Functional Development of the Hypothalamo-Hypophyseal-Adrenal Cortex Axis in the Chick Embryo, *Gallus domesticus* [1]

PHYLLIS M. WISE [2] AND B. E. FRYE [3]
Department of Zoology, University of Michigan, Ann Arbor, Michigan 48104

ABSTRACT Basal plasma levels of corticosterone increased gradually during the second half of incubation, reaching a peak around the time of hatching. Stress resulted in a very significant elevation of corticosterone above the basal level in embryos 16 days or older, but not in younger embryos. Newly hatched chicks did not respond to stress, but a typical stress response was evident one week after hatching. Adrenocorticotropin (100 mU) elicited a significant rise in plasma corticosterone in 14-day embryos and in newly hatched chicks, demonstrating that the adrenal is capable of responding to pituitary stimulation at these times when a stress response does not occur.

Decapitated 16-day embryos had significantly lower basal levels of corticosterone than normal, and showed no rise in corticosterone in response to stress. Basal levels of corticosterone were unaffected by decapitation of 14-day embryos. Grafting ten-day embryonic pituitaries to the chorioallantoic membrane on day 9 of incubation restored normal basal hormone levels on day 16 in decapitated embryos, but did not restore the ability to respond to stress.

This study demonstrates that the hypothalamo-hypophyseal-adrenal axis is functional before hatching in chicks. The adrenal exhibits significant autonomous functional capability prior to day 14, and the pituitary becomes important in maintaining both the resting level of hormone and the stress response between days 14 and 16 of incubation. The hypothalamus does not appear to control normal resting levels of corticosterone, but is essential for the stress response.

Analysis of the functional development of the adrenal cortex is of interest for two reasons. First, as physiological regulators, adrenal steroids play key roles in the regulation of water and mineral balance, in the metabolism of carbohydrate, fat, and protein, and in stress tolerance, and in many other functions. The development of these functions is obviously important in the development of the capacity of an organism for its free-living post-embryonic existence, and may also be important in the regulation of the metabolism of the embryo. Second, adrenal steroids have some specifically developmental effects, such as the known effects on growth (Evans, '53; Karnofsky et al., '51; Moog and Richardson, '55; Sames and Leathem, '51) and on the functional differentiation of the small intestine (cf. Moog, '59). The full extent to which such regulatory and developmental functions are initiated by and under the control of the adrenal cortex in the embryo can be ascertained only in the light of precise information about the pattern of steroid secretion in the developing animal.

Complete characterization of the functional development of the adrenal cortex involves not only analysis of the capacity for hormone secretion by the adrenal itself, but the functional development of the entire neuroendocrine axis, including the hypothalamus and the pituitary as well as the adrenal. Both normal resting levels of steroid secretion and elevated

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[2] Present address: Reproductive Endocrinology Program, Department of Obstetrics and Gynecology, University of Michigan Hospital, Ann Arbor, Michigan 48104.

[3] Address all reprint requests to this author.

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

In the past several different approaches have been used to analyze the functional status of the adrenal cortex and the associated hypothalamo-hypophyseal axis. These have included (1) morphological and histochemical studies of the adrenal, (2) analysis of functional changes in target tissues during development that are indicative of functional changes in the adrenal itself, and (3) analysis of the effects of hypophysectomy and pituitary transplantation upon the normal course of adrenal functional differentiation as characterized by the first two methods. The exact pattern of developmental changes suggested by such studies varies among species. In the chick, they indicate four particular times during development when important changes in the functional status of the adrenal appear to occur. First, histochemical tests for lipids, cholesterol, carbonyl groups, and ascorbic acid suggest that the gland may become capable of steroid synthesis and accumulation between days 10 and 12 of incubation (Dawson, '53; Moog, '59; Sivaram, '68). Second, the initiation of adrenocorticotropin (ACTH) secretion and thus of hypophyseal control of the adrenal may begin around the fifteenth day of incubation (Betz, '67; Case, '52; Moog, '59). Third, changes in the concentration of duodenal alkaline phosphatase which are dependent upon glucocorticoids indicate that there may be a significant rise in the level of adrenal activity between the seventeenth and the nineteenth days of incubation (Moog, '59). Fourth, if one can extrapolate from studies done on mammals, in which a sharp increase in steroid secretion is seen at birth, significant changes in adrenal function in the chick might be expected to occur around the time of hatching.

There is need to establish more precisely the details of adrenal functional development by direct measurement of hormone levels in blood and tissues. Until recently the methods of assay available required large amounts of blood or tissue, and even then lacked precision and sensitivity, and thus were not applied to embryos of small species with much success. The most recently developed protein-binding radioassay for corticosteroids (Murphy, '67) has provided the necessary sensitivity and precision to permit determination of adrenal glucocorticoids in small quantities of blood, and has thus prompted the present study.

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

1. To measure plasma levels of corticosterone (the major glucocorticoid of the chicken) during embryonic and post-hatching development and to correlate these data with critical physiological and developmental changes described above and presumed to be under adrenal control.

2. To determine to what extent the normal pattern of plasma corticosterone levels in the chick embryo is dependent upon the intact hypothalamo-hypophyseal-adrenal cortex axis, as determined by hypophysectomy and by transplantation of the pituitary to the chorio-allantoic membrane.

3. To determine when the developing animal is capable of responding to stress by an increase in plasma steroid above the normal resting levels. The objective of this portion of the study is to utilize the stress response as an indication of the functional status of the complete neuroendocrine circuit; thus it is an extension of the second objective.

MATERIALS AND METHODS

Animals

Fertile eggs and day-old cockerels (White Leghorn, DeKalb Strain 131) were used. The eggs were incubated in a David-Bradley Incubator at a dry temperature of 38 $\pm$ 1°C and a wet bulb temperature of 31°C. The chicks were kept in a battery brooder under constant light at a temperature of about 32°C. They were fed Purina Startena and water *ad libitum*. The age of embryos and post-hatching chicks was designated according to the standard scheme described by Moog ('61).

For all pre-hatching stages and newly hatched chicks, blood was pooled without knowledge of the sex of individuals. Males were used in all experiments dealing with the seven day-old chickens.

Hypophysectomy and pituitary replacement therapy

Partial decapitation was done on embryos which were incubated without turning until embryos reached stage 10 or 11 (Hamburger and Hamilton, '51), using Fugo's technique (Fugo, '40). Albumen was removed and a "window" was made and sealed without surgical manipulation for those embryos serving as controls.

Pituitary replacement was done by grafting single pituitaries from ten-day donors onto the chorio-allantoic membrane of nine-day decapitated host embryos according to the method of Betz ('67).

Stress and ACTH administration

The stress stimulus consisted of opening the shell and breaking the shank of one leg 20 minutes before collecting blood. This procedure was done without removing the embryo from the egg. During the waiting period after stress, the opening in the shell was covered with a piece of cotton moistened with saline. Post-hatching chicks were stressed in a similar manner: a leg was broken and the chicks were kept in a warm box until the blood was collected 20 minutes later. Control embryos or chicks were left undisturbed in the incubator or brooder until blood was collected.

Sigma powdered porcine ACTH was dissolved in distilled water. One hundred milliunits were given intravenously to embryos and subcutaneously to newly hatched chicks 20 minutes before blood was collected. Controls received equivalent amounts of distilled water 20 minutes before collecting blood. During the 20 minutes the animals were treated the same way as were stressed animals.

Sample collection

All blood was collected between 7:30 AM and 12 noon since there is evidence in the literature for a circadian rhythm of corticotropin releasing factor (CRF), adrenocorticotropic hormone (ACTH), and glucocorticoids in some species (Bajpayee and Brown, '72; Cheifetz et al., '68; David-Nelson and Brodish, '69; Galicich et al., '65; Guillemin et al., '69). Embryonic blood was collected from a major chorio-allantoic vein or artery using a fine tipped glass pipette coated with heparin. The blood was centrifuged immediately after it was collected and plasma was separated from red blood cells. 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 ('67). 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, '67; DeRoos, '61; DeRoos and DeRoos, '63; Frankel, Graber and Nalbandov, '67; Frankel, Graber, Cook and Nalbandov, '67; Nagra et al., '60, '63; Resko et al., '64). 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 in duplicate 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 AM and 10 AM and centrifuged, and separated. The plasma was divided into aliquots and frozen. Radioactive samples were counted in a Packard Tri-Card Liquid Scintillation Counter Model 3003.

Thorough discussions of the theoretical and practical aspects of the assay appear in the literature (Murphy, '67). 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 ten seconds and then incubated in a water bath at 45°C for five minutes.

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

4. Fifteen milligrams of Florisil were added to each tube to remove unbound corticosterone. The tubes were shaken by inversion for two minutes, centrifuged for ten minutes at 2500 rpm and returned to the ice bath for five minutes.

5. One-tenth milliliter 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 ten minutes each time.

An example of a standard curve appears in figure 1. The assay was found to be most sensitive and precise when measuring corticosterone levels between 1 and 20 ng. Therefore varying amounts of plasma (1–2 ml), pooled from a varying number (2–15) of animals, were used from different experimental groups.

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 of these samples were carried out based on the lowest value measurable on the curve.

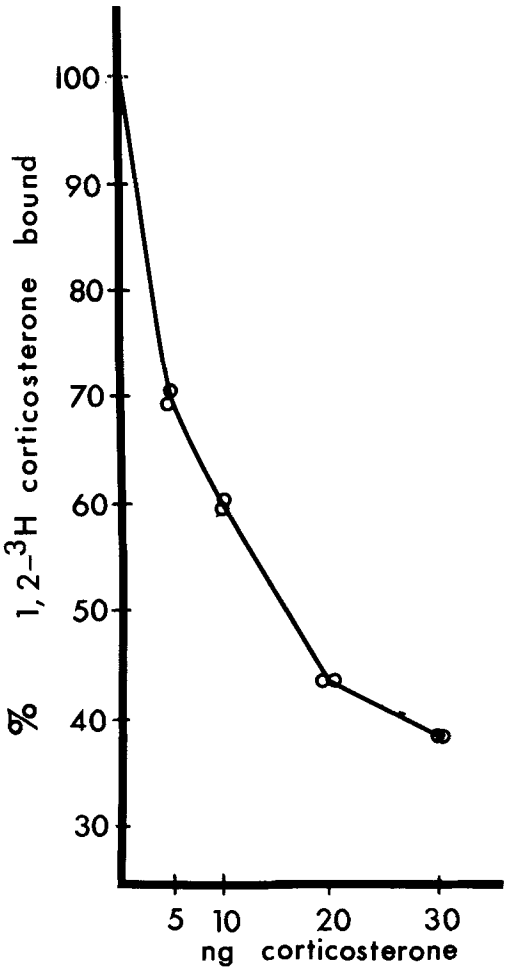

Fig. 1 Standard curve from protein binding radioassay. This curve represents the results from a typical assay in which duplicate standards were determined at 0, 5, 10, 20 and 30 ng.

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 ($p < 0.01$). Regression analysis and covariance analysis were applied to the data from the first experiment.

RESULTS

Normal resting corticosterone levels (table 1)

Corticosterone was present in detectable amounts at ten days of incubation, the earliest age sampled, and rose steadily through the twentieth day of incubation. Linear regression analysis of the data from days 10–20 of incubation revealed that the slope of the line is different from zero ($p < 0.01$; fig. 2). T-test comparison of the means of successive days indicated that the change is significant only between days 14 and 16. Because of this, the data from days 10–14 and 16–20 were compared to determine whether linear regressions of these two groups of data differed from one another in slope or in intercept. Covariance analysis showed no significant difference between either the slopes or the intercepts.

During the early post-hatching period corticosterone levels fell. Hormone levels

TABLE 1

Normal resting levels of plasma corticosterone during embryonic development collected during 1971

Age	Number of samples per group	Plasma corticosterone ng/ml Mean ± S.E. [1]
10 days incubation	5	$<0.7 \pm 0.2$
12 days incubation	5	$<1.1 \pm 0.2$
14 days incubation	5	1.2 ± 0.1
16 days incubation	5	1.8 ± 0.1
18 days incubation	5	2.1 ± 0.4
20 days incubation	5	2.3 ± 0.1
newly hatched	8	1.7 ± 0.3
1 week post-hatching	10	1.3 ± 0.2

Statistical comparisons

Comparison	Level of significance
10 days vs. 12 days	NS [2]
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
20 day embryo vs. newly hatched	NS
newly hatched vs. 1 week post-hatching	NS
20 day embryo vs. 1 week post-hatching	$p<0.01$

Covariance analysis

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

[1] S.E., standard error.
[2] NS, not significant.

of seven day-old chicks are not significantly different from those of chicks on the day of hatching, but are significantly below values obtained on the twentieth day of incubation.

Response of intact animals to stress (figure 3)

The stress of opening the shell and breaking a leg did not affect corticosterone levels in 12 day-old embryos, but caused a significant elevation of plasma corticosterone levels 20 minutes after the stimulus was administered on day 14 ($p < 0.04$), and a very significant increase ($p < 0.01$) on days 16, 18 and 20. When expressed as percentage increase in hormone levels of stressed embryos, the stress response becomes more intense as the embryos get older. On day 14 stress caused a 58% increase; on day 16 a 67% increase; and on day 18 an 86% increase in corticosterone. Stress did not cause a significant rise in plasma corticosterone concentration in newly hatched chicks, but caused a highly significant rise of 275% on day 7 after hatching. This large per cent increase is due both to somewhat higher absolute stress values and to lower resting values in the seven-day chick.

Response of intact animals to ACTH (table 2)

Since 14 day-old embryos did not give as great a response to stress as older embryos, a group of 14-day embryos was injected with 100 mU of ACTH in order to assess their capability to respond. Similarly, ACTH was injected into newly hatched chicks to determine whether the lack of response to stress at this age might be due to an absence of sensitivity to ACTH. In both groups the response to ACTH was highly significant.

Effect of decapitation upon resting corticosterone levels (figure 4)

To test the role of the brain and/or the pituitary gland in determining normal plasma corticosterone levels, embryos were decapitated at 33 hours of incubation, and then sampled at 14 or 16 days of incubation. At 14 days no significant effect of decapitation was seen ($0.05 < p < 0.06$). However, on the sixteenth day there was a significant ($p < 0.02$) decline in plasma corticosterone concentration of decapitated embryos relative to controls. Corticosterone values of decapitated embryos are equivalent at the two ages, and it thus appears that the effect of decapitation is to prevent the rise that normally occurs during this period.

Response of decapitated embryos to stress (figure 4)

In order to ascertain whether the elevation in corticosterone which occurs in response to stress in normal embryos depends upon the pituitary and/or the forebrain (including the hypothalamus),

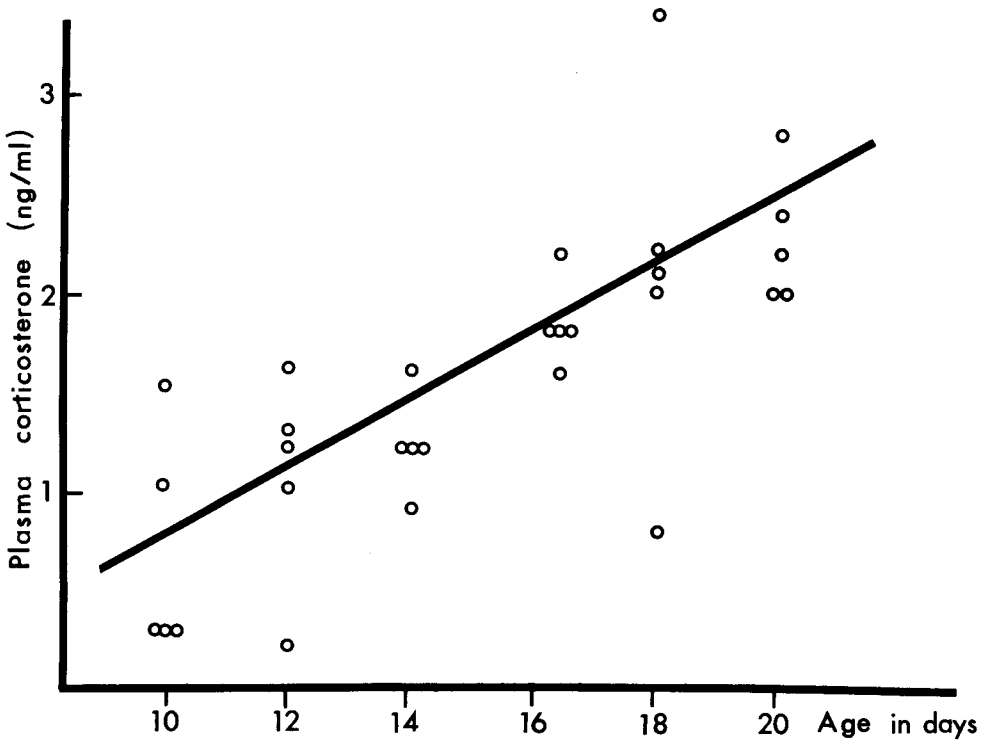

Fig. 2 Linear regression analysis of normal resting levels of plasma corticosterone in embryos. Each point represents a single determination of a pooled sample.

embryos decapitated at 33 hours of incubation were stressed and sampled on the fourteenth and sixteenth days of incubation. After decapitation there was no significant rise in plasma corticosterone in response to stress at either 14 days ($p < 0.08$) or 16 days ($p < 0.8$) of incubation.

Corticosterone levels in decapitated embryos with pituitary grafts (figure 5, table 3)

In order to distinguish between the role of the pituitary gland and the role of the hypothalamus and/or forebrain in determining normal corticosterone levels, pituitaries from donors incubated for ten days were transplanted onto the chorioallantoic membrane of nine day-old decapitated hosts. Blood was collected on the sixteenth day of incubation. Plasma corticosterone levels of decapitated embryos with pituitary grafts were significantly higher ($p < 0.04$) than in decapitated embryos without grafts, and not significantly different ($0.2 < p < 0.3$) from intact controls.

Stress response of decapitated embryos with pituitary grafts (table 3)

Just as with decapitated embryos without a graft, no elevation of plasma corticosterone occurred following stress of decapitated animals bearing a pituitary graft. Thus, the hypothalamus and/or forebrain is essential for the stress response to occur in the 16-day old chick embryo.

DISCUSSION

Normal resting levels of plasma corticosterone

Corticosterone is already detectable in the blood of chick embryos on day 10 of incubation. Because of the difficulty of obtaining adequate samples for assay at younger stages, we cannot specify the earliest age at which corticosterone secretion by the embryonic adrenal begins. Pedernera ('72) has recently demonstrated that adrenals and pituitaries from eight-day chick embryos, but not from six-day embryos, have secretory capability after culture in vitro for 48 hours. Although

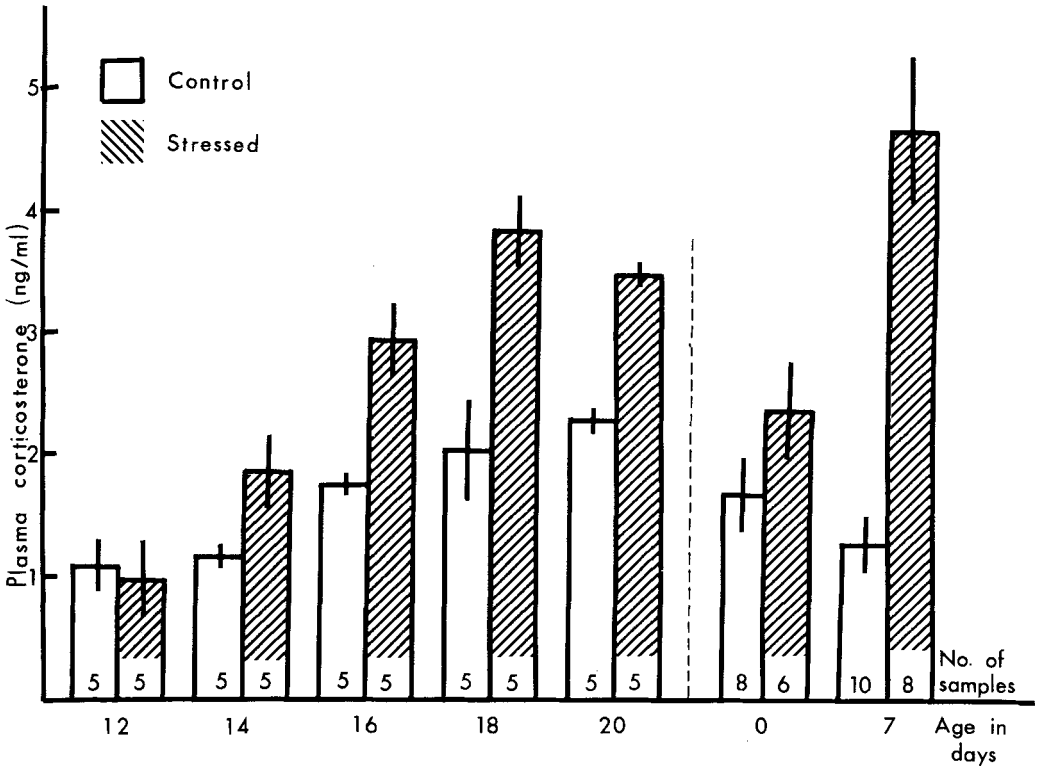

Fig. 3 Effect of stress upon plasma corticosterone levels during the embryonic period and post-hatching period. Means ± standard errors.

TABLE 2

Effect of ACTH upon plasma corticosterone levels on 14 day-old embryos and newly-hatched chicks

Age	Number of samples per group	Treatment	Plasma corticosterone ng/ml Mean ± S.E. [1]
14 day embryo	6	Water injected control	2.1 ± 0.3
14 day embryo	5	ACTH injected	4.7 ± 0.2
newly hatched	7	Water injected	1.8 ± 0.3
newly hatched	10	ACTH injected	3.4 ± 0.4

Statistical comparisons

Comparison	Level of significance
14 day control vs. 14 day ACTH injected	$p < 0.01$
newly hatched control vs. newly hatched ACTH injected	$p < 0.01$

[1] S.E., standard error.

this would appear to suggest that corticosterone secretion begins between the eighth and tenth days of incubation, the in vitro data are not directly comparable to normal in vivo development in view of the possibility that in vitro culture conditions could alter the normal rate of functional differentiation.

Regression analysis shows that from day 10 to hatching there is a gradual

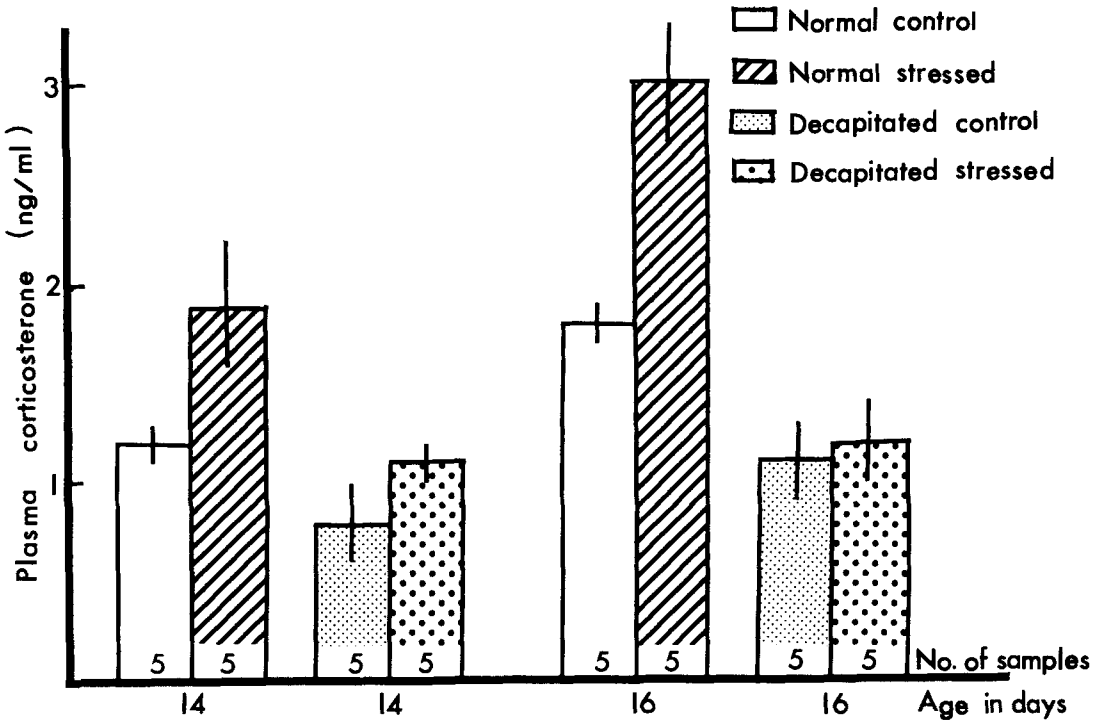

Fig. 4 Effect of decapitation on plasma corticosterone levels before and after a stressful stimulus is administered. Means ± standard errors.

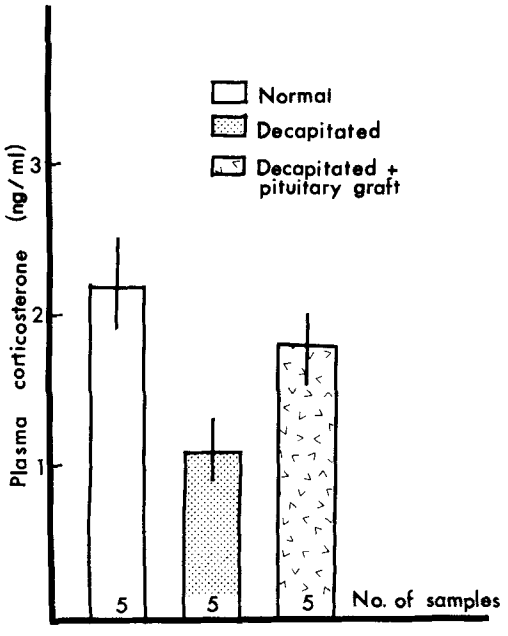

Fig. 5 Comparison of plasma corticosterone levels under control conditions in normal, decapitated and decapitated with pituitary graft embryos. Means ± standard errors.

increase in resting levels of plasma corticosterone. In addition, T-tests demonstrate that there is a very significant rise in hormone levels between days 14 and 16, and that there is no such significant difference between consecutive days sampled at any other time during the last half of 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 thirteenth and the fifteenth days. After hypophysectomy on the second day of incubation, adrenals of controls and experimentals continue to grow at the same rate until the fifteenth day; 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 (Case, '51, '52). The pattern of adrenal ascorbic acid accumulation also remains unaltered in hypophysectomized

TABLE 3

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 per group	Treatment	Plasma corticosterone ng/ml Mean ± S.E. [1]
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 [2]
16 day decapitated with pituitary transplant vs. 16 day decapitated with pituitary transplant stressed	NS

[1] S.E., standard error.
[2] NS, not significant.

embryos until the fifteenth day, after which ascorbic acid levels of normal embryos continue to rise while the levels of hypophysectomized embryos remain steady (Case, '51, '52).

Covariance analysis of the data from the interval before (days 10, 12 and 14) and after (days 16, 18 and 20) the 14–16 day rise shows that the slopes and the intercepts of the two periods do not differ. Thus, although there is an increase in the absolute level of hormone secretion, apparently due to ACTH, there is no change in the *rate* of the rise of basal hormone levels which occurs during normal development. This suggests that one limiting factor controlling steroid secretion rate may be some intrinsic property of the developing adrenal itself, which limits both the autonomous steroid secretion rate (before day 16) and the ACTH dependent steroid secretion rate (after day 16) in equivalent, proportionate ways. One such factor could be the mass of adrenal tissue present at any given day of development. Case's study ('51) shows that the largest increase in adrenal weight occurs between days 14 and 16. A recent study by Girouard and Hall ('73) demonstrates a peak of mitotic activity in embryonic chick adrenals on day 14 which is significantly lower in decapitated embryos or in those treated with dexamethasone. It may be that the main effect of endogenous ACTH is on adrenal growth rather than steroid secretion *per se*.

Woods and his colleagues studied glucocorticoid levels in chorio-allantoic fluid of the chick embryo between the tenth and seventeenth days of incubation (Woods et al., '71). 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 our study. There are two probable reasons for these differences: (1) Woods et al. 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, '61; Frankel, Cook, Graber and Nalbandov, '67; Frankel, Graber and Nalbandov, '67; Frankel, Graber, Cook and Nalbandov, '67; Nagra et al., '60). 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, '67). It has been found that several brief fluorometric methods for the determination of glucocorticoids are grossly inaccurate when applied to avian adrenal plasma (Frankel, Cook,

Graber and Nalbandov, '67). The particular advantage of the protein binding radioassay 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. (2) 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. Whereas glucocorticoid concentrations were measured in arterial and venous blood of embryos in the present study, Woods and his group measured hormone concentrations from chorio-allantoic fluid. Since the allantoic sac serves mainly to store waste products accumulated during the embryonic period, the concentration of corticosteroids measured may reflect all the hormones and metabolites which have accumulated up to that time. Measuring glucocorticoid levels in peripheral plasma gives a more accurate indication of the functional state of the adrenal at that point in time.

In the chicken, plasma hormone levels are highest around hatching and then decrease by the time the chicks are a week old. A pattern of gradually increasing hormone levels during gestation with a peak around birth has also been reported in several mammalian species (rat: Holt and Oliver, '68; Milkovic and Milkovic, '63; sheep: Alexander et al., '68; Bassett and Thorburn, '69; human: Gemzel, '54; Kawahara, '60; Migeon et al., '56). Since steroids can pass through the placenta, elevated glucocorticoid levels during the perinatal period in mammals 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 around hatching.

Functional significance

The presence of the hormone and the pattern of change raise the question of whether adrenocortical hormones have a function during the developmental period. Studies by Moog and associates show that the adrenal participates in embryonic physiological changes which occur in duodenal epithelium (Moog, '59; Moog and Ford, '57; Moog and Richardson, '55; Moog and Thomas, '57). These changes proceed most rapidly between days 17 and 20, during which time alkaline phosphatase concentrations increase a hundredfold. 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.

It is not known whether the adrenal has any other developmental function unique to the embryo. Relatively large doses of corticoids administered to eight to ten day-old embryos retard growth (Evans, '53; Karnofsky et al., '51; Sames and Leathem, '51). 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, '55). Clawson and Domm ('64) found that administration of cortisol causes a decrease in liver glycogen within six hours when administered between the sixth and eleventh days, but causes an increase in levels when administered to 17 day-old embryos.

The pituitary-hypothalamic axis

The literature contains contradictory data regarding the role of the hypothalamo-hypophyseal axis in the regulation of the adrenal in birds. A number of experiments suggest that birds have a typical hypothalamo-hypophyseal-adrenal axis similar to that seen in mammals (Chan et al., '72; Legait, '55a,b). However, other work, notably that of Nalbandov and his associates (Ma and Nalbandov, '63; Nalbandov and Card, '43; Shirley and Nalbandov, '56) indicates that the adrenals of the chicken may be somewhat independent of hypophyseal and hypothalamic control. Assenmacher ('58) has reached a similar conclusion from his work with the domestic duck. Thus, those experiments pertain-

ing to the neuroendocrine axis of the adrenal in the present study are pertinent not only to the question of the development of functional competency of the system, but also to the question of whether a typical neuroendocrine relationship exists in the chicken.

a. The stress response. It is widely accepted that the typical stress response, characterized by an increase in plasma corticosteroids, depends upon the intact hypothalamo-hypophyseal axis (Fortier, '66; Ganong, '63; Mangili et al., '67). In the present study a very significant response to stress was seen only in embryos 16 days or older, with a significant response ($p < 0.04$) in 14 day embryos. Twelve-day-old embryos gave no response to stress. Nerve fibers enter the leg and knee joint as early as the seventh day of incubation, but proliferation of branches continues past the fifteenth day (Romanoff, '60). Thus, it is possible that the failure of the younger embryos to respond to the stress of leg fracture is due to immaturity of the afferent pathway. However, as discussed below, the results of this study collectively suggest that it is immaturity of the neuroendocrine axis at some level that causes the lack of the stress response.

Leg fracture had no effect upon levels of circulating glucocorticoids in newly hatched chicks. The capacity to respond to such stimuli returns 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, '50; Levine et al., '67; Milkovic and Milkovic, '59). Freeman ('67) found that three-week-old cockerels are capable of responding to stress as demonstrated by depletion of adrenal ascorbic acid. The mechanism of the "non-responsive" period is not known. Milkovic and Milkovic ('63) found that adrenals of neonatal rats 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 newborn rats to stress. Hiroshige and his colleagues discovered that in adult rats the response pattern of CRF is characteristically biphasic: first a rapid phase consisting of a prompt increase in hypothalamic CRF content followed by a depletion 20 minutes after the onset of stress, and a second slow phase 40 minutes after stress stimuli are begun. In newborn rats only the second delayed response is apparent (Hiroshige and Sato, '70; Hiroshige et al., '71). If the adrenal response is dependent upon the hypothalamus in chickens and if the pattern of CRF secretion is similar to that of neonatal rats, then any measurement of glucocorticoid secretion taken in the present study would not show increased adrenocortical hormone levels.

b. Response to ACTH. Although neither 14-day embryos nor newly hatched chicks showed a very significant response to stress, both responded to ACTH injection by highly significant elevations in plasma corticosterone levels. A similar phenomenon has been described in newborn rats, which also do not respond to stress, but show adrenal ascorbic acid depletion, elevation of plasma corticosterone, and a rapid decrease in P^{32} uptake by the adrenal following ACTH administration (Bukovsan, '69; Levine et al., '67; Milkovic and Milkovic, '63). Thus, the inability to respond to stress at these times is apparently due to the functional status of the pituitary-hypothalamic axis rather than incompetence of the adrenal *per se*.

c. Decapitation. The role of the neuroendocrine axis in regulation of the chick adrenal, suggested by the ACTH experiments, is directly confirmed by the effects of decapitation. These experiments show that the pituitary and/or hypothalamus are necessary for the full development of the steroidogenic capabilities of the adrenal. Decapitation had no effect on plasma corticosterone levels in 14-day-old embryos. However, decapitated 16-day embryos did not show the normal rise in corticosterone, which remained at the 14-day level. Similarly Case ('51, '52) has shown that adrenal growth, which is autonomous during early development, becomes dependent on the pituitary around days 14–16. Hinni and Watterson ('63) also showed that in hypophysectomized embryos, adrenal development is arrested at the 14-day stage. Thus, there is an autonomous phase of adrenal development, through day 14,

when steroid secretion does not depend on hypothalamo-hypophyseal regulation. The subsequent normal rise in plasma corticosterone concentration is dependent upon the pituitary and/or hypothalamus.

These results corroborate the general observation of other workers that normal morphological development of the chick embryo in general, as well as that of the thyroid, gonads, and adrenals, is inhibited by decapitation *in ovo* (Bellware and Betz, '70; Benoit, '62; Betz, '67, '68; Brasch and Betz, '71; Case, '52; Fugo, '40; Love and Konigsberg, '58; Manwell and Betz, '66; Martindale, '41; Moszkowska, '58; Stalsberg, '65; Tixier-Vidal, '58).

d. Pituitary grafts. Decapitation as a mode of hypophysectomy necessarily involves removal of both the hypothalamus and the pituitary. Therefore, grafting of the pituitary to the chorio-allantoic membrane of the decapitated embryos was used as a means of determining the degree of dependence of the pituitary upon neural regulation during development. Although the role of the hypothalamus in maintaining the basal level of function of the pituitary-adrenal axis is well established in mammals, as discussed above, this relationship is less clearly established in the chicken (*op cit.*).

In the present study pituitary grafts were capable of restoring normal resting levels of corticosterone in previously decapitated (16-day) embryos. A similar conclusion has been reached by Betz and his coworkers who showed that similar treatment restored normal adrenal histology and normal duodenal differentiation (Bellware and Betz, '70; Betz, '67). Mess ('68) likewise reported that subcutaneous pituitary grafts transplanted into previously decapitated recipients show seemingly normal basal secretions of ACTH and capacity to increase the secretion of this hormone in response to metapyrone treatment. These results are most readily explained by the assumption that the basal level of ACTH secretion in the chicken embryos is autonomous, i.e., independent of the hypothalamus or other parts of the forebrain. In experiments analyzing the function of pituitary grafts in adult organisms, it is controversial whether sufficient CRF might reach the graft through the general circulation (Ganong, '63). In the present study this is unlikely since the entire forebrain is removed by decapitation. However, since other sources of CRF-activity cannot be totally ruled out (Stainer and Holmes, '69), the present experiment, though strongly suggestive of it, does not necessarily prove autonomy of the pituitary insofar as basal or "resting" levels of ACTH secretion are concerned.

The results of the present study 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 extra-hypothalamic sources of CRG exist in the chicken, these sources do not respond to environmental stimuli.

General conclusions

The present investigation demonstrates that during the development of the chicken, adrenal function is controlled at three different levels: the adrenal itself, the pituitary, and the hypothalamus. These levels appear to form a hierarchy, the lower levels possessing a considerable degree of autonomy, but each level being subject to control by the next higher level.

First, the adrenal appears to be capable of autonomous secretion of glucocorticoids. This autonomy is revealed by the persistence of a significant level of corticosterone secretion after decapitation. Moreover, the adrenal apparently accounts entirely for the basal levels of circulating corticosterone during the earlier phases of functional differentiation of the gland (14 days of incubation or younger) when decapitation does not depress corticosterone below the basal level. Substantial adrenal autonomy persists after pituitary control begins (16 days) since decapitation results not in total disappearance of plasma corticosterone, but in subsidence to values approximately equal to normal 12 or 14 day hormone levels.

Second, the pituitary level of control is superimposed upon the adrenal after 14 days of incubation. The pituitary is necessary for the progressive rise in plasma corticosterone which occurs after day 14. This is shown by the effect of decapitation and pituitary grafts in 16-day em-

bryos. Although the pituitary is inferred to be *necessary* for the gradual elevation of basal hormone concentration after day 14, it may not be the limiting factor in determining the *rate* of this increase, which is the same as that which was established prior to the beginning of pituitary control, i.e., during the period of autonomy.

Third, the hypothalamic level of control also becomes functional between the fourteenth and sixteenth days of incubation. Thus the pituitary and the hypothalamic levels are not separable in time. Their independence is shown by comparison of the results of the decapitation and the decapitation plus pituitary graft experiments. The hypothalamus does not appear to influence normal basal corticosterone levels, which are maintained by pituitary grafts in decapitated 16-day embryos, but is necessary for a response to stress, and presumably other afferent input. The extent to which the hypothalamic component of the adrenal neuroendocrine axis in the chick embryo is physiologically important is uncertain, since there is no information concerning the possible nature and significance of environmental input into the adrenocortical system before hatching.

These results establish that the chicken has a qualitatively typical hypothalamo-hypophyseal-adrenal neuroendocrine axis, which becomes functional several days prior to hatching. They suggest further that the neural component of this axis is concerned primarily with modulation of responses to afferent input, and that substantial autonomy in basal secretion rates exists at both the adrenal and the pituitary level.

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