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The Effects of Exogenous ACTH on 5-3b-hydroxysteroid Dehydrogenase Activity in the Embryonic Avian Adrenal Gland

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THE EFFECTS OF EXOGENOUS ACTH ON Δ^5-3B-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN THE EMBRYONIC AVIAN ADRENAL GLAND

by

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BIOGRAPHY

Grover Charles Ericson was born in Oak Park, Illinois, on February 17, 1941. He was graduated from the Naperville Community High School, Naperville, Illinois in June, 1959. He entered North Central College, Naperville, Illinois, in September, 1959, and was awarded the Bachelor of Arts degree in June, 1964.

While attending North Central College, the author married a fellow student, the former Miss Barbara Joye Moede.

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ABSTRACT

This investigation concerned itself primarily with the development of adrenal $\triangle^5$-3B-hydroxysteroid dehydrogenase ($\triangle^5$-3B-HSD) activity and the effects of exogenous ACTH on this activity in the embryonic chicken.

A preliminary investigation was undertaken to determine the normal development of the adrenal of brown and white Leghorn chick embryos. Since chronological and morphological ages are not always closely correlated, morphological characteristics, as established by Hamburger and Hamilton ('51), were employed to determine developmental age. There was no significant difference in development of the adrenal glands in the two varieties of Leghorn studied. Only minor variations between our observations and those of other investigators were observed.

A modification of the histochemical technique of Levy and coworkers ('59) was used to determine $\triangle^5$-3B-HSD activity. Such activity was observed in the primordial adrenocortical cells of stage 23 (day 4) brown and white Leghorn embryos. There was a significant difference in the development of $\triangle^5$-3B-HSD activity between brown and white Leghorn embryos. Differences in the intensity of the enzyme reaction when sections of the same embryo were incubated with the two substrates, dehydroepiandrosterone (DHA) and pregnenolone, suggest the possibility of two enzymes rather than one.

Brown Leghorn embryos of stages 23, 23-24 and 24 showed an increase in enzyme activity after the administration of porcine ACTH. The time interval between the last ACTH injection and the time of sacrifice was found to be significant. Embryos sacrificed prior to three hours following injection ex-

hibited no increase in activity as did those sacrificed between 21 and 46 hours following injection. A significant increase in activity was observed in the adrenals of embryos sacrificed three to 11 hours after the final injection.

The occurrence of $\triangle^5$-3B-HSD activity in the adrenals of the chick embryo lead to the conclusion that the adrenocortical cells have the capacity for steroid hormone synthesis by stage 23 (day 4). Treatment of such embryos with exogenous ACTH resulted in an increase in adrenal $\triangle^5$-3B-HSD activity indicating that the embryonic chicken adrenal is susceptible to ACTH stimulation in its early stages of development.

INTRODUCTION

It is now known that the adrenocorticotrophic hormone (ACTH) stimulates the formation and elaboration of cortical hormones in the avian adrenal gland, (Zarrow, et al., '62; Nagra, et al., '63). The avian pituitary, like that of mammals, contains ACTH but its relative potency has not been established (Sturkie, '65). The initiation of ACTH production in the embryonic pituitary of the domestic fowl has been reported as early as the eighth day of development (Toth, et al., '58; Szekely, et al., '58) however, most investigators feel that the endocrine activity of the adrenal cortex is controlled by ACTH somewhat later in development. From a study of the ascorbic acid content of the chick adrenal gland, Case ('51) concluded that the pituitary gland may exert its adrenocorticotrophic effects at least by the 13th day. Watterson and coworkers ('59) felt that the endocrine activity of the adrenal cortex is controlled by ACTH by the 16th day of incubation.

Various histochemical reactions have been employed in an attempt to determine the time at which adrenal cortical function begins. Dawson ('53) used a group of histochemical reactions characterized by lipid droplets of steroid producing organs to determine the time at which differentiation of the embryonic adrenal cortical cells first occurred. The earliest positive histochemical response in the cortical tissue of embryos was observed at the age of six days and seven hours.

The appearance of a steroidogenic potential in the embryonic adrenal cortex has been used as an indicator of adrenal steroid hormone biosynthesis.

Wattenberg ('58) developed a method to indicate the cellular areas of steroid synthesis by deposition of the formazan (reduction product) of a tetrazolium salt at the sites of one of the enzymes (Δ^5-3B-hydroxysteroid dehydrogenase) involved in the biogenesis of the steroid hormones. Activity of Δ^5-3B-hydroxysteroid dehydrogenase (Δ^5-3B-HSD) has been found to be essential in steroidogenic endocrine tissue in the early biosynthesis of all biologically active steroid hormones (Bongiovanni and Root, '64). However, the presence of the enzyme is only indicative of steroidogenic activity because the presence of a single enzyme in the biosynthetic pathway does not necessarily insure successful hormone synthesis (Lobel, et al., '62).

There is at present no general agreement as to the time at which development of Δ^5-3B-HSD activity in the embryonic avian adrenal occurs. Straznicky et al., ('66) first observed the enzyme in the 14 to 15 day old embryo; Sivaram ('64) in the ten day embryo; Boucek et al., ('66) in the five day embryo and Chieffi et al., ('64b) in the four day embryo.

The purpose of this investigation was to determine the effects of exogenous ACTH on Δ^5-3B-HSD activity in the embryonic adrenal of the single comb, light-brown Leghorn fowl during its early stages of development before the pituitary-adrenal axis was established.

A preliminary investigation was also undertaken to determine the normal adrenocortical development of chick embryos, using morphological characteristics as established by Hamburger and Hamilton ('51) to determine developmental age. Previous investigators who have worked with the embryonic avian adrenal (Hays, '14; Sivaram, '64, '65; Chieffi, et al., '64b; and others) have used total incubation time to determine the age of the embryo. It is now well

known that chronological and morphological age may not always be closely correlated.

REVIEW OF THE LITERATURE

Early development of the adrenal glands

As in mammals, the avian adrenal consists of two components, namely the interrenal tissue and the chromaffin tissue (Hartman and Brownell, '49). In mammals the adrenals are divided into an outer cortex consisting of interrenal tissue and an inner medulla of chromaffin tissue.

In the domestic fowl the arrangement of the interrenal and chromaffin tissues differs from that found in mammals. The interrenal cells are distributed throughout the gland in the form of irregularly arranged, frequently anastomosing, columns interspersed with groups of chromaffin cells without distinct cortical or medullary zones. The terms "cortex" and "medulla" as applied to the avian adrenal refer only to the interrenal tissues and chromaffin tissue, respectively.

The cortical and medullary components of the adrenal gland are anatomically and embryologically separate structures. The cortical component arises from mesoderm and the medullary from ectoderm (Romanoff, '60).

The adrenal cortex. The cells forming the cortical substance of the avian adrenal gland were first observed in the 78 hour chick embryo (Hamilton, '52) as a thickening of the peritoneal epithelium close to the mesentery, ventral and medial to the mesonephros, ventral to the dorsal aorta, and dorsal to the hind-gut (Hays, '14). These cells form solid bodies on each side of the base of the mesentery and later folds in the peritoneal epithelium force the cells laterally from this position (Hays, '14). At this stage in development a

groove in the thickening peritoneal epithelium begins about one-half millimeter behind the pronephros and extends posteriorly for about one millimeter where it becomes continuous with the antero-dorsal part of the germinal epithelium (Hamilton, '52). Proliferating cells from this groove migrate dorsally into the mesenchyme (with a majority passing lateral to the subcardinal vein, Hays, '14) to an area between the antero-medial surface of the mesonephros and the dorsal aorta (Hamilton, '52). The proliferating cells increase in size, become rounded in shape, and their less granular nuclei stain lighter than those of the peritoneal cells (Hays, '14). A chain of cells can be traced from the peritoneal epithelium to a point in the mesenchyme between the mesonephros and the dorsal aorta (Hays, '14). This connection ends by the end of the fourth day of incubation when the dorsal migration of cortical cells is complete (Hamilton, '52).

In the four day embryo, the cortical cells are arranged in scattered groups in the mesenchyme and nearly fill the entire region dorsal to the subcardinal veins, lateral and ventral to the aorta, medial and ventral to the posterior cardinal veins, and posterior to the level of the anastomosis of the subcardinal veins in the median line ventral to the dorsal aorta (Romanoff, '60).

The mass of the cortical substance in the five day embryo increases by the concentration of cells into large groups and by mitotic division (Hays, '14; Romanoff, '60). Cells of the cortical mass are arranged in large groups lying dorso-medial to the subcardinal veins, ventral to the mesonephric arteries which run over the anterior ends of the cell groups and between the aorta and mesonephros (Hays, '14). The cortical cell bodies are separated from the

mesonephros by a thin layer of mesenchyme (Hays, '14; Romanoff, '60; Sivaram, '65). The close arrangement of the cells causes them to lose their regular shape but the nuclei remain spherical (Hays, '14; Romanoff, '60).

The mass of the cortical tissue in the embryo of six days continues to enlarge and the cells become arranged into cords and have a somewhat hexagonal shape. The cylindrical cortical cords have light staining cells radiating from the center and they have no lumen (Romanoff, '60). The nuclei continue to become more granular (Hays, '14). At this time connective tissue, which eventually forms the adrenal capsule, begins to grow around the cortical cells (Romanoff, '60).

The cords are well developed in the seven day embryo. A central section of the cortical mass is roughly circular and has approximately the same cross sectional area as the aorta (Hays, '14). Development of the mesonephros in a ventral direction has resulted in only the dorso-medial angle of the mesonephros being in contact with the adrenal (Hays, '14). The subcardinal veins at this time are still the ventral border of the glands (Hays, '14).

The cross sectional area of the cortical mass of the eight day embryo is twice that of the aorta (Hays, '14). The beginning of vascularization is indicated by the presence of blood cells in the openings between the cords of cortical cells (Hays, '14). The connective tissue capsule has formed by this time.

In the embryo of nine days incubation there is little change in the form and size of the gland. The greatest change has occurred in the internal arrangement of the cortical cells. These have become very dense and compact, making it difficult to see the outline of individual cells. Vascularization

between the cords has increased (Hays, '14).

The adrenal medulla. The chromaffin tissue arises from cells that have migrated from the sympathetic chains (Hamilton, '52). In three and four day embryos, large, oval, deep staining cells, from the sympathetic chains, migrate ventrally between the aorta and the adrenal cortical cell group. Most of these sympathoblasts migrate to the ventral side of the aorta to form the prevertebral sympathetic plexuses but in the five day embryo some of these cells turn off ventrally and enter the adrenal cortical masses or become attached to the surface of the cell groups (Hays, '14; Romanoff, '60). Morphologically, there is no difference between the cells which penetrate the gland and those which form the prevertebral sympathetic plexuses (Hays, '14). In most cases the penetrating cells collect in groups of two or three although single cells are found throughout the cortical mass (Hays, '14).

In the six day embryo, after penetrating the cortical mass, the chromaffin cells change from large circular cells with round, clear nuclei to irregularly-shaped, small cells with oval granular nuclei (Hays, '14; Romanoff, '60). They are easily distinguished from the cortical cells by their strong affinity for basic stains (Hays, '14). Cords of chromaffin cells are formed between the cortical cords (Hays, '14).

The adrenal capsule is formed by the eighth day and the invasion of the cortical mass by the sympathoblasts is complete (Romanoff, '60).

In all of the above papers on the development of the adrenal, the embryos were staged according to their chronological or incubation age. Since the stages were described in terms of days and hours of incubation it is difficult to define accurately the stage in development when the first evidence of a

histochemical differentiation of the gland appeared. It is now well known that chronological and morphological age may not always be closely correlated (Dawson, '53) and that there is a great deal of variation in the degree of development of embryos of the same age, especially during the first week (Hamilton, '52). Hamburger and Hamilton ('51) point out the following as some of the factors responsible for the lack of correlation between chronological and morphological age: genetic differences in the rate of development of different breeds, seasonal differences in the viability and vigor of embryos, differences in the stage of development when incubation was started, differences in the "freshness" of eggs, i.e., the lapse of time between laying and incubation, differences in the temperature of eggs when placed in the incubator and in the size of individual eggs, differences in the temperature of incubation, and in type and size of incubator. Thus, a comparison of observations with those made in other laboratories must be made with caution due to the variables inherent in comparisons based on chronological age even though local conditions have been vigorously standardized (Dawson, '53).

Adrenal steroid production

There is a considerable body of knowledge concerning the biosynthetic pathways of the adrenocortical steroids of mammals and especially of man (Grant, '62). However, considerably less is known concerning these pathways in birds, but it seems likely that they are similar to those of mammals (Chester Jones, et al., '62).

Corticosterone and aldosterone have been identified in the adrenals of birds with corticosterone as the major adrenocortical steroid produced (de Roos, '61).

Hall and Koritz ('66) showed that chicken adrenal slices were capable of converting cholesterol-7a-^{3}H into corticosterone-^{3}H *in vitro*. They maintain that their observations demonstrated that avian adrenal tissue *in vitro* behaves like the mammalian adrenal, according to the parameters investigated.

Sandor, Lamoureux and Lanthier ('63) incubated adrenal slices from young female white Pekin ducks and Leghorn chickens with pregnenolone-7-H^3 and progesterone-4-C^{14}. From the incubation media C^{14}- and H^3-labeled corticosterone, aldosterone, 18-hydroxycorticosterone and 11-desoxycorticosterone were identified. Their results indicate that progesterone and pregnenolone are the precursors of the steroids isolated.

In addition to the steroids with functions characteristic of the adrenal cortex, androgenic steroids have been isolated from mammalian adrenals (Dorfman, '59) and have also been detected in the adrenals of the domestic fowl (McGowen, '36; Arrington, Fox and Bern, '52). McGowen ('36) observed virilism in a hen with an adreno-cortical tumor and Arrington, Fox and Bern ('52) showed that adrenals from young chicks implanted into an incision in the base of the comb of three-day old host chicks produced an effect only slightly inferior to that of testicular tissue.

The production *in vitro* of adrenal androgens from acetate by human adrenal glands is well established (Short, '60). Two of the androgenic steroids known to be secreted by the adrenal cortex in man are dehydroepiandrosterone (DHA) and androstenedione. DHA is usually regarded as exclusively of adrenal origin (Grant, '62). The available information suggests that the pathways involved in the formation of androstenedione are similar to those demonstrated for the gonads (Grant, '62).

Fevold and Eik-Nes ('62, '63) demonstrated that the testis of the English sparrow (Passer domesticus) can convert progesterone into testosterone and androstenedione in vitro by a pathway chemically identical to that for mammalian testis.

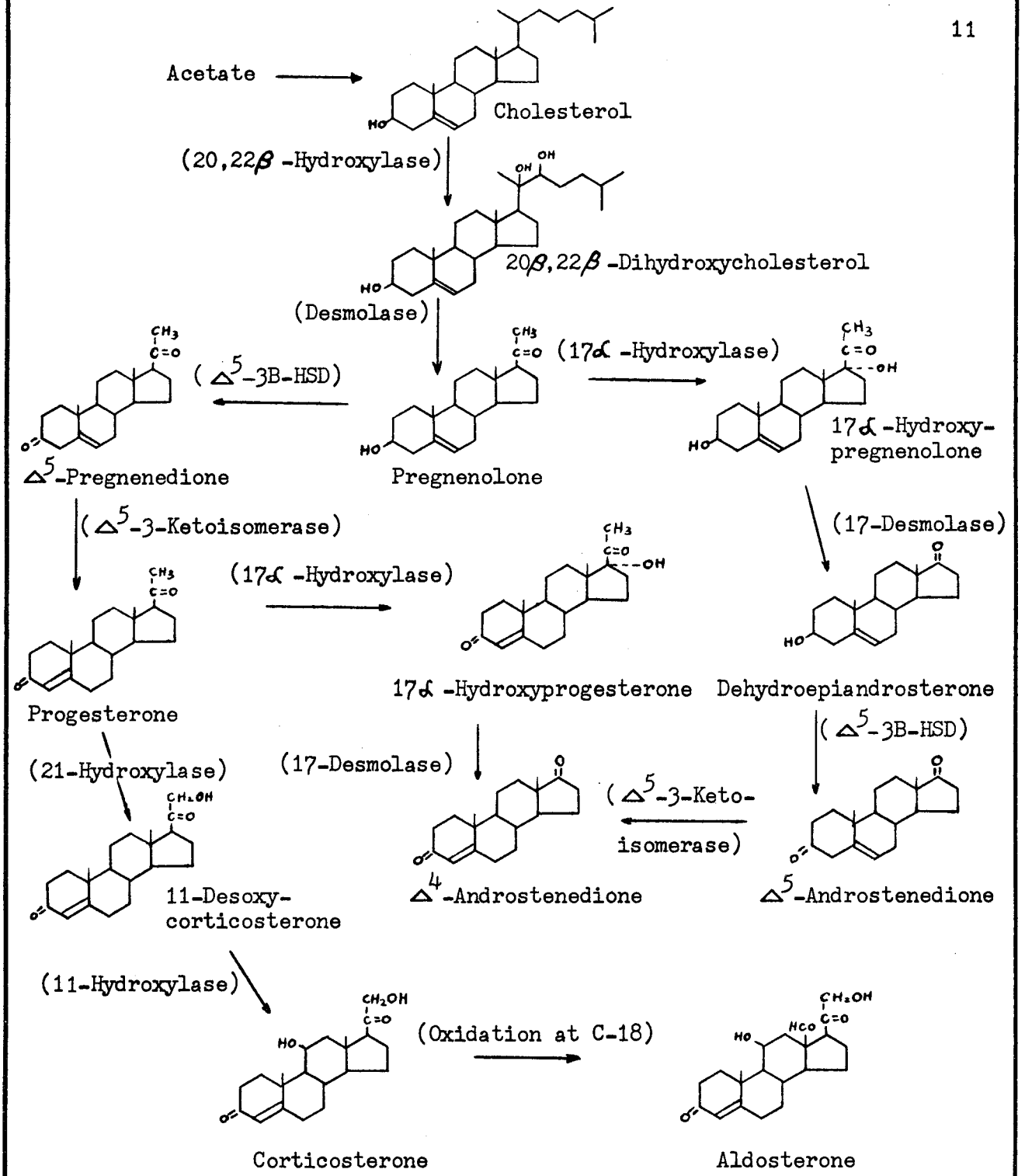
Assuming that the biosynthetic pathways of adrenal cortical steroids in birds are similar to those of mammals, the diagram shown on the following page would indicate the metabolic pathways involved in the biogenesis of these steroids.

Histochemistry

The characteristic distinguishing chemical properties of avian adrenocortical cells include: (1) a high lipid content with a large fraction consisting of cholesterol esters (Dawson, '53; Taylor, et al., '56; Howard and Constable, '58); (2) a high concentration of ascorbic acid (Case, '52; Jailer and Boas, '50; Howard and Constable, '58; and Perek, et al., '59) and (3) the presence of enzymes that take part in the synthesis of steroids and the specific cortical hormones (Chieffi, et al., '64b; Narbaitz and Kolodny, '64 and others).

Dawson ('53) used a group of histochemical reactions characteristic of the lipid droplets of steroid producing organs to determine the time at which histochemical differentiation of the embryonic adrenocortical cells occurred. These lipid droplets react positively in tests for carbonyl groups (Schiff test and the naphthoic acid hydrazine method of Ashbel and Siligman, '49) as well as staining with Sudan black B and osmic acid. When cholesterol or its esters are present in the lipid droplets, a blue-green color results following exposure of tissue sections to concentrated sulfuric acid and glacial acetic

Major metabolic pathways involved in biogenesis of adrenocortical steroids.



(Sandor, et al., '63; Samuels and Uckekawa, '67; Gorbman and Bern, '62).

acid (Schultz, '24). Cholesterol and its esters display a birefringence when viewed with a polarizing microscope (Lillie, '66). Dawson ('53) observed the earliest positive histochemical responses (osmiophilia, sudanophilia, Schultz reaction for cholesterol, Schiff reaction and birefringence) in cortical tissue of embryos at six days and seven hours incubation. Using the Ashbel-Seligman ('49) method, Dawson also observed carbonyl groups in nine day embryos, the youngest embryos studied by this method. In the early stages of cortical development, positive reactions appeared to be confined to cortical tissue that had become arranged into definite cords. Positive histochemical reactions were not uniformly and consistently present until 11 to 12 days of incubation at which time ascorbic acid also appeared.

Castane Decoud, Pedernera and Narbaitz ('64) observed the presence of sudanophilia in the adrenal glands of five day embryos and birefringence in six day embryos.

In 1958 Wattenberg developed a histochemical method to indicate cellular sites of steroid hormone synthesis. This method indicates the presence of the enzyme reaction in which pregnenolone is converted to progesterone or dehydroepiandrosterone (DHA) to Δ^4-androstenedione. Two enzymes, Δ^5-3B-hydroxysteroid dehydrogenase (Δ^5-3B-HSD) and Δ^5-3-ketoisomerase, are involved in this transformation. Δ^5-3B-HSD oxidizes the 3B-hydroxyl group and requires diphosphopyridine nucleotide (DPN), and Δ^5-3-ketoisomerase catalyzes the migration of the double bond from the 5-6 position to the 4-5 position (Samuels and Uchikawa '67). All hormonally active steroids are synthesized biologically by pathways involving such a synthetic step, and Δ^5-3B-HSD activity is essential in steroidogenic endocrine tissues in the early biosynthesis of these steroid

hormones (Bongiovanni and Root, '64).

Since Δ^5-3B-HSD is involved in one of the initial steps of steroid hormone biosynthesis, its presence in a tissue has been used as an indication of such synthesis. However, it must be noted that the presence of the enzyme is only indicative of steroidogenic activity because the presence of a single enzyme in the biosynthetic pathway does not necessarily imply successful hormone synthesis (Lobel, et al., '62).

Wattenberg ('58) incubated unfixed frozen sections in a medium containing the substrate (DHA, pregnenolone and others) dissolved in acetone, plus DPN, nicotinamide, buffer (pH 8.0) and a tetrazolium salt (Nitro BT). Hydrogen ions removed from the substrate by Δ^5-3B-HSD are transferred to DPN, then from DPNH to the tetrazolium salt. The tetrazolium salt is colorless and water soluble in the unreduced form, but when reduced it becomes colored and insoluble, and is deposited in the form of fine formazan granules at the site of the enzyme reaction.

There is at present no general agreement as to the time at which development of Δ^5-3B-HSD activity occurs in the embryonic avian adrenal. Straznicky, Hajos and Bohus ('66) first observed this enzyme in the 14 to 15, Boucek, Gyori and Alvarez ('66) in the five and Chieffi, Manelli, Botte and Mastrolia ('64b) in the four and one-half day old embryo.

Adrenal-pituitary axis

It is now known that adrenocorticotrophic hormone (ACTH) stimulates the growth and development of adrenal cortical tissue and the elaboration of adrenocortical hormones. The avian pituitary, like that of mammals contains ACTH (deRoos and deRoos, '64). Chemically pure ACTH has been prepared from the

pituitaries of a number of mammalian species, but little is known of the chemical nature of ACTH from avian pituitaries (Sturkie, '65). DeRoos and deRoos ('64) incubated avian adrenal tissue with mammalian ACTH and with acid extracts of chicken pituitaries and observed a significant increase in the secretion of corticosterone and aldosterone. Since the steroidogenic response of the chicken adrenal to the two hormone preparations was essentially the same they suggested that both hormones possess similar biological properties. These workers also showed that acid extracts of the pituitaries of several chondrichthyean fish stimulated corticoid production by the chicken adrenal in vitro and it therefore appeared to them that species specificity may be absent in the biological activity of ACTH obtained from different vertebrate pituitaries. They were not able to determine accurately the amount of ACTH activity present in the chicken pituitary, however, their "approximate" determinations were similar to the average yields reported by Fortier ('59) using a similar method of acid extraction in the laboratory rat.

Szekely and coworkers ('58) used Endroczi's ('54) quantitative bioassay for ACTH to detect ACTH in pituitary extracts from chick embryos on the eighth day of development.

Much work has been undertaken to determine the onset of pituitary control in the embryonic adrenal cortex. Toth, Simon and Szekely ('58) observed an increase in mitotic activity in the embryonic chicken adrenal cortex at the time of the initiation of ACTH production (eighth day), which returned to its original level within two days. However, hypophysectomized embryos showed no increase in mitotic activity during the eighth day. It was concluded that histological differentiation of the adrenal cortex was dependent on the anterior

pituitary as early as the eighth day of development but it was not established that the mitotic index is an adequate measure of functional activity (Castane Decoud, et al., '64).

Experiments have shown that the anterior pituitary exerts its adrenocorticotrophic effects during the last third of the embryonic period of the chick. Case ('51) noted a decrease in the adrenal ascorbic acid content in decapitated chick embryos and suggested that the pituitary exerted its adrenocorticotrophic influence by at least the 13th day. Case ('52) found that adrenal weights of normal and decapitated embryos did not differ during the 14th and 15th days. These weights were increasingly divergent after the 16th day. Adrenal weights of the decapitated embryos remained very close to the weights of the normal 15 day adrenal. Case also observed a decrease in the amount of lipid-containing cortical tissue in the adrenals of decapitated 18 day embryos. ACTH treatment in such embryos resulted in an increase in the amount of lipid-containing cortical tissue when compared with decapitated controls. Despite the apparent increase in cortical tissue, there was no apparent increase in the total size of the gland.

The differentiation of the duodenal epithelium, with particular reference to its phosphatase content, has been used as an indicator of cortical secretion (Moog, '59). Chick embryos injected with hydrocortisone (Moog and Richardson, '55) and ACTH (Moog and Ford, '57) showed an accelerated rate of differentiation of duodenal epithelium and a precocious production of alkaline phosphatase. Watterson and coworkers ('59) did not observe alkaline phosphatase in the duodenal epithelium of most of their hypophysectomized chick embryos at 16 to 20 days of incubation. In the few hypophysectomized specimens that showed this

enzyme, the amount observed was always less than that of normal 17 days embryos. These investigators conclude that their results indicate an ACTH control of the adrenal cortex by 16 days of incubation.

Since no chemically pure avian ACTH has thus far been prepared mammalian ACTH has been used for experimental work involving the effects of exogenous ACTH in the bird. Zarrow and coworkers ('62) demonstrated the ability of mammalian ACTH to stimulate growth of the avian adrenal gland. They treated 14 to 16 day old cockerels daily with one to ten USP units of porcine ACTH for seven days and observed a 40 to 193 percent increase in relative weights of the adrenal gland. Adrenal hypertrophy was also noted following ACTH administration in male chickens by Brown and coworkers ('58) and in the quail by Zarrow and Baldini ('52) and in baby chicks by Jailer and Boas ('50). Such adrenal weight increases were not seen in chicks by Conner ('59) or in quail by Flickinger ('59) following similar treatment. Moog and Ford ('57) treated 11 day chick embryos with ACTH and observed that the adrenals were significantly heavier than those of saline-injected controls and that this difference was maintained to 19 days, the latest stage studied.

Perek, Eckstein and Eshkol ('59) found that the adrenals of adult chickens were depleted of adrenal ascorbic acid following ACTH treatment, but those of young chicks or older sexually immature chickens were not. Bhattacharyya and coworkers ('67) observed a decrease in adrenal ascorbic acid in the adults of three species (pigeon, egret and myna) following treatment with ACTH. However, other investigators (Wolford and Ringer, '62; Howard and Constable, '58; Jailer and Boas, '50; Zarrow and Zarrow, '50; Zarrow and Baldini, '52) noted no significant differences in adrenal ascorbic acid content or concentration follow-

ing ACTH treatment.

Mazina ('65) observed a decrease in adrenal ascorbic acid content and concentration in 13 to 18 day old chick embryos three hours after a single injection of ACTH. The reduction in concentration was greater in treated 15 days and older embryos due to the increase in adrenal weight. No reduction in either total content or concentration of ascorbic acid was observed in the adrenals of two and five day old baby chicks after injection of ACTH. Six hours after the ACTH injection, adrenal ascorbic acid concentration was near normal in 13 and 14 day old embryos but still low in 15 to 18 day embryos. The weights of adrenal glands six hours after ACTH injection increased as did the ascorbic acid content. However, the increase in ascorbic acid lagged behind the increases in weight. Twelve hours following the injection, the ascorbic acid content was still high but the adrenal weights were within normal limits. These changes were still present but less pronounced after 24 hours but they had disappeared by the 48th hour.

Administration of ACTH results in a depletion of adrenal cholesterol in the young and adult chicken (Howard and Constable, '58; Siegel and Beane, '61; Wolford and Ringer, '62) and in the adult pigeon, egret and myna (Bhattacharyya, et al., '67). The depletion of adrenal cholesterol was believed to be the result of an accelerated conversion into cortical hormones (Bhattacharyya, et al., '67). Castane Decoud, Pedernera and Narbaitz ('64) injected chick embryos daily with ACTH during the third and fourth days of incubation. The embryos were sacrificed on the fifth and sixth day and their adrenals were studied histochemically for lipids (Sudan black B) and for cholesterol and its esters (birefringence). Both sudanophilia, which appeared on the fifth day,

and birefringence, which appeared on the sixth day, were increased in frequency and intensity following ACTH treatment. These investigators suggested two possible explanations for the accumulation of adrenal lipids and cholesterol: (1) In mammals, the injection of ACTH produces a preliminary stage of lipid and cholesterol depletion followed by a second stage of recuperation in which lipids and cholesterol accumulate (Sayers and Sayers, '48). Assuming that the embryonic chick adrenal responds to ACTH in a manner similar to that of the mammal, the chick embryos in the experiment were sacrificed during the second stage of recuperation. (2) The embryonic chicken adrenals in the early stages of development behaved differently from those of the adult chicken. Thus, their results indicate that the embryonic chicken adrenal is susceptable to ACTH stimulation in its early stages of differentiation.

Nagra and coworkers ('63) observed a decrease in the corticosterone level of adrenal venous blood of chickens hypophysectomized when 7.5 weeks of age. The intravenous administration of ACTH in such birds elevated the amount of corticosterone in the venous blood of adrenals. The incubation of avian adrenal tissue with mammalian ACTH significantly increased the secretion of corticosterone and aldosterone (deRoos and deRoos, '64).

Hall and Koritz ('66) incubated adrenal tissue from six week old chickens with cholesterol-7a-^{3}H with and without ACTH. The adrenal tissue converted cholesterol-7a-^{3}H into corticosterone-^{3}H and it was observed that this conversion was increased by the addition of ACTH.

Samuels and Helmreich ('56) used a biochemical method to detect a decrease in Δ^{5}-3B-HSD activity in homogenated adrenal glands from hypophysectomized rats. Levy et al., ('59) and Fuhrmann ('63) demonstrated a decrease in Δ^{5}-3B-HSD

activity in the adrenals of hypophysectomized rats and rabbits. They found that treatment of such animals with ACTH prevented or reversed the reduction of adrenal Δ^5-3B-HSD activity.

Cavallero and Chiappino ('62) noted an increase in Δ^5-3B-HSD activity in the human adrenal gland following exogenous ACTH treatment. Pearson and co-workers ('64) injected mice with ACTH and observed an increase in adrenal Δ^5-3B-HSD activity within four hours after a single injection and by 72 hours the enzyme activity returned to normal.

On the other hand, Rubin and Dorfman ('57) did not observe any significant change in the concentration of Δ^5-3B-HSD of homogenates of guinea pig adrenal glands incubated with ACTH while Freses et al., ('65) noted a reduction in Δ^5-3B-HSD activity in rat adrenals after ACTH administration.

Manelli and Mastralia ('64) observed the effects of ACTH <u>in vitro</u> on Δ^5-3B-HSD activity in the embryonic chicken adrenal. The adrenals of 12 day old embryos were cultured in media with and without ACTH. The reaction for Δ^5-3B-HSD was equal in ACTH treated and control adrenals cultured for six days. By the seventh day the reaction for the enzyme decreased in control adrenals but remained almost unvaried in the ACTH treated. After ten days of culturing, the reaction for Δ^5-3B-HSD was nearly absent in the control adrenal but very clearly positive in ACTH treated adrenals.

MATERIALS AND METHODS

Light-brown Leghorn eggs from a purebred colony maintained at the laboratory of Dr. Domm and white Leghorn eggs from a local commercial breeder were used in these experiments. The eggs were permitted to set at room temperature for at least 24 hours before they were placed in an incubator maintained at a temperature of 37.8±0.3°C. The age of the embryos at the time of sacrifice represents the actual time the eggs remained in the incubator. However, since there is a great deal of variation in degree of development of embryos of the same age, especially during the first week (Hamilton, '52), all embryos were staged according to the morphological characteristics as established by Hamburger and Hamilton ('51).

The purpose of the first part of this investigation was to determine the normal early adrenocortical development. Brown and white Leghorn embryos were sacrificed between 96 and 170 hours of incubation (Hamburger-Hamilton stages 21 through 30). After candling the egg to locate the embryo, a small hole was made through the shell and shell membranes on the side of the shell opposite the embryo (Harkmark and Graham, '51). The egg was turned so that the hole was uppermost and then returned to the incubator until the embryo had come to lie directly under the hole. A hole was then made in the shell and outer shell membrane at the blunt end of the egg in order to permit air to escape from the air cell and form a new air chamber beneath the inner shell membrane at the uppermost side of the egg. The embryo was thus lowered from the shell and shell membrane which could then be removed without injuring the embryo. Embryos were staged in the shell while still alive, and were then placed in

Bouin's fixative for 24 hours, dehydrated in ethanol and embedded in paraffin. Eight μ serial cross sections of the embryos were stained with hematoxylin and eosin.

The second part of this investigation was designed to study the development of adrenal Δ^5-3B-hydroxysteroid dehydrogenase (Δ^5-3B-HSD) activity in brown and white Leghorn embryos. These embryos, between 94 and 141 hours of incubation, were also staged according to the above procedure. The embryos were removed from the shell and immediately quick frozen in an extended position on the freezing stage of a cryostat maintained at -30°C. The frozen embryos were then wrapped in multiple layers of plastic wrap and stored in sealed plastic vials at -30°C until they were sectioned.

The technique for the histochemical visualization of Δ^5-3B-HSD activity employed in this investigation was a minor modification of Levy, Deane and Rubin's ('59) modification of Wattenberg's method ('58). Wattenberg incubated unfixed frozen sections in a medium containing the substrate (dehydroepiandrosterone, pregnenolone and others) dissolved in acetone, plus diphosphopyridine nucleotide (DPN), nicotinamide, a tetrazolium salt (Nitro-BT) and buffer (pH 8.0). Hydrogen ions removed from the substrate by Δ^5-3B-HSD are transferred to DPN forming DPNH, then from DPNH to the tetrazolium salt. The tetrazolium salt is colorless and water soluble in the unreduced form, but when reduced it becomes colored and insoluble, and is deposited in the form of fine formazan granules at the site of the enzyme reaction.

Levy and coworkers ('59) modified Wattenberg's method by using propylene glycol as the substrate solvent, lowering the pH of the buffer (pH 7.1-7.4) and changing the concentration of DPN, Nitro-BT and the substrate.

Frozen embryos were mounted on a microtome chuck and sectioned at 10μ (temperature -20°C) on a Cryo-cut cryostat microtome. Spaced serial sections were mounted on three consecutive No. 1 coverslips by thawing so as to have three almost identical coverslips. All sections were air dried for 15 minutes at room temperature in order to attach the sections to the coverslips.

The following technique was employed for the observation of Δ^5-3B-HSD activity. Sectioned embryos were placed in 0.1M phosphate buffer, pH 7.1, for 15 minutes at room temperature to remove endogenous substrates (Levy, et al., '59). The sections on two of the coverslips (experimental sections) were then incubated for two hours in Columbia jars containing the substrate medium. Dehydroepiandrosterone (DHA) was used as the substrate for most of the embryos examined, however, pregnenolone was used for some. Acetone and propylene glycol were both used to solubilize the substrate. When propylene glycol was used as the solvent, the substrate dissolved in acetone was put in a dry Columbia jar and the acetone evaporated off prior to the addition of propylene glycol and the other constituents. The composition of the substrate medium was as follows:

Constituent	Volume	Weight	Final Concentration
DHA			0.0988 mM
or		0.2 mg	
Pregnenolone			0.0903 mM
DPN soln., 3.00 mg/ml	0.8 ml	2.4 mg	0.0518 mM
Nitro-BT soln., 1 mg/ml	1.0 ml	1.0 mg	0.175 mM
Phosphate buffer, 0.1M pH7.1	4.4 ml		0.057 M
Acetone			0.974 M
or	0.5 ml		
Propylene glycol			0.978 M

The sections of the third coverslip (controls) were incubated for two hours in a control media which lacked the substrate.

Following incubation sections were fixed for 15 minutes in a mixture consisting of 10% formalin, 5% glacial acetic acid, 60% ethyl alcohol and 25% distilled water. They were then rinsed in distilled water to remove residual fixative. Control sections and one coverslip with experimental sections were stained for 10 minutes in Grenacher's Alum Carmine. The experimental sections on the second coverslip remained unstained. After a second wash in distilled water all sections were dehydrated in a graded series of alcohols, cleared in xylene and mounted with Harleco synthetic resin (HSR). Sections treated in this manner show embryonic cells with carmine stained nuclei and fine, dark-blue, formazan granules.

The intensity of the formazan deposition in the embryonic adrenal was rated according to a 0 to +6 scale, where 0 was no formazan deposition and +6 an extremely heavy deposition. An attempt was also made to determine the optical density of the formazan deposition in the embryonic adrenocortical mass, of unstained experimental sections, of some embryos, with the use of a photometer (Densichron 1, Welch Scientific Company, Skokie, Illinois).

The third part of this investigation was to determine the effects of exogenous ACTH on adrenal Δ^5-3B-HSD activity in the young brown Leghorn embryo. At 48 hours incubation, a small hole was made through the shell and shell membranes, in the manner described above, so as to have an air chamber beneath the inner shell membrane and over the embryo. Embryos of such eggs were injected with one International Unit (IU) of ACTH (Castane Decoud, et al., '64) at 24 hour intervals starting on the 48th hour of incubation and continu-

ing through the 96th. Porcine ACTH (Corticotropin-gel 40 IU per cc., Wilson Laboratories, Chicago, Illinois) was administered to the embryonic vascular area through the small hole in the shell above the embryo. Controls consisted of: (1) normal embryos, (2) embryos receiving saline injections, and (3) manipulated embryos (those receiving all manipulations to which treated were subjected except actual injection). After each injection the hole was sealed with cellophane tape and the egg returned to the incubator. Embryos were staged between 93 and 119 hours of incubation then sacrificed by quick freezing on the freezing stage of the cryostat. Embryos sacrificed between 93 and 96 hours received only two ACTH injections as did a few between 97 and 101 hours. Frozen cross sections of the embryos were examined histochemically for Δ^5-3B-HSD activity using the above technique. The medium substrate was DHA for all embryos examined in this part of the investigation.

Photomicrographs were made using an American Optical Microstar microscope with photographic attachment and 35 mm Kodachrome and Kodacolor film.

OBSERVATIONS

Early development of the adrenal gland

A total of 12 brown Leghorn and 17 white Leghorn embryos representing Hamburger-Hamilton ('51) stages 21 through 30 were studied in this part of the investigation.

Our observations indicated a lack of uniformity in development of embryos of the same chronological age (incubation time). This was especially true of the embryos of the earlier stages studied (table 1). The morphological stages for embryos incubated 96 hours was 21 through 25 (84 to 120 hours). With increased incubation there was a closer correlation between chronological age and morphological age.

There was a significant difference in the degree of adrenal development in embryos of the same chronological age but no significant difference in those of the same morphological age. No difference was observed in the development of the adrenal glands between brown and white Leghorn embryos of the same morphological age.

In stage 21 embryos (fig. 1) at the level of the origin of the omphalomesenteric artery, there was a thickening of the peritoneal epithelium lateral to the mesentery and usually immediately ventral to the subcardinal veins. A groove was generally present in the thickened peritoneum. Cells which eventually form the adrenal cortex were proliferated from this thickened peritoneal epithelium and were seen in the mesenchyme immediately dorsal to the epithelium. These cells were round in shape and larger than the peritoneal cells

and their cytoplasm and nuclei stained lighter than those of the peritoneum.

By stage 22 (figs. 2, 3) some of these proliferated cells had begun migrating dorsally to a point in the mesenchyme dorsal to the subcardinal vein and between the dorsal aorta and mesonephros. The number of proliferated cells continued to increase so that by stage 23 (figs. 4, 5) there was a chain of cortical cells lateral to the subcardinal vein and extending from the groove in the peritoneal epithelium to a point in the mesenchyme, ventrolateral to the dorsal aorta and ventromedial to the mesonephros, where they formed scattered groups composed of two or three cells. A few cells were seen on the medial side of the subcardinal vein. The dorsal extent of the cortical groups was the ventral level of the aorta and they extended posteriorly past the origin of the omphalomesenteric artery. The number of cells in the scattered groups at the dorsal extent of their migration increased to three or four. Numerous mitotic figures were seen among the cortical cells. Deeply stained chromaffin cells were observed in the mesenchyme between the aorta and mesonephros, medial to the cortical cells.

In stage 24 embryos (figs. 6, 7) a connection between the cortical cells in the mesenchyme and the thickened peritoneal epithelium was still evident. The position of the cortical cell groups in the mesenchyme remained, relatively, the same as in stage 23 with the exception that the dorsal extent of these groups was the level of the lower one-third of the aorta. In embryos of this stage there was a small anastomosis between the right and left subcardinal veins, and it was to this level that the cells extended posteriorly. Both the number of cells in the cell groups and the number of cortical cells undergoing mitosis had increased at this stage.

There was no connection between the cortical cells in the mesenchyme and the peritoneal epithelium in stage 25 embryos. The location of the cortical cells remained the same but the size of the cortical cell mass had enlarged (fig. 8). The cortical cell groups now contained as many as eight or nine cells.

The cortical cell groups of stage 26 embryos (fig. 9) contained more cells than those of previous stages and these were situated medial to the mesonephros and ventrolateral to the aorta. The change in position with respect to the mesonephros was due to development of the mesonephros in a ventral direction. A few mesenchymal cells separated the cortical cell groups from one another. The cortical mass extended slightly dorsal to the mesonephric arteries which passed through this mass. Groups of chromaffin cells were observed on the medial surface of the cortical cell groups.

The cortical cell groups of stage 27 embryos (fig. 10) formed solid bodies in the mesenchyme between the aorta and the mesonephros. The cortical mass extended craniad to the posterior tip of the lung and caudad to the anastomosis of the subcardinal veins. The cross sectional area of this mass was slightly larger than that of the aorta. Chromaffin cells were observed on the medial surface of the cortical mass and inside this mass between the cortical cell groups.

The number of cells in the cortical groups was much greater by stage 28 (figs. 11, 12). The first indication of the formation of the adrenal capsule was seen at this stage. A large number of cortical cells were undergoing mitosis and numerous chromaffin cells were seen within the cortical mass separating the cortical cell groups.

The cortical cells had started to form cords by stage 29 (fig. 13). In cross section the latter were circular to oval and had about six or seven cells arranged radially. There was no lumen in any of the cords.

The number of cells in cross sections of cortical cords of stage 30 embryos (fig. 14) had increased to about ten or 12 cells. The presence of blood cells in the spaces between the cords indicated the beginning of vascularization. The connective tissue capsule was more prominent at this stage.

Development of adrenal $\triangle^5$-3B-HSD activity

Fourteen brown and 16 white Leghorn embryos were examined histochemically for the presence of adrenal $\triangle^5$-3B-HSD activity. A series of pilot studies was undertaken to determine the optimal buffer pH and the optimal incubation time. Sectioned embryos were incubated in media containing buffers with pH values ranging from 7.08 to 7.71. The optimal buffer pH was found to be 7.10 and the optimal length of incubation two hours.

Histochemical method for $\triangle^5$-3B-HSD using DHA as the substrate

Scattered deposits of formazan were observed in the adrenocortical cells of stage 23 brown and white Leghorn embryos. These deposits were found to increase in amount and density with increased development (table 2). The intensity of the formazan deposition was rated according to the 0 to +6 scale described previously.

Due to difficulty in distinguishing migrating adrenocortical cells from mesenchymal cells in frozen sections, we were not able to definitely locate formazan in these primordial adrenocortical cells. Formazan deposits of +1 intensity were observed in the cells of the peritoneal epithelium and in the region where the migrating adrenocortical cells would normally be located in

stage 22 embryos. Adrenocortical cells were observed in stage 23 embryos and these cells contained formazan deposits of +2 intensity in both brown and white Leghorn embryos (fig. 15). The adrenal was larger in stage 24 than in 23 embryos and showed a formazan intensity of +3 in the brown Leghorn embryo (fig. 19) and +2 in the white Leghorn (fig. 21). The formazan deposition intensity ranged from +4 to +5 in stage 25 brown Leghorn embryos. The size of the formazan granules at this stage ranged from very fine deposits, which gave the cytoplasm a light blue color, to large, oblong, crystal-like deposits which were dark blue in color (fig. 22). The formazan intensity of the stage 25 white Leghorn adrenocortical cells ranged from +2 to +3 and was +3 at stage 26. At stage 28 both the brown and white Leghorn adrenocortical cells had a formazan intensity of +5 and contained both very fine and large oblong formazan granules.

An attempt was made to determine the optical density of the adrenal formazan deposition of unstained sections using the Densichron 1 photometer but this instrument was not sufficiently sensitive to detect slight changes in optical density.

In addition to the formazan deposition in the adrenals, a generalized pattern of formazan granule deposition was observed in several other tissues of embryos of stages 22 through 25. Formazan granules of an intensity of +1 or +2 were present in the ependymal layer of the neural tube, intestinal epithelium, peritoneal epithelium, mesonephros and mesenchyme tissue. With advancing development, the intensity of the deposition in the mesenchyme decreased but it remained about the same or increased in the other tissues.

Control sections incubated in media lacking the substrate but containing

the substrate solvent propylene glycol showed an absence of formazan deposition in all cases. Control sections incubated in media lacking the substrate but containing the substrate solvent acetone occasionally had a general formazan deposition of about +1 intensity in the ependymal layer of the neural tube, intestinal epithelium, peritoneal epithelium, mesonephros, mesenchymal tissue as well as in the adrenocortical cells. All embryos which had positive control sections with the acetone control media had experimental sections in which the adrenal formazan intensity was +2 or greater.

Histochemical method for $\triangle^5$-3B-HSD using pregnenolone as the substrate

Some brown Leghorn embryos were examined histochemically for the presence of $\triangle^5$-3B-HSD using pregnenolone as the medium substrate (table 2). Propylene glycol was used as the substrate solvent in all cases. Formazan granules were first observed in the adrenocortical cells at stage 23. These were seen in only a few sections of the adrenal and when present had an intensity of only +1 (fig. 25). This was also the case in the adrenals of stage 25 embryos. However, at stage 27 the adrenal formazan deposition intensity was +5 and the size of the granules ranged from very fine and round, to large oblong and crystal-like (fig. 27).

A generalized pattern of formazan granule deposition as seen with DHA was not observed at stages 23 and 25. Formazan deposits were observed in the peritoneal epithelium and intestinal epithelium of stage 27 embryos when pregnenolone was used but not in the ependymal layer of the neural tube, mesonephros or mesenchymal tissue as seen with DHA. Control sections showed an absence of formazan granules in all cases.

Effects of exogenous ACTH on adrenal $\triangle^5$-3B-HSD activity

Forty four brown Leghorn embryos ranging in age from stage 22 to 25 were studied in this part of the investigation. Twenty six were treated with ACTH, four with saline, three manipulated (those receiving all manipulations to which treated were subjected except actual injection) and 11 kept intact. The saline treated, manipulated and intact embryos served as controls. All embryos were examined histochemically for the presence of Δ^5-3B-HSD using DHA as the medium substrate.

Stage 22 embryos. We were unable to make a positive identification of adrenocortical cells in frozen sections of embryos at this stage. The embryos were sacrificed before 96 hours of incubation and, therefore, the experimental group did not receive a third ACTH injection (table 3). A total of 22 hours elapsed between the last injection and sacrifice. Formazan granules of +1 intensity were observed in the region where migrating adrenocortical cells would normally be located. There appeared to be no increase in formazan deposition in this area 22 hours after the last ACTH injection.

Stage 23 embryos. The adrenocortical cells of seven treated and six control stage 23 embryos were examined histochemically for the presence of Δ^5-3B-HSD activity. Each treated embryo received an injection of 1 IU of ACTH at 48 hours incubation. Six embryos received a second injection of ACTH at 72 hours and three a third at 96 hours incubation (table 4). All embryos were sacrificed between 93 and 101 hours of incubation. The period between the last injection and the time of sacrifice was noted.

At stage 23, evidence of adrenal Δ^5-3B-HSD activity was observed in the adrenocortical cells of intact control embryos which had a formazan deposition of +2 (fig. 15). Formazan depositions of +2 and +3 were observed in the

adrenals of manipulated and saline treated control embryos, respectively. The intensity of the adrenal formazan deposition of ACTH treated embryos varied from +2 to +5 depending on the time interval between the last ACTH injection and the time of sacrifice. The adrenals of embryos sacrificed one to two hours after the last injection contained a formazan deposition of +2 intensity. Five hours after the last injection, the intensity had increased to +5 (fig. 16)and 21 to 46 hours post injection the formazan intensity was +3.

Stage 23-24 embryos. One treated and two control embryos were sacrificed at a stage between 23 and 24 (table 5). The treated embryo received 1 IU of ACTH at 48, 72 and 96 hours of incubation and was sacrificed three hours after the last injection. The adrenal formazan deposition intensity of this treated embryo was +5 (fig. 18) while that of the two intact control embryos was +3 (fig. 17).

Stage 24 embryos. Nine treated and four control embryos were sacrificed at stage 24. Eight of the treated received 1 IU of ACTH at 48, 72 and 96 hours of incubation and one received 1 IU of ACTH at 48 and 72 hours incubation. The embryos were sacrificed between one and 22 hours after the final ACTH injection (table 6).

A formazan deposition intensity of +2 to +3 was observed in the adrenals of the control embryos (fig. 19). The adrenals of treated embryos sacrificed one to two hours after the final injection of ACTH contained a formazan deposition intensity equal to that of the controls. A large increase in adrenal formazan deposition intensity was observed in embryos sacrificed three to eight hours after the last injection. These adrenals had cells showing a deposition of +4 to +5 and had formazan granules ranging in size from very

fine to large, oblong and crystal-like ones (fig. 20). The formazan in embryos sacrificed 21 or 22 hours after the final injection was +2.

Stage 25 embryos. Eight treated and five control embryos were sacrificed at stage 25. All treated embryos received 1 IU of ACTH at 48 and 72 hours incubation and five treated embryos received a third ACTH injection at 96 hours (table 7).

Intact and saline treated controls had an adrenal formazan intensity of +4 to +5 (fig. 22). A formazan intensity of +4 to +5 was also observed in the adrenals of embryos sacrificed four to 11 hours after the last ACTH injection (fig. 23). The intensity in embryos sacrificed between 22 and 28 hours after the last injection was +2 to +3 which was below the level of the controls. The formazan deposits of the adrenal cells of the treated and control embryos ranged from very fine and round to large, oblong and crystal-like granules.

DISCUSSION

The purpose of this investigation was to determine the effects of exogenous ACTH on Δ^5-3B-hydroxysteroid dehydrogenase activity in the embryonic adrenal of the domestic fowl before the pituitary-adrenal axis was established.

Previous investigators who have studied the adrenals of the chick embryo (Hays, '14: Sivaram, '64, '65; Chieffi, et al., '64a, '64b; Narbaitz and Kolodny, '64; Boucek, et al., '66; Straznicky, et al., '66; and others) used chronological age, or total incubation time, to determine the age of embryos. Hamilton ('52) showed that there is a great deal of variation in the degree of development of embryos of the same age, especially during the first week of development. Hamburger and Hamilton ('51) list eight factors which may contribute significantly to variability in the rate of development of the chick embryo. Hence, a comparison of observations from various laboratories using chronological, rather than morphological, age must be made with caution even though local conditions have been rigorously standardized (Dawson, '53). Therefore, it was necessary to undertake a preliminary study of the early development of the embryonic chick adrenal using the morphological characteristics established by Hamburger and Hamilton ('51) to determine developmental age.

Our observations revealed a lack of uniformity in the development of embryos of the same chronological age (table 1) and we also observed a significant difference in the degree of adrenal development of embryos of the same chronological age. However, no such difference was observed in adrenal devel-

opment of embryos of the same morphological age. Furthermore, we found no significant difference in the development of the adrenals in the two varieties of Leghorn fowl studied.

The thickened peritoneal epithelium, from which the primordial adrenocortical cells are formed, was present in stage 21 embryos (3½ days, 84 hours), the youngest embryos studied in this investigation. Rabl (1891) and Soulie ('03) first observed cortical cells in 78 hour chick embryos. However, Hays ('14), Willier ('30) and Sivaram ('65), did not observe these cells prior to the fourth day of incubation (96 hours).

A few of the primordial cortical cells had completed their migration from the peritoneal epithelium to a point in the mesenchyme dorsal to the subcardinal vein and between the aorta and mesonephros by stage 22 (84 to 96 hours) and had formed scattered groups of cells in the mesenchyme by stage 23 (96 hours). The dorsal migration of these cells was completed by stage 25 (108 to 120 hours). Hays ('14) observed completion of migration after 120 hours. Our observations on development of the adrenals between stage 26 (120 hours) and stage 30 (156 to 168 hours) were generally in agreement with those of other investigators who used chronological rather than morphological age to stage their embryos.

By using steroid dehydrogenase histochemical techniques, it is possible to identify the cells with steroidogenic potential. The enzyme Δ^5-3B-hydroxysteroid dehydrogenase (Δ^5-3B-HSD) is responsible for the transformation of pregnenolone to progesterone and dehydroepiandrosterone (DHA) to Δ^4-androstenedione. This enzyme has been identified in the adrenals of the chick embryo but there is at present no general agreement as to the time at which

it first occurs. Straznicky, Hajos and Bohus ('66) first observed this enzyme in 14 to 15 day old chick embryos, Sivaram ('64) at ten days, Boucek, Gyori and Alvarez ('66) at five days, and Chieffi, Manelli, Botte and Mastrolia ('64b) at four and one-half days. The results of our investigation, in which a modification to the technique of Levy, Deane and Rubin ('59) was used, revealed the presence of Δ^5-3B-HSD in the adrenocortical cells of stage 23 (4 days) brown and white Leghorn embryos. This was the stage in which some of the adrenocortical cells, having completed their migration from the peritoneal epithelium, were located as scattered groups in the mesenchyme dorsal to the subcardinal vein and between the aorta and the mesonephros. Formazan granules, indicating the presence of Δ^5-3B-HSD activity, were observed in the cells of the peritoneal epithelium and in those of the mesenchyme between the peritoneal epithelium and the scattered groups of adrenocortical cells. Owing to difficulties in identifying individual cell types in frozen sections, we were unable to determine if these granules were associated with the migrating adrenocortical or the mesenchymal cells. Since the adrenocortical cells arose from the peritoneal epithelium which revealed Δ^5-3B-HSD activity, it would not be surprising to find this enzyme in the migrating adrenocortical cells. The presence of the enzyme in the adrenals of the 12 day mouse embryo suggested steroid biosynthesis in the adrenocortical anlage as soon as it became recognizable (Hart, '66).

Although there appeared to be no difference in the development of the embryonic adrenals of the brown and white Leghorn, there nevertheless was a difference in the development of adrenal Δ^5-3B-HSD activity. The enzyme was evident in the adrenals of both varieties of Leghorn at stage 23 at which time

a reaction intensity of +2 was observed (table 2). However, a maximum intensity of +5 was seen in the adrenals of stage 25 brown Leghorn embryos whereas in the white the maximum intensity did not occur until stage 28.

The adrenocortical cells of stage 23 and 25 embryos revealed Δ^5-3B-HSD activity when DHA and pregnenolone were employed as substrates but the intensity of the formazan deposition was considerably less with pregnenolone than with DHA (figs. 25, 26). However, at stage 27 the reverse was true since there was greater intensity with pregnenolone than with DHA (figs. 27, 28). This difference may be the result of a poor solubility of pregnenolone (Levy et al., '59). It could also be the result of the presence of two enzymes rather than one. Baillie and Griffiths ('64) postulated the existence of different substrate-specific 3B-hydroxysteroid dehydrogenases in the Leydig cells of the fetal mouse. Their results suggested that in these cells the 3B-hydroxysteroid dehydrogenase concerned in the conversion of pregnenolone to progesterone was the first to develop at about 11 days followed five days later by the enzyme concerned with the metabolism of DHA to Δ^4-androstenedione. The results of Boucek et al. ('66) also suggest the presence of two enzymes in the gonad tissues of the chick embryo.

Boucek and coworkers ('66) also found the first evidence of adrenal Δ^5-3B-HSD activity in 6μ sections of five day embryos using pregnenolone dissolved in acetone-buffer as the substrate. The five day embryo is about stage 26 or 27. In our investigation we found evidence of Δ^5-3B-HSD in 10u sections of stage 23 and 25 embryos using pregnenolone dissolved in propylene glycol as the substrate, however, the reaction intensity was very low (+1) and was not present in every section of the adrenal. At stage 27 (5 to 5½ days) the in-

tensity of the reaction was +5 and here it was evident in every section. The presence of acetone in the media, the thickness of the tissue sections and the length of incubation may be key factors in detecting small amounts of the enzyme.

Wattenberg ('58) and Levy et al. ('59) washed tissue sections in acetone to give a more accurate enzyme localization by removal of lipids. They observed a finer deposition of formazan in acetone treated sections since crystallization did not occur on the surface of lipid droplets but Levy and coworkers also observed a decrease in Δ^5-3B-HSD activity in rat tissues treated with acetone, as did Boucek et al. ('66) in chick embryos. For this reason the tissues were not washed in acetone in our investigation. Since lipid droplets were found to be first evident in the adrenals of five day chick embryos (Castane Decoud et al., '64) there was no need to wash the tissue sections in acetone. The formazan granules in adrenals of stage 23 and 24 control embryos were generally fine and round but those of stage 25 and older embryos ranged from fine and round to large and oblong. The morphological age of stage 25 embryos is four and one-half to five days. We came to the conclusion that the variation in size and shape of the formazan granules in the adrenals of stage 25 embryos was due to formazan crystallization on the surface of lipid droplets. These droplets were subsequently removed from the tissue in the process of clearing when the sections were being mounted thus leaving the formazan deposition in the tissue.

When acetone was used as the substrate solvent, formazan granules were observed in the control sections. This was the "nothing dehydrogenase" reaction of Pearse ('61). This difficulty was eliminated by using propylene

glycol instead of acetone as the solvent.

The results of the administration of ACTH on adrenal $\triangle^5$-3B-HSD activity are listed in tables 3, 4, 5, 6 and 7. This activity in the adrenals of embryos sacrificed one to two hours after the final ACTH injection was equal to that of controls. Embryos of stages 23, 23-24 and 24 sacrificed three to eight hours after the last injection showed a noticeable increase in enzyme activity. This activity in stage 25 control embryos was equal to that of stage 25 treated embryos sacrificed four to 11 hours after the final injection of ACTH. The enzyme activity of stage 23 and 24 embryos sacrificed 21 to 46 hours following terminal injection had returned to the control level while the activity of stage 25 embryos had decreased to a level below that of the controls.

It is noteworthy to call attention to the similarity in results of this investigation on the chick embryo with those of Pearson et al. ('64) in the mouse. These investigators injected mice with ACTH and noted an increase in adrenal $\triangle^5$-3B-HSD activity four hours later with a return to a level slightly below normal when examined at 18 and 72 hours.

SUMMARY AND CONCLUSIONS

The early development of the adrenals was studied in brown and white Leghorn chick embryos. A significant difference in the degree of development was observed in embryos of the same chronological age but not between those of the same morphological age. No difference in adrenal development between brown and white Leghorn embryos of the same morphological age was detected.

Primordial adrenocortical cells were found just dorsal to the peritoneal epithelium in stage 21 embryos (84 hours), the youngest stage studied. By stage 23 (96 hours) some of these cells had migrated to a point in the mesenchyme dorsal to the subcardinal veins and between the aorta and the mesonephros. By stage 25 (108 to 120 hours) the dorsal migration of these cells was complete.

Development of adrenal $\triangle^5$-3B-hydroxysteroid dehydrogenase ($\triangle^5$-3B-HSD) activity was studied in both varieties of Leghorn embryos using a modification of the histochemical technique of Levy et al. ('59). Pregnenolone and dehydroepiandrosterone (DHA) were employed as the substrates. Activity of the enzyme $\triangle^5$-3B-HSD was observed in the adrenocortical cells of stage 23 embryos with both substrates in both varieties of Leghorn.

A significant difference in the development of $\triangle^5$-3B-HSD activity was seen between brown and white Leghorn embryos. This activity in the former reached its maximum intensity before that of the latter. In brown Leghorn embryos of stages 23 and 25, the intensity of the formazan deposition was less with pregnenolone than with DHA as the substrate, whereas, at stage 27 it was

greater with pregnenolone than with DHA. This difference was believed to result from the presence of two enzymes rather than one.

Porcine ACTH was found to effect Δ^5-3B-HSD activity in the adrenocortical cells of stage 23 through 25 brown Leghorn embryos. Embryos of stages 23, 23-24 and 24, sacrificed three to eight hours after receiving the last of three injections of ACTH, at 48, 72 and 96 hours, showed a significant increase in the activity of this enzyme. However, this activity in stage 25 control embryos was equal to that of stage 25 treated embryos sacrificed four to 11 hours after the final injection, probably due, in part, to its conspicuous increase in normals at this stage. The enzyme activity of stage 23 and 24 embryos, sacrificed 21 to 46 hours following injection, had returned to the control level whereas that of stage 25 treated embryos had decreased to a level below that of controls.

It is tentatively concluded that the adrenocortical cells of the chick embryo have the capacity for steroid hormone synthesis by stage 23 (96 hours). This is the stage at which some of these cells have completed their migration from the peritoneal epithelium to a point in the mesenchyme between the mesonephros and the aorta. Treatment of such embryos with exogenous ACTH, resulted in an increase in adrenal Δ^5-3B-HSD activity, indicating that the adrenals have the potential to respond to this hormone prior to the establishment of the pituitary-adrenal axis.

LITERATURE CITED

Arrington, L. R., M. H. Fox and H. A. Bern 1952 Androgen content of testis and adrenal of white Leghorn cockerels. Endocrinology, 51: 226-236.

Ashbel, R., and A. M. Seligman 1949 A new reagent for the histochemical demonstration of active carbonyl groups. A new method for staining ketonic steroids. Endocrinology, 44: 565-583.

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

Bhattacharyya, T. K., A. K. Sarkar, A. Ghosh and A. Ganguli 1967 A comparative study on avian adrenocortical response to exogenous and endogenous corticotropin. J. Exp. Zool., 165: 301-307.

Bongiovanni, A. M., and A. W. Root 1964 The adrenogenital syndrome. New Eng. J. Med., 268: 1283-1289, 1342-1351, 1391-1399.

Boucek, R. J., E. Gyori and R. Alvarez 1966 Steroid dehydrogenase reactions in developing chick adrenal and gonadal tissues: A histochemical study. Gen. Comp. Endocrinol., 7: 292-303.

Brown, K. I., D. J. Brown and R. K. Meyer 1958 Effect of surgical trauma, ACTH and adrenal cortical hormones on electrolytes, water balance and gluconeogenesis in male chickens. Am. J. Physiol., 192: 43-50.

Case, J. F. 1951 Influence of pituitary on adrenal chemodifferentiation in the chick embryo. Anat. Rec., 109: 277-278.

_____ 1952 Adrenal cortical-anterior pituitary relationships during embryonic life. Ann. N. Y. Acad. Sci., 55: 147-158.

Castane Decoud, A., E. Pedernera and R. Narbaitz 1964 Accion del ACTH sobre la suprarrenal del embrion de pollo. Rev. Soc. Argent. Biol., 40: 181-186.

Cavallero, C., and G. Chiappino 1962 Histochemistry of steroid-3B-01 dehydrogenase in the human adrenal cortex. Experientia, 18: 119-120.

Chester Jones, I., J. G. Phillips and D. Bellamy 1962 The adrenal cortex throughout the vertebrates. Brit. Med. Bull., 18: 110-113.

Chieffi, G., H. Manelli, V. Botte and L. Mastrolia 1964a Il differenziamento istochimico dell'interrenale e dei tessuti somatici della gonade embrionale di pollo: compoitamento della steroide 3B-olo-deidrogenasi.

Acta Embryol. Morphol. Exptl., 7: 89-91.

_____ 1964b Osservazioni sul precoce differenziamento funzionale dell' interrenale nell'embrione di pollo. Atti Accad. Naz. Linci, Ser, 8, 37: 82-85.

Conner, M. H. 1959 Effect of various hormone preparations and nutritional stress in chicks. Poultry Sci., 38: 1340-1343.

Dawson, A. B. 1953 Histochemical evidence of early differentiation of the suprarenal gland of the chick. J. Morph., 92: 579-595.

deRoos, R. 1961 The corticoids of the avian adrenal gland. Gen. Comp. Endocrinol., 1: 494-512.

deRoos, R., and C. C. deRoos 1964 Effects of mammaliam corticotropin and chicken adrenohypophysial extracts on steroidogenesis by chicken adrenal tissue in vitro. Gen. Comp. Endocrinol., 4: 602-607.

Dorfman, R. I. 1959 Comparative biochemistry of adrenocortical hormones. In Comparative Endocrinology. Editor, A. Gorbman, pp. 613-623. John Wiley & Sons, Inc., New York.

Endroczi, E. 1954 A new biological method for adrenocorticotropic hormone (ACTH) estimation. Acta Physiol. Hung., 6: 397-401.

Fevold, H. R., and K. B. Eik-Nes 1962 Progesterone metabolism by testicular tissue of the English sparrow (Passer domesticus) during the annual reproductive cycle. Gen. Comp. Endocrinol., 2: 506-515.

_____ 1963 Progesterone metabolism by testicular tissue of the English sparrow (Passer domesticus). Gen. Comp. Endocrinol., 3: 335-345.

Flickinger, D. D. 1959 Adrenal responses of California quail subject to various physiological stimuli. Proc. Soc. Exp. Biol. Med., 100: 23-25.

Fortier, C. 1959 Note on the extraction and storage of the rat pituitary prior to invitro assay for ACTH. Can. J. Biochem. Physiol., 37: 608-611.

Freses, A. T., J. Diaz and J. Falen 1965 Efecto de ACTH, dexzmetusona y progesterona sobre la actividad de 3B-ol-esteroide deshidrogenasa de la suprarrenal de rata albino. Estudio histoquimico. Rev. Iber. Endocrinol., 12: 5-14.

Fuhrmann, K. 1963 Histochemical investigation on hydroxysteroid dehydrogenase activity in the adrenal and ovary of the rat and rabbit. Arch. Gynaekol., 197: 583-600.

Gorbman, A., and H. A. Bern 1962 In: A Textbook of Comparative Endocrinology. pp. 230-246, John Wiley and Sons, Inc., New York.

Grant, J. K. 1962 Studies on biogenesis of the adrenal steroids. Brit. Med. Bull., 18: 99-105.

Hall, P. F., and S. B. Koritz 1966 Action of ACTH upon steroidogenesis in the chicken adrenal gland. Endocrinology, 79: 652-654.

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 In: Lillie's Development of the Chick. Henry Holt and Company., New York.

Harkmark, W., and K. Graham 1951 A method for opening and closing eggs with an experimental demonstration of its advantages. Anat. Rec., 110: 41-48.

Hart, D. M. 1966 Hydroxysteroid dehydrogenase in mouse adrenal and gonads. J. Anat., 100: 801-812.

Hartman, F. A., and K. A. Brownell 1949 In: The Adrenal Gland. Lea and Febiger, Philadelphia.

Hays, V. J. 1914 The development of the adrenal glands of birds. Anat. Rec., 8: 451-474.

Howard, A. N., and B. J. Constable 1958 The metabolism of adrenocorticotrophic hormone and ascorbic acid in the chick. Biochem. J., 69: 501-505.

Jailer, J. W., and N. F. Boas 1950 The inability of epinephrine or adrenocorticotrophic hormone to deplete the ascorbic acid content of the chick adrenal. Endocrinology 46: 314-318.

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

Lillie, R. D. 1966 In: Histopathologic Technic and Practical Histochemistry. McGraw-Hill Book Co., New York.

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. Gynec., 83: 295-299.

Manelli, H., and L. Mastrolia 1964 Azione dell'ACTH e reazione della steroide 3B-olodeidrogenasi nelle surrenali embrionali di pollo coltivate in vitro. Atti Accad. Naz. Linci, Ser. 8, 35: 598-602.

Mazina, T. I. 1965 Effects of ACTH and insulin injections on ascorbic acid content of chick embryo adrenals. Fed. Proc. (Trans. Suppl.), 24: T387-T389.

McGowen, J. P. 1936 Suprarenal virilism in a domestic hen: Its possible significance. J. Exp. Biol., 13: 377.

Moog, F. 1959 The development of function in the adrenal cortex. In: Comparative Endocrinology. Editor, A. Gorbman, pp. 624-638. John Wiley and Sons, Inc., New York.

Moog, F., and E. Ford 1957 Influence of exogenous ACTH on body weight, adrenal growth, duodenal phosphatase, and liver glycogen in the chick embryo. Anat. Rec., 128: 592.

Moog, F., and D. Richardson 1955 The influence of adrenocortical hormones on differentiation and phosphatase synthesis in the duodenum of the chick embryo. J. Exp. Zool., 130: 29-56.

Nagra, C. L., J. G. Birnie, G. J. Baum and R. K. Meyer 1963 The role of the pituitary in regulating steroid secretion by the avian adrenal. Gen. Comp. Endocrinol., 3: 274-280.

Narbaitz, R., and L. Kolodny 1964 Δ^5-3B-hydroxysteroid dehydrogenase in differentiating chick gonads. Zeit. Zellforsch., 63: 612-617.

Pearse, A. G. E. 1961 Diaphorases and dehydrogenases. In: Histochemistry: Theoretical and Applied, 2nd ed. pp. 583-584, Little Brown and Co., Boston, Mass.

Pearson, B., P. Wolf, F. Grose and M. Andrews 1964 The histochemistry of 3B-hydroxysteroid dehydrogenase. I. With special reference to adrenal glands, as affected by ACTH, cold, trauma and tumor. Am. J. Clin. Path., 41: 256-265.

Perek, M., B. Eckstein and Z. Eshkol 1959 The effect of ACTH on adrenal ascorbic acid in laying hens. Endocrinology, 64: 831-832.

Rabl, H. 1891 Die Entwickelung und structur der nebennieren bei den vogeln. Arch. Anat. Entwmech., 38: 492-523.

Romanoff, A. L. 1960 In: The Avian Embryo: Structural and Functional Development. The Macmillan Company, New York.

Rubin, B. L., and R. I. Dorfman 1957 Quantitative assay of 3B-ol dehydrogenase system in whole adrenal homogenate. Endocrinology, 61: 601-610.

Samuels, L. T., and M. L. Helmreich 1956 The influence of chorionic gonadotropin in the 3B-ol dehydrogenase activity of testes and adrenals. Endo-

crinology, 58: 435-442.

Samuels, L. T., and T. Uchikawa 1967 Biosynthesis of adrenal steroids. In: The Adrenal Cortex, Ed. A. B. Eisenstein, pp. 61-102. Little, Brown and Co., Boston, Mass.

Sandor, T., J. Lamoureux and A. Lanthier 1963 Adrenocortical function in birds: In vitro biosynthesis of radioactive corticosteroids from pregnenolone-7-H^3 and progesterone-4-C^{14} by adrenal glands of the domestic duck (Anas platyrhynchos) and the chicken (Gallus domesticus). Endocrinology, 73: 629-636.

Sayers, G., and M. A. Sayers 1948 The pituitary-adrenal system. In: Recent Progress In Hormone Research, 2: 81-115.

Schultz, A. 1924 Eine methode des mikrochemischen Cholesterinnachweises am Gewebsschnitt. Zentr. Allgem. Pathol. Anat., 35: 314-317.

Short, R. V. 1960 The secretion of sex hormones by the adrenal gland. Symp. Biochem. Soc., 18: 59-84.

Siegel, H. S., and W. L. Beane 1961 Time responses to single intramuscular doses of ACTH in chickens. Poultry Sci., 40: 216-219.

Sivaram, S. 1964 Histochemistry of the developing adrenal gland of the chick. Anat. Rec., 148: 336,

_____ 1965 Structure and development of the adrenal gland of Gallus domesticus. Can. J. Zool., 43: 1021-1031.

Soulie, A. 1903 Recherches sur le developpment des capsules surrenales chez les vertebres superieurs. J. Anat. et Physiol., 39: 197-293.

Straznicky, K., F. Hajos and B. Bohus 1966 Relationship between ultrastructure and cortical activity of the embryonic adrenal gland in the chicken. Acta Biol. Hung., 16: 261-274.

Sturkie, P. D. 1965 In: Avian Physiology, 2nd ed., Comstock Publishing Associates, Cornell University Press, New York.

Szekely, Gy, E. Endroczi and J. Szentagothia 1958 Erscheinen der argentaffinen substanz und beginn der sekretion adrenocorticotrophen hormons in der hypophyse von huhnerembryonen. Acta Biol. Hung., 8: 283-287.

Taylor, D. J., J. Greenberg, E. S. Josephson and E. M. Nadel 1956 Histochemical sudanophilia and cholesterol concentration in the adrenal glands of chicks parasitized with Plasmodium gallinaceum. Acta Endocrinol., 22: 173-178.

Toth, N., P. Simon and Gy Szekely 1958 Abhangigkeit der fruhen geweblichen differentiation der nebennierenrinde von der hypophyse bei huhnerembryonen. Acta Biol. Hung., 8: 289-294.

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

Watterson, R. L., D. A. Brown and A. Bartha 1959 Reduced endocrine activity of the adrenal cortex of hypophysectomized chick embryos as indicated by modified development of the duodenum. Anat. Rec., 133: 347-348.

Willier, B. H. 1930 A study of the origin and differentiation of the suprarenal gland in the chick embryo by chorioallantoic grafting. Physiol. Zool., 3: 201-225.

Wolford, J. H., and R. K. Ringer 1962 Adrenal weight, adrenal ascorbic acid, adrenal cholesterol and differential leucocyte counts as physiological indicators of "stressor" agents in laying hens. Poultry Sci., 41: 1521-1529.

Zarrow, M. X., and J. F. Baldini 1952 Failure of adrenocorticotrophic and various stimuli to deplete the ascorbic acid content of the adrenal gland of the quail. Endocrinology, 50: 555-561.

Zarrow, M. X., D. L. Greenman, J. Kollias and D. Dalrymble 1962 The pituitary adrenal axis in the bird. Gen. Comp. Endocrinol., 2: 177-180.

Zarrow, M. X., and I. G. Zarrow 1950 Ascorbic acid in the adrenal gland of the duck. Anat. Rec., 108: 600-601.

TABLE 1

MORPHOLOGICAL AND CHRONOLOGICAL AGES OF BROWN AND WHITE LEGHORN EMBRYOS

Morphological stage		Brown Leghorn embryos		White Leghorn Embryos	
Number	Age (hrs)	Animal Number	Incubation Time (hrs)	Animal Number	Incubation Time (hrs)
21	84	C163	100	C003	96
				C005	96
				C016	100
21-22	84-96			C001	96
22	84-96	C161	98	C009	97
				C015	99
23	96	C160	98	C014	99
		C162	98		
		C164	100		
		C165	100		
24	108	C158	97	C113	115
				C018	116
25	108-120	C168	96	C017	116
26	120			C106	120
				C107	120

TABLE 1 (cont'd)

Morphological stage		Brown Leghorn embryos		White Leghorn Embryos	
Number	Age (hrs)	Animal Number	Incubation Time (hrs)	Animal Number	Incubation Time (hrs)
27	120-132	C172	118	C074	139
28	132-144	C173	119	C027	141
		D001	144		
		D002	145		
29	144-156			C078	146
30	156-168			C076	165
				C080	170

TABLE 2

INTENSITY OF ADRENAL FORMAZAN DEPOSITION IN Δ^5,3B-HSD PREPARATIONS

Morphological stage		Brown Leghorn embryos			White Leghorn embryos	
Number	Age (hrs)	Animal Number	DHA[a]	Preg.[b]	Animal Number	DHA[a]
22	84-96	D005	*		C121	*
23	96	C128	+2		C124	+2
		C129	+2			
		C132	+2			
		D025	+2	+1		
		D052		0		
23-24	96-108	C130	+3		C118	+1
		C131	+3			
24	108	D036	+3		C111	+2
24-25	108-120				C091	+3
25	108-120	C126	+4		C092	+2
		C134	+4		C108	+3
		C138	+5		C112	+3
		D034		+1	C114	+3
					C115	+2
					C116	+2

TABLE 2 (cont'd)

Morphological stage		Brown Leghorn embryos			White Leghorn embryos	
Number	Age (hrs)	Animal Number	DHA[a]	Preg.[b]	Animal Number	DHA[a]
					C117	+2
26	120				C093	+3
					C109	+3
					C120	+3
27	120-132	C171	+4	+5		
28	132-144	C170	+5		C103	+5

[a] Adrenal formazan deposition as estimated from visual microscope examination of tissue sections incubated in (DHA) media. Intensity scoring on a 0 to +6 scale.

[b] Adrenal formazan deposition as estimated from visual microscopic examination of tissue sections incubated in pregnenolone media. Intensity scoring on a 0 to +6 scale.

* Adrenocortical cells not identified.

TABLE 3

TREATMENT OF STAGE 22 EMBRYOS

Animal Number	Total Incubation Time (hrs)	Treatment 48 hrs	Treatment 72 hrs	Treatment 96 hrs	Interval[a] (hrs)	Formazan Deposition Intensity[b]
D003	94	ACTH	ACTH	-	22	Adrenal Cortical Cells not seen.
D005	95	I	I	I	-	Adrenal Cortical Cells not seen.

[a] Time interval between last injection and sacrifice.

[b] Adrenal formazan deposition as estimated from visual microscopic examination of tissue sections. Intensity scoring on a 0 to +6 scale.

ACTH = Injection of 1 IU of ACTH.

I = Intact control embryo.

TABLE 4

TREATMENT OF STAGE 23 EMBRYOS

Animal Number	Total Incubation Time (hrs)	Treatment 48 hrs	Treatment 72 hrs	Treatment 96 hrs	Interval[a] (hrs)	Formazan Deposition Intensity[b]
D016	97	ACTH	ACTH	ACTH	1	+2
D018	98	ACTH	ACTH	ACTH	2	+2
C143	101	ACTH	ACTH	ACTH	5	+5
D007	93	ACTH	ACTH	-	21	+3
D004	94	ACTH	ACTH	-	22	+3
D012	95	ACTH	ACTH	-	23	+3
D013	94	ACTH	-	-	46	+3
C128	97	I	I	I	-	+2
C129	97	I	I	I	-	+2
C132	98	I	I	I	-	+2
D025	98	I	I	I	-	+2
D133	101	S	S	S	5	+3
D008	96	M	M	M	-	+2

[a] Interval between last injection and sacrifice.

[b] Adrenal formazan deposition as estimated from visual microscopic examination of tissue sections. Intensity scoring on a 0 to +6 scale.

ACTH = Injection of 1 IU of ACTH.

I = Intact control embryo.

S = Injection of 0.05 cc saline.

M = Manipulated embryo.

TABLE 5

TREATMENT OF STAGE 23-24 EMBRYOS

Animal Number	Total Incubation Time (hrs)	Treatment 48 hrs	72 hrs	96 hrs	Interval[a] (hrs)	Formazan Deposition Intensity[b]
C135	99	ACTH	ACTH	ACTH	3	+5
C130	98	I	I	I	-	+3
C131	98	I	I	I	-	+3

[a] Time interval between last injection and sacrifice.

[b] Adrenal formazan deposition as estimated from visual microscopic examination of tissue sections. Intensity scoring on a 0 to +6 scale.

ACTH = Injection of 1 IU of ACTH.

I = Intact control embryo.

TABLE 6

TREATMENT OF STAGE 24 EMBRYOS

Animal Number	Total Incubation Time (hrs)	Treatment 48 hrs	Treatment 72 hrs	Treatment 96 hrs	Interval[a] (hrs)	Formazan Deposition Intensity[b]
D035	97	ACTH	ACTH	ACTH	1	+2
C154	97	ACTH	ACTH	ACTH	1	+2
D027	98	ACTH	ACTH	ACTH	2	+3
C136	99	ACTH	ACTH	ACTH	3	+4
C140	100	ACTH	ACTH	ACTH	4	+5
C145	103	ACTH	ACTH	ACTH	7	+5
C146	104	ACTH	ACTH	ACTH	8	+5
C125	117	ACTH	ACTH	ACTH	21	+2
D010	94	ACTH	ACTH	-	22	+2
D036	97	I	I	I	-	+3
C156	98	S	S	S	2	+2
D022	100	M	M	M	-	+3
D019	99	M	M	M	-	+3

[a] Time interval between last injection and sacrifice.

[b] Adrenal formazan deposition as estimated from visual microscopic examination of tissue sections. Intensity scoring on a 0 to +6 scale.

ACTH = Injection of 1 IU of ACTH.

I = Intact control embryo.

S = Injection of 0.05 cc of saline.

M = Manipulated embryo.

TABLE 7

TREATMENT OF STAGE 25 EMBRYOS

Animal Number	Total Incubation Time (hrs)	Treatment 48 hrs	72 hrs	96 hrs	Interval[a] (hrs)	Formazan Deposition Intensity[b]
C139	100	ACTH	ACTH	ACTH	4	+4
C142	101	ACTH	ACTH	ACTH	5	+5
C144	102	ACTH	ACTH	ACTH	6	+4
C148	106	ACTH	ACTH	ACTH	10	+5
C149	107	ACTH	ACTH	ACTH	11	+5
D009	94	ACTH	ACTH	-	22	+3
D021	97	ACTH	ACTH	-	25	+2
D048	100	ACTH	ACTH	-	28	+2
C126	117	I	I	I	-	+4
C134	118	I	I	I	-	+4
C138	102	I	I	I	-	+5
C150	107	S	S	S	11	+5
C137	99	S	S	S	3	+4

[a] Time interval between last injection and sacrifice.

[b] Adrenal formazan deposition as estimated from visual microscopic examination of tissue sections. Intensity scoring on a 0 to +6 scale.

ACTH = Injection of 1 IU of ACTH.

I = Intact control embryo.

S = Injection of 0.05 cc of saline.

PLATE 1

EXPLANATION OF FIGURES

1 Cross section of a stage 21 brown Leghorn embryo showing primordial adrenocortical cells (AC) in the mesenchyme immediately dorsal to the peritoneal epithelium (PE). The subcardinal vein (SV), mesonephros (M) and aorta (A) are also shown. X 250.

2 Cross section of a stage 22 brown Leghorn embryo at the level of the origin of the omphalomesenteric artery (O) showing the location of the thickened peritoneal epithelium (PE), primordial adrenocortical cells (AC), mesonephros (M), aorta (A) and subcardinal vein (SV). X 100.

3 A higher magnification of the stage 22 brown Leghorn embryo shown in figure 2. Primordial adrenocortical cells (AC) are shown migrating from the peritoneal epithelium (PE) to a point in the mesenchyme dorsal to the subcardinal vein (SV) and between the aorta (A) and mesonephros (M). X 250.

4 Cross section of a stage 23 brown Leghorn embryo showing a chain of migrating adrenocortical cells (AC') lateral to the subcardinal vein (SV) and extending from the peritoneal epithelium (PE) to a point in the mesenchyme between the mesonephros (M) and aorta (A) where they form scattered groups of adrenocortical cells (AC''). Migrating chromaffin cells (CC) are also shown. X 100.

5 A higher magnification of the stage 23 brown Leghorn embryo shown in figure 4 showing the chain of migrating adrenocortical cells (AC') and the scattered groups of two of three adrenocortical cells (AC''). The thickened peritoneal epithelium (PE), mesonephros (M), subcardinal vein (SV) and migrating chromaffin cells (CC) are also shown. X 250.

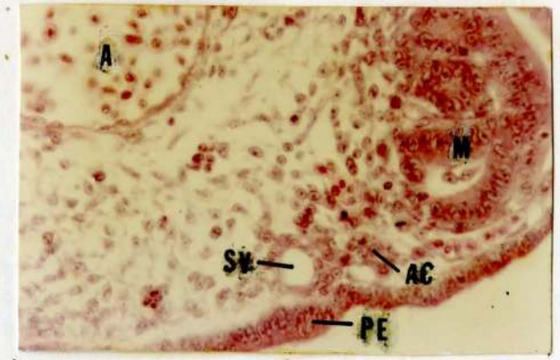


FIGURE 1.

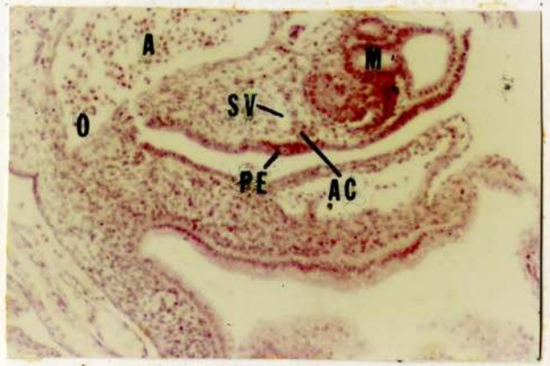


FIGURE 2

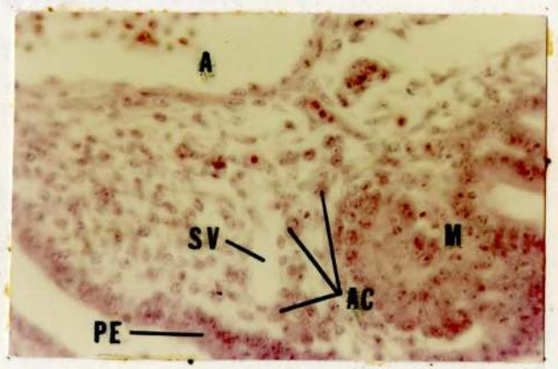


FIGURE 3.

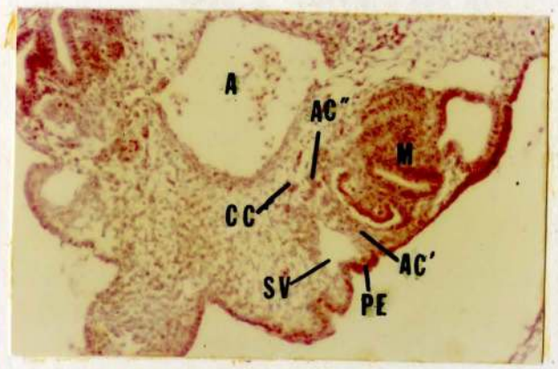


FIGURE 4.

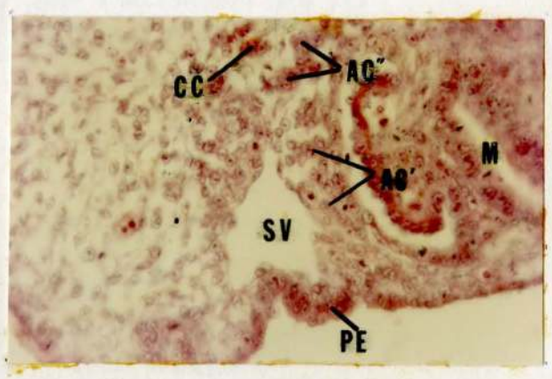


FIGURE 5.

PLATE 2

EXPLANATION OF FIGURES

6 A cross section of a stage 24 brown Leghorn embryo at the level of the origin of the omphalomesenteric artery (O) showing the adrenocortical cell groups (AC) ventral to the mesonephric artery (MA) and dorsal to the subcardinal vein (SV). The aorta (A) and mesonephros (M) are also shown, X 100.

7 A higher magnification of the stage 24 brown Leghorn embryo shown in figure 6 showing the increase in the number of cells in the cortical cell groups (AC) from the previous stage (fig. 5). There is still a chain of migrating adrenocortical cells (AC) lateral to the subcardinal vein (SV) connecting the peritoneal epithelium (PE) and the cortical cell groups (AC). The aorta (A), mesonephros (M) and chromaffin cells (CC) are indicated. X 250.

8 A cross section of a stage 25 brown Leghorn embryo at the level of the origin of the omphalomesenteric artery (O) showing the increase in size of the cortical mass (AC) from the previous stage (fig. 6). The aorta (A), notochord (N), mesonephros (M) and subcardinal vein (SV) are also indicated. X 50.

9 A cross section of a stage 26 white Leghorn embryo showing the cortical mass (AC) medial to the mesonephros (M) and extending dorsal to the mesonephric artery (MA). The aorta (A) and subcardinal vein (SV) are also shown. X 100.

10 A cross section of a stage 27 brown Leghorn embryo at the level of the origin of the omphalomesenteric artery (O) showing solid bodies of cortical cell groups (AC) in the mesenchyme between the aorta (A) and mesonephros (M). Dark staining chromaffin cells (CC) are on the medial surface of the cortical mass and between the cortical cell groups. The gonad (G) is seen ventral to the subcardinal vein (SV). X 100.

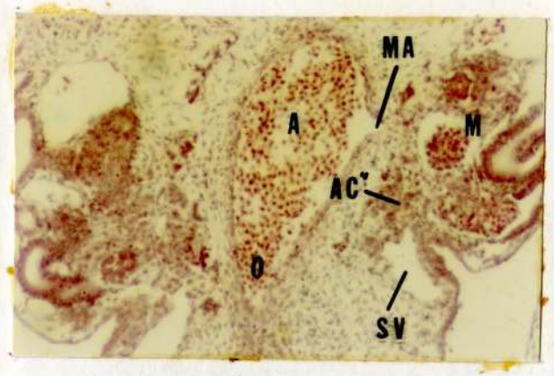


FIGURE 6.

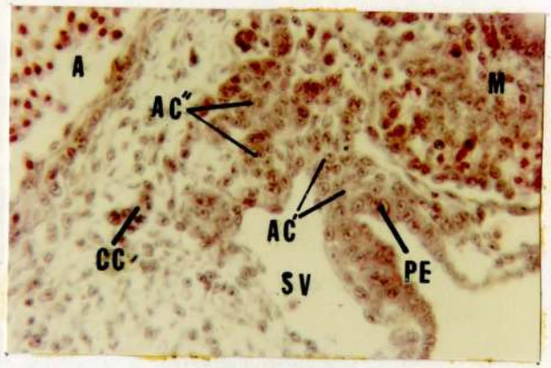


FIGURE 7.

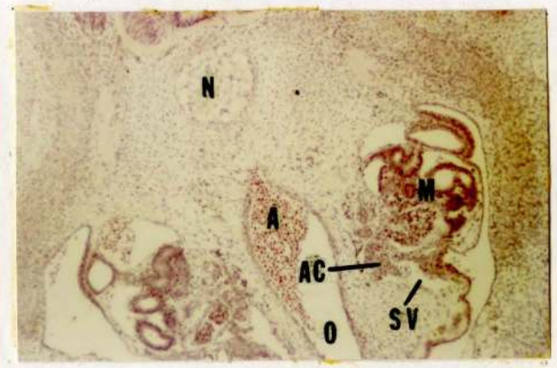


FIGURE 8.

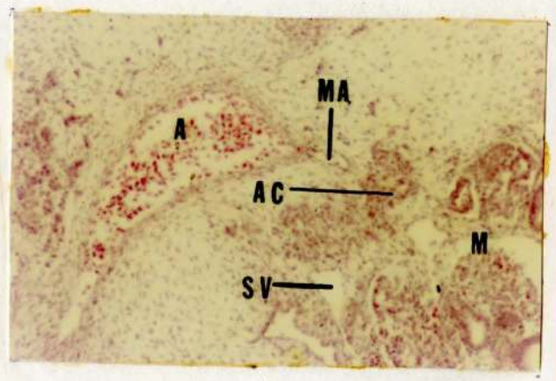


FIGURE 9.

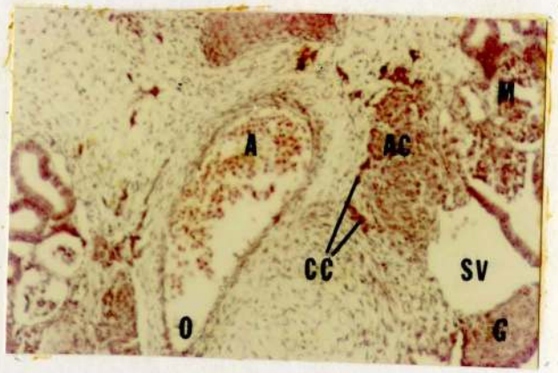


FIGURE 10.

PLATE 3

EXPLANATION OF FIGURES

11 A cross section of a stage 28 brown Leghorn embryo showing the increase in the size of the cortical mass (AC) from the previous stage. The notochord (N), aorta (A), mesonephros (M) and subcardinal vein (SV) are also shown. X 100.

12 A higher magnification of the adrenal of the stage 28 brown Leghorn embryo shown in figure 11, showing adrenocortical cells undergoing mitosis (MI) and chromaffin cells (CC) within the mass. X 450.

13 Cross section of the adrenal of a stage 29 white Leghorn embryo showing the cortical cells beginning to form cords (C) consisting of six or seven cells radially arranged. X 450.

14 Cross section of the adrenal of a stage 30 white Leghorn embryo showing an increase in the number of cells in the cortical cords (C) from the previous stage (fig. 13), and the presence of blood cells (B) and chromaffin cells (CC) between the cords. X 450

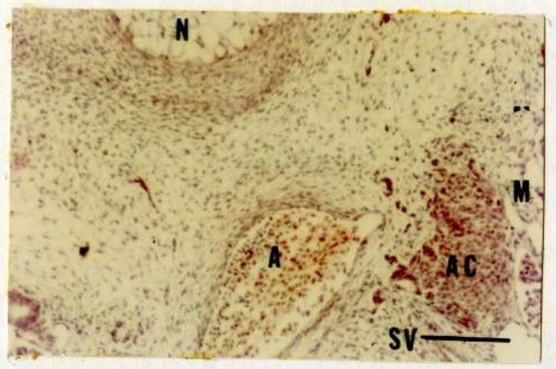


FIGURE 11.

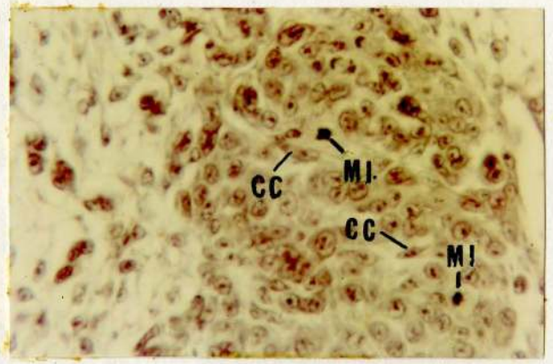


FIGURE 12.

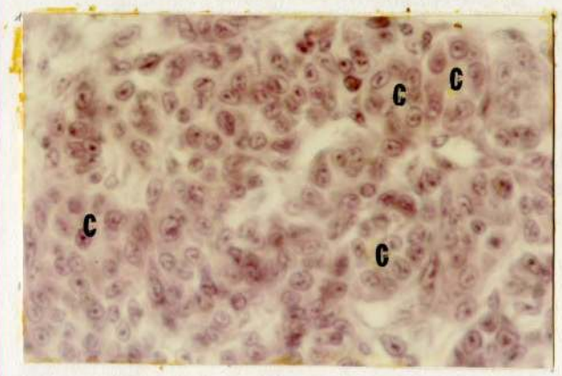


FIGURE 13.

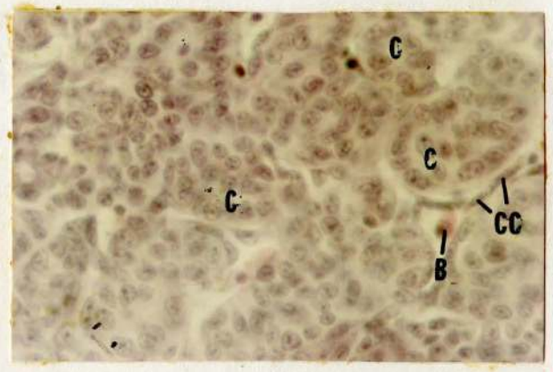


FIGURE 14.

PLATE 4

EXPLANATION OF FIGURES

15 Cross section of the adrenocortical cell mass of a stage 23 control brown Leghorn embryo incubated with dehydroepiandrosterone (DHA) showing a +2 formazan deposition in the form of fine dark blue granules. X 450.

16 Cross section of the adrenocortical cell mass of a stage 23 ACTH treated brown Leghorn embryo sacrificed five hours after the last ACTH injection and incubated with DHA showing a +5 formazan deposition intensity. Note increase in dark blue granules. X 450.

17 Cross section of the adrenocortical cell mass of a stage 23-24 control brown Leghorn embryo incubated with DHA showing a +3 formazan deposition intensity. X 450.

18 Cross section of the adrenocortical cell mass of a stage 23-24 ACTH treated brown Leghorn embryo sacrificed three hours after the last ACTH injection and incubated with DHA showing a +5 formazan deposition intensity. (Compare fig. 17). X 450.

19 Cross section of the adrenocortical cell mass of a stage 24 control brown Leghorn embryo incubated with DHA showing a formazan deposition intensity of +3. X 450.

20 Cross section of the adrenocortical cell mass of a stage 24 ACTH treated brown Leghorn embryo sacrificed four hours after the last ACTH injection and incubated with DHA showing a formazan deposition intensity of +5. (Compare fig. 19). X 450.

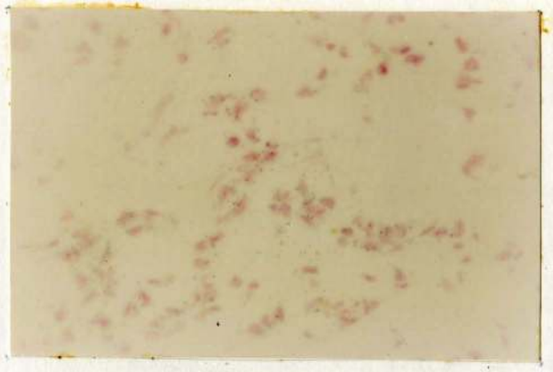


FIGURE 15.

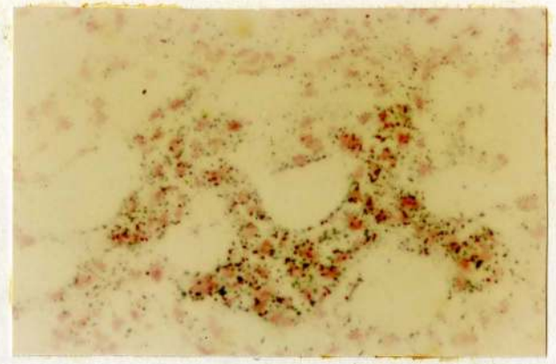


FIGURE 16.

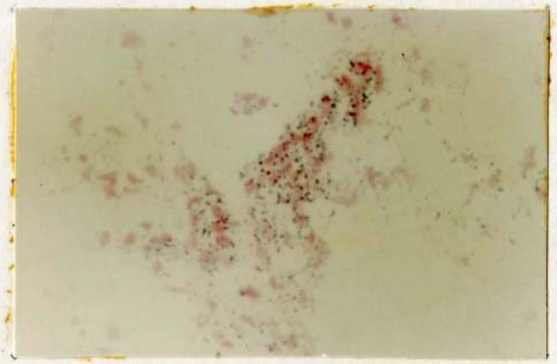


FIGURE 17.

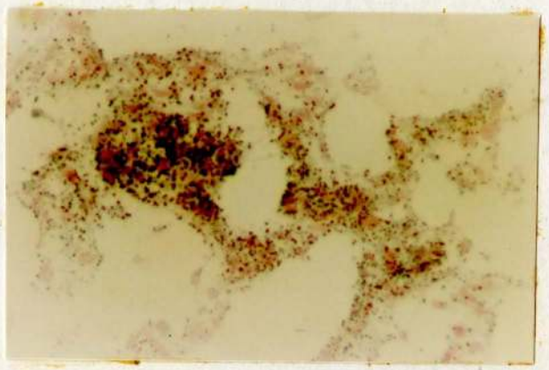


FIGURE 18

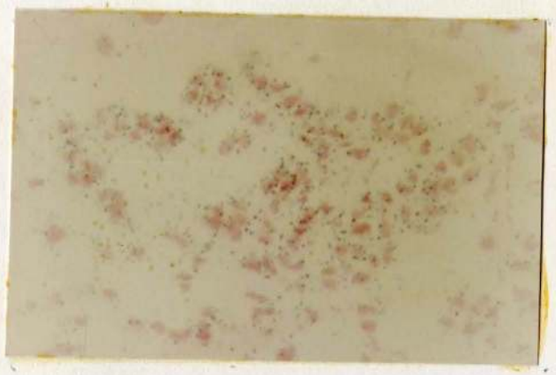


FIGURE 19.

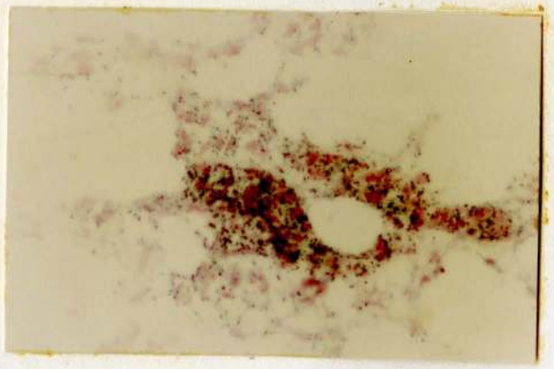


FIGURE 20.

PLATE 5

EXPLANATION OF FIGURES

21 Cross section of the adrenocortical cell mass of a stage 24 control white Leghorn embryo incubated with DHA showing a formazan deposition intensity of +2. X 450.

22 Cross section of the adrenocortical cell mass of a stage 25 control brown Leghorn embryo incubated with DHA showing a formazan deposition intensity of +4 and the various sizes and shapes of the formazan deposits. (Compare fig. 21). X 1000.

23 Cross section of the adrenocortical cell mass of a stage 25 ACTH treated brown Leghorn embryo sacrificed five hours after the last ACTH injection and incubated with DHA showing a formazan deposition of +5. X 450.

24 Cross section of the adrenocortical cell mass of a stage 25 ACTH treated brown Leghorn embryo sacrificed 25 hours after the last ACTH injection and incubated with DHA showing a formazan deposition intensity of +2. (Compare fig. 23). X 450.

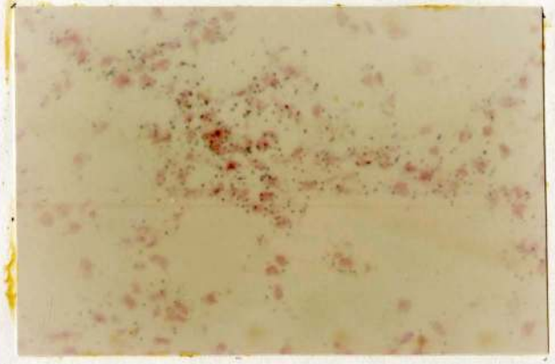


FIGURE 21.

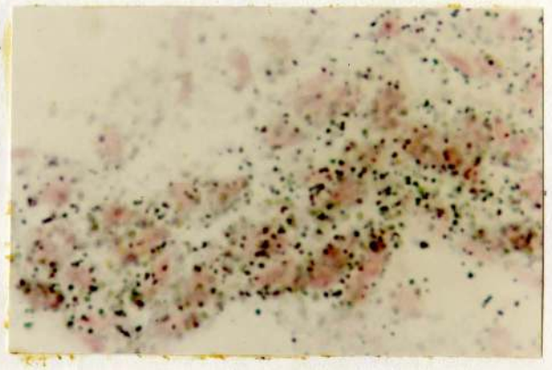


FIGURE 22.

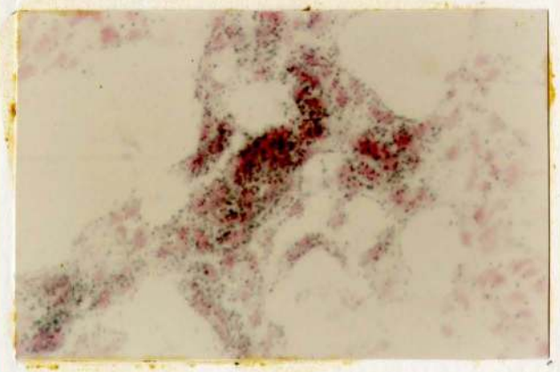


FIGURE 23

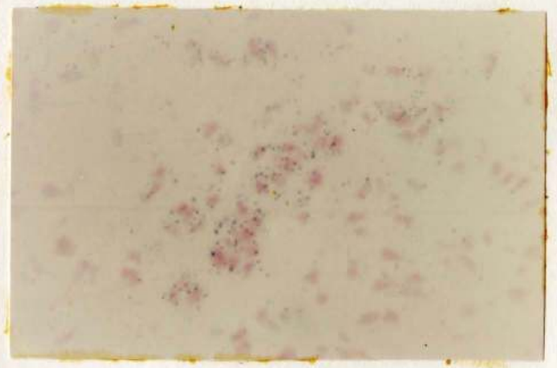


FIGURE 24

PLATE 6

EXPLANATION OF FIGURES

25 Cross section of the adrenocortical cell mass of a stage 23 control brown Leghorn embryo incubated with pregnenolone showing a formazan deposition intensity of +1. The arrow is pointing to the formazan granules. X 450.

26 Cross section of the adrenocortical cell mass of the same stage 23 control brown Leghorn embryo of figure 25 incubated with DHA showing a formazan deposition intensity of +2. (Compare fig. 25). X 450.

27 Cross section of the adrenocortical cell mass of a stage 27 control brown Leghorn embryo incubated with pregnenolone showing a formazan deposition intensity of +5. X 450.

28 Cross section of the adrenocortical cell mass of the same stage 27 control brown Leghorn embryo of figure 27 incubated with DHA showing a formazan deposition intensity of +4. (Compare fig. 27). X 450.

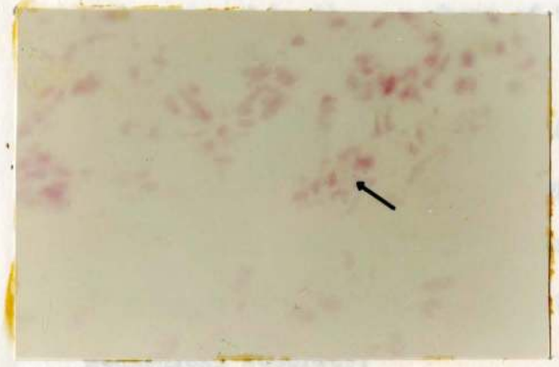


FIGURE 25.

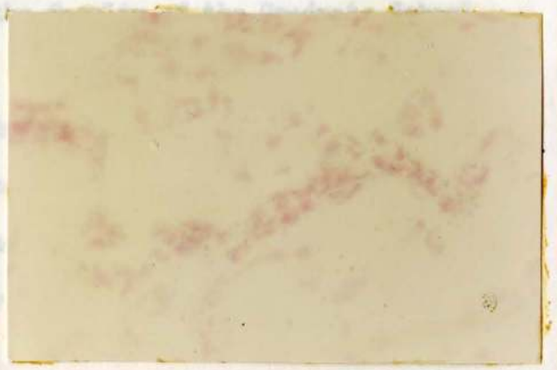


FIGURE 26.

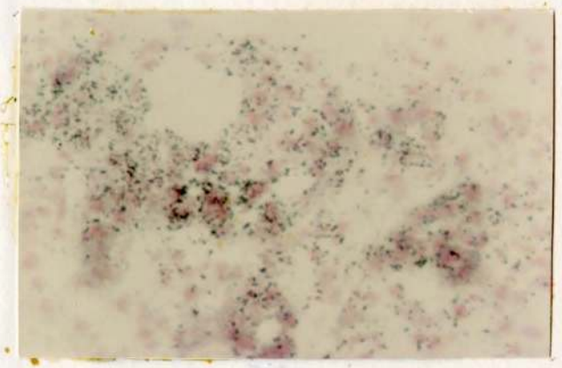


FIGURE 27.

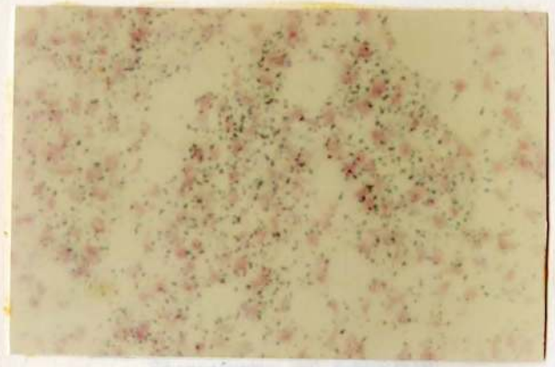


FIGURE 28.

APPROVAL SHEET

The thesis submitted by Grover Charles Ericson has been read and approved by three 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.

1-22-68
Date

Signature of Advisor