THE EFFECT OF A CRUDE HYPOTHALAMIC PROTEIN EXTRACT UPON *IN VITRO* STEROIDOGENESIS BY COCKEREL ADRENALS

by

Paul L. Williams

A Thesis Submitted to the Faculty of the

DEPARTMENT OF BIOLOGICAL SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN ZOOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 6 9

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Paul L. Williams

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Robert B. Chiasson
Professor of Zoology

17 January 1969
Date

TABLE OF CONTENTS

ABSTRACT

The introduction of a crude hypothalamic protein extract into ten *in vitro* cockerel adrenal bisect preparations failed to significantly stimulate adrenal release of corticosterone into the incubation media. The hypothesis that an extra-hypophyseal source of an ACTH-like substance exists in the avian hypothalamus gains no support from this study.

INTRODUCTION

Resko, Norton, and Nalbandov (1964) suggested that an extra-hypophyseal ACTH-like factor was present in the hypothalamus of the chicken and that this factor was released *de novo* in hypophysectomized birds with no damage to the median eminence.

The hypothesis that an extra-hypophyseal ACTH-like factor exists in the chicken hypothalamus was tested in this study by introducing a hypothalamic protein extract into 10 *in vitro* cockerel adrenal preparations and measuring the corticosterone released.

MATERIALS AND METHODS

Eleven 21 to 24 week-old White Leghorn cockerels from a single flock served as donors of adrenal tissue. Three weeks prior to adrenal excision, all of the 11 cockerels were subjected to a photoperiod regimentation of 8 hours light and 16 hours darkness. A preliminary investigation by this author had indicated that the state of sexual maturation of the cockerels may have influenced adrenal sensitivity to exogenous ACTH. This preliminary study also indicated that adrenal sensitivity might be increased by reducing the photoperiod. The cockerels were housed at room temperature in groups of 2-3 in a special battery of cages inside a photoperiod chamber. Limited movement was possible and food and water was provided ad libitum.

All of the cockerels were sacrificed by cervical dislocation. Two or three cockerels were used at a time. Immediately after sacrificing, the body cavity was opened and the adrenal glands were extracted simultaneously and in an intact condition. All extraneous tissue was removed and the adrenals were then bisected with a pair of fine surgical scissors and weighed.

Sixty 1 year-old White Leghorn laying hens from one flock were used as sources for hypothalamic, pituitary and cerebellar tissues. The birds were sacrificed under chloroform. Fifteen birds were used at a time. Immediately after sacrificing, the craniums of the birds were opened, whole brains were extracted and the anterior pituitaries were removed from the sella turcica. Hypothalamic sections, whole cerebelli and anterior pituitaries were then pooled separately and homogenized in acetone.

Extraction of the protein from the respective tissues was accomplished by minor variations of the Koch and Wolf (1955) technique. The concentrations of the crude protein extracts prepared from hypothalamic, anterior pituitary and cerebellar tissues were equivalent to 5 of each of the extracted tissues per ml.

Four experiments were performed each week during the period 4/17 to 5/10/1968. Each of the experiments consisted of 2 to 3 trials performed concurrently. A total of 11 trials were performed in the four experiments. With the exception of the last, four different adrenal bisects were used in each <u>in vitro</u> trial. These bisected adrenals were incubated in a Dubnoff incu-shaker with the various crude protein extracts and a mammalian ACTH (Acthar, Armour Pharmaceutical Co.) preparation. The incubating fluids were prepared by the technique of DeRoos (DeRoos and

DeRoos, 1964) except that the Krebs-Ringer bicarbonate solution contained only 20 gm of dextrose per liter and the pH of the incubating fluid was 7.4.

The bath temperature was held at 37°C. Incubation proceeded for 4 hours and there was no pre-incubation period.

The first of these preparations was a hypothalamic-adrenal preparation consisting of a 0.5 ml addition of the crude hypothalamic protein extract to 2.5 ml of incubating fluid containing a single adrenal bisect. The final crude protein concentration was, therefore, equivalent to 0.8 extracted hypothalamus per ml.

The second adrenal bisect was incubated with 0.5 ml of the crude pituitary extract in 2.5 ml of incubating fluid. The third preparation consisted of a 0.5 ml addition of the crude cerebellar protein solution to 2.5 ml of incubating fluid containing an adrenal bisect. The final crude protein concentration in both instances was equivalent to 0.8 extracted pituitary or cerebellum per ml.

The fourth preparation consisted of a 0.5 ml addition of the mammalian ACTH preparation to 2.5 ml of incubating fluid containing an adrenal bisect. The final ACTH concentration was equivalent to 0.55 USP units per ml.

The crude protein extracts and the ACTH solution were examined for interfering fluorogenic impurities. The

same concentrations and methodology were employed with these solution controls as were utilized for the adrenal bisect preparations except no adrenal tissue was present. These controls were run concurrently with the adrenal bisect preparations during each experiment.

The concentrations of released corticosterone were measured by the technique of Silber and Porter (1954) as adapted for fluorometric analysis by Sweat (1954). The modifications of Silber, Busch, and Oslapas (1958) to the Silber and Porter (1954) technique were also adopted. The amount of fluorescing corticosterone within the incubating fluids, after solution control correction, was read from a corticosterone standardization slope which fit the regression equation $y=a+bx$. These readings were then expressed in terms of a corticosterone production rate (ug of corticosterone per ml of incubating fluid per 100 mg of adrenal tissue per hour). Statistical analysis of the data was by the method of analysis of variance (Simpson, Roe, and Lewontin, 1960).

RESULTS

The individual trial preparation results are presented in Table 1. The hypothalamic-adrenal preparation in trial 1 and the pituitary-adrenal, cerebellar-adrenal and ACTH-adrenal preparations in trial 2 were lost during centrifugation. No cerebellar-adrenal preparation was run in trial 11.

The mean responses (± 2 standard errors) for the pituitary-adrenal, hypothalamic-adrenal and cerebellar-adrenal preparations are presented in Figure 1. The 9 cerebellar-adrenal (control) preparations produced fluorescence equivalent to a mean corticosterone production rate of .002 ug/ml/100mg/hr with a standard error of ±.002. Ten hypothalamic-adrenal preparations produced fluorescence equivalent to a mean corticosterone production rate of .005 ±.003 ug/ml/100mg/hr. These means were not statistically different from zero.

In contrast, when adrenal glands were incubated with the crude pituitary protein extract they produced fluorescence considerably above that of the adrenals incubated with the cerebellar and hypothalamic crude protein extracts. The pituitary-adrenal incubations produced a secretion comparable to 0.16 ±.02 ug/ml/100 mg/hr which was

Table I. Preparation responses - corticosterone production rate (ug/ml/100 mg/hr).

	trial	ACTH-adrenal	Pituitary-adrenal	Hypothalamic-adrenal	Cerebellar-adrenal
EXP. 1	1	1.70	0.11	*	0
	2	*	*	0	*
	3	1.00	0.23	0	0
EXP. 2	4	0.46	0.23	0.02	0
	5	0.33	0.18	0	0.02
	6	1.35	0.14	0	0
EXP. 3	7	0.33	0.20	0	0
	8	0.04	0	0	0
	9	0.19	0.18	0.03	0
EXP. 4	10	0.29	0.11	0	0
	11	0.09	0.23	0	not run
mean ± S. E.		0.578 ± 0.18	0.161 ± 0.073	0.005 ± 0.003	0.002 ± 0.002

* lost during centrifugation

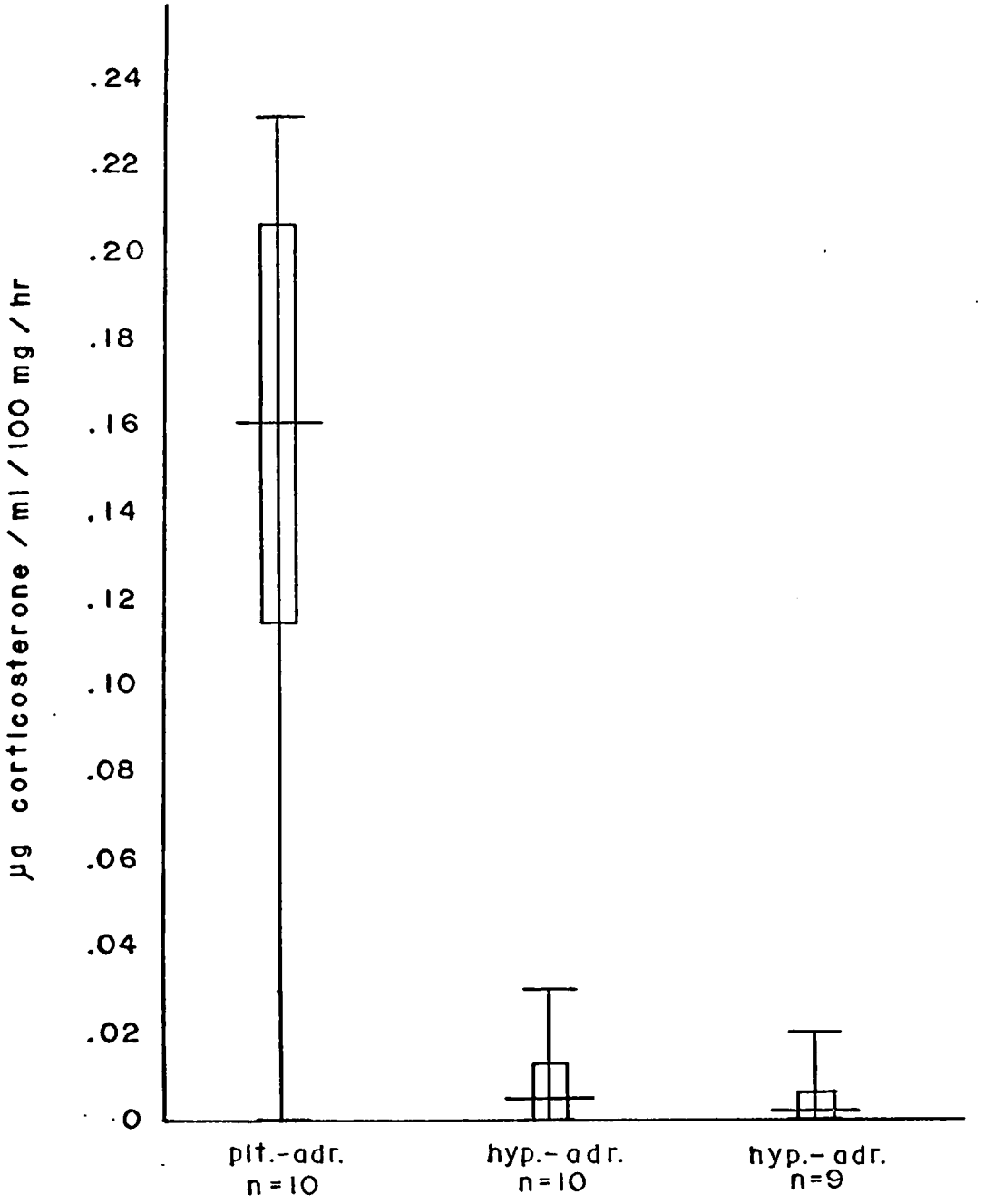

Figure 1. Mean preparation responses (ACTH-adrenal preparation not shown).

statistically different from the mean cerebellar-adrenal secretion at the 0.01 level of probability.

To test the ability of the adrenal glands to produce measurable corticosterone under the influence of mammalian ACTH of known biological activity, ten ACTH-adrenal preparations were employed. These preparations produced fluorescence equivalent to an average corticosterone production rate of 0.58 $\pm$.18 ug/ml/100mg/hr. The standard error value for the ACTH-adrenal response ($\pm$.18) was considerable and was, of course, a manifestation of the large range of the individual trial responses.

DISCUSSION

The addition of a crude hypothalamic protein extract equivalent to 0.8 extracted hypothalamus per ml failed to elicit significant steroidogenesis in ten *in vitro* cockerel adrenal bisects. Likewise, the presence of a crude cerebellar protein extract equivalent to 0.8 extracted cerebelli failed to stimulate significant steroidogenesis in nine other *in vitro* adrenals. Thus, it would appear that no ACTH-like factor was present in either preparation. The adrenals did respond significantly to the presence of a crude anterior pituitary protein extract, indicating the presence of an avian ACTH-like factor in the pituitary.

Frankel, Graber, and Nalbandov (1967b) reported that hypothalamic lesions in the ventral tuberal area of intact chickens caused a decrease in adrenal venous corticosterone concentrations. Similar lesions in adenohypophysectomized birds were reported to cause an elevation in corticosterone concentration when compared with the intact lesioned response. These authors concluded that the hypothalamus was not the site of an extra-hypophyseal source of an ACTH-like substance.

Frankel et al. (1967c) reported that a hypothalamic homogenate from chicken source failed to elicit steroidogenesis in 5 cockerel *in vitro* adrenal preparations. However, these authors stated that it was possible that the *in vitro* system used to detect ACTH was not sensitive enough. No pituitary-adrenal preparation (control) was employed in their study.

In hypophysectomized mammals, the adrenal gland quickly atrophies (Deane and Greep, 1946) and loses its ability to both synthesize adrenal steroids and respond to stress (Guillemin et al., 1958).

In contrast, hypophysectomy has only limited effect on adrenal histology in birds (Miller and Riddle, 1942; Nalbandov and Card, 1943; Resko et al., 1964; Frankel et al., 1967b) and there is considerable evidence now available that the adrenal glands of birds may continue to produce corticosteroids in the absence of the adenohypophysis (Brown, 1961; Nagra et al., 1963; Resko et al., 1964; Frankel, Graber, and Nalbandov, 1967a; Frankel et al., 1967c).

The ability of the bird adrenal to adjust rapidly to adenohypophysectomy by maintaining a significant production of corticosterone could conceivably be explained by one or a combination of three possibilities. The adrenal gland of the bird could function autonomously, the posterior pituitary might have adrenocorticotrophic

properties or finally, an extra-hypophyseal source of an ACTH-like substance might serve to maintain the adrenal.

There appears to be considerable evidence in the literature which would rule against posterior pituitary involvement including the reports that neurohypophyseal atrophy does not effect in vivo steroidogenesis (Frankel et al., 1967b) and that pitressin, pitocin and arginine vasotocin appear to have no adrenocorticotrophic properties in adenohypophysectomized birds (Resko et al., 1964; Frankel et al., 1967a).

It has been reported (Frankel et al., 1967a) that adrenal function in the adenohypophysectomized chicken can be totally inhibited by an ACTH inhibitor (dexamethasone phosphate) which has no inhibitory effect upon the adrenal directly. In addition, it has been reported that the adrenal gland of the adenohypophysectomized chicken can respond positively to stress (Nagra et al., 1963; Frankel et al., 1967a). This evidence argues against the possibility of adrenal autonomy and indicates that an extra-hypophyseal source of ACTH may exist in the bird, although the evidence gained from this study would indicate that this factor probably does not reside in the hypothalamus.

The hypothalamus, however, would seem to be involved in the control of the pituitary-adrenal axis in the bird although, due to conflicting lesioning reports, its

contribution is unclear. In intact chickens, lesions in the ventral tuberal area of the hypothalamus have been reported to reduce adrenal venous corticosterone levels to that seen in hypophysectomized chickens (Frankel et al., 1967b). Aspiration of the median eminence of adeno-hypophysectomized pigeons has been reported to cause adrenal hypertrophy (Miller, 1961). Resko et al. (1964) reported that lesions in the median eminence of chickens resulted in a significantly reduced rate of corticosterone production when compared with hypophysectomized birds with no such lesions. In contrast to this, Frankel et al. (1967b) reported that there was no change in the release of corticosterone in hypophysectomized birds lesioned in the median eminence but that lesions in the ventral tuberal area increased *in vivo* steroidogenesis when compared with intact lesioned birds.

The most recent model proposed for explaining the control of the avian adrenal in adenohypophysectomized chickens is that of Frankel et al. (1967c). In this model, the role of the ventral tuberal area of the bird hypothalamus would be modified when the hypophysis was removed and would mediate control of an ACTH-like factor produced elsewhere. According to the authors, this model would explain how stress and dexamethasone phosphate influence adrenal response as well as explain the apparent

inhibitory role the ventral tuberal area plays in adeno-hypophysectomized birds. However, there would appear to be specific areas of fault for this model. First, it fails to take into account the discussed conflicting lesioning reports of Resko et al. (1964) and Miller (1961). Second, it does not explain why lesions in various areas of the hypothalamus apparently have no effect upon adrenal corticosterone content in the intact bird (Egge and Chiasson, 1963).

The ACTH-adrenal preparations in this study probably responded on a maximal basis. The concentration of the Acthar used in this study (.55 USP units) was designed to produce maximal adrenal stimulation and was probably excessive. DeRoos and DeRoos (1964) have reported that 0.1 USP unit produced maximal stimulation in vitro.

The range of the individual adrenal bisect responses to Acthar cannot be readily attributed to a lack of specificity on the part of the mammalian ACTH preparation as DeRoos and DeRoos (1964) have reported that both mammalian ACTH and an avian ACTH-like substance extracted from chicken anterior pituitaries possessed similar biological activity and that species specificity to ACTH may be absent. Also, the range cannot be attributed solely to differences in individual adrenal bisect sensitivity to ACTH as the pituitary-adrenal preparations did not exhibit the erratic

responses displayed by the ACTH-adrenal preparations. It is also possible that the actual quantity of Acthar administered in each experiment was different in spite of the uniform preparation method employed. Conceivably, in spite of the photoperiod regimentation, it could be possible that the state of sexual maturation of the cockerels was adversely effecting maximal adrenal responsiveness. However, a conclusion on this matter cannot be drawn on the evidence at hand.

The range of the pituitary-adrenal responses was appreciable although considerably less than the ACTH-adrenal range. It was not possible to determine whether or not this range was due to individual differences in adrenal bisect sensitivity, to a potential sexual development influence, to differences in the amounts of the ACTH-like factor actually extracted from the fifteen anterior pituitaries used in each experiment or to a combination of the above.

In light of the conflicting lesioning reports in the literature, a systematic reexamination of the effects of electrolytic lesions in various areas of the bird hypothalamus upon <u>in vivo</u> adrenal function would be profitable. Should the apparent inhibitory role of the ventral hypothalamus in the adenohypophysectomized bird be born out by such a study, it would be of interest to determine

whether or not dexamethasone phosphate would be capable of causing an inhibition of *in vivo* adrenal steroidogenesis in such a bird. In such an eventuality, the possibility of adrenal autonomy could be ruled out and the nature of the ventral tuberal area contribution would be further elucidated. The next logical step would be to determine the exact location of the extra-hypophyseal ACTH-like factor the ventral tuberal area would be controlling. Perhaps an autoradiographic approach employing labeled dexamethasone would be fruitful in pinpointing the exact location.

It is also suggested that there may be a possibility that the physiological state of male birds undergoing sexual maturation may exert a deleterious effect upon adrenal sensitivity. This possibility is worthy of investigation.

REFERENCES

Brown, K. I. 1961. The validity of using plasma corticosterone as a measure of stress in the turkey. Proc. Soc. Exptl. Biol. Med. 107:538-542.

Deane, H. W. and R. O. Greep. 1946. A morphological and histochemical study of the rat's adrenal cortex after hypophysectomy with comments on the liver. Amer. J. Anat. 79:117-137.

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

Egge, A. S. and R. B. Chiasson. 1963. Endocrine effects of diencephalic lesions in the White Leghorn hen. Gen. Comp. Endocrinol. 3:346-361.

Frankel, A. I., J. W. Graber, and A. V. Nalbandov. 1967a. Adrenal function in cockerels. Endocrinology. 80:1013-1019.

Frankel, A. I., J. W. Graber, and A. V. Nalbandov. 1967b. The effect of hypothalamic lesions on adrenal function of intact and adenohypophysectomized cockerels. Gen. Comp. Endo. 8:387-396.

Frankel, A. I., J. W. Graber, B. Cook, and A. V. Nalbandov. 1967c. The duration and control of adrenal function in adenohypophysectomized cockerels. Steroids. 10:699-707.

Guillemin, R., G. W. Clayton, J. D. Smith, and H. S. Lipscomb. 1958. Measurement of free corticosteroids in rat plasma: physiological validation of a method. Endocrinology 63:349-358.

Koch, W. T. and F. J. Wolf. 1955. Purification of corticotropin. J. Am. Chem. Soc. 77:489-490.

Miller, R. A. 1961. Hypertrophic adrenals after lesions in the medium eminence of totally hypophysectomized pigeons and their response to stress. (abstract). The Anat. Rec. 139-254.

Miller, R. A. and O. Riddle. 1942. The cytology of the adrenal cortex of normal pigeons and experimentally induced atrophy and hypertrophy. Am. J. Anat. 71:311-335.

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

Nalbandov, A. V. and L. E. Card. 1943. Effects of hypophysectomy of growing chicks. J. Exptl. Zool. 94:387-409.

Resko, J. A., H. W. Norton, and A. V. Nalbandov. 1964. Endocrine control of the adenohypophysis in chickens. Endocrinology 75:192-200.

Silber, R. H. and C. C. Porter. 1954. The determination of 17,21-Dihydroxy-20-ketosteroids in urine and plasma. J. Biol. Chem. 210:923-932.

Silber, R. H., R. D. Busch, and R. Oslapas. 1958. Practical procedure for estimation of corticosterone or hydroxycorticosterone. Clin. Chem. 4:278-285.

Simpson, G. L., A. Roe, and R. C. Lewontin. 1960. Analysis of variance. pp. 266-271 in Quantitative Zoology, Harcourt, Brace and Co., Inc.

Sweat, Max L. 1954. Sulfuric acid induced fluorescence of corticosteroids. Anal. Chem. 26:773-776.