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THE BINDING AND METABOLISM OF TESTOSTERONE BY THE CHICK OVIDUCT

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SUMMARY

A testosterone receptor was identified in cytosol from the oviducts of estrogen stimulated chicks. Testosterone binding was not detected in serum or in cytosol prepared from heart tissue. The sucrose gradient profile of the receptor was 4 S under conditions of high ionic strength and 8 S under low ionic strength conditions. Incubation of intact oviducts at 37°C with labeled testosterone resulted in the rapid appearance of cytosol binding initially and then nuclear binding. The nuclear receptor was a 4 S protein under conditions of either high or low ionic strength. The identity of the steroid bound to the cytosol and nuclear receptor following incubations of whole oviducts was determined by t.l.c. More than 75% of the steroid bound to the cytosol receptor was unchanged testosterone. In contrast, more than 75% of the steroid bound to the nuclear receptor was 5α-dihydrotestosterone. The observations reported in this paper indicate that testosterone binding and metabolism in the chick oviduct and in male target organs is similar.

The chick oviduct has been widely used in studies of the mechanism of steroid hormone action because exposure to estradiol leads to striking oviduct growth, glandular differentiation and synthesis by the oviduct of specific proteins such as ovalbumin, conalbumin, lysozyme, etc. [1, 2, 3]. Several studies have shown that estrogen-induced growth and ovalbumin synthesis are further stimulated by the simultaneous administration of testosterone [3, 4]. Since the actions of steroid hormones are generally thought to be mediated by intracellular receptors the chick oviduct was examined to determine if cytosol receptors for testosterone were detectable and a testosterone receptor was detected [5]. This paper further describes the characteristics of the oviduct testosterone receptor and reports the metabolism of testosterone by the oviduct.

MATERIALS AND METHODS

[1,2 ^{3}H]-testosterone (43.5 Ci/mmol) and [^{14}C]-formaldehyde (10 Ci/mmol) were obtained from New England Nuclear Corporation. Unless otherwise indicated, unlabeled steroids and other chemicals were purchased from the Sigma Chemical Co. One week old female Rhode Island Red chicks were obtained from Acme Farms (Nashville, Tennessee) and given daily subcutaneous injections of 5 mg diethylstilbestrol (DES) in sesame oil, usually for 2 weeks before use.

The following buffers were used: A. 50 mM Tris–HCl, 1 mM EDTA, 10 mM thioglycerol, pH 7.5; B. 10 mM Tris–HCl, 1 mM EDTA, 10 mM thioglycerol, pH 7.5; C. 10 mM Tris–HCl, 1 mM EDTA, pH 7.5; D. 0.5% Charcoal (Norit A), 0.05% Dextran-80 (Pharmacia) in Buffer C; E. 0.5 M sucrose, 50 mM Tris–HCl, 25 mM KCl, 2 mM $MgCl_2$, pH 7.5; F. 1.8 M sucrose, 50 mM Tris–HCl, 25 mM KCl, 2 mM $MgCl_2$, pH 7.5.

Preparation of cytosol. Chicks were killed by cervical dislocation and the oviduct magna removed and placed in ice-cold saline. All subsequent procedures were performed at 4°C unless otherwise indicated. The tissues were minced, placed in 5 vol. (w/v) of Buffer A and homogenized with a polytron PT-10 (Brinkman Inst.). The homogenate was centrifuged at 150,000 *g* for 1 h. the floating fat removed and the clear supernatant, termed cytosol, was used in these experiments. When prepared in this manner the protein concentration of all cytosols was 20–25 mg/ml.

Preparation of nuclei. This procedure was based on that described by Blobel and Potter[6]. The effectiveness of the method has recently been extensively analyzed [7]. The oviducts were minced, placed in 10 vol. (w/v) of Buffer E and homogenized using a motor-driven Teflon–glass homogenizer. The homogenate was poured through 2 layers of cheese-cloth and centrifuged at 6000 *g* for 10 min. This pellet was resuspended in 5 vol. (w/v) of Buffer F with 3–5 up and down strokes of the Teflon–glass homogenizer and centrifuged at 25,000 *g* for 20 min. The supernatant was rehomogenized and centrifuged again. The two pellets were then combined and resuspended for a second time in 5 vol. (w/v) of Buffer F. The suspension was filtered through one layer of organza and spun at 25,000 *g* for 20 min. This final pellet was usually white or light tan. Phase-contrast microscopy of the final preparation showed no intact cells and the nuclei were free of observable attached cytoplasmic remnants.

Conventional sucrose gradients. Sucrose gradients (5–20%) were prepared from stock solutions of 0.5% and 28% (w/v) sucrose in Buffer B using a Beckman gradient former. Cytosol was labeled with $2 \times 10^{-9} M$ [^{3}H]-testosterone for 2 h and 0.2 ml layered on the 4.6 ml gradient. [^{14}C]-ovalbumin was included in each sample and the position of the 3.55 S protein [8] was used to determine the sedimentation constant of the testosterone binding protein [9]. After ultracentrifugation in a Spinco SW 50.1 rotor at 40,000 rev/min for 16 h, 16-drop fractions were obtained by piercing the tube bottom, and analyzed for radioactivity.

Charcoal adsorption method. This assay was performed essentially as described by Korenman[10]. Aliquots (0.5 ml) of charcoal suspension (Buffer D) were added to 0.4 ml of cytosol containing labeled hormone. After 10 min. incubation the tubes were centrifuged at 600 *g* for 10 min. and the clear supernatant, containing protein-bound testosterone was counted. The incubation times were chosen to provide less than 5% non-specific binding. The neutral pH and low temperature were chosen because in separate experiments they resulted in maximal receptor stability and maximal specific binding.

Steroid extraction and t.l.c. The cytosol or nuclear extract was extracted five times with equal volumes of ethyl acetate [11]. After evaporation under nitrogen, the lipid residue was dissolved in 70% methanol and stored at −20°C overnight. The insoluble material was then removed by centrifugation at 2000 *g* for 10 min. The remaining methanol solution was then diluted with 2 vol. of water and the steroids removed from this aq. solution by an ethyl acetate extraction. This mixture was evaporated under nitrogen, redissolved in a small vol. of methanol and ether (1:1 v/v) and spotted on silica-gel plates. The samples, plus authentic testosterone and 5α-dihydrotestosterone standards were run once in an unsaturated Benzene–ethyl acetate (50/50 v/v) system. The areas on the plate corresponding to the standards were scraped, eluted with ether and methanol and their radioactivity determined.

Miscellaneous. Radioactivity of the aqueous samples was determined by combining 0.5 ml of sample with 5 ml scintillation fluid consisting of toluene (Baker), Triton X-100 (RPI) and Spectrafluor (Amersham–Searle), 2366:1320:100 (v/v) and counting in a Beckman LS-233 scintillation counter at an average efficiency of 40%. Samples eluted from silica were dried under air stream and counted in 10 ml of a scintillation fluid consisting of toluene and Spectrafluor (2366:150, v/v). Quantitative measurement of protein was performed according to Lowry *et al.*[12] using B.S.A. (Sigma) as standard. [^{14}C]-ovalbumin was prepared using [^{14}C]-formaldehyde as described by Rice and Means[13].

RESULTS

Binding specificity of the receptor. Competitive binding analysis utilizing the charcoal assay technique

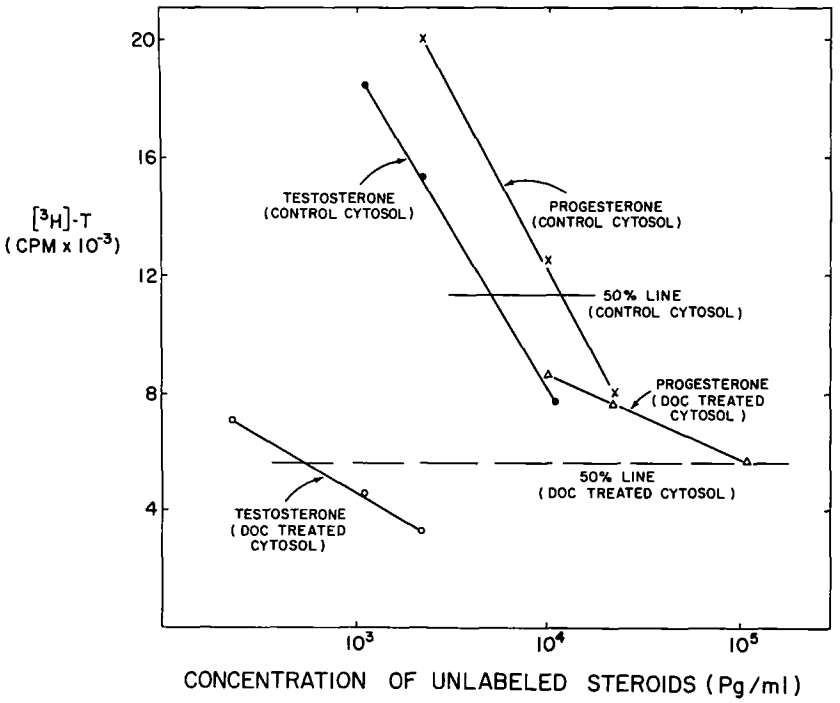

Fig. 1. *Effect of desoxycorticosterone on the competitive binding analysis of testosterone binding.* Oviduct cytosol was used either untreated or after having desoxycorticosterone added to a concentration of 4×10^{-8} M. Aliquots of both cytosol preparations received 2×10^{-9} M [^{3}H]-testosterone in addition to various concentrations of unlabeled testosterone or progesterone. After an overnight incubation at 4°C the amount of bound [^{3}H]-testosterone was determined by charcoal assay.

TABLE 1

Relative Binding Affinity (RBA) of Various Steroids

Competing Steroid	RBA
Testosterone	1.000
5α-dihydrotestosterone	3.300
Progesterone	0.009
Estradiol	0.005
Cortisol	<0.001
Androsterone	<0.001

Various concentrations of the unlabeled steroids listed above were added simultaneously with labeled testosterone to samples of cytosol containing 4 x 10^{-8}M desoxycorticosterone. Following an overnight incubation at 4°C the amount of bound [^{3}H]-testosterone was determined by charcoal assay. The relative binding affinity is the molar concentration of unlabeled steroid required to reduce [^{3}H]-testosterone binding by 50% divided by the concentration of unlabeled testosterone required to cause a similar reduction.

showed variable results with occasional experiments in which progestins competed with [^{3}H]-testosterone nearly as well as testosterone (Fig. 1). Based on the assumption that some testosterone was binding to large amounts of progesterone receptor [14], cytosol was pre-treated with unlabeled desoxycorticosterone. Desoxycorticosterone has a higher affinity for the progesterone receptor than testosterone [14]. Cytosol pre-treated in this way consistently shows androgen specificity such as is shown in the lower part of Fig. 1. A more extensive survey of binding specificity is shown in Table 1. Treatment with desoxycorticosterone did not alter the sucrose density gradient profile of androgen binding in the 8 S region. Figure 2 shows

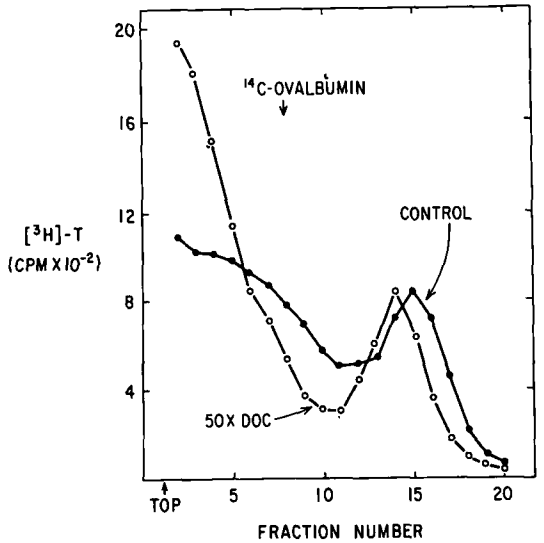

Fig. 2. *Effect of desoxycorticosterone on the sucrose density gradient profile of* [^{3}*H*]*-testosterone binding.* Control cytosol (●———●) and cytosol incubated for 2 h at 4°C with 4 x 10^{-8} M desoxycorticosterone (O———O), before labeling with 2 x 10^{-9} M [^{3}H]-testosterone. The mixtures were layered on 5–20% sucrose density gradients with a [^{14}C]-ovalbumin internal standard and centrifuged in a SW 50.1 rotor at 150,000 *g* for 16 h. For details of fractionation and analysis, see Methods.

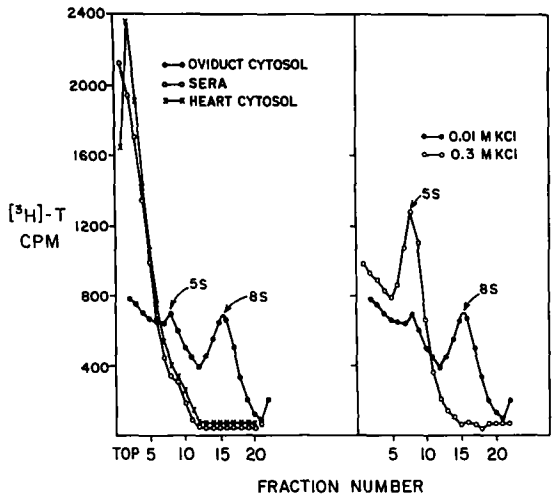

Fig. 3. *Sucrose density gradient analysis of* [^{3}*H*]*-testosterone binding to heart cytosol, oviduct cytosol and serum.* **Left panel.** Heart cytosol was prepared exactly as previously described for oviduct. Serum was diluted 1:10 to result in a similar protein concentration to the cytosol samples. The three samples were labeled for 2 h at 4°C with 2 x 10^{-9} M [^{3}H]-testosterone. Details of sucrose gradient analysis are in Methods. **Right panel.** Cytosol samples were labeled at 4°C with 2 x 10^{-9} M [^{3}H]-testosterone. One sample was layered on a 5–20% sucrose gradient which contained 0.01 M KCl. The other sample was layered on an identical gradient except that it contained 0.3 M KCl. Other details are identical to the description in Methods.

a profile of cytosol labeled with [^{3}H]-testosterone with or without the prior addition of desoxycorticosterone. The untreated profile shows a distinct peak in the 8 S region and some slurred binding in the 4–6 S region of the gradient. The only apparent effect of desoxycorticosterone treatment was to cause some diminution of binding in the 4–6 S region of the gradient.

Demonstration of organ specificity and ionic dependence of testosterone binding. Cytosol was prepared from the heart and oviduct of a chick and serum taken from the same animal was diluted with buffer A. All three samples contained equivalent protein concentrations of approx. 20 mg/ml as determined by the Lowry procedure (see Methods). The experiment depicted in the left panel of Fig. 3 showed that neither heart cytosol nor serum contained an androgen binding protein.

The right panel of Fig. 3 shows the effect of ionic strength on the sedimentation characteristics of the androgen receptor. Under the usual low salt conditions (0.01 M KCl), the receptor sedimented in the 8 S region of the gradient. When the ionic strength was raised by increasing the KCl concentration to 0.3 M KCl, binding in the 8 S region disappeared, suggesting dissociation of the receptor aggregates [15], and there was a concurrent increase of binding in the 4 S region.

Evidence for the protein nature of the receptor. Figure 4 shows the androgen binding profile of cytosol samples which have undergone various treat-

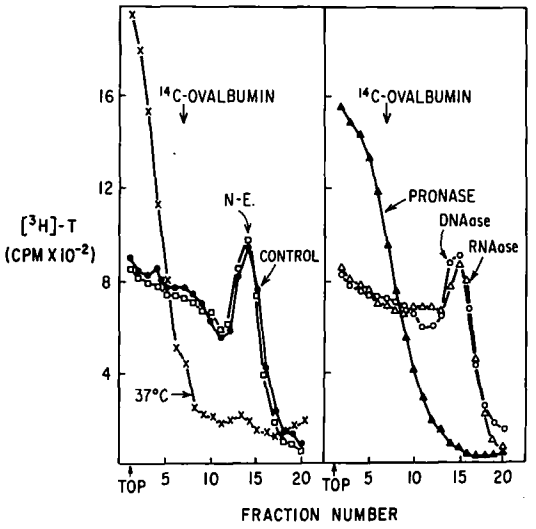

Fig. 4. *Effect of various treatments on testosterone binding.* Cytosol samples were labeled for 2 h at 4°C with 2×10^{-9} M [^{3}H]-testosterone. Identical aliquots then received the following treatments: 1 h at 25°C (control); 1 h heating at 37°C; 1 h exposed to 3 mg/ml (24 mM) N-ethylmaleomide (N-E); 1 h at 25°C exposed to 1 mg/ml Pronase, 5 mg/ml RNAase or 5 mg/ml DNAase. Sucrose density gradient analysis was then performed as described in Methods.

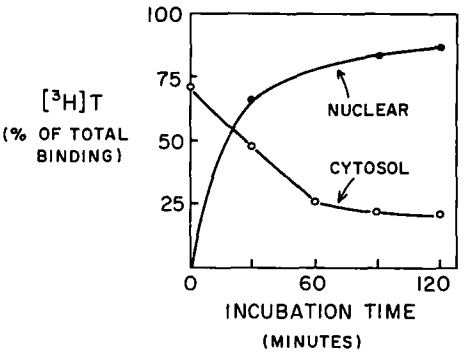

Fig. 5. *Incubation of oviduct explants with [^{3}H]-testosterone-cytosol and nuclear binding.* Chick oviducts were slit lengthwise and placed one per 50 ml flask containing 20 ml Basal Eagle's Medium, 10^{-8} M desoxycorticosterone and 2×10^{-9} M [^{3}H]-testosterone under a 95% O_2-5% CO_2 atmosphere. Incubations were performed at 37°C in a Dubnoff shaking water incubator. At the indicated times, an oviduct was removed from its incubation mixture and cooled in a beaker of ice-cold Tris-buffered saline. A small piece of the oviduct was used to prepare cytosol and the larger piece used to prepare nuclei. The nuclei were then extracted at 4°C for 30 min. using 0.4 M KCl Buffer C. The nuclear extract and cytosol were then analyzed by the charcoal assay. The points represent cytosol or nuclear extract derived from a single tissue and charcoal assayed in duplicate. The results of this experiment are typical of those results obtained in two other, similar experiments.

ments. The gradient profile of the untreated sample is shown in the left-hand panel as are the profiles of samples treated with N-ethylmaleomide (N-E) or heated at 37°C for one hour. N-ethylmaleomide treatment had no effect on 8 S binding while heating virtually abolished androgen binding in this region. Treatment with DNAase and RNAase had no effect on binding but treatment with pronase abolished binding completely (right-hand panel).

Nuclear translocation of the cytosol receptor. Since nuclear translocation of the cytosol receptor is thought to be biologically significant, the cytosol uptake and nuclear translocation of testosterone by the intact oviduct was studied.

The general experimental protocol was to incubate slit oviducts in Basal Eagle's Medium under 95% O_2-5% CO_2, at 37°C in the presence of 1×10^{-8} M [^{3}H]-testosterone. Following the incubation the oviducts were divided and homogenized either in Buffer A for cytosol or Buffer E for the preparation of nuclei. Both buffers contained 10^{-7} M unlabeled testosterone to prevent spurious formation of steroid–receptor complexes post-homogenization [16]. Otherwise, both cytosol and nuclei were prepared as described in Methods. The nuclei were extracted with 4 vol. of Buffer C + 0.4 M KCl and both the nuclear extract and cytosol analyzed by the charcoal assay. The results of a typical experiment are depicted in Fig. 5 and show that nuclear translocation was time-dependent and that as the amount of nuclear binding increased cytosol binding decreased. Detectable nuclear binding did not occur during the brief time intervals studied, if the oviducts were incubated at 4°C. The addition of a large excess of unlabeled testosterone prior to incubation at 37°C reduced binding indicating that binding in both cytosol and nuclear compartments was to a saturable component (Table 2).

Sucrose density gradient analysis of the nuclear extract showed binding in the 4 S region with evidence of considerable aggregation (Fig. 6). Ultracentrifugation of the extract in a low salt gradient resulted in an increase of the apparent aggregation but did not result in the appearance of an 8 S form. Control experiments showed that if the 4 S form had resulted merely from exposure of the cytosol receptor to 0.4 M KCl, then ultracentrifugation in a low-ionic strength gradient would have resulted in the return of an 8 S peak.

TABLE 2

TESTOSTERONE - RECEPTOR COMPLEX BINDING TO OVIDUCT NUCLEI

Conditions	CPM/ml of Nuclear Extract
37°C + [^{3}H]-Testosterone	2800
37°C + [^{3}H]-Testosterone + 100 x unlabeled testosterone	420
4°C + [^{3}H]-Testosterone	100

Chick oviducts were slit lengthwise and incubated in Basal Eagle's Medium under a 95% O_2 - 5% CO_2 atmosphere at either 37°C or 4°C. The medium also contained 4×10^{-8}M Desoxycorticosterone and 2×10^{-9}M [^{3}H]-testosterone. One incubation mixture also contained 2×10^{-7}M unlabeled testosterone as indicated. All incubations were for 30 minutes. Nuclei were then isolated (Methods), the 0.4 M KCl extract obtained and the charcoal assay performed.

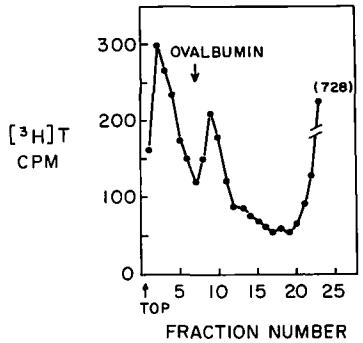

Fig. 6. *Sucrose density gradient analysis of the nuclear receptor.* Nuclei were isolated from an oviduct which had been incubated for 60 min. under conditions similar to those in Fig. 5. The 0.4 M KCl nuclear extract was then layered on a 5–20% sucrose gradient which contained 0.3 M KCl, centrifuged at 150,000 *g* for 16 h, fractionated and counted. The position of the internal marker [^{14}C]-ovalbumin is also noted. For details of the sucrose density gradient technique, see Methods.

The metabolism of testosterone by the chick oviduct. Androgen metabolism is an important facet of the mechanism of action of this class of steroids. Having demonstrated testosterone binding and nuclear uptake in the oviduct, the next important question concerned the nature of the radioactivity bound to the cytosol receptor and the nuclear receptor. Accordingly, free hormone was removed from cytosol or nuclear extracts by extraction with charcoal. The remaining protein-bound radioactivity was extracted and analyzed by t.l.c. (see Methods and Table 3). The results, depicted in Table 3, show that the steroid involved in cytosol binding is predominantly testosterone but 5α-dihydrotestosterone is the major steroid bound to the nuclear receptor.

DISCUSSION

A previous report by one of us suggested that the chick oviduct contains an estrogen-dependent protein which has the characteristics of an androgen receptor [5]. It was necessary to add desoxycorticosterone to the cytosol to abolish interference caused by testosterone binding to the extremely high levels of progesterone receptor [14] also induced by estrogenization (Fig. 1). This treatment did not interfere with the authentic androgen receptor as defined by sucrose gradient analysis nor did it interfere with nuclear translocation of the androgen receptor. Testosterone binding was not affected by glucocorticoids or estrogens thus excluding the possibility that binding to a sex steroid-binding globulin was being observed.

TABLE 3

TESTOSTERONE METABOLISM BY THE INTACT OVIDUCT

Sample	Percentage of Total Counts in Sample	
	Testosterone	5α-Dihydrotestosterone
A - Unspun Homogenate	75	25
B - Cytosol - Charcoal Treated	75	25
C - Nuclear Extract - Charcoal Treated	25	75

Chick oviducts were incubated in Basal Eagle's Medium at 37°C in the presence of 4×10^{-8}M DOC and 2×10^{-9}M [^{3}H]-testosterone. Following incubation for 1 hour, portions of the oviducts were used to prepare cytosol or nuclei (see Methods). A portion of the cytosol homogenate was removed before centrifugation. The steroids present were extracted and chromatographed. Lastly, a portion of cytosol and a portion of 0.4 M KCl nuclear extract were charcoal-extracted and the supernatants containing only protein-bound radioactivity, were then extracted and chromatographed.

The androgen receptor is tissue specific and sensitive to changes in buffer ionic strength in a manner characteristic of steroid receptors (Fig. 3). The receptor is sensitive to both proteolysis and heat but not RNAase or DNAase (Fig. 4) suggesting that it is a protein. However, unlike receptors in several other tissues [17, 18], binding was not sensitive to treatment with N-ethylmaleomide.

A general observation made concerning most responsive tissues thus far studied, is that at physiologic temperatures, the steroid–receptor complex translocates into the nucleus [17, 18, 19]. In addition, many androgen-sensitive tissues appear to metabolize testosterone to 5α-dihydrotestosterone as a part of the nuclear binding step [17, 18]. In this paper the oviduct testosterone receptor has been shown to undergo a similar translocation and the steroid is concurrently reduced to 5α-dihydrotestosterone. Our data suggests, but does not conclusively prove, that the conversion of testosterone to 5α-dihydrotestosterone occurs while the steroid is associated with the receptor and being translocated into the nuclear compartments. Our experiments did not, however, provide insight into whether this conversion occurred before or after the translocation step.

Another female reproductive tissue, the immature rat uterus, contains a cytosol receptor with binding characteristics distinctly different from the oviduct [20, 24]. This tissue was found to bind testosterone with a higher affinity than 5α-dihydrotestosterone and did not appear to contain significant 5α-reductase activity. On the other hand, human breast tissue preferentially binds 5α-dihydrotestosterone and has significant 5α-reductase activity [22–24]. Thus, the androgen responsiveness of the oviduct and human breast tissue appears to be mediated by a similar mechanism.

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