40. Binding of Sex Steroid to Oviduct Cytosol of the Newt, *Cynops pyrrhogaster ensicauda*

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It has been demonstrated that either estradiol or testosterone administered together with prolactin stimulates the synthesis of protein and mucopolysaccharide in the oviduct of the newt, *Cynops pyrrhogaster ensicauda*.⁶⁾ If estradiol and testosterone are given simultaneously, a cumulative effect is observed. The experiments were undertaken to characterize binding proteins for the two steroids in the newt oviduct.

Materials and methods. *Buffers.* The following buffers (pH 7.4) were used. Ten mM Tris-HCl containing 1.5 mM EDTA (TE buffer); TE buffer plus 1 mM 2-mercaptoethanol (TEM buffer); TEM buffer containing 10% glycerol (TEMG buffer).

Preparation of samples. Oviduct taken from 5–10 newts, *Cynops p. ensicauda*, were homogenized in 2–4 volumes of TE or TEMG buffer. Cytosol was prepared by centrifugation of the homogenate at 100,000×g for 60 min. The cytosol preparation was diluted to a concentrations of 2–5 mg protein/ml with an appropriate buffer. All procedures were carried out at 0–4°C.

Charcoal adsorption assay. Specific steroid binding was determined by a modification of the charcoal adsorption assay of Korenman and Rao.⁷⁾ In competition studies, 0.1 ml of cytosol was incubated with 1 nM [2,4,6,7-³H]estradiol-17 β (sp. activity 96.0 Ci/m mol, NEN) or [1,2,4,6-³H]testosterone (sp. activity 98.8 Ci/m mol, NEN) in the presence or absence of 1,000-fold unlabeled steroid for 2 hr at 0°C. The incubation was terminated by the addition of an equal volume of dextran-coated charcoal (0.5% Norit A charcoal and 0.05% Dextran T70 in TE buffer). The samples were vortexed and centrifuged immediately at 2,000×g for 5 min. Radioactivity of aliquot of the supernatant was measured. For the determination of binding affinity and capacity for estradiol or testosterone, cytosol was incubated with 0.1–10.0 nM labeled estradiol or 3.3–12.4 nM of labeled testosterone in the presence or absence of 1,000-fold unlabeled hormone for 2 hr at 0°C. Specific binding was calculated by subtracting the nonspecific binding from the total binding. Association constant (K_a) and binding capacity were determined according to Scatchard.¹⁵⁾ Protein content in the cytosol fraction was measured by the method of Lowry *et al.*⁸⁾

Sucrose density gradient centrifugation. Cytosol which had been incubated with 1 nM labeled steroid in the presence or absence of 1,000-fold unlabeled steroid was layered on the 4.8 ml sucrose gradient (5–20% in TE buffer). After centri-

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fugation at $131,000\times g$ for 17.5 hr, 25 fractions were obtained by perforating the bottom of the cellulose nitrate tubes using an ISCO-640 fractionator. Each fraction (0.2 ml) was transferred into scintillation vials to determine the radioactivity. Sedimentation coefficient was estimated by the method of Martin and Ames⁹⁾ using BSA as an external marker.

Polyacrylamide gel electrophoresis at steady state condition. Binding of steroid hormone to oviduct cytosol was analyzed by the method of Ritzen *et al.*¹²⁾ using a steady state polyacrylamide gel electrophoresis. Oviduct cytosol was prepared with 2.5 volumes of TEMG buffer and the cytosol (0.55 ml) was mixed with 0.02 ml of TEMG buffer containing 5 mg Norit A and 0.5 mg Dextran T70 and kept for 6 hr at 0°C to remove endogenous steroids. The 7% polyacrylamide gel (containing 2 nM labeled steroid with or without 1000-fold unlabeled steroid and 10% glycerol) was polymerized at 4°C in a glass tube. The charcoal-dextran-treated sample (0.1 ml) was incubated for 1 hr at 0°C with 2 nM labeled steroid and layered directly on top of the running gel. Electrophoresis was performed for 1.75 hr at 4°C for a distance of 40 mm. The current was initially 1 mA per tube, being increased to 5 mA after the samples entered the running gels. After electrophoresis, gels were sliced transversely into 1 mm thickness to measure the radioactivity.

Radioactivity measurement. The aqueous radioactive solutions and sliced gels were kept in 3–5 ml of liquid scintillation fluid (toluene/Triton X-100 containing PPO and POPOP) with addition of 0.2–0.3 ml of DW overnight, then the radioactivity was determined in a Beckman model LS-100C liquid scintillation counter with a counting efficiency of 30–40%.

Results. The specificity of the steroid-binding components in the oviduct cytosol was evaluated by competitive binding experiments. As shown in Table I,

Table I. Relative ability of various steroids to displace either [³H]estradiol or [³H]testosterone from oviduct cytosol

Unlabeled steroid (10 ⁻⁹ M)	Relative binding (%)	
	[³ H]estradiol (10 ⁻⁹ M)	[³ H]testosterone (10 ⁻⁹ M)
None	100	100
Estradiol	13	49
Testosterone	26	23
DHT	19	31
Progesterone	89	102
Cortisol	101	99
DES	97	103

estradiol had the strongest ability to displace [³H]estradiol. Both testosterone and 5 α -dihydrotestosterone (DHT) also markedly displaced the labeled estradiol. On the other hand, unlabeled testosterone was the strongest competitor to binding of [³H]testosterone with oviduct cytosol. Estradiol and DHT exhibited a considerable competitive effect. In both cases, progesterone, cortisol and diethylstilbestrol (DES) appeared to have little competitive effect. When the oviduct cytosol labeled with [³H]estradiol or [³H]testosterone was applied to the sucrose density gradient, a radioactive peak was recovered at 6–7S region in both cases (Fig. 1). The cytosol was incubated with [³H]estradiol or [³H]testosterone and fractionated using polyacrylamide gel electrophoresis. As shown in Fig. 2, both estradiol- and testosterone-binding components migrated to the similar position. To determine affinity and capacity of binding of estradiol or testosterone in the

Fig. 1. Sucrose density binding profile of [^3H]estradiol (A) or [^3H]testosterone (B) to oviduct cytosol. ○, absence of unlabeled steroid; ●, presence of unlabeled steroid.

Figs. 2-3. 2 (left): Polyacrylamide gel electrophoresis of cytosol fraction. Cytosol was previously incubated with [^3H]estradiol (A) or [^3H]testosterone (B). ○, absence of unlabeled hormone; ●, presence of unlabeled hormone. 3 (right): Scatchard plot of binding of [^3H]estradiol (●) or [^3H]testosterone (○) to cytosol of newt oviduct. Protein concentration of the cytosol was 5 mg/ml.

oviductal cytosol, binding data were analyzed according to Scatchard (Fig. 3). The K_a was $2.2 \times 10^8 \text{ M}^{-1}$ for estradiol binding and $1.2 \times 10^8 \text{ M}^{-1}$ for testosterone binding. Binding capacity was $3.7 \times 10^{-13} \text{ mol/mg}$ protein for estradiol and $3.5 \times 10^{-13} \text{ mol/mg}$ protein for testosterone.

Discussion. The present experiment demonstrated the presence in the oviduct cytosol of proteins which bind estradiol and testosterone. However, neither sedimentation analysis nor electrophoretic analysis provided an evidence that two discrete proteins exist in the newt oviduct capable of binding estradiol and testosterone, respectively. Scatchard analysis revealed that the binding capacity for estradiol is close to that for testosterone. Moreover, the results of competition experiment indicate that estradiol and testosterone have a considerable binding affinity for testosterone-binding protein and estradiol-binding protein, respectively. These results suggest that these two steroids may bind to a common binding component in the oviduct cytosol.

In amphibians, receptors for estrogen and androgen were evidenced in various tissues such as skin,^{1),3)} mullerian duct,¹⁰⁾ thumb pad²⁾ and liver,^{4),11),16)} K_a being $1-3 \times 10^9 \text{ M}^{-1}$ (see Ref. 5)). These K_a values are somewhat higher than those obtained in the present experiment. The results of competition experiment indicate that DES, a nonsteroidal synthetic estrogen, has no ability to displace [³H]estradiol. This is characteristic of the steroid-binding protein (SBP) in the plasma observed in other animals. In amphibians, sex steroid-binding globulins are known to exist, their K_a values being 10^8-10^9 M^{-1} (see Ref. 13)). Salhanick *et al.*¹⁴⁾ has reported that in the turtle (*Chrysemys picta*) oviduct, an SBP (K_a , 10^8 M^{-1} ; capacity, $10^{-12} \text{ mol/mg protein}$) which is similar to that found in the plasma exists. After the removal of the plasma SBP-like protein by DNA affinity chromatography, they demonstrated a high affinity (10^9 M^{-1}) and low capacity ($10^{-14} \text{ mol/mg protein}$) estrogen receptor. We have reported that estradiol, testosterone or combination of both hormones administered together with prolactin induces oviducal development in the newt, *Cynops p. ensicauda*.⁶⁾ This suggests that receptors for estrogen and androgen exist in the oviduct. However, further studies are required to define the receptors for the steroid hormones in the newt oviduct.

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