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The University of Michigan, Ph.D., 1972
Zoology

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# FUNCTIONAL DEVELOPMENT OF THE HYPOTHALAMO-HYPOPHYSEAL-ADRENAL CORTEX AXIS IN THE CHICK EMBRYO, GALLUS DOMESTICUS

by

Phyllis Mary Wang Wise

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Zoology)
in The University of Michigan
1972

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# INTRODUCTION

Studies of the development of the endocrine system of vertebrates are important for two reasons. First, understanding of the onset and maturation of endocrine functions is of paramount importance to the understanding of the development of the physiological capabilities of an organism during the period of later embryonic life when the organism becomes capable of an independent, coordinated existence. This phase of development has been appropriately called the phase of reintegration (Weiss, 1939). Second, in addition to their role as physiological regulators, many hormones have specific developmental effects (for example, on growth, sexual differentiation, metamorphosis). Knowledge of the kinds and amounts of hormones secreted at various stages of development is essential to the understanding of the role of hormones as regulators of developmental processes. This role of hormones has been viewed as an extension of the sequence of embryonic inductions which begins with the primary induction of neural tube by the chordo-mesoderm (Needham, 1950).

Analysis of the functional development of the adrenal cortex is of interest for both of the above reasons. As physiological regulators, adrenal steroids play key roles in the regulation of water and mineral balance, in the metabolism of carbohydrate, fat, and protein, in stress tolerance, and

many other functions. These functions are not only important during the post-embryonic, free-living existence of an organism, but may be important also in the regulation of the metabolism and survival of the embryo. In addition, adrenal steroids are essential for growth, and have at least one specific developmental effect, the control of the functional differentiation of the small intestine (Moog, 1959). The extent to which such functions are initiated by, and under the control of, adrenal steroids in the embryo can only be ascertained in the light of information about the pattern of steroid secretion in the developing animal.

Complete characterization of the functional development of the adrenal cortex, as with any gland which functions as a component of a complex neuro-endocrine reflex, involves not only analysis of the capacity for hormone secretion by the adrenal itself, but the functional development of the entire neuroendocrine axis. The neurosecretory axis includes the hypothalamus, the anterior pituitary, and the adrenal cortex, and operates in the following manner: A hormone, the corticotropin releasing factor (CRF) is produced by neurosecretory cells in the hypothalamus. This hormone is secreted at the axonal terminals in the median eminence into the hypophyseal portal blood vessels and transmitted to the anterior pituitary. There it elicits the secretion of ACTH which in turn stimulates the synthesis and secretion of adrenal steroids, particularly the glucocorticoid corticosterone in the case of the chicken. Both normal resting levels of steroid secretion as well as elevated rates of secretion

in response to appropriate sensory input, such as stress, depend upon the intact neurosecretory pathway. Therefore, the development of the normal pattern of steroid secretion may reflect not only developmental changes in the adrenal itself, but also the functional development of the anterior pituitary corticotrophs and/or the development of the neurosecretory neurons in the hypothalamus which secrete CRF.

In the light of the above remarks, two major objectives of this investigation may be stated:

1. First, to characterize the pattern of corticosterone secretion in the chick during embryonic and post-hatching development and to correlate this pattern with certain critical physiological and developmental changes which have been presumed to be under adrenal control.

2. Second, to determine to what extent the normal pattern of corticosterone secretion by the chick embryo is dependent upon the intact hypothalamo-hypophyseal-adrenal cortex axis. This has been approached through studies of the effect of removal of the hypothalamus and pituitary (by decapitation) and the effect of pituitary replacement therapy upon the pattern of corticoid secretion in normal and stressed animals.

In the past, several different approaches have been used to analyse the functional status of the developing adrenal. But for the most part the methods, which are reviewed below, have not been sufficiently precise or sensitive to provide accurate information about hormone levels. The

present investigation makes use of the recently developed protein-binding radioassay for plasma corticosteroids which has the necessary sensitivity and precision to permit determination of corticosterone levels in small quantities of blood (Murphy, 1967).

Based upon previous work, three particular times during the embryonic period are of particular importance. First, the experiments of Case (1951, 1952) and Moog (1959) have suggested that pituitary ACTH secretion may begin around the 15th day of incubation. Thus, measurement of glucocorticoid levels around this time is of interest. Second, the concentration of duodenal alkaline phosphatase, which is dependent upon glucocorticoids, rises abruptly from the 17th to the 19th day. Consequently, a rise in corticoid secretion might be expected during this period. Third, birth of mammalian fetuses is accompanied by a several-fold increase in corticosterone levels. Since hatching is an analogous event, possibly involving stresses on the animal which might elicit corticosteroid secretion, measurement of corticosterone levels at this time is of interest.

The following review of the literature provides further background necessary for a clear understanding of the rationale for conducting the present study and will help in evaluating the interpretation of the conclusions.

## Morphological Development of the Adrenal

Classical studies relied upon observations of changes in the morphology of the adrenal cortex as the basis for

deducing patterns in the development of adrenal function. These studies suggest that steroidogenic activity could be expected during the late stages of embryonic development.

In the chick embryo the primordial adrenal cortical cells, which arise by proliferation from the mesothelium medial to the mesonephros, are distinguishable by the end of the 4th day of incubation (incubation period = 21 days). Rapid increase in the size of the gland occurs during the next several days. By the 7th day the cortical cells are arranged in the cords characteristic of the avian adrenal. Medullary cells can first be seen as they become associated with cortical elements between the 4th and 5th day of incubation. Encapsulation of the gland occurs by the 10th day (Dawson, 1949, 1953; Lillie, 1956; Romanoff, 1960). High concentrations of ascorbic acid appear and are maintained in the cords from the 10th day on (Sivarain, 1968). During this time histochemical tests for lipids, cholesterol, and carbonyl groups become irregularly positive, but do not become uniformly so until the 12th day (Dawson, 1949, 1953.) Sudanophilia becomes steadily more intense during the last two-thirds of the embryonic period (Moog, 1959).

Not enough studies have been done to know whether this pattern of morphological change is common to other vertebrates. However, several studies have been done in rats and mice. In these animals the mesothelial thickening, which gives rise to the adrenal cortex, can be clearly seen on the 12th day (gestation period = 21 days). Sudanophilic granules can be seen on day 16. Histochemical reactions indicate active

secretion by day 18 (Josimovich et al., 1954; Kahri, 1966). The arrangement of adrenal cortical cells into three distinct zones, which is unique to the mammalian gland, appears during the fetal period. However, the exact timing of this zonation is disputed (Christianson and Chester-Jones, 1957; Cohen, 1954, 1955; Josimovich et al., 1954; Lever, 1955; Mitchell, 1948).

Morphological studies have been done on other species: human (Johannisson, 1969; Luse, 1967; McNutt and Jones, 1970; Milner and Villee, 1970; Ross, 1962) and monkey (Benirschke and Richart, 1964). The pattern of morphological development is the same as that described above for rats and mice; however, the time of morphological changes differs since the period of intrauterine development varies for the different species.

These morphological studies are preliminary indications of the functional state of the adrenal cortex during various stages of development and suggest that steroidogenic activity can be expected during the last half of the embryonic period.

Functional Development

The physiological and developmental responses of target structures have been used to demonstrate that the developing adrenal cortex has a functional role during the embryonic period. Such studies of the physiological actions of adrenal steroids during embryonic development are relevant to the present study because the onset of the adrenal regulated functions is indicative of adrenal steroid secretion. The

two most important target organ responses to adrenocortical secretions are: 1) duodenal differentiation and 2) liver glycogen accumulation.

The studies of Moog (1959) provide indirect but strong indications of the functional status of the adrenal. In the chick, epithelial cells of the duodenum undergo differentiation during the last week of incubation. One characteristic of this differentiation is a hundred-fold increase in alkaline phosphatase activity during the last three days in ovo (Moog, 1950). Moog and her co-workers found that glucocorticoids can influence the timing of this increase. When eggs were injected with cortisone acetate at 10 days, alkaline phosphatase increased slightly at 16 to 17 days of incubation to a level comparable to that normally attained at 19 days. Injections at 14 days produced an effect by the 17th day. Injections made on day 17 show an effect within 24 hours (Moog, 1959; Moog and Ford, 1957; Moog and Richardson, 1955; Moog and Thomas, 1957). Thus, glucocorticoids administered earlier and in larger quantities than occur normally, accelerate the normal pattern of accumulation of alkaline phosphatase. These observations suggest that glucocorticoids normally influence the later stages of duodenal differentiation. Evidence of duodenal differentiation give some indication of onset of functional activity of the adrenal. Work with exogenous steroids does not constitute proof. However, equivalent studies in the mouse have demonstrated that steroid deprivation through surgical adrenalectomy prevents duodenal

differentiation. Thus, these studies confirm, in principle, the interpretation of the data obtained from the chick embryo (Moog, 1953).

An analogous approach to studying the initiation of adrenal function is using liver glycogen accumulation as an indication of the presence of adrenal cortical secretion. Studies in the rat have shown that liver glycogen levels are low until the 19th day of gestation, when they quadruple abruptly within 24 hours (Jacquot, 1955, 1959a). Jacquot (1959b) found that adrenalectomy of an 18 day old fetus in an adrenalectomized mother prevents the abrupt increase normally seen between the 19th and 20th days. Thus, on the basis of liver glycogen accumulation, it is evident that the adrenal has become functional during the final days of gestation; but the data does not exclude function at an earlier period as the target may not be capable of responding.

By far the most direct and potentially most useful approach to the question is the use of direct biochemical assay of the hormone present. Several biochemical methods are available to study adrenal function: 1) <u>in vivo</u> and <u>in vitro</u> measurements of steroid in the adrenal gland, 2) quantitative and qualitative measurement of glucocorticoids in plasma, 3) measurement of enzyme activity in the adrenal for those enzymes necessary in the synthesis of glucocorticoids, 4) measurement of enzyme activity in the liver for those enzymes necessary in the metabolism of adrenal steroids, and 5) perfusion of the adrenal with labelled precursors

followed by analysis of the products of synthesis and metabolism in the gland, plasma, and urine.

For the most part, these methods require large amounts of blood or tissue, and thus have not been applied to embryos with much success. Nonetheless, many studies have attempted measurements of adrenal steroids, since qualitative and quantitative data are of importance in answering questions regarding the differentiation of the adrenal cortex and its role in the development of the organism.

Kamoun and his associates isolated corticosterone in rat adrenals during the last five days of gestation (Kamoun et al., 1964). They found twice as much corticosterone in the fetal adrenal gland than in the newborn, except on the 18th day of gestation when the hormone content was four times as high as in the neonate. Shapiro and Geller (1964) described corticosterone content of rat embryos just before birth as being the same as in adults. In vivo studies done by Milkovic and Milkovic (1962) demonstrated that in the rat, plasma corticosterone levels increase between the last day of embryonic development and the first day after birth from 21 µg/100 ml. to 30.5 µg/100 ml. plasma, then decrease to about 10 µg/100 ml. plasma by the 5th day postnatally, where it remains steady. Holt and Oliver (1968), using another assay, found very different values, but basically the same pattern appeared. Milkovic and Milkovic (1962) demonstrated the production of corticosterone from endogenous precursors in adrenal slices from 21 day old rat fetuses cultured in vitro. They found that corticosterone production increased

if the slices were incubated with fetal hypophyses or ACTH.

Jackson and Piasecki (1969) studied cortisol and corticosterone secretion rates by examining effluent adrenal and peripheral venous blood of 56 to 63 day old dog fetuses (gestation period = about 70 days). They found cortisol to be the major glucocorticoid. The secretory rates of this hormone increased after administration of ACTH.

Several studies have examined glucocorticoid concentrations and secretion rates in sheep (Alexander et al., 1968; Bassett and Thorburn, 1969; Chester-Jones et al., 1964; Jackson, 1967; Jones et al., 1964). Quantitatively, the results vary greatly from one research group to another. This is probably due to slightly different surgical techniques and different assays. The data establish that the adrenals of prenatal lambs secrete glucocorticoids by the last third of pregnancy (Alexander et al., 1968) and that these steroids circulate in increasing concentration from day 130 to day 148 (gestation period = 148 days) (Bassett and Thorburn, 1969).

In the human, aldosterone and androgens can be isolated from fetal adrenal tissue after nine weeks (gestation = 40 weeks). At this time androgenic steroids are found in higher concentrations than aldosterone. The ratio of glucocorticoids to androgenic steroids becomes greater than one at 16 weeks of age and continues to increase with the age of the fetus (Bloch et al., 1956). In homogenates of human fetal adrenal tissue, a similar shift in pattern of steroid synthesis from androstenedione to cortisol and corticosterone was observed

(Bloch, 1965, 1967, 1968;Ghilain and Schwers, 1957; Villee et al., 1959; Villee and Villee, 1965). The enzyme systems necessary to convert acetate to steroids are detectable from week 12 on, with a sharp increase in 3 β-hydroxysteroid dehydrogenases around week 13 (Bloch and Benirschke, 1959; Goldman et al., 1966; Niemi and Baillie, 1965). Isolation of metabolites following perfusion with steroids or their precursors also supports the view that the human fetal adrenal is capable of forming and metabolizing steroids (Bengtsson et al., 1964; Bloch et al., 1955; Solomon et al., 1964; Stachenko, 1959; Zander, 1961).

More fragmentary information regarding the synthesis, concentration, and secretion rates of glucocorticoids is known for many other species (armadillo: Bloch, 1967, 1968; Bloch and Benirschke, 1965; cow and pig: Druzhinima, 1958; dog, rabbit, and guinea pig: Milkovic and Milkovic, 1959; monkey: Kittinger and Beamer, 1971; Wolf and Bowman, 1963).

In interpreting biochemical studies on mammalian embryos it is important to bear in mind that maternal hormones may cross the placenta, thus influencing hormone levels in fetal blood. First, although there is some evidence that the placenta is impermeable to anterior pituitary hormones (Christianson and Chester-Jones, 1957; Foster, 1970; Gitlin et al., 1965; Goodman and Wislocki, 1933; Jost, 1966a; Wislocki and Snyder, 1932), other evidence shows that ACTH as well as other anterior pituitary hormones can pass through the placenta when levels of these hormones are particularly high

(Knobil and Briggs, 1955; Milkovic and Milkovic, 1962, 1966). To what extent this contributes to fetal hormone levels is not known.

Secondly, steroid hormones do cross the placenta (Eguchi, 1969; Finlay and Cox, 1970; Foster, 1970; Jacquot, 1959; Jost, 1966a; Linzell and Heap, 1968; Migeon et al., 1956; Mitskevich, 1962; Schmidt and Hoffman, 1954). Thus, any situation facing the mother which would cause a rise in maternal glucocorticoids can be expected to affect fetal hormone titers as well. Surgical laparotomy is one stress to the mother sufficient to affect fetal adrenal endocrinology (Cohen, 1963). It is known that the human placenta actively participates in the metabolism of steroids of maternal or of fetal origin and in the synthesis of estrogens (Diczfalusy et al., 1965). Excretion of estrogen metabolites in pregnant women also depends upon the functional state of the fetal adrenal cortex (Frandsen and Stakemann, 1964). Therefore, any manipulation of the mother which influences her placental metabolism could indirectly affect the biosynthetic status of the fetal adrenal cortex. If determinations of fetal hormone levels are done when the fetus itself is capable of responding to stress, surgical manipulations necessary to physically reach the fetus may be sufficiently stressful to elevate levels of adrenal cortical hormones.

Embryonic Pituitary Regulation of Adrenal Cortex Function.

In adult mammals the function of the adrenal cortex is regulated by the pituitary. In adult birds of several

species it appears that the adrenal may have a slight degree of functional autonomy; however, studies have shown that the pituitary still influences adrenal function under certain physiological conditions (Assenmacher, 1958; Egge and Chiasson, 1963; Ma and Nalbandov, 1963; Nalbandov and Card, 1943; Shirley and Nalbandov, 1956). For example, hypophysectomy of domestic fowl leads to an initial decrease in adrenal weight and an atrophic appearance; however, after an extended period of time the morphology and many indirect assays of the functional state of the gland indicate return to the normal state. Other studies indicate that the adrenal of post-hatching chickens and ducks remains functionally abnormal after hypophysectomy (Bradley and Holmes, 1971; Frankel et al., 1967b,c).

Many studies have established that the embryonic pituitary is necessary in several mammalian and avian species for the normal development of the embryonic adrenal cortex (Angervall and Lundin, 1964; Bearn, 1967, 1968; Eguchi, 1960, 1961; Jost, 1948, 1953, 1966b, 1969; Kitchell and Wells, 1952b; Reid, 1960; Wells, 1947, 1948). In birds and in mammals, decapitation of the embryo provides clear evidence that the pituitary is essential to normal adrenal development. Hypophysectomy of the mouse embryo causes decreased adrenal size, measured both in absolute volume and in the ratio of adrenal volume to body weight. The cortex is narrower, cortical cells are smaller, less eosinophilic, and more vacuolated. The younger the embryo is when it is hypophysectomized, the more exaggerated are the volumetric and histological changes

(Eguchi, 1961). Similar results have been found in other mammalian species examined (Christianson and Chester Jones, 1957; Eguchi, 1960; Cohen, 1954, 1955, 1963; Jost, 1948, 1954, 1956; Picon, 1961, 1962; Raynaud and Frilley, 1950; Wells, 1947, 1948). Case (1951, 1952) found that in chicks hypophysectomy on day 2 causes a lowered adrenal weight compared to the adrenals of normal embryos on day 16 of incubation and after. In fact, adrenal weights increase normally until day 15; then there is no significant change in adrenal weights of decapitated embryos between the 16th day and 20th day, during which time the adrenals of normal chick embryos almost double in weight (Case, 1951). The adrenals of decapitated 17 day embryos have fewer cords of cells and fewer and smaller lipid droplets, which tend to be distributed around the cell membrane as opposed to randomly through the cytoplasm.

Another indication of pituitary regulation of the development of the adrenal cortex is the observation that decapitation of fetuses has an effect upon ascorbic acid levels. Cohen (1958) found that rat embryos which have been decapitated at 18 days have lower adrenal ascorbic acid levels on day 21 than controls of the same age. If the fetuses are decapitated and injected with ACTH, the levels of ascorbic acid at 21 days are higher than the decapitated, though the levels are not always comparable to unoperated controls. In chick embryos adrenal ascorbic acid levels differ between controls and decapitated animals after the 15th day of incubation (Case, 1951).

Decapitation of the fetus, like adrenalectomy of

mother and fetus, prevents the abrupt rise in liver glycogen observed during the last several days of gestation. Jacquot (1956, 1959a) found that decapitation of the rat fetus on day 18 lowers glycogen levels to approximately 60% of controls when the mother is normal and to approximately 15% of controls when the mother has been adrenalectomized. Cortisone given to either the mother or to the fetus restores glycogen levels to the normal range. Picon and Bouhnik (1966) observed that decapitation of the rat fetus on day 18 significantly reduces the amount of glycogen in the ventricular muscle of the heart on the 21st day, provided that the mother has been adrenalectomized. Giving ACTH to the decapitated fetus restores the normal level of glycogen. In non-adrenalectomized mothers, the myocardial glycogen of the decapitated fetus remains normal without injection of ACTH.

As discussed previously, in the chick embryo, the early experiments of Moog and her colleagues implicated adrenal influence upon duodenal differentiation (Moog, 1950, 1959; Moog and Ford, 1957; Moog and Richardson, 1955; Moog and Thomas, 1957). The involvement of the pituitary-adrenal axis in this aspect of development was confirmed by the experiments of other workers (Hinni and Watterson, 1963; Manwell and Betz, 1966; Watterson et al., 1959). Decapitation on the second day of incubation causes the differentiation of the duodenal epithelium, including the accumulation of alkaline phosphatase, to be arrested on day 16 of development.

Tissue culture studies have shown that ACTH is necessary

in order for cultivated adrenal cells to have their specialized morphological and functional characteristics of the differentiated cell type *in vivo*. Proliferation and differentiation of the membranes of the smooth endoplasmic reticulum and mitochondria, correlated with an increase in the steroidogenic activity of the cortical cells, occurs only if the cells are cultured with ACTH (Kahri, 1966; Milner, 1971; Milner and Villee, 1970; Schaberg and deGroot, 1958).

In addition to these basic observations, the existence of a feedback mechanism confirms a pituitary-adrenal interdependence during development. Adrenalectomy of the rat fetus causes an increase in the size of the pituitary and an increase in the number of acidophils (Coetzee, 1956; Coetzee and Wells, 1957; Jacquot, 1959; Tobin, 1939). On the other hand, an excess of glucocorticoids reduces the size of the pituitary and the adrenal (Coetzee, 1956; Coetzee and Wells, 1957; Jost *et al.*, 1955; Yakaitis and Wells, 1956.) Unilateral adrenalectomy of the rat fetus results in compensatory hypertrophy of the remaining adrenal (Eguchi *et al.*, 1964; Kitchell, 1950; Kitchell and Wells, 1952b; Walaas and Walaas, 1944). Adrenalectomy of, or the administration of excess corticosteroid to, a pregnant mother animal also affects the fetal adrenal (Courrier *et al.*, 1951; Davis and Plotz, 1954; D'Angelo and Wall, 1971; Goldman, 1967; Holland, 1958; Ingle and Fisher, 1938; Jost *et al.*, 1955; Picon, 1957; Schmidt and Hoffman, 1954).

Thus, it appears that during embryonic development,

the adrenal cortex grows and differentiates autonomously at first and subsequently comes under the regulation of the anterior pituitary.

Embryonic Hypothalamic Regulation of Pituitary-Adrenal Cortex Function.

Much evidence has been collected which indicates that in the adult mammal, secretion of anterior pituitary hormones is regulated by hormones secreted from the hypothalamus (deGroot and Harris, 1950; Fortier, 1966; Ganong, 1963; Greer et al., 1970; Hume and Wittenstein, 1950; Mangili et al., 1967). The role of the hypothalamus in the control of avian target organs has not been clearly established (Assenmacher, 1958; Egge and Chiasson, 1963; Farner et al., 1967; Ma and Nalbandov, 1963; Miller, 1961).

The role the hypothalamus plays in the development of the functional capabilities of the embryonic anterior pituitary has not yet been clearly established. The capacity to respond to a stress by elevating glucocorticoid levels is known to be mediated by the central nervous system (deGroot and Harris, 1950; Fortier, 1966; Ganong, 1963; Greer et al., 1970; Hume and Wittenstein, 1950; Mangili et al., 1967; Selye, 1950.) Several aspects of this capacity to respond to stress have been studied during the embryonic period of many species: 1) fetuses are capable of responding to stressful stimuli or to administration of ACTH (dog: Jackson and Piasecki, 1969; mice: Eguchi and Wells, 1965; rat: Milkovic and Milkovic, 1959, 1961, 1962, 1963, 1966; Milkovic et al., 1968, 1970),

2) birth is a stressful event as reflected in increased circulating levels in the fetus at that time (dog: Kinsella et al., 1970; human: Migeon et al., 1956; rat: Holt and Oliver, 1968), 3) there is a period of non-responsiveness of the pituitary adreno-cortical system to stressful stimuli during the early postnatal period (dog: Jailer, 1950; Milkovic and Milkovic, 1959; mice: Eguchi and Wells, 1965; rabbits: Milkovic and Milkovic, 1959; rats: Allen and Kendall, 1967; Jailer, 1950; Levine et al., 1967; Milkovic and Milkovic, 1959; Shapiro, 1962; Shapiro and Geller, 1964; Shapiro et al., 1962).

Human anencephalism, a pathological condition in which the brain is absent but the anterior pituitary is present, results in abnormal development and function of the adrenals, testes, and external genitalia (Bearn, 1968; Benirschke, 1956; Clayton, 1968; Hatakeyama, 1969; Moeri, 1951; Tuchmann-Duplessis, 1959; Zondek and Zondek, 1965a,b). A similar condition can be produced experimentally in rat fetuses by a surgical procedure in which the whole brain is removed, leaving the adenohypophysis in situ in the sella turcica (Jost, 1966a,b, 1967, 1969). Encephalectomy performed on day 16 or later does not prevent further morphological development of the pituitary. Despite the presence of the anterior pituitary, the size of the adrenals is as small as those in which the fetus has been hypophysectomized (Jost, 1966b). Adrenal and testicular functions are both abnormally low (Jost, 1969). Encephalectomized fetuses treated with either purified beef corticotropin

releasing factor (CRF) or crude extracts of rat hypothalami, have normal adrenal weights on day 21. The same treatment given to hypophysectomized (by decapitation) fetuses does not prevent adrenal atrophy (Jost, 1966a, 1967, 1969; Jost et al., 1966).

Fugita and his colleagues destroyed the hypothalamic area of 20 day old rat fetuses using an electrocoagulator. This procedure leaves much of the rest of the brain undisturbed. At term, the adrenals were very small with conspicuous shrinkage of cortical cells (Fugita et al., 1970).

These results support the concept that hypothalamic influence on the pituitary is an important factor during the embryonic period in at least some species. Experiments in other mammalian fetuses support these results (armadillo: Wurster and Benirschke, 1964; cow: Kivalo and Talanti, 1957; human: Levina, 1968, 1970; sheep: Foster, 1970; Liggins, 1969b; rabbit: Hilliard et al., 1966).

Experiments in the avian embryo show a different pattern of hypophyseal control than exists in mammalian fetuses. Betz (1965, 1967) found that adrenal, gonadal, and thyroidal morphology is restored to a normal morphological state when pituitary grafts were transplanted onto the chorioallantoic membrane of decapitated embryos. Restoration of normal endocrine function was indicated by normal duodenal differentiation and normal skin and feather development (Bellware and Betz, 1970; Brasch and Betz, 1971). Experiments with metapyrone demonstrated the capacity of the decapitated embryo

with a transplanted pituitary to increase secretion of ACTH in response to metapyrone (Mess, 1968).

These experiments support the hypothesis that in mammalian embryos the hypothalamic-pituitary-adrenal cortex axis functions as an integrated unit before birth. In the avian embryo, it appears that the hypothalamus is not necessary for normal development of the pituitary-adrenal cortex axis.

## MATERIALS AND METHODS

### Animals

Fertile eggs and day-old cockerels were obtained from Klager Hatchery, Bridgewater, Michigan. Both were White Leghorns, Dekalb Strain 131. Considerable variation in corticosterone levels was found and much of this was shown to be attributable to variation between different flocks of this strain and to seasonal variation occassioned by the extended period required to do the first series of experiments. Therefore, after 1970, all the eggs were secured from a single flock, and whenever feasible, one entire experiment was done using eggs laid on one day.

The eggs were incubated in a David-Bradley Incubator at a dry temperature of 38±1°C and a wet bulb temperature of 31°C. The cockerels obtained from the hatchery were kept in a battery brooder under constant light at a temperature of about 32°C for the first week, 27°C for the second and third week. They were fed Purina Startena and water ad libitum. Mature birds were maintained on a 17:7 light:dark cycle at a temperature of 22°C and were fed a 14% protein mash (Washtenaw Farm and Garden Center, Ann Arbor, Michigan) and water ad libitum.

Designation of age in embryos and post-hatching chicks was according to the standard scheme described by Moog (1961). Accordingly, embryos in the incubator between 0 and 24 hours

were considered to be 0 days old, between 24 and 48 hours they were considered to be 1 day old and so on. Hatched chicks were considered to be 0 days old for the first 24 hours, 1 day old for the second 24 hours and so on. The Hamburger and Hamilton method of staging embryos was used to determine when an embryo was at the proper stage to be hypophysectomized since this operation had to be done at a precise time during embryonic development (Hamburger and Hamilton, 1951).

For all prehatching stages and newly hatched chicks, blood was pooled without knowledge of the sex of individuals. Males were used in all experiments dealing with 7 day, 14 day, 21 day, and adult chickens.

Hypophysectomy and Pituitary Replacement Therapy

Hypophysectomy was done on embryos which were incubated without turning until embryos reached stage 10 or 11, also called the 33 hour stage (Hamburger and Hamilton, 1951), using a modification of Fugo's technique (Fugo, 1940). This method involved 4 steps: 1) a hole 1-2 mm in diameter was made at the narrow end of the egg. Approximately 2 ml of albumen were withdrawn and the hole was sealed with a mixture of melted beeswax and paraffin; 2) a square opening or "window" was made, approximately 1.5 cm on a side, at the uppermost part of the shell. This part of the shell was removed; 3) using glass rodding drawn out to 50 μ diameter, the anterior region of the head including the forebrain was excised and removed; 4) the opening in the shell was closed by covering it with Saran Wrap which had been soaking in ether for several

hours, and then was sealed using beeswax-paraffin mixture. Though this procedure entails severing the anterior part of the head and the brain anterior to the mesencephalon, many studies have shown that the abnormalities which occur are attributable to the absence of the pars distalis as opposed to the absence of the hypothalamus, the epiphysis, or the surgical trauma (Benoit, 1962; Betz, 1967; Fugo, 1940; Love and Konigsberg, 1958; Martindale, 1941; Moszkowska, 1958, Stalsberg, 1965; Tixier-Vidal, 1958). Albumen was removed and a "window" was made and sealed without surgical manipulation for those embryos serving as controls.

Pituitary replacement therapy was done using the method of Betz (1967). When decapitated embryos were 9 days old, one pituitary from a 10 day old donor was placed on a chorioallantoic vein of the host. Betz (1965) demonstrated that the pars distalis is first clearly recognizable when embryos are 10 days old and that the chorioallantois is capable of supporting grafts when embryos are 9 days old.

Stress Stimuli

The stress stimulus consisted of breaking one leg and waiting for 20 minutes before collecting blood. In the case of the embryos, this procedure was done without removing the embryo from the egg. During the waiting period after stress, the opened shell was covered with a piece of cotton moistened with saline. In post-hatching chicks stress was carried out in a similar manner: a leg was broken and the chicks were kept in a warm box until the blood was collected. Control

embryos and control post-hatching chicks were left undisturbed in the incubator and brooder respectively until blood was collected.

In experiments with adrenocorticotropic hormone, Sigma powdered porcine ACTH dissolved in distilled water was used. One hundred milliunits were given intravenously to embryos and subcutaneously to newly hatched chicks 20 minutes before blood was collected. Controls for these experiments received equivalent amounts of distilled water 20 minutes before collecting blood. During the 20 minute wait the animals were treated the same way as were stressed animals.

Sample Collection

All blood was collected between 7:30 A.M. and 12 noon since, though appropriate studies have not yet been done in chickens, there is much evidence in the literature for a circadian rhythm of corticotropin releasing factor (CRF), adrenocorticotropic hormone (ACTH), and glucocorticoids in other species (Cheifetz et al., 1968; Critchlow et al., 1963; David-Nelson and Brodish, 1969; Galicich et al., 1965; Guillemin et al., 1959; Halberg et al., 1965; Retiene et al., 1968). Embryonic blood was collected from a major chorioallantoic vein or artery using a drawn out glass pipette coated with liquid heparin. The assay used to determine corticosterone levels is sensitive and precise over a limited range; therefore depending on age and size blood from a varying number of animals was pooled to get a sample. The minimum pool size was 3 animals but 10-15 animals comprised the pool in the case of

smaller embryos and hypophysectomized animals. Plasma was prepared immediately after the blood was collected. Corticosterone was extracted from the plasma using dichloromethane. The dichloromethane was subsequently removed by evaporation under air.

Assay Techniques

Corticosterone levels were determined using the protein-binding radioassay developed by Murphy (1967). No purification of the plasma or the dichloromethane extract was performed since previous investigators found corticosterone to be the major glucocorticoid in chicks and the only glucocorticoid to increase after stress of ACTH treatment (Bonhommet and Weniger, 1967; DeRoos, 1961; DeRoos and DeRoos, 1963; Frankel et al., 1967b,c; Nagra et al., 1960, 1963; Resko et al., 1964). Doubly labelled corticosterone (1,2-$^{3}$H; New England Nuclear) was repurified using paper chromatography on a Bush C system. Non-radioactive corticosterone (Sigma) was stored with no further purification. When it was used, it was dissolved in 95% ethanol and standards were prepared containing 5, 10, 20, and 30 nanograms (ng) of corticosterone. The ethanol was subsequently removed by evaporation under air. Florisil (60-100 mesh) was bought from the Floridin Company and was stored without further purification.

Male human plasma was used as a source of transcortin (corticoid binding globulin, CBG). The blood was collected from two donors between 9:00 A.M. and 10:00 A.M., centrifuged, and separated. The plasma was divided into aliquots and

frozen.

Radioactive samples were counted in a Packard Tri-Carb Liquid Scintillation Counter Model #3003.

Thorough discussions of the theoretical and practical aspects of the assay appear in the literature (Murphy, 1964, 1967, 1969, 1970). The steps in the assay are briefly outlined below.

1. A transcortin solution was prepared by combining 1,2-$^{3}$H-corticosterone (2%), male human plasma (2.5%) and distilled water (95.5%). One milliliter of this solution was added to each standard and sample tube.

2. All tubes were agitated gently for 10 seconds and then incubated in a water bath at 45°C for 5 minutes.

3. Tubes were transferred to an ice bath at a temperature of 4° - 10°C for 10 minutes.

4. Fifteen mg of Florisil were added to each tube to remove unbound corticosterone. The tubes were shaken by inversion for 2 minutes, centrifuged for 1 minute at a low speed, and returned to the ice bath for 5 minutes.

5. One tenth ml aliquots were taken from each tube, combined with 3.0 ml absolute ethanol, then with 10.0 ml scintillation fluid. The standards and the samples were counted twice for 10 minutes each time.

In two instances, though normal assay procedures were used, the levels of corticosterone were less than the minimum level of the assay's sensitivity. These low values were taken as biologically real and calculations on these samples were

carried out based on the lowest value measurable on the curve.

Statistical Analysis

In accordance with standard statistical convention, differences between means were classified as not significant ($p > 0.05$), significant ($0.01 < p < 0.05$), and highly significant ($p < 0.01$). A probability level of 0.01 was used as the criterion for rejecting the null hypothesis that there was no difference between means. When the variances about the means were highly significantly different from each other ($p < 0.01$), an adjusted T-test was used to determine the level of significance between means. Regression analysis and covariance analysis were applied to the data from the first experiment.

Experimental Protocol

Six sets of experiments were included in the research.

1. Normal Plasma Corticosterone Levels--Levels of plasma corticosterone were monitored in normal control chick embryos every other day of incubation from the time embryos were 10 days old. The minute amounts of blood available from younger embryos were too small to allow the pooling necessary to obtain enough material for the assay. Plasma was collected from normal control post-hatching chicks to determine the pattern of hormone levels during this period.

2. Response to Stress--Blood from normal but stressed embryos was collected concurrent with the normal controls. This was done in order to determine if and when chicks were

capable of responding to stress. Blood from normal stressed post-hatching chicks was collected to determine whether a "non-responsive" period occurs following an earlier responsive period.

3. Response to ACTH--ACTH was administered to embryos and post-hatching chicks not responding to stress to determine whether the absence of the normal response to stress was attributable to a deficiency of ACTH or to a depression of sensitivity on the part of the target organ.

4. Effect of Hypophysectomy--Hypophysectomy (by decapitation) was performed on the youngest embryos which responded to stressful stimuli to determine whether control levels were dependent upon the secretion of hormones by the pituitary and/or the hypothalamus.

5. Stress Response of Hypophysectomized Embryos--Concurrent with experiment 4, hypophysectomized animals were stressed to determine whether stress levels were dependent upon the presence of a pituitary and/or hypothalamus.

6. Decapitated Embryos with Pituitary Graft--Since previous experiments by other workers suggested that the hypothalamus was not functional during the embryonic period in the chick, pituitary glands were transplanted onto decapitated animals resulting in an animal with a pituitary but no hypothalamus or forebrain. Corticosterone levels were monitored under control and stress conditions to determine whether the presence of a pituitary alone was sufficient to result in the same results as were attained in experiments 1 and 2.

# RESULTS

## Experiment 1--Normal Plasma Corticosterone Levels

Study of normal patterns of plasma corticosterone was repeated in two successive years. Tables 1 and 2, and Figures 1 and 2, respectively, show the results from pre- and post-hatching chicks from the 1970 flock. Hormone levels during the embryonic period from day 12 through day 18 remained constant. Student T tests and regression analysis revealed no significant difference or change in hormone levels between days 12 and 18. Plasma hormone levels on day 20 were significantly different at the 0.01 level from any other day sampled during the embryonic period. During the first 24 hours after hatching hormone levels remained high, but were significantly lower ($p < 0.01$) by the time the chicks were a week old. Glucocorticoid levels from 2 week, 3 week, and adult chickens were not significantly different from each other and were significantly lower ($p < 0.01$) than both 0 day and 1 week levels.

Plasma samples collected during the last half of the embryonic period and the initial post-hatching period during 1971 are shown in Tables 3 and 4. These results are depicted in Figures 3 and 5. Normal levels rose during the entire portion of the embryonic period tested. Linear

TABLE 1

NORMAL RESTING LEVELS OF PLASMA CORTICOSTERONE DURING EMBRYONIC DEVELOPMENT COLLECTED DURING 1970

| Age | Number of Samples | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|
| 12 days | 5 | 3.4 ± 0.2 |
| 14 days | 10 | 3.8 ± 0.5 |
| 16 days | 9 | 3.9 ± 0.2 |
| 18 days | 9 | 4.4 ± 0.4 |
| 20 days | 4 | 10.0 ± 1.2 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 12 days vs. 14 days | NS[b] |
| 14 days vs. 16 days | NS |
| 16 days vs. 18 days | NS |
| 18 days vs. 20 days | $p < 0.01$ |
| 12 days vs. 18 days | NS |

[a] S.E. = Standard Error

[b] NS = Not Significant

TABLE 1--continued

| REGRESSION ANALYSIS OF DAYS 12, 14, 16, 18 | |
|---|---|
| Level of Significance of Slope | NS |

Figure 1--Normal resting levels of plasma corticosterone in embryos from the 1970 flock. Means and standard errors represent data in Table 1.

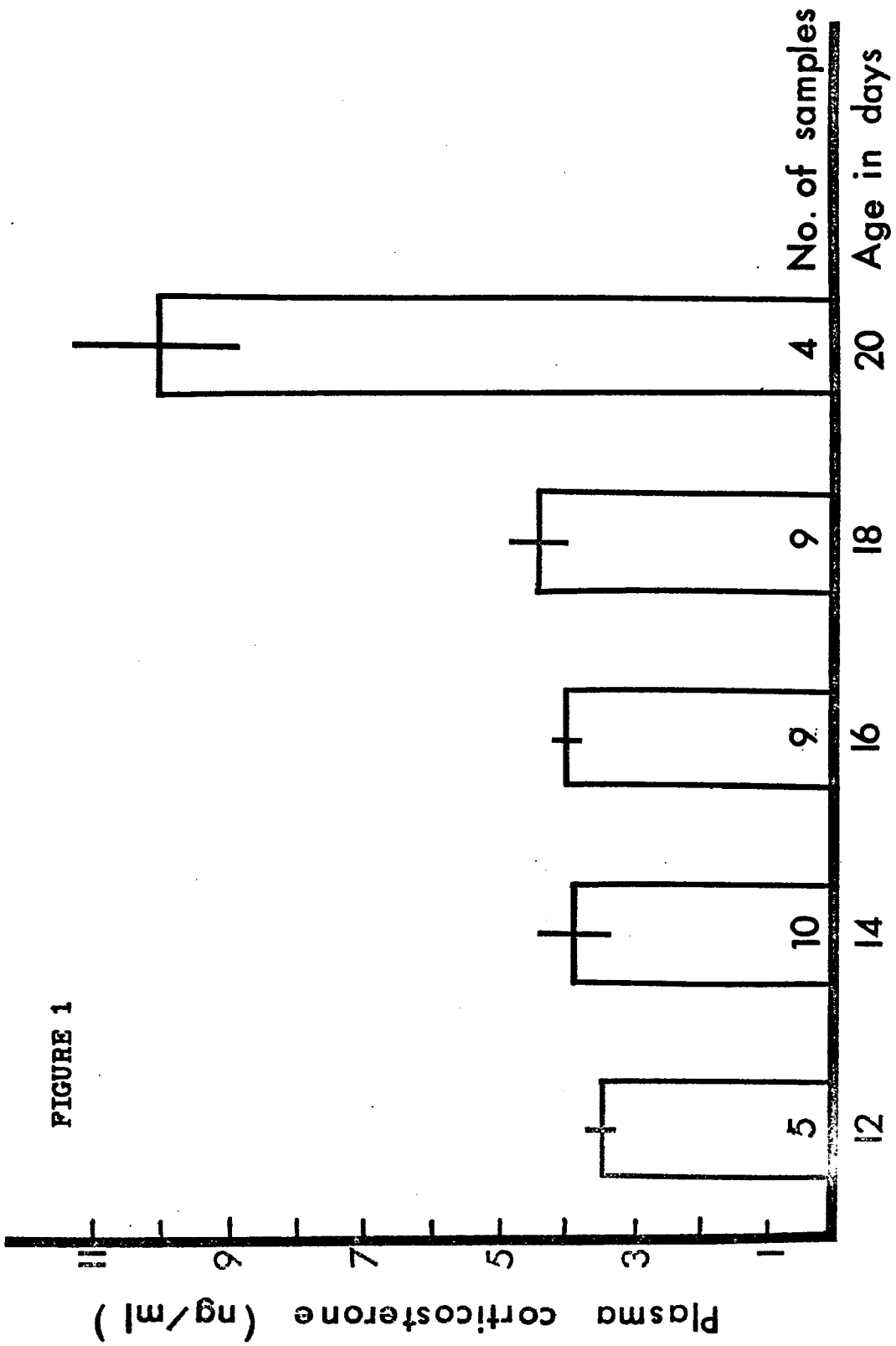

Plasma corticosterone (ng/ml)
11
9
7
5
3
1
12
14
16
18
20
Age in days
5
10
9
9
4
No. of samples

FIGURE 1

TABLE 2

NORMAL RESTING LEVELS OF PLASMA CORTICOSTERONE DURING THE POST-HATCHING PERIOD COLLECTED DURING 1970

| Age | Number of Samples | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|
| 0 days | 10 | 7.2 ± 1.4 |
| 1 week | 10 | 3.2 ± 0.5 |
| 2 weeks | 10 | 1.0 ± 0.1 |
| 3 weeks | 5 | 0.9 ± 0.3 |
| adult[b] | 4 | 0.6 ± 0.2 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 0 days vs. 1 week | $p < 0.01$ |
| 1 week vs. 2 weeks | $p < 0.01$ |
| 2 weeks vs. 3 weeks | NS[c] |
| 3 weeks vs. adult | NS |

[a]S.E. = Standard Error

[b]adult = six months and older

[c]NS = Not Significant

Figure 2--Normal resting levels of plasma corticosterone during the embryonic and post-hatching period from the 1970 flock. Means and standard errors represent data from Tables 1 and 2. Data from the embryonic period have been presented in Figure 1.

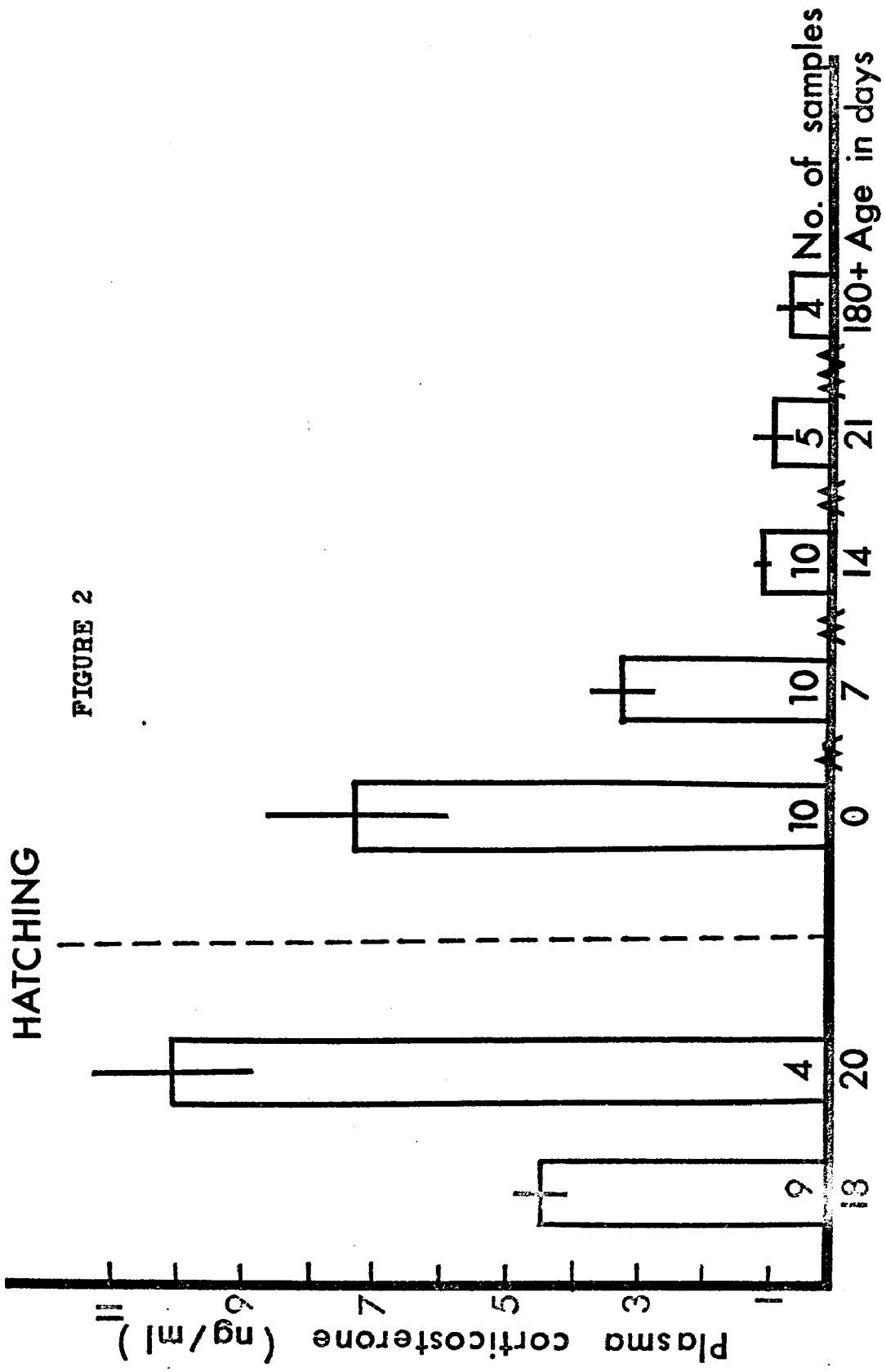

Plasma corticosterone (ng/ml)
1
3
5
7
9
11
HATCHING
9
4
10
10
10
5
10
4
-13
-20
0
7
14
21
180+
Age in days
No. of samples

FIGURE 2

TABLE 3

NORMAL RESTING LEVELS OF PLASMA CORTICOSTERONE DURING EMBRYONIC DEVELOPMENT COLLECTED DURING 1971

| Age | Number of Samples | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|
| 10 days | 5 | < 0.7 ± 0.2 |
| 12 days | 5 | < 1.1 ± 0.2 |
| 14 days | 5 | 1.2 ± 0.1 |
| 16 days | 5 | 1.8 ± 0.1 |
| 18 days | 5 | 2.1 ± 0.4 |
| 20 days | 5 | 2.3 ± 0.1 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 10 days vs. 12 days | NS[b] |
| 12 days vs. 14 days | NS |
| 14 days vs. 16 days | $p < 0.01$ |
| 16 days vs. 18 days | NS |
| 18 days vs. 20 days | NS |

[a] S.E. = Standard Error

[b] NS = Not Significant

TABLE 3--continued

COVARIANCE ANALYSIS

| Comparison | Level of Significance |
|---|---|
| Slope of 10, 12, and 14 days vs. Slope of 16, 18, and 20 days | NS |
| Intercept of 10, 12, and 14 days vs. Intercept of 16, 18, and 20 days | NS |

REGRESSION ANALYSIS

Slope = 0.168 Y intercept = -0.986

Level of Significance: $p < 0.01$

Correlation Coefficient = 0.767

Figure 3--Normal resting levels of plasma corticosterone in embryos from the 1971 flock. Means and standard errors represent data in Table 3.

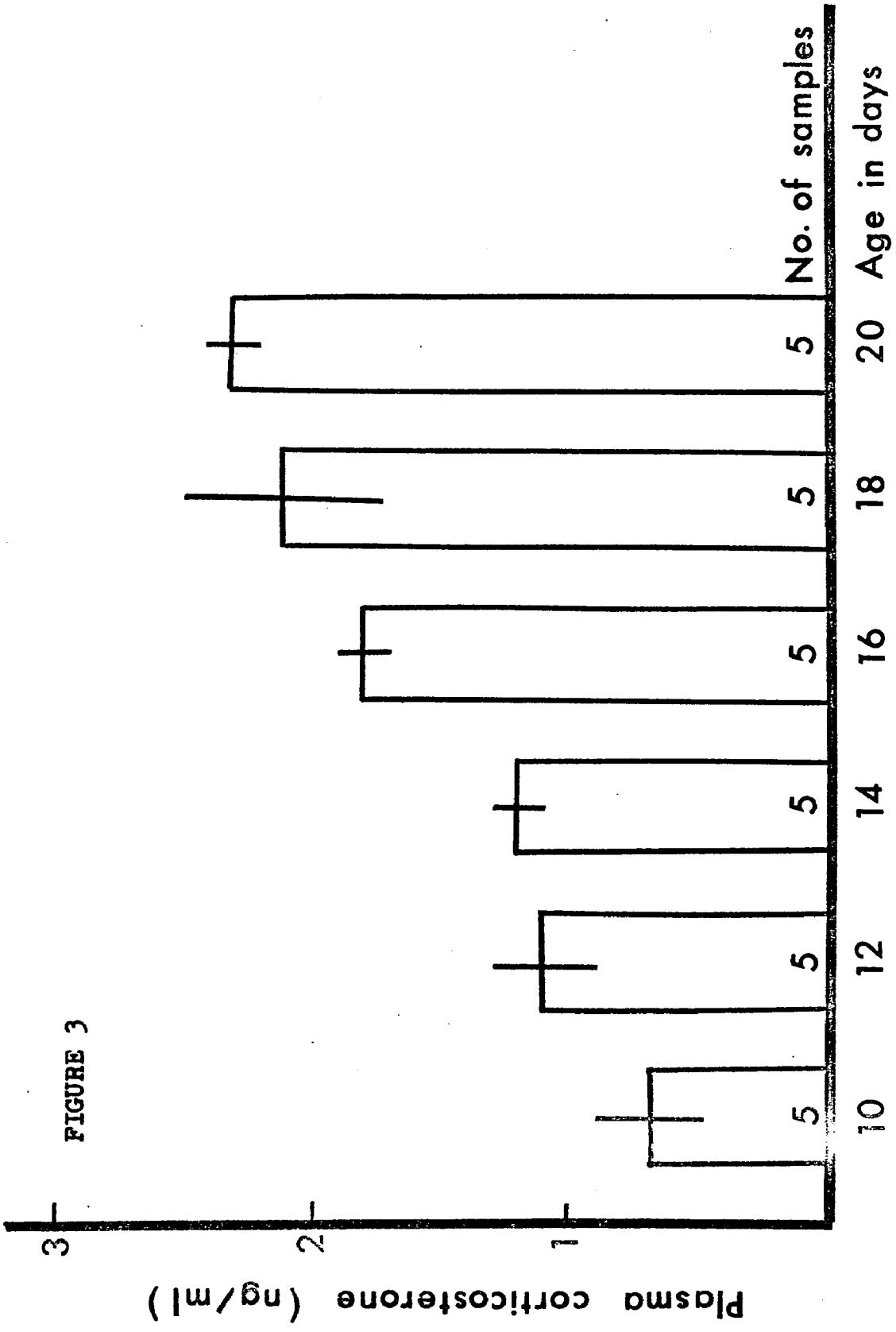

Plasma corticosterone (ng/ml)
3
2
1
10
12
14
16
18
20
Age in days
5
5
5
5
5
5
No. of samples

FIGURE 3

Figure 4--Linear regression analysis of normal resting levels of plasma corticosterone in embryos. Data can be found in Appendix and Table 3.

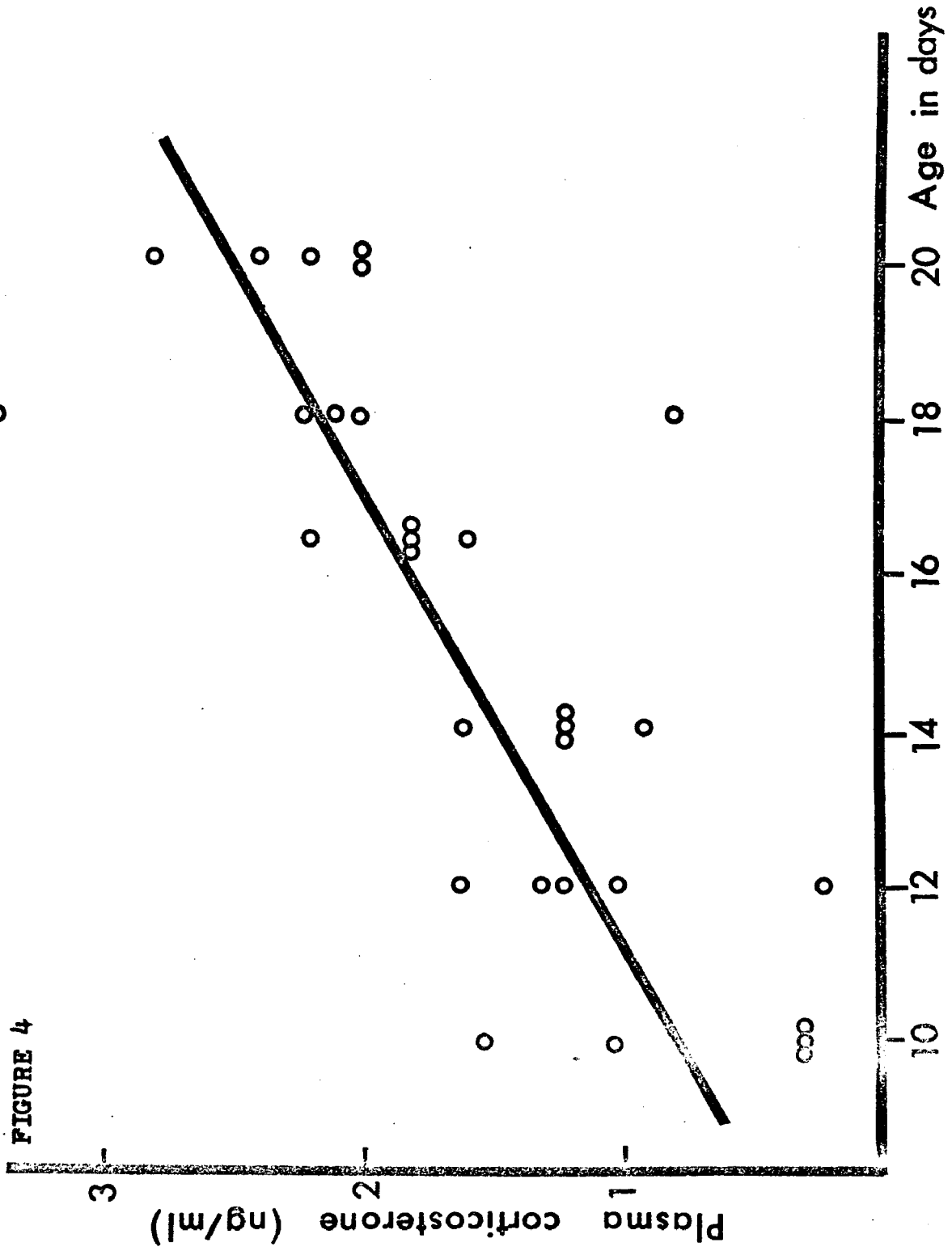



FIGURE 4

TABLE 4

NORMAL RESTING LEVELS OF PLASMA CORTICOSTERONE DURING THE POST-HATCHING PERIOD COLLECTED DURING 1971

| Age | Number of Samples | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|
| 0 days | 8 | 1.7 ± 0.3 |
| 1 week | 10 | 1.3 ± 0.2 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 20 day embryo vs. 0 day post-hatching | NS[b] |
| 0 day vs. 1 week | NS |
| 20 day embryo vs. 1 week post-hatching | $p < 0.01$ |

[a] S.E. = Standard Error

[b] NS = Not Significant

Figure 5--Normal resting levels of plasma corticosterone during the embryonic and post-hatching period from the 1971 flock. Means and standard errors represent data from Tables 3 and 4. Data from the embryonic period have been presented in Figure 3.

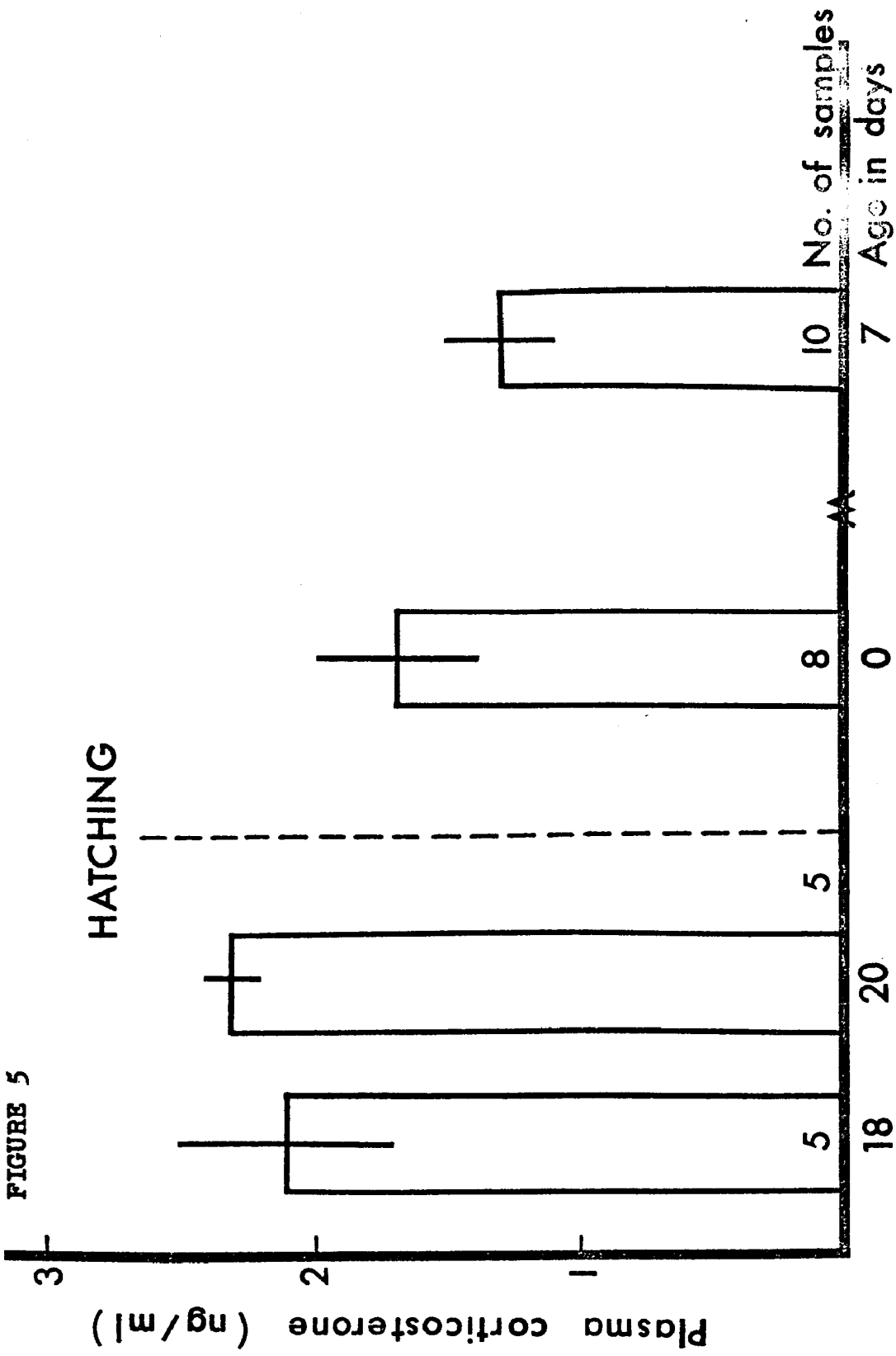

Plasma corticosterone (ng/ml)
3
2
1
HATCHING
18
20
0
7
Age in days
5
5
8
10
No. of samples

FIGURE 5

regression analysis of the data revealed that the slope of the line differed significantly from zero at the 0.01 level (Figure 4). T-tests comparing the means of successive days were not significantly different with the exception of the difference between the mean hormone levels on days 14 and 16. The means between these two days were significantly different at the 0.01 level. The data from days 10, 12, 14 were compared with the data from days 16, 18, and 20 to see if linear regressions of these two groups of data differed in slope or in intercept from each other. A covariance analysis uncovered no significant difference between the slopes ($0.8 < p < 0.9$) or the intercepts ($0.6 < p < 0.7$) of these two groups (Table 3).

Normal resting corticosterone levels from the initial post-hatching period demonstrate that hormone levels on the day of hatch were not significantly different than the last embryonic day ($0.09 < p < 0.1$). Hormone levels of week old chicks were not significantly different from that of day old chicks ($0.2 < p < 0.3$) but were significantly different from the last embryonic day tested at the 0.01 level (Table 4 and Figure 5).

Experiment 2--Response to Stress

Stressful stimuli applied to embryonic chicks caused significant increases in plasma corticosterone levels 20 minutes after the stimulus is administered on day 14 ($p < 0.04$) and a very significant increase in hormone levels on every day thereafter. The percent increase

of hormone levels of stressed embryos over control levels steadily rises between days 14 and 18. On day 14 stress caused a 58% increase, on day 16 a 67% increase, and on day 18 an 86% increase. There was no significant effect of stress on hormone levels in 12 day old embryos. These results appear in Table 5 and Figure 6. During the post-hatching period the same type of stress was incapable of producing a significant rise in plasma hormone levels in newly hatching chicks, but caused elevated levels in cockerels at one week of age (Table 6, Figure 7).

Experiment 3--Response to ACTH

Since 14 day old embryos did not respond to stress at as high a level of significance as was found in older embryos, 100 mU of adrenocorticotropin (ACTH) was administered to these embryos to determine whether they were sensitive to ACTH at this age. ACTH was also injected into newly hatched chicks to determine whether the lack of response to stress at this age was attributable to an absence of sensitivity to ACTH. The results of this experiment appear in Table 7 and Figure 8. In both age groups the response was very significant ($p < 0.01$).

Experiment 4--Effect of Hypophysectomy

To test the role of the pituitary and/or brain in the stress response, embryos were decapitated at the 33 hour stage and incubated to 14 and 16 days of age. Hormone levels of these animals were compared with those of normals

TABLE 5

EFFECT OF STRESS UPON PLASMA CORTICOSTERONE LEVELS DURING THE EMBRYONIC PERIOD

| Age | Number of Samples | Plasma Corticosterone ng/ml Mean ± S.E.[a] | |
|---|---|---|---|
| | | Control[b*] | Stressed |
| 12 days | 5 | <1.1 ± 0.2 | 1.0 ± 0.3 |
| 14 days | 5 | 1.2 ± 0.1 | 1.9 ± 0.3 |
| 16 days | 5 | 1.8 ± 0.1 | 3.0 ± 0.3 |
| 18 days | 5 | 2.1 ± 0.4 | 3.9 ± 0.3 |
| 20 days | 5 | 2.3 ± 0.1 | 3.5 ± 0.1 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 12 day control vs. 12 day stressed | NS[c] |
| 14 day control vs. 14 day stressed | $p < 0.04$ |
| 16 day control vs. 16 day stressed | $p < 0.01$ |
| 18 day control vs. 18 day stressed | $p < 0.01$ |
| 20 day control vs. 20 day stressed | $p < 0.01$ |

[a] S.E. = Standard Error

[b] All control data used for comparisons was collected in 1971.

[c] NS = Not Significant

*Control data appear in Table 3.

Figure 6--Effect of stress upon plasma corticosterone levels during the embryonic period. Means and standard errors represent data from Table 5. Control data have been presented in Figure 3.

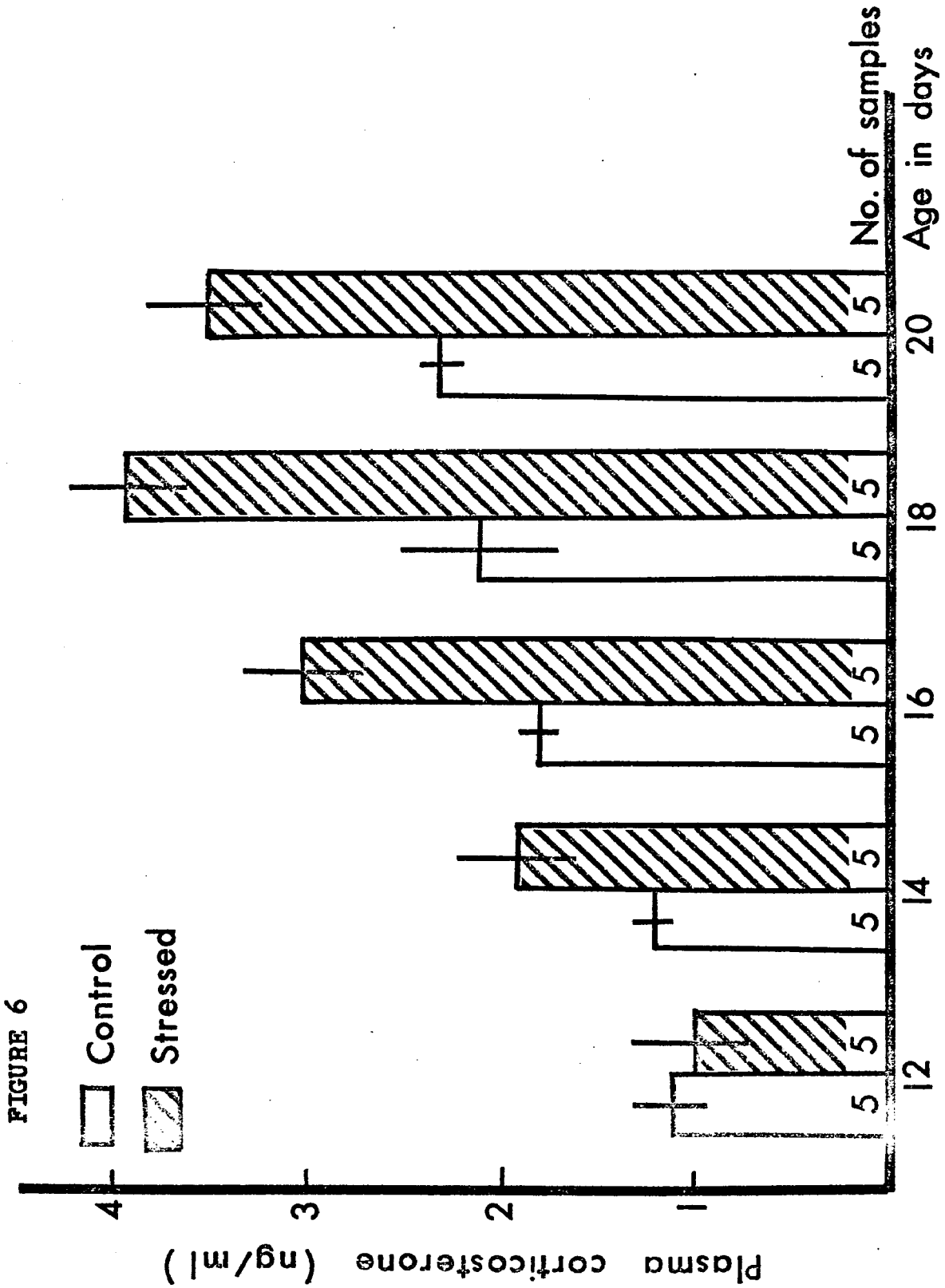

Plasma corticosterone (ng/ml)
4
3
2
1
Control
Stressed
No. of samples
Age in days
12
14
16
18
20
5
5
5
5
5
5
5
5
5
5

FIGURE 6

TABLE 6

EFFECT OF STRESS UPON PLASMA CORTICOSTERONE LEVELS DURING THE POST-HATCHING PERIOD

| Age | Number of Samples | Treatment | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|---|
| 0 days | 8 | Control | 1.7 ± 0.3 |
| 0 days | 6 | Stressed | 2.4 ± 0.4 |
| 1 week | 10 | Control | 1.3 ± 0.2 |
| 1 week | 8 | Stressed | 4.7 ± 0.6 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 0 day control vs. 0 day stressed | NS[b] |
| 1 week control vs. 1 week stressed | $p < 0.01$ |

[a] S.E. = Standard Error

[b] NS = Not Significant

Figure 7--Effect of stress upon plasma corticosterone levels during the embryonic and post-hatching period. Means and standard errors represent data from Tables 5 and 6. Data from embryos have been presented in Figure 6.

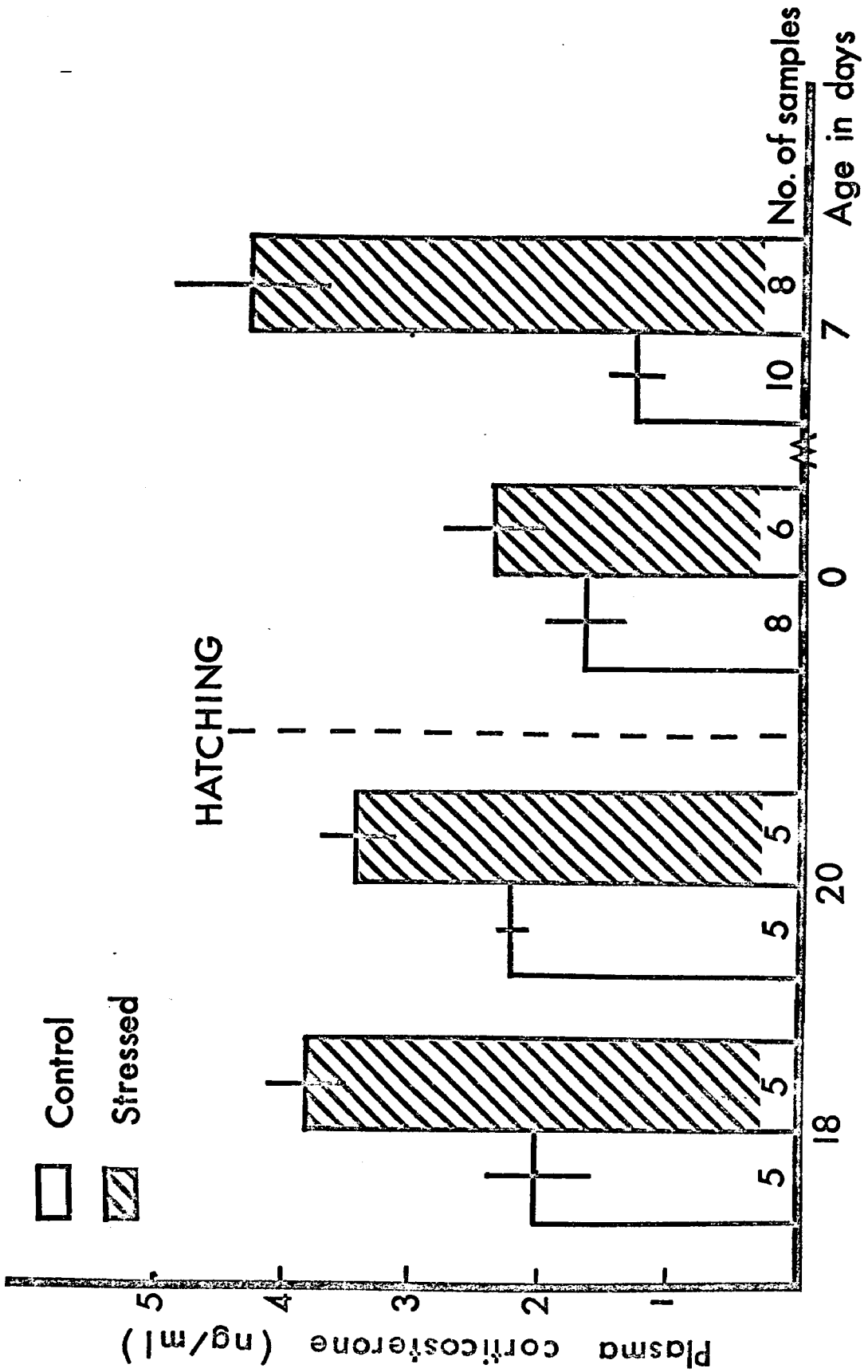

Plasma corticosterone (ng/ml)
1
2
3
4
5
Control
Stressed
HATCHING
18
20
0
7
Age in days
No. of samples
5
5
5
5
8
6
10
8

FIGURE 7

TABLE 7

EFFECT OF ACTH UPON PLASMA CORTICOSTERONE LEVELS ON FOURTEEN DAY-OLD EMBRYOS AND NEWLY-HATCHED CHICKS

| Age | Number of Samples | Treatment | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|---|
| 14 day embryo | 6 | Water injected Control | 2.1 ± 0.3 |
| 14 day embryo | 5 | ACTH injected | 4.7 ± 0.2 |
| 0 day post-hatch. chick | 7 | Water injected Control | 1.8 ± 0.3 |
| 0 day post-hatch. chick | 10 | ACTH injected | 3.4 ± 0.4 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 14 day control vs. 14 day ACTH injected | $p < 0.01$ |
| 0 day post-hatch. control vs. 0 day post-hatch. ACTH injected | $p < 0.01$ |

[a] S.E. = Standard Error

Figure 8--Effect of ACTH upon plasma corticosterone levels in fourteen day old embryos and newly hatched chicks. Means and standard errors represent data from Table 7.

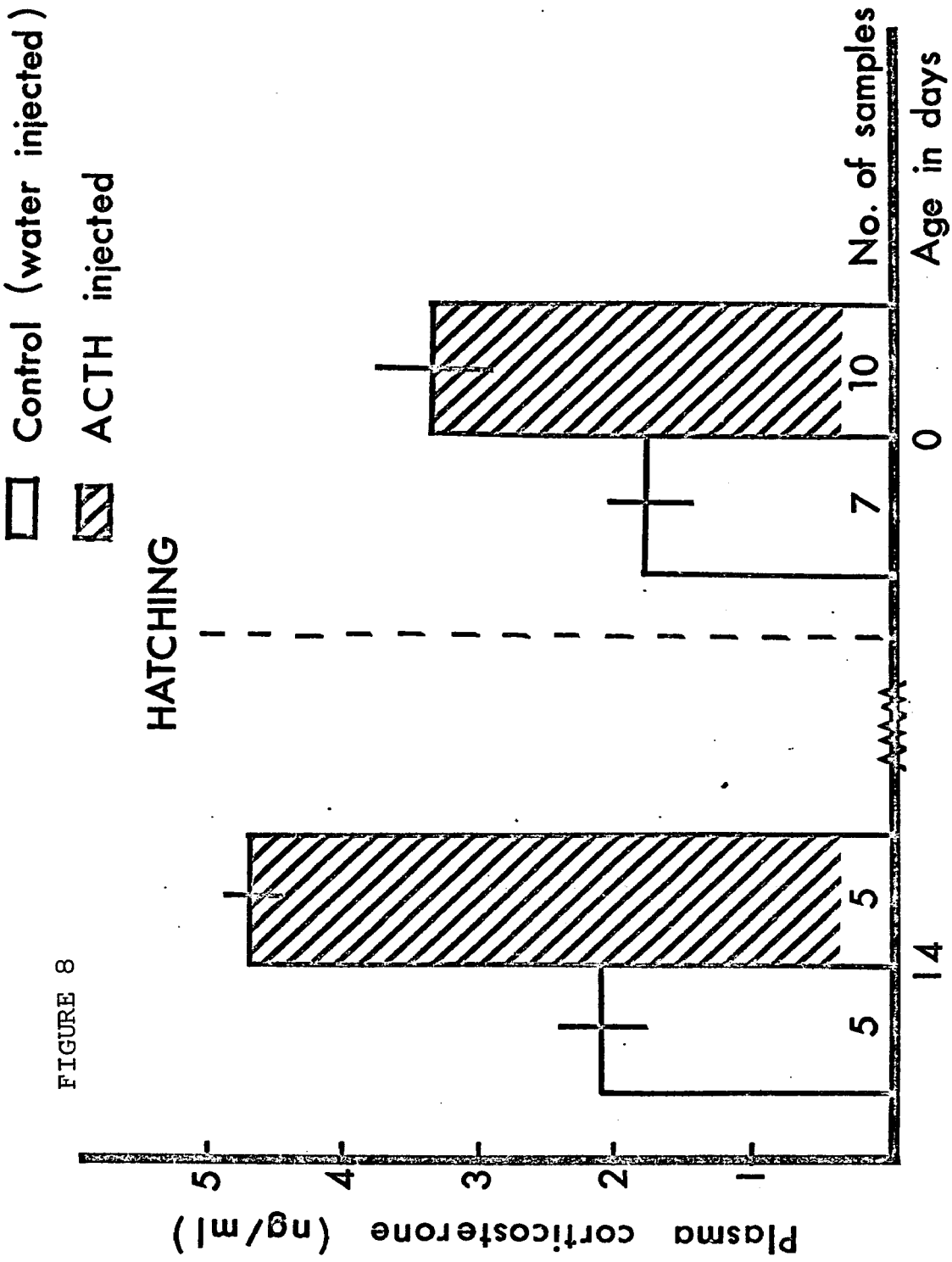



FIGURE 8

Fig 8 3c

(Table 8 and Figure 9). Hormone levels of decapitated embryos were not significantly different from normal controls at 14 days ($0.05 < p < 0.06$) but were significantly different at 16 days ($p < 0.02$).

Experiment 5--Stress Response of Hypophysectomized Embryos

Decapitated embryos were subjected to stressful stimuli to determine whether the stress response is mediated by the pituitary and/or hypothalamus. Comparison between the normal response and the response of decapitates on day 14 and on day 16 is shown in Table 9 and Figure 10.

Though normal 14 day old embryos showed a significant response to stress ($p < 0.04$), decapitated animals were incapable of any significant response to the same stimulus ($0.08 < p < 0.09$). At 16 days decapitated animals still showed no increase in hormone levels after stress ($0.8 < p < 0.9$) though normal embryos demonstrated a very significant response to a stress stimulus ($p < 0.01$).

Experiment 6--Decapitated Embryos with Pituitary Transplants

Hormone levels of embryos having a pituitary but no brain or hypothalamus were compared to those having neither pituitary nor hypothalamus and brain, and to those having both pituitary and brain. The results, shown in Table 10 and Figure 11, demonstrate that the hypothalamus was not necessary to maintain normal resting hormone levels on day 16. Thus, hormone levels of decapitates were significantly different from normal controls ($p < 0.02$) and

TABLE 8

EFFECT OF HYPOPHYSECTOMY (BY DECAPITATION) ON PLASMA CORTICOSTERONE LEVELS OF FOURTEEN AND SIXTEEN DAY EMBRYOS

| Age | Number of Samples | Treatment | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|---|
| 14 days | 5 | Normal | 1.2 ± 0.1 |
| 14 days | 5 | Decapitated | 0.8 ± 0.2 |
| 16 days | 5 | Normal | 2.2 ± 0.3 |
| 16 days | 5 | Decapitated | 1.1 ± 0.2 |

STATISTICAL COMPARISONS

| Comparison | Levels of Significance |
|---|---|
| 14 day normal vs. 14 day decapitated | NS[b] |
| 16 day normal vs. 16 day decapitated | $p < 0.02$ |

[a] S.E. = Standard Error

[b] NS = Not Significant

Figure 9--Effect of decapitation on plasma corticosterone levels of fourteen and sixteen day embryos. Means and standard errors represent data from Table 8.

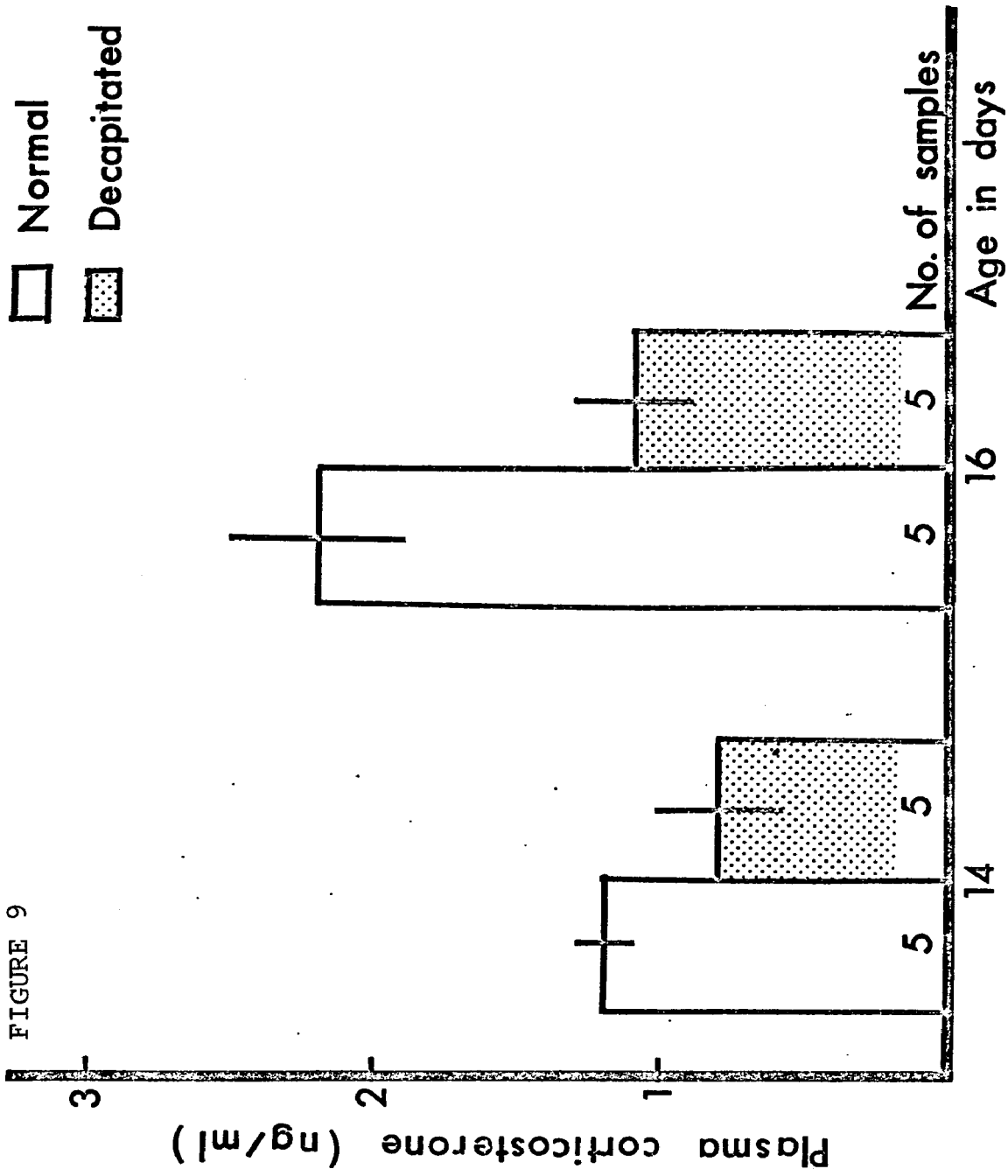

Normal
Decapitated
Plasma corticosterone (ng/ml)
3
2
1
No. of samples
Age in days
5
5
5
5
5
14
16

FIGURE 9

TABLE 9

EFFECT OF HYPOPHYSECTOMY (BY DECAPITATION) ON PLASMA CORTICOSTERONE LEVELS BEFORE AND AFTER A STRESSFUL STIMULUS IS ADMINISTERED

| Age | Number of Samples | Treatment | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|---|
| 14 days | 5 | Normal Control* | 1.2 ± 0.1 |
| 14 days | 5 | Normal Stressed* | 1.9 ± 0.3 |
| 14 days | 5 | Decapitated Control* | 0.8 ± 0.2 |
| 14 days | 5 | Decapitated Stressed | 1.1 ± 0.1 |
| 16 days | 5 | Normal Control* | 2.2 ± 0.3 |
| 16 days | 5 | Normal Stressed* | 3.0 ± 0.3 |
| 16 days | 5 | Decapitated Control* | 1.1 ± 0.2 |
| 16 days | 5 | Decapitated Stressed | 1.2 ± 0.2 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 14 day normal control vs. 14 day normal stressed | $p < 0.04$ |
| 14 day decapitated control vs. 14 day decapitated stressed | NS[b] |
| 16 day normal control vs. 16 day normal stressed | $p < 0.01$ |
| 16 day decapitated control vs. 16 day decapitated stressed | NS |

[a] S.E. = Standard Error

[b] NS = Not Significant

* Data appear previously in Tables 5 and 8.

Figure 10--Effect of decapitation on plasma corticosterone levels before and after a stressful stimulus is administered. Means and standard errors represent data from Table 9. Data from normal -- control and stressed, and decapitated control have been presented in Figures 6 and 9.

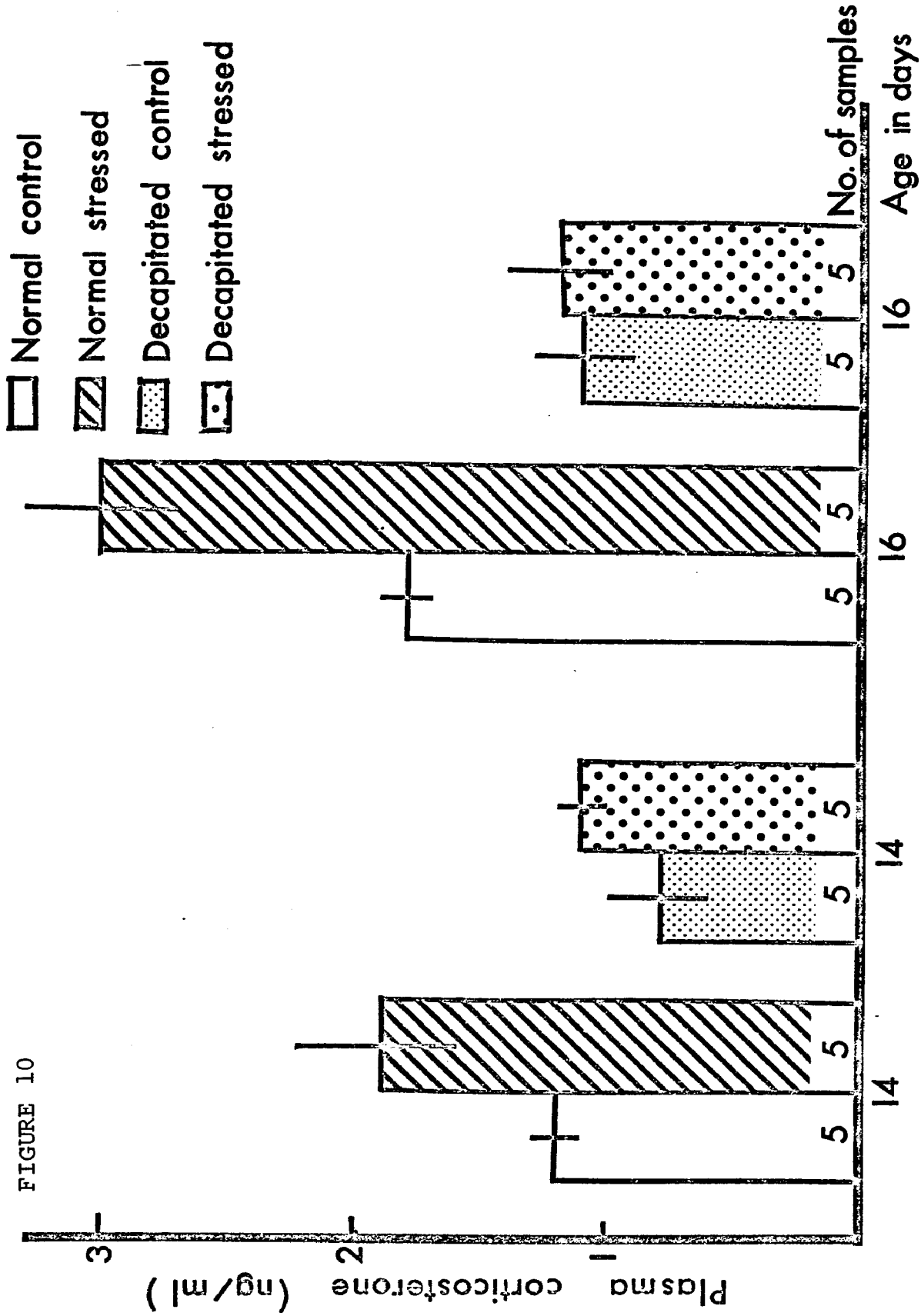

Normal control
Normal stressed
Decapitated control
Decapitated stressed
Plasma corticosterone (ng/ml)
1
2
3
No. of samples
Age in days
5
5
5
5
5
5
5
5
14
14
16
16

FIGURE 10

TABLE 10

COMPARISON OF PLASMA CORTICOSTERONE LEVELS UNDER CONTROL CONDITIONS IN NORMAL, DECAPITATED, AND DECAPITATED WITH PITUITARY GRAFT EMBRYOS

| Age | Number of Samples | Treatment | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|---|
| 16 days | 5 | Normal* | 2.2 ± 0.3 |
| 16 days | 5 | Decapitated* | 1.1 ± 0.2 |
| 16 days | 5 | Decapitated with Pituitary Graft | 1.8 ± 0.2 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 16 day normal vs. 16 day decapitated | $p < 0.02$ |
| 16 day normal vs. 16 day decapitated with pituitary graft | NS[b] |
| 16 day decapitated vs. 16 day decapitated with pituitary graft | $p < 0.04$ |

[a] S.E. = Standard Error

[b] NS = Not Significant

* Data appear previously in Table 8.

Figure 11--Comparison of plasma corticosterone levels under control conditions in normal, decapitated, and decapitated with pituitary graft embryos. Means and standard errors represent data from Table 10. Normal and decapitated data have been presented in Figure 9.

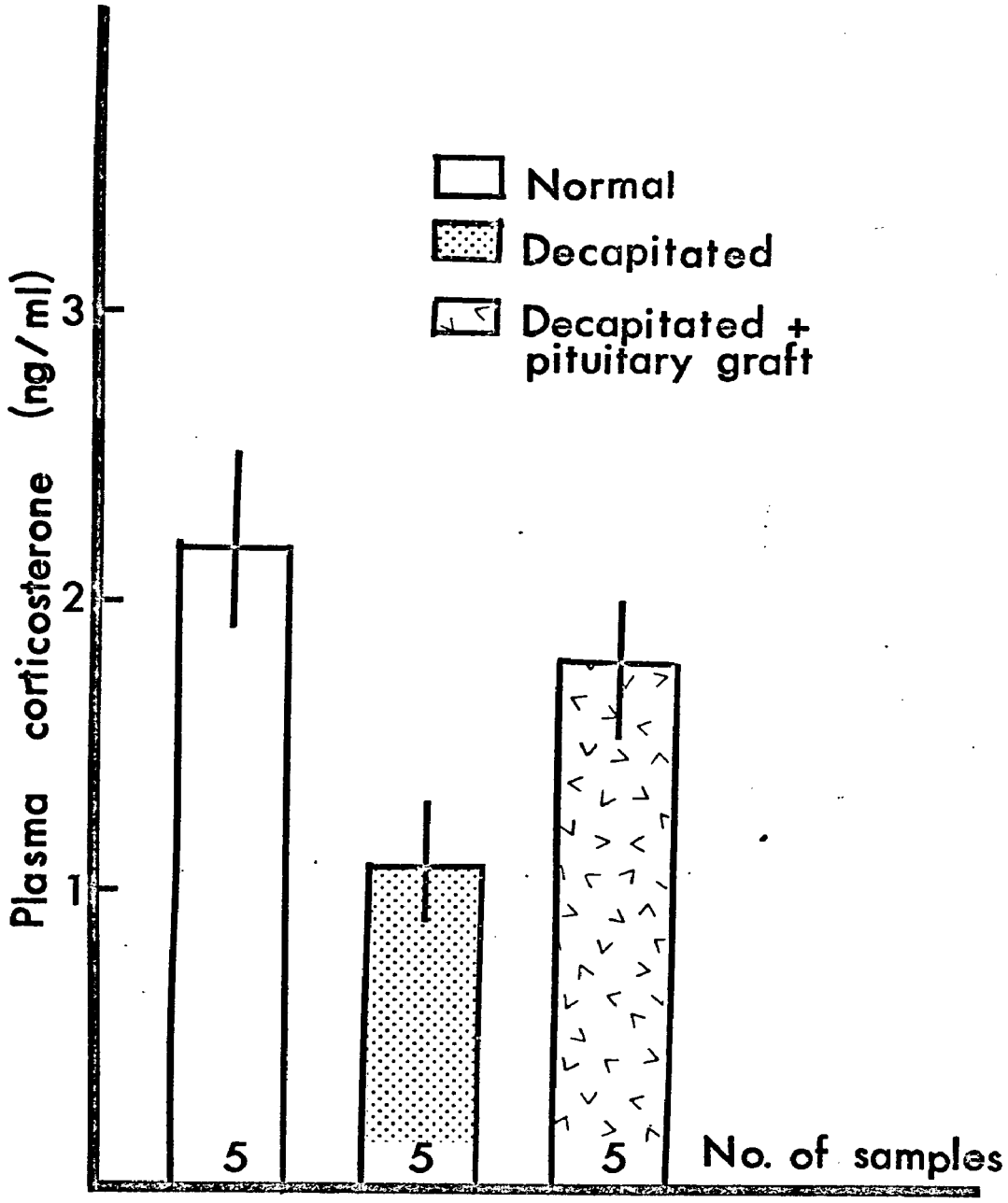

Normal
Decapitated
Decapitated + pituitary graft
Plasma corticosterone (ng/ml)
3
2
1
5
5
5
No. of samples

FIGURE 11

from decapitates receiving pituitary grafts ($p < 0.04$). Further, corticosterone levels of normals were not significantly different from decapitates with pituitary grafts ($0.2 < p < 0.3$).

The role of the hypothalamus in the capacity to respond to stress appears in Table 11 and Figure 12. Normal 16 day old embryos were capable of responding to stressful stimuli ($p < 0.01$); however, decapitates with or without pituitary grafts were not capable of responding in a similar manner ($0.9 < p < 1.0$, $0.9 < p < 1.0$). Thus, the hypothalamus is necessary for a response to stressful stimuli in the chick embryo at 16 days.

TABLE 11

EFFECT OF DECAPITATION AND DECAPITATION WITH PITUITARY GRAFT REPLACEMENT ON PLASMA CORTICOSTERONE LEVELS BEFORE AND AFTER A STRESS STIMULUS IS ADMINISTERED

| Age | Number of Samples | Treatment | Plasma Corticosterone ng/ml Mean ± S.E.[a] |
|---|---|---|---|
| 16 days | 5 | Normal Control* | 1.8 ± 0.1 |
| 16 days | 5 | Normal Stressed* | 3.0 ± 0.3 |
| 16 days | 5 | Decapitated Control* | 1.1 ± 0.2 |
| 16 days | 5 | Decapitated Stressed* | 1.2 ± 0.2 |
| 16 days | 5 | Decapitated with Pituitary Transplant Control* | 1.8 ± 0.2 |
| 16 days | 5 | Decapitated with Pituitary Transplant Stressed | 1.9 ± 0.1 |

STATISTICAL COMPARISONS

| Comparison | Level of Significance |
|---|---|
| 16 day normal control vs. 16 day normal stressed | $p < 0.01$ |
| 16 day decapitated control vs. 16 day decapitated stressed | NS[b] |
| 16 day decapitated with pituitary transplant vs. 16 day decapitated with pituitary transplant stressed | NS |

[a] S.E.= Standard Error

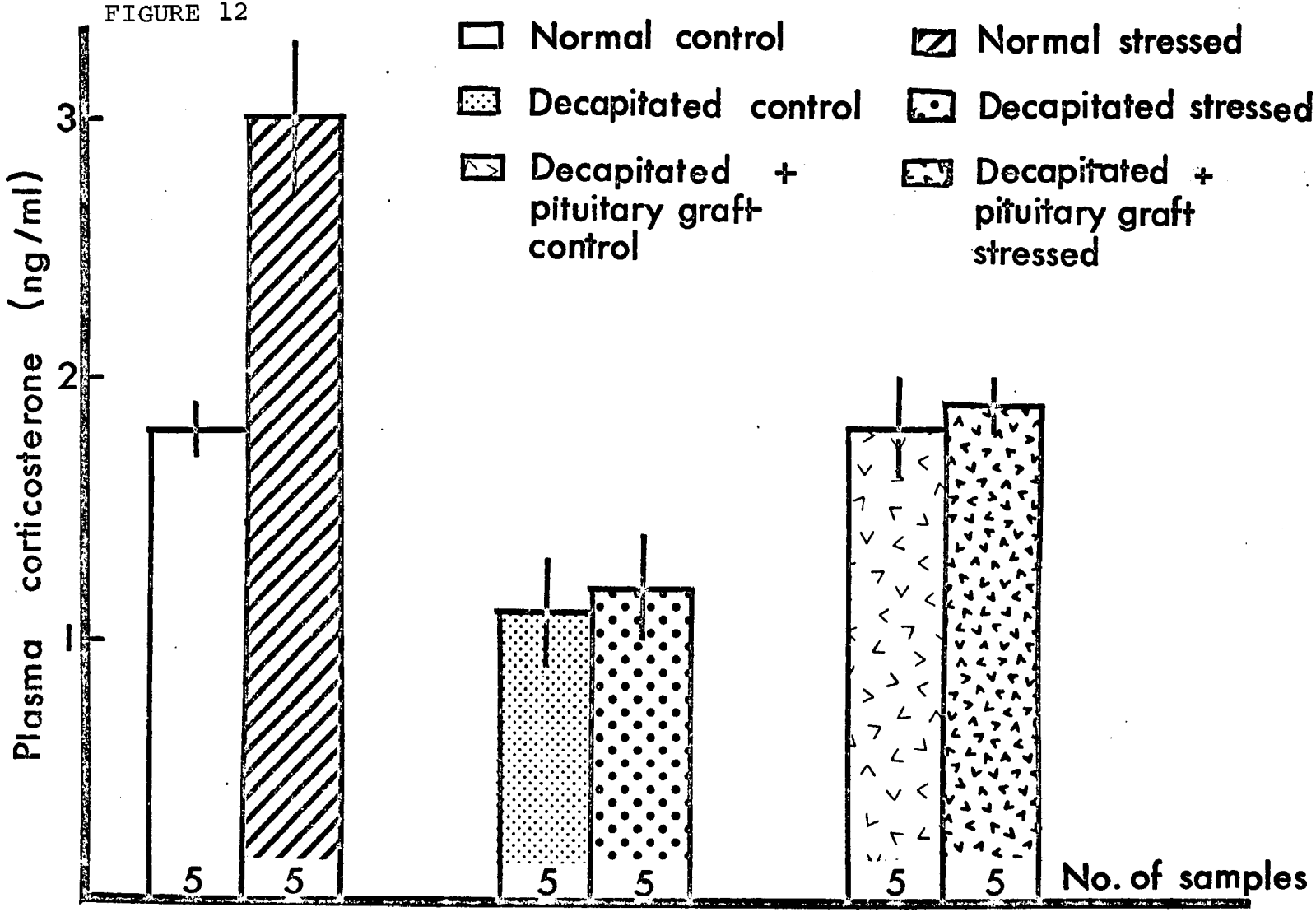
[b] NS = Not Significant

*Data appear in Tables 9 and 10.

Figure 12--Effect of decapitation and decapitation with pituitary graft replacement on plasma corticosterone levels before and after a stressful stimulus is administered. Means and standard errors represent data from Table 11. Data from normal -- control and stressed, decapitated -- control and stressed, and decapitated with pituitary graft -- control have been presented in Figures 10 and 11.

Fig 12 70.

FIGURE 12



# DISCUSSION

The results of the present study characterize the temporal pattern of glucocorticoid secretion in the chick embryo under normal conditions. In addition, the data from decapitated embryos and decapitated embryos receiving pituitary grafts give some indication of the extent of the participation of the hypothalamus and pituitary in the function of the adrenal during development.

## Normal Resting Levels of Plasma Corticosterone During Development

Measurement of plasma corticosterone levels is the most direct means available to assess the functional status of the adrenal cortex during development. The formulation of hypotheses regarding the participation of glucocorticoids in target organ development and differentiation, and the regulation and control of the adrenal by the pituitary and the hypothalamus, depend upon information on normal basal secretion levels of the hormone.

Since plasma corticosterone levels were monitored in chick embryos, newly hatched chicks, and older post-hatching cockerels during two different years, a comparison can be made for those days on which measurements were made both in 1970 and 1971. Several differences are apparent in both the

absolute hormone concentrations and the pattern of hormone levels over time between the series for these two years.

There are several possible causes for the variability: 1) Although Klager Hatchery received its eggs from the same egg farms during both years, one group of hens was the source of fertile eggs for all the data from 1970, while a totally different group from the same strain were used in 1971. This resulted from the husbandry practice of using laying hens for only one year and replacing them each year by young offspring of the layers of the previous year. Thus, though the strain used for all the experiments was the same, the individuals in the group of laying hens were different from 1970 to 1971. Slight differences in results occurred when Moog changed the breed of chick she was using (Moog, 1961), and it appears from the present study that just a change in the flock, even when the strain of chick is unchanged, might result in significantly different hormone levels. 2) Seasonal variation in concentration of adrenal steroid hormones has been reported for adult White Throated Sparrows (Dusseau and Meier, 1971). No such study has been done in chick embryos. However, since certain samples were not collected in corresponding months of the different years, it is possible that seasonal variation may have affected the hormone determinations observed in this study. 3) The investigator and the assay used were the same. Every parameter of the assay was checked for possible inaccuracy. None were uncovered. Nevertheless, due to increased familiarity with the steps in the research,

it is possible that measurements made during the second year of the research were more accurate than those made at the beginning of the study.

In 1971 all the embryos and post-hatching chicks came from one farm; no eggs from any of the other farms which supply Klager Hatchery were accepted. Large groups of eggs were purchased at once, allowing many parts of one experiment to be done using eggs collected from one morning. Thus, a more uniform diet for the hens, a more uniform schedule of collecting newly laid eggs, and a more uniform policy for storing eggs could be expected. Since these parameters of the study were more stringently controlled in 1971 and since all other experiments in this study were done in 1971, all further discussion will be based on data collected in 1971 whenever it is available.

Regression analysis of the data shows that from day 10 to hatching there is a gradual increase in resting levels of plasma corticosterone of normal embryos during the entire period monitored. In addition, T-tests demonstrate that there is a very significant rise in hormone levels between day 14 and day 16, and that there is no such significant difference between consecutive days at any other time during embryonic development. Thus, a significant change in the control over corticosterone secretion may be occurring in the 14-16 day interval. One possibility is that pituitary ACTH secretion begins or is significantly elevated at this time. Evidence from other workers suggests that the pituitary begins

to influence adrenal development between the 13th and 15th day. Thus, after hypophysectomy on the 2nd day of incubation, adrenals of controls and experimentals continue to grow at the same rate until the 15th day (Case, 1951, 1952). After this time the adrenals of hypophysectomized embryos cease to gain weight, whereas those of normal embryos double their weight by the time of hatching. The pattern of adrenal ascorbic acid accumulation also remains unaltered in hypophysectomized embryos until the 15th day, after which ascorbic acid levels of normal embryos continue to rise while the levels of hypophysectomized embryos remain steady (Case, 1951, 1952).

An additional intriguing aspect of the change in steroid secretion pattern is revealed by covariance analysis of the data from the two periods: before (days 10, 12, and 14) and after (days 16, 18, and 20) the 14-16 day rise. This analysis shows that the slopes and thus the rate of increase in corticosterone secretion in the two periods is the same. Hence, the limiting factor in steroid secretion rate may be some intrinsic property of the developing adrenal, which is the same during the ACTH dependent period (day 16 on) and the autonomous period (before day 14). One such factor could be the mass of adrenal tissue. Examination of the data from Case's study shows that the largest increase in weight of the adrenal occurs between 14 and 16 days (Case, 1951). It may be that ACTH stimulates the "abrupt" increase in adrenal size during the 14 to 16 day interval. It is possible then, that the effect of endogenous ACTH is mainly on growth rather

than steroid output per se.

Woods and his colleagues studied glucocorticoid levels in chorioallantoic fluid of the chick embryo between the 10th and 17th day of incubation (Woods et al., 1971). The quantity of hormone found by these investigators, as well as the pattern of change of hormone levels during the developmental period studied, differ from the results of this study. On day 10 of incubation, the levels reported by Woods and his group are 500 times higher than those found in the present investigation, and the differences increase as the embryos grow older. There are two probable reasons, discussed below, for these differences. 1) The non-specific nature of the assay used by Woods and his group compared with the specificity of the protein binding radioassay. 2) Differences in the physiological properties of the fluids used for the determination of hormone levels.

First, regarding the assay techniques: Woods and his group used a brief fluorometric assay with cortisol as the standard. In the present studies the protein binding radioassay was used, with corticosterone as the standard. Studies using adult chickens, both in vivo and in vitro, indicate that corticosterone is the major glucocorticoid in chickens (DeRoos, 1961; Frankel et al., 1967a,b,c; Nagra et al., 1960). Corticosterone has also been identified as the major glucocorticoid in the culture medium of adrenals of 16 and 18 day old embryos (Bonhommet and Weniger, 1967). It has been found that several brief fluorometric methods for

the determination of glucocorticoids are grossly inaccurate when applied to avian adrenal plasma (Frankel et al., 1967a). Residual fluorescence caused by interfering fluorogens could not be eliminated despite various efforts to prepurify the plasma and the reagents. Thus all the brief fluorometric methods tested by these investigators show high concentrations of "corticosterone" in plasma presumed to be free of the hormone. The specificity of the protein-binding radioassay depends upon the high affinity and specificity of transcortin for corticosterone. The particular advantage of this method is the absence of effects from steroid metabolites and non-steroidal substances, and the small group of steroid hormones which are highly competitive under ideal assay conditions.

Second, regarding the source of material: whereas glucocorticoid concentrations were measured in arterial and venous blood of embryos in the present study, Woods and his group measured hormone concentrations from chorioallantoic fluid. Since the allantoic sac serves mainly to store waste products accumulated during the embryonic period, the concentration of corticosteroids and their metabolites found may not represent the levels being secreted by the embryo at the time of sampling, but may consist of all the hormones and metabolites which have accumulated up to that time, including those recently secreted. Measuring glucocorticoid levels in peripheral plasma gives an indication of the functional state of the adrenal at that point in time. Since metabolites of steroids are continually being excreted into the chorioallantoic fluid, the higher concentrations of hormone found in Woods' study on

day 10 and the increasing differences with age can partially be accounted for by the fact that different physiological fluids having totally different functions were sampled in the two studies.

Adrenocortical hormone levels during the last part of fetal development have been monitored in sheep and rats. By sampling blood *in utero*, Bassett and Thorburn (1969) were able to determine glucocorticoid levels in lamb fetuses from day 130 to birth on day 148. They found steadily increasing levels of the hormone in the fetal circulation during this entire period. Holt and Oliver (1968) measured corticosterone levels in the rat fetus during the last 3 days before birth and found hormone levels peaked just before birth. Although neither of these studies cover as large a fraction of the developmental period as the present one, both show that hormone levels in these two species steadily rise during the last part of embryonic development as they do in chicks.

The presence of hormone and the pattern of change raise the question of whether adrenocortical hormones have a function during the developmental period. Studies by Moog and her group show that the adrenal participates in embryonic physiological changes which take place in duodenal epithelium (Moog, 1950, 1959; Moog and Ford, 1957; Moog and Richardson, 1955; Moog and Thomas, 1957). These changes proceed most rapidly between days 17 and 20, during which time alkaline phosphatase concentrations increase a hundred fold. Since the present study indicates that no drastic change in plasma hormone concentrations occurs during this period, it appears

that the abrupt change in intestinal chemistry depends upon a gradual, not a sudden, increase in hormone levels. Hence, Moog's studies and the present one suggest that gradually increasing hormone levels over an extended period of time are necessary for 1) the differentiation of the duodenal epithelium and 2) the abrupt increase in alkaline phosphatase levels.

Few studies have examined the question of whether the adrenal participates in the same regulatory functions in both the embryo and adult. This is probably due to the fact that a method of adrenalectomy of the chick embryo has not yet been developed. Relatively large doses of corticoids administered to 8 to 10 day old embryos retards growth (Evans, 1953; Karnofsky et al., 1951; Sames and Leathem, 1951). The same concentration administered to 16 day embryos, causes a slight weight increase by the time the embryos are 19 days old (Moog and Richardson, 1955). Clawson and Domm (1964) found that administration of cortisol causes a decrease in liver glycogen within 6 hours when administered between the 6th and 11th day, but causes an increase in levels when administered to 16 day old embryos.

Plasma hormone levels for post-hatching chicks in this study show that levels are highest around hatching and then decrease to lower levels by the time the chicks are a week old. Elevated hormone levels around the time of birth have also been reported in several mammalian species (rat: Holt and Oliver, 1968; Milkovic and Milkovic, 1963; sheep:

Alexander et al., 1968; Bassett and Thorburn, 1969; human: Gemzel, 1954; Kawahara, 1960; Migeon et al., 1956). Thus, it appears that elevated adrenal activity is associated with both the birth of mammals and the hatching of birds. Since steroids are known to pass through the placenta, elevated glucocorticoid levels during the perinatal period in mammalian animals could be of a maternal origin. In the chick, with no immediate maternal influence possible, elevated hormone levels can only be caused by a hyperactive adrenal or a lowered turnover rate during the period around hatching.

Post-hatching hormone levels beyond a week were monitored only during 1970. Corticosterone levels appear to reach an equilibrium between the first and second week after hatching. In rats, a constant resting level is reached by the time the animals are 5 days old (Milkovic and Milkovic, 1963).

In general, the hormone levels found in this study for post-hatching chicks are lower than those reported by other investigators (Frankel et al., 1967a,b,c; Nagra et al., 1960). There are several probable reasons for the difference. 1) Since Freeman (1967) demonstrated that ascorbic acid depletion occurs within 10 minutes of handling a group of chicks, great care was taken not to disturb the chicks before the blood was collected. Thus, it is hoped that "control" levels of hormone are truly basal secretion levels and not those which reflect a stressful situation. 2) Anesthesia has been used in many other studies in order to draw blood from a vein. Such a procedure is known to cause a stress response

in older chickens and may be responsible for the higher levels reported. 3) The assays used by other groups differ from that used for the present study and therefore may be measuring a slightly larger group of glucocorticoids.

The Effect of Leg Fracture on Plasma Corticosterone Levels During Development.

Leg fracture was used in this study to elicit a "stress response". The theory of a stress response was developed by Selye (1950). It had been observed that a variety of non-specific damaging stimuli such as cold, infection, or fracture of a part of the body cause a complex adaptive reaction by the organism. One or more of several changes take place in the organism after such stress, such as involution of the thymus, hyperglycemia, and disappearance of lymphatic apparatus. One characteristic response to various types of stress is an increase in adrenal corticoid production. That the typical increase in hormone levels after stressful stimuli is dependent upon the pituitary and hypothalamus has been widely accepted. Thus, experiments were designed to determine whether injury to chicks would cause the classical increase in glucocorticoid titers and to reveal the role of the pituitary and hypothalamus.

Only embryos 16 days and older demonstrate a statistically highly significant response to stress, as measured by elevated plasma corticosterone levels. It is possible that 12 day old embryos do not respond to leg fracture because their nervous apparatus is not developed enough to interpret

the stimulation. Nerve fibers can be seen passing into the leg and knee joint as early as the 7th day; however, differentiation and proliferation of branches continue to develop past the 15th day (Romanoff, 1960). Unlike the response of older embryos, the response to stress of 14 day old embryos is significant only at the 0.04 probability level. The lower level of significance in these embryos can be attributed to three possible factors. First, it is possible that the development of the stress response takes several days in each individual embryo and thus does not reach the same functional status for several days. Or the developmental process may be relatively fast, but due to the fact that the real age of the embryos in each group may vary up to 12 hours, the speed of the process is hidden. Thirdly, the maturation process may be fast or slow but may occur at slightly different ages in each individual.

Stressful stimuli are known to have some effect on adrenal activity during the fetal period in rats. The injection of epinephrine or leg fracture cause elevated corticosterone levels and adrenal ascorbic acid depletion in 20 day old rat embryos (Milkovic and Milkovic, 1963). No studies testing whether the fetus is capable of responding to stressful stimuli before this time appear in the literature.

A most intriguing aspect of this study is the disappearance of a stress response during the first days after hatching. Thus, leg fracture has no effect upon circulating glucocorticoid levels in newly hatched chicks. The capacity

to respond to such stimuli is apparent again when the chicks are one week old. Such non-responsiveness of the adrenocortical system to stressful stimuli during the early post-natal period has also been reported in rats, dogs, and rabbits (Jailer, 1950; Levine et al., 1967; Milkovic and Milkovic, 1959). The non-responsive period found in chicks in this study is comparable to that found in other studies (Milkovic and Milkovic, 1963). Freeman (1967) found that 3 week old cockerels are capable of responding to stress as demonstrated by depletion of adrenal ascorbic acid. No other studies have concentrated around the initial post-hatching period in chickens.

Both the mechanism and the physiological significance of the "non-responsive" period are important aspects of this developmental stage. Studies attempting to explain the non-responsive period have examined each component of the hypothalamic-hypophyseal-adrenal cortex axis. Milkovic and Milkovic (1963) found that rat adrenals are capable of responding to elevated levels of ACTH both in vivo and in vitro, and therefore proposed that it is the release or production of ACTH by the pituitary which is rate limiting in the response of the newborn rats to stress. Hiroshige and his colleagues discovered that the response pattern of corticotropin releasing factor (CRF) to stress is very different in adults than that of neonatal rats (Hiroshige and Sato, 1970; Hiroshige et al., 1971). In adult rats the pattern is characteristically biphasic: first a rapid

phase consisting of a prompt increase in hypothalamic CRF content followed by a depletion twenty minutes after the onset of stress, and a second slow phase forty minutes after stress stimuli are begun. In newborn rats only the second delayed response is apparent. If the adrenal response is dependent upon hypothalamic stimulation, any measurement of glucocorticoid concentration taken in the newborn rats of the Milkovic and Milkovic (1963) study and the chickens of the present study within the first forty minutes would not show increased adrenocortical hormone levels even if the adrenal contained the hormone to be released. Hiroshige and his group did not include any measurement of CRF activity during the embryonic period in rats. The question of whether a rapid phase takes place at this stage, and if so, why it disappears in neonates, remains unanswered.

The present data indicate that the use of the term "non-responsive" period may be misleading. It is important to consider that since "basal" levels of plasma corticosterone reach their highest concentrations around hatching, chicks may be already responding to the stress of hatching to the fullest extent. It is possible that the hypothalamo-hypophyseal-adrenocortical axis is hyperactive and additional stimuli, such as leg fracture, do not result in an even higher rate of CRF, ACTH, or glucocorticoid release.

The ability of an organism to respond to stressful stimuli through an increase in glucocorticoid secretion has classically been interpreted to mean that the hypothalamo-

hypophyseal-adrenal cortex axis is mature. The theory that environmental stimuli acting upon the nervous system induce important functional changes through the mediation of the anterior pituitary was first hypothesized by Harris (1948). Direct evidence for the involvement of the hypothalamus in the pituitary-adrenocortical response to stress was provided by deGroot and Harris' (1950) finding that ACTH release can be induced in the rabbit by electrical stimulation of the floor of the third ventricle, and that ACTH response to stress can be prevented by electrolytic lesion of the same area. Since these basic observations were made, there has been considerable progress in solving related problems regarding the relationship of the hypothalamo-hypophyseal-adrenal cortex axis (see Fortier, 1966; Ganong, 1963; and Mangili et al., 1967 for reviews and extensive bibliographies). Jost (1966b, 1968) presents evidence that the axis is functional during embryonic development in rats. His studies show that the adrenals of fetuses with no hypothalamus or with no hypothalamus or pituitary develop abnormally. No studies on the ability of these decapitated or encephalectomized fetuses to respond to stressful stimuli have been performed.

Since the avian literature regarding the role of they hypothalamus and/or pituitary in the adrenocortical stress response is contradictory, the remainder of the experiments in this study were undertaken to examine whether or not the hypothalamus and/or pituitary has a role in adrenal function.

The Effect of ACTH on Plasma Corticosterone Levels.

The response to leg fracture in 14 day old embryos was not as significant as is attained by the time the embryos are 16 days. Newly hatched chicks show no significant increase in plasma corticosterone levels in response to stressful stimuli. When these two age groups were given ACTH, both responded with highly significant elevations in hormone level. Thus, during both periods the adrenal is capable of responding to ACTH.

That the adrenal is responsive to ACTH at the same time it is not responsive to stressful stimuli has been reported previously under different experimental conditions. Bukovsan (1969) reported a rapid and sustained increase in $^{32}P$ uptake by the adrenal of newly hatched chicks within one-half hour of ACTH injection. Milkovic and Milkovic (1963) reported adrenal ascorbic acid depletion in newborn rats after ACTH injection although animals of the same age do not respond to stressful stimuli consisting of leg fracture. In other experiments done in rats, newborn show no significant elevation in plasma corticosterone in response to stressful stimuli until 18 days, though increased plasma levels of the hormone are found after ACTH injection as early as the third day (Levine et al., 1967).

As discussed above, the studies of Hiroshige and his colleagues have shown that in neonatal rats CRF levels are not elevated until 40 minutes after stress is administered (Hiroshige and Sato, 1970; Hiroshige et al., 1971). Blood

was collected for glucocorticoid determination 20 minutes after leg fracture in the present study. Therefore, if the pattern of CRF elevation is the same in the chick as in the rat, whether or not the adrenal is responsive to elevated ACTH levels, none would have been experienced by these animals before they were killed.

Effect of Decapitation on Plasma Corticosterone Levels.

The literature contains many contradictions regarding the role of the pituitary in the regulation of the avian adrenal cortex. Evidence that the pituitary regulates adrenocortical function in a manner similar to that in mammals comes from experiments of Miller and Riddle (1942). They found that hypophysectomy of pigeons leads to atrophy of the adrenals. Newcommer (1958) reported hypophysectomy of young post-hatching chicks inhibits the normal response to stress. Many other early reports recorded that decreased activity of pituitary target organs results from hypophysectomy of several species of birds (Hill and Parkes, 1934; Hill et al., 1934; Mitchell, 1929; Schooley et al., 1941). Most recently Bradley and Holmes (1971) found that hypophysectomy of the duck decreases plasma corticosterone concentrations, secretion rates, and metabolism of adrenal steroids.

However, other experiments suggest that the adrenals of adult birds, in contrast to mammals, are somewhat independent of hypophyseal support. Nalbandov and Card (1943) reported that although hypophysectomy of growing chickens results initially in complete atrophy of the gland, partial

recovery occurs after a short period of time. Ma and Nalbandov (1963) confirmed these results in laying hens. Though adrenal steroids were not measured directly in that study, Ma found that radioactive phosphorus ($^{32}P$) uptake by the adrenal is greater in hypophysectomized hens than in controls and that adrenal ascorbic acid is no different in hypophysectomized birds 20 days after the operation.

Investigations in avian embryos have established that normal morphological development of the embryo in general, as well as that of the thyroid, gonads, and adrenals, is inhibited by decapitation in ovo (Bellware and Betz, 1970; Benoit, 1962; Betz, 1965, 1967, 1968; Brasch and Betz, 1971; Case, 1951, 1952; Fugo, 1940; Love and Konigsberg, 1958; Manwell and Betz, 1966; Martindale, 1941; Moszkowska, 1958; Stalsberg, 1965; Tixier-Vidal, 1958). None of these studies has established the effect of decapitation on the development of steroidogenic capabilities and few studies have studied the timing of any effect the pituitary might have.

The results from experiment 4 demonstrate that the pituitary and/or hypothalamus are necessary for the full development of steroidogenic capabilities. Hypophysectomy does not cause a significant decrease in corticosterone levels on day 14, but causes a significant decrease in hormone levels on day 16. This means that the rise in normal resting glucocorticoid levels up to day 14 is independent of the pituitary, but any further increase is dependent upon the presence of the pituitary and/or hypothalamus.

These results corroborate those of Case (1951, 1952). He found that the weight of paired adrenals is the same in normals and in decapitated embryos through day 14. After this time, the adrenals of normals continue to grow, whereas the adrenals of decapitated embryos do not gain any more weight. These data taken together suggest that the embryonic adrenals in the chick undergo a period of autonomous growth and differentiation until around day 14. Further growth and differentiation are dependent upon stimulation from the embryonic pituitary and/or brain.

Similar results have been reported in the rat fetus (Cohen, 1963). Cohen found that decapitation of the fetus alters the growth of the adrenal from day 17 until term, but decapitation has no effect upon adrenal weight before this time.

The present experiments further demonstrate that whereas the ability to respond to stress is significant at day 14 and highly significant on day 16, no elevated hormone levels occur in decapitated stressed embryos at either age. These results are interpreted to mean that during the embryonic stages, the capacity to respond to stress is dependent upon stimulation from the pituitary and/or hypothalamus. This capacity never resides in the adrenal alone, but is dependent upon the regulation and interaction of at least two, possibly three, hormonal systems.

Effect of Decapitation and Pituitary Grafts on Plasma Corticosterone Levels.

The role of the hypothalamus in maintaining basal pituitary-adrenocortical function, as well as its regulatory role in the response to the environment, is acknowledged in mammals. Many studies in birds have suggested that adrenal functions are much less rigidly controlled by neuroendocrine mechanisms.

Shirley and Nalbandov (1956) reported that interruption of neural and humoral connections between the hypothalamus and pituitary gland by transection of the hypophyseal stalk does not have any effect upon adrenal weights in chicks. Though secretion of ACTH was not measured in a precise quantitative manner, adequate concentrations must have been secreted to prevent regression of the target gland. Assenmacher (1956) confirmed these results in the domestic drake. Ma and Nalbandov (1963) found that hypophysectomy, or hypophysectomy followed by autotransplantation of the pituitary, leads to a decrease in adrenal weight in male domestic fowl. But the adrenals of both experimental groups regain their original weight within 30 days. Further, though hypophysectomized males can not withstand environmental stresses, fowl with autotransplanted pituitaries are as resistant to abrupt changes in ambient temperatures and nocturnal fasting as unoperated controls.

These experiments have been interpreted to mean that in the bird, neuroendocrine mechanisms do no exert as tight control over the pituitary-adrenocortical unit as they do in mammals. It is important to consider other alternative

possibilities. Stalk section experiments are complicated by the problem of regeneration of portal vessels. It is possible that the results of Shirley and Nalbandov are due to such regeneration. Whether CRF is produced in sufficient quantities to pass through the general circulation to the pituitary is still controversial (Ganong, 1963). A response to surgical stress is still present in the dog after stalk section at a time when regeneration is unlikely (Hume and Egdahl, 1959). Adrenal atrophy is not marked in monkeys or humans after stalk section (Holmes et al., 1959; Van Wyk et al., 1960). Rats with pituitary transplants in the eye continue to respond to stress (Cheng et al., 1949; Fortier, 1951; Fortier and Selye, 1949; Goldberg and Knobil, 1957; Hertz, 1960; Martini et al., 1959).

There is some evidence that a neurosecretory role exists in birds. Legait (1955a,b) found that the administration of cortisone reduces the activity of the hypothalamic neurosecretory cells in Rhode Island Red hens. Releasing factors for several of the pituitary trophic hormones have been isolated in birds; in particular, compounds having CRF activity have been isolated in chickens and ducks (Chen et al., 1968; Jackson, 1971a,b; Jackson and Nalbandov, 1969; Muller et al., 1967; Salem et al., 1970; Stainer and Holmes, 1969).

Previous experiments have investigated the role of the hypothalamus in the chick embryo. Pituitary grafts onto the chorioallantoic vein of a previously decapitated embryo

restores normal adrenal histology and normal duodenal differentiation (Bellware and Betz, 1970; Betz, 1965, 1967). Mess (1968) reported subcutaneous pituitary grafts transplanted into previously decapitated recipients show seemingly normal basal secretions of ACTH and the capacity to increase the secretion of this hormone in response to metapyrone treatment.

The results of the present study demonstrate that the presence of the pituitary alone restores normal resting levels of plasma corticosterone on day 16. There are two possible explanations for these results. First, a source of a sufficient concentration of CRF may still exist in the decapitated embryo carrying a pituitary graft to allow the secretion of ACTH from the graft which in turn allows normal resting levels of plasma corticosterone to be maintained. Stainer and Holmes (1969) detected CRF activity in the hypothalamus, cerebrum, and spinal cord of the duck. In fact it appears that other pituitary releasing factor activity may have a more general distribution in lower vertebrates. Nicoll and Fiorindo (1969) reported the presence of substances which stimulate the release of prolactin in the hypothalamus and cerebrum of the turtle. In rats substances with such activity only appear in hypothalamic extracts. Melanophore stimulating hormone inhibiting factor (MSHIF) may also occur widely in the nervous tissue of frogs (Ralph and Sampath, 1966). The hypothesized extrahypothalamic sources of CRF in the chick embryo, then, would be capable of maintaining

basal plasma levels of corticosterone. A second explanation for the results may be that maintenance of normal basal levels depends only upon the presence of a pituitary and not upon the hypothalamus. One would have to hypothesize that the secretion of ACTH, under normal resting conditions, is not controlled by any factor from the brain.

The results of the present study also clearly establish that the hypothalamus plays an important role following stress in embryonic chicks. The presence of only a pituitary and no hypothalamus or brain is not sufficient to reestablish the capacity to increase hormone levels in response to leg fracture. It appears that, if the extra-hypothalamic sources of CRF hypothesized as one of the alternatives above is sufficient to maintain basal secretion levels of ACTH, these sources do not respond to environmental disturbances.

The present investigation demonstrates that, during development, plasma corticosterone concentration is regulated and controlled at three different levels.

First, the adrenal appears to be capable of autonomous secretion of glucocorticoids during the initial stages of embryonic development. During this time hormone levels are low but nevertheless measurable. To what extent such low hormone levels participate in the development and differentiation of the chick is difficult to evaluate, since adrenalectomy of embryos is not yet a feasible surgical procedure. Abnormally high doses of glucocorticoids administered to intact embryos do affect the growth rate, yolk

retraction, and liver glycogen levels. Since such high doses of hormone were used in the above experiments, it is questionable whether these can be considered physiological or pharmacological effects.

At the second level of control, adrenal function becomes integrated with the pituitary when embryos are between 13 and 15 days old. Two aspects of pituitary regulation are of interest. First, it appears that the gradual increase in levels of plasma corticosterone which occurs after day 14 is dependent upon the pituitary, since hypophysectomy of 16 day embryos causes return of hormone levels to the normal basal levels seen on day 14. Second, from the data available, it appears that though the pituitary allows the continuous and gradual elevation of basal hormone concentrations on days 16, 18, and 20, its regulation does not affect the pattern of increase which was established prior to its integration. That is to say, there is no shift in the pattern of secretion correlated with the entry of the pituitary as a regulatory influence. This interpretation of the role of the pituitary is supported by indirect evidence from other experiments. Thus, Case (1951, 1952) found that adrenal weight and adrenal ascorbic acid remain at the 14 day levels in hypophysectomized embryos. Hinni and Watterson (1963) found that in hypophysectomized embryos, development of the duodenal epithelium is arrested at the 14 day stage.

Between the 13th and 16th day of incubation, a third

level of control, that of the hypothalamus, becomes important. The hypothalamic component does not appear to influence normal basal hormone levels. This is demonstrated by the fact that corticosterone levels in embryos having only a pituitary and adrenal do not differ from those in intact embryos. The hypothalamus does play an important role in the stress response as indicated by increased corticosterone levels. That the hypothalamus is essential to the ability to respond is demonstrated by the fact that embryos possessing only a pituitary and adrenal are incapable of responding to stress on day 16, whereas embryos having a hypothalamus, pituitary, and adrenal cortex show elevated corticosterone levels after leg fracture. Since no abrupt increase in glucocorticoid levels occurs when the hypothalamus exerts its influence, it appears that the importance of this third component of the axis lies in its ability to permit more information to be fed into the system. Environmental input can now be interpreted and responded to by the adrenocortical system.

Thus, it appears that the hypothalamo-hypophyseal-adrenal cortex axis has a functional role before hatching.

## SUMMARY

Basal plasma levels of corticosterone (th principal adrenal steroid in chicks) were monitored during the second half of incubation and the initial week after hatching. Hormone levels were found to increase gradually and to peak around hatching. They then decreased again by the time the chicks were a week old.

Corticosterone levels were also monitored after stressful stimuli were administered. It was found that elevated hormone levels ($p < 0.01$) resulted from this procedure from the time embryos were 16 days old. Newly hatched chicks did not respond to stress but the typical response was again evident when they were a week old. ACTH (100mU) was given to 14 day embryos and newly hatched chicks to determine whether the adrenals of these age groups were responsive to pituitary stimulation. ACTH resulted in an increase in glucocorticoid levels.

To determine the role of the pituitary and hypothalamus in the stress response, embryos were decapitated or decapitated and given replacement therapy consisting of pituitary grafts on the chorioallantoic membrane. Corticosterone concentrations were monitored under normal and stress conditions in both groups. It was found that decapitation caused significantly lower corticosterone levels

on day 16 and resulted in the absence of a stress response. When decapitated embryos of the same age had pituitary grafts, basal hormone levels were restored; however, they were incapable of responding to stress.

Hence, the present study demonstrates that the hypothalamo-hypophyseal-adrenal cortex axis is functional before hatching in chicks. The pituitary plays an important role in the maintenance of resting hormone levels from around day 14 on. The hypothalamus does not appear to affect normal resting levels, but plays an important role in the stress response.

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# A P P E N D I X

I. Data from 1970

| Age | Corticosterone ng/ml. | Age | Corticosterone ng/ml. |
|---|---|---|---|
| 12 days | 2.5 | 0 day post-hatching | 2.6 |
| | 3.5 | | 4.0 |
| | 3.4 | | 7.0 |
| | 3.6 | | 5.4 |
| | 4.0 | | 8.4 |
| 14 days | 5.6 | | 16.4 |
| | 4.5 | | 10.8 |
| | 6.2 | | 10.6 |
| | 4.5 | | 3.1 |
| | 3.9 | | 3.5 |
| | 1.2 | | |
| | 2.3 | 1 week post-hatching | 6.8 |
| | 4.1 | | 2.7 |
| | 1.9 | | 4.0 |
| | 3.4 | | 2.2 |
| 16 days | 3.4 | | 3.4 |
| | 4.2 | | 2.5 |
| | 3.5 | | 1.4 |
| | 4.9 | | 1.9 |
| | 4.0 | | 2.5 |
| | 3.1 | | 4.7 |
| | 4.3 | | |
| | 4.3 | 2 week post-hatching | 0.9 |
| | 3.1 | | 1.2 |
| 18 days | 4.3 | | 1.4 |
| | 4.8 | | 1.0 |
| | 5.8 | | 1.2 |
| | 6.4 | | 1.0 |
| | 3.3 | | 0.8 |
| | 3.6 | | 0.8 |
| | 4.3 | | 1.0 |
| | 5.0 | | 1.0 |
| | 2.5 | 3 week post-hatching | 0.4 |
| 20 days | 13.0 | | 0.7 |
| | 7.8 | | 2.0 |
| | 8.6 | | 0.6 |
| | 10.6 | | 0.9 |
| | | adult | 0.2 |
| | | | 1.4 |
| | | | 0.5 |
| | | | 0.3 |

II. Data from 1971

| Age | Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Stress | Water Injected | ACTH Injected | Hypophysectomy Control | Hypophysectomy + Pituitary Transplant Control | Hypophysectomy Stress | Hypophysectomy + Pituitary Transplant Stress | Open Window Control |
| | Plasma Corticosterone (ng/ml) | | | | | | | | |
| 10 days | 1.0 | | | | | | | | |
| | 0.3 | | | | | | | | |
| | 0.3 | | | | | | | | |
| | 1.5 | | | | | | | | |
| | 0.3 | | | | | | | | |
| 12 days | 1.0 | 0.3 | | | | | | | |
| | 1.2 | 1.7 | | | | | | | |
| | 1.6 | 1.3 | | | | | | | |
| | 1.3 | 1.2 | | | | | | | |
| | 0.2 | 0.6 | | | | | | | |
| 14 days | 1.2 | 1.8 | 2.0 | 4.9 | 1.0 | | 0.8 | | 1.7 |
| | 1.2 | 2.8 | 1.4 | 5.1 | 1.0 | | 1.5 | | 1.3 |
| | 1.6 | 2.1 | 3.4 | 4.7 | 0.5 | | 1.3 | | 0.8 |
| | 1.2 | 1.4 | 2.1 | 4.0 | 0.3 | | 1.3 | | 1.1 |
| | 0.9 | 1.5 | 1.8 | 5.0 | 1.0 | | 0.9 | | 1.2 |
| 16 days | 2.2 | 3.5 | | | 1.7 | 1.7 | 1.7 | 1.8 | 1.8 |
| | 1.8 | 1.8 | | | 1.5 | 2.4 | 1.7 | 2.1 | 2.2 |
| | 1.6 | 3.3 | | | 1.3 | 2.0 | 0.5 | 1.6 | 2.2 |
| | 1.8 | 3.5 | | | 0.7 | 1.6 | 0.9 | 1.8 | 1.8 |
| | 1.8 | 3.1 | | | 0.4 | 1.5 | 1.2 | 2.0 | 3.2 |

II. Data from 1971 (cont'd.)

| Age | Control | Stress | Water Injected | ACTH Injected | Hypophysectomy Control | Hypophysectomy + Pituitary Transplant Control | Hypophysectomy Stress | Hypophysectomy + Pituitary Transplant Stress | Open Window Control |
|---|---|---|---|---|---|---|---|---|---|
| | Treatment | | | | | | | | |
| | Plasma Corticosterone (ng/ml) | | | | | | | | |
| 18 days | 0.8 | 3.8 | | | | | | | |
| | 2.1 | 3.8 | | | | | | | |
| | 2.0 | 3.0 | | | | | | | |
| | 3.4 | 4.6 | | | | | | | |
| | 2.2 | 4.1 | | | | | | | |
| 20 days | 2.0 | 2.9 | | | | | | | |
| | 2.2 | 3.2 | | | | | | | |
| | 2.8 | 4.6 | | | | | | | |
| | 2.0 | 3.6 | | | | | | | |
| | 2.4 | 3.1 | | | | | | | |
| 0 day post-hatching | | | | 4.7 | | | | | |
| | 1.6 | 3.1 | 1.0 | 5.4 | | | | | |
| | 2.9 | 2.9 | 2.4 | 3.4 | | | | | |
| | 1.1 | 3.2 | 2.4 | 3.0 | | | | | |
| | 0.8 | 0.9 | 1.1 | 3.8 | | | | | |
| | 1.7 | 1.7 | 2.6 | 2.5 | | | | | |
| | 1.1 | 2.7 | 2.4 | 3.4 | | | | | |
| | 1.6 | | 1.5 | 1.5 | | | | | |
| | 2.8 | | | 4.1 | | | | | |
| | | | | 2.4 | | | | | |

II. Data from 1971 (cont'd.)

| Age | Control | Stress | Water Injected | ACTH Injected | Hypophysectomy Control | Hypophysectomy + Pituitary vTransplant Control | Hypophysectomy Stress | Hypophysectomy + Pituitary Transplant Stress | Open Window Control |
|---|---|---|---|---|---|---|---|---|---|
| | Treatment | | | | | | | | |
| | Plasma Corticosterone (ng/ml) | | | | | | | | |
| 1 week | 0.6 | 0.8 | | | | | | | |
| | 2.1 | 4.3 | | | | | | | |
| | 1.6 | 5.0 | | | | | | | |
| | 2.3 | 5.7 | | | | | | | |
| | 1.7 | 6.6 | | | | | | | |
| | 0.7 | 4.3 | | | | | | | |
| | 0.9 | 2.8 | | | | | | | |
| | 0.8 | 4.6 | | | | | | | |
| | 0.4 | | | | | | | | |
| | 1.5 | | | | | | | | |