Studies on Sex-Organ Development

OESTROGENIC EFFECT ON ORNITHINE DECARBOXYLASE ACTIVITY IN THE DIFFERENTIATING MÜLLERIAN DUCTS AND OTHER ORGANS OF THE CHICK EMBRYO

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The activity of ornithine decarboxylase in the differentiating left and right Müllerian ducts was assayed and compared with that in other embryonic organs, i.e. the liver and the brain throughout the stages of development. In general the enzyme activity was high in the early stages and decreased extensively in the late stages of development. Specifically, in the left and right Müllerian ducts, the enzyme activity was high from day 8 to day 9 of incubation. In the right duct the enzyme activity started to decline on day 9 and then continuously decreased to an almost undetectable value on day 18 of incubation. In the left duct the enzyme activity also decreased slightly from day 9 to day 12; however, it increased from day 13 to day 15 and finally decreased to a constant value from day 18 until hatching. The alteration in enzyme activity in the Müllerian duct as assayed *in vitro* during development is not due to the effect of the size of the endogenous ornithine pool. When the enzyme activity was subjected to oestrogen stimulation, an increase of 5–10-fold for the left duct and of 5–3-fold for the right duct was observed during the course of development. No such stimulation was observed with the treatment of progesterone. Testosterone consistently caused a 25–30% inhibition of the enzyme activity in the Müllerian duct. Oestrogen slightly stimulated the enzyme activity in the developing liver but inhibited that of the brain. The concentration of the three polyamines measured in the Müllerian duct corresponds to the activity of the enzyme determined.

A high activity of the polyamine-synthesizing enzyme ornithine decarboxylase has been reported in the embryos of the chick (Raina, 1963; Calderara *et al.*, 1965; Russell & Lombardini, 1971), the rat foetus (Russell & McVicker, 1972) and the human foetus (Sturman & Gaull, 1974). Ornithine decarboxylase is the major enzyme responsible for the synthesis of putrescine, which subsequently serves as the precursor for other polyamines, i.e., spermidine and spermine. All these polyamines are closely correlated with the biochemical synthetic activity for cellular growth and functions (Jänne, 1967; Heby *et al.*, 1975).

We are interested in understanding the biochemical mechanisms behind the embryonic organ differentiation and the responsiveness of the developing organ to the hormonal effect. We have selected the embryonic female chick urogenital organ as a model system for these studies. In the female embryo, the right Müllerian duct starts to regress on day 9 of incubation and appears as a tiny non-functional rudiment by the time of hatching. The left Müllerian duct continues to develop and becomes the future functional oviduct of the mature female chick. The system provides the example of dual responses in

organ development, i.e. organ regression and organ growth, both occurring in the same organism.

The amount of ornithine decarboxylase activity in the growing or regressing organ could reflect the state of differentiation of the organ. The different rates of ornithine decarboxylase activity induced by the oestrogenic hormone during development serves as a good biochemical parameter for measuring the organ responsiveness toward the hormone.

Materials and Methods

Animals and chemicals

White Leghorn chicks (embryos or newborn female chicks) at various stages of development were obtained as described previously (Teng & Teng, 1975). 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, progesterone, putrescine, spermidine, spermine and ninhydrin spray were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); NCS tissue solubilizer and aqueous radioactivity counting scintil-

lant 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 mCi/mmol) 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 Brinkmann Inst. (Des Plaines, IL, U.S.A.). All other chemicals were of analytical grade.

Assay of ornithine decarboxylase activity

Enzyme activity was determined by a modified technique of Russell & Snyder (1968) and Stasny & Cohen (1970). Pooled Müllerian ducts (or other organs) (approx. 80–100 mg), which had been removed from chick embryos, were immediately homogenized in 10 vol. of 0.05 M-sodium/potassium phosphate buffer, containing 5 mM-dithiothreitol at pH 7.2 (or other value specified). The homogenate was centrifuged at 15000g for 30 min. Samples of the supernatant, representing 0.25–0.30 mg of protein from the Müllerian duct or 0.5–1.0 mg of protein from the liver or brain, were assayed in a final volume of 0.5 ml with the above buffer and preincubated with 0.5 mM-pyridoxal phosphate in a 6 ml glass counting vial (ISO-Tex, Friendswood, TX, U.S.A.) for 10 min at 37°C with gentle shaking. Within these ranges of protein concentration, ornithine decarboxylase activity was linear with respect to the amount of protein added.

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 vial. The vial was equipped with a rubber stopper supporting a polyethylene centre well that contained 0.3 ml of a 1:1 (v/v) mixture of NCS tissue solubilizer and toluene. Incubation was carried out for 30 min at 37°C with shaking. The reactions were stopped by injecting 0.25 ml of 2 M-citric acid through the rubber stopper. The vials were shaken for an additional 30 min at 37°C to permit the absorption of the released CO₂ into the NCS solution. The centre wells were removed and placed in a mini counting vial (Rochester Scientific Co., Rochester, NY, U.S.A.) containing 4 ml of the aqueous scintillant. Samples were assayed in a Beckman model LS-250 liquid-scintillation spectrometer.

Enzyme blanks were determined by heating the enzyme at 100°C for 15 min before preincubation; this step was followed by the addition of similar chemical ingredients in the reaction mixture. The radioactivity (approx. 35–40 c.p.m.) obtained from this preparation was subtracted from all other enzyme determinations.

Exposure of Müllerian duct or chick oviduct to steroid hormones in vivo

The steroid hormone was dissolved in sesame oil and applied to the allantois of the chick embryos through the shell hole as described previously (Teng & Teng, 1976). A single dose (40 μ g in 0.1 ml) of steroid hormone (or other concentration specified) was injected. The control group received the same amount of the vehicle minus the steroid. After the injection was completed, the hole on the shell was sealed with Scotch tape, and the eggs were returned to the incubator. After 3 h (or different lengths of time), the embryos were dissected and the embryonic organs were removed for enzyme assay. Immature female chicks were injected subcutaneously with 2 mg of the steroid (or other dose specified) in 0.2 ml of sesame oil and were decapitated 3 h after the injection.

Determination of ornithine pool size

The size of the ornithine pool of the developing Müllerian duct was determined at four different stages of embryonic development by the procedure of Silver & Comb (1967) as modified by Teng (1974). Müllerian ducts (approx. 200 mg) were minced finely with scissors. The minced tissue was suspended in 10 vol. (or 2.2 ml) of 5% (w/v) trichloroacetic acid solution at 0°C and homogenized for 12 strokes with a Teflon/glass homogenizer. The homogenate was stirred for 18 h at 0°C and then centrifuged at 3000g for 15 min; the precipitate was discarded. The supernatant was extracted with 3 $\times$ 4 vol. of diethyl ether and heated at 65°C with a heating block (Lab-Line Instrument, Melrose Park, IL, U.S.A.) to remove traces of the ether. Acid-washed charcoal (5 mg) 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 (0.2 M, pH 2.2). A portion (0.2 ml) was analysed for amino acid content on the Beckman model 121 amino acid analyser as described by Spackman *et al.* (1958).

Isolation and quantitation of polyamines

The isolation of polyamines from Müllerian ducts was essentially as described by Inoue & Mizutani (1973). The tissue (approx. 80–150 mg) was minced and homogenized (30 strokes) in 10 vol. of 2% (w/v) HClO₄ with a Dounce homogenizer equipped with a loose pestle (type A). The homogenate was further treated for three strokes in the same homogenizer with a tight pestle (type B). The homogenate was centrifuged at 10000g for 15 min, and the precipitate was washed with 2 $\times$ 1 ml of 2% HClO₄. The supernatants

were combined and applied to a column (0.8 cm × 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 adsorbed 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 polyamines was dissolved in 100 µl of 0.05 M-HCl.

Polyamines were quantified as described by Hammond & Herbst (1968). In accordance with this technique, a sample (25 µl) was applied to a silica-gel plate and, as a standard, 25 µl of a polyamine mixture, containing 12.5, 25, 37.5 nmol each of putrescine, spermidine and spermine, was simultaneously subjected to chromatography on the same plate. The plate was chromatographed in diethylene glycol monoethyl ether/propionic acid/water solvent (14:3:3, by vol., with the saturation of NaCl) for 18 h. After drying 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% ethanol for 60 min. The extract was then centrifuged at 2000g for 10 min, and the A_{575} of the supernatant read. A standard curve for each polyamine was made by the same procedure.

General procedures

These were as previously described (Teng & Teng, 1977).

Results and Discussion

Assay of ornithine decarboxylase activity and its response to steroid hormones

Ornithine decarboxylase activity was assayed in the Müllerian ducts, liver and brain of the chick embryo by measuring the production of CO₂ from DL-[1-¹⁴C]ornithine after incubation with the tissue extract. The assay was linear with respect to both time of incubation and the concentration of tissue extract used.

The optimum pH values for extraction and assay of ornithine decarboxylase activity in the Müllerian ducts, liver and brain are 7.2, 7.2 and 6.9 respectively. The optimum concentration of ornithine to serve as the substrate for the enzyme reaction in Müllerian duct is 0.1 mM. At this concentration the enzyme was substrate-saturated. In the liver and brain, the optimum concentration of ornithine is 0.05 mM (results not shown); therefore all subsequent enzyme assays were based on this defined condition.

The time course of the oestrogenic hormone effect on ornithine decarboxylase activity in the left Müllerian duct is linear with time from 1 to 3 h after hormone administration. The hormonal stimulation was 8.5-fold that of the control and remained at the same value after 3–5 h of stimulation. A similar oestrogenic effect, but with less stimulation, was found in the right Müllerian duct and the liver (with 6.5- and 1.25-fold stimulation respectively). A slight and consistent inhibition of enzyme activity was observed in the brain (Fig. 1a). The selective effect of oestrogen on ornithine decarboxylase activity in Müllerian ducts paralleled the effects found in other

Fig. 1. Effect of oestrogen on the ornithine decarboxylase activity in various embryonic organs

(a) Time-course of oestrogenic effect. A single dose of oestradiol-17β (40 µg/embryo) was administered to the 15-day (or 11-day) embryonic female chick as described in the Material and Methods section. After various periods of time, the left and right Müllerian ducts (from 15-day embryo), liver and brain (from 11-day embryo) were removed for assay. (b) Effect of oestrogen concentration. Various amounts of oestradiol-17β were administered to the embryo for 3 h; the control group received the vehicle without oestrogen. The ornithine decarboxylase activity [in terms of pmol of CO₂/30 min per mg of protein ($\times 10^{-2}$)] in the control group is 5.4–6.2, 0.8–0.9, 0.7–0.75 and 2.0–3.4 for the left Müllerian duct, right Müllerian duct, liver and brain respectively. Values are means $\pm$ S.D. for three determinations. ●, Left Müllerian duct; ○, right Müllerian duct; ▲, liver; △, brain.

hormone-responding tissues, i.e. lutropin (luteinizing hormone) in the ovary (Kobayashi *et al.*, 1971; Kaye *et al.*, 1973), the growth hormone in the rat liver (Jänne & Raina, 1969; Russell *et al.*, 1970), testosterone in the ventral prostate (Pegg *et al.*, 1970), and

oestradiol in the uterus and oviduct (Cohen *et al.*, 1970; Kaye *et al.*, 1971).

The optimum dose of oestrogen for eliciting maximum enzyme activity in the Müllerian duct is 40 µg/embryo. In previous reports we demonstrated that this dose of oestrogen could effectively translocate the cytoplasmic oestrogen receptor and oestrogen complex into the nucleus and subsequently bind to the chromatin in the Müllerian duct (Teng & Teng, 1976, 1978). Various concentrations of oestrogen have been administered to the embryo; however, it could neither change the stimulation nor the inhibition patterns of ornithine decarboxylase in the liver or the brain (Fig. 1b).

The effects of different steroid hormones have been tested on ornithine decarboxylase activity in the Müllerian duct, and it was found that testosterone at the dose range of 20–40 µg/embryo, causes approx. 25–30% inhibition of the enzyme activity. No significant effect of testosterone was found at the dosage of 5 µg/embryo. Progesterone, at the dose of 40 µg/embryo, caused a slight inhibition of the enzyme activity (approx. 18% inhibition), but no significant effect was found at 20 µg/embryo (Table 1). The molecular mechanism behind the inhibition of this enzyme by these steroids is still unknown.

Table 1. Comparison of the steroidal effect on ornithine decarboxylase activity in Müllerian duct

Chick embryos (15 days old) were injected with 40 µg (or other dosage as indicated) of oestradiol, testosterone, progesterone or 0.1 ml of sesame oil for 3 h; the left Müllerian ducts were then removed for enzyme assay. The results presented are means ± s.d. for six determinations.

Steroid	Concentration (µg/embryo)	Ornithine decarboxylase activity (pmol of CO ₂ /30 min per mg of protein)
Control	None	602 ± 14
Oestradiol	40	5120 ± 150
Testosterone	40	407 ± 6
	20	452 ± 7
	5	537 ± 10
Progesterone	40	494 ± 10
	20	601 ± 14

Fig. 2. Developmental alteration of ornithine decarboxylase activity and its response to oestrogenic stimulation in various embryonic organs

(a) Enzyme activity observed in the left Müllerian duct. (b) Enzyme activity in the right Müllerian duct. The oestrogen-induced enzyme activity is shown on the right ordinate. (c) Enzyme activity in the developing liver. (d) Enzyme activity in the brain tissue. The values in (a) and (b) are means ± s.d. for four experiments (eight determinations). Values in (c) and (d) are means ± s.d. for three experiments (six determinations). ●, Control; ▲, oestrogen-stimulated. (The data points for the oestrogen response are derived from hormone administered at each particular age and assayed after 3 h.)

Table 2. *Ornithine pools in the cell of Müllerian duct of various developmental stages*
 Values shown are means $\pm$ s.d. for three determinations.

Age (days)	Ornithine content						
	Duct	(nmol/mg of DNA)		(nmol/mg of protein)		(nmol/assay)	
		Left	Right	Left	Right	Left	Right
10		2.85 $\pm$ 0.5	2.80 $\pm$ 0.5	0.43 $\pm$ 0.08	0.41 $\pm$ 0.08	0.11 $\pm$ 0.02	0.10 $\pm$ 0.02
12		3.40 $\pm$ 0.6	3.14 $\pm$ 0.5	0.52 $\pm$ 0.09	0.49 $\pm$ 0.08	0.13 $\pm$ 0.02	0.12 $\pm$ 0.02
15		3.50 $\pm$ 0.6	2.25 $\pm$ 0.5	0.53 $\pm$ 0.08	0.35 $\pm$ 0.08	0.13 $\pm$ 0.02	0.09 $\pm$ 0.02
6 (newborn)		16.00 $\pm$ 1.2	—	2.67 $\pm$ 0.45	—	0.67 $\pm$ 0.12	—

Fig. 3. *Developmental responsiveness to oestrogen for ornithine decarboxylase stimulation in differentiating Müllerian ducts*

The values shown are summarized from Figs. 2(a)–2(c). ●, Left Müllerian duct; ○, right Müllerian duct; ▲, liver.

Developmental change in ornithine decarboxylase activity and its response to oestrogen

During the early stages of Müllerian-duct differentiation (8–9 days), the ornithine decarboxylase activity [in terms of pmol of CO₂/30 min per mg of protein ($\times 10^{-2}$)] was high (7.4–7.5), then declined to 3.8 from days 9 to 12. In the developing left duct the activity increased to 6.0 at day 15, and then declined to a constant value of 1.4–1.8. No such increase was observed in the involuting right duct (Figs. 2a, 2b). One could argue that the change in enzyme activity during development in the Müllerian ducts, or the difference in enzyme activity between the left and right duct is due to the fluctuation of the size of the ornithine pool in the duct. However, in Table 2 we have shown that the ornithine concentration in the extract is only 0.09–0.13 nmol/assay (this concentration constituted one-hundredth of the substrate concentration). This concentration could not significantly effect the enzyme assay.

In other embryonic organs, i.e. the liver and brain, the developmental changes of ornithine decarboxylase activity are comparable with Müllerian ducts. They all show higher activity in the early developmental stages than the late stages. In the liver and brain there is a stage (at day 11) in which the enzyme activity rises and then declines (Figs. 2c, 2d).

This observation indicates that the pattern and timing of the enzyme appearance for each organ are different. We postulate that the early increase in ornithine decarboxylase activity in the embryonic organ is responsible for ‘priming’ the tissues that are ready to undergo proliferation or rapid growth. Other examples that demonstrate similar patterns are in the developing sea urchin, the toad and chick embryo (Barros & Giudice, 1968; Russell, 1970; Caldarera *et al.*, 1971), and the regenerating rat liver (Raina *et al.*, 1970). The observed high enzymic activity in the early developmental stages of the Müllerian duct is concomitant with a high template activity and high rate of RNA synthesis at that stage (Teng & Teng, 1978).

The second wave in the increase in enzymic activity of the embryonic organ is probably related to the programming of the differentiation and future growth of the organ. There have been no second waves of increase observed in the involuting right Müllerian duct. The causal relationship of enzyme activity and organ growth or death has not yet been defined.

The administration of an optimum dose of oestradiol to a 9–10-day-old chick embryo increased ornithine decarboxylase activity approx. 5-fold in both left and right ducts. If an optimum dose of oestradiol was administered to the 15–18-day-old chick embryo, the enzyme activity increased 8–10-fold in the left duct. This responsiveness remained until day 6 after birth. There was significantly less response observed in the right duct (7–3.5-fold increase) (Figs. 2a, 2b and 3). The injection of oestradiol caused a higher rate of enzyme induction in the left duct than the right duct in the late stages of differentiation. This information probably reflects the difference in the genomic structure of these two ducts. Indeed, in our previous observation we have shown the

Table 3. *Oestrogenic effect on the ornithine decarboxylase activity in the embryonic organs*

The procedures for enzyme assay were described in the Material and Methods section. Values shown are means $\pm$ s.d. for six determinations (from three experiments). (a) Control; (b) oestrogen-stimulated.

Age (days)	Ornithine decarboxylase activity (pmol of CO ₂ /30 min per mg of DNA)							
	Müllerian duct				Liver		Brain	
	Left		Right					
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
9	4620 $\pm$ 221	25200 $\pm$ 1120	4611 $\pm$ 219	24814 $\pm$ 1191	3066 $\pm$ 127	3390 $\pm$ 146	5618 $\pm$ 235	4377 $\pm$ 183
12	2400 $\pm$ 117	20400 $\pm$ 985	1260 $\pm$ 58	8700 $\pm$ 435	1766 $\pm$ 79	1938 $\pm$ 81	1708 $\pm$ 62	1590 $\pm$ 57
15	3600 $\pm$ 137	31500 $\pm$ 1323	480 $\pm$ 20	3360 $\pm$ 129	439 $\pm$ 17	499 $\pm$ 23	825 $\pm$ 32	803 $\pm$ 30
18	480 $\pm$ 12	4770 $\pm$ 152	180 $\pm$ 5.6	258 $\pm$ 7.4	302 $\pm$ 6.8	336 $\pm$ 7.2	731 $\pm$ 28	734 $\pm$ 27
6 (newborn)	300 $\pm$ 6.6	3900 $\pm$ 82	—	—	279 $\pm$ 6.4	290 $\pm$ 6.5	153 $\pm$ 5.5	156 $\pm$ 5.6

decrease of chromosomal protein and oestradiol binding capacity in the chromatin of the regressing right duct (Teng & Teng, 1978). These changes in the chromosomal content could affect the processes of enzyme induction. In the left duct, a gradual increase in the capacity for enzyme induction by oestradiol is probably determined by the proper state of genomic maturation, tissue-hormone interaction and physiological need of the organ.

Throughout development the embryonic liver responded to the oestrogen far less intensively than the Müllerian duct. The maximum response occurred on days 10–12, when 30–36% stimulation was observed. In the embryonic brain, oestradiol consistently demonstrated its inhibitory effect on enzymic activity (Figs. 2c, 2d).

In Table 3 the ornithine decarboxylase activity has been calculated in terms of pmol of CO₂/30 min per mg of DNA, and the results agree with the enzyme activity calculated on a per mg of protein basis (Figs. 2a–2d).

Polyamines in the developing Müllerian ducts

We have considered the possibility that the content of polyamines in the Müllerian duct could affect the activity of ornithine decarboxylase through the induction of the synthesis of an inhibitory protein (Fong *et al.*, 1976; Heller *et al.*, 1976). The polyamine content (in terms of nmol/mg of DNA) in the differentiating left and right ducts was determined. Two general observations can describe this content. (1) The concentration of spermidine is highest among the three polyamines, and that of putrescine is the least. (2) The changes of polyamine concentration during development correspond to the enzyme activity; the higher the enzyme activity, the higher the polyamine concentration (Fig. 4). Similar observations were made of the embryo of the South African clawed toad (*Xenopus laevis*), the rat foetus, and the human

Fig. 4. Polyamine concentration in the Müllerian ducts of the 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. ▲, Putrescine from left duct; △, putrescine from right duct; ●, spermine from left duct; ○, spermine from right duct; ■, spermidine from left duct; □, spermidine from right duct.

foetus during development (Russell, 1970; Russell & Lombardini, 1971; Sturman & Gaull, 1974).

On day 9 of development there is no obvious difference in the concentration of polyamines in the left and right ducts. The difference starts on day 12 of development. From days 12–18 the concentrations of spermidine, spermine and putrescine in the right duct are respectively 22–50%, 30–55% and 18–48% lower than in the left duct (Fig. 4). The functional significance of the relative concentrations of cellular polyamines has not yet been fully defined (Raina & Jänne, 1975). However, a previous report suggested a positive correlation between these concentrations and the tissue activity (Tabor & Tabor, 1964). The relation of

polyamines to the growth potential of the tissue has been demonstrated in aging tissue, which was accompanied by a marked decrease in polyamine concentration (Jänne *et al.*, 1964).

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