Biochem. J. (1977) **162**, 123–134
Printed in Great Britain

Studies on Sex-Organ Development

THE HORMONAL REGULATION OF STEROIDOGENESIS AND ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE IN EMBRYONIC-CHICK OVARY

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(*Received* 2 *June* 1976)

1. We investigated the production of steroid hormones by the ovaries of the developing embryonic chick under conditions of organ culture. Radioimmunoassay techniques were used to measure the amount of steroid hormone released into the culture medium. Stimulation of the production of steroid hormones by choriogonadotropin from the urine of pregnant human was dose-dependent. Oestradiol and testosterone production was optimal when 20 i.u. of gonadotropic hormone was present in the culture medium. 2. During development, both left and right ovaries responded to gonadotropic hormone stimulation with a 2.5–3-fold increase in oestrogen production. However, the right ovary was twice as efficient in testosterone production as the left one. The presence of dibutyryl cyclic AMP in the culture medium of the embryonic ovaries mimicked the effect of the gonadotropic hormone. 3. The human choriogonadotropic hormone stimulated cyclic AMP production in the embryonic ovarian tissue. Thyrotropin, growth hormone and insulin had no stimulating effect. 3-Isobutyl-1-methylxanthine potentiated the gonadotropic hormone effect by increasing the concentration of cyclic AMP in the ovarian tissue. 4. The amount of cyclic AMP synthesized in the embryonic ovary was gradually increased (from 1.2 to 6.5 pmol/mg of tissue) when incubated with increasing doses of human choriogonadotropic hormone *in vitro*. The newly synthesized cyclic AMP reached the maximum concentration after 30 min of incubation, then decreased at 2 h of incubation. A portion of the newly synthesized cyclic AMP was released into the culture medium. 5. At various developmental stages, both left and right embryonic-chick ovaries responded to stimulation by gonadotropic hormone with an increase in cyclic AMP production. The cyclic AMP concentration in the right ovary was 80% higher than that in the corresponding left ovary.

Asymmetrical development of the embryonic ovaries is a characteristic feature of the avian species (Swift, 1915; Brode, 1928). In the chick embryo, the left ovary begins to grow before hatching. Growth continues after hatching, and eventually it becomes the functional ovary of the hen. The right ovary grows for a short period of time during the early stages of development and then regresses. It subsequently becomes a rudimentary non-functional organ. The ovarian secretion of steroids was believed to be responsible for the respective growth and regression of left and right Müllerian ducts (Wolff, 1959; Jost, 1970).

Our previous observations showed the presence of oestrogen receptors in the Müllerian ducts (Teng & Teng, 1975*a*,*b*). The translocation of the oestrogen receptor into the nucleus by oestradiol-17β and the increase of endogenous oestrogen-binding sites in the nucleus of the developing Müllerian duct have also been demonstrated (Teng & Teng, 1976). Little is known, however, of the regulatory mechanism in the embryonic ovaries that controls secretion of the steroid hormones. How the secretions of the embryonic ovaries affect differentiation and development of the urogenital tract is also unknown. This formed the basis of the present study.

Observations of steroidogenesis in embryonic-chick gonads have been reported from many laboratories (Weniger & Zeis, 1971; Guichard *et al.*, 1973; Galli & Wasserman, 1973; Haffen, 1975). These previous studies provided semiquantitative measurements only, and determination of the total secretion of the embryonic organ was performed in the present study. The influence of gonadotropic hormone on the stimulation of steroidogenesis in the differentiating embryonic left and right ovaries is not fully understood. It has been suggested that polypeptide hormones affect their respective target tissues by regulating the intracellular concentration of cyclic AMP (Haynes *et al.*, 1960; Hall & Eik-Nes, 1962; Marsh & Savard, 1964*a*,*b*). However, the influence of polypeptide hormones on steroidogenesis in the embryonic

ovarian tissue is still unknown. Part of this work has already been published in a preliminary form (Teng & Teng, 1975*c*).

Materials and Methods

Animals and chemicals

Fertile white Leghorn eggs, obtained from Rich-Glo Farms, Houston, TX, U.S.A., were incubated at 38°C in a Petersime model 4 humidified incubator. The age of the embryos was determined by the criteria of Hamburger & Hamilton (1951). Embryos at different stages of development were decapitated and dissected mid-ventrally. The embryonic ovaries were placed in a Petri dish containing Hanks' balanced salt solution (Hanks & Wallace, 1949) before being incubated. The total time from decapitation to the ovary incubation was limited to 1h.

Choriogonadotropin (from urine of pregnant human) [50i.u. (international units) as defined by Leech (1939)], thyrotropin (from ox pituitary, 1i.u./mg), growth hormone (from pig pituitary, 1i.u./mg), insulin (from ox pancreas, 26.4i.u./mg), unlabelled oestradiol, testosterone, dibutyryl cyclic AMP and cyclic AMP were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The sheep luteinizing hormone (NIH, S-18) was obtained from Dr. A. R. Means (Cell Biology Department, Baylor College of Medicine, Houston, TX, U.S.A.). All gonadotropic hormones were dissolved in 0.9% NaCl before use. Steroid hormones were made up in ethanol as stock solution and diluted with phosphate-buffered saline (0.01M-sodium phosphate buffer, pH7.2/0.14M-NaCl) containing 0.1% gelatin before use.

[6,7-^{3}H]Oestradiol-17β (sp. radioactivity 45Ci/mmol) and [1β,2β-^{3}H]testosterone (sp. radioactivity 90Ci/mmol) from New England Nuclear Corp. (Boston, MA, U.S.A.), were supplied in a solution of benzene/methanol (9:1, v/v); they were evaporated under N_2 to dryness and re-dissolved in ethanol before use.

The following chemicals were purchased from the sources indicated: cyclic [^{3}H]AMP (sp. radioactivity 21Ci/mmol in 50%, v/v, ethanol) (Schwarz/Mann, Orangeburg, NY, U.S.A.), Hanks balanced salt solution (Gibco, New York, NY, U.S.A.), antibody against oestradiol-17β and testosterone (Radioassay System Lab., Carson, CA, U.S.A.), 3-isobutyl-1-methylxanthine (Aldrich Chemical Co., Milwaukee, WI, U.S.A.), Dowex AG-50W-X4 (Bio-Rad Laboratories, Richmond, CA, U.S.A.), Dextran T-70 (Pharmacia, Uppsala, Sweden), activated Charcoal (Mallinckrodt, St. Louis, MO, U.S.A.) and protein kinase (from rabbit skeletal muscle, provided by Mr. D. Miller, Cell Biology Department, Baylor College of Medicine).

The radioactivity was determined at 60% efficiency in a Beckman model LS-250 scintillation spectrometer with 4ml of scintillation fluid [Liquifluor (New England Nuclear)/Triton/toluene, 83:500:917] in mini vials (Rochester Scientific Co., Rochester, NY, U.S.A.).

Incubation of embryonic ovaries

Freshly excised intact ovaries (approx. 15–25mg of tissue) were incubated in a Falcon model 3001 tissue-culture dish (Oxnard, CA, U.S.A.) containing 1ml of Hanks balanced salt solution in an atmosphere of O_2+CO_2 (95:5) at 41°C in a model CO-20 incubator (New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.) as described by Connell *et al.* (1966). Standard incubation time for the steroid-hormone assay was 4h and for the cyclic AMP assay was 30min, unless otherwise specified. Various additions to the incubation medium are indicated in the legend of each Figure.

Radioimmunoassay for steroid hormone

[^{3}H]Oestradiol-17β and [^{3}H]testosterone were dried under N_2 to remove benzene, and re-dissolved in ethanol for storage. Non-radioactive oestradiol-17β and testosterone were dissolved in ethanol in a final concentration of 1μg/ml as stock solution. Phosphate-buffered saline solution containing 0.1% gelatin, pH7.2, was used throughout the assay procedure. Steroid working standards (3–1000pg) were prepared by diluting the 1μg/ml non-radioactive oestradiol or testosterone solution in ethanol with phosphate-buffered saline containing 0.1% gelatin. Radioactive oestradiol or testosterone solution was prepared by diluting the stock solution with phosphate-buffered saline containing 0.1% gelatin to 3500c.p.m./0.1ml (tracer mass is 15pg). Oestradiol-17β was measured by radioimmunoassay with an antiserum (E17) against 6-oxo-oestradiol-17β-oxime–human serum albumin. According to the supplier's test report, the antiserum has negligible cross-reactivity (0.06%) with a wide variety of C_{21}, C_{19} and C_{18} steroids. No chromatographic purification step was involved in the assay because of the high specificity of the antiserum. Testosterone was measured by radioimmunoassay with an antiserum (TD3) against testosterone-3-oxime–human serum albumin. The cross-reactivities to cholesterol, C_{21} and C_{18} steroids, androstenedione, androsterone and aetiocholanolone are less than 0.01% except for a 70% reaction with dihydrotestosterone. In the present studies no chromatography was involved for the separation of testosterone and dihydrotestosterone in the culture medium. The antibody for oestradiol-17β or testosterone was diluted with the assay buffer according to the specification of the supplier. Usually a 40–50% binding of the total labelled

steroid present in the assay was obtained. The assay was performed by the following sequence: to 0.5ml of assay buffer containing an unknown or a standard, 0.1ml of [^{3}H]oestradiol-17β (or [^{3}H]testosterone) and 0.1ml of diluted antibody were added, mixed, and incubated at 4°C overnight. After the incubation, 0.2ml of cold charcoal/dextran suspension (0.625% charcoal and 0.0625% dextran in phosphate-buffered saline solution) was added to each tube. The tubes were stirred with a vortex mixer for 20s and left at 4°C for 20min. After centrifugation at 2300rev./min for 10min, the supernatant containing the antibody-bound oestradiol or testosterone was decanted into a counting vial and added to scintillation fluid for radioactivity assay. Each sample was stabilized overnight at 4°C and counted for radioactivity for 5min. A blank (no antibody) and a zero tube (without standard or unknown) were included with every assay as controls.

Preliminary experiments indicated that the presence of Hanks' balanced salt solution (up to 0.2ml) does not interfere in the radioimmunoassay system. Therefore the presence of the steroid in the incubation medium can be measured directly. In the standard assay conditions, duplicated samples of 25μl of culture medium were assayed for the oestradiol concentration, and 50μl of culture medium for the testosterone concentration.

Cyclic AMP assay

After culture *in vitro* for 30min, the cyclic AMP that was present in the ovarian tissue or culture medium was extracted with 5% (w/v) trichloroacetic acid by homogenizing the tissue in 1ml of 5% trichloroacetic acid with a Teflon/glass homogenizer. The culture medium was adjusted to a final concentration of 5% by adding concentrated trichloroacetic acid. The above extraction procedures were performed at 4°C.

Cyclic AMP present in the trichloroacetic acid extract was purified immediately by elution through a Dowex 50-X4 column (Krishna *et al.*, 1968) at room temperature (24°C).

Before chromatography, the trichloroacetic acid-extracted sample was mixed with a known amount of cyclic [^{3}H]AMP (approx. 125000c.p.m./ml of the extract), as an internal indicator. The recovery of the radioactive tracer after the chromatography was generally about 35–50%. The amount of cyclic AMP present in the eluate from the Dowex column was measured by the protein-binding assay method as described by Gilman (1970). The binding protein used for cyclic AMP assay was prepared by the affinity-chromatographic technique originally developed by Cuatrecasas (1970) and modified by Means *et al.* (1974). The binding-protein preparation used in the experiments presented here bound 0.3pmol of cyclic AMP/μg of this binding protein, and could be quantitatively adsorbed by a single Millipore filter (0.45μm pore size).

The protein-binding assay reaction was conducted in a final volume of 100μl in 50mM-sodium acetate buffer, pH4.0, which contained 2.5pmol of cyclic [^{3}H]AMP, 50μl of the eluate obtained from the Dowex column and protein kinase as the binding protein to bind less than 30% of the nucleotides. After incubation at 0°C for 60min, the reaction was terminated by addition of 2ml of stopping solution (20mM-potassium phosphate, pH5.0/2mM-EDTA). After 5min the mixture was passed through a Millipore filter, and the protein-bound cyclic AMP that was retained on the filter was washed with 10ml of the same potassium phosphate/EDTA buffer. The filters were dried and placed in 15ml of scintillation fluid. The radioactivity was determined by a Beckman model SL-250 liquid-scintillation spectrometer.

General procedures

The wet weights of ovaries excised from embryos of various developmental stages were determined immediately. Approx. 5–20 ovaries per group were weighed in a Mettler model H-54 balance. Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard. DNA was determined by the diphenylamine reaction (Giles & Myers, 1965) with calf thymus DNA (Schwarz/Mann) as a standard. RNA was determined by the procedure of Munro & Fleck (1966).

Results

Embryonic ovary development and sex-hormone production

The developmental patterns of the embryonic chick ovaries are presented in Table 1. Changes in the wet weights and the contents of DNA, RNA and protein show that the left and right ovaries developed concurrently from days 8 to 10 of incubation. During this period the four observed biological parameters increased approximately twice as fast in the left ovary as in the right ovary. The left ovary continued to grow after day 10 of incubation, but the right one remained at about the same size between days 10 and 12. A significant regression of the right ovary was observed at day 15 of incubation. During the time of hatching the right ovary became a tiny piece of rudiment.

The radioimmunoassay technique was used to measure the steroid-hormone production in the embryonic ovaries during development. The standard curves of radioimmunoassay obtained for both oestradiol and testosterone, as shown in Fig. 1, parallel those obtained by Auletta *et al.* (1974). The

Table 1. *Wet weight, DNA, RNA and protein concentrations in the developing embryonic-chick ovaries*
Each value represents the mean±S.D. for ten measurements (each including 30–40 embryonic gonads).

Age (days)	Wet weight (mg/ovary)		DNA (μg/ovary)		RNA (μg/ovary)		Protein (μg/ovary)	
	Left	Right	Left	Right	Left	Right	Left	Right
8	0.25±0.08	0.21±0.10	1.36±0.82	0.98±0.33	11.2±3.3	8.5±1.0	28.9±2.3	22.1± 3.4
10	1.35±0.15	0.75±0.05	5.30±1.20	2.67±0.52	29.8±2.2	17.5±5.1	125±2.0	57.1± 5.2
12	2.63±0.14	0.73±0.07	9.80±2.30	2.50±0.84	34.1±3.2	9.8±2.1	222±7.0	50.0±12.0
15	4.60±0.92	0.50±0.12	20.90±2.15	2.40±0.27	44.9±4.3	9.0±3.3	406±1.0	50.1± 9.5
18	6.10±0.31	—	22.50±1.44	—	50.0±4.0	—	545±6.2	—

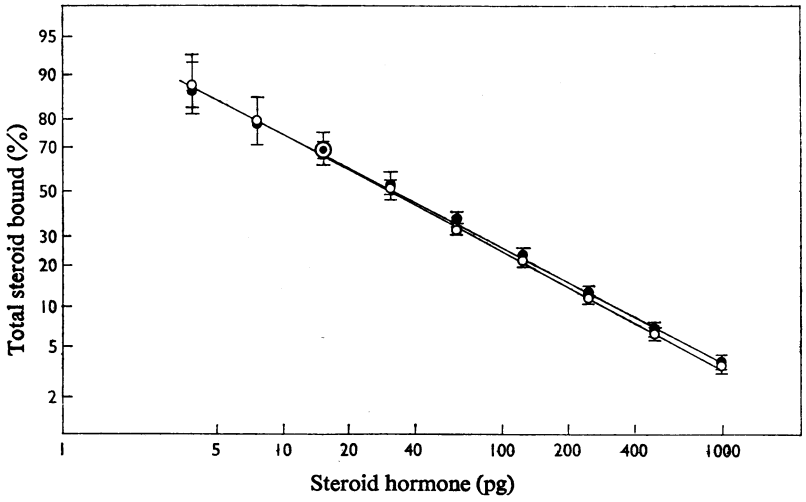

Fig. 1. *Standard curves for testosterone and oestradiol*
The Figure shows a typical standard curve obtained when the amount of steroid (series dilution) is plotted against the percentage of steroid bound. The percentage of the steroid bound was calculated by the following equation:

$$\text{Steroid bound (\%)} = \frac{\text{Radioactivity (c.p.m.) in sample} - \text{radioactivity (c.p.m.) in blank}}{\text{Radioactivity (c.p.m.) in zero control} - \text{radioactivity (c.p.m.) in blank}}$$

The data were plotted on logarithmic logistic probability paper (3 logarithmic cycles×60 divisions). ●, Testosterone; ○, oestradiol.

sensitivity of this assay was at the picogram level. The linear relationships between oestradiol and testosterone concentration in the culture medium and the volume of the culture medium being assayed are presented in Figs. 2(*a*) and 2(*b*). On the basis of these results, 10–25 μl of the cultured medium was used for the assay of oestradiol, and 50 μl of the medium was used for the assay of testosterone. The concentration of the steroid hormone in the given volume of the medium should be within the reliable range (10–200 pg) as indicated in the standard curve.

Various tissues from the 15-day chick embryo were cultured *in vitro*. The results indicated that the gonads from both sides of the embryo were the major organs responsible for steroid-hormone production. Oestrogen was found to be produced by both the left and right ovaries. After culture for 24 h, oestrogen production in the left ovary was 48% higher than that in the right ovary. Trace amounts of oestrogen were observed in the adrenal and the kidney, but no oestrogen was detectable in the liver or testis. Testosterone production was found in both the left and right ovaries, but the latter produced 32% more than the former after 24 h of culture. The amount of testosterone produced by the right ovary is equivalent to that produced by the testis. Only trace amounts of

testosterone were detectable in the adrenal and the kidney, but no detectable amount was observed in the liver (Table 2).

Effect of choriogonadotropin concentration on ovarian steroid-hormone production

Fig. 3 shows the effect of choriogonadotropin concentration on the stimulation of steroid-hormone production in the embryonic ovary. The amount of steroid hormone measured was expressed as pg of steroid/ovary. Oestrogen production increased threefold with treatments of choriogonadotropin from 5 to 20i.u. Further additions of choriogonadotropin (55–100i.u.) to the culture medium did not cause greater increases in oestrogen production. Ovarian production of testosterone was greatly enhanced by treatments with choriogonadotropin *in vitro*. When 5–20i.u. of choriogonadotropin was added, the amount of testosterone produced was almost tenfold higher than in the control. However, a less efficient stimulation (fivefold) was observed if the choriogonadotropin dose was increased to 100i.u.

Effect of choriogonadotropin on production of steroid hormones in the developing ovaries

Ovarian production of steroid hormones and its susceptibility to choriogonadotropin stimulation at various stages of development are summarized in Table 3. The amount of oestradiol (in pg/ovary) released into the culture medium by the left embryonic ovaries increased from 50 at day 8 of incubation to 350 at day 14 after birth, an increase of sevenfold. The oestradiol production in the developing left ovary was in three stages: (*a*) high rate of increase from day 8 until day 10 of incubation (from 50 to 160, an increase of 220%), (*b*) slow rate of increase from day 10 to day 15 of incubation (from 160 to 175, an

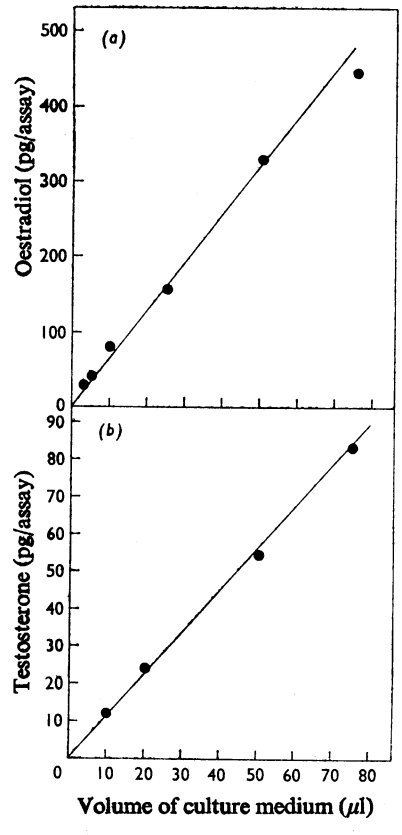

Fig. 2. *Linear relationship between steroid-hormone concentration and the volume of culture medium being assayed* For this, 25 15-day embryonic-chick left ovaries were cultured under the standard conditions described in the Materials and Methods section. Various volumes of cultured medium were taken directly for radioimmunoassay for oestradiol (*a*) and testosterone (*b*) concentration.

Table 2. *Release of steroids into the culture medium from various tissues of 15-day chick embryo*
Portions (20mg) of tissue were removed from various organs of the 15-day chick embryo immediately after decapitation. The tissues were cultured under the standard incubation conditions described in the Materials and Methods section. At the end of the incubation, duplicate samples of 50μl of culture medium from each incubation dish were removed and assayed for the presence of oestradiol and testosterone. Each value represents the mean ± S.D. of six measurements. N.D., not detectable.

	Oestradiol (pg/mg of tissue)		Testosterone (pg/mg of tissue)	
Tissue Incubation time (h) ...	4	24	4	24
Adrenal	3.6±0.4	6.4± 0.32	1.3±0.2	2.0±0.22
Kidney	2.4±0.8	N.D.	2.7±0.3	1.3±0.15
Liver	N.D.	N.D.	N.D.	N.D.
Testis	N.D.	N.D.	1.6±0.5	15.5±0.24
Left ovary	27.8±0.6	141.2± 6.3	2.3±0.4	4.6±0.40
Right ovary	64.2±6.2	95.5±15.3	6.1±1.6	14.5±3.25

increase of 9%), and (*c*) a rapid rate of increase from day 15 of incubation to day 14 after birth (from 175 to 350, an increase of 100%). When the left ovary was tested at various stages of development for responsiveness to choriogonadotropin, the oestradiol production increased steadily from day 8 to day 18. The results indicated that left ovaries at different developmental stages all respond to choriogonadotropin with a 2.5–3-fold increase in oestradiol secretion. Although a 2-week-old immature chick ovary produced more oestradiol than did the embryonic ovaries, it lost the responsiveness to the choriogonadotropin effect.

The involuting right ovary produced oestradiol during development (32–82pg/ovary), reaching its maximal value at day 10 of incubation. Even during regression, the right ovary still retained its responsiveness to choriogonadotropin, with a two- to three-fold increase in oestradiol production *in vitro*.

Testosterone production in the developing chick ovaries is presented in Table 3. In both the left and right ovaries, production was not detectable in the early stages of development (e.g. days 8, 10 and 12 of incubation), yet it was measurable after day 15 of incubation. The right ovary almost disappeared at day 15 of incubation, but its testosterone production was sufficiently high for detection. The left ovary of the 2-week-old chick released three times more testosterone than that of the 15-day and 18-day embryonic chick.

The addition of choriogonadotropin to the culture medium caused, after 4h, a significant stimulation of testosterone production in the left and right ovaries of the 8–12-day-old embryonic chick. Testosterone secretion by the left ovary of the 15-day chick embryo was increased sevenfold after choriogonadotropin stimulation, and that in the right ovary increased about 14-fold. The immature chick ovary responded to choriogonadotropin stimulation with a twofold increase in testosterone production.

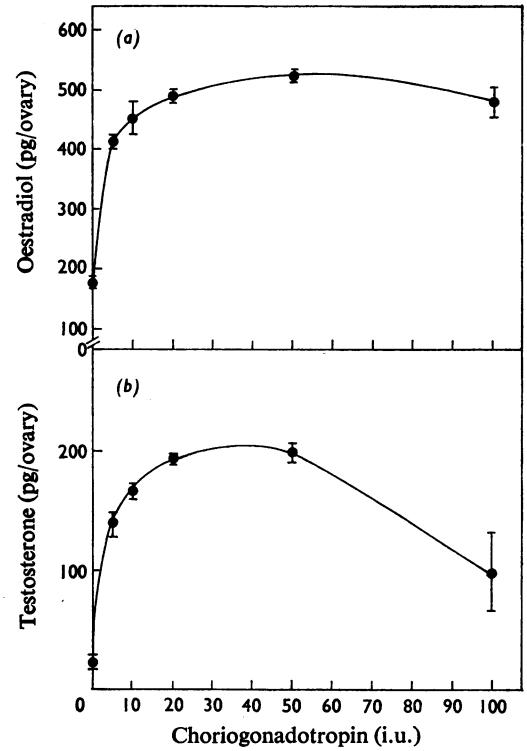

Fig. 3. *Effect of choriogonadotropin on steroid hormone released into the culture medium by the 15-day embryonic-chick left ovary*

Five ovaries (approx. 20–30mg of tissue) from the left side of female chick embryo at 15 days of gestation were cultured under the standard incubation conditions described in the Materials and Methods section. At the end of incubation, duplicate samples of culture medium were taken from each culture dish and assayed for the presence of steroid hormone. (*a*) Radioimmunoassay for oestradiol concentration; (*b*) radioimmunoassay for testosterone concentration. Each point represents the mean value from triplicate cultures.

Table 3. *Effect of choriogonadotropin on steroid-hormone production during the development of embryonic chick ovaries*

Ovarian tissue (20mg) from both sides of the female chick embryo during various developmental stages was incubated as described in the Materials and Methods section. (*a*) Incubation without choriogonadotropin; (*b*) incubation with choriogonadotropin (20i.u./ml of culture medium). Each value is the mean ± S.D. of three determinations.

Period of incubation (days)	Oestradiol (pg/ovary)				Testosterone (pg/ovary)			
	Left ovary		Right ovary		Left ovary		Right ovary	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
8	50± 3	208± 7	32±4	158± 6	N.D.	10±0.8	N.D.	12±0.9
10	160± 6	285±16	82±5	183±12	N.D.	20±0.9	N.D.	22±0.9
12	168± 6	435±15	32±5	120± 6	N.D.	54±6.0	N.D.	25±1.2
15	175±14	518±10	35±3	107± 8	24±2	178±8.0	3.5±0.4	49±1.5
18	252± 6	644± 7	—	—	22±2	118±11	—	—
Immature chick (14-day-old)	350±11	350±10	—	—	86±2	182±3.0	—	—

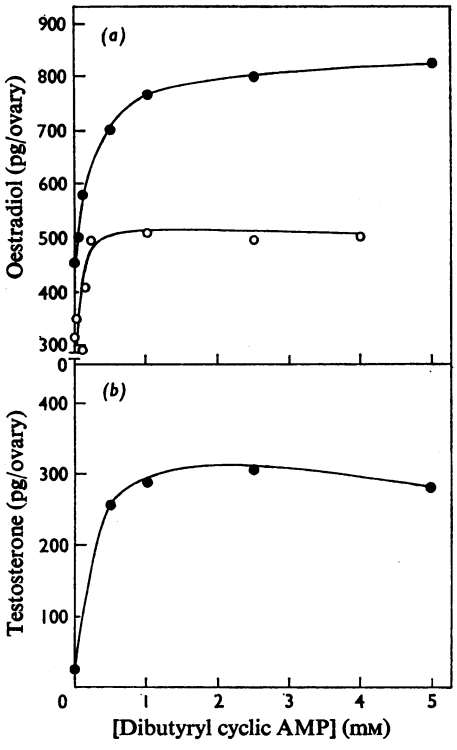

Fig. 4. *Effect of dibutyryl cyclic AMP concentration on steroid-hormone production in the 15-day embryonic-chick left ovary*

Five ovaries from the left side of the female embryo at 15 days of gestation were cultured as described in the Materials and Methods section in the presence of various concentrations of dibutyryl cyclic AMP. At the end of incubation, duplicate samples of culture medium were removed from each culture dish and assayed for the presence of (*a*) oestradiol and (*b*) testosterone. ●, 0.5mM-3-Isobutyl-1-methylxanthine in each culture dish; ○, no 3-isobutyl-1-methylxanthine. Each point represents the mean value from duplicate culture dishes.

Effect of exogenous cyclic AMP on steroid synthesis by the embryonic ovary

After the addition of exogenous dibutyryl cyclic AMP to the culture medium of the 15-day embryonic ovaries, an increase in oestradiol production was observed (Fig. 4*a*). A maximum stimulation was obtained by adding 0.5mM-dibutyryl cyclic AMP; this increase is close to that produced by a maximally stimulatory amount of choriogonadotropin (20i.u./ml). When the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was also included in the culture medium at different concentrations of the cyclic nucleotides, oestradiol production increased by about 50%. Exogenous dibutyryl cyclic AMP also stimulated testosterone production in the 15-day embryonic ovary. The maximum stimulatory dosage was in the range 0.5–1.0mM (Fig. 4*b*). The production of steroids remained constant when the higher doses of exogenous cyclic AMP were added.

Time-course experiments indicated a similarity in the patterns of oestradiol and testosterone production as caused by choriogonadotropin and dibutyryl cyclic AMP. However, the latter had a greater effect (Fig. 5*a*). 3-Isobutyl-1-methylxanthine can cause the same amount of increase in oestradiol production as choriogonadotropin does within 1h of incubation; at 4h of incubation, 3-isobutyl-1-methylxanthine caused an increase in steroid-hormone production which was lower than the increase by choriogonadotropin, but still twice as much as the control. 3-Isobutyl-1-methylxanthine had no effect on testosterone production within 1h of incubation; after 3h of incubation however, it shows the stimulatory effect. In the presence of 20i.u. of choriogonadotropin or dibutyryl cyclic AMP (plus 3-isobutyl-1-methylxanthine) at 3h of incubation, production of testosterone was stimulated by about 380–480% above that observed when only 3-isobutyl-1-methylxanthine was added (Fig. 5*b*).

Effect of choriogonadotropin on ovarian cyclic AMP production

The results with exogenous dibutyryl cyclic AMP suggest that this nucleotide was a mediator of the action of choriogonadotropin on steroidogenesis in the embryonic ovaries. But such a hypothesis rests on the assumption that the addition of choriogonadotropin would bring about an increase in the endogenous concentration of cyclic AMP in the embryonic ovarian tissue. Table 4 shows that cyclic AMP was present in the tissue of the 15-day embryonic ovary. When the tissue was cultured in the presence of 3-isobutyl-1-methylxanthine, an increase of approx. 46% in cyclic AMP was observed; in the absence of phosphodiesterase inhibitor there was no detectable increase in cyclic AMP, but when both choriogonadotropin and 3-isobutyl-1-methylxanthine were present a threefold increase in cyclic AMP was obtained, both per mg of tissue and per mg of protein.

A number of other hormones were tested for their effects *in vitro* on the accumulation of cyclic AMP in the 15-day embryonic ovaries. Table 5 shows that sheep lutenizing hormone caused a marked increase in cyclic AMP accumulation of about 18-fold above that of the control. Thyrotropin, growth hormone and insulin were also individually tested on the embryonic ovary, but no stimulatory effect was observed. Heat-denatured choriogonadotropin totally lost its ability to stimulate cyclic AMP accumulation.

Fig. 6 shows that graded doses of choriogonadotropin added to the culture medium of ovarian tissue

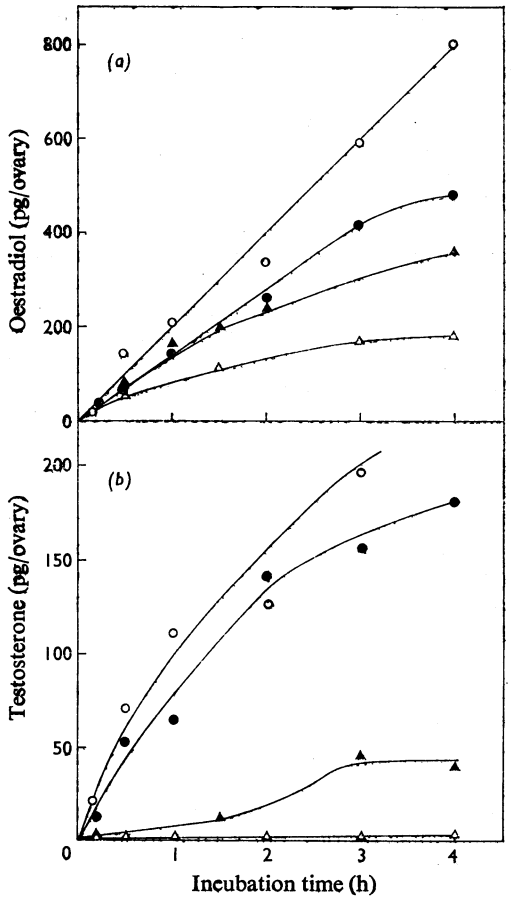

Fig. 5. *Time-course for steroid-hormone production in response to choriogonadotropin, 3-isobutyl-1-methylxanthine and dibutyryl cyclic AMP*

Five ovaries per culture dish were obtained from the left side of 15-day embryonic female chicks. Ovaries were incubated as described in the Materials and Methods section for various lengths of time. Triplicate cultures were used for each time-point. At the end of incubation, duplicate samples of culture medium were removed and assayed for the presence of (*a*) oestradiol and (*b*) testosterone. △, Control (no addition); ●, 20i.u. of choriogonadotropin; ▲, 0.5mM-3-isobutyl-1-methylxanthine; ○, 0.5mM-3-isobutyl-1-methylxanthine and 1mM-dibutyryl cyclic AMP.

for 30min resulted in a gradual increase in the amount of cyclic AMP. Doses of choriogonadotropin ranging from 1 to 10i.u./ml did not increase the cyclic AMP in the 15-day embryonic ovary. However, when the doses were increased from 10 to 25i.u./ml, the cyclic AMP production increased linearly, and then reached a plateau when the doses were further increased from 35 to 70i.u./ml. Therefore the choriogonadotropin dose range of 35–50i.u./ml was used throughout these experiments.

When the 15-day-old chick ovaries were incubated with 50i.u. of choriogonadotropin/ml, within 15min the presence of choriogonadotropin led to an increase of cyclic AMP in the tissue from 1.30 to 1.60pmol/mg of tissue, an increase of 20% over control tissue. When the culture of the embryonic organs was continued for 30–60min, the amount of cyclic AMP in the choriogonadotropin-stimulated tissue reached a maximal value of 2.5pmol/mg, an increase of 100% (Fig. 7*a*). In the absence of choriogonadotropin, the cyclic AMP accumulated in the tissue, but no detectable cyclic AMP was released into the medium. However, 5min after the addition of choriogonadotropin, a portion of the newly synthesized cyclic AMP (approx. 15–20% of the total cyclic AMP produced) was released to the medium. After 120min of incubation, about 36% of the total cyclic AMP was detected in the medium (Fig. 7*b*). After 3h of incubation, the concentration of cyclic AMP in both the tissue and culture medium began to decrease.

Effect of choriogonadotropin on cyclic AMP accumulation during the development of embryonic ovaries

In the presence of 0.5mM-3-isobutyl-1-methylxanthine in the culture medium, both left and right embryonic ovaries, at all stages of development, responded to choriogonadotropin stimulation with an increase in cyclic AMP accumulation (Table 6). This result would be expected if cyclic AMP mediates the action of gonadotropin on steroidogenesis. We showed above (Table 3) that choriogonadotropin does stimulate steroid-hormone production at all stages of development, even in the involuting right ovary.

During the course of development, the capacity of both ovaries for cyclic AMP synthesis could be expressed in the following ways. (1) In terms of pmol/mg of tissue, the left ovary is 1.2, 1.3, 1.3 and 2.1 at days 10, 12, 15 and 18 of incubation. The right ovary ranges from 0.8 to 1.5 at days 10 and 12 of incubation. After the stimulation by choriogonadotropin, the content of cyclic AMP in the left ovary increased about 190–250%, whereas in the right ovary it increased about 350–520%. The capacity of the right ovary for responding to choriogonadotropin is 84–108% higher than the left one. (2) In terms of pmol/ovary, the left ovary produced 2, 3.5, 5.5 and 12 at days 10, 12, 15 and 18 of incubation. The right ovary produced 1.0 and 1.5 for days 10 and 12 of incubation. The addition of choriogonadotropin caused a 170–250% increase in cyclic AMP in the left ovary and a 270–350% increase in the right ovary.

Both methods of calculation show that overall, the right ovary has a 40–60% higher capacity for responding to choriogonadotropin stimulation and producing cyclic AMP than the left one.

Table 4. *Effect of choriogonadotropin and 3-isobutyl-1-methylxanthine in vitro on cyclic AMP concentration in the 15-day embryonic-chick left ovary*

Pooled ovaries (approx. 20mg of tissue) were cultured under the standard vulture conditions described in the Materials and Methods section. Cyclic AMP was determined immediately after the incubation. The incubation was terminated by the addition of trichloroacetic acid directly to the culture. The assay of cyclic AMP concentration in the trichloroacetic acid extract of the tissue was described in the Materials and Methods section. Values are expressed as means ± S.D. of triplicates from three experiments.

Additions	Cyclic AMP (pmol/mg of tissue)	Cyclic AMP (pmol/mg of protein)
None	0.903 ± 0.10	11.2 ± 1.0
Chorlogonadotropin (35i.u./ml)	1.070 ± 0.05	12.0 ± 1.2
3-Isobutyl-1-methylxanthine (0.5mM)	1.330 ± 0.15	15.8 ± 3.0
Choriogonadotropin (35i.u./ml)+ 3-isobutyl-1-methylxanthine (0.5mM)	3.500 ± 0.26	28.6 ± 4.0

Table 5. *Effect of various hormones in vitro on cyclic AMP accumulation in the 15-day embryonic-chick left ovary*

Five embryonic ovaries were incubated as described in the Materials and Methods section, except that 0.5mM-3-isobutyl-1-methylxanthine was present in each incubation. Choriogonadotropin was heat-denatured by heating it in boiling water for 30min before use. Values are expressed as means ± S.D. of triplicate samples from three experiments.

Hormone	Cyclic AMP (pmol/mg of tissue)
None	1.19 ± 0.07
Choriogonadotropin (50i.u./ml)	4.70 ± 0.07
Heat-denatured choriogonadotropin (50i.u./ml)	1.03 ± 0.50
Thyrotropin (0.5i.u./ml)	1.03 ± 0.44
Growth hormone (10μg/ml)	1.30 ± 0.30
Insulin (10μg/ml)	1.01 ± 0.24
Sheep luteinizing hormone (10μg/ml)	18.20 ± 0.50

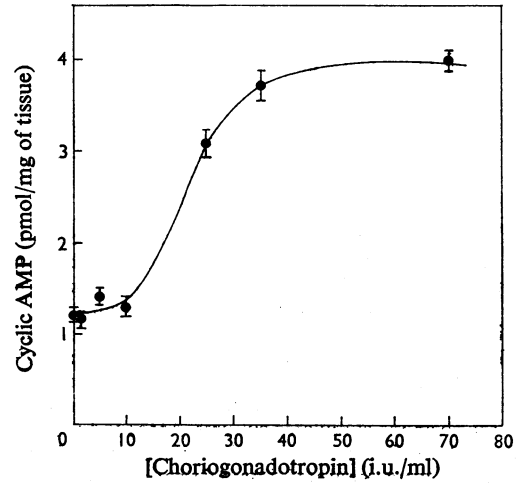

Fig. 6. *Effect of choriogonadotropin concentration on cyclic AMP in the 15-day embryonic left ovary*

Five ovaries were cultured under the standard conditions described in the Materials and Methods section, in the presence of various concentrations of choriogonadotropin plus 0.5mM-3-isobutyl-1-methylxanthine for 30min. After incubation, concentrated trichloroacetic acid was added to the medium to give a final concentration of 5% (w/v). The ovarian tissues were then homogenized in a Teflon/glass homogenizer. After centrifugation at 900*g* for 10min, the cyclic AMP present in the supernatant was assayed by the protein-binding assay. Each value represents the mean ± S.D. of three determinations.

Discussion

The radioimmunoassay technique provided us with a sensitive way to measure quantitatively the steroid hormones released from the embryonic ovary *in vitro*. This assay technique has been proved to be suitable for the study of minute quantities of embryonic tissue in the organ-culture condition. Our results are consistent with previous observations that steroid production is probably confined to the steroidogenic target tissue, such as adrenal (Haynes *et al.*, 1954), testis (Dufau *et al.*, 1971) and ovary (Dorrington & Kilpatrick, 1967). The capacity of the right ovary to secrete the steroid hormone is not affected by regression. This observation is similar to that reported by Wolff & Haffen (1952) and Weniger (1961), who demonstrated the phenomenon of feminization of male gonads when cultured together with the female right ovary. They concluded that the feminization is a result of oestrogen secretion from the right ovary.

Our study demonstrated that the embryonic right ovary responded to choriogonadotropin with a greater percentage of testosterone production than the left one. The percentage of stimulation by choriogonadotropin of oestrogen production re-

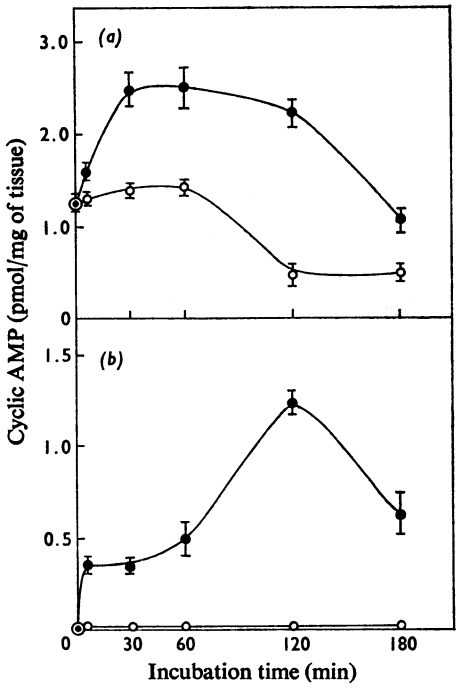

Fig. 7. *Effect of incubation time on cyclic AMP accumulation in the left ovary of the 15-day embryonic chick and the release of cyclic AMP into the culture medium during stimulation by choriogonadotropin in vitro*

Five ovaries were cultured as described in the Materials and Methods section in the presence of 0.5mM-3-isobutyl-1-methylxanthine in all culture dishes; 50i.u. of choriogonadotropin was present in the hormone-stimulated cultures. At the end of each incubation time-period, ovarian tissue and culture medium were separated immediately. Cyclic AMP was extracted by the addition of cold trichloroacetic acid to a final concentration of 5% (w/v). Cyclic AMP present in the trichloroacetic acid extract was purified and assayed immediately. Each point represents the mean ± S.D. of quadruplicate determinations from two experiments. ●, Choriogonadotropin; ○, control.

mained constant, however, in the developing left and right ovaries. A similar observation was made by Woods & Podczaski (1974), who found a greater amount of androgenic hormone in the presumptive right ovary than in the left one of a 5½-day embryo by using an immunofluorescent detection method. This relatively higher degree of stimulation of testosterone production after choriogonadotropin treatment in the right ovary (particularly in the later developmental stage at 15 days) reflects a basic difference in structure between the right and left ovaries. Previous observations by Swift (1915), Meyer (1964) and van Limborgh (1968) support this view. They found that from day 3 of incubation the distribution of primordial germ cells was greater in the left ovary of the chick embryo than in the right ovary. During days 9 and 11 of incubation, in the left gonad the germinal epithelium proliferates to form the cortex and the sex cords, but it fails to develop into a functional cortex in the right gonad. The right ovary is therefore 'ovotestis' in nature (Gaarenstroom, 1939; Hamilton, 1963).

The left ovary's production of oestrogen progressively increases, according to the age of the embryo. A similar observation has been reported by Guichard *et al.* (1973). The capacity of the early embryonic ovary to respond to choriogonadotropin indicated that the mechanisms for receiving gonadotropin were already available. The observed decline in responsiveness to choriogonadotropin stimulation for oestrogen production in the immature chick ovary parallels observations of the adult rat testis (Sandler & Hall, 1966). It has been suggested that adult tissue receiving stimulation by a high concentration of endogenous gonadotropic hormone loses its sensitivity to the exogenous gonadotropic hormone stimulation (Hall, 1970). It is possible, however, that damage to the ovarian tissue during the processes of tissue-slide preparation could lead to the lowered sensitivity to the exogenous hormone (Savard *et al.*, 1963).

In both ovaries we observed that testosterone production is significantly lower than oestrogen production in the same tissues when cultured in the absence of choriogonadotropin. After exposure to choriogonadotropin, in both ovaries the percentage increase in testosterone (five-to-ten-fold above control) is higher than that for oestrogen (2.5–3-fold above control). This phenomenon underlines the importance of gonadotropin triggering the production or release of testosterone. Previous observations demonstrated that in the early embryonic stages, development of the ovary is free from pituitary influence (Fugo, 1940; Jost, 1947; Wolff & Haffen, 1952). Gonadotropin begins to exert its influence on day 13 of incubation, when the hypothalamus–pituitary axis becomes connected (Woods & Weeks, 1969). The release of gonadotropin on day 13 could increase the production of testosterone in the ovarian tissue. This phenomenon is particularly prominent in the right ovary; a higher ratio of testosterone to oestrogen is evident in the ovarian tissue. The consequence of this change in testosterone/oestrogen ratio corresponding to the physiological condition of the embryo is yet unknown. However, the right Müllerian duct of females usually starts to regress by day 11–12 of incubation. The involution of the right Müllerian duct could be attributed to the high concentration of testosterone (Willier, 1942; Wolff, 1959; Romanoff, 1960; Burns, 1961; Groenendijk-Huijbers, 1962; Hamilton & Teng, 1965). Since the right ovary is

Table 6. *Effect of choriogonadotropin on cyclic AMP concentration in the embryonic-chick ovary during various developmental stages*

Ovarian tissue (20mg) from both sides of the femal chick embryo was obtained at various developmental stages and incubated as described in the Materials and Methods section; 0.5mM-3-isobutyl-1-methylxanthine was present in all incubations and 35i.u. of choriogonadotropin was included in hormone-treated cultures. At the end of incubation, cyclic AMP present in the tissue and medium was extrcted with 5% (w/v) trichloroacetic acid and assayed. (*a*) Incubation without the hormone; (*b*) incubation with the hormone. Results are means ± S.D. of three determinations.

Period of incubation (days)	Cyclic AMP (pmol/mg of tissue)				Cyclic AMP (pmol/ovary)			
	Left ovary		Right ovary		Left ovary		Right ova	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
10	1.20±0.05	2.35±0.07	0.84±0.10	4.40±0.60	2.1±0.18	3.4±0.15	1.0±0.15	3.5±0.16
12	1.30±0.05	3.20±0.50	1.50±0.33	5.24±0.54	3.5±0.18	8.0±0.22	1.5±0.15	4.5±0.17
15	1.28±0.07	3.42±0.30	—	—	5.5±0.20	14.2±0.21	—	—
18	2.07±0.45	4.83±0.50	—	—	12.3±2.50	24.3±2.60	—	—

ovotestis in nature, one would expect that a higher concentration of testosterone in the right ovarian tissue would stimulate the synthesis of certain factors, such as the Müllerian-duct-inhibiting factor, which has been observed in the testes of the male chick during the post-hatching period (Maraud *et al.*, 1966; Groenendijk-Huijbers & Burggraaff, 1974).

In our present study the exogenous cyclic AMP (dibutyryl cyclic AMP) was observed to promote steroid-hormone production. The concentration of dibutyryl cyclic AMP required to stimulate embryonic-chick ovarian tissue for steroid-hormone production *in vitro* is much higher, however, than the endogenous concentration of the nucleotide normally found in the choriogonadotropin-stimulated chick ovarian tissue. Similar findings were observed in bovine corpus luteum (Savard *et al.*, 1965), rabbit ovarian tissue (Dorrington & Kilpatrick, 1967) and adrenal glands (Haynes, 1958). These results could be attributed to the following possible causes. (*a*) The penetration of the exogenous nucleotides into the ovarian cells *in vitro* is inefficient (Rall & Sutherland, 1961). (*b*) The added cyclic nucleotides are destroyed by cyclic AMP phosphodiesterase already present in the embryonic ovarian tissue (Dorrington & Kilpatrick, 1967). The presence of high concentrations of phosphodiesterase in the embryonic tissue was reflected in an increase in oestradiol production when 3-isobutyl-1-methylxanthine alone was added.

In our experimental system we observed that the effect of dibutyryl cyclic AMP (in the presence of 3-isobutyl-1-methylxanthine) on steroid-hormone production is greater than that of choriogonadotropin. The smaller capacity of the embryonic chick's adenylate cyclase system to respond to choriogonadotropin may be responsible for the lowered efficiency of choriogonadotropin. Nevertheless, the observed facts indicate that cyclic AMP is the intermediary in the action of choriogonadotropin on steroidogenesis in the embryonic chick ovary: (*a*) the exogenous cyclic AMP mimicked the action of choriogonadotropin, (*b*) the phosphodiesterase inhibitor alone was able to stimulate steroid production in the embryonic ovaries, and (*c*) choriogonadotropin stimulated the increase in endogenous cyclic AMP in the embryonic ovarian tissue, and the increase preceded the increase of steroid synthesis. The intricacies of the action of cyclic AMP on steroidogenesis are still unclear.

We have observed that at low concentrations of choriogonadotropin (5–10i.u./ml), the increase in steroid-hormone production can occur without a detectable increase in cyclic AMP. A similar finding was reported in the rat testis (Dufau *et al.*, 1973) and the bovine adrenal gland (Carchman *et al.*, 1971; Beall & Sayers, 1972). These observations lead one to question whether the increase in cyclic AMP is the obligatory step for steroidogenesis, or whether the current assay method for cyclic AMP is too insensitive to detect the minute increase (Carchman *et al.*, 1971; Dufau *et al.*, 1972; Beall & Sayers, 1972).

During the course of sex-organ differentiation, the embryonic right ovary responds to choriogonadotropin stimulation with a higher percentage of cyclic AMP accumulation than the left ovary. Whether the increase in the right ovary is only related to steroid production or to some other physiological role pertinent to the cell death is unclear. An observation related to this finding has been reported in the mouse lymphoma cell (Coffino *et al.*, 1975), in which the promotion of cell death by cyclic AMP was evident. The phenomenon of cell death and its subsequent cause of organ regression is a unique part in the normal course of organogenesis. Our present observations might indicate that cyclic AMP plays an important role in the induction of organ regression.

In conclusion, during the development of embryonic chick ovaries both the growing left ovary and

regressing right ovary respond to choriogonadotropin stimulation with an increase in steroid production and an accumulation of cyclic AMP, indicating that the adenylate cyclase system is present and functional as early as 8 days of gestation. The stimulatory effect of the gonadotropins on gonadal steroidogenesis in the embryonic chick appears to be mediated through cyclic AMP.

We thank Dr. A. R. Means and Dr. A. M. Novotny for assistance with the cyclic AMP assay, Dr. S. McCormack for assistance with radioimmunoassay of oestradiol and testosterone, Mr. D. Miller for providing the protein kinase. Mr. G. Andrews and Mrs. Celine Yu for technical assistance. This study was supported by NIH grant HD-08218 and HD-09467.

References

Auletta, F. J., Caldwell, B. V. & Hamilton, G. L. (1974) in *Methods of Hormone Radioimmunoassay* (Jaffe, B. M. & Behrman, H. R., eds.), pp. 347–370, Academic Press, New York

Beall, R. J. & Sayers, G. (1972) *Arch. Biochem. Biophys.* **148**, 70–83

Brode, M. J. (1928) *J. Morphol.* **46**, 1–57

Burns, R. K. (1961) in *Sex and Internal Secretion* (Young, W. C., ed.), pp. 76–158, Williams and Wilkins, Baltimore

Carchman, R. A., Jaanus, S. D. & Rubin, R. P. (1971) *Mol. Pharmacol.* **7**, 491–499

Coffino, P., Bourne, H. R. & Tomkins, G. M. (1975) *Am. J. Pathol.* **81**, 199–204

Connell, G. M., Connell, C. J. & Eik-Nes, K. B. (1966) *Gen. Comp. Endocrinol.* **7**, 158–165

Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065

Dorrington, J. H. & Kilpatrick, R. (1966) *J. Endocrinol.* **35**, 53–63

Dorrington, J. H. & Kilpatrick, R. (1967) *Biochem. J.* **104**, 725–730

Dufau, M. L., Catt, K. J. & Tsuruhara, T. (1971) *Biochim. Biophys. Acta* **252**, 574–579

Dufau, M. L., Tsuruhara, T., Watanabe, K. & Catt, K. J. (1972) *Excerpta Med. (Int. Congr. Endocrinol.)* **256**, 199–200

Dufau, M. L., Watanabe, K. & Catt, K. J. (1973) *Endocrinology* **92**, 6–11

Fugo, N. W. (1940) *J. Exp. Zool.* **85**, 271–297

Gaarenstroom, J. H. (1939) *J. Exp. Zool.* **82**, 31–46

Galli, F. & Wasserman, G. F. (1973) *Gen. Comp. Endocrinol.* **21**, 77–83

Giles, K. W. & Myers, A. (1965) *Nature (London)* **206**, 93

Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305–312

Groenendijk-Huijbers, M. M. (1962) *Anat. Rec.* **142**, 9–20

Groenendijk-Huijbers, M. M. & Burggraaff, J. M. (1974) *Anat. Anz.* **135**, 43–46

Guichard, A., Cedard, L. & Haffen, K. (1973) *Gen. Comp. Endocrinol.* **20**, 16–28

Haffen, K. (1975) *Am. Zool.* **15**, 257–272

Hall, P. F. (1970) in *The Testis* (Johnson, A. D., Gomes, W. R. & Vandemark, N. L., eds.), vol. 2, pp. 1–57, Academic Press, New York

Hall, P. F. & Eik-Nes, K. B. (1962) *Biochim. Biophys. Acta* **63**, 411–422

Hamburger, V. & Hamilton, H. L. (1951) *J. Morphol.* **88**, 49–58

Hamilton, T. H. (1963) *Proc. Inst. Ornithol. Congr. 13th*, 1004–1040

Hamilton, T. H. & Teng, C. S. (1965) in *Organogenesis* (DeHann, R. L. & Ursprun, H., eds.), pp. 681–700, Holt, Rinehart and Winston, New York

Hanks, J. H. & Wallace, R. E. (1949) *Proc. Soc. Exp. Biol. Med.* **71**, 196–200

Haynes, R. C. (1958) *J. Biol. Chem.* **233**, 1220–1222

Haynes, R. C., Savard, K. & Dorfman, R. I. (1954) *J. Biol. Chem.* **207**, 925–938

Haynes, R. C., Sutherland, E. W. & Rall, T. W. (1960) *Recent Prog. Horm. Res.* **16**, 121–138

Jost, A. (1947) *C. R. Hebd. Séances Acad. Sci. Ser. D* **225**, 322–324

Jost, A. (1970) *Adv. Exp. Med. Biol.* **10**, 11–18

Krishna, G., Weiss, B. & Brodie, B. (1968) *J. Pharmacol.* **163**, 379–385

Leech, P. N. (1939) *J. Am. Med. Assoc.* **113**, 2418–2419

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Maraud, R., Stoll, R. & Couland, H. (1966) *C. R. Séances Soc. Biol.* **160**, 964–966

Marsh, J. M. & Savard, K. (1964*a*) *J. Biol. Chem.* **239**, 1–7

Marsh, J. M. & Savard, K. (1964*b*) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **23**, 462

Means, A. R., MacDougall, E., Soderling, T. R. & Corbin, J. D. (1974) *J. Biol. Chem.* **249**, 1231–1238

Meyer, D. B. (1964) *Dev. Biol.* **10**, 154–190

Munro, H. M. & Fleck, A. (1966) *Methods Biochem. Anal.* **14**, 113–176

Rall, T. W. & Sutherland, D. W. (1961) *Cold Spring Harbor Symp. Quant. Biol.* **26**, 347–354

Romanoff, A. L. (1960) in *The Avian Embryo* (Romanoff, A. L., ed.), pp. 781–862, Macmillan, New York

Sandler, R. & Hall, P. F. (1966) *Endocrinology* **79**, 647–649

Savard, K., Marsh, J. M. & Howell, P. S. (1963) *Endocrinology* **73**, 554–563

Savard, K., March, J. M. & Rice, B. F. (1965) *Recent Prog. Horm. Res.* **21**, 285–365

Swift, C. H. (1915) *Am. J. Anat.* **18**, 441–470

Teng, C. S. & Teng, C. T. (1975*a*) *Biochem. J.* **150**, 183–190

Teng, C. S. & Teng, C. T. (1975*b*) *Biochem. J.* **150**, 191–194

Teng, C. T. & Teng, C. S. (1975*c*) *J. Cell Biol.* **67**, 430A

Teng, C. S. & Teng, C. T. (1976) *Biochem. J.* **154**, 1–9

van Limborgh, J. (1968) *Arch. Anat. Microsc. Morphol. Exp.* **57**, 79–90

Weniger, J. P. (1961) *Arch. Anat. Microsc. Morphol. Exp.* **50**, 269–288

Weniger, J. P. & Zeis, A. (1971) *Gen. Comp. Endocrinol.* **16**, 391–395

Willier, B. H. (1942) *Cold Spring Harbor Symp. Quant. Biol.* **10**, 135–144

Wolff, E. (1959) in *Comparative Endocrinology* (Gorbman, A., ed.), pp. 568–581, Wiley, New York

Wolff, E. & Haffen, K. (1952) *C. R. Séances Soc. Biol.* **146**, 1772–1774

Woods, J. E. & Weeks, R. L. (1969) *Gen. Comp. Endocrinol.* **13**, 242–254

Woods, J. E. & Podczaski, E. S. (1974) *Gen. Comp. Endocrinol.* **24**, 413–423