EFFECT OF PROLONGED CORTISONE TREATMENT ON THE CORTICOTROPIN RELEASING ACTIVITY OF THE RAT HYPOTHALAMUS*

By

C.D. BAGUL

Department of Biochemistry, All-India Institute of Medical Sciences, New Delhi

It has been shown that ACTH output by the anterior pituitary gland can be inhibited by the administration of corticosteroids, under basal conditions or under conditions of stress (20, 13, 18, 10). Prolonged treatment with cortisone is known to cause adrenal atrophy in dogs, rats, and in man (11,9), due to inhibition of ACTH secretion from the anterior pituitary gland. Such an inhibitory action has been demonstrated by Sydnor (23) who showed that the release of ACTH from the pituitary in response to a stress is prevented if a dose of cortisol is administered to the animal just prior to the application of stress. Farrell and Laqueur (7) observed reduction in pituitary ACTH content after cortisone treatment to dog. The site and mechanism of blocking action of corticosteroids is still an unsolved problem. The inhibitory effect might be exerted at the level of the central nervous system or the anterior pituitary gland or at both these levels.

It is generally believed that ACTH secretion is regulated by a neurohumour, corticotropin releasing factor (CRF), released from the hypothalamus (4,5, 16, 19, 17,). Therefore, effect of conditions which reduce release and content of pituitary ACTH on corticotropin releasing activity of the hypothalamus was studied. The data are presented here.

MATERIALS AND METHODS

Male rats (about 300 *g*) were obtained from Hormone Assay Laboratories, Chicago, Illinois, and were acclimatized for 1-2 weeks to the local animal house conditions. Animals were fed Purina rat Chow and given water *ad libitum*. All injections were given by the subcutaneous route except where stated otherwise.

Cortisone treatment —Each rat of the experimental group received injections of 12.5 *mg* cortisone acetate as 0.5 *ml* aqueous suspension (Upjohn, Kalamazoo, Michigan) every day, for 24 days. After the completion of the above period, body weights were determined, the rats decapitated and adrenal and pituitary weights measured. Control rats were given 0.5 *ml* of 0.9% sodium chloride solution per day, for 24 days before they were sacrificed.

Preparation of the hypothalamic extracts—The hypothalamic area around the pituitary stalk 2 *mm* in anteroposterior plane, 1 *mm* in lateral plane and 1 *mm* in ventrodorsal plane (weight about 6-8 *mg*) was excised and immediately crushed in a glass homogenizer containing cold (about 4°C) 0.1 N hydrochloric acid. The hypothalamic areas from the control and treat-

*This work was carried out in the Department of Physiology, School of Medicine, Western Reserve University, Cleveland, U.S.A.

ed group of animals were separately pooled and then homogenized. Thirty animals were used per group. The extracts thus prepared were heated to 100°C for 10 min. in order to destroy toxic substances (21). Volumes of the extracts were adjusted so that 0·5 *ml* of the extract represented hypothalamus from 1 animal, hereafter referred to as a hypothalamic unit (H.U.). Extracts were centrifuged for 1/2 hour at 20,000 r.p.m. in a preparative head (No. 40) of a Spinco ultracentrifuge. The corticotropin releasing activity of the supernatant was estimated by the method of Briggs and Munson (1). Male rats (120-140 *g*) were given pentobarbitone sodium (40 *mg/kg*) in 0.9% sodium chloride solution by the intraperitoneal route. Ten minutes later morphine sulfate (15 *mg/kg*) in 0·9% sodium chloride solution was injected by the intraperitoneal route. Ten minutes after the morphine injection, the left adrenal gland was removed via a dorsal approach, cleaned, weighed and put into centrifuge tube containing 10 *ml* of 4% trichloracetic acid and a little sand and was crushed by a glass rod. This was used for ascorbic acid estimation. The left femoral vein was exposed and hypothalamic extract was infused over a period of 60 to 90 seconds. The injections of the hypothalamic extracts were made at 2 dose levels (0.25 H.U. and 1 H.U.); the volume injected was kept constant at 0.5 *ml*. In another series of assay rats standard ACTH (USP standard) in solution was infused at two dose levels, 0.2 mU and 0.8 mU ACTH. The ACTH solution was prepared in solution containing 0.1 N HCl, 0.1% W/V Bovine serum albumin (Nutritional Biochemicals, Cleveland, Ohio), and 0.9% sodium chloride. Bovine albumin was added to prevent sticking of micro quantities of ACTH to the wall of glass containers.

Seven to 10 assay animals were used for each dose of hypothalamic extract or of ACTH. Half an hour after the injection of the hypothalamic extract or of ACTH, the right adrenal gland was removed and processed like the left one. The ascorbic acid content of individual adrenal glands was separately estimated by the method of Sayers *et al.*(22).

The assay rats were also infused with either non-specific (cerebral) or specific (hypothalamic) extracts (Table 1.)

TABLE I

Ascorbic Acid Depletion in Pentobarbital-Morphine Blocked Rats 30 Minutes after the Stimulus. Normally, 100 gm adrenal wet weight contains about 350 mg ascorbic acid.

Stimulus	*Ascorbic Acid Depletion (mg per 100g Adrenal wet weight)*	*Number of Animals*
Laparotomy+Unilateral Adrenalectomy	16±7.0	5
Laparotomy+Unilateral Adrenalectomy+0.5 *ml* 0.1 N HCl	15±6.0	15
Laparotomy+Unilateral Adrenalectomy+0.5 *ml* Cerebral Extract in 0.1 N HCl	8±3.0	14
Laparotomy+Unilateral Adrenalectomy+0.5 *ml* Hypothalamic Extract in 0.1 N HCl	146±8.0	6
Laparotomy+Unilateral Adrenalectomy+25 mU Vasopression in 0.5 *ml* 0.1 N HCl	9±9.7	7
Laparotomy+Unilateral Adrenalectomy+100 mU Vasopression in 0.5 *ml* 0.1 N HCl	128±8.0	8

Index of precision of the CRF-ACTH assay

Standard ACTH (obtained from the U.S.P. office, New York) was infused in 3 groups of assay rats, each containing 7 to 10 animals, at 3 dose levels 0.2 mU, 0.4 mU and 0.8 mU. A linear relationship was observed between the log dose and the response. Index of precision of the assay (λ) was calculated (22). Index of precision is the ratio of standard deviation to the slope of the fitted regression line. If the value of λ is below 0·20, the regression line is considered to be quite good to use for the bioassay. In this case λ was found to be 0.2026 (Fig. 1).

To exclude the possibility that the hypothalamic extracts could have been contaminated with ACTH, the hypothalamic extracts were tested for ACTH activity according to the ascorbic acid depletion method of Sayers *et al.*(22). One half *ml* of the extract was infused into 24 hour hypophysectomized rats and the ascorbic acid depletion caused was noted (Table 4).

TABLE 2

Effect of Prolonged Treatment with Cortisone Acetate on the Body weight, Adrenal weight and Pituitary weight of Rats

	Normal (Saline treated)	*Cortisone Treated*	*p***
Body weight, in *g*	364.00±2.00 (63)*	209.00±2.00 (58)	0.001
Combined Adrenal Weight, in *mg*	38.90±0.70 (63)	15.54±0.34 (58)	0.001
Anterior Pituitary Weight, in *mg*	5.03±0.14 (63)	4.80±0.12 (58)	0.5

*Number in the parentheses indicates number of animals in the group.
**For the difference between the means.

Vasopressin in the extracts—Vasopressin is known to release ACTH. Vasopressin content of the hypothalamic extract was determined by employing the 'Rat Pressor Bioassay' technique of De Kanski (6) (Table 5).

TABLE 3

Comparison between the Corticotropin Releasing Activity of Hypothalamic Extracts Obtained from Normal and from Cortisone-Treated Rats.

	Ratio of Potency	*Fiducial Limits for the Ratio of Potency p=0.67*
Cortisone-Treated : Normal	0.59	0.58 to 0.60

'p' for the darallelism of slopes was>0.5

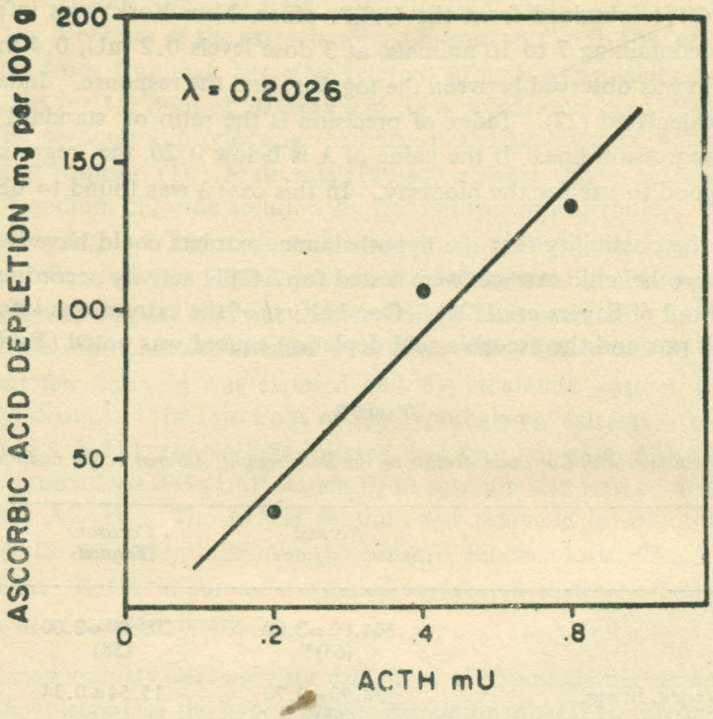

Fig. 1.

Log-dose X response relationship between ACTH infused and the ascorbic acid depletion induced in pentobarbital—morphine blocked rats.

RESULTS

The corticotropin releasing activity in the hypothalamic extracts of cortisone-treate animals was about half of that found in the hypothalamic extracts of the normal animals(Table 3 Such a comparison is valid since the slopes for the above two groups were parallel to each oth ($p>0.5$) (Fig. 2). Similarly, the method of assay of released ACTH might be considered vali since the slopes for cortisone-treated group and the normal controls were parallel to the slop for ACTH. ($p>0.4$ and $p>0.2$ respectively, Fig. 2).

Cerebral extract did not release ACTH in the assay animals. Similarly, ACTH wa found to be released only on infusion of hypothalamic extract or infusion of 100 mU of vaso pressin (Table 1). The activity of ACTH in the hypothalamus of cortisone-treated animals in the normal controls was negligible (Table 4). Similarly, the vasopressin content in th hypothalamic extracts was too low to release ACTH and cause ascorbic acid depletion in th assay rats (Table 5).

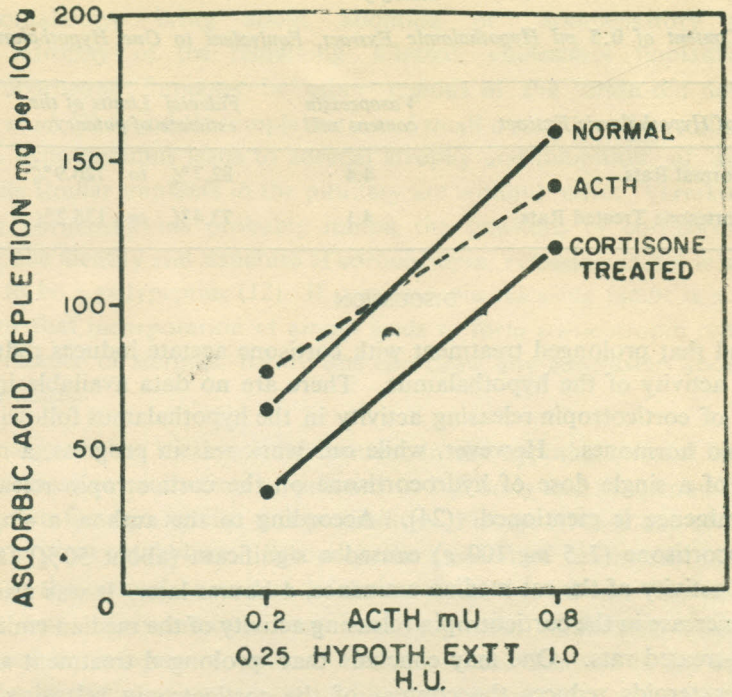

Fig. 2.

Ascorbic acid depletion induced in pentobarbital--morphine blocked assay rats following the administration of Standard (U.S.P.) ACTH or hypothalamic extract. (The hypothalamic extract was obtained from normal or cortisone-treated rats).

TABLE 4

ACTH Activity in the Hypothalamic Extracts Obtained from Normal and Cortisone-Treated Rats

Ascorbic Acid Depletion in 24-Hour Hypophysectomized Rats 30 Minutes after the Injection of 0.5 *ml* Hypothalamic Extract in 0.1 N Hydrochloric Acid (equivalent to one Hypothalamus)

Source of Hypothalamic Extract	*Ascorbic Acid Depletion mg per 100 g*	*Number of Animals*
Normal Rats	—3 ±22	6
Cortisone-Treated Rats	—4 ±28	6

The body weights of the cortisone-treated rats were significantly less than those of the saline-treated controls (Table 2). Cortisone treatment also caused adrenal atrophy in the animals. Thus the average combined weight of the two adrenals of the individual rat was 38.90±0.70 *mg* in the normal saline treated group whereas it was 15.54±0.34 *mg* in the cortisone-treated group (Table 2). On the other hand, anterior pituitary weights of the normal controls and those of the cortisone-treated animals were comparable (Table 2).

TABLE 5

Vasopressin Content of 0.5 ml Hypothalamic Extract, Equivalent to One Hypothalamus

Source of Hypothalamic Extract	*Vasopressin content mU*	*Fiducial Limits of the estimate of potency*
Normal Rats	4.4	82.7% *to* 120.9%
Cortesone Treated Rats	4.1	73.4% *to* 136.2%

DISCUSSION

It was found that prolonged treatment with cortisone acetate induces reduction in corticotropin releasing activity of the hypothalamus. There are no data available in the literature as to the amount of corticotropin releasing activity in the hypothalamus following treatment of animals with steroid hormones. However, while our work was in progress, a report appeared wherein an effect of a single dose of hydrocortisone on the corticotropin releasing activity of the rat median eminence is mentioned (24). According to the author, a single intravenous injection of hydrocortisone (7.5 *mg*/100 *g*) caused a significant (about 50%) fall in the corticotropin releasing activity of the rat median eminence, 4 hourss later. It was also observed that stress induced an increase in the corticotropin releasing activity of the median eminence in normal but not in steroid-treated rats. One may conclude that prolonged treatment as well as single injection of corticosteroids reduces the content of the corticotropin releasing activity in the hypothalamus.

Results presented here on the effect of prolonged treatment with corticosteroid on the adrenals confirm those reported by other investigators. Ingle *et al.* (14) for example, have shown that the administration of adrenocortical extract leads to adrenal atrophy. Kitay *et al.* (15) found that cortisone acetate (5 *mg*/120 to 140 *g*/day) induced a significant decrease in adrenal weight.

Fortier (8) found that 10 days of hydrocortisone acetate treatment (3 *mg*/100 *g*/day) resulted in 40% decrease in the pituitary ACTH content in the rat. Further that the same treatment if prolonged for 24 days induced 90% decrease in pititary ACTH content. Fortier (8) also found that 10 and 24 days of treatment with the steroid led to 50% and 70% reduction in adrenal weight, respectively.

Kitay *et al.* (15) found that the pituitary weights in the steroid-injected group and in the saline control group did not differ from each other although pituitary ACTH content was less by about 60% in the cortisone-treated group. The results of other workers are in agreement with this finding (8, 15). We did not find significant difference between the weights of the anterior pituitaries obtained from cortisone-treated and saline-treated rats. We did not measure pituitary ACTH content in these two groups.

Davidson and Feldman (3) feel that the hypothalamus rather than the pituitary should be regarded as the primary site of feed back inhibition of ACTH by hydrocortisone.

The argument is based on their observations that hydrocortisone implants in the anterolateral hypothalamus bring about abolition of compensatory adrenal hypertrophy and even atrophy of the remaining adrenal; cholesterol implants were without effect. Also, hydrocortisone implants in other regions of the brain did not produce such effects. Chowers *et al.* (2) found that implantation of small quantities of crystalline hydrocortisone acetate in the hypothalamus leads to adrenal atrophy and inhibition of adrenal ascorbic acid depletion, while similar implants in the pituitary are without effect. Vernikos-Danellis (24) has suggested that corticosteroids probably inhibit the synthesis of corticotropin releasing factor. At present, the identity and structure of corticotropin releasing factor is not fully known, though it is likely to be a polypeptide (12). If corticotropin releasing factor is a polypeptide, it is necessary to show that incorporation of amino acids to form corticotropin releasing factor is prevented in the presence of steroids, before one can draw any conclusion about the effect of steroids on its synthesis.

It might be argued that the low CRF content in the hypothalamus of cortisone-treated rats may be due to massive catabolism of proteins in the body and hence non-specific. However, the following two facts go against this possibility. Firstly, CNS is one of the organs that suffers least during protein catabolism. Secondly, the amount of vasopressin in the hypothalamus (polypeptide) in the normal as well as in the cortisone-treated rats was nearly identical in the experiments in here. This indicates that massive protein catabolism following cortisone treatment did not reduce vasopressin content. Therefore, the decrease in the content of CRF in the hypothalamus of cortisone-treated rats is likely to be specific.

SUMMARY

Cortisone treatment to the rats (12.5 *mg*/day; subcutaneously for 24 days) led to 40% reduction in the corticotropin releasing activity of the hypothalamic median eminence region. The ACTH activity and vasopressin content of this region were found to be too low to interfere with the assay of corticotropin releasing activity. The results suggest that cortisone inhibits the synthesis of corticotropin releasing factor.

Cortisone treatment also caused a 42% and 60% fall in the body weight and the adrenal weight of the cortisone-treated group though pituitary weight did not differ significantly from those in the normal controls.

ACKNOWLEDGEMENT

The author is grateful to Professor George Sayers under whose guidance the work was carried out. The author is indebted to U.S.P.H.S., U.S.A. for the grant of a fellowship under PI-574.

REFERENCES

1. Briggs, F.N. and P.L. Munson. Studies on the mechanism of stimulation of ACTH secretion with the aid of morphine as a blocking agent. *Endocrinol.* **57**:205, 1955.

2. Chowers, I., S. Feldman, and J.M. Davidson. Effect of intrahypothalamic crystalline s roids on acute ACTH secretion. *Am. J. Physiol.* **205**:671, 1963.

3. Davidson, J.M., and S. Feldman. Cerebral involvement in the inhibition of ACTH sec tion by hydrocortisone. *Endocrinol.* **72**:936, 1963.

4. De Groot, J., and G.W. Harris. Hypothalamic control of anterior pituitary gland and blo lymphocytes. *J. Physiol.* (*London*) **111**:335, 1950.

5. De Groot, J., and G.W. Harris. Hypothalamic control of ACTH secretion by the pituit gland. In Ciba Foundation Colloquia on Endocrinology. **4**:103, 1952.

6. Dekanski, J. The quantitative assay of vasopressin. *Brit. J. Pharmacol.* **7**:567, 1952.

7. Farrell, G.L. and G. Laqueur. Reduction of pituitary content of ACTH by cortiso *Endocrinol.* **56**:471, 1955.

8. Fortier, C. Pituitary ACTH and plasma free corticosteroids following bilateral adrenal tomy in the rat. *Proc. Soc. Exp. Biol. Med.* **100**:13, 1959.

9. Fortier, C. Effect of hydrocortisone on pituitary ACTH and adrenal weight in the r *Proc. Soc. Exp. Biol. Med.* **100**:16, 1959.

10. Fraschini, F., G. Mangili, M. Motta, and L. Martini. Midbrain and feedback control adrenocorticotropia secretion. *Endocrinol.* **75**:765, 1964.

11. Ganong, W.F., and D.M. Hume. Effect of hypothalamic lesions on steroid-induced atrop of the adrenal cortex in the dog. *Proc. Soc. Exp. Biol. Med.* **88**:528, 1955.

12. Guillemin, R. Control of pituitary hormone secretion ; Hypothalamic factors releasi pituitary hormones. *Rec. Prog. Hor. Res.* **20**:89, 1964.

13. Hermann, M. and G. Winkler. Zellkernvolumen der nebennierenrinde und 17-OH cor costeroid ausscheidung beim meerschweinchen nach lang-fristiger cortisonovorbehandlu und nach diphtherietoxin vergiftung. *Acta Neuroveg.* (*Vienna*) **20**: 39, 1959.

14. Ingle, D.J. ,G.M. Higgins, and E.C. Kendall. Atrophy of the adrenal cortex in the r produced by the administration of large amounts of cortin. *Anat. Rec.* **71**:363, 1938.

15. Kitay, J.I., D.A. Holub, and J.W. Jailer. Hormonal regulation of pituitary adrenocor cotropin. *Proc. Soc. Exp. Biol. Med.* **97**:165, 1958.

16. MacCann, S.M. Effect of hypothalamic lesions on the adrenal cortical response to stress the rat. *Am. J. Physiol.* **175**:13, 1953.

17. McCann, S.M., and J.R. Brobeck. Evidence for the role of the supraopticohypophys system in the regulation of adrenocorticotropic secretion. *Proc. Soc. Exp. Biol. Med.* *318, 1954.*

18. Peron, F.G. and R.I. Dorfman. A method for the evaluation of adrenocorticotropic h mone suppressing action of corticoids. *Endocrinol.* **64**:431, 1959.

19. Porter, R.W. The central nervous system and stress induced eosinopenia. *Rec. Prog. Hor. Res.* **10**:1, 1954.

20. Porter, R.W. and J.C. Jones. Effect of plasma from hypophyseal-portal vessel blood on adrenal ascorbic acid. *Endocrinol.* **58**:62, 1958.

21. Royce, P.C., and G. Sayers. Corticotropin releasing activity of a pepsin labile factor in the hypothalamus. *Proc. Soc. Exp. Biol. Med.* **98**:677, 1958.

22. Sayers, M.A., G. Sayers, and L.A. Woodbury. The assay of adrenocorticotropic hormone by the adrenal ascorbic acid depletion method. *Endocrinol.* **42**:379, 1948.

23. Sydnor, K.L. Blood ACTH in the stressed adrenalectomized rat after intravenous hydrocortisone. *Endocrinol.* **56**:204, 1955.

24. Vernikos-Danellis, J. Effect of stress, adrenalectomy, hypophysectomy and hydrocortisone on the corticotropin releasing activity of rat median eminence. *Endocrinol.* **76**:122, 1965.