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Purification and Characterization of a Steroid Receptor from Chick Embryo Liver†

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ABSTRACT: The high-speed supernatant fraction of the 16-day-old chick embryo liver homogenate was found to contain a macromolecular species that could form tight complexes with steroid hormones. This hormone receptor was purified by isoelectric precipitation, high-speed centrifugation, DEAE-cellulose column chromatography, and preparative disc electrophoresis. The purified receptor migrated as one diffuse band on analytical acrylamide gel electrophoresis and had an isoelectric point of 5.3, as shown by electrofocusing. The hormone–receptor complex sedimented as a single 4S species in sucrose gradient centrifugation in several concentrations of KCl. The binding of hydrocortisone to the receptor has a temperature optimum at 37°, and saturation is reached within 10 min of incubation. The binding was insensitive to changes in pH within the range of 4.5–9.4. By the use of equilibrium dialysis it was estimated that the association constant for the binding of hydrocortisone was 7.5×10^8 M^{-1} at 4°, while that for the binding of corticosterone was 3.6×10^8 M^{-1} at 4°. The protein nature of the receptor was suggested by its susceptibility to proteolytic enzymes. Estradiol and testosterone were found to bind to the crude receptor preparation (the high-speed supernate). This binding, however, would not reach saturation even when high concentrations of hormones were used. In addition, neither estradiol nor testosterone would compete with the binding of hydrocortisone or corticosterone by the receptor. After the receptor was purified by preparative disc electrophoresis, the binding of hydrocortisone and corticosterone was at least 3000-fold higher than that of estradiol and testosterone, when compared in the range where the binding of hydrocortisone was linear with respect to hormone concentrations.

Several approaches have been used for the elucidation of the mechanism of hormone action. Injection of radioactive hormone into animals showed concentration of radioactivity in specific target organs (Bellamy *et al.*, 1962; Litwack and Baserga, 1967). Induction of RNA and protein synthesis after *in vivo* injection of hormone into an animal has also been demonstrated (Kenney, 1962; Schimke *et al.*, 1965; Teng and Hamilton, 1968).

The specificity of action manifested by the hormone is incompatible with its being a low molecular weight substance. It has been hypothesized (Jensen and Jacobson, 1960) that the target tissue may play an important role in the final physiological manifestation of hormone action. More recently, receptor molecules for specific hormones have been isolated from their target organs. Such a receptor was initially isolated from the rat or calf uterus, which bound estrogen hormones (Toft and Gorski, 1966; Jensen *et al.*, 1967; Erdos, 1968; Puca *et al.*, 1971). The hormone–receptor complex was isolated from the soluble supernate of the uterine homogenate and was found to sediment as an 8S molecule on sucrose gradient. This 8S molecule dissociated into a 4–5S species upon treatment with 0.3 M KCl. Similar receptor molecules have been reported for other steroid hormones (Edelman and Fimognari, 1968; Gardner and Tomkins, 1969; Sherman *et al.*, 1970). There is evidence that these receptor molecules enter the nucleus of the target tissue in the presence of hor-

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mone (Shyamala and Gorski, 1969; Musliner *et al.*, 1970; O'Malley and Toft, 1971).

The definition of the role of such a receptor in the overall process of hormonal regulation must await the thorough purification of the receptor and the subsequent elucidation of the mechanism of its interaction with the specific hormone on one hand and the target cell on the other. Partial purification of such receptors from rat uterus cytosol (DeSombre *et al.*, 1969) and hepatoma cells (Gardner and Tomkins, 1969) has been carried out. We now report the isolation and purification of a receptor for corticosteroids from 16-day-old chick embryo livers. Analytical acrylamide gel electrophoresis reveals the purified hormone receptor as one diffuse band. The purified receptor sediments exclusively as a 4S molecule in sucrose gradient both in the presence and absence of salt. Some of its properties and binding behavior with various steroid hormones are also presented.

Materials and Methods

Source of Hormone Receptor. Chick embryos (16 days old) were obtained from Truslow Farms, Chestertown, Md. The embryos were dissected midventrally to obtain the livers. The livers were blotted on tissue paper to free them of excess blood before being pooled into the standard buffer of 0.3 M sucrose–0.0015 M EDTA–0.02 M Tris-HCl, pH 7.6. For each preparation, 36–48 chick embryos were used, yielding a wet weight of 10–12 g of liver. The isolation of the receptor is described in the Receptor Purification Procedure later in this section.

Other Proteins. Digestive enzymes were obtained from Worthington Biochemical Corp. Purified neuraminidase from *Clostridium perfringens* was the gift of Dr. Ed McGuire of this department. Protein molecular weight markers were purchased from Mann Research Laboratories, Inc.

Steroids. Unlabeled steroids were obtained from Mann Research Laboratories, Inc. [1,2-^{3}H]Hydrocortisone (12–44 Ci/mmol), [1,2-^{3}H]corticosterone (39.1 Ci/mmol), [6,7-^{3}H]-estradiol (31.7 Ci/mmol), and [6,7-^{3}H]testosterone (44 Ci/mmol) were purchased from New England Nuclear. All radioactive hormones supplied in benzene–methanol (9:1) solution were first evaporated to dryness and redissolved in absolute or 50% ethanol before use. Radiochemical purity of the steroids was periodically checked by paper chromatography, using benzene–methanol–water (4:2:1) or benzene (paper treated with 30% formamide in acetone) as the solvent systems (New England Nuclear brochure).

Measurement of Radioactivity. Radioactivity was measured with a Packard Tri-Carb scintillation spectrometer Model 2002. Tritium efficiency was 25% for 1.5 ml of aqueous sample in 10 ml of Bray's fluor.

Sucrose Gradient Centrifugation. Linear sucrose gradients (5–20%) were prepared with a Buchler gradient maker. Ultrapure sucrose was used (Schwarz/Mann Co). Prior to centrifugation, incubation of the liver receptor or plasma hormone receptor with radioactive hormone was carried out for 20–30 min at 37°. Centrifugations were performed at 4° in a Beckman Model L ultracentrifuge, using the Spinco SW 50L rotor. Fractions of 0.1–0.2 ml were collected after centrifugation and assayed for radioactivity. The sedimentation coefficient of the liver hormone receptor was determined, using bovine serum albumin (4.6 S), γ-globulin (6.5 S), and yeast alcohol dehydrogenase (7.4 S) as standards.

Electrofocusing. A LKB-8100 ampholine electrofocusing apparatus was used. Electrofocusing was carried out at 0–2° in a 110-ml ampholine column with a solution of 1% ampholytes in a 0–50% sucrose gradient. The receptor–tritiated hydrocortisone complex was focused for 72 hr at 250–400 V in a pH gradient (pH 3–10), and 2-ml fractions were collected at the end of the run. The pH was measured at 0° and 1-ml aliquots were used to determine radioactivity.

Acrylamide Gel Electrophoresis. Analytical acrylamide gel electrophoresis was carried out after the method of Davis (1964). Running gel (7.5%) was used. In general, no stacking gels were used. Electrophoresis was run at 1 mA/tube. Preparative disc electrophoresis was performed on a Shandon preparative acrylamide apparatus. The gel column was made up of 60 ml of 7.5% acrylamide gel mixture (Davis, 1964). The electrode buffer consisted of 6 g of Tris and 14.4 g of glycine/l. of water, pH 8.4. The elution buffer consisted of 52 g of Tris and 14 ml of glacial acetic acid/l., pH 8.2. In some experiments, the gel was preelectrophoresed overnight at 50–100 V with elution buffer.

In Vitro Standard Binding Assay. Throughout the isolation and purification procedure, the hormone receptor was assayed as a tritiated hydrocortisone–macromolecule complex. To 0.2 ml of sample was added 3.5×10^{-3} μg of tritiated hydrocortisone. The mixture was incubated at 37° for 10 min. The sample was then applied onto a small Sephadex G-50 column (1.8 × 24 cm) at 24° to separate the hormone–receptor complex from free hormone. The excluded volume was collected into scintillation vials and counted for radioactivity. Specific activity of the hormone receptor throughout the purification steps was expressed as disintegrations per minute of tritiated hormone bound per milligram of protein. Alternatively, specific activity was expressed as picomoles of hormone bound per microgram of protein. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Equilibrium Column Analysis. A Sephadex G-50 column (1.2 × 28 cm) was equilibrated with 1.2 pmol/ml of tritiated hydrocortisone. The hormone receptor was incubated with a final concentration of tritiated hydrocortisone equal to that in the equilibrated column. The complex was layered onto the column and eluted with 0.01 M Tris-HCl, pH 7.4, with 1.2 pmol/ml of tritiated hydrocortisone. Fractions (1 ml) were collected and assayed for radioactivity.

Equilibrium Dialysis. Equilibrium dialysis was carried out in double-chambered glass cells with a screw cap on each end. A volume of 2.5 ml was used on each side of the membrane. The screw caps were lined with Teflon which was previously tested and found to adsorb less than 1% of the total radioactive hormone added. Dialysis membranes were disks cut from dialysis tubings obtained from Thomas Co. The membrane disks were also found not to adsorb significant amounts of hormone. Radioactive hormones were initially put in both sides of the membrane to expedite equilibrium. Dialysis was performed at 4° with constant shaking for 18–30 hr. At the end of dialysis, 0.1- and 0.5-ml aliquots were removed from each side of the cells and counted in Bray's fluor. The amount of bound hormone was obtained by subtracting the radioactivity on one side of the membrane from that of the side containing the hormone receptor.

Receptor Purification Procedure. After several washes with standard buffer, the pooled livers were homogenized with a Teflon homogenizer in 10 volumes of the same buffer. The homogenate was centrifuged at 2000*g* for 7 min and then at 30,000*g* for 30 min. The supernate was passed through a glass wool filter to free it of the yolky scum. This supernatant fraction was adjusted to pH 5.6 with 1 N HCl and centrifuged at 105,000*g* for 3 hr. The clear solution resulting from the high-

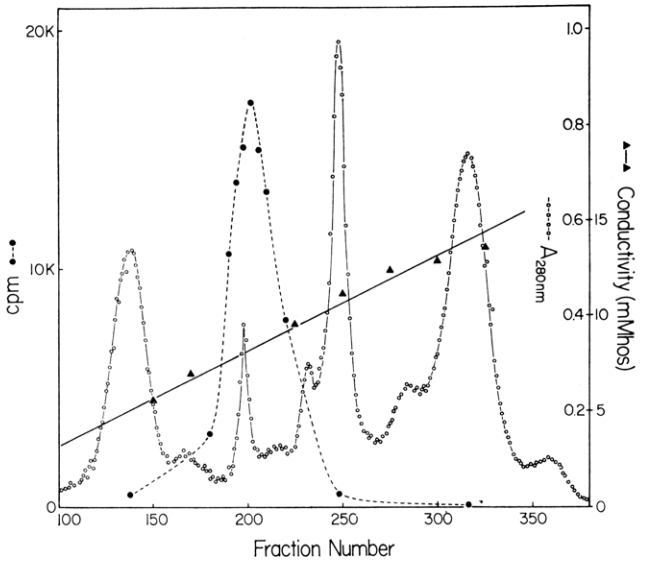

FIGURE 1: Elution profile of the hormone–receptor fraction through a DEAE-cellulose column. The 105,000*g* supernate was readjusted to pH 7.4 and placed onto a DEAE column (3.5 × 19 cm). After washing with 0.02 M NaCl, protein was eluted with a gradient of 0.02–0.2 M NaCl (pH 7.4). The 0.02 M NaCl wash eluate and the beginning of the gradient are not shown on the figure: (●) binding activity; (○) protein profile monitored by optical density; (▲) ionic strength of the salt gradient measured by conductivity.

speed centrifugation was readjusted to pH 7.5 with 1 N NaOH.

The sample was then applied onto a DEAE-cellulose column (3.5 × 20 cm) equilibrated with 0.02 M Tris-HCl–0.02 NaCl, pH 7.4. The column was washed with equilibrating buffer until the eluate had an OD_{280} of less than 0.05; usually this required about 1 l. of buffer. A linear gradient of 0.02–0.2 M NaCl in 0.02 M Tris-HCl, pH 7.4, was applied. *In vitro* binding assays were conducted to locate active fractions. The active fractions were pooled, dialyzed against water adjusted to pH 7.5 with Tris, lyophilized, and stored at −20°.

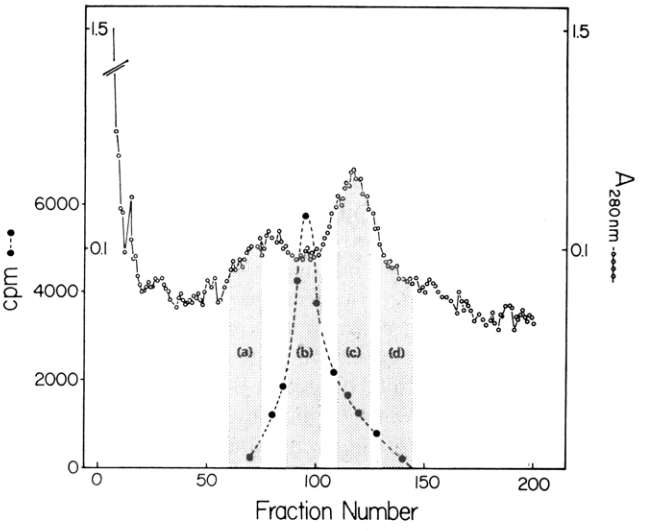

FIGURE 2: Subfractionation of the receptor by preparative disc electrophoresis. Electrophoresis was run at 200 V for the first hour and then at 400 V for 1.5 hr. Two-minute fractions were collected after the tracking dye was eluted and the voltage was increased to 600 V. Fractions under each shaded area were pooled, dialyzed, and concentrated for further analysis: (○) optical density at 280 nm for protein; (●) distribution of the hormone-binding activity assayed by the use of radioactive hormone.

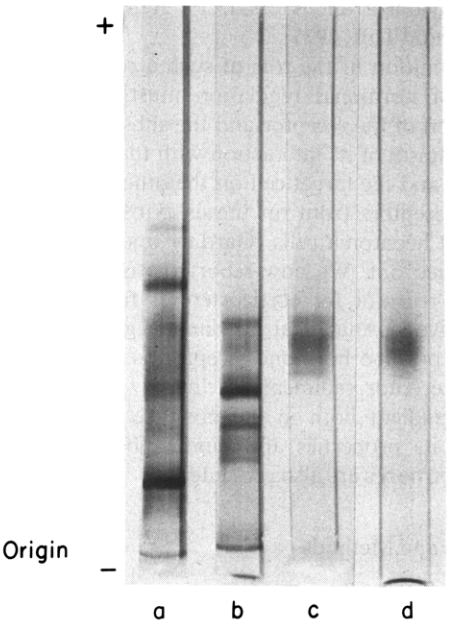

FIGURE 3: Acrylamide gel electrophoresis of the hormone receptor at different steps of purification. Aliquots of the receptor fractions at each step of purification were electrophoresed as described under Materials and Methods: (a) acidified high-speed supernate of the liver extract; (b) lyophilized DEAE-purified material; (c) an earlier preparation of receptor which had been purified by preparative disc electrophoresis; (d) preparative disc purified receptor when sharper cuts were made on pooling the active fractions.

The final step of purification of the hormone receptor was accomplished by preparative disc electrophoresis. A volume of sample not exceeding 2 ml was loaded onto the gel column with a few drops of 2 M sucrose and a drop of Bromophenol Blue as tracking dye. Electrophoresis was conducted under circulating ice–water to cool the gel column. With the anode at the lower reservoir, electrophoresis was run at 200 V for the first hour and then at 400 V for the next 1.5 hr. When the tracking dye was about to be eluted, voltage was increased to 600 V. Elution buffer was kept flowing from the start of the run. Fractions were collected at 2- or 5-min intervals with a Buchler polystaltic pump regulating the flow rate at 2–2.5 ml/fraction.

Results

Purification. Before the crude extract was subjected to high-speed centrifugation, its pH was lowered to 5.6. This acidification step precipitated 40% of the protein of the unacidified 105,000*g* supernate without any significant loss in binding activity. The high-speed supernatant fraction contained 3–3.5 mg/ml of protein and 75–80% of the total binding activity of the crude extract. Figure 1 illustrates a representative separation obtained from DEAE-cellulose column chromatography. The total protein applied onto the column for each experiment was about 400–600 mg. The flow-through fractions were found to be devoid of any hormone-binding activity. The protein under the binding activity peak represents 5–10% of the total protein applied onto the column. There is only one peak of binding activity which is eluted at about 0.06 M NaCl. Rechromatographing the fractions with binding activity on a shallower gradient of 0.02–0.1 M NaCl did not yield any further increase in specific activity. The binding activity still remained as a single peak upon rechromatographing.

Fractions with binding activity eluted from the preparative disc electrophoresis were found to contain three–four protein bands on an analytical acrylamide gel. To find out which protein band was responsible for the hormone-binding activity, material under the binding activity peak eluted from the preparative disc electrophoresis was pooled into subfractions, as shown by the shaded areas in Figure 2. The fastest moving band in the analytical gel was found not to have any binding activity, while the middle diffuse band contained 80% of the total binding activity recovered. By making sharper cuts at the front and trailing edges of the binding activity peak, the hormone receptor could be recovered as one major diffuse band in most of the preparations. Figure 3 shows the acrylamide gel pattern of the hormone receptor at different stages of purification. Figure 3c shows protein bands obtained from preparative disc electrophoresis purified receptor at earlier preparations, while Figure 3d shows the purified hormone receptor when sharper cuts were made when pooling the binding fractions from the preparative disc electrophoresis eluate.

Table I summarizes the typical results obtained from the three-step purification procedure. The relatively low purification factor obtained was in contrast to the protein patterns seen in gel electrophoresis in the purification steps (Figure 3). This could be accounted for by the loss of activity during the protein concentrating process. The overall recovery was about 10–20%. With purer preparations, 1 mg of protein was obtained from 12 g of wet tissues. Because of the low purification factor, it was necessary to determine whether purification steps had grossly modified the hormone receptor. Sucrose gradients were performed on the hormone–receptor complex at each step of purification, namely, the crude extract, the extract after acidification, lyophilized DEAE eluate, and the lyophilized preparative disc electrophoresis fraction. The tritiated hydrocortisone–receptor complex was shown to sediment at the same position in the sucrose gradients. Consequently, the various purification steps did not lead to any qualitative changes of the molecule with respect to sedimentation and hormone binding activity.

Sucrose Gradient Centrifugation and Electrofocusing. When an aliquot of the hormone receptor was mixed with tritiated hydrocortisone or the receptor–tritiated hydrocortisone complex which had been purified by the Sephadex assay column was centrifuged through a 5–20% sucrose gradient, the radioactive peak sedimented slightly slower than the bovine serum albumin (4.6S) marker (Figure 4). The isoelectric point of the protein–hormone complex was determined by electrofocusing

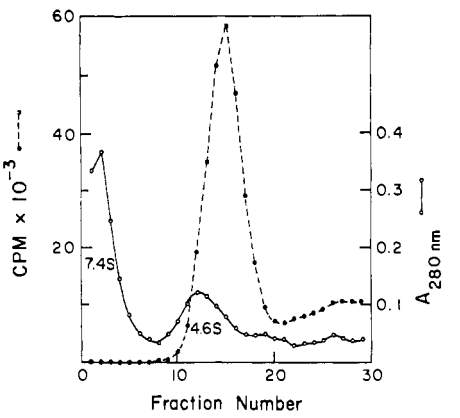

FIGURE 4: Sucrose gradient centrifugation of the hormone receptor. An aliquot of 0.3 ml of the hormone receptor purified by preparative disc electrophoresis was incubated with 3×10^{-3} μg of tritiated hydrocortisone at 4° for 8 hr. The receptor was loaded on a 5–20% sucrose gradient in 0.01 M Tris–0.01 M KCl–0.001 M EDTA, pH 7.4. In a separate gradient, a mixture of bovine serum albumin and yeast alcohol dehydrogenase was added. Centrifugation was carried out at 47,000 rpm in a SW 50L rotor for 12 hr. Twelve-drop fractions were collected from the bottom of each tube; 0.3 ml of 0.01 M Tris-HCl (pH 7.4) was added to each fraction for the marker gradient and the OD_{280} (○) was measured. The gradient with the receptor was counted for radioactivity (●).

and was found to be at pH 5.3 (Figure 5). The receptor from chick embryo liver thus has one hormone-binding component with a sedimentation coefficient of 4 S and is acidic in nature.

Effect of Salt on Binding. DeSombre *et al.* (1969) observed that the rat uterus estrogen receptor sedimented as an 8S component in sucrose gradients. In the presence of 0.3–0.4 M KCl, however, the receptor sedimented as a 4S species. To see if salts have any effect on the receptor–tritiated hydrocortisone complex of the chick liver, the purified complex was incubated at 0.05, 0.15, 0.3, 0.5, and 0.8 M KCl. The complexes in the different salt solutions were centrifuged through sucrose gradients of the respective salt concentrations. Figure 6 demonstrates the results obtained. No faster or slower sedimenting component was found present within the range of salt concentration tested; the receptor–tritiated hydrocortisone complex still appeared as a single species as in the absence of KCl. Also, a single radioactive peak with a 4S value was observed (data not shown) in the presence or absence of salt even when the crude supernate (30,000*g*, 30-min centrifugation) was used in the assay.

TABLE I: Purification Scheme of the Liver Hormone Receptor.

Expt	Steps	Total Bound (dpm × 10^4)	Protein (mg)	Sp Act. ((dpm/mg) × 10^4)	Recovery (%)
I	Acidified 105,000*g* supernate	3150	612	5.1	100
	DEAE pool	2580	53	49.0	84
	DEAE lyophilized	1500	32	47.0	50
	Prep disc electrophoresis	860	*a*	*a*	25
	Prep disc lyophilized	700	4	175.0	20
II	Acidified 105,000*g* supernate	4800	620.0	7.7	100
	DEAE pool	2600	36.0	72.2	54.1
	DEAE lyophilized	1300	21.7	60.8	27.5
	Prep disc electrophoresis	720	1.8	400.0	15.0

[a] Concentration too low to be determined.

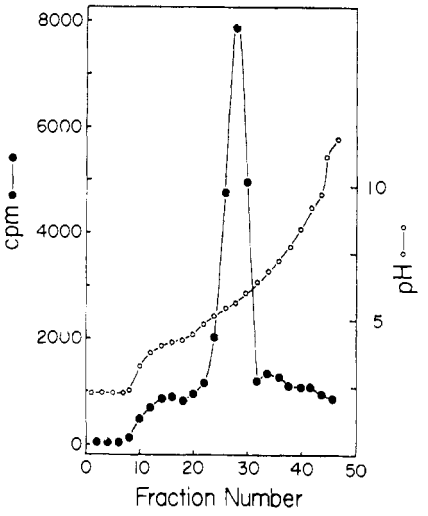

FIGURE 5: Electrofocusing of the tritiated hydrocortisone-receptor complex. The receptor used in this experiment was previously purified by chromatography on DEAE-cellulose and Bio-Gel A-0.5m columns. The hormone-receptor complex was mixed with 1% Ampholine solution in 0–50% sucrose gradient. Electrofocusing was carried out at 0° with a LKB-8100 apparatus for 72 hr at 400 V. Fractions (2 ml each) were collected at the end of the run: (○) the pH gradient; (●) radioactivity of the hormone–receptor complex.

Optimum Conditions for Binding. The kinetics of tritiated hydrocortisone binding to the liver receptor were carried out by incubating the cortisol receptor with a constant amount of hydrocortisone at 37° for varying amounts of time. Binding reached saturation level within 10 min of incubation. Longer incubation up to 1.5 hr did not increase binding activity. Incubation at 0° showed the same kinetic profile of binding, except lower specific activity was observed. Saturation was also reached after 10 min of incubation at 0°. If the increase in binding at 37° was due to a faster binding rate at elevated temperature, the binding at 0° should eventually reach the same level as that observed at higher temperature after a longer incubation time. Since this was not observed, the increase in binding at 37° must be due to the increased availability of binding sites on the receptor molecule. The optimum temperature for binding was found to be 37°. There was appreciable binding at 0 and 25°, while at 60° most of the binding activity was lost. The *in vitro* binding of hormone to the receptor seemed to be insensitive to pH changes over a wide range. When the hormone receptor was incubated at 37° with tritiated hydrocortisone in 0.02 M Tricine at different pH values, ranging from 4.5 to 9.4, the binding activity remained quite constant. This relative insensitivity to pH, however, may be a reflection of the method employed, since the receptor is exposed to the particular pH for only a short time during incubation with hydrocortisone. When the crude extract is acidified to a pH below 5.6 and then subjected to high-speed centrifugation, there is a significant loss in binding activity in the resultant supernate which is not recoverable in the pellet fraction. The receptor is thus sensitive to changes in pH to a certain extent.

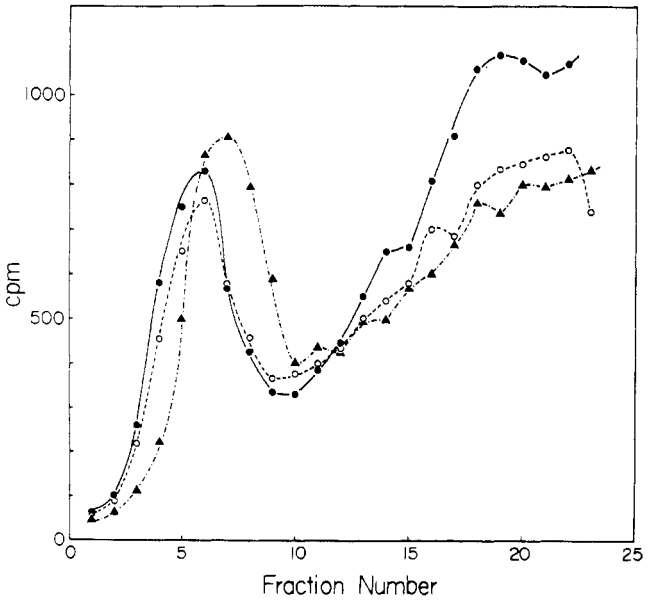

FIGURE 6: Effect of KCl on the tritiated hydrocortisone-receptor complex. An aliquot of 0.3 ml of preparative disc electrophoresis purified receptor was incubated with 1.5×10^{-2} μg of tritiated hydrocortisone at 37°. The hormone–receptor complex was purified by the standard assay. Aliquots of 0.4 ml of the complex were adjusted to different KCl concentrations and centrifuged on sucrose gradients, each adjusted to the corresponding KCl concentration. Centrifugation was carried out in a SW 50L rotor at 48,000 rpm for 24 hr at 4°: (●) complex without added KCl. (○) complex in the presence of 0.5 M KCl; (▲) complex in 0.8 M KCl. Not shown in the figure but found to sediment on the same position of the gradient were complexes in 0.05, 0.15, and 0.3 M KCl.

TABLE II: Digestion of the Hormone Receptor by Enzymes.[a]

Expt	Treatment	[^{3}H]Hydrocortisone Bound (cpm)	% Control
I	No addition	7620	100.0
	Trypsin, 20 μg	480	6.3
	Pronase, 20 μg	360	4.2
	RNase, 5 μg	7150	96.7
	Phospholipase A, 40 μg	6490	85.2
II	No addition	1800	100.0
	Neuraminidase, 40 μl	1780	98.9

[a] Receptors used for both experiments were preparative disc electrophoresis purified. In experiment I, digestive enzymes in final concentrations as indicated were added to the receptor protein in the presence of 100 μg of bovine serum albumin. The total volume in each assay was 0.2 ml in 0.01 M Tris-HCl, pH 7.0. Incubation was carried out at 37° for 10 min; the tritiated hydrocortisone–receptor complex was determined by the standard assay. In experiment II, the incubation mixture contained 20 μl of hormone receptor, 40 μl of neuraminidase, and 140 μl of 0.1 M NaAc with 70 μg of bovine serum albumin, pH 5.5. Incubation was carried out at 37° for 30 min before tritiated hydrocortisone was added for the standard binding assay. The specific activity of the neuraminidase used was 0.7 μmol/ml per min.

Chemical Nature of the Hormone Receptor. An ultraviolet (uv) absorption spectrum of the purified hormone receptor in the absence of hormone exhibited a typical protein absorption maximum at 278–280 nm, with an A_{280}:A_{260} ratio of 1.5. A fluorescent emission spectrum of the same protein also showed a peak at 350 nm, characteristic of tryptophan fluorescence. Enzyme digestions of the binding protein gave further evidence of its protein nature (Table II). Incubations were carried out as close to the optimum conditions of the digestive

enzymes as possible (Hummel, 1959). A 20-min digestion by Pronase and trypsin destroyed more than 90% of the binding activity, while RNase and neuraminidase had no significant effect on the binding. The slightly lower level of binding after digestion with phospholipase A might be due to a small amount of proteolytic enzymes present in the crude preparation of phospholipase. Since the effectiveness of enzyme digestions was measured by the assay of tritiated hydrocortisone binding to the receptor, the presence of nonprotein components, which was not crucial in the binding *per se*, could not be ruled out.

Chemical Identification of the Bound Hydrocortisone. Radioactive hydrocortisone incubated with the hormone receptor at 37° for 10 min was layered onto the standard assay column to separate the complex from free hormone; only the front of the excluded volume was collected. The purified complex was then incubated at 60° for 30 min, which released more than 95% of the radioactive hormone from the receptor. The released hydrocortisone was then identified by ascending paper chromatography. The bound hormone recovered was identified as hydrocortisone and was not a metabolite of the hormone under the *in vitro* assay conditions used.

Hormone Specificity of Binding. It was found that other steroids, in addition to hydrocortisone, would bind to the acidified high-speed supernate of the liver extract. By studying the mode of binding of these hormones, two groups were established. The hormones of the first group (hydrocortisone and corticosterone) exhibited characteristics of specific interactions with the receptor, while the binding of the hormones in the second group (estradiol and testosterone) could be explained in terms of nonspecific interactions. Hydrocortisone and corticosterone binding reached saturation level at low concentrations of hormones added. At saturation value, hydrocortisone binding had higher loading values than corticosterone, as measured by the standard assay column. Estradiol and testosterone binding, however, did not reach saturation even at high concentrations of hormones added in the incubation mixture (Figure 7). Binding increased linearly up to 250 pmol of radioactive hormones added with no sign of leveling off. Testosterone binding showed lower specific activity than estradiol binding.

Experiments were carried out to test whether there is competition in the binding of the two hormone groups. Excess amounts of cold estradiol in the tritiated hydrocortisone incubation mixture or cold hydrocortisone in the tritiated estradiol mixture did not affect tritiated hydrocortisone binding in the first case or tritiated estradiol binding in the latter. Furthermore, the binding was found to be additive when both tritiated hydrocortisone and tritiated estradiol were incubated in the same mixture. This gave good evidence that hydrocortisone and estradiol had different binding sites on the receptor.

An attempt was made to assess the relative abundance of hydrocortisone and estradiol binding activity in an early and the final step of the purification procedure. When the high-speed supernate was incubated with low concentrations of hormone, *i.e.*, 2×10^{-10} M, where hydrocortisone binding was linear, the ratio of hydrocortisone/estradiol binding per picomole of hormone added was 10. In the preparative disc electrophoresis purified material, however, estradiol binding could not be detected at this low hormone concentration. It can be seen in Figure 7A that estradiol binding to the preparative disc electrophoresis purified receptor can be detected if the hormone concentration is raised in the incubation mixture. This binding did not reach saturation and remained

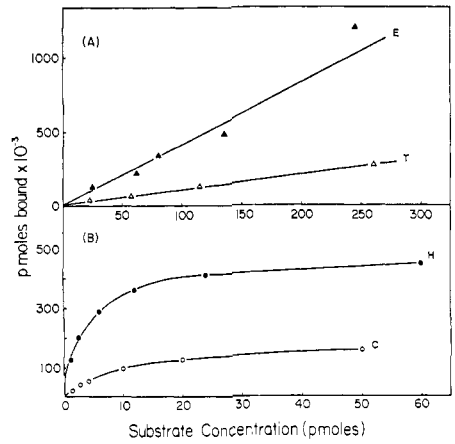

FIGURE 7: Saturation curves of the binding of different steroids to the receptor. Preparative disc electrophoresis purified receptor was used. Standard binding assays were carried out with different steroid hormones at the concentrations indicated. Open and closed triangles in (A) represent testosterone and estradiol binding, respectively. In (B), open circles represent corticosterone binding and closed circles represent hydrocortisone. The values on the ordinates represent binding by a sample of 0.2-ml volume. For a comparison of parts (A) and (B), see section on Hormone Specificity of Binding. The values from this figure should not be used to calculate the number of hormone-binding sites on the receptor for the reasons given in the text.

linear within the concentration range tested. Assuming that this linearity holds true even at low estradiol concentration, *e.g.*, 10^{-10} M (an assumption not necessarily correct), a value can be obtained, by extrapolation, for the extent of estradiol binding to the receptor at an estradiol concentration of *ca.* 10^{-10} M. Using this "extrapolated value," the ratio of hydrocortisone/estradiol binding of the purified receptor is calculated to be 3000. This should be considered as a lower limit, because the assumptions made for its derivation were designed to maximize the significance of estradiol binding. With the available data, it cannot be decided whether we have purified away some molecular species responsible for the binding of estradiol or have modified the hormone receptor during the purification process such that its affinity for estradiol was greatly reduced. In any event, we have succeeded in obtaining a highly purified receptor fraction specific for hydrocortisone–corticosterone. This specificity of the purified receptor toward these hormones concurs with the specificity of the hormonal action of the organ from which the receptor is isolated, *i.e.*, the liver.

Equilibrium Column Analysis. If the affinity of the receptor for estradiol was very weak, the assay column used in the present studies would not be able to detect this type of binding, since such a complex would be dissociated before it had been eluted out of the column. Equilibrium columns were set up to test this possibility. Under the conditions of the equilibrium column, the receptor would be in the presence of hormone at all times; any binding that occurred, no matter how unstable, would be detected (Fairclough and Fruton, 1966). The receptor–tritiated hydrocortisone complex was eluted as a sharp radioactive peak, followed by a trough of lower radioactivity, which is a typical binding phenomenon on an equilibrium column (Fairclough and Fruton, 1966) (Figure 8). The extent of binding activity obtained from such a column was much higher than that obtained by the standard assay column, indicating that, in the latter case, a large percentage of the complex *was dissociated in the course of elution* at 24°. Because of this, the data in Figure 7 should not be used to cal-

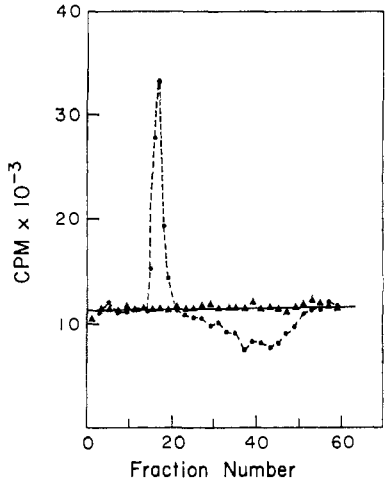

FIGURE 8: Equilibrium column chromatography of tritiated hydrocortisone and tritiated estradiol binding to the receptor. Preparative disc electrophoresis purified receptor was incubated with tritiated hydrocortisone or tritiated estradiol at 4×10^{-4} μg/ml. At the end of incubation, the mixture was passed through a G-50 column (1.2 × 28 cm) equilibrated with hormone at 4×10^{-4} μg/ml and eluted with 0.01 M Tris-HCl (pH 7.4) containing the same concentration of hormone. Fractions of 1 ml each were collected and assayed for radioactivity: (●) tritiated hydrocortisone; (▲) tritiated estradiol.

culate the number of binding sites on the receptor since they do not represent equilibrium values. Estradiol *did not bind* even under the more stringent conditions of this assay.

Thus, the results obtained from the standard binding column appeared to be *qualitatively* valid; however, they should not be compared *quantitatively* with the results obtained from equilibrium dialysis experiments to be presented below.

Equilibrium Dialysis. Because the standard column assays for binding did not represent true equilibrium condition, and extensive analyses by equilibrium column would be uneconomical, equilibrium dialysis was carried out to determine association constants for the binding of different hormones and their interrelationship to the binding process. Experiments were conducted varying the concentration of tritiated hormone added, while the concentration of binding protein

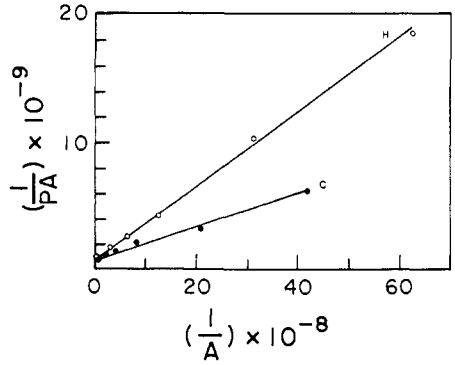

FIGURE 9: Double reciprocal plot of tritiated hydrocortisone and tritiated corticosterone binding to the receptor. Equilibrium dialysis was carried out with a constant amount of purified receptor (16 μg) and varying concentrations of tritiated hydrocortisone or corticosterone. Dialysis was carried out at 4° for 38 hr. The inverse of the total amount of bound hormone was plotted against the inverse of the total amount of hormone added: (○) corticosterone binding; (●) hydrocortisone binding.

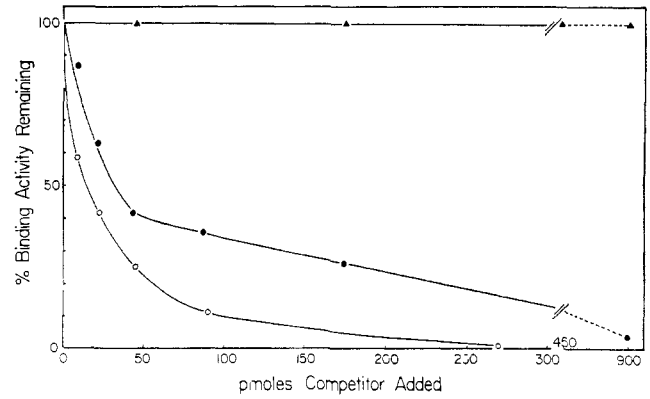

FIGURE 10: Competition curves of the binding of various hormones to the receptor protein. Standard binding assays were carried out with preparative disc electrophoresis purified receptor in the presence of 12 pmol of tritiated hydrocortisone and increasing concentrations of cold corticosterone or estradiol. The reverse experiment was also performed with 10 pmol of tritiated corticosterone and increasing amounts of cold hydrocortisone: tritiated corticosterone binding competed by cold hydrocortisone; (○) tritiated hydrocortisone binding competed by cold corticosterone; (▲) tritiated hydrocortisone binding competed by cold estradiol.

was kept constant in each dialysis chamber. In competition experiments, the concentrations of protein and radioactive hormones were kept constant while varying concentrations of cold competitor hormone were added. Figure 9 is a reciprocal plot of hydrocortisone and corticosterone binding, calculated from equilibrium dialysis data. Both hydrocortisone and corticosterone binding were extrapolated to the same total concentration of binding sites, 1×10^{-9} mol/ml (1 μM). The association constant for hydrocortisone binding was 7.5×10^{8} M^{-1} at 4° and that for corticosterone binding was 3.6×10^{8} M^{-1} at 4°. Estradiol showed no binding in these experiments where purified protein and lower concentrations of estradiol were used. Competition experiments showed that hydrocortisone and corticosterone would compete against each other, with hydrocortisone being a more efficient competitor than corticosterone (Figure 10). This is in agreement with the observation that the association constant for hydrocortisone binding was stronger than that for corticosterone. Again, hydrocortisone binding was not affected by the addition of a high concentration of estradiol in the dialysis medium.

Liver vs. Plasma Hormone Receptor. Experiments were conducted to determine the relationship of the isolated liver receptor and the corticosteroid-binding globulin which had been reported earlier (Seal and Doe, 1962). Blood from 16-day-old chick embryos was drained, and plasma was obtained by a low centrifugation to pellet the coagulated red blood cells. Plasma was then diluted 1:5 (v/v) with standard buffer, and the procedure used for purification of the liver receptor was carried out for the plasma. The peak fractions of binding activity obtained from the DEAE chromatography were skewed toward a higher salt elution, peaking at 0.07 M NaCl instead of at 0.06 M as seen for the liver receptor. This slight difference, however, was reproducible. Preparative disc electrophoresis also showed the plasma receptor eluting slightly ahead of the liver receptor. Sedimentation by sucrose gradient centrifugation, however, revealed that both the purified liver and plasma receptors migrated to the same position on the gradient (Figure 11). Gel filtration chromatography of the liver and plasma receptor–hormone complexes on a Bio-Gel A-0.5m column also showed the two radioactive peaks to be coin-

cidental. The temperature optimum for binding of tritiated hydrocortisone to plasma was 37–45°. This thermal stability is in contrast to that reported by Seal and Doe (1962) for the chick corticosteroid binding globulin. At 37° the corticosteroid binding globulin lost 76% of the binding activity present at 0°. The association constant of tritiated hydrocortisone binding to a crude preparation of plasma was 1.3×10^8 M^{-1} at 4°. This is about five times less than the hydrocortisone binding to the liver receptor. Lineweaver–Burk or Scatchard plots, however, extrapolate the hydrocortisone binding in the plasma to the same number of binding sites as those in the liver.

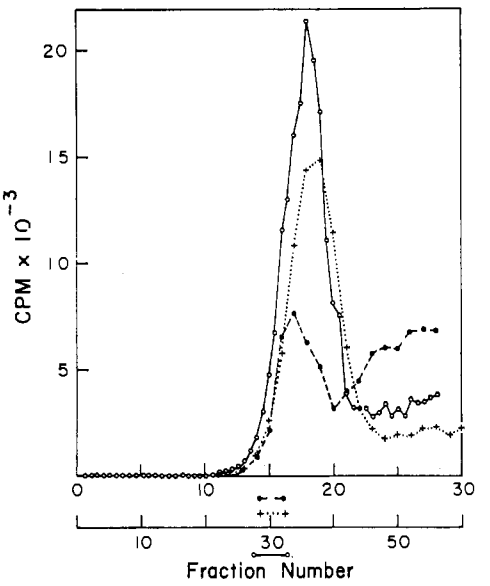

FIGURE 11: Sucrose gradient analysis of liver- and plasma-binding proteins. Preparative disc electrophoresis purified receptors from liver and plasma were each incubated with 3×10^{-3} μg of tritiated hydrocortisone and the hormone–receptor complex was analyzed on 5–20% sucrose gradients. Centrifugation was carried out at 47,000 rpm for 13 hr in a SW 50L rotor at 4°: (●) liver-receptor binding; (+) plasma-receptor binding. All gradients were collected in 12-drop fractions from the bottom. Open circles represent the liver and plasma receptors cosedimenting on the same gradient. Six-drop fractions were collected for this gradient.

Discussion

Most of the earlier investigations carried out on hormone binding proteins were with crude extracts obtained from high-speed supernates. Several laboratories that have attempted to purify specific hormone receptors faced difficulties due to labilities of the receptors and their tendency to spontaneously aggregate. We found that the majority of the binding activity was lost during the concentration step. When the lyophilized material was redissolved in water or standard buffer, there was a great loss of binding activity due to precipitation. This property of the receptor may account for the apparent low purification factor obtained in the purification process, while the purity of the molecular species involved in the binding is actually increasing. Alternate methods of concentration of protein such as vacuum dialysis, pressure dialysis, and concentration by Lyophogel also resulted in precipitation and in loss of binding activity. A variety of protective agents were tried to minimize irreversible aggregation of the hormone receptor also without any success. It seemed that when the receptor reached a certain concentration, it would tend to aggregate. Despite all these problems, enough material was accumulated for the biochemical studies and physical characterization of the protein.

DeSombre *et al.* (1969) partially purified a uterine estrogen receptor. They stabilized the receptor in the presence of 0.3 M KCl by converting the labile 8S component into a more stable 4S molecule. Sherman *et al.* (1970) also observed the aggregation phenomenon of the 8S component of the progesterone receptor. However, Fang *et al.* (1969) reported on a dihydrotestosterone receptor of 3.7 S. They did not note a heavier component present when centrifuging the receptor–hormone complex through a sucrose gradient. The same observation was made in the rat liver, where the receptor was present *exclusively* as a 4S molecule (Beato *et al.*, 1969). A still smaller hydrocortisone receptor (2.7 S) has been reported (Gardner and Tomkins, 1969). In the chick embryo liver extracts, the hydrocortisone receptor sedimented as a 4S species, whether salt was present or absent in the sedimentation medium. No larger aggregate was observed at any time during the purification procedure. The variation in sedimentation coefficients of hormone receptors from different sources may reflect a target-organ-specific heterogeneity. Whether this heterogeneity has also some functional significance cannot be decided at the present time.

Apparently not only can the hormone receptor isolated from certain systems exist in different sedimenting forms as a function of ionic strength, but it may also be present in the cytosol in multiple forms. A number of steroid-binding fractions have been isolated from the rat liver cytosol by Litwack *et al.* (1972). Beato and Feigelson (1972) and Koblinsky *et al.* (1972) were able to identify and characterize three glucocorticoid-binding proteins from the rat liver cytosol. One of these proteins, which binds dexamethasone (a synthetic, not naturally occurring glucocorticoid) with high affinity and which is very labile, was claimed by these investigators to be the most plausible hepatic glucocorticoid receptor in relation to biological activity. Exactly what the functional relationships are among these binding proteins is unclear at the present time. They may be parts of an aggregate in the cell, the same protein in various states of configurations, or truly independent in their molecular identity and function, but these must remain speculations until more direct evidence becomes available that would link the hormone-binding property of such receptors to their physiological function(s).

Again, in the chick embryo liver extracts, we were able to detect only one form of hydrocortisone receptor. Throughout the purification procedure, from the crude extract (the 30,000*g*, 30-min supernate) to the material purified by disc acrylamide electrophoresis, the hormone receptor was observed as a single functional entity. There is only one binding species revealed by sucrose gradient, electrofocusing, and column chromatography. This, however, does not necessarily mean that the purified molecular entity involved in the interaction with the hormone is monodisperse (*e.g.*, one polypeptide only). There are indications which point to the contrary. The most highly purified fraction of the hormone receptor obtained from preparative acrylamide electrophoresis showed one *diffuse* band in subsequent analytical acrylamide gel electrophoresis. In the presence of 5 mM dithiothreitol, the appearance of a slower migrating band could be induced without any effect on the binding activity. Electrophoresis in sodium dodecyl sulfate–acrylamide gels (results to be published elsewhere) showed at least three distinct components which appeared as one diffuse band in acidic gels. In the gel filtration experiment, the hormone receptor appeared to have

molecular weights of greater than 100,000, whether the protein was layered onto the column either as a receptor–hormone complex or as receptor in the absence of hormone. The analyses of sedimentation data, however, suggested a smaller molecule, *i.e.*, mol wt 50,000 if the protein is assumed to be globular. This size discrepancy had also been noted in other systems (Sherman *et al.*, 1970). We are now faced with an apparent paradox. The hormone receptor appears homogeneous by functional criteria (*i.e.* binding), but *heterogeneous* or *polymorphic* by structural criteria. This discrepancy can be explained if we assume that one of the following alternatives, or both, are correct. The receptor molecule is indeed equivalent with one covalently held entity (*e.g.*, polypeptide), capable of existing in a number of structural states, thus giving rise to a narrowly dispersed population of conformationally related molecules. The second alternative is that the receptor is composed of a number of interacting subunits (similar or dissimilar) and it can exist in different states of molecular association. This issue cannot be settled with the data available at present.

Whether there is any correlation between the plasma receptor isolated with the present procedure and the corticosteroid-binding globulin isolated by Seal and Doe (1962) cannot be determined. The difference in thermal stability between the two and the slight differences between the liver and plasma receptors on the basis of electrophoretic and chromatographic criteria do not strongly support the notion that they are different proteins. Purification of the plasma receptor by the procedure developed for the liver receptor did not show an increase in specific activity. Sodium dodecyl sulfate gel electrophoresis showed two major bands which do not correspond to any of the major bands of the liver receptor protein. It is possible that the plasma fraction contains a small quantity of hormone receptor with the characteristics of the liver receptor, but is is masked by the presence of large amounts of other plasma proteins. Obviously more work is needed to clarify this point.

One of the general concepts of the molecular sequence on the mechanism of hormone action is the retention of hormone by target tissue, leading to derepression of RNA synthesis and finally to the stimulation of protein synthesis (Karlson, 1963). The presence of specific hormone receptors in the different systems is now well documented. Little if any is known, however, on the functional and physiological aspects of such receptors. It is conceivable that such receptors could fit in any step of the hormone mechanism scheme. This could be a transport vehicle for hormone to reach its target tissue or serve as a protective agent in the target tissue for the hormone, preventing it from being metabolized before it carries out its function. Acting as a derepressor or repressor for RNA synthesis is not an improbable assignment for such hormone receptors. Of course, any one or several of such functions could be carried out by the receptor molecule.

All investigations on the hormone receptors have been dependent on its hormone-binding activity as an assay. It is conceivable that hormone-binding activity constitutes only one of the properties of such a receptor. Its other functional activities could be independent and may be exclusive from the hormone-binding activity. We therefore feel that though hormone binding is an important property in relation to hormone, it is equally important that the receptor should be isolated and purified free from its substrate to facilitate the study of its other probable functions. We have succeeded in obtaining such a highly purified fraction of liver hydrocortisone receptor in the chick embryo system. It is now feasible to test some of the above hypotheses relating the receptor molecule to the regulatory mechanism of hormone action.

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