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Progesterone "Receptors" in the Cytoplasm and Nucleus of Chick Oviduct Target Tissue*

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Abstract. This report demonstrates that the chick oviduct, a specific target organ for progesterone, contains both cytoplasmic and nuclear macromolecules which bind progestins. These binding molecules can be clearly distinguished from transcortin by centrifugation through sucrose gradients of low ionic strength and by agarose gel filtration. The cytoplasmic progesterone-binding molecules also bind 5-α-pregnane-3,20-dione, but have significantly lower affinity for cortisol, estrone, or aldosterone. They are absent from blood and nontarget organs such as lung and spleen. The tissue-specific binding components appear to be heat-labile proteins with an average dissociation constant for progesterone of about 8×10^{-10} M at 2°C. These results are consistent with the identification of the progesterone-binding molecules as the functional hormone receptors. In further support of this concept is the finding that treatment of the chicks with estrogen coordinately induces a 20-fold increase in the number of progesterone-binding molecules and enhances the capacity of progesterone to induce avidin synthesis.

A progesterone-"receptor" complex can be detected in both the cytoplasm and nuclei of oviduct tissue after an injection of [^{3}H]progesterone to estrogen-treated chicks. By contrast, incubation of oviduct tissue with [^{3}H]progesterone *in vitro* at 2°C for 5 min leads to labeling of the cytoplasmic "receptor" only. Transfer of the "receptor"-steroid complex into the nucleus then appears to occur upon subsequent incubation *in vitro* at 37°C. This observation suggests that the transfer of bound progesterone across the nuclear membrane may be an energy-requiring enzymatic process.

Most of the recent theories on the mechanism of action of steroid hormones involve the initial combination of the hormones with a specific receptor in the target tissue. Reports from several laboratories show the existence of tissue-specific macromolecules which can interact with estrogens in rat uterus, with androgens in prostate, and with aldosterone in kidney.[1–8] In these three hormonal systems, "receptor" molecules have been identified and partially characterized in both cytoplasmic and nuclear fractions of the target tissues. Since many of the biological effects of steriod hormones appear to involve alterations at the gene level, the nuclear localization of hormone-receptor complexes is of major importance. Recent investigations on the binding of estradiol in the rat uterus show that this hormone first binds to a cytoplasmic receptor protein which is

subsequently transported into the cell nucleus.[2,9,10] A similar occurrence has been reported for the interaction of dihydrotestosterone with the ventral-prostate target tissue of rat.[6,11]

During the past four years we have utilized the chick oviduct as a model to study the mechanism of action of progesterone.[12-15] In the estrogen-stimulated chick oviduct, progesterone specifically induces the synthesis of avidin. This induction is inhibited by actinomycin D. Progesterone also causes early changes in nuclear rapidly-labeled RNA,[14] RNA polymerase,[16] nearest-neighbor dinucleotide composition of nuclear RNA,[12] and hybridizable species (populations) of nuclear RNA.[17] These data suggest that progesterone selectively alters oviductal gene expression to effect new RNA transcriptions. To date, we have reported only preliminary evidence to support an initial action of progesterone with an intracellular receptor.[12] We have demonstrated that within 15 min after injection of [^{3}H]progesterone into estrogen-pretreated chicks, the steroid is bound to a soluble macromolecule which can be identified by gel filtration on G-200 sephades.[12] The present study reports the preliminary characterization of cytosol- and nuclear-binding proteins for progesterone in the oviduct and offers evidence for the transfer of the cytoplasmic complex of progesterone receptor into the nucleus.[22]

Materials and Methods. The materials utilized for these experiments were obtained as follows: [^{3}H]progesterone (33.5 Ci/mmol) and [^{3}H]cortisol (45 Ci/mmol) from New England Nuclear Corp.; diethylstilbestrol from Merck and Co.; agarose beads from Bio-Rad Laboratories; RNase and DNase from Worthington; pronase from Calbiochem; Sephadex G-50 from Pharmacia; and Rhode Island Red chicks from Acme Corp., Nashville.

Preparation of tissue supernatant and nuclear extracts: Female Rhode Island Red chicks were injected subcutaneously with 5 mg of diethylstilbestrol in sesame oil for 15 days beginning at the sixth day of life. The chicks were then sacrificed and the oviduct magnum was removed, rinsed in saline, weighed, and homogenized (Polytron, Pt-10, Brinkman) in Tris–EDTA buffer (0.01 M Tris–1.5 mM EDTA, pH 7.4) at 0°C. The homogenate was centrifuged at 850 $\times$ g and the nuclear pellet removed. The supernatant extract was then centrifuged at 120,000 $\times$ g for 1 hr at 2°C.

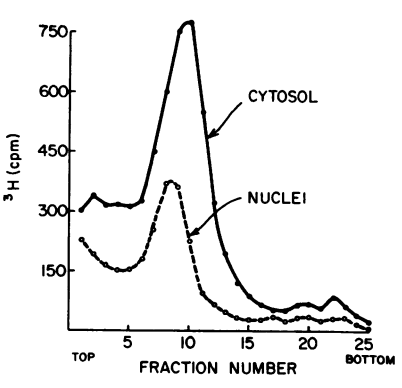
Nuclear extracts were prepared from the 850 $\times$ g pellet after resuspending and centrifuging twice in Tris–EDTA buffer. The pellet was then extracted with 0.3 M KCl in Tris–EDTA, centrifuged, and the supernatant extract was analyzed for progesterone-binding component. In additional experiments, the washed nuclear pellet was further lightly homogenized in buffer N (0.25 M sucrose, 0.05 M Tris, 0.025 M KCl, 0.002 M Ca Cl_2, 0.024 M thioglycerol, and 0.25% Triton X-100) resuspended and centrifuged twice in the same buffer; the nuclei were then centrifuged for 60 min at 60,000 $\times$ g through 2.2 M sucrose in buffer N without Triton X-100. These highly purified nuclei were then extracted with 0.3 M KCl in Tris–EDTA.

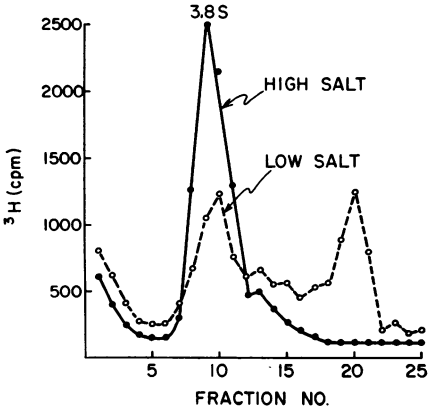
Sucrose gradient centrifugations: Aliquots (0.2 ml) of cytosol or nuclear extracts were applied to 4.8 ml sucrose gradients (5–20% sucrose in Tris–EDTA buffer) in the presence or absence of 0.3 M KCl and centrifuged for 16 hr at 1°C in the Spinco SW-50 rotor at 50,000 rpm. The gradients were fractionated and collected on an ISCO Gradient Analyzer (Model D, Instrumentation Specialties Co.) and counted in toluene–Triton–Liquifluor (625:333:42) with a counting efficiency of 31% for ^{3}H. Sedimentation coefficients ($s_{20,w}$) of the progesterone-binding components were estimated by comparison with ovalbumin (3.67S).

Agarose gel filtration: Agarose beads (Bio-Gel A--0.5m) were equilibrated in 0.01 M Tris, 1.5 mM EDTA, 0.3 M KCl, pH 7.4 at 0°C and packed into a 1.27 $\times$

110 cm column. Aliquots (0.2–1.0 ml) of cytosol or chick plasma were applied to the column at 1°C; the absorbance of the eluate was monitored by a Beckman DB-G spectrophotometer and 1.1-ml fractions were collected in a Gilson Fraction collector.

Results. Macromolecular binding of [^{3}H]progesterone injected *in vivo:* [^{3}H]progesterone (90 μCi) was administered intravenously into estrogen-treated chicks and the animals were sacrificed 8 min after the injection. Nuclear extracts and cytoplasmic supernatant (cytosol) were centifuged through 5–20% sucrose gradients containing 0.3 M KCl. Both preparations revealed the presence of macromolecules (~4S) capable of binding [^{3}H]progesterone (Fig. 1).





(*Left*) FIG. 1. Sucrose gradient centrifugation of progesterone-binding components of chick oviduct cytoplasm (cytosol) and nuclei. Animals were sacrificed and oviduct preparations made at 8 min after injection of the steroid (90 μCi of [^{3}H]progesterone, intravenously). Tris–EDTA 5–20% sucrose gradients containing 0.3 M KCl in Tris–EDTA were layered with 0.2-ml extracts of cytosol or nuclei and centrifuged for 16 hr at 1°C in the Spinco SW-50 rotor at 50,000 rpm.

(*Right*) FIG. 2. Sucrose gradient analysis of cytoplasmic progesterone-binding components of chick oviduct in the presence (high salt) or absence (low salt) of 0.3 M KCl.

Considerably less [^{3}H]progesterone was bound in the nucleus 1 min after injection, and the amount of nuclear binding appeared maximal 25 min following administration of the steroid (data not shown). We next attempted to study the interaction of progesterone with the cytosol- and nuclear-binding molecules separately, and under controlled conditions *in vitro.*

Cytoplasmic binding of [^{3}H]progesterone *in vitro:* Sucrose gradient centrifugation of oviduct cytosol incubated with [^{3}H]progesterone (10^{-8} M) at 0°C revealed a single, 3.8S, peak of bound radioactivity in gradients containing 0.3 M KCl (Fig. 2). From similar experiments at different [^{3}H]progesterone concentrations, a dissociation constant of about 8×10^{-10} M was estimated under these conditions.[22] In the absence of KCl, two separate binding components (5S and 8S) were detected in the crude oviduct cytosol (Fig. 2). Experiments are in progress to evaluate the relationship among these components, in particular to ascertain whether the 3.8S component observed in KCl is a subunit or simply a less compact form of the 5S component, and whether the 8S component is a dimer of the 5S form.

Chick plasma transcortin (CBG) binds progesterone with a high affinity and could be present as a contaminant in the oviduct extract. However, CBG has a sedimentation coefficient of approximately 4 S in both high salt and low salt gradients. In addition, the oviduct-binding component can be separated from CBG by gel filtration on Agarose A-0.5m.[18,22] Four peaks of radioactivity are resolved by this method (Fig. 3). The initial peak of radioactivity is small and

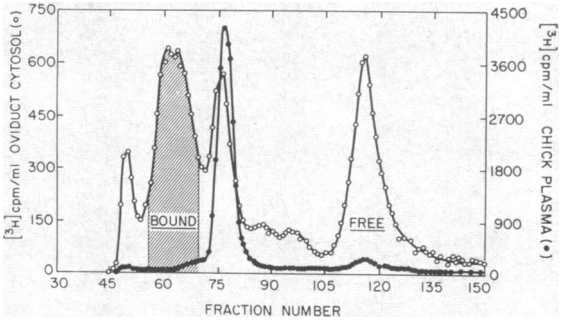


FIG. 3. Gel filtration (Agarose A, 0.5m) of chick oviduct cytosol after *in vitro* incubation with 4.7×10^{-8}M [^{3}H]-progesterone at 1°C for 45 min. The elution pattern, from the same column, of chick plasma similarly incubated is superimposed for reference.

variable and represents substances excluded from the gel (molecular weight $>5 \times 10^5$). The major peak of bound progesterone (shaded area) chromatographs as a broad peak encompassing two or more components. These components are found only in oviduct target tissues and are absent from serum, lung, or spleen tissue. The major peak of bound progesterone is eluted sooner than the bulk of oviductal cytoplasmic proteins, as measured by absorbance at 280 nm.

The next sharp peak seen in Fig. 3 is eluted at about the same position as the chick plasma CBG marker. This peak is nearly eliminated by extensive washing of the fresh oviduct in cold saline (0.154 M NaCl) before homogenization. Refiltration of the peak of progesterone bound by chick plasma produces no artifactual aggregation into the area of the specific progesterone-binding components, and refiltration of the major peak of cytosol-bound progesterone does not shift its elution position. The final radioactive peak shown in Fig. 3 contains unbound [^{3}H]progesterone.

More than 93% of the bound radioactivity isolated by either gradient centrifugation or gel filtration chromatographs as progesterone in the Bush A-1 (ligroin–methanol–water, 10:9:1) paper chromatography system. This demonstrates that no significant metabolism of the bound [^{3}H]progesterone occurs under the condition of these *in vitro* experiments (0°C).

The results of experiments to determine the nature of the macromolecular-steroid complex are shown in Fig. 4. The bound [^{3}H]progesterone (3.8S component) was recovered after sucrose gradient centrifugation in 0.3 M KCl, and incubated at 0°C alone or in the presence of pronase, RNase, or DNase. The bound [^{3}H]progesterone was then separated from free steroid by passage through a Sephadex G-50 column. Only pronase, of the enzymes tested, caused a significant release of bound [^{3}H]progesterone, indicating a protein nature for the molecular binding site.

The steroid binding specificity of the oviduct cytosol was investigated by

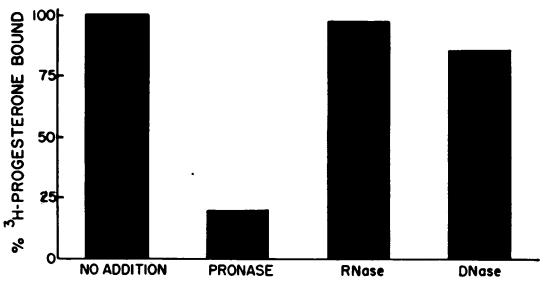


FIG. 4. Release of [³H]progesterone from oviduct-cytosol binding component. Oviduct cytosol was incubated with [³H]-progesterone (as in Fig. 3), centrifuged through a sucrose gradient, and fractionated. Aliquots (0.4 ml) of pooled fractions containing bound radioactivity were then incubated for 30 min at 1°C with no addition, addition of 0.5 mg pronase, 0.5 mg bovine RNase, or 0.5 mg of DNase plus 5 mM magnesium acetate. The samples were then passed through Sephadex G-50 columns to separate bound from free radioactivity. A total of 1348 cpm ³H was utilized in each incubation and recovery of radioactivity through the entire procedure was about 80%.

comparing the relative capacities of unlabeled progesterone, 5-α-dihydroprogesterone (5-α-pregnane-3,20-dione), and cortisol to compete with [³H]progesterone for sites on the oviductal binding protein (Fig. 5). [³H]progesterone (3.2 ng) plus a 1000-fold excess of competing steroid were added to oviduct cytosol extracts. These samples were then centrifuged in sucrose gradients to determine [³H]progesterone binding. Unlabeled progesterone or 5-α-pregnane-3,20-dione eliminates the binding of [³H]progesterone but cortisol has very little effect (Fig. 5). Similar experiments to determine the binding of progesterone metabolites and other biologically active steroids reveal minimal competition using 5-α-pregnane-3β,20α-diol; 3β,16α-dihydroxy-5α-pregnane-20-one; *d*-aldosterone; estrone; estriol; androstenedione; dehydroisoandrosterone; and cholesterol.

Nuclear binding of [³H]progesterone: In previous studies, [³H]progesterone was shown in both the nuclear and cytoplasmic fractions of the chick oviduct after injection of labeled steroid to chicks, or after *in vitro* incubation of oviduct in medium containing [³H]progesterone.[12] We are now able to solubilize the macromolecular component from the nuclear fraction that binds progesterone.

In the following experiments, oviduct tissue segments were incubated *in vitro* with 3.2 ng of [³H]progesterone for 15 min in 2 ml Basal Eagle's Medium at 37°C. The tissue was then cooled immediately to 0°C and homogenized in Tris–EDTA buffer. The nuclear pellet was resuspended and centrifuged twice with Tris–

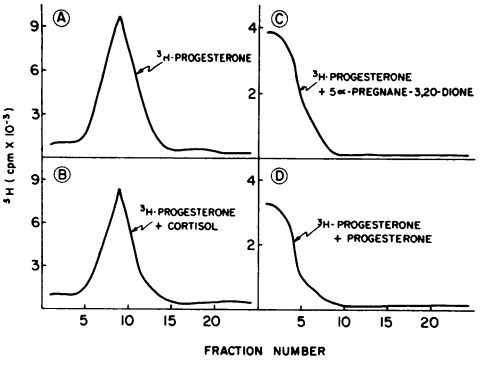


FIG. 5. Steroid-binding specificity of oviduct cytosol *in vitro*, studied by sucrose gradient centrifugation. Cytosol (1 ml) was incubated with 3.2 ng of [³H]progesterone alone (A), or in the presence of 3.2 μg of cortisol (B), 5-α-pregnane-3,20-dione (C), or progesterone [D]. Aliquots of the incubation mixture were then centrifuged through sucrose gradients containing 0.3 M KCl.

EDTA buffer and the pellet was extracted with the same buffer containing 0.3 M KCl. Sucrose gradient analysis of the nuclear extract revealed a sharp peak of bound progesterone in the 4S area of the gradient which could not easily be distinguished from the cytosol binding component as it exists in 0.3 M KCl (Fig. 6). The nuclear binding protein was saturated at a concentration of 15.0 ng progesterone per gram oviduct in 1 ml of incubation medium. No progesterone binding component was eluted from the nuclear chromatin pellet when it was extracted with low-salt buffers.

To demonstrate that the nuclear binding protein is an intranuclear molecule we purified the nuclei by treatment with 0.25% Triton X-100 in buffer N to remove the outer nuclear membrane, rinsed the nuclei twice in buffer, and sedimented them through 2.2 M sucrose. Extraction of the purified nuclei again revealed an intranuclear progesterone-binding protein which was extractable with 0.3 M KCl.

We next attempted to define the kinetics of progesterone distribution between cytoplasmic and nuclear cell compartments upon *in vitro* uptake of trace amounts of the steroid. Oviduct segments were preincubated with [^{3}H]progesterone at 0°C for 5 min to allow passive diffusion of the steroid into the cell, then incubated at 37°C for the periods of time shown in Fig. 7. The tritium contents of cyto-

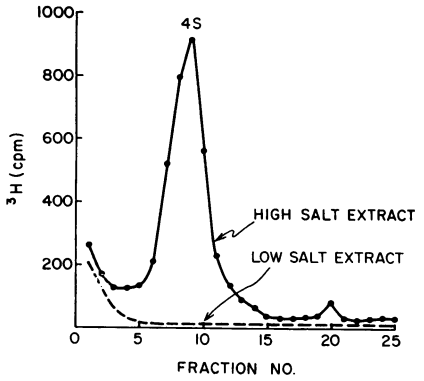


FIG. 6. Sucrose gradient analysis of a 0.3 M KCl extract (high salt) of oviduct nuclei. The nuclear binding protein was labeled with [^{3}H]progesterone as described in the text.

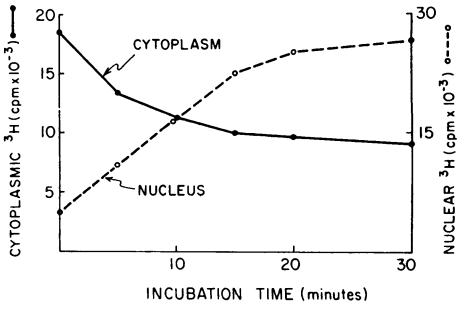


FIG. 7. Transfer of [^{3}H]progesterone from oviduct cytoplasm to nucleus. Incubations carried out as described in the text were stopped by freezing and the tissue was homogenized and extracted with ether. The ordinate is expressed as cpm/0.5 g tissue extract.

plasm and nuclear fractions were then determined. Initially, [^{3}H]progesterone was present almost exclusively in the cytoplasm and relatively little was seen in the nuclear fraction. Upon continued incubation, there was a progressive dimunition in cytoplasmic-^{3}H, and a concomitant increase in nuclear-^{3}H, as the progesterone was transferred to the nucleus (Fig. 7). Thin layer chromatography (benzene–ethyl acetate, 70:30) revealed that >70% of the ^{3}H was unmetabolized progesterone at the end of the 37°C incubation.

To determine if this intracellular transfer of hormone requires and involves the entire progesterone-receptor complex, we repeated the *in vitro* incubation

in the same manner but now isolated both the cytoplasmic and nuclear receptors using the sucrose gradient method (Fig. 8). At zero time (5 min at 0°C; 0 min at 37°C), the steroid has entered the cell and formed a complex with the cytoplasmic receptor but none of this complex has appeared in the nucleus. After subsequent incubation at 37°C, a progressive increase in the proportion of [^{3}H]progesterone bound to the nuclear receptor.

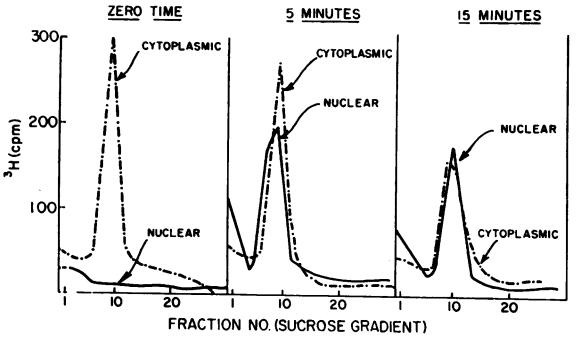


FIG. 8. Transfer of the [^{3}H]progesterone–receptor complex from oviduct cytoplasm to nucleus. Incubations and gradient centrifugation were carried out as described in the text.

Discussion. The present studies demonstrate that the chick oviduct, a specific target organ for progesterone, contains both cytoplasmic and nuclear macromolecules which bind progestins. These binding molecules can be clearly distinguished from transcortin by centrifugation through sucrose gradients of low ionic strength and by agarose gel filtration.[22] The cytoplasmic progesterone-binding molecules also bind 5-α-pregnane-3,20-dione, but have significantly lower affinity for cortisol, estrone, or aldosterone. They are absent from blood and from non-target organs such as lung and spleen. The tissue-specific binding components appear to be heat-labile, acidic, proteins with an average dissociation constant for progesterone of about 8×10^{-10} M at 2°C. These results are consistent with the identification of the progesterone-binding molecules as the functional hormone receptors. In further support of this concept is the finding that pretreatment of the chicks with estrogen coordinately induces a 20-fold increase in the number of progesterone-binding molecules[19] and enhances the capacity of progesterone to induce avidin synthesis.[20]

A progesterone-receptor complex can be detected in both the cytoplasm and nuclei of oviduct tissue 8 min after injection of [^{3}H]progesterone into estrogen-treated chicks. By contrast, incubation of oviduct tissue with [^{3}H]-progesterone *in vitro* at 2°C for 5 min leads to labeling of the cytoplasmic receptor only. Transfer of the receptor–steroid complex then appears to occur upon subsequent incubation *in vitro* at 37°C. This observation suggests that the transfer of bound progesterone across the nuclear membrane may be an energy-requiring enzymatic process.

Experiments that employ addition of excess [^{3}H]progesterone to the cytoplasmic and nuclear extracts, obtained at various times during the *in vitro* incubation, suggest that: (1) the cytoplasmic receptor is depleted during the transfer of progesterone to the nucleus; (2) neither the depletion of cytoplasmic receptor nor appearance of an extractable nuclear receptor occurs in the

absence of steroid; (3) the cytoplasmic and nuclear receptors cannot be distinguished from one another by sucrose gradient or gel filtration techniques.

The simplest explanation for the above data may be that the nuclear receptor is in fact the cytoplasmic receptor transported into the nucleus. Alternatively, it is possible that the nuclear receptor is a fragment of the cytoplasmic receptor, another conformational state of the cytoplasmic receptor, or a completely different protein. The major hazard in investigating these hypotheses is that the molecular parameters of the intracellular receptors may be altered by the conditions of extraction and *in vitro* analysis and thus may bear no absolute relationship to the size or configuration of the receptors in the unbroken cell. Nevertheless, techniques such as sucrose gradient analysis permit the detection of protein-bound hormone and its movement between subcellular compartments.

The present data for the interaction of progesterone with oviduct tissue are consistent with the two-step mechanism for the interaction of estradiol with rat uterus proposed by Jensen[2] and Gorski.[10,21] A similar mechanism has recently been proposed for the interaction of androgen with prostate tissue by Fang *et al.*[6] The similarity of the results of the combined studies on these three different steroid hormones suggests a common biochemical sequence of events in the mechanism of action of all steroid hormones.

Abbreviation: CBG, chick plasma transcortin

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