, we have used similar techniques to purify the hen oviduct nuclear estrogen receptor to homogeneity (Smith & Schwartz, 1979; Taylor & Smith, 1979). Our methodology is in contrast to that described by Sica & Bresciani (1979), who reported the successful purification of the calf estrogen receptor by using a high-capacity affinity resin containing 1–2 μmol of estrogen/mL of resin. At these concentrations, proteins having a relatively low affinity for estradiol would be expected to be retained by the resin. Thus their success must have depended greatly on the special affinity of the calf estrogen receptor for heparin–Sepharose, which was used as a preliminary purification step. Such an affinity for heparin–Sepharose was not evident with the human progesterone receptor. The other major problem generally encountered that was not addressed by these authors is the difficulty in elution of receptors from high-capacity resins. Since elution is accomplished with excess steroid, the limit of solubility of the steroid becomes an important parameter. These authors, however, lessened the problem by the use of chaotropic buffers to increase both the solubility of the steroid and the dissociation rate of the receptor. Because the success of their particular method appeared to rely heavily on specific properties of the biologic system, we feel that for general purposes better success will be achieved by using the lower capacity resins described herein.

To confirm that we had indeed purified the receptor rather than some nonspecific protein, we compared the properties of the receptor in crude cytosol with the alleged pure receptor. These properties, which are summarized in Table III, are consistent with the fact that the progesterone binding protein found in cytosol is identical with that of the purified form. The low molecular weight progesterone binding protein (M_r 25 000) was not found consistently in all cytosol preparations and may reflect the heterogeneity of the human uterine specimens. Crude cytosol, in addition to protein of M_r 43 000, always contains aggregated forms of the receptor (7 S; M_r 247 000; 82 Å) which do not exist when the R5020 photoaffinity labeled receptor is denatured on $NaDodSO_4$–polyacrylamide gels when only a peak of M_r 42 000 is evident. Dure et al. (1980) successfully photoaffinity labeled the crude chick progesterone receptor in a similar manner. However, in addition to labeling of a protein of M_r 39 000, receptor proteins of M_r 106 000 and 76 000 were labeled. Because a major protein band was evident at a molecular weight of 39 000, these investigators considered that this peak was probably not receptor-like but a contaminant. The molecular weight of the pure receptor is at variance with that (M_r 100 000) reported by us previously (Smith et al., 1975). This lower molecular weight species that we now report may prove to be the result of proteolytic degradation of the high molecular weight form similar to that described previously for the chick oviduct progesterone receptor (Sherman et al., 1976; Vedeckis et al., 1980). Indeed the pure receptor described by us has identical molecular weight, Stokes' radius, and sedimentation coefficient to the type IV chick oviduct progesterone receptor. Whether the type IV receptor is indeed the biologically active form of the human receptor or whether it is a product of receptor mediated down regulation of hormone action remains to be established. Regardless of its function, the receptor in its present form is still a suitable substrate for affinity labeling studies since its progesterone binding site is still intact. In fact, although we were unsuccessful in photoaffinity labeling the pure receptor with [^{3}H]R5020, we were successful in affinity labeling the pure receptor by displacing progesterone with 11-deoxycorticosterone bromo[2-^{3}H]acetate (Smith et al., 1978; Holmes et al., 1981). After the receptor was denatured and $NaDodSO_4$–polyacrylamide gel electrophoresis was performed, a single band of radioactivity migrated with the single band of M_r 42 000 (Smith et al., 1978; Holmes et al., 1981). The above results indicate that the pure product is indeed the progesterone receptor previously described in human uterine cytosol (Janne et al., 1975; Smith et al., 1974, 1975).

Acknowledgments

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Comparison of the Interactions of a Specific Neurotoxin (α-Bungarotoxin) with the Acetylcholine Receptor in *Torpedo californica* and *Electrophorus electricus* Membrane Preparations†

Pierre Leprince,‡ Richard L. Noble,§ and George P. Hess*

ABSTRACT: α-Bungarotoxin, a snake neurotoxin, binds irreversibly and specifically to the acetylcholine receptor isolated from the electroplax of *Electrophorus electricus* and *Torpedo* species and has been an important tool in the study of the receptor–ligand binding mechanism. Two distinct kinetic processes have been observed in studies with membranes from *E. electricus*. A minimum mechanism for the toxin reaction involves (i) the reversible binding of two toxin molecules to the receptor prior to the irreversible formation of toxin:receptor complexes and (ii) a toxin-induced conformational change of the receptor which leads to an increase in the affinity of the receptor binding sites for toxin [Hess, G. P., Bulger, J. E., Fu, J.-j. L., Hindy, E. F., & Silberstein, R. J. (1975) *Biochem. Biophys. Res. Commun. 64*, 1018–1027]. Only one process has been detected in *Torpedo* membranes. Here, we determine whether the receptors in *Torpedo californica* and *E. electricus* membranes have different properties or whether the measurements and their interpretation were responsible for the different results. Two methods which are frequently used in binding studies to separate free and bound toxin, a CM-52 cellulose minicolumn assay and DE-81 filter disk assay, have been compared. The results obtained indicate that the interaction of toxin with receptor from *T. californica* is similar to that observed with receptor from *E. electricus*. The apparent differences which have been reported in the literature are shown to have arisen from the design of the experiments in which *T. californica* membranes were used.

The binding of an activating ligand to the acetylcholine receptor protein is a triggering event for the initiation of electrical signals in nerve and muscle cells. Many studies have been directed toward the characterization of this interaction, and snake neurotoxins that specifically inhibit the nicotinic acetylcholine receptor have been essential tools in this field (Chang & Lee, 1963; Weiland et al., 1976; Blanchard et al., 1979; Weber & Changeux, 1974; Maelicke et al., 1977; Brockes & Hall, 1975; Bulger & Hess, 1973; Hess et al., 1975; Bulger et al., 1977; Quast et al., 1978). In many studies, a toxin from *Bungarus multicinctus*, α-bungarotoxin, is used because it binds irreversibly to the receptor of muscle cells and of the electric organ of *Electrophorus electricus* and *Torpedo* species, and it competes with receptor–ligands for this binding (Albuquerque et al., 1979). Many different results and interpretations have been reported for the interaction of this toxin with the receptor in *E. electricus* and *Torpedo* species membrane fractions.

On the basis of electrophysiological measurements, Katz & Thesleff (1957) suggested that exposure of the receptor to acetylcholine induces a conformational change and results in an increase in the affinity of the receptor toward this ligand. A reaction that consists of two phases and results in an increase in the affinity of the receptor toward the ligand has been observed in the binding of α-bungarotoxin to both soluble and membrane-bound receptor protein isolated from *Electrophorus electricus* [Hess et al., 1975; for a review, see Heidmann & Changeux (1978) and Eldefrawi & Eldefrawi (1979)], and in the receptor-controlled translocation of inorganic ions across the plasma membrane of vesicles prepared from *E. electricus* (Hess et al., 1978, 1979, 1980; Aoshima et al., 1980; Cash & Hess, 1980; Cash et al., 1980). A preliminary investigation of the reaction of α-bungarotoxin with the receptor in *T. marmorata* membranes indicated a simple bimolecular process preceded by a fast initial phase of low amplitude (Franklin & Potter, 1972).

The exception to these results appeared to be the reaction of α-bungarotoxin with the membrane-bound acetylcholine receptor isolated from the electric organ of *T. californica* (Quast et al., 1978). In these investigations covering a range of initial toxic concentrations, only a simple first-order reaction

† From the Section of Biochemistry, Molecular and Cell Biology, Division of Biological Studies, Cornell University, Ithaca, New York 14853. *Received December 10, 1980; revised manuscript received April 7, 1981.* This work was supported by grants from the National Institutes of Health (GM 04842 and NS 08527) and the National Science Foundation (PCM 78-09356) awarded to G.P.H.; a National Institute of Health Cancer Center grant (CA 14454) and a National Institutes of Health training grant (5 T32 GM0723) provided financial support for P.L.

‡ Present address: Biochimie Générale et Comparée, Université de Liege, Belgium.

§ Recipient of a Muscular Dystrophy Association fellowship. Present address: Diabetes and Endocrinology Research Center, University of Iowa, Iowa City, IA 52240.

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