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A Histochemical Study of Steroid Hormone Synthesis in the Gonads of the Brown Leghorn Chick Embryo

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A HISTOCHEMICAL STUDY OF STEROID HORMONE SYNTHESIS IN THE GONADS OF THE BROWN LEGHORN CHICK EMBRYO

by

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BIOGRAPHY

David Glenn Rabuck was born on December 30, 1940 in Geneva, Illinois.

He was graduated from Evanston Township High School, Evanston, Illinois, in June 1958. He attended North Central College, Naperville, Illinois, and was awarded the Bachelor of Arts degree in June, 1963.

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INTRODUCTION

It is well known that, in at least some of the vertebrates, sex hormones are elaborated by the gonads during the embryonic period. However, the exact time in development at which these hormones are first produced is not known with certainty. Histochemical and biochemical methods should reveal their presence earlier in development than bioassay techniques. However, this is not the case with regard to the chick embryo gonad.

In the chick embryo *in vivo* transplantation experiments have indicated the initial synthesis and release of sex hormones by the gonads at eight days (Weniger, 1965). On the other hand, biochemical techniques have not disclosed the presence of estrogens in embryonic chick gonad extracts until the tenth day of incubation (Gallien and Le Foulgoc, 1957).

Histochemical techniques have not revealed steroid hormone synthesis prior to the eighth day of incubation in chick embryos. The presence of histochemically demonstrable cholesterol has been reported at eight days in the gonads of both sexes (Chieffi *et al.*, 1964), and at eight days in the ovary and ten days in the testis (Narbaitz and Sabatini, 1963). Δ^5-3β-Hydroxysteroid dehydrogenase (Δ^5-3β-HSD) activity has been reported in the embryonic gonads of both sexes from eight days onward by Chieffi (1964), while Narbaitz and Kolodny (1964) first observed the presence of this enzyme at eight days in the ovary and at ten days in the testis.

The absence of histochemically demonstrable Δ^5-3β-HSD activity in the gonads of the chick embryo before eight days of incubation may possibly be

ascribed to the fact that this method is a threshold reaction and the enzyme may be present before this time but not available in sufficient quantities for reduction of the tetrazolium salt. Also, other investigators may not have cut their sections thick enough, or incubated them long enough in the substrate medium.

A recently developed immunofluorescent technique for the histological visualization of androgenic steroids (Woods and Domm, 1966) is probably, like many immunological procedures, a sensitive and specific indication for this one class of steroids and the exclusion of others.

The Δ^5-3β-HSD technique, demonstrating as it does an essential step in the synthesis of steroid hormones, generally can provide a broader spectrum of information concerning steroid hormone synthesis than the immunofluorescent method. It is recognized, however, that the presence of a single enzyme in a bio-synthetic pathway does not necessarily insure successful synthesis nor release of formed hormone (Lobel et al., 1962). Moreover, this technique does not provide information as to the character of the elaborated steroid hormone.

Since the time of onset of sex hormone synthesis in the gonad of the chick embryo, as demonstrated histochemically, does not precede in time the initial secretion of formed hormone as determined by bioassay, a re-examination of the histochemical aspects of this problem would seem to be indicated. In the present study, chick embryo gonad sections were examined for the presence of Δ^5-3β-HSD in order to determine the period during embryonic development when steroid hormone synthesis first occurs in these organs.

REVIEW OF THE RELATED LITERATURE

A. Early Observations and Theories

The first recorded hypothesis attributing sex differentiation to hormones emanating from the embryonic gonads was that of Bouin and Ancel (1903). These investigators observed interstitial cells of Leydig in a secretory state in the testis of the 30 mm pig embryo.

The first evidence in support of this hypothesis was reported by Lillie (1916, 1917) and Keller and Tandler (1916) with regard to the phenomenon of freemartinism in cattle. They observed that the embryonic membranes of twins occasionally were so closely associated that anastomoses of membranes and blood vessels occurred, thus permitting a mixing of their blood. If the twins were a heterosexual pair, the male developed normally while the reproductive system of the female was masculinised or appeared to be an intersex. The normal development of the ovarian cortex and of the Mullerian ducts were inhibited and development and differentiation of the Wolffian ducts were facilitated in the female. In an attempt to explain this condition these investigators postulated that in such cases the male hormone was produced in the embryo prior to that of the female hormone and thus exerted its influence earlier in developmental time. The phenomenon did not occur in the absence of vascular anastomoses of embryonic membranes.

A similar phenomenon occurs, but to a lesser extent, in the pig (Hughes, 1929). Petskoi (1953, 1955) also noted the freemartin phenomenon in cattle,

but did not observe it in sheep or goats, apparently due to a comparatively late establishment of vascular anastomosis of embryonic membranes in the latter.

Lillie (1917) stated that it would be necessary to prove his theory experimentally before it could be accepted.

B. Experimental Verification and Testing of Theories

Since the present investigation has been limited to the bird, the following literature review is also so limited.

Proof of the presence of embryonic sex hormone synthesis is basic to the establishment of the hormonal theory of sex differentiation. Several approaches have been employed by a host of investigators in an effort to establish the production of these hormones during the embryonic period.

1. Transplantation

Gonads or pieces of gonads were grafted onto the chorio-allantoic membrane of chick embryos (Minoura, 1921; Greenwood, 1925; Kemp, 1927; Willier, 1927; Bradley, 1941). Willier (1927) grafted undifferentiated, as well as differentiated, gonads onto the chorio-allantoic membrane. Minoura was the only investigator to report that the grafts exerted a hormonal effect. He reported that like gonads stimulated one another's growth and differentiation, while unlike gonads inhibited each other.

Bradley, (1941), noticed occasional feminization of testes under the influence of an ovary grafted onto the chorio-allantoic membrane of host embryos. Minoura's and Bradley's results, therefore, contributed evidence

for the theory that sex hormones are responsible for sex differentiation, while the work of Greenwood, of Kemp and of Willier does not support this theory.

Wolff (1947) transplanted gonads from 6-11 day embryos into the coelom of 48-52 hour embryos. He obtained feminization of host testes (development of a cortex) by transplanted ovaries and inhibition of development of the Mullerian ducts of female hosts by transplanted testes.

Groenendijk-Huijbers (1960) transplanted embryonic chick gonads from 3-6 day donors into the coelom of 4-8 day hosts. Since no data was gathered before the eleventh day of incubation of any host, the results indicate that with the youngest grafts hormone production took place at an undetermined time before the twelfth day of incubation. This investigator also made chorio-allantoic transplants; however, only an incomplete reduction of Mullerian ducts was obtained. This condition was considered to be due to the fact that it was impossible to transplant enough testis onto the chorio-allantoic membrane to cause complete reduction of this duct system.

2. Tissue Culture and Castration

The results of the in vitro culture of gonads side by side, and of gonads and target organs demonstrate strictly traditional feminization by ovarian and masculinization by testicular secretions. These results are similar to those obtained from castration experiments. Whether the action of sex hormones on target organs is interfered with by interrupting their secretion through castration (Wolff and Wolff, 1949, 1951) or by removing the target organs and cultivating them in vitro the results are the same. Target

organs removed before sex differentiation and cultured alone, as well as those present in castrated embryos, evolved in the same way regardless of the genetic sex of the embryo (Wolff and Wolff, 1951-53). These neutral forms were the male form of the syrinx and genital tubercle of the duck embryo and the female form of the Mullerian ducts of the chick embryo. When the duck syrinx and genital tubercle were cultivated with estrogens and the chick Mullerian duct with androgens, the influence of these hormones caused development in the direction of the sex of the culture hormone (Wolff et al., 1952; Luts-Ostertag, 1954).

Experiments were also performed associating gonads heterosexually and associating gonads of the chick embryo with Mullerian ducts (Wolff and Haffen, 1952; Wenniger, 1962). When gonads were associated heterosexually, the testes were feminized, while Mullerian ducts regressed when associated with testes.

Later Wenniger (1964, 1965a, b) cultured 7-12 day old chick embryo ovaries in vitro and afterward tested the medium for estrogens by the Allen-Doisy method. Estrogens were first detected in a medium in which seven day old ovaries had been cultured for 24 hours, i.e., on the eighth day of incubation.

3. Administration of Hormones

The results of this type of research are rather complex and somewhat contradictory. They will be categorized here according to class of hormone administered.

Utilizing estrogens, results were obtained that are similar to those of

ovarian influence in transplant and tissue culture experiments. Although there are some differences, the similarity is quite evident.

In general, estrogens when administered in early embryonic life feminized the testes by bringing about the differentiation of a cortex, and in extreme cases, by changing the morphological characteristics of the testis so that it was indistinguishable from an ovary. In females, the right Mullerian duct, which normally undergoes involution, is maintained and both right and left duct may hypertrophy depending on the dosage administered (Dantchakoff; Willier, Gallagher and Koch; Wolff and Ginglinger, 1935). Normal differentiation of the ovaries was not affected or changed. Benoit (1923), Domm (1927), Taber, (1954) and Kornfeld (1958), stated that estrogens seem to be responsible for the inhibition of growth and differentiation of the right ovary. Wolff and Wolff (1951), on the other hand, reported that the germinal epithelium was maintained and differentiated into an ovarian cortex on the right side when they injected estrogens into the embryo before the fourth day. Lewis (1946) and Lewis and Domm (1948) treated duck embryos of 4-10 days incubation with estrogens and obtained a modification of the syrinx and genital tubercle in the female direction, indicating that the normal female character is determined by estrogens. Since these results are similar to those observed in normal development, they appear to indicate that the embryonic gonads exert an influence on sex differentiation as a result of the synthesis and release of sex hormones.

The results of experiments in which androgens were administered are less clear-cut and not easily explained. Testosterone and androsterone were observed to stimulate the Wolffian ducts of both sexes, (Dantchakoff; Willier,

Gallagher and Koch; Wolff and Ginglinger, 1935) as well as the Mullerian ducts in females. Furthermore, androsterone was observed to cause the incipient cortex of the left testis to differentiate into a genuine cortex, thus bringing about the development of a typical ovotestis.

Although the results are not too well defined, there are indications that the effects of administering hormones resemble those of the association of gonads in vitro and in vivo, particularly with reference to estrogens (Koszelka and Gallagher, 1934; Dantchakoff, 1935; Wolff and Ginglinger, 1935; Willier et al., 1937; Lewis, 1946). The fact that the results do not correspond completely may indicate that the hormonal effects seen in vitro and in vivo are the results of an interaction of androgens and estrogens on the accessory sex characters. The purity of the administered hormones may also have been a critical factor.

4. Biochemical Findings

Leroy (1948) extracted a substance from chick embryos of 14-19 days incubation which was active on the comb of the capon, indicating the presence of androgens. Using biochemical methods, Stoll and Meraud (1956) analyzed the allantoic and amniotic fluids of chick embryos for 17-ketosteroids. Traces of these steroid metabolism end-products were first observed at six days, the amount increasing from seven to 13 days. Gallien and Le Foulgoc (1957) used colorimetry and fluorimetry to detect and measure phenolic steroids extracted from ovaries of 10, 13 and 21 day old chick embryos. These steroids were observed to increase in amount with increasing age.

5. Histochemical Observations

The first histochemical investigation concerning hormone production by the embryonic gonad involved the identification of lipids, and cholesterol, the primary precursor of all steroids.

Initially Scheib (1958a, b) investigated total lipid content in 8, 12, 14, 18 and 20 day old chick embryos using Sudan B. Lipids were visualized in the gonads at all ages examined and were confined largely to the medullary component of both gonads. This investigator later (Scheib, 1959) examined the gonads of embryos from seven days of incubation to hatching by means of Sudan staining. He noted that lipids first appeared in the medullary cords of the testis on day seven, and in the medullary component of the ovary, "at the time of morphological sex differentiation."

Narbaitz and Sabatini (1963a) used the Sudan Black reaction for lipids and the digitonin reaction for cholesterol on the gonads of chick embryos of 7-12 days of incubation. In the ovary both substances were seen on the eighth day of incubation, while in the testis lipids were observed on day eight, but cholesterol was not seen until the tenth day.

Narbaitz and Sabatini (1963b) removed gonads from chick embryos at six and seven days and cultured them <u>in vitro</u> for four days. They were then examined by means of the digitonin technique for cholesterol. The results differed somewhat depending on which of two culture media were employed. Cholesterol specific precipitation was not seen on the sixth day in any of the gonads cultured in Wolff and Haffen's (1952) standard medium, but it was seen in 59% of the cases cultured beginning on the seventh day. On the other hand,

a specific precipitation was seen in 50% of the cases cultured in Stenger-Haffen's No. 46 synthetic medium, (Stenger and Haffen 1957) beginning with the sixth day, and in 83% of the cases cultured beginning with the seventh day. The positive reaction was generally distributed throughout the testis, but in the ovary it was limited to the medullary component. These investigators were not able to determine the sex of the gonads in all instances, but they attributed the lack of complete consistency of their results to a finding previously described by Narbaitz and Sabatini (1963a), namely, that the ovary shows the presence of cholesterol two days earlier than the testis. Thus it was shown that, depending on the medium used for culture, the presence of cholesterol can be demonstrated in six or seven day old embryonic chick gonads which have been cultured *in vitro* for four days. Chieffi *et al.*, (1964) also used the Sudan B technique for lipids. He tested gonads of chick embryos of 4 to 7, 7½, 8, 10, 12, 15, 17 and 21 days of age and observed positive reactions beginning with the eighth day of incubation.

Narbaitz and Kolodny (1964), and Chieffi *et al.*, (1964) examined gonads of chick embryos for the presence of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD). This enzyme catalyzes the conversion of Δ^5-3β-hydroxy steroids to Δ^4-3-ketosteroids (Samuels *et al.*, 1951). Since the active form of all steroid hormones is the Δ^4-3-keto form, or is derived from a precursor of this form, it is apparent that Δ^5-3β-HSD is an essential enzyme in the biosynthetic pathway of steroid hormones. Therefore, it is present in all tissues where steroid hormone synthesis takes place. Narbaitz and Kolodny reported that the enzyme was seen in the ovary at eight days of incubation and in the testis beginning at ten days thus confirming previous reports

concerning the presence of lipids and cholesterol (Narbaitz and Sabatini, 1963a, b). Chieffi et al., (1964), however, reported its presence in both ovary and testis beginning at eight days of incubation.

a. Techniques Employed

The technique for histologically visualizing the presence of Δ^5-3β-HSD activity employed in this investigation was that of Wattenberg (1958) as modified by Levy et al., (1959). He sectioned frozen tissues, then incubated them in a medium containing steroid substrate (dehydroepiandrosterone, pregnenolone and others), nicotine adenine dinucleotide (NAD), buffer (pH 8.0), a tetrazolium salt (Nitro-BT) and solvent (acetone). The hydrogen ions removed from the substrate by the dehydrogenase were transferred to the NAD, then from the NADH to the tetrazolium salt which, following the reduction, changed its form and precipitated as a granular formazan, indicating cellular localization of the enzyme. Wattenberg described the presence of a positive reaction in the cells of the adrenal cortex, interstitial cells of Leydig of the testis, stromal cells of the ovary, and weakly in the cells of the liver parenchyma.

Levy et al., (1959) modified Wattenberg's method by substituting propylene glycol for acetone, maintaining the medium at a lower pH (7.1-7.4), and changing the concentrations of NAD, Nitro-BT and substrate. Studies were made on rat adrenals, gonads and the adnexa of both sexes. These investigators confirmed Wattenberg's findings, but they also observed a positive reaction in the epithelial cells of the oviduct.

A great deal of Δ^5-3β-HSD work has been done by a number of investigators (Baillie and Griffiths, 1964; Chieffi et al., 1964; Narbaitz and Kolodny, 1964;

Preal et al., 1965; Rubin et al., 1965; Baillie et al., 1965-1966; Goldman et al., 1966). Of special interest is the study of Baillie and Griffiths (1964), in which both dehydroepiandrosterone (DHA) and pregnenolone were used as substrates for the histochemical demonstration of Δ^5-3β-HSD activity in the interstitial cells of Leydig of fetal mouse testes. When pregnenolone was employed as a substrate, a positive histochemical reaction was observed four days earlier in developmental time (11 days) than when DHA was used as the substrate (15 days). Thus, it appears that either pregnenolone is a more specific substrate for Δ^5-3β-HSD, or that there are actually two enzymes involved, the one which utilizes pregnenolone being present at a younger embryonic age, at which time the enzyme which utilizes DHA as substrate is absent.

MATERIALS AND METHODS

All embryos were obtained from single comb, light-brown Leghorn eggs incubated at a temperature of 38.3 ±0.3°C. in a forced draft incubator. The age of the embryos at the time of sacrifice represents the actual time the eggs remained in the incubator. Embryos were sacrificed from day three through hatching. The ages of the earliest embryos were verified by use of the staging technique of Hamburger and Hamilton (1951). Sacrifices were performed within four hours of the specified number of days. All staging and handling of tissues used for experimental purposes was carried out as rapidly as possible, and, in fact, an effort was made to carry out this manipulation within five minutes of time of sacrifice.

Gonads were prepared in three ways: (1) The first of these was by freezing the entire embryo in an extended position on the freezing stage of a cryostat. (2) Gonads were recovered attached to the dorsal body wall. A rectangular section of the dorsal body wall, with the viscera ventral to the gonads and the skin of the dorsal surface removed to obtain better liquid contact for freezing, was frozen immediately dorsal side down on the freezing stage of a cryostat. These tissue blocks were then wrapped with multiple layers of flexible plastic wrap and stored in sealed bottles at - 20°C. for subsequent sectioning. (3) The gonads were dissected, being careful to separate them from adjacent tissues. The first gonad removed was placed on a stainless steel weighing spatula and flooded with a drop of cold isotonic saline. The second one was removed and placed in the same drop of saline.

The spatula was then placed in the cryostat, where excess moisture was removed just prior to freezing by blotting the edge of the drop of saline with absorbant paper. The gonads were transferred to the freezing stage, either by lifting them from the spatula with the corner of a piece of dry paper towel or on the points of a pair of "watch makers" forceps. In certain cases they were placed upon the top surface of the freezing stage while hanging by capillary adhesion from the spatula. In all cases after being transferred to the freezing stage the gonads were frozen within about one second's time.

Since the isolated gonads were too small to mount directly on a cryostat chuck, a method of support that would allow for rapid and easy handling of the tissue was devised. The use of large pieces of tissues was considered but discarded because of a lack of uniformity of the mount and ease of handling. Gelatin or gelatin-related materials, molded and frozen to shape directly over the gonads on the stage, were utilized. These substances possess the characteristics of both uniformity and unlimited availability. Several such materials were used for this purpose. A 5% solution of gelatin in water is recommended by workers using gelatin block methods. However, when this concentration was employed, the sections were either compressed or could not be obtained in serial order. Since the problem seemed to be one of too great a flexibility of the block, the concentration of gelatin was reduced to 2.5%. This provided somewhat more satisfactory properties when blocked, but these blocks melted too easily and were more fragile.

A commercial embedding medium made by Lab-Tek Corporation for frozen sectioning ("O.C.T.") was found to give the best results. This medium is

available in three grades: I, II and III, in decreasing order of temperatures recommended for its use. In our work both O.C.T. I (for temperatures of 0° to -15°C.) and O.C.T. II (for temperatures of -15 to -30°C.) were used and proved very satisfactory when used within the recommended temperature range. The O.C.T. I medium was somewhat more satisfactory than the O.C.T. II for three reasons: (1) the temperature range of 0° to -15°C. gave optimal cutting results with gonadal material, (2) these blocks did not melt as readily when handled, and (3) the higher temperature was more easily maintained by the refrigerating apparatus of the cryostat. To prepare the blocks, a mold made from a strip of polyethylene was placed around the gonads on the freezing stage and the embedding medium poured into the mold. A heat extractor attached to the wall of the cryostat was then lowered onto the top of the mold. After the molded block had frozen, it was broken loose from the stage, the mold stripped away and the block wrapped in multiple layers of plastic wrap. It was then labeled and stored in a sealed bottle at -20°C. for future sectioning.

Frozen blocks were initially cut on a Lipshaw freezing microtome. However, an American Optical "Cryocut" was used for the major portion of our investigation. Sections of 2 to 40 micra thickness were cut singly or, when possible, in ribbons. These sections were either lifted onto a pre-chilled slide or coverslip, which was then removed from the chamber and the section allowed to dry on the slide, or a warm slide was brought into contact with the cut tissue adhering to the knife. The tissue slice adhered to and melted on the slide. All sections were air-dried for at least fifteen minutes.

The following technique was employed to examine gonads for Δ^5-3β-HSD

activity. Slides with adherent sections were placed in a warm (37°C.) buffer solution (pH 7.2 ±0.1) for 15 to 30 minutes in order to remove endogenous substrates (Wattenberg, 1958) and to dissolve the material used as a support for the tissue while cutting. Following this, sections were incubated in the substrate medium for periods of one-half to twenty-eight hours. In the majority of cases the incubation time was one hour. Prolonged incubation periods were used for gonads from younger embryos in order to compensate for the smaller quantity of enzyme present. Dehydroepiandrosterone (DHA) was the substrate employed with most gonads examined. However, pregnenolone was used as an alternate substrate for some of the gonads. The incubation medium was prepared according to the method of Levy et al., (1959), as follows:

CONSTITUENT	AMOUNT	CONCENTRATIONS
Dehydroepiandrosterone (DHA) or Pregnenolone	0.2 mg	0.1mM
Nicotine-adenine dinucleotide (NAD)	2.4 mg	0.54mM
Nitro-BT	1.0 mg	0.16mM
Phosphate buffer (pH 7.2)	4.0 ml	0.057M
Acetone	0.5 ml	

When incubations were complete, sections were fixed for 15 to 30 minutes in a mixture consisting of 10% formalin, 5% glacial acetic acid and 85% of 70% ethyl alcohol, (F.A.A.), rinsed in tap water to remove residual fixative and stained for 5-10 minutes in Grenacher's Alum Carmine. After a second tap water rinse, tissue sections were dehydrated in a graded series of alcohols, cleared in xylene and mounted with Harleco synthetic resin.

Ovaries and testes were also embedded in paraffin and stained with Harris' Hematoxylin and Eosin for purposes of general histological identification. Histological examination and photography were carried out by means of an American Optical Microstar microscope at 40, 100, 450, and 1000 X magnifications. Photomicrographs were made using 35 mm. Kodachrome and Kodacolor film and exposure times of one fifth to one twenty-fifth of a second.

OBSERVATIONS

A total of 49 preparations of chick embryo gonads were examined. They ranged in age from three to 20 days of incubation. Eight of these were testes, 13 ovaries, and 27 of gonads prior to sex differentiation.

For the examination of chick embryos younger than ten days, cross sections of the entire embryo were found to be the most convenient form of preparation, in terms of orientation of the gonads as well as their association with other tissues in the embryo.

The times found to be optimal for fixation in F.A.A. were 15 minutes for sections up to 15 μ and 30 minutes for those thicker than 15 micra.

Sex differentiation is said to begin on the fifth or sixth day of incubation (Swift, 1916; Essenberg and Garwacki, 1938), the seventh day (Laulanie, 1886; Firket, 1914), or the eighth day (Firket, 1920). In this study it was first found to be noticeable grossly toward the end of the seventh day and microscopically at about nine days. The criteria found most useful in distinguishing the sex of the embryos examined were: (1) The presence in ovaries of a cortex which was absent or greatly reduced in testes, and a two layered medulla, the inner layer of which has many large lacunae, giving it a loose appearance, the outer layer consisting of compact tissue. After nine days the cortex shows a secondary proliferation of cords which have invaginated from the germinal epithelium. (2) The testes present a considerably more homogeneous picture. They consist entirely of a connective tissue stroma into which primary (medullary) cords have invaginated from the germinal epithe-

lium prior to the eighth day. These medullary cords become wavy by day eight, convoluted by day 11, and finally branch, anastomose, and form a reticulum by the 13th day. (3) The surface of testes is smooth and covered by a somewhat thickened tunica albuginea, which becomes increasingly prominent with advancing age, while the surface of the ovary is uneven, giving it a roughened appearance.

A. The Histochemical Method for Δ^5-3β-Hydroxysteroid Dehydrogenase

The results following the use of this method are most meaningful when divided into three periods according to incubation age: (1) the period prior to sex differentiation, (2) the period from sex differentiation to day 13 and (3) the period from day 13 to hatching. The time of sex differentiation would appear to be a significant initial point of division, since following this period the gonads are different from one another, as well as from their pre-differentiation form. Thirteen days of incubation is a significant second point of division because it divides the incubation period, from the time of sex differentiation to hatching, into two equal time intervals and also is the age at which interstitial tissue, often said to be involved in steroid hormone synthesis, is believed to appear (Swift, 1916).

B. The Use of Dehydroepiandrosterone (DHA) as the Substrate

1. The Period of Three Days to Sex Differentiation

This period was characterized by an unexpected finding. A somewhat generalized pattern of formazan granule deposition was seen throughout most of the tissues in cross-sections of three day embryos, becoming less general-

ized in distribution on the following days of incubation up through the period of sex differentiation. Tissues other than the genital ridges exhibiting formazan granules were the gut, notochord, neural tube, spleen primordium, and the general mesenchymal tissue (figs. 1 and 2). The genital ridges showed formazan deposition to a lesser extent than the other tissues mentioned and to the same extent as the surrounding mesenchymal tissue and the closely adjacent dorsal mesentery (fig. 1). The control slides showed an absence of formazan deposition in all cases (figs. 3 and 4). The intensity of reaction in the sections examined was rated according to a 0 to ++++ scale, (table 1). The gonads and adjacent reactive tissues exhibited an intensity of ++, while the strongly reacting duct epithelia showed a +++ degree of reactivity. These tissue comparisons were made on whole embryo sections which were cut at a thickness of 30 μ and incubated for approximately six hours in the histochemical medium.

On day five, the reaction had become more localized and was seen with an intensity of ++ in the gonads (fig. 5), epithelial lining of the stomach, ependymal layer of the neural tube, Wolffian duct and diffusely in the mesonephros, and with an intensity of +++ in the adrenals. Again, the control sections showed a complete absence of granule deposition. Sections of day four embryos had an appearance intermediate between those of days three and five.

After day five, the mesonephros, including the gonads, was usually dissected free of other tissues in preparation for examination. It continued to show a fine and very diffuse formazan deposition. This reactiveness was

also occasionally seen in the mesonephros of control slides, thus minimizing its significance in this study. Adrenal tissue often accompanied sacral segments and usually showed an intense granulation. Sections of gonads of six through nine days of incubation showed a ++ reactiveness when cut at ten μ and incubated in the histochemical medium for three hours. No granules were seen in the dorsal mesentery, neural tube or mesenchyme at these ages (figs. 6 and 7).

2. The Period From Sex Differentiation to Day 13

In ovaries granule deposition showed a tendency, with advancing age, to become localized in patches, presumably in the cords, within the medulla of the ovary. Testes exhibited a granule deposition of + to ++, limited largely to the medullary cords. No substantial increase in the amount of activity in ovaries and testes was seen during this age range. The control slides showed no reaction. Thus, it seems that a relatively stable amount of enzyme is present in the ovary between the period of sex differentiation and 13 days.

3. The Period of Day 13 to Hatching

The ovary shows a steady increase in the amount of reactivity throughout this period (table 1), (figs. 8 and 15). The reactivity was seen almost exclusively in the medullary component of the ovary, although there also were some formazan deposits in the cortex. The deposition was extremely heavy in the outer, more compact portion of the medullary component in the 14 day ovary, but it was also seen in small groups of cells within the primary cords of the

inner loose portion of the medulla (figs. 8 and 9).

A generalized reactiveness was seen throughout the stroma of the testis with the greatest intensity in the cords. This generalized reaction had an intensity judged at ++. An especially intense deposit of formazan granules was seen in occasional cells within the sex cords of testes. This corresponds to observations in the female, which could perhaps be anticipated since the testis and the ovarian medulla are homologous structures. This deposit was seen at about +++ intensity in a 16 day testis (figs. 10 and 11), 17 day ovary and testis (figs. 12 and 13), and in a 20 day ovary and testis (figs. 14 and 15). The 16 day testis was cut at 4 μ and incubated for one hour while the 17 and 20 day gonads were cut at 10 micra and incubated for three hours.

C. The Use of Pregnenolone as the Substrate

Pregnenolone was used as a substrate for Δ^5-3β-HSD with the hope that the activity of this enzyme could be traced to a younger incubation age than that observed when DHA was used, thus extending to another species the observations reported by Baillie and Griffiths (1964) on the testis of the mouse embryo. The results, however, were not as anticipated. A reactive granule deposition was seen only in certain experimental slides, whereas an extremely intense reaction (++++) was observed when DHA was employed as the substrate. An example of this is the case of an adrenal from a 19 day female embryo which revealed a + reaction with pregnenolone, while with DHA the section appeared almost completely opaque as a result of formazan deposits. The controls for both the pregnenolone and DHA experiments were negative.

DISCUSSION

This investigation was undertaken for the purpose of determining, by histochemical means, the time of initial steroid hormone synthesis in the gonads of the chick embryo.

The results obtained with gonads between three days of incubation and the period of sex differentiation (approximately 6-7 days) were not as expected. Δ^5-3β-HSD activity, employing DHA as a substrate, was not limited to those tissues which are known to contain this enzyme i.e., gonads, adrenals, mesonephros and liver (Levy et al., 1959). Formazan granules were also seen throughout the gut, neural tube, as well as in the notochord, spleen primordium and mesenchyme from three to five days of incubation. The deposits decreased in intensity with advancing age until during the fifth day they had disappeared from the notochord, spleen primordium, the neural tube, except for the ependymal layer, and the mesenchyme. These deposits had also disappeared by this time in the gut, except for the epithelial lining. This granulation in these tissues is an unusual finding and two possible explanations may be considered. First, there is the possibility that this could be a phenomenon of enzyme induction. The fact that the tissues are less differentiated in nature and therefore less specialized in young embryos may allow the tissues to produce a Δ^5-3β-hydroxysteroid dehydrogenating enzyme in response to introduced substrate. A second possibility is that the Δ^5-3β-HSD is normally found in more generalized locations in very young embryos, again because of the less specialized nature of their tissues. This would imply a generalized

potentiality of the tissue to synthesize steroid hormones, many of which no doubt lose this potential as they become specialized. Steroid hormone synthesis in the gonads alone probably takes place gradually and may be indicated by the increase in granule deposition in the gonads seen in our investigation shortly before the onset of sex differentiation, that is, at about six days.

The results with embryonic gonads at ages between sex differentiation and hatching are straightforward in nature. An apparently steadily increasing concentration of enzyme was observed in the gonads with advancing age.

The fact that the sex hormones seem to be synthesized mostly by the medullary component of the ovary or testis appears to confirm the results of *in vitro* studies in which chick gonads were cultured in heterosexual combination and gonads and accessory sex organs were associated (Wolff and Haffen, 1952; Weniger, 1962). It was found that the principal hormones involved in differentiation of the reproductive system in the chick are the androgens. The Müllerian duct grew and differentiated normally in the absence of hormones, while the presence of androgens caused degeneration, as normally occurs when differentiation takes place in the male direction. Thus, the direction of differentiation can be determined by the presence or absence of male hormones. The fact that the testis is feminized by female hormones is probably not as important as the Müllerian duct phenomenon, since a high concentration of estrogens is not normally encountered in the male.

Our results, then, confirm the findings of Chieffi *et al.*, (1964), and Narbaitz and Kolodny (1964) who observed the presence of Δ^5-3β-HSD in the

embryonic gonads immediately after gonad differentiation and thus shortly prior to differentiation of the Mullerian ducts. However, we have also observed Δ^5-3β-HSD activity in the undifferentiated gonad, which would appear to indicate that the enzymatic mechanisms for steroid hormone synthesis are present in these organs some time in advance of secretion of the sex hormones. This is a particularly important point in that it is a verification of the timing of one event in a sequence of events. Before the hormones can be released they must first be synthesized by the gonads and before they can by synthesized, the enzymes involved in their synthesis must be present. All of these events should precede in time the actual secretion of formed hormone as determined by bioassay techniques.

The significance of this work, then, lies in the fact that, while up to the present the above considerations were apparent, the actual sequence of events had not been experimentally demonstrated, i.e., the results of biochemical and histochemical techniques on the one hand and bioassay results on the other, did not reveal a proper time sequence. It now appears that the synthetic mechanisms for steroid hormone formation are present prior to differentiation of the gonads.

Regarding the failure of the substrate pregnenolone to yield the expected results, it is to be noted that the solvent used in our investigation was different from that used on fetal mouse gonads by Baillie and Griffiths (1964). These workers used propylene glycol in both their pregnenolone and dehydroepiandrosterone (DHA) experiments, employing the modification of Levy et al., (1959) of Wattenberg's (1958) original medium. We used acetone, as

did Wattenberg in his investigation. In the acetone-pregnenolone medium a moderate amount of precipitate was seen on the bottom of the staining jar, which was not seen in the DHA jar. It is conceivable that this precipitate could be pregnenolone since the medium contained a high percentage of water. Pregnenolone and DHA are both water insoluble; however, pregnenolone is probably less soluble in the medium used, than is DHA, possibly to the point where it is not available in sufficient concentration for utilization as a substrate. It might be available in greater concentration if a different solvent were used thereby permitting complete solubilization.

SUMMARY AND CONCLUSIONS

1. Forty-nine embryonic gonads of the single comb, light-brown, Leghorn fowl from three days of incubation to hatching were examined by the technique of Wattenberg (1958) for the presence of Δ^5-3β-hydroxysteroid dehydrogenase, an enzyme necessary for the synthesis of steroid hormones. Two substrates were employed, dehydroepiandrosterone and pregnenolone.
2. Virtually no enzyme specific granulation was seen in the tissue incubated with pregnenolone probably due to its relative insolubility in the acetone medium employed. Propylene glycol was the solvent used in previous investigations (Baillie and Griffiths, 1964).
3. Results obtained in three day embryos, with the use of dehydroepiandrosterone as the substrate, showed formazan granulation in the gut and Wolffian ducts, as well as the ependymal layer of the neural tube, dorsal mesentery, spleen primordium, surrounding abdominal mesenchymal tissue, and the genital ridges.
4. Between days three and five the generalized reactiveness became more restricted to the epithelia of the Wolffian ducts, neural tube, gut and to the genital ridges.
5. Subsequent to day five, formazan deposits became more intense in localized patches of cells around lacunae in the medullary component of the ovary and weakly generalized in the ovarian cortex and the medullary cords of the testis.
6. It is assumed that the generalized tissue reactiveness, which is seen in

the embryo from day three to five, is due to the relatively undifferentiated nature of embryonic structures during this period of development because of which all tissues may: (1) possess the potential for steroid synthesis by virtue of the presence of certain enzymes involved in the biogenesis of steroid hormones including Δ^5-3β-HSD or (2) respond to the test substrate by producing a Δ^5-3β-HSD.

7. It is tentatively concluded that the results of this investigation indicate a capacity for steroid hormone synthesis in the gonad of the chick embryo beginning on about the sixth day of incubation. At this time the generally distributed reactiveness, seen in most of the tissues during the early embryonic period, begins to disappear and reactiveness in the gonads begins to increase. After the sixth day, reactiveness in the gonads appeared to show a steady increase up to the time of hatching.

LITERATURE CITED

Baillie, A. H., and K. Griffiths 1964 3β-Hydroxysteroid dehydrogenase in the foetal mouse Leydig cell. J. Endocrin., 31: 63-66.

Baillie, A. H., K. C. Calman, M. M. Ferguson and D. McK. Hart 1966 Histochemical utilization of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21α- and 24α-hydroxysteroids. J. Endocrin., 34: 1-12.

Baillie, A. H., E. H. D. Cameron, K. Griffiths and D. McK. Hart 1965 3β-hydroxysteroid dehydrogenase in the adrenal gland and placenta. J. Endocrin., 31: 227-233.

Benoit, J. 1923 Transformation experimental du sexe par ovariotomie precoce chez la poule domestique. C. R. Acad. Sci., 178: 341-344.

Bouin, P., and P. Ancel 1903 Sur la signification de la glande interstitielle du testicule embryonnaire. C. R. Soc. Biol., 55: 1682-1684.

Bradley, E. M. 1941 Sex differentiation of chick and duck gonads as studied in homoplastic and heteroplastic host-graft combinations. Anat. Rec., 79: 507-528.

Chieffi, G., H. Mancelli, V. Botte and L. Mastrolia 1964 Sulla distribuzione del colesterolo e della Δ^5-3β-idrossisteroide deidrogenasi nella gonade embrionale di pollo. Ric. Sci., IIB, 34: 77-84.

Dantchakoff, V. 1935 Sur l'inversion sexuelle experimentale de l'ebauche testiculaire chez l'embryon de poulet. C. R. Acad. Sci., 200: 1983-1985.

Domm, L. V. 1927 New experiments on ovariotomy and the problem of sex inversion in the fowl. J. Exp. Zool., 48: 31-173.

Essenberg, J. M., and J. K. Garvacki 1938 On the origin and development of the definitive germ cells in the domestic fowl. Western J. Surg. Obst. Gynecol., 46: 145-152.

Firket, J. 1920 Recherches sur l'organogenese des glandes sexuelles chez les oiseaux. II. Arch. Biol., 30: 393-516.

Gallien, L., and M. T. Le Foulgoc 1957 Detection par fluorimetrie et colorimetrie de steroides sexuels dans les gonades embryonnaires de Poulet. C. R. Soc. Biol., 151: 1088-1089.

Goldman, A. S., W. C. Yakovac and A. M. Bongiovanni 1960 Development of activity of 3β-hydroxysteroid dehydrogenase in human fetal tissues and in two anencephalic newborns. J. Clin. Endocrin. Metab., 26: 14-22.

Greenwood, A. W. 1925 Gonad grafts in embryonic chicks and their relation to sexual differentiation. Brit. J. Exp. Biol., 2: 165-187.

Groenendijk-Huijbers, M. M. 1960 Functional characteristics of the testicular hormone in the chick embryo. Anat. Rec., 137: 237-249.

Hamburger, V. and H. L. Hamilton 1951 A series of normal stages in the development of the chick embryo. J. Morph., 88: 49-92.

Hamilton, H. L. 1952 "Lillie's" Development of the chick. Holt, Rinehart and Winston, Chap. 13, 488-501.

Hughes, W. 1929 The freemartin condition in swine. Anat. Rec., 41: 213-245.

Keller, K., and J. Tandler 1916 Über das Verhalten der Eihaute bei der Zwillingstrachtigheit des Rindes. Wien. Tierarztl. Wochenschr., 2: 513-526.

Kemp, T. 1927 Studier over Konskarakterer Hos Fostre. København. 1-117.

Kornfeld, W., and A. V. Nalbandov 1958 Endocrine influences upon the growth of the rudimentary gonad of fowl. Anat. Rec., 130: 619-637.

Koszelka, A. W., and T. F. Gallagher 1934 Effect of male hormone extracts theelin and theelol on the chick embryo. Proc. Soc. Exp. Biol. and Med., 31: 1143-1144.

Laulanie, M. 1886 Sur la mode d'evolution et la valeur de l'epithelium germinatif dans le testicule embryonnoire dus poulet. C. R. Soc. Biol., 38: 87-89.

Leroy, M. 1948 Effet androgene d'extraits embryonnaires de pulet sur la crete du Chapon. C. R. Acad. Sci., 226: 520-522.

Levy, H., H. W. Deane and B. L. Rubin 1959 Visualization of steroid-3β-oldehydrogenase activity in tissues of intact and hypophysectomized rats. Endocrinology, 65: 932-943.

Lewis, L. B. 1946 A study of some effects of sex hormones upon the embryonic reproductive system of the white pekin duck. Physiol. Zool., 19: 282-329.

Lewis, L. B. and L. V. Domm 1948 Sexual transformation of the osseus bulla in the duck embryo following administration of estrogens. Physiol. Zool., 31: 65-69.

Lillie, F. R. 1916 The theory of the freemartin. Science, 43: 611-613.

Lillie, F. R. 1917 The freemartin: a study of the action of sex hormones in the foetal life of cattle. J. Exp. Zool., 23: 371-452.

Lobel, B. L., H. W. Deane and S. L. Romney 1962 Enzymic histochemistry of the villous portion of the human placenta from six weeks of gestation to term. Am. J. Obst. Gynecol., 83: 295-299.

Lutz-Ostertag, Y. 1954 Contribution a l'etude de development et de la regression des canaux de Muller chez l'embryon d'oiseau. Bull. Biol., 88: 334-412.

Minoura, T. 1921 A study of testis and ovary grafts on the hen's egg and their effects on the embryo. J. Exp. Zool., 33: 1-61.

Moore, C. R. 1944 Gonad hormones and sex differentiation. Am. Natur., 78: 97-130.

Narbaitz, R., and L. Kolodny 1964 Δ^5-3β-Hydroxysteroid dehydrogenase in differentiating chick gonads. Z. Zellforsch., 63: 612-617.

Narbaitz, R., and M. T. Sabatini 1963a Histochemical demonstration of cholesterol in differentiating chick gonads. Z. Zellforsch., 59: 1-5.

Narbaitz, R., and M. T. Sabatini 1963b Histochemical demonstration of cholesterol in embryonic chick gonads differentiated in vitro. Z. Zellforsch., 61: 52-55.

Petskoi, P. G. 1953a Conditions of the intra-uterine development of Bos taurus, causing phenomena of freemartinism. DAN SSSR Proc. Acad. Sci. U.S.S.R., 90: 693.

Petskoi, P. G. 1953b Embryonic parabiosis in goats and sheep. Proc. Khirov Agri. Inst., 9: 63.

Petskoi, P. G. 1955 Embryonic parabiosis and multifetation of farm animals. Trans. Inst. Morph. Animals Acad. Sci. U.S.S.R., 14: 44.

Presl, J., J. Jirasek, J. Horsky and M. Henzl 1965 Observations on steroid-3β-ol-dehydrogenase activity in the ovary during early postnatal development in the rat. J. Endocrin., 31: 293-294.

Romanoff, A. L. 1960 The Avian Embryo. MacMillian Co., New York, N. Y. Chap. 10.

Rubin, B. L., J. A. Hamilton, F. J. Karlson and R. I. Tufaro 1965 Effect of the age of the rat at the time of hemicastration on the weight, estimated secretory activity and Δ^5-3β-hydroxysteroid dehydrogenase activity of the remaining ovary. Endocrinology, 77: 909-916.

Samuels, L. T., M. L. Helmreich, M. B. Lasater and H. Reich 1951 An enzyme in endocrine tissues which oxidizes Δ^5-3β-hydroxysteroids to α,β unsaturated ketones. Science, 113: 490-491

Scheib, D. 1958a Sur la genese et la repartition des lipides dans les gonades embryonnaires du Poulet. I. Gonades femelles. C. R. Soc. Biol., 152: 728-729.

Scheib, D. 1958b Sur la genese et la repartition des lipides dans le gonades embryonnaires du Poulet. II. Gonades Males. C. R. Soc. Biol., 152: 762-763.

Scheib, D. 1959 Sur la detection des lipides figures dans les gonades de l'embryon de Poulet. Ann. Histochem., 4: 33-50.

Stenger-Haffen, K. 1957 Etude des besoins nutritifs des gonades embryonnaires d'oiseau cultivees en milieux synthetiques. Arch. Anat. Micr. Morph. Exp., 46: 521-607.

Stoll, R., and R. Maraud 1956 Existence d'hormones steroliques dans l'organisme de l'embryon de Poulet au cours de sa differenciation sexuelle, C. R. Acad. Sci., 242: 1235-1237.

Swift, C. H. 1916 Origin of the sex-cords and definitive spermatogonia in the male chick. Am. J. Anat., 20: 375-410.

Taber, E., and K. W. Salley 1954 The effects of sex hormones on the development of the right gonad in female fowl. Endocrinology, 54: 415-424.

Wattenberg, L. 1958 Microscopic histochemical demonstration of steroid-3β-ol-dehydrogenase in tissue sections. J. Histochem. Cytochem., 6: 225-232.

Weniger, J-P. 1962 Diffusion des hormones gonadiques de l'embryon de Poulet dans le milieu de culture. Arch. Anat. Micr. Morph., 51: 325-335.

Weniger, J-P. 1964 L'ovaire d'embryon de Poulet cultive in vitro secrete une hormone oestrogene. C. R. Soc. Biol., 158: 175-178.

Weniger, J-P. 1965a Extraction d'une substance oestrogene de milieux surlesquels ont ete cultivees des gonades droites femelles, atrophiques, d'embryon de Poulet. C. R. Soc. Biol., 159: 464-466.

Weniger, J-P. 1965b Extraction d'une substance oestrogene (test d'Allen et Doisy positif) de milieux sur lesquels les gonades d'embryons de Poulet femelles de 7 jours furent cultivees pendant 24th. C. R. Acad. Sci., 261: 809-812.

Willier, B. H. 1927 The specificity of sex, of organization and of differ-

entiation of embryonic chick gonads as shown by grafting experiments. J. Exp. Zool., 46: 409-465.

Willier, B. H., T. F. Gallagher and F. C. Koch 1935 Sex modifications in the chick embryo resulting from injections of male and female hormones. Proc. Nat. Acad. Sci., 21: 625-631.

Wolff, E. 1947 Recherches sur l'intersexualite experimentale produite par la methode des greffes de gonades a l'embryon de poulet. Arch. Anat. Micr. Morph. Exp., 36: 69-90.

Wolff, E., and K. Haffen 1952 Sur la regression et la necrose in vitro des canaux de Muller de l'embryon de poulet sous l'action directe des hormones males. C. R. Soc. Biol., 146: 1793-1795.

Wolff, E., Y. Lutz-Ostertag and K. Haffen 1952a Sur la culture in vitro des canaux de Muller de l'embryon de poulet. C. R. Soc. Biol., 146: 1791-1793.

Wolff, E., Y. Lutz-Ostertag and K. Haffen 1952b Sur la regression et la necrose in vitro des canaux de Muller de l'embryon de poulet sous l' action directe des hormones males. C. R. Soc. Biol., 146: 1793-1795.

Wolff, E., and E. Wolff 1949 Application de la methode de castration a l'embryon de canard: sur deux tests de l'activite precoce des gonades embryonnaires, la syrinx et le tubercule genital. C. R. Soc. Biol., 143: 529-531.

Wolff, E., and E. Wolff 1951 The effects of castration on bird embryos. J. Exp. Zool., 116: 59-98.

Wolff, E., and E. Wolff 1952a Le determinisme de la differentiation sexuelle de la syrinx du canard cultivee in vitro. Bull. Biol., 86: 325-350.

Wolff, E., and E. Wolff 1952b Action de l'hormone femelle sur la differenciation sexuelle de la syrinx de canard cultivee in vitro. C. R. Soc. Biol., 146: 111-113.

Wolff, E., and E. Wolff 1953 Cultivation and differentiation of the syrinx of the duck embryo cultivated in vitro. Poult. Sci., 32: 348-351.

Woods, J. E., and L. V. Domm 1966 A histochemical identification of the androgen-producing cells in the gonads of the domestic fowl. Anat. Rec., 154: 443-444. also: Gen. Comp. Endocrin, (In press).

TABLE 1

THE INTENSITY OF FORMAZAN DEPOSITION IN Δ^5-3β-HSD PREPARATIONS

DAYS 3 to 6

Days of Incubation	No. of Specimens	Undifferentiated Gonads	Ovaries	Testes	Thickness (Micra)	Incubation Period (Hours)
3	1	++G2			30	6
	1	+G2			10	2 3/4
4	1	++G2			30	18 1/2
	1	+G2			10	3 1/2
5	4	+–++G2			20-40	2-4
	1	Gonad not seenG1			10	4 1/2
	1	+G1			10	3
6	4	+–++			12	1-3 (one 17)
	1	++			40	28
	1	++			10	3 3/4
	2	0			40	2 1/2-4

0 No formazan deposition
+ Barely detectable, but definite deposition
++ Moderate deposition
+++ Heavy deposition
++++ Extremely heavy deposition (tissue opaque)
G1 Generalized deposition present in several tissues other than gonad, mesonephros or adrenal
G2 Deposition generalized through most tissues

TABLE 2

THE INTENSITY OF FORMAZAN DEPOSITION IN Δ^5-3β-HSD PREPARATIONS

DAYS 7 to 9

Days of Incubation	No. of Specimens	Undifferentiated Gonads	Ovaries	Testes	Thickness (Micra)	Incubation Period (Hours)
7	4	++-+++			30	1
	1	++			10	2 1/2
	1	0			30	1
	1	++			10	4
	1	0			20	1
	1	+			12	1
8	2		++	++	12	1
	1			++	10	3
9	3		+-+++		6&10	1

0 No formazan deposition
+ Barely detectable, but definite deposition
++ Moderate deposition
+++ Heavy deposition
++++ Extremely heavy deposition (tissue opaque)
G1 Generalized deposition present in several tissues other than gonad, mesonephros or adrenal
G2 Deposition generalized through most tissues

TABLE 3

THE INTENSITY OF FORMAZAN DEPOSITION IN Δ^5-3β-HSD PREPARATIONS

DAYS 11 to 20

Days of Incubation	No. of Specimens	Undifferentiated Gonads	Ovaries	Testes	Thickness (Micra)	Incubation Period (Hours)
11	1		+		6	1
	1			++	10	3
12	1		+		6	1
14	1			+++-++++	2&10	1
	1		+++		10	3
	1			+	2&4	1
	1		+++-++++		40	2 3/4
15	1		+		10	1 1/3
16	1			++	2&4	1
17	1		++-+++		10	1 1/3
	2		++	++	10	3
18	1		+-++		2	1
19	1		+++		10	1 1/3
20	2		+++	+++	10	3

0 No formazan deposition
+ Barely detectable, but definite deposition
++ Moderate deposition
+++ Heavy deposition
++++ Extremely heavy deposition (tissue opaque)
G1 Generalized deposition present in several tissues other than gonad, mesonephros or adrenal
G2 Generalized deposition through most tissues

PLATE 1

EXPLANATION OF FIGURES

1 Experimental cross-section of a 3 day embryo cut at 30 micra and incubated for 6½ hours showing +++ reaction of Δ^5-3β-HSD in genital ridge (G), Wolffian duct (W), adjacent mesenchymal tissue (M), coelomic epithelium (C), and dorsal mesentery (D). X 450.

2 Experimental cross-section of a 3 day embryo shown in figure 1 showing ++ reaction throughout neural tube, (Nt), notochord (Nd), and lining of gut (G). X 450.

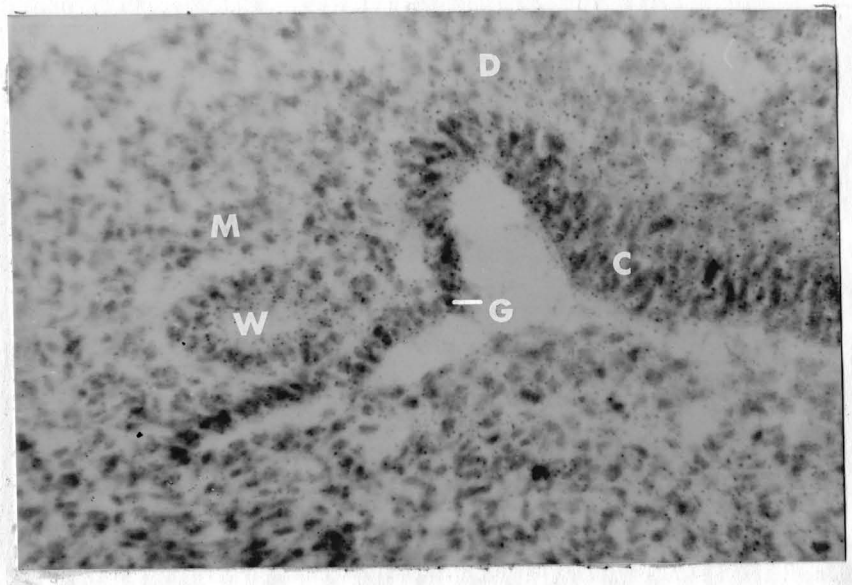

FIGURE 1

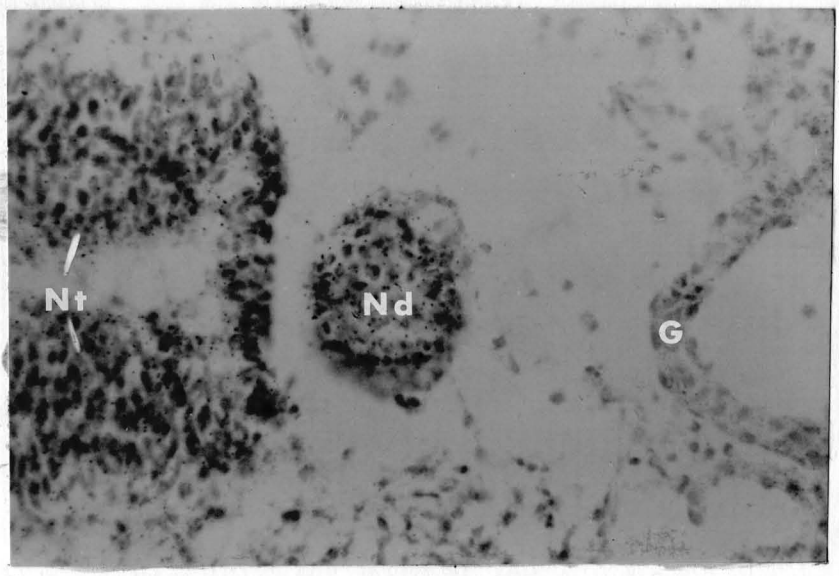

FIGURE 2

PLATE 2

EXPLANATION OF FIGURES

3 A control cross-section of 3 day embryo shown in figures 1 and 2 to show absence of Δ^5-3β-HSD in genital ridges (g) and their relationship to the gut (G), notochord (Nd), and neural tube (Nt). X 100.

4 A higher magnification of upper genital ridge shown in figure 3 to confirm absence of formazan granules. The genital ridge (G), Wolffian duct (W) and dorsal mesentery (D) are shown. X 450.

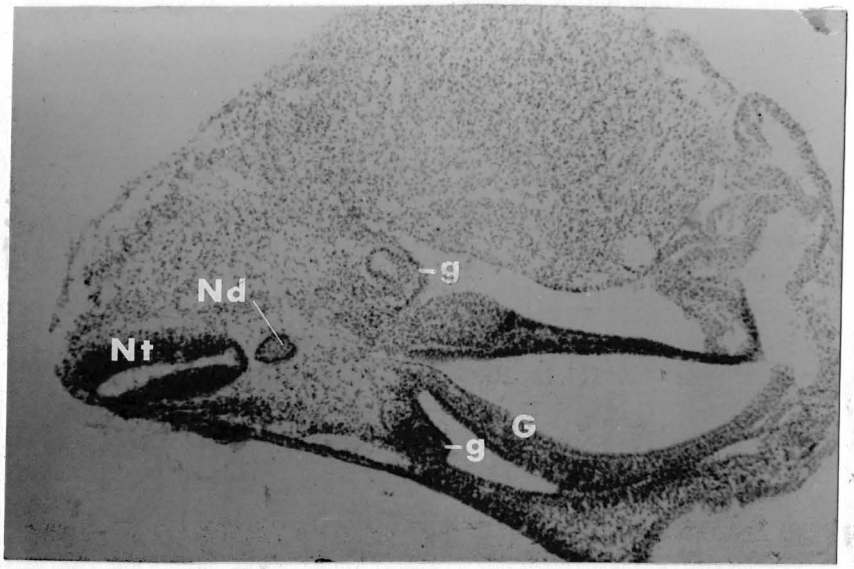

FIGURE 3

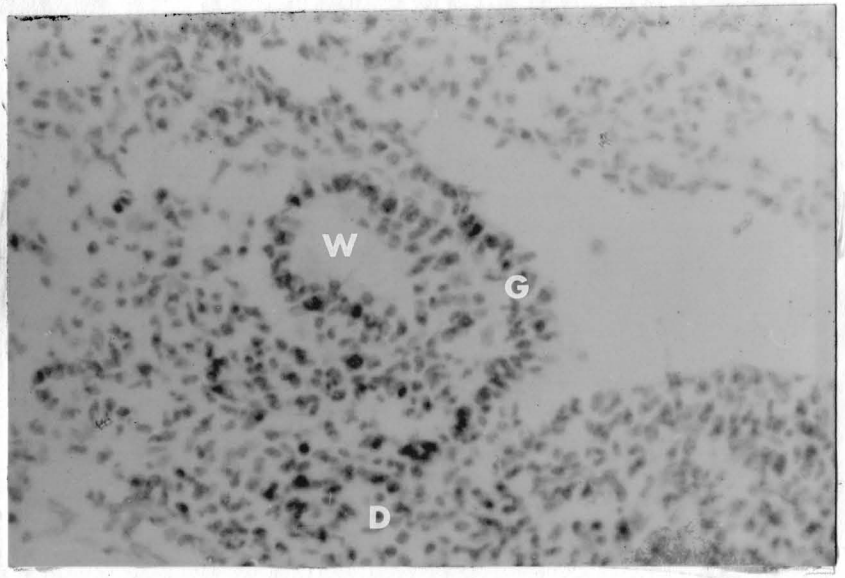

FIGURE 4

PLATE 3

EXPLANATION OF FIGURES

5 Experimental cross-section of a gonad (G) of a 5 day embryo cut at 10 micra and incubated at 4½ hours showing scattered formazan granules throughout the genital ridge and gut epithelium (E) at ++ intensity. X 450.

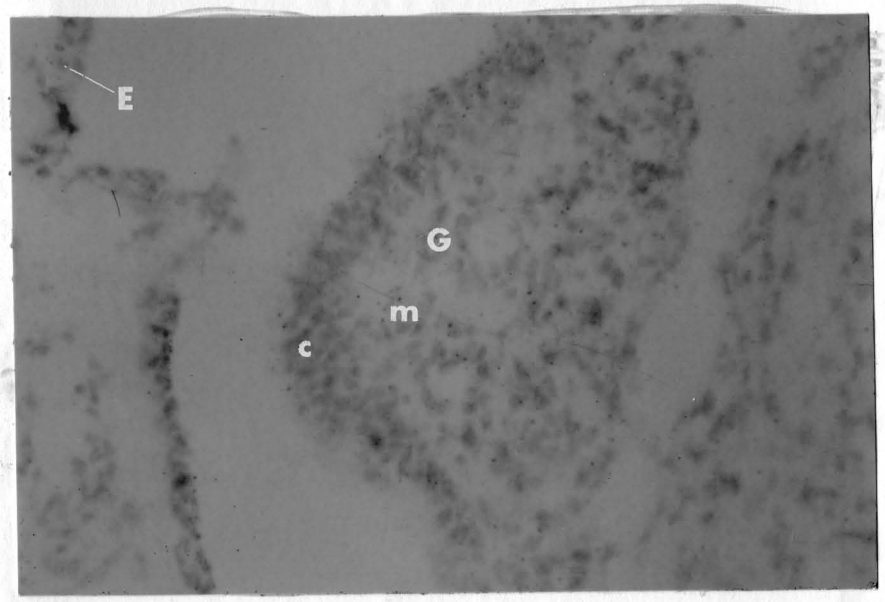

FIGURE 5

PLATE 4

EXPLANATION OF FIGURES

6 Experimental cross-section of a 7 day gonad cut at 12 micra and incubated 1 hour showing diffuse but definite granulation judged at ++ intensity. X 450.

7 Experimental cross-section of a 9 day gonad cut at 6 micra and incubated 1 hour showing reaction (R) in what may be the medullary component of an ovary. X 100.

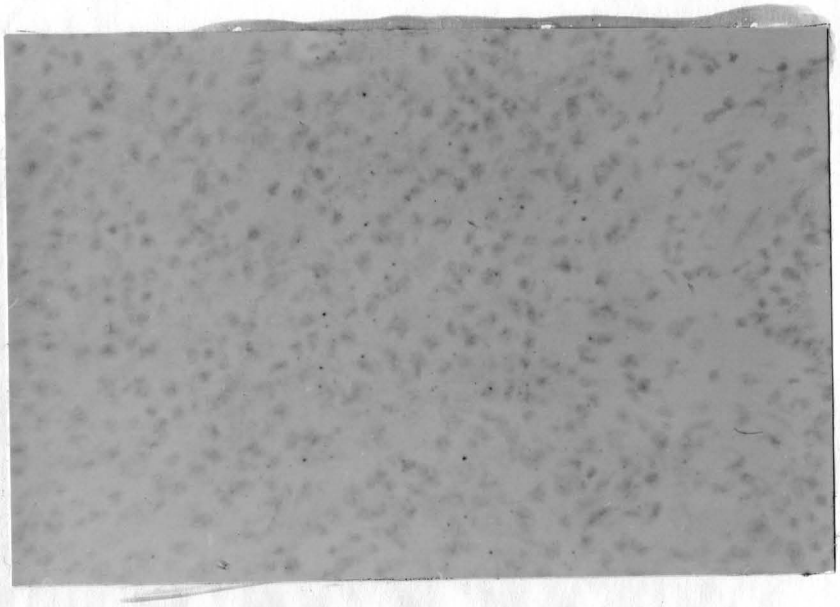

FIGURE 6

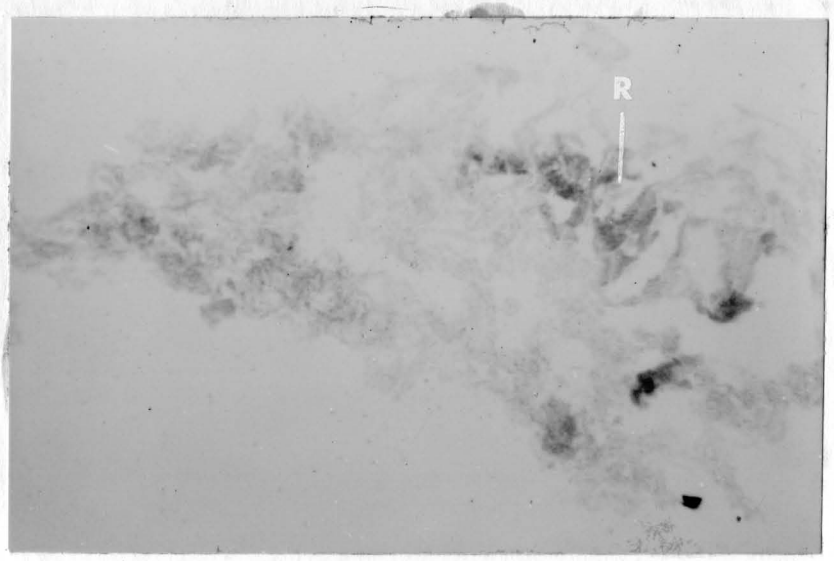

FIGURE 7

PLATE 5

EXPLANATION OF FIGURES

8 Experimental cross-section of a 14 day ovary cut at 40 micra and incubated 2 3/4 hours showing a patchy reaction in loose portion of medullary component (Ml), and a very dense reaction in the compact portion (Mc). There is a small amount of reaction in the cortex (C). X 100.

9 Higher magnification of a cross-section of loose medullary portion shown in figure 8 showing medullary lacuna (L), and the reaction (R) in the interstices between medullary lacunae and cords. X 450.

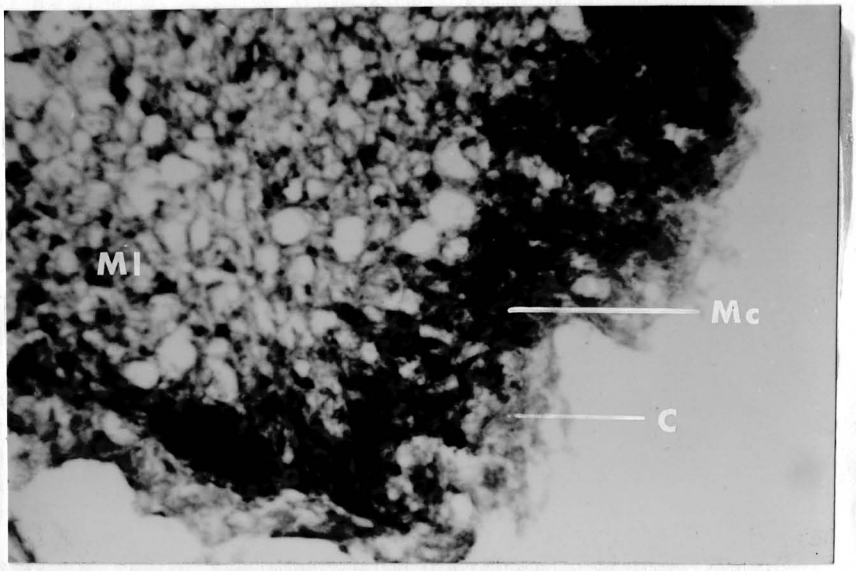

FIGURE 8

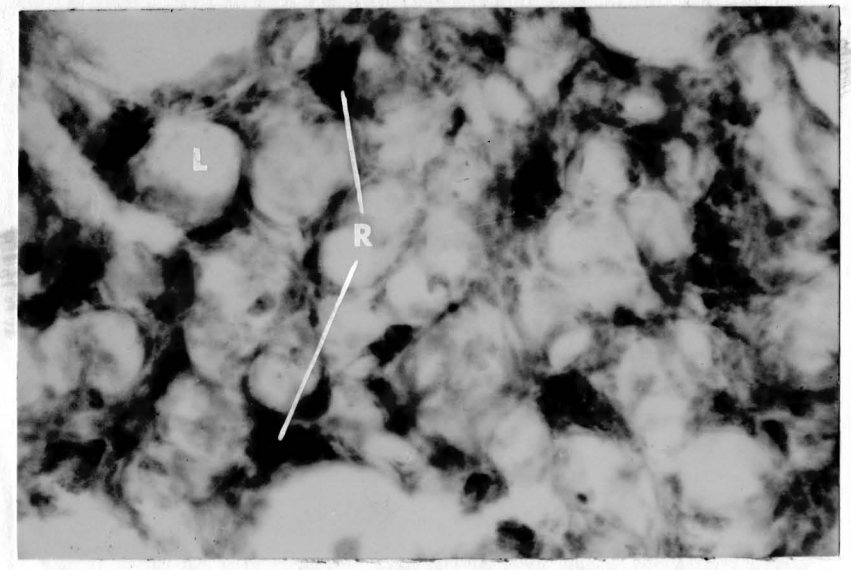

FIGURE 9

PLATE 6

EXPLANATION OF FIGURES

10 Experimental cross-section of a 16 day testis cut at 4 microns and incubated 1 hour showing a ++ reaction throughout the organ as well as several reactive +++ patches (R). X 450.

11 Patch of intense formazan deposition (D) in same tissue section as shown in figure 10. The intracellular localisation of the deposits may be seen. Oil immersion X 1000.

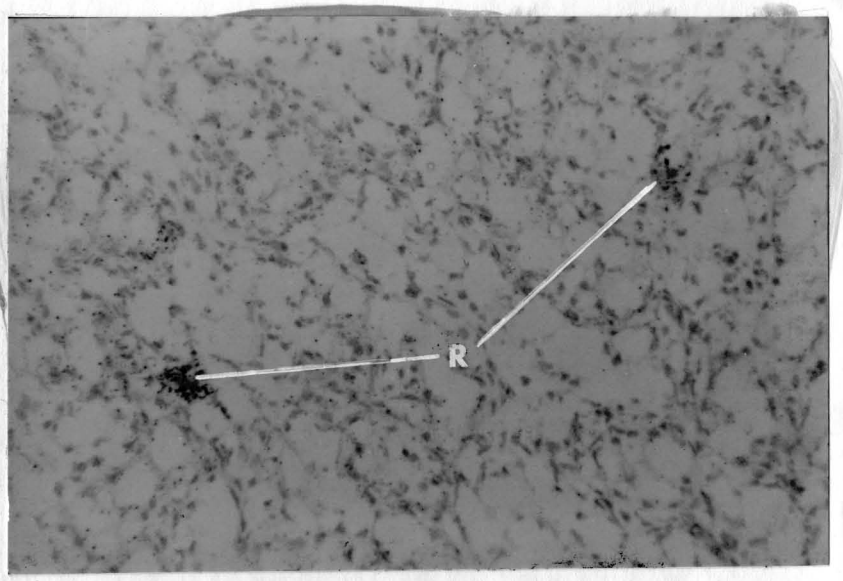

FIGURE 10

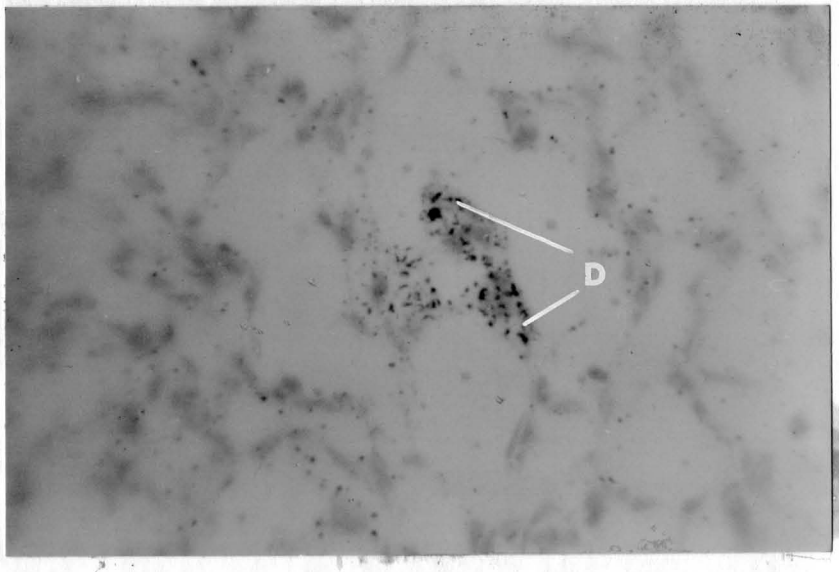

FIGURE 11

PLATE 7

EXPLANATION OF FIGURES

12 Experimental cross-section of the sacral portion of a 17 day embryo cut at 10 micra and incubated 1½ hours showing the ovary (O) with deposits (D) in patches in the medulla and the adrenals (A). X 100.

13 Higher magnification of section of ovary shown in figure 12 showing cortex (C) and medulla (M) with patches of granule deposition (D). X 450.

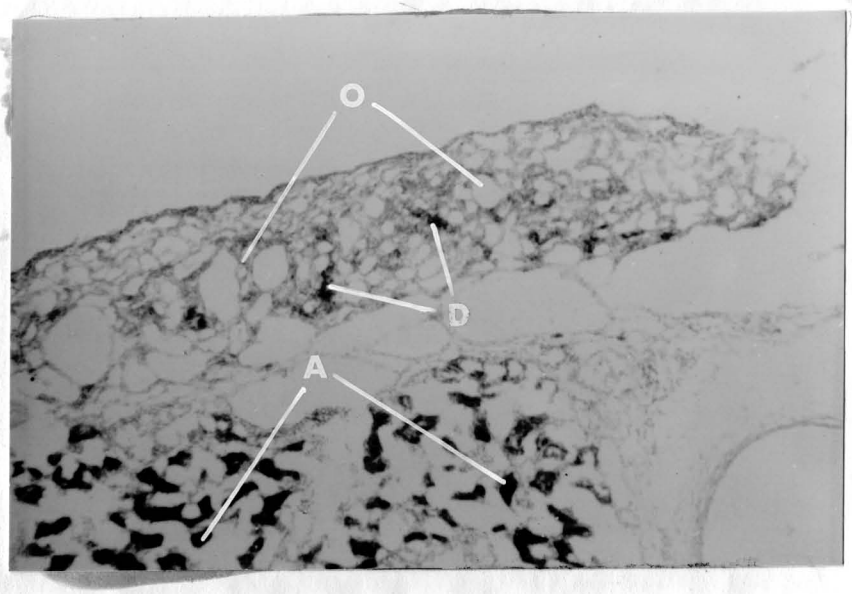

FIGURE 12

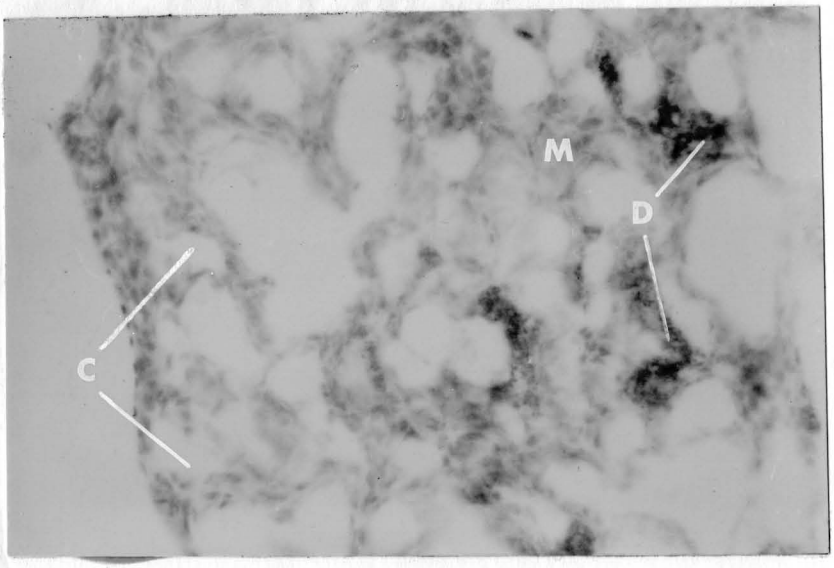

FIGURE 13

PLATE 8

EXPLANATION OF FIGURES

14 Experimental cross-section of a 20 day testis cut at 10 micra and incubated 3 hours showing granulation (G) mostly in center of this organ. X 100.

15 Experimental cross-section of a 20 day ovary cut at 10 micra and incubated 3 hours showing relatively compact cortex (C) and loosly arranged medulla (M) with a patchy formazan granule deposition (D). X 100.

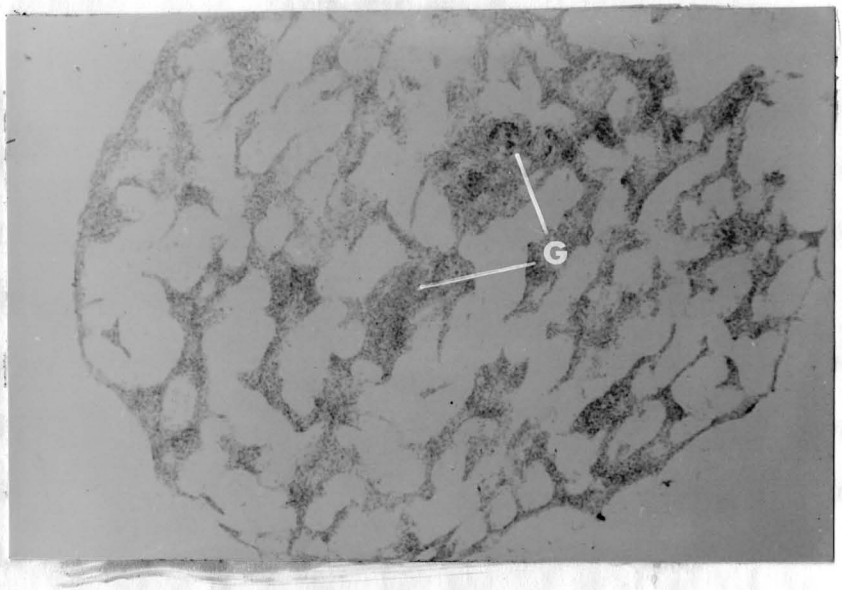

FIGURE 14

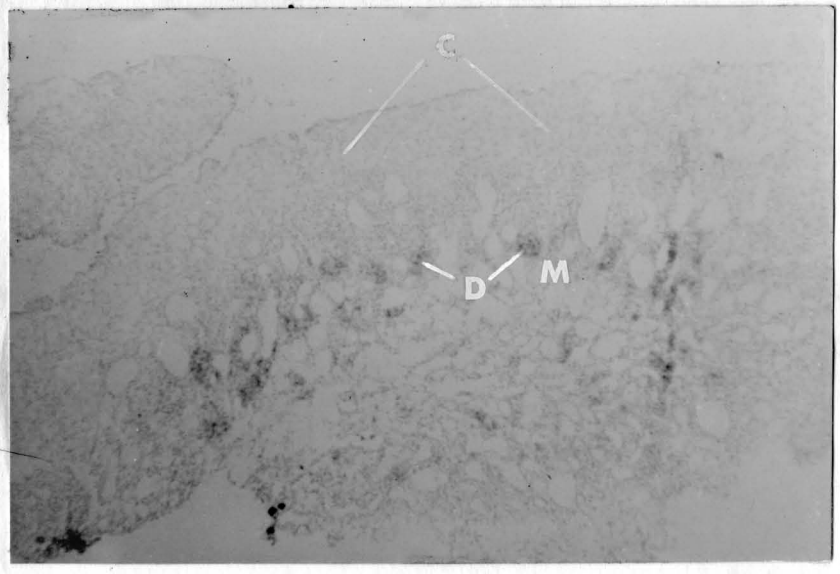

FIGURE 15

APPROVAL SHEET

The thesis submitted by David Glenn Rabuck has been read and approved by four members of the faculty of the Graduate School of Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

June 1, 1966
Date

L. K. Downes
Signature of Advisor