Studies on Sex Organ Development

EFFECT OF HORMONES ON ORNITHINE DECARBOXYLASE ACTIVITY IN THE DEVELOPING CHICK OVARY

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The response of ornithine decarboxylase activity to hormones in the embryonic left ovary was measured throughout the stages of development. During the early stage of ovarian development (9th day of incubation), the ornithine decarboxylase activity (in terms of pmol CO₂/30 min per mg of protein) was high (766); it decreased from the 10th to the 12th day (575–239), increased slightly from the 13th to the 15th day (306) and finally fell to a low value (192–20) from the 18th day of development to birth. Administration of an optimal dose of oestrogen to the 9–10-day embryo stimulated the ovarian ornithine decarboxylase activity by 48–53%. If the same dose of oestrogen was administered to the 15–18-day embryo, the ovarian enzyme activity was suppressed by 32–43%. This biphasic response to oestrogen for enzyme induction is characteristic of the developing ovary and is not observed in other genital organs of the chick. In the early developmental stage (9–10th day) testosterone has no effect on ovarian ornithine decarboxylase activity, but in the late stage testosterone inhibits the activity by 41%. Organ culture techniques have been used to test the ovarian response to lutropin (luteinizing hormone). Lutropin stimulated ornithine decarboxylase activity by approx. 99–155% in the ovary of the early embryonic stage (10–13th day), and by 175–200% in the ovary of the late embryonic stage (15–18th day). The alteration in enzyme activity in the ovary as assayed *in vitro* during development is not due to the effect of the size of the endogenous ornithine pool. The relationship of ornithine decarboxylase activity to the morphological and biochemical changes in the developing ovary is discussed.

The biochemical mechanisms behind the growth of embryonic ovary are not well known. It has been observed that steroidal and gonadotropic hormones are required for the proper differentiation and development of the embryonic ovary. However, the questions of how the hormones affect the ovary and how ovary responds to these hormones are still unclear. We have selected the left ovary of the embryonic female chick as a model system and the enzyme ornithine decarboxylase as the biochemical marker for investigating its response to the effect of hormones.

Ornithine decarboxylase is the major enzyme responsible for the synthesis of putrescine, which is the precursor for other polyamines such as spermidine and spermine. Polyamines are correlated with some basic biochemical synthetic activities required for cellular growth and function (Jänne, 1967; Cohen, 1971; Heby *et al.*, 1975). The ontogenetic

appearance of ornithine decarboxylase activity in the embryonic ovary could reflect its state of differentiation and development. The different rates of ornithine decarboxylase activity as induced by hormones during development provide a sensitive parameter for measuring the responsiveness of the organ toward the hormones.

Materials and Methods

Animals and chemicals

White Leghorn chick embryos at various stages of development were obtained as described previously (Teng & Teng, 1975). The ovaries (or testes) freshly removed from the embryos were used immediately for enzyme assay. The following chemicals and hormones were obtained from the sources indicated: potassium phosphate and sodium phosphate were from Mallinckrodt (St. Louis, MO, U.S.A.);

anhydrous citric acid, pyridoxal 5'-phosphate, DL-ornithine hydrochloride, oestradiol-17 β , testosterone, putrescine, spermidine, spermine and ninhydrin spray were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ovine lutropin (S-19) was from the National Institutes of Health, Bethesda, MD, U.S.A.; Coon's F-12 tissue culture medium, penicillin/streptomycin (1000 i.u. of penicillin/ml and 1000 μ g of streptomycin/ml) were from Gibco (Grand Island, NJ, U.S.A.); NCS tissue solubilizer and aqueous radioactivity-counting scintillant were from Amersham/Searle (Arlington Heights, IL, U.S.A.); sesame oil and toluene were from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.); dithiothreitol was from Calbiochem (San Diego, CA, U.S.A.); DL-[1-¹⁴C]ornithine monohydrochloride (specific radioactivity 45 Ci/mol) was from New England Nuclear Corp. (Boston, MA, U.S.A.); polyethylene centre wells were from Kontes Glass Co. (Vineland, NJ, U.S.A.); cation-exchange resin AG 50W (X4; 200–400 mesh) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.); silica-gel glass plates were from Brinkman (Des Plaines, IL, U.S.A.). All other chemicals were of an analytical grade.

Assay of ornithine decarboxylase activity

The enzyme activity in ovary and other organs was assayed as described by Teng & Teng (1978). In the standard incubation mixture 0.5 mg of protein (in 0.5 ml of 0.05 M-sodium/potassium phosphate buffer containing 5 mM-dithiothreitol, pH 7.2) from the tissue extract was preincubated with 0.5 mM-pyridoxal phosphate for 10 min at 37°C. The reaction was initiated by the addition of 0.1 mM-L-ornithine and 0.125 μ Ci of DL-[1-¹⁴C]ornithine to the mixture and was carried out for 30 min at 37°C. The reaction was stopped by injecting 0.25 ml of 2 M-citric acid and the ¹⁴CO₂ released was absorbed into the NCS solution in a polyethylene centre well and assayed for radioactivity.

Enzyme blanks were determined by heating the enzyme at 100°C for 15 min before preincubation; this step was followed by the addition of chemical ingredients similar to those in the reaction mixture. The radioactivity obtained from this interpretation was subtracted from all other enzyme determinations.

Exposure of ovary and other organs to steroid hormones in vivo

The steroid hormone was dissolved in sesame oil and applied to the allantois of the chick embryo through the shell hole as described previously (Teng & Teng, 1976, 1978). A single dose (40 μ g/0.1 ml or other concentration as specified) of steroid hormone was injected. The control group received the same amount of solvent only. After the injection was

completed, the hole in the shell was sealed and the eggs were returned to the incubator. After 3 h (or different lengths of time), the embryos were dissected and the organs were removed for enzyme assay.

Incubation of embryonic ovaries

Left ovaries (approx. 20–25 mg of tissue from different ages of embryo) were incubated in a Falcon model 3001 tissue culture dish (Oxnard, CA, U.S.A.) containing 1 ml of Coon's F-12 tissue culture medium with 1000 i.u. of penicillin/ml and 1000 μ g of streptomycin/ml. Incubation was in an atmosphere of air/CO₂ (19:1) at 41°C in a model CO-20 incubator (New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.) for 4 h as described by C. T. Teng & C. S. Teng (1977, 1979). Ovine lutropin (50 ng/ml of culture medium) was added for incubation.

Isolation and quantification of polyamines

The isolation of polyamines from ovarian tissue was as described by Inoue & Mizutani (1973) and modified by Teng & Teng (1978). The tissue (approx. 100–150 mg) was minced and homogenized in 10 vol. of 2% (w/v) HClO₄ with a Dounce homogenizer. The homogenate was centrifuged at 10000 g for 15 min, and the precipitate was washed with 2 $\times$ 1 ml of 2% (w/v) HClO₄. The supernatants were combined and applied to a column (0.8 cm $\times$ 4.5 cm) of AG 50W resin and subsequently washed with 30 ml of 0.1 M-sodium phosphate buffer, pH 8.0, containing 0.7 M-NaCl, and then with 10 ml of 1 M-HCl. The polyamines absorbed on the column were eluted with 9 ml of 6 M-HCl, and the eluate was evaporated to dryness in a heating block at 55°C. The residue, containing the polyamines, was dissolved in 100 μ l of 0.05 M-HCl.

The polyamines were quantified by the procedure of Hammond & Herbst (1968). A sample (25 μ l) isolated by the previous procedures was applied to a silica-gel plate and, as a standard, 25 μ l of a polyamine mixture, containing 12.5, 25 and 37.5 nmol of putrescine, spermidine and spermine respectively, was simultaneously subjected to chromatography on the same plate. The plate was chromatographed in diethylene glycol monoethyl ether/propionic acid/water (14:3:3, by vol., saturated with NaCl) for 18 h. After being dried at 60°C for 15 min, the plate was sprayed with ninhydrin reagent. Colour was developed by heating at 70°C for 60 min. The bands corresponding to polyamines were individually removed and extracted with 1.5 ml of 70% (v/v) ethanol for 60 min. The extract was then centrifuged at 200 g for 10 min, and the A₅₇₅ was measured. A standard curve for each polyamine was made by the same procedure.

Determination of ornithine pool size

The size of the ornithine pool of the developing ovary was determined by the procedures described previously (Teng & Teng, 1978). Ovarian tissues (approx. 250–300mg) were finely minced and homogenized in 10 vol. of 5% (w/v) trichloroacetic acid solution at 0°C with a Teflon/glass homogenizer. The homogenate was stirred for 18 h at 0°C and then centrifuged at 3000g for 15 min; the supernatant was saved and extracted with 3 × 4 vol. of diethyl ether. The ether in the extract was removed by heating at 65°C with a heating block (Lab-Line Instrument, Melrose Park, IL, U.S.A.). Acid-washed charcoal (5mg) was added to the extract, which was then centrifuged at 300g for 15 min in a Beckman model J-21B centrifuge. The supernatant was evaporated to dryness under N₂ and redissolved in sodium citrate solution (0.2M, pH 2.2). A portion (0.2ml) was analysed for amino acid content on the Beckman model 121 amino acid analyser as described by Spackman *et al.* (1958).

General procedures

The procedures for DNA and protein determination were as previously described (Teng & Teng, 1977).

Results and Discussion

Ovarian ornithine decarboxylase activity and its response to hormones

The optimum pH value for extraction and assay of ornithine decarboxylase activity in the ovary (and testis) is 7.0. The optimum concentration of ornithine as substrate for the reaction is 0.1 mM. At this concentration the enzyme was saturated by substrate (results not shown). Various concentrations of oestrogen were administered to the embryo, and it was found that oestrogen at the dose range 40–60 µg/embryo causes

approx. 48–53% stimulation of ovarian ornithine decarboxylase activity in the embryos of early developmental stages (at the 9th and 10th day of incubation), whereas the same dose of oestrogen caused an approx. 32–43% inhibition of ornithine decarboxylase activity in the late stages (15th and 18th day of incubation) (Table 1). This observation indicated that the effect of oestrogen on the enzyme in the developing ovary is stage-specific. The reason behind this specificity can be understood by analyzing the developmental changes within the left ovary as follows. In early developmental stages (at 7–10 days of incubation) the tissue and cell types in the embryonic ovary are engaged in rapid morphogenesis and division (Swift, 1915, 1916; Brode, 1928; Zavadvosky & Zubina, 1928; Willier, 1939; Rahil & Narbaitz, 1972), including (1) the proliferation of germinal epithelium, which provided the space for the multiplication and distribution of primordial germ cells; (2) transformation of primordial germ cells into oogonia; (3) formation of cortical cords and the medulla layer in the left ovary; and (4) appearance of lining bodies, which signal the differentiation of prefollicular cells. Naturally, the synthesis of nucleic acids and proteins is active in ovarian tissue at these early developmental stages. It has been reported that the synthesis of these macromolecules is controlled by polyamines, whose synthesis is initiated by ornithine decarboxylase (Nakagawa *et al.*, 1967; Dion & Herbst, 1967; Moruzzi *et al.*, 1968; Pegg & Williams-Ashman, 1968; Oka, 1974; Russell *et al.*, 1976). The observed oestrogenic stimulation of ornithine decarboxylase activity in the embryonic ovary indicated the possibility of oestrogen involvement in the process of cellular growth and proliferation in the early embryonic stages. In the late developmental stages (at the 15th to 20th day of incubation), the proliferation of germinal epithelium and primordial germ cells in embryonic ovary is finished. The germinal epithelium in the left

Table 1. Responses of ovarian ornithine decarboxylase activity to different concentrations of oestrogen
Chick embryos (9, 10, 15 and 18 days) were injected with various concentrations of oestradiol in 0.1 ml of sesame oil. The control received the same volume of sesame oil without oestrogen. After 3 h, the left ovaries were removed for enzyme assay. The results presented are means ± S.D. for six determinations. Activity of ornithine decarboxylase is expressed as pmol of CO₂/30 min per mg of protein.

		Ornithine decarboxylase						
Oestrogen injected ...		None (control)	20 µg/egg		40 µg/egg		60 µg/egg	
Age (days)		Activity	Activity	Percentage of control	Activity	Percentage of control	Activity	Percentage of control
9		766 ± 19	1064 ± 32	139	1130 ± 35	148	1125 ± 35	147
10		575 ± 14	742 ± 16	129	882 ± 21	153	880 ± 22	153
15		306 ± 12	199 ± 8	66	195 ± 7	64	175 ± 7	57
18		192 ± 8	142 ± 7	74	130 ± 6	68	115 ± 4	60

ovary is reduced to a single layer of columnar cells and the oogonia are differentiating toward the stages of meiotic prophase. The chromatin of the oogonia is in a thick heavy thread-like diplotene stage, and no transcription activity was observed in the ovary (Hollander, 1904; Romanoff, 1960). Under this circumstance, the biological activity in the ovary is probably lower than that in the early stages. On the other hand, the maturation of steroid-secreting interstitial cells in the medullary cord has begun (Willier, 1939). The administration of oestrogenic hormone has been reported to inhibit the growth and functional activity of the interstitial cells in the medullary cord region (Taber & Salley, 1954; Taber *et al.*, 1958, 1964). This inhibiting effect could probably be attributed to the suppression of ornithine decarboxylase activity by oestrogen as reported here. In addition, the ovarian enzyme activity in the early developmental stage (9-day embryo) is four times higher than that in the later stages (18-day embryo) (Table 1). This observation supports our prediction that the biological activities in the ovary of early embryonic stages are high, and may surpass that in the later stages.

In 10-day embryo, no stimulation of ornithine decarboxylase in ovary by the administration of testosterone *in vivo* was observed (Fig. 1). In 15-day embryo, testosterone caused a constant inhibition (41%) in ovarian enzyme activity (Fig. 1). This is consistent with a previous report that androgenic hormone is not directly involved in the growth and differentiation of female gonads (Wolff, 1946; Haffen, 1975). The time course of the effects of oestrogenic hormone (either stimulation or inhibition) on ornithine decarboxylase activity in the ovarian tissue is linear with time from 1–3 h and remains constant from 3–5 h after steroid administration (Fig. 1).

In embryonic chick, the response of ornithine decarboxylase activity in the genital organs to oestrogenic hormones has a different magnitude.

Observation of three organs (ovary, testis and Müllerian duct) indicated that the basal enzyme activity is generally higher in the early stage and gradually decreased in the later stage (Table 2). Oestrogen consistently inhibited the testicular enzyme activity throughout the stages of development, which probably provides the answer to a previous observation that prenatal oestrogen treatment leads to the inhibition of growth and secretion of the

Fig. 1. Time course of effect of steroids on ornithine decarboxylase activity

A single dose of oestradiol-17 β or testosterone (40 μ g/embryo) was administered to 10-day (or 15-day) embryonic female chicks as described in the Materials and Methods section. After various periods of time, the left ovaries were removed for enzyme assay. The assay conditions are described in the Materials and Methods section. Values shown are means $\pm$ S.D. for three experiments (from six determinations), ○, ●, control; △, ▲, oestrogen-treated; □, ■, testosterone-treated. The open symbols represent 10-day embryos and the closed symbols represent 15-day embryos.

Table 2. Effect of oestrogen on ornithine decarboxylase activity in the ovary and other genital organs

Chick embryos were treated with oestrogen (40 μ g/egg) as described in Table 1. The left and right testes were removed and combined for enzyme assay. Left Müllerian ducts were used for enzyme assay (Teng & Teng, 1978). The results presented are means $\pm$ S.D. for six determinations. Ornithine decarboxylase activity is expressed as pmol of CO₂/30 min per mg of protein.

Age (days)	Ornithine decarboxylase activity in					
	Ovary		Testis		Müllerian duct	
	Control	+Oestrogen	Control	+Oestrogen	Control	+Oestrogen
9	766 $\pm$ 19	1130 $\pm$ 35	348 $\pm$ 11	208 $\pm$ 8	782 $\pm$ 18	4020 $\pm$ 140
10	575 $\pm$ 14	822 $\pm$ 21	373 $\pm$ 12	185 $\pm$ 7	535 $\pm$ 12	3100 $\pm$ 115
15	306 $\pm$ 12	195 $\pm$ 7	153 $\pm$ 7	119 $\pm$ 6	545 $\pm$ 12	5320 $\pm$ 164
18	192 $\pm$ 8	130 $\pm$ 6	85 $\pm$ 3	78 $\pm$ 2	95 $\pm$ 3	965 $\pm$ 26

embryonic testis (C. S. Teng & C. T. Teng, 1979). Ornithine decarboxylase activity has a higher response to oestrogen induction in the Müllerian duct than in the other two organs investigated. In the Müllerian duct, the biochemical mechanisms that can be correlated with enzyme induction by oestrogen have been described (Teng & Teng, 1978).

Ovarian ornithine decarboxylase activity was stimulated 99, 155, 175 and 206% over control in 10-, 13-, 15- and 18-day embryos respectively after 3 h of stimulation with lutropin *in vitro* (Table 3). Similar stimulation by lutropin has been demonstrated in the mammalian ovary *in vivo* and in the ovarian granulosa cells *in vitro* (Kobayashi *et al.*, 1971; Williams-Ashman *et al.*, 1972; Kaye *et al.*, 1973; Nuredin, 1977; Johnson & Sashida, 1977; Osterman & Hammond, 1977). It was suggested that the stimulating effect of gonadotropin on ornithine decarboxylase in granulosa cells of porcine ovarian follicles was mediated by cyclic AMP (Osterman *et al.*, 1978).

In embryonic-chick ovary, cyclic AMP synthesis gradually increased on incubation with human choriogonadotropic hormone *in vitro*. The stimulation of cyclic AMP production was 96, 146, 167 and 133% over that in the control in 10-, 12-, 15- and 18-day embryo respectively (Teng & Teng, 1977). This pattern of hormonal response coincided with that observed with ornithine decarboxylase activity, with higher response to hormonal effect in the later stages of development. In embryonic ovary no correlation of cyclic AMP with induction of ornithine decarboxylase activity is known. However, a direct link between these two biological events probably exists and should be investigated. Our studies on enzyme induction by gonadotropin confirmed a previous finding that the gonadotropin-res-

ponsive system is in existence in the early stages of embryonic ovary development and the system gradually reaches full functional capacity in the later stages of development (Teng & Teng, 1977).

Developmental change in ornithine decarboxylase activity and its response to steroids

During the early stages of embryonic ovary differentiation (9–12 days), the ornithine decarboxylase activity (in terms of pmol of CO₂/30 min per mg of protein) was originally high (766), then declined to 575–192 from days 9 to 12 (Fig. 2). The activity increased to 306 pmol/30 min per mg of protein at day 15, then declined to 40 at day 6 of hatching. One could argue that this pattern of change in enzyme activity during development is due to the fluctuation of the size of the ornithine pool in the ovary. However, we found that the ornithine pool size in the developing chick ovary is constant. The corresponding ornithine concentrations are 11.8, 12.3, 11.5 and 11.2 nmol/mg of DNA, for the 10-, 12-, 15- and 18-day embryo respectively. The amount of ornithine extracted from the sample into the assay mixture is only 0.2–0.4 nmol/assay (this concentration constituted one-thousandth of the substrate concentration). This concentration could not significantly affect the enzyme assay.

In other embryonic organs, i.e. the liver, brain, testis and Müllerian duct, the developmental changes in ornithine decarboxylase activity are comparable with those in the ovary. They all show higher activity

Table 3. Stimulation of ovarian ornithine decarboxylase activity by lutropin *in vitro*

Ovaries were cultured in Coon's F-12 medium with or without lutropin at 41°C in an atmosphere of air/CO₂ (19:1) for 3 h as described in the Materials and Methods section. After culture the organs were used immediately for enzyme assay. The results presented are means ± s.d. for three experiments (six determinations). Ornithine decarboxylase activity is expressed as pmol of CO₂/30 min per mg of protein.

Age (days)	Ornithine decarboxylase in			
	Control ovaries	Hormone-treated ovaries		
	Activity	Activity	Percentage of control	Increase (fold)
10	463 ± 13	921 ± 22	199	0.99
13	220 ± 13	562 ± 13	255	1.55
15	280 ± 11	769 ± 14	275	1.75
18	167 ± 6	511 ± 12	306	2.06

Fig. 2. Effect of steroids on ornithine decarboxylase activity in ovary during development

Chick embryos at various stages of development were injected with 40 µg of oestradiol or testosterone or 0.1 ml of sesame oil; after 3 h the ovaries were removed for enzyme assay. Values presented are means ± s.d. for six determinations. ●, Control; ▲, oestradiol-treated; ■, testosterone-treated.

in the early developmental stages than in the later stages. In the liver, brain and Müllerian duct there is a stage (at day 11, 11 and 15 respectively) at which the enzyme activity rises and then declines (Teng & Teng, 1978). The observed high activity in the early developmental stages of embryonic ovary has been correlated with early morphological development and germ-cell proliferation within the ovary as described above. The second wave of increase in enzyme activity in the embryonic ovary (at day 15) is probably related to the maturation of a large population of follicular cells, which start association with oögonia at this particular stage. The stage-specific response of embryonic ovary to the effect of oestrogen has been stated above.

In 12-day to 18-day embryo, testosterone caused a constant inhibition (20–41%) of ovarian enzyme activity. The inhibiting effect of testosterone on ovarian growth and function has been reported in Rhesus monkey and rat (Zuckerman, 1937; Scott, 1956; Scott & Wharton, 1959; Gorski & Barraclough, 1962). In neonatal (or prenatal) rat, it was shown that early exposure to testosterone could permanently alter the hypophyseal-ovarian axis and inhibit the follicular growth and luteinization in the ovary. Consequently, ovulation did not occur in the treated animals (Everett, 1964; Barraclough, 1967; Flerko *et al.*, 1967). In chick embryonic ovary, inhibition of ornithine decarboxylase activity by testosterone could initiate the morphologic changes and malfunction of the ovary.

Polyamines in the developing ovary

One of the major factors that determines orni-

Fig. 3. Polyamine concentration in the ovary of developing chick embryo

Polyamines were isolated and quantified as described in the Materials and Methods section. Values shown are means $\pm$ s.d. for three determinations. Δ , Putrescine; $\circ$, spermine; $\square$, spermidine.

thine decarboxylase activity in embryonic tissue is its biochemical end product, polyamines. Polyamines could induce the synthesis of a protein that inhibits the enzyme (Fong *et al.*, 1976; Heller *et al.*, 1976). In order to ensure that the low enzyme activity detected in the late stages of development is not due to this effect, the polyamine content (in terms of nmol/mg of DNA) in the embryonic ovary was measured (Fig. 3). Of the three polyamines, spermidine concentration is the highest and putrescine concentration is the lowest. During development, the changes in polyamine concentration correspond to the changes in enzyme activity; the higher the enzyme activity, the higher the polyamine concentration (Figs. 2 and 3). This is parallel to other embryonic systems, i.e. the African clawed toad (*Xenopus laevis*) embryo, the rat foetus, human foetus and chick Müllerian duct (Russell, 1970; Russell & Lombardini, 1971; Sturman & Gaull, 1974; Teng & Teng, 1978).

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