Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 989–999. Pergamon Press. Printed in Great Britain.

MOLECULAR MECHANISMS OF STEROID HORMONE ACTION

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SUMMARY

The differentiated chick oviduct is a target tissue for progesterone. Administration of the hormone to estrogen-primed chicks causes a rapid induction of new messenger RNA, resulting in the appearance in cytoplasm of specific mRNA species. One of these species is the mRNA coding for the egg white protein avidin, which is produced only in response to progesterone.

The cytoplasm contains receptors which bind progesterone specifically and transport it as a complex into oviduct nuclei. Only a small fraction of the receptors are active *in vitro*; this fraction is increased to 30% by brief warming of the receptor-hormone complexes before incubation with the nuclei. The binding reaction is slow, and the receptors cannot be released from nuclei by DNase treatment. The reaction occurs preferentially with oviduct nuclei, which contain at least twice as many acceptor sites (8000/nucleus) as other chick tissues (< 2000/nucleus). The binding constants are the same in all tissues ($K_d \sim 10^{-8}$ M), and are identical to the constant for receptor binding to isolated chromatin.

We have isolated the progesterone receptor component which binds to the chromatin. Oviduct cytosol from laying hens was prepared and progesterone receptors were precipitated with ammonium sulfate (30% sat.). The re-dissolved pellet was eluted from a steroid-affinity column (Sepharose 4B-BSA-deoxycorticosterone) with 3M urea. The receptors were reconstituted by dialysis and labeled with [^{3}H]-progesterone. The [^{3}H]-progesterone-receptor complexes were then purified by sequential chromatography and elution with the indicated slats at pH 7·4 from DEAE-cellulose (0·2 M KCl), phosphocellulose (0·26 M KCl), and hydroxylapatite (0·15 M K_xPO_4). The peak fraction was finally chromatographed on an agarose A-1·5 M column ($K_{av} = 0·28$). Yield was 1%, and purity approached the theoretical maximum specific activity, 10^9 d.p.m./mg protein.

INTRODUCTION

We have been investigating the mechanism of progesterone action in the chick oviduct. This system has been used to great advantage for this purpose since the tissue contains a high titer of specific progesterone receptor proteins[1, 2]. The tissue differentiates under the influence of estrogens[3–6] into several cell types. One of these cell types, the goblet cells, responds to progesterone by synthesizing the egg white protein avidin[7, 8]. Many of the early molecular events of progesterone action were developed in this system. Unmetabolized progesterone enters the cells and binds receptors which localize in the nuclear chromatin. Details of this process have been the subject of a series of publications from this laboratory[9–12].

In general, the molecular events of steroid hormone action appear to be strikingly similar for all of the tissue systems studied. The central importance of the receptor proteins for these processes has been noted in studies of estrogens[13–15], androgens[16–18] and corticoids [19–22]. The processes of steroid mediated gene activation have been shown to be similar both *in vivo* and *in vitro* for the glucocorticoids studied in hepatocytes, hepatoma cells or thymocytes. Thus it is likely that further studies of these processes in all of the above systems will continue to expose similarities of events at the molecular level.

In the present communication we report some of our recent efforts regarding three aspects of our progesterone receptor studies. Several new methods of purification have been applied to the receptor proteins. Second, chromatographic studies have been performed on the activation of receptors for nuclear uptake. Third, a kinetic study of receptor binding to nuclei *in vitro* has been used to quantitate nuclear acceptor sites.

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MATERIALS AND METHODS

Reagents

All chemicals were reagent grade and were used without further purification. Tris buffer, ammonium sulfate and sucrose were from Schwarz-Mann (Orangeburg, New York, U.S.A.). Ion-exchange resins DE-52 and P-11 were from Reeve-Angel (Clifton, New Jersey, U.S.A.). Non-radioactive steroids were from Steraloids (Pawling, New York, U.S.A.). Agarose A-1·5 M beads were obtained from Bio-Rad (Rockville Centre, New York, U.S.A.). Spectrafluor was obtained from Amersham–Searle (Arlington Heights, Illinois, U.S.A.). Radioactive [1, 2-$^3H_2$]progesterone (50·1 Ci/mmole) was purchased from New England Nuclear (Boston, Mass., U.S.A.).

Animals and tissue

White leghorn chicks 7 days of age were fed Purina feed and water *ad lib.* Each received 5 mg diethylstilbestrol in sesame oil daily by subcutaneous injection for 14–21 days. The chicks were killed, and oviducts (magnum portion) were removed and washed in ice-cold 0·9% NaCl. Oviducts averaged 1–2 g each. White leghorn laying hens were also used. Each animal's oviduct averaged 25–30 g.

Buffers

All experiments were performed in Buffer A (0·01 M Tris–HCl, 0·001 M Na_2EDTA, 0·012 M 1-thioglycerol pH 7·4) containing additions of sucrose or KCl as noted in the text.

Subcellular fractions

Nuclei. Preparation of oviduct and other tissue nuclei and crude receptor preparations have been reported in detail elsewhere[23–25]. The method involves homogenization in Buffer A containing 0·5 M sucrose, followed by several washes and a pelleting through 1·75 M sucrose. The nuclei are free of cytoplasmic debris.

Cytoplasmic fractions: Tissue is homogenized in Buffer A containing 0·15 M KCl, and centrifuged first at low speed (20,000 ***g***) and then at 140,000 ***g*** for 1 h to prepare soluble cytoplasmic fraction (cytosol) containing progesterone receptors. Chicks contain essentially no endogenous progesterone, and hence all receptor sites are available for assay in this fraction. Laying hens contain high levels of circulating progesterone, which blocks many of the sites. Subsequent steps involving precipitation of receptors with ammonium sulfate effectively free most but not all of these sites of endogenous steroid.

Column chromatography

Methods for DEAE-cellulose, hydroxylapatite, DNA cellulose and agarose gel filtration column chromatography have been described in detail elsewhere[2, 23].

Phosphocellulose chromatography was done using Whatman P-11 equilibrated in Buffer A containing 0·05 M KCl. The column (2·6 cm × 20 cm) was loaded with a receptor preparation, washed in the equilibrating buffer and then eluted with a linear KCl gradient in Buffer A. Salt concentrations in the eluted fraction were determined using a Radiometer conductivity cell. Protein in the fraction was monitored by measuring the absorbance of 0·1 ml aliquots of the fractions at 280 nm.

Chromatin-affinity chromatography was performed using oviduct chromatin prepared from estrogen-primed chicks by the methods of Spelsberg *et al.*[10] and Schrader *et al.*[24]. About 10 mg of chromatin was suspended in 10·0 ml Buffer A containing 0·05 M KCl with the aid of a teflon-glass homogenizer (Glenco Instruments, Houston, Texas, U.S.A.). This suspension was added to Agarose A-1·5 M (10 ml bed volume) and the chromatin was dispersed in the gel beads by stirring. This material was then poured into a column 1·6 × 5 cm and washed with Buffer A containing 0·05 M KCl until no further material eluted absorbing at 260 nm. Labeled progesterone receptors prepared by ammonium sulfate precipitation at 30% saturation were applied in the equilibrating buffer and the column was washed extensively. It was then eluted using a KCl gradient up to 0·5 M KCl. Fractions (5 ml) were collected and assayed for ^3H.

Radioactivity counting

Tritium was counted using a Beckman LS-233 scintillation spectrometer. Aqueous samples (0·7 ml) were counted in a toluene based POPOP-PPO fluor cocktail containing 33% Triton X-100 (Beckman Instruments, Palo Alto, California, U.S.A.). Counting efficiency was 33%.

Binding-site determinations

Three standard methods were used for assay of [^3H]-progesterone bound to receptors. These were a modification of the charcoal-dextran adsorption method of Korenman[25]; precipitation of receptor complexes using ammonium sulfate; and gel filtration on short Sephadex G-75 columns. All three methods have been described in detail elsewhere[2, 23].

Sucrose-gradient ultracentrifugation

Receptor sedimentation values were obtained on 5 ml of 5–20% sucrose gradients in Buffer A containing various concentrations of KCl. Gradients were run in

Beckman SW-50·1 rotors at 2°C for 16 h at 45,000 rev/min. This technique is also described elsewhere[2].

Steroid-affinity chromatography

Affinity resins consisting of deoxycorticosterone hemisuccinate bound to denatured bovine serum albumin immobilized on Sepharose 4B were prepared and washed as described by Sica *et al.*[27]. A 5 ml settled volume of this resin was combined in a slurry with receptors precipitated with ammonium sulfate, incubated 16 h at 0° and then washed by centrifugation. Counter-elution using [^{3}H]progesterone (10^{-6} M) was done at 25°C for 60 min. Labeled receptor-hormone complexes were washed through the gel by filtration and freed of excess labeled steroid by gel filtration at 0° on Sephadex G-75.

RESULTS

The first step used in purification was steroid-affinity chromatography. Progesterone receptors from chicks were precipitated in 30% saturated ammonium sulfate and then applied to the DOC-Sepharose column. The binding constant for receptor adsorption is $K_d = 10^{-8}$ M, roughly two orders of magnitude weaker than the K_d for receptor-progesterone binding. The adsorption to the column requires the presence of adsorbed DOC, since sham columns prepared with activated Sepharose-BSA but no DOC are inactive. The binding reaction involves the receptor steroid binding site, since the binding rate at 0° is nearly the same as the rate for receptor-progesterone complex formation in solution.

Elution of the receptors from the column has been accomplished in two ways. First, receptors can be removed by purging the column with 3·0 M buffered urea, followed by gradient dialysis to remove the urea. After dialysis nearly 100% of the steroid-binding sites are recovered. Considerable aggregation of this material is noted in subsequent steps, as such; the method has not yet been developed further to date.

The second elution method employs an incubation of [1, 2-^{3}H]-progesterone with the adsorbed receptors at 25° for 60 min. At this elevated temperature the receptors are released by the solid-phase DOC, and are sequestered by the free [^{3}H]-progesterone. Yield of receptors by this technique is about 70–80% of the sites in the cytosol; purification is about 2000-fold. A sample purification is shown in Table 1. Several procedural problems have been encountered and dealt with successfully during our application of this technique. First, the value of using a large BSA side-chain to couple DOC to the Sepharose has been confirmed in our hands. If a di-isopropyl side-chain is used with the DOC, the column will bind receptors but with reduced efficiency. Yields by competition elution with [^{3}H]-progesterone are far below 1%. This is due to constant hydrolic cleavage of the side-chains, causing release of the DOC-receptor complexes into solution before competition with the label. The BSA side-arm, however, is joined to the Sepharose beads by multipoint attachment. This renders the DOC–BSA–Sepharose resin much more stable.

Receptor-progesterone complexes prepared in this way were studied on SDS–polyacrylamide gel electrophoresis. Several bands were seen, varying in molecular weight from 80,000 to 180,000 daltons. Two major bands were seen at 110,000 and 117,000 daltons which were tentatively identified as the receptor complexes.

Our earlier studies had shown the existence of two receptor forms, which could be separated on DEAE-cellulose columns by salt gradient elution[2, 9]. These forms were found to bind differently to nuclear constituents. The less acidic form (A) bound to DNA but not to chromatin, whereas the more acidic form (B) bound to chromatin but not to DNA. In view of the elution of two major bands from the affinity column, we attempted an analysis of the material prepared by this technique to determine the relationship of these two bands to receptors A and B prepared by classical techniques.

Receptor components from the affinity-column extract were chromatographed on a DEAE-cellulose

Table 1. Steroid-affinity chromatography of chick progesterone receptors

Step	Total protein* (mg)	Total receptor sites† (10^{-6} × d.p.m.)	Specific activity‡ d.p.m./mg ($\times 10^{-6}$)	Yield (%)	Purification (−fold)
Cytosol	2788	185	0·066	100	1 ×
30% Pellet	25·2	45·1	1·79	24	27
Affinity eluate	0·19	27·1	142·5	14·6	2150

* Protein was determined by the method of Lowry *et al.*[31].
† Receptor sites were determined by Scatchard-plot method, or by total d.p.m. of progesterone bound in Affinity eluate.
‡ Theoretical maximum specific activity estimated at 10^9 d.p.m./mg.

column by stepwise KCl elution at 0·15 M KCl (component A) and 0·4 M KCl (component B). These receptors were both 4S on sucrose gradients. When aliquots of the two samples were run on SDS-polyacrylamide gels, receptor A fraction ran as a single band at a molecular weight of 110,000 daltons. Receptor B fraction again contained a prominent band at 117,000 daltons, together with several minor contaminating bands.

In order to compare receptors made by this technique with those our laboratory had described earlier, a more complete purification by classical techniques was required to permit detection of the receptor proteins themselves in electrophoresis gels. Additional steps beyond the DEAE-cellulose method were required. Since DEAE-chromatography separates on the basis of a negative charge on the protein, a method was chosen based upon resolution involving positive charges. The first additional method was phosphocellulose chromatography. Receptor complexes from the ammonium sulfate step were applied to the column and eluted as shown in Fig. 1. As was the case for DEAE-cellulose, the receptor elution profile indicated the presence of multiple receptor forms. On this step KCl gradient, the forms tended to run together. Rechromatography of the major peak resolved two fractions, one eluting at 0·26 M KCl and the other at 0·3 M KCl. Dilution and chromatography of these two fractions on DEAE-cellulose showed that the 0·26 M peak was receptor B, while the 0·3 M peak was receptor A. As can be seen from Fig. 1, this step affords nearly a 50-fold purification, since most of the proteins are eluted at KCl molarities below 0·2 M Yields average about 50% for both receptor forms.

The next step was hydroxylapatite column chromatography. Either receptor preparation A or B from PC could be applied alone to a 10 ml HAP column equilibrated in 0·001 M K_xPO_4 pH 7·4 containing 0·1 M KCl and 0·012 M 1-thioglycerol. This step was a convenient one, since HAP is virtually unaffected by KCl concentrations. Thus, the receptors could be eluted from PC with high KCl and applied directly to the HAP columns. The receptors were eluted using 0·4 M K_xPO_4 buffer. Yield of receptor was over 80%, and the volume of the extract was reduced to 5 ml. Purification was about 4-fold. Since the contaminants were of varying molecular weights, a gel filtration purification step was added as the final step as described below.

Receptors A or B from the hydroxylapatite step were applied to a 5·0 cm × 50 cm agarose A-1·5 column in Buffer A containing 0·3 M KCl. The two progesterone receptor forms eluted differently. Both chromatographed behind the void volume of the column. Receptor A eluted at $K_{av} = 0{\cdot}41$, while receptor B eluted at $K_{av} = 0{\cdot}28$ as shown in Fig. 2. The two receptor peaks were separately pooled, dialyzed against distilled water and lyophilized. The lyophilized proteins were then characterized by SDS-gel electrophoresis. The gels showed that the two preparations still contained some minor contaminants. However, the major bands corresponded to those observed in the affinity chromatography preparative technique.

The B protein prepared by the two methods thus has a molecular weight of 117,000 daltons, and the A protein a molecular weight of 110,000 daltons. These values are in contrast to our earliest estimates from crude cytosol of about 100,000 daltons[1].

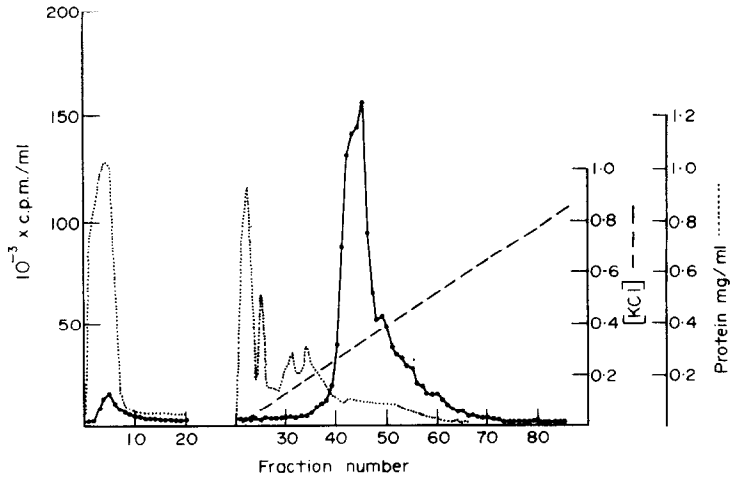

Fig. 1. Phosphocellulose chromatography of labeled progesterone receptor complexes from an ammonium sulfate precipitate. Receptor in Buffer A (10 ml) applied and the column washed with 40 ml Buffer A. Fractions (2·0 ml) were collected and 0·1 ml aliquots were counted for ^3H (●——●). Protein was determined by absorbance at 235 nm (. . . .). The KCl gradient used to elute the column was generated using Buffer A and Buffer A containing 1·0 M KCl. KCl concentrations were determined by conductivity (- - - -).

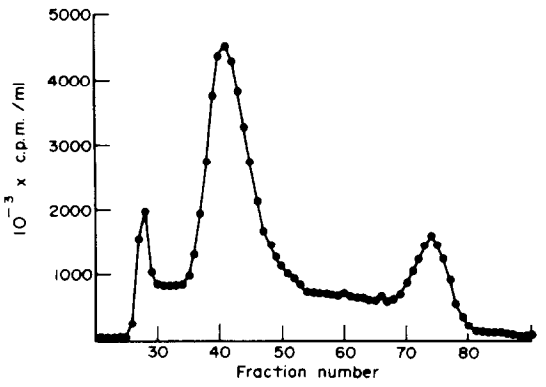

Fig. 2. Agarose A-1·5 M chromatography of purified receptor B protein. Column was equilibrated in Buffer A containing 0·3 M KCl. Fractions (1·5 ml) were collected at 0° (100 ml/h) and aliquots counted for ^3H (●——●). Void volume was in fraction 29, included volume in fraction 74.

Whenever a labile material is to be isolated, a central problem is that of stability of the purified product. Due to the fact that the receptors can only be detected during isolation by their association with a radioactive ligand, this problem is particularly difficult in this case. Several aspects of receptor stability and storage have been examined.

Various parameters were studied for their effect on receptor–hormone complex stability. Oviduct cytosol progesterone receptors were labeled with [^3H]-progesterone, and then incubated under various conditions. After the experimental period, the preparations were diluted back to the initial conditions, and the bound hormone remaining as receptor complex was determined by analytical gel filtration on 5 ml sephadex G-75 columns.

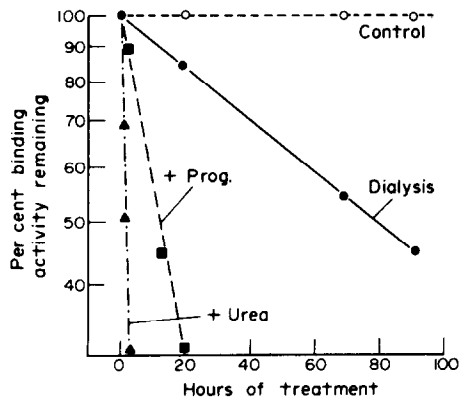

Fig. 3. Stability of receptor–hormone complexes to various treatments. Labeled receptors from an ammonium sulfate precipitate were treated with several agents at 0° and the bound ^3H loss from receptors followed with time by charcoal adsorption assay. Control (○- - - -○) sample was untreated and kept at 0°. Dialysis against Buffer A (●——●); addition of excess nonradioactive progesterone (■- - - -■); addition of 3·0 M urea (▲- - -▲).

Receptor-hormone complexes can be dialyzed for up to 12 h with only a 10% loss of bound hormone. There is no significant difference in stability between dialysis of 0·3 M KCl and that in buffer lacking KCl. Thus, the binding site is not greatly perturbed in 0·3 M KCl.

Figure 3 shows the effect of dialysis on the complexes. The Figure compares this dissociation rate to that previously determined for pseudo first-order dissociation using excess non-radioactive progesterone. The rate of hormone loss during dialysis is significantly slower than that observed with excess cold progesterone. Apparently, the dissociation of complexes is a complicated process. Also shown is the stability of intact, non-dialyzed complexes kept at 0°. Although there are significant functional changes in the complexes, there is no detectable loss of hormone-binding activity under the control conditions. For comparison, the rate of denaturation is shown for treatment with 3 M urea. This process clearly involves effects on conformation, since the dissociation rate is an order of magnitude faster than the normal first-order rate constant.

The conditions discussed above have been evaluated for their effects upon receptor complexes as assayed by the maintenance of bound progesterone. However, it appears that there are many subtle changes in receptor properties which do not result in release of bound progesterone. One of these changes is the conversion of crude cytosol receptor complexes to a new, "sticky" form. A study of this phenomenon is shown in Fig. 4. Receptor-hormone complexes from cytosol were prepared by chromatography of labeled cytosol on Sephadex G-75. Receptors chromatograph in the void volume of this column. The void volume receptor complexes were then stored either in ice or frozen at −20°C. Samples were thawed at various times and re-chromatographed on G-75 columns. There was no release of progesterone from the complexes. However, receptors did not pass through the columns quantitatively after storage. As shown in the Figure, there was a time-dependent conversion of the receptors to a form which would not elute through G-75. Significantly, the process occurred to the same degree in either the frozen state or when stored unfrozen at 0°. This process does not involve merely conversion of receptors to highly aggregated forms. Sucrose-gradient analysis of the stored receptors showed that the expected 4S form was still present after storage (data not shown). Freezing and thawing by themselves do not produce this change, as shown by the squares in Fig. 4. These two points represent assays on G-75 of fresh receptor complexes frozen and thawed quickly twice or four times on the day they were prepared. Even four cycles produced relatively

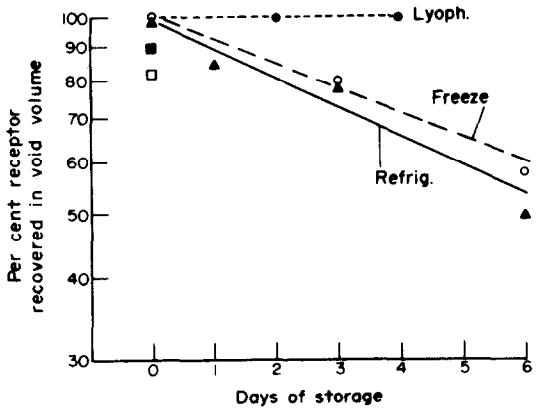

Fig. 4. Effect of storage on aggregation state of progesterone-receptor complexes. Labeled complexes prepared as in Fig. 3 were assayed for ^3H bound to macromolecules by Sephadex G-75 elution after various times of treatment. A lyophilized sample (● ···· ●) was redissolved in an equivalent volume of water and assayed. Samples frozen on day 0 (○ - - - ○) were thawed quickly by swirling in a 37° bath on the indicated days. Samples refrigerated 0° (▲ - - - - ▲) were assayed on the indicated days. Samples were also subjected to either two (□) or four (■) freeze-thaw cycles on the same day.

little change in chromatographic properties. This conversion can be prevented by proper choice of storage method. As shown in the Figure, receptors in the frozen state can be lyophilized and stored as dry powders. When re-dissolved, the complexes behave normally with no dissociation or conversion to abnormal forms. We have previously reported the usefulness of storing receptors as ammonium sulfate precipitated pellets[2], and now use this procedure together with lyophilization to store the purified materials.

The next parameter studied for its effect on stability was the requirement for a reducing environment. Receptor-hormone complexes were prepared in Buffer A containing 1-thioglycerol or in buffer lacking this constituent. After three hours, the complexes were chromatographed on agarose A-1·5 M columns which were equilibrated in Buffer A containing 0·3 M KCl and either no thioglycerol or 0·012 M 1-thioglycerol. The amount of [^3H]-receptor complexes in the macromolecular fractions was determined in both cases. The two chromatograms showed more bound ^3H in the column containing the thioglycerol, indicating the receptors were stabilized by the presence of this constituent. This stability change affected both receptor components. When receptors A and B were isolated by DEAE-cellulose chromatography in buffer with or without thioglycerol, the results shown in Table 2 were obtained. The Table shows that the receptor retained by DEAE was drastically reduced when thioglycerol was omitted. This did not involve a conversion of A to B form. However, as we have demonstrated in other experiments not presented here, the stabilization produced by this agent is not the same as that afforded by 10–40% glycerol reported by other laboratories. A test of the effective molarity of 1-thioglycerol showed that 0·012 M was nearly optimal. Less stabilization occurred at 1·2 mM; 60 mM was also less effective. Consequently, the 1-thioglycerol concentration of 0·012 M has been used routinely in our work.

We have continued our experiments designed to evaluate the binding of receptors to nuclei and nuclear constituents. To facilitate these studies, a cell-free system prepared from the estrogen-primed chick oviduct was developed and used to study the uptake of cytoplasmic progesterone-receptor complex by isolated nuclei. The receptor and purified nuclei were shown to be stable at 25°, but not at 37°. Thus, nuclear incubations were routinely performed at 25°. Incubations at this temperature revealed greater nuclear uptake of the cytoplasmic hormone-receptor complex than did incubations performed at 0°. The uptake process showed a quantitative preference for oviduct nuclei[32, 33]. These data show that chick oviduct progesterone receptor binds in a saturable fashion to purified oviduct, colon, and reticulocyte nuclei. Moreover, while the apparent binding affinity was the same for all tissues ($K_d \sim 10^{-8}$ M), more acceptor sites were present in the oviduct nuclei than in colon or erythrocyte nuclei. Of course it is unknown whether these non-target tissue acceptor sites are functionally equivalent to those of the oviduct.

The kinetics of interaction of chick oviduct progesterone receptor with highly purified nuclei have been studied in detail with respect to the influence of various parameters on the kinetic constants K_d and n, the number of "acceptor" sites per nucleus. We have further characterized the influence of ionic strength on

Table 2. Effect of thioglycerol on receptor DEAE-cellulose chromatography

Sample buffer	Thioglycerol molarity	Per cent of ^3H in breakthrough	A peak	B peak	A/B Ratio A peak	B peak
K_xPO_4	0	72	13	15	46	54
K_xPO_4	1.2 mM	32	23	44	35	65
K_xPO_4	12 mM	15	37	49	43	57
K_xPO_4	60 mM	29	26	45	37	63
Tris-EDTA	12 mM	25	30	44	41	59

nuclear binding by showing that "non-specific" nuclear binding is constant and independent of salt concentration. Significantly, the decrease in nuclear binding capacity which accompanied an increased ionic strength was shown to be due to the stepwise elution of a single predominant class of nuclear acceptor sites. The time necessary to reach equilibrium was a function of receptor concentration. At 10^{-8} M steady state was rapidly achieved and the predominant class of nuclear acceptor sites ($K_d \sim 10^{-8}$ M) was filled.

We have continued our experiments designed to evaluate the binding of receptors to nuclei and nuclear constituents. To this end, a technique was developed which may be termed "chromatin-affinity" chromatography. Chick oviduct chromatin was suspended in agarose beads and washed as described in Methods. When oviduct progesterone-receptor complexes from an ammonium sulfate precipitate were applied to the column, some of the receptors adsorbed, as shown in Fig. 5. This receptor fraction eluted with a KCl gradient at about 0·15 M KCl. Subsequent analysis of this collected peak by DEAE-cellulose chromatography showed that it consisted of receptor component B, the form of receptor shown in previous studies to bind to oviduct chromatin. Thus, this technique yields a fraction enriched in receptors of chromatin interaction. Such fractions are prime candidates for study of receptor effects on chromatin transcription *in vitro*.

DISCUSSION

The studies outlined above now provide a protocol for isolation of these interesting receptor molecules from chick oviduct. The methods are suitable for scaling up to prepare bulk amounts of receptors for physical and chemical studies. There are now three main questions which need to be answered regarding the function of the steroid receptors.

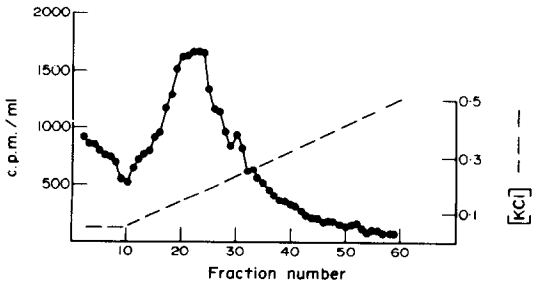

Fig. 5. Chromatin-affinity chromatography of receptor complexes from ammonium sulfate precipitate. Receptors were applied to the chromatin agarose 1·5 M suspension packed as a column and washed in Buffer A containing 0·05 M KCl. A KCl gradient (- - - -) was used to elute 2 ml fractions which were assayed for ^3H (●——●).

First, are the receptor–hormone complexes directly involved in the process of gene regulation in target cells? Despite intensive research in this area for several years, the answer to this question is still not proven to be in the affirmative. Correlative studies of receptor uptake by nuclei with time and autoradiographic studies have shown that steroid hormones do indeed accumulate in nuclei at the appropriate times to account for increased nuclear activity. Recent studies from our laboratory have shown that one consequence of this intranuclear steroid is the elaboration of newly-synthesized messenger RNA's for the proteins directed by that steroid[28, 29]. Never the less, direct proof of the involvement of receptors in other than a transport role awaits the availability of an *in vitro* chromatin transcription system responding to the complexes.

Second, the existence of multiple forms of the receptors with different binding activity toward DNA and chromatin nucleoprotein, opens to question the molecular events by which these receptors interact with the genome. Any number of models can be postulated to account for the occurrence of these forms. However, tests of these models invariably require purified receptor components for the assays. Binding of receptors to nuclear constituents *in vitro* has so far only been studied extensively in relatively crude receptor preparations. These contain large amounts of other proteins, some of which are undoubtedly capable themselves of conflicting interactions with the nuclear material. The sensitive assays derived for studying gene regulating proteins in prokaryotes, for example[30], require highly purified proteins to prevent non-specific DNA interactions from interfering. It is hoped that such studies can now begin with receptors, in order to determine whether they may act directly on RNA polymerases (perhaps as directors of template selection) or, more likely, whether they act at regulatory regions of the DNA gene itself. A model such as the gene repressor proteins of bacterial systems might be active.

Third, the role of the hormone ligand in receptor activity remains obscure. By choice of the name receptor, we tend to think of these proteins as carriers for the hormone. In fact, it may be more appropriate to think of the hormone as a facilitator of the biologic activity of the receptor protein. The requirement of hormone on the receptor to make it bind to DNA or chromatin, or even be taken up by whole nuclei *in vitro* points to this possibility. Thus, it is important to understand the conformational and electrostatic changes induced on the receptor by the hormone. These studies will lead to mapping of the hormone-binding site(s) on the receptor, with three important results to be determined: (1) How does the receptor bind the hormone so tightly, as much as 10^6 times as strong an

association as most enzymes have for their substrates? (2) What is the nature of the hormone-binding site's recognition of the proper ligand? (3) Can our understanding of this binding process lead to the development of suitable synthetic hormone analogs having desirable hormonal properties but a minimum of side effects?

Attempts to answer these questions have, in the past, been severely hampered by a lack or purified receptor-hormone complexes. The sort of approach outlined in this communication has now provided stored materials for study. Experiments of the sort discussed above are now in progress in our laboratory. As these materials become available for other hormones and other systems, it should be possible to greatly advance our understanding of the biologic function of these intriguing macromolecules.

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DISCUSSION

Munck:

Have these A and B proteins been found with other steroid hormone receptors?

Schrader:

Sullivan and Strott (*J. biol. Chem.* **248** (1973) 3202–3208) reported a DEAE cellulose elution profile of the rat ventral prostate androgen receptor which was indistinguishable from mine. As far as I know, no one else has attempted to use this technique directly to show the differential specificity of the binding.

Jensen:

Liao has what he calls α and β proteins. The β protein is the receptor but is that anything analogous to your A and B? Can you comment on this?

Schrader:

If we administer tritiated progesterone *in vivo* by intravenous injection, into a chick, we can observe the movement of both of these components into the nuclei in equal amounts, so presumably both of them, in our hands, are receptors and they certainly are kinetically identical as far as the steroid binding site.

Jensen:

When you extract the nucleus, do you get a nuclear complex corresponding to both A and B proteins or is your nuclear complex a single entity?

Schrader:

Nucleus contains both materials.

Vorob'ev:

As far as I understand, you use 3 M Urea for elution of receptors in affinity chromatography. Don't you think that this procedure results in denaturation?

Schrader:

I don't know that it denatures entirely. It does, however, denature enough to release proteins from the bound steroid and allow the elution of the receptor protein from the column. The curious thing which apparently is a general phenomenon now is that the receptor protein can be reconstituted undenatured by gradient dialysis. It cannot be done, in my hands, by dilution.

Vorob'ev:

Have you measured any physical constant before and after gradient dialysis?

Schrader:

No, I have not, except to say that the behaviour of the material on sucrose gradients and on all of these ion exchange columns just seems the same. Clearly, the hormone binding site itself has not been dramatically changed.

Korenman:

Have you purified the A component well enough to study the kinetics of assocation and dissocation of individual and combined fractions to see if there's any interaction between the two components?

Schrader:

We have been using partially purified receptors. I have never had enough of the purified receptor protein to do nuclear or chromatin binding studies using competition methods. We cannot detect: (1) any re-association of the two molecules together, for example, on sucrose gradients. (2) any recombination of the two on chromatin or on DNA. The curious fact that the two preparations are present in equal amounts in cytosol and go into the nucleus and appear in the nuclear fractions in equal amounts would seem to indicate that the two are coupled. We cannot reproduce that coupling if it exists.

Korenman:

It would be possible even though the proteins are completely charged with progesterone during the entire process of purification to do an exchange type dissociation experiment to see if the dissociation of the hormone–receptor complexes is bi-phasic or uni-phasic and whether the 2 components contribute differently to this dissocation rate reaction. That might be of some interest.

Schrader:

The components both have the same dissociation rates as the crude cytosol. However there is a break in the dissociation curve for the A component and not the B and I have no explanation for that.

Korenman:

Have you done affinity chromatography with components of chromatin rather than whole chromatin to see about purification?

Schrader:

I've attempted to make what I've termed an affinity column of chromatin and wash it initially with varying levels of salt to wash off the histones and leave the rest of the chromatin intact but I was unsuccessful. Enough of the chromatin just washed through the column when I went to 0·35 M salt.

Hansson:

Since you all are talking about purification, I would like to show a slide from the purification of testicular androgen binding protein (ABP) from rabbit. Cytosol from caput epididymis homogenate obtained from 100 adult rabbits was fractionated by ammonium sulfate precipitation (40%), Sephadex G-200 filtration, DEAE-cellulose ion exchange chromatography and preparative gel electrophoresis. As you can see from the figure, the purified ABP moved as one single band by polyacrylamide electrophoresis. The band of binding activity moved together with the protein band (Fig. 1). We have obtained about 250 μg of this apparently homogeneous ABP preparation; however, the yield has been rather poor. We hope, that modifications of the procedures, including affinity chromatography will increase the yield.

Rousseau:

You have shown that the apparent binding capacity of isolated nuclei for receptor-progesterone complex depends on the salt concentration, and you have chosen to work at 0·15 M NaCl, if I'm not mistaken. Then you go on to give us values for dissociation constants and number of sites. Do you have data on whether the salt concentration affects the number of sites or apparent dissociation constants?

Schrader:

Yes, we do. We did saturation binding of nuclei with receptor

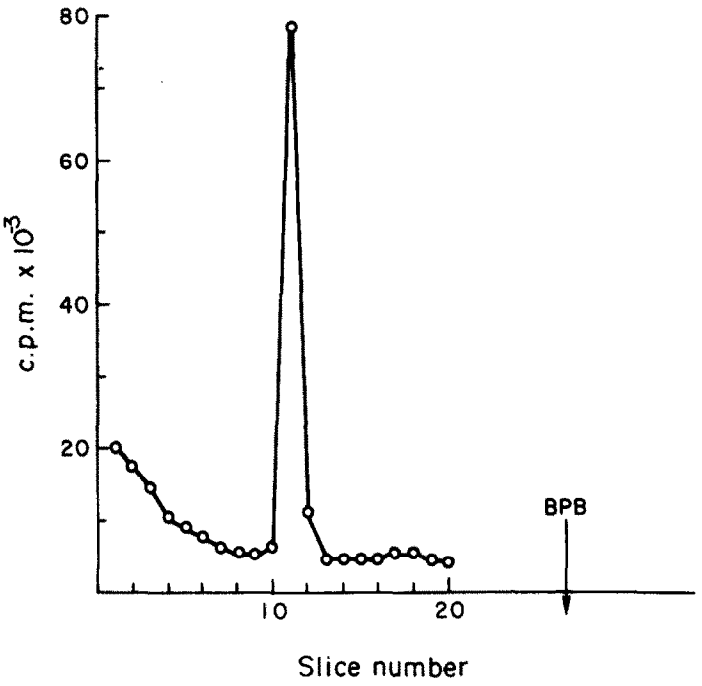

Fig. 1. (Hansson).

at all of these salt concentrations that I showed. What I was showing there was the last point on a whole series of curves. Then we take the data from the saturation experiments at every salt concentration and plot them by the double-reciprocal method; we obtain, in extrapolation, the dissociation constant for binding at that salt molarity. The dissociation constant, within the limits of experimental error, does not change. The number of sites changes.

Villee:

When the two subunits of the progesterone receptor were first described they were called, I think, subunits of a single receptor for while they appeared to be independent receptors they both took progesterone into the nucleus. Then in your last slide today, you indicated that the "a" type is converted into the "b" type on warming. What is the situation?

Schrader:

The original observation that the receptor population for progesterone in the oviduct was a heterogeneous one was made by Dr. Mary Sherman in the original publication by Dr. O'Malley's group on the subject in 1970. They adopted the term "components" to identify the small peaks that seemed to appear on gel filtration patterns and also on gel electrophoresis. We continued the nomenclature of calling them "components" when we purified them. In one of our experiments we slipped and called them subunits. At the present time, however, I have no evidence to prove that the two components I talked about are in fact subunits of a large macromolecule in the true biochemical sense of the word. I would like to point out, however, that the two receptor activities which can be separated from the tissue seem to appear in about equal amounts. For example, they both appear in nuclei and yet you noticed I talked about binding to nuclei involving the "b" component and yet they both occur in there. Perhaps they act in concert. Maybe one of the subunits, the "b" component, for example, finds the acceptor site which says "I am a progesterone-responsive gene' and then the "a" component reacts with the DNA to allow transcription but that's wild speculation at this point. I think a lot more work has to be done.

Jensen:

On the same subject, in your last slide you seemed to imply that the "a" component might be the native form and the "b" component the transformed form and you were converting one into the other. Did I misunderstand you?

Schrader:

No, you did not. We have been reluctant to go along, as you know, with assignment of the precursor-product relationship in the progesterone oviduct system in exact analogy to what you have described in the uterus predominantly because of the fact that regardless of how one got receptor out one always saw some of both of the receptor forms. This was true whether we had EDTA present or not. However, now we have some analytical techniques using ion exchange columns for isolating particular fractions which are enriched for what we would think was a precursor or a product or whatever. I think we can begin to take a look at this question. I would not be at all surprised if it turns out that our "a" preparation corresponds to what you call the precursor or untransformed material and that the "b" preparation corresponds to what you call the nuclear 5S form, but that remains to be seen. I might point out one other fact about receptor transformation. When we warm the ammonium sulfate fraction, we get the transformation that I showed on phosphocellulose. But if you take the isolated materials and try to warm them, they don't seem to convert. That would suggest that there is in

fact something else present which is doing this transformation which is lost in the purification.

McKerns:

I imagine, and I'd like some clarification on this, that you are equating progesterone action with avidin synthesis because avidin is a convenient protein to follow. In this regard, when you're equating numbers of sites, that is, a decrease with increasing salt concentration, I suppose that you're not assuming that this is a decrease in sites just for avidin? Or could it mean that there are more sites available on the gene at low salt and they're not necessary just for avidin expressions?

Schrader:

Well, this depends upon how you view the concept of how hormones and receptors might be controlling things. Dr. Jensen alluded to the fact that the receptor could be acting essentially as a regulatory factor for RNA polymerase. That's certainly a possibility. However, my objection to that argument would be based upon the fact that the number of receptors retained in nuclei is far lower than the number of RNA polymerase molecules. You would have to imagine heterogeneity of RNA polymerases or something like that. As far as anyone knows, the avidin gene is a single copy gene although no one has actually measured that yet. Let's assume for the moment that it is. Clearly progesterone is doing an awful lot of things so presumably it does not take all these thousands of receptor molecules to affect the one gene. My feeling is that all the progesterone responsive genes might have the same acceptor site telling the system that here is where the receptor is to go and do something. The acceptors would be the same on each gene regulated by that hormone.

Exley:

Could you elaborate a little bit more on the estrogen induction of your progesterone sites which you get in certain mammals. Do you get this in the chick oviduct system?

Schrader:

Estrogen administration causes a rise in the total number of sites in the tissue. However, estrogen causes the cells to grow and to divide. There is progesterone receptor present in the newly hatched untreated animal. However, there are estrogenic events in the embryo's Mullerian duct, as well, so it may be that what I call an unstimulated oviduct, one which is one day post-hatching, may already have been acted upon by estrogen.

Exley:

I was really asking if there were more progesterone sites per cell after administration of oestrogen.

Schrader:

The number of progesterone sites per cell does increase but not more than a factor of 4 or 5 or something like that. So the number of sites per oviduct grows enormously but the oviduct grows about 10,000 fold in a period of a couple of weeks.