AN IN VIVO ASSAY METHOD FOR CORTICOTROPHIN-RELEASING FACTOR (CRF) IN DEXAMETHASONE-NEMBUTAL TREATED RATS

ZEN ITOH
First Department of Surgery and Institute of Endocrinology, Gunma University School of Medicine, Maebashi

SYNOPSIS

The effect of dexamethasone on pituitary-adrenal function was studied in rats for the purpose of developing a new assay method for corticotrophin-releasing factor (CRF) in vivo. Intraperitoneal administration of 0.2 mg of dexamethasone and 5.0 mg of Nembutal per 100 g body weight lowered the plasma corticosterone concentration in saline injected rats (3.5 ± 0.3 µg) and suppressed the effect of a wide variety of stressful stimuli under the level of 10.0 µg/100 ml of plasma. This treatment also inhibited the corticotrophic activity of vasopressin in doses less than 80 mU of activity. Partially purified CRF from the posterior pituitary was tested at several dose levels; a linear dose response curve was obtained between 0.04 to 2.2 µg of CRF and plasma corticosterone level.

It is proposed that the dexamethasone and Nembutal treatment is a new assay system for CRF, which is simple and suitable for routine assay procedure.

The relationship between CRF and vasopressin and the blocking mechanism of dexamethasone are briefly discussed.

Since Saffran and Schally (1955 a and b) reported the existence of corticortrophin-releasing factor (CRF) in the posterior pituitary lobe as a neurohormon, a number of workers have presented various methods for assaying CRF. Originally, Saffran and Schally (1955 a) offered an in vitro method, assaying ACTH release from the pituitary in response to various substances. The in vitro assay method has a great advantage in that the effect of endogenous neurohormon release is completely excluded. The method, however, has some theoretical and practical shortcomings (Fortier, 1958 a, and b; Fortier and Ward, 1958; Guillemin et al., 1959 b) and the specificity of this method has now been questioned (Barrett and Sayers, 1958).

In vivo assay methods for CRF have also been widely studied. Pharmacologically, in Nembutal-morphine treated rats the acute effect of stressful stimuli on the rate of ACTH secretion is blocked and the effect of various stressful drugs on the pituitary-adrenal system is suppressed (Briggs and Munson, 1955; Munson and Briggs, 1955; Guillemin et al., 1959 a). Destruction of the median eminence has also been found to inhibit the release of ACTH following a wide variety of noxious stimuli. Rats with the hypothalamus or median eminence lesioned by means of stereotaxic instruments have been used for the assay of CRF (McCann, 1953; Ganong and Hume, 1954; McCann, 1957; de Wied et al., 1958; Slusher, 1958;
McCann and Harberland, 1960; de Wied, 1961).

On the other hand, the secretion of ACTH from the adenohypophysis is under negative feed-back control of the adrenocortical hormones. Therefore, a large amount of corticosteroids is capable of inhibiting the effects of pituitary-adrenal activities. This mechanism has been utilized for the assay of CRF. (McCann, 1957; McCann et al., 1958; de Wied and Mirsky, 1959; Leeman et al., 1962; Rerup, 1964 a; de Wied., 1964; Carbin et al., 1965).

In this laboratory, it became necessary to estimate corticotrophin-releasing activity quantitatively for the purpose of purifying CRF from the posterior pituitary. At first, the in vitro method of Saffran and Schally and in vivo method of Guillemin (1959 b) were followed, but without satisfactory results. In view of difficulties encountered in other hitherto reported methods when applied to our purpose, a new procedure had to be worked out.

The purpose of the present paper is to describe a new in vivo method for the bioassay of CRF which is simple, easy and suitable as a routine procedure.

**MATERIALS AND METHODS**

(1) *Animals*

Male albino rats of an inbred strain weighing 150 to 180 g, imported from Holtzman Rat Co., Madison, Wisconsin, U.S.A. in 1963, were used throughout the experiments. The rats were housed in individual metal cages in air-conditioned animal quarters maintained at 25±1°C and lighted artificially from 8:00 a.m. to 8:00 p.m. Food and water were given ad libitum. To accustom the rats to the manipulations during experiments, they were subjected daily to handling and mock intraperitoneal injection for at least 5 days before experiments.

(2) *Stress procedures*

Surgical:
1) A bilateral fracture of the femur.
2) Laparotomy.

Pharmacological:
1) Histamine dihydrochloride, 0.5 mg/100 g B.W., intraperitoneally (i.p.)
2) L-Epinephrine hydrochloride, 0.03 mg/100 g B.W., subcutaneously (s.c.)
3) Serotonin creatinine sulfate, 0.05 mg/100 g B.W., intravenously (i.v.)

(3) *Blood sampling and measurement of plasma corticosterone (Cpd. B) level*

Blood was collected in heparinized beakers from the trunks of rats quickly decapitated 15 min. after stress or injection. For determination of the non-stressed resting level of plasma corticosterone, rats were quickly taken from the cage to the next room and was decapitated within 20 secs.

Plasma corticosterone determination was carried out according to the method of Zenker and Bernstein (1958) with some modifications. Plasma was separated from heparinized blood by centrifugation at 3,000 r.p.m. for 5 min. and stored in a deep-freezer at -30°C. One ml aliquot of each plasma was extracted with 30 ml of chloroform. The extract, after being washed with 4.0 ml of 0.1 N NaOH, was added to 3.0 ml of 50% ethanol sulfuric acid (1:2.4, v:v). The fluorometric determination on the sulfuric acid layer was carried out after incubation at 25°C for 2.5 hrs. Incubation under these conditions gave a linear dose-response relationship with corticosterone standard.
(4) Administration of drugs and hormonal preparations

Heparin sodium (Novo), 10,000 USP/vial, was dissolved in 50 ml of normal saline and 0.1 ml aliquot was used for each blood specimen.

Pentobarbital sodium, Nembutal (Abbott), was injected intraperitoneally in a dose of 5.0 mg /100 g B.W.

Dexamethasone-21-phosphate disodium salt, "Decadron", was injected intraperitoneally at various doses after dilution with normal saline.

Histamine (Sanko Junyaku), L-Epinephrine (Tokyo Kasei) and Serotonin (Nutritional Biochemicals Corporation) were dissolved in normal saline for use.

Synthetic lysine-8 vasopressin was administered intravenously after being diluted to a desired concentration with normal saline.

ACTH, commercial preparation of Organon, was dissolved in normal saline and administered intravenously.

Corticotrophin-releasing factor (CRF) was extracted from the hog posterior pituitary and precipitated with acetone and ether. The preparation was further purified by carboxymethyl-cellulose chromatography and partition chromatography on "Sephadex" G-25. The partially purified preparation used in the present study still contained a small amount of vasopressin (15.1 IU/mg).

(5) Bioassays

ACTH was assayed in Nembutal anesthetized rats (Rerup and Hedner, 1962) or 24-hour hypophysectomized rats. Hypophysectomy was performed by the transauditory canal method (Koyama, 1962) and the completeness of the operations was verified at autopsy.

Vasopressin was assayed by the method of Dekanski (1952).

RESULTS

(1) Plasma corticosterone level of normal and Nembutal treated rats

Resting, non-stressed level of plasma corticosterone in male rats was shown to be 14.6±0.3 µg/100 ml (mean±SE) in the afternoon (1:00-4:00 p.m.). The level was found to rise to 31.5±0.8 µg/100 ml 20 mins. after removal of animals from the animal room to the laboratory (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Control resting and elevated level of plasma corticosterone in rats between 1:00 and 4:00 p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Control resting in animal room</td>
</tr>
<tr>
<td>Twenty mins. after removal from animal room to laboratory</td>
</tr>
</tbody>
</table>

* Mean±SE

Figure 1 shows the pattern of the level of plasma corticosterone in rats changing after injection of 5.0 mg of Nembutal. Deeply anesthetized rats were brought to the laboratory and kept in an oxygen tent at 32°C. An intraperitoneal injection of Nembutal caused a minimal but definite increase in plasma corticosterone.
Fig. 1. Changing pattern of the plasma level of corticosterone in rats after intraperitoneal injection of Nembutal (5.0 mg/100 g).
Bars denote the standard error of the means.

concentration, but the plasma level returned to the original level and decreased to 10 µg/100 ml, 40 and 60 mins. after injection, respectively. This low level of plasma corticosterone persisted for a further 40 mins. period at least, without recovery from anesthesia. It was concluded that the procedure of stress and blood sampling was to be performed between 40 and 100 mins. after the injection of Nembutal.

(2) Effect of dexamethasone in Nembutal-treated rats
Effect of various doses of dexamethasone on the adrenal responsiveness to ACTH and inhibition of vasopressin effect were tested. Figure 2 shows that with the increase of dexamethasone dose, the inhibiting effect of corticosteroid on ACTH secretion became more evident. On the contrary, the adrenal responsiveness for the moderate doses of ACTH decreased with an increase in the dose of dexam-
A NEW BIOASSAY FOR CRF IN VIVO

Fig. 2. Blocking effect of dexamethasone in the Nembutal anesthetized rats on plasma corticosterone level stimulated by 50 mU of synthetic lysine vasopressin and 0.5 mU of ACTH. Each point and vertical line denote the means of 10-15 animals and the standard errors.

Table 2. Inhibiting effect of dexamethasone on corticosterone concentration in rat 15 min after stress consisting of 5.0 mg Nembutal i.p. injection

<table>
<thead>
<tr>
<th>Time after dexamethasone injection i.p. (hr)</th>
<th>1/2</th>
<th>1</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpd. B, µg/100 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min after stress consisting of 5.0 mg Nembutal i.p. injection</td>
<td>15.3±2.2*</td>
<td>9.2±0.5</td>
<td>7.2±1.8</td>
<td>6.8±0.4</td>
</tr>
</tbody>
</table>

* Mean±SE.

Cf. Without pretreatment with dexamethasone, level of Cpd. B 15 min. after 5.0 mg Nembutal injection was 17.5±0.8 µg/100.

It was found that the most suitable dose of dexamethasone was 0.2 mg per 100 g body weight when animals were treated with 5.0 mg of Nembutal.

In an attempt to investigate the stress-blocking effect of dexamethasone, rats were treated with 0.2 mg of dexamethasone and subjected to stress with i.p. Nembutal injections after 1/2, 1, 2 and 4 hrs. Table 2 demonstrates that the stress
blocking effect was manifested 2 hrs. after the administration of dexamethasone and lasted for at least 2 hrs. thereafter.

(3) Standard schedule for CRF assay

Figure 3 shows the standard schedule for the assay method of CRF. After the administration of 0.2 mg of dexamethasone i.p., rats were returned to individual cages and left free from all stimuli. Two hrs. after injection, they were subjected to a second injection of 5.0 mg of Nembutal and kept in the oxygen tent at 32°C. Test material was injected by way of tail vein one hr. after anaesthesia and the rats were decapitated for blood sampling 15 mins. after i.v. injection of the test material.

(4) Responses of plasma corticosterone to various stress in dexamethasone-Nembutal treated rats

Table 3 summarizes the data on the effect of saline, histamine, epinephrine and serotonin on the plasma corticosterone level in rats treated with dexamethasone and Nembutal following the schedule shown above in Figure 3. The stimulating effect of these drugs on ACTH release were effectively inhibited, the plasma corticosterone concentration was always under 10.0 μg/100 ml, and the saline injected control level was 3.5±0.3 μg/100 ml; approximately the same level as that of hypophysectomized or adrenalectomized rats.

Table 3. Effects of various stressful stimuli on plasma corticosterone concentration in dexamethasone-Nembutal treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Cpd. B. μg/100 ml plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline 0.3 ml, i.v.</td>
<td>10</td>
<td>3.5±0.3*</td>
</tr>
<tr>
<td>Histamine 0.5 mg, i.v.</td>
<td>10</td>
<td>9.4±1.2</td>
</tr>
<tr>
<td>Serotonin 0.05 mg, i.v.</td>
<td>10</td>
<td>8.3±1.2</td>
</tr>
<tr>
<td>L-Epinephrine 0.03 mg, s.c.</td>
<td>10</td>
<td>5.9±0.6</td>
</tr>
<tr>
<td>Bilateral fracture of the femur</td>
<td>10</td>
<td>6.8±1.9</td>
</tr>
<tr>
<td>Laparotomy</td>
<td>10</td>
<td>15.4±2.3</td>
</tr>
</tbody>
</table>

* Mean±SE.
The blocking effect of dexamethasone was also determined on more stressful stimuli, i.e., a bilateral fracture of the femur or the standardized surgical procedure involving laparotomy. A midline incision approximately 3 cm in length, was made for inspection of the bilateral adrenal glands, and closed with silk sutures, which took about 1 min. The results shown in Table 3 indicate that the treatment with dexamethasone and Nembutal was capable of blocking the ACTH release considerably, even with such intense stimuli. It was found that the stimuli caused by surgical procedure of laparotomy was much more intense than that of histamine or epinephrine in this system.

(5) Effect of vasopressin

The antidiuretic hormone, vasopressin, is a potent stimulant on the hypothalamo-hypophysio-adrenal system in intact or Nembutal treated rats. Figure 4

![Bar chart](image_url)

Fig. 4. Effect of increasing dose of synthetic lysine vasopressin on the plasma corticosterone in rats treated with dexamethasone and Nembutal (filled column) and with Nembutal alone (blank column).
shows the results of vasopressin administration in Nembutal or dexamethasone-Nembutal treated rats. The data show that the stimulating effect of vasopressin upon ACTH release was inhibited by the treatment of 0.2 mg dexamethasone if the pressor activity was kept under 80 mU per rat. However, if the pressor activity reached 100 mU, the plasma corticosterone concentration was significantly raised. When rats were treated with Nembutal alone, a large increase in plasma corticosterone was observed even if a small amount of vasopressin (10 mU) was administered.

(6) Response of corticotrophin-releasing factor

Figure 5 shows a typical log dose response curve obtained with partially purified CRF. When treated with various doses of CRF, a linear log dose relationship was obtained between 0.04 to 2.2 µg of CRF and Cpd. B µg/100 ml response. Plasma corticosterone responses to 2.2 and 4.3 µg of CRF were 34 ± 2 and 35 ± 3 µg/100 ml, respectively; there was no statistically significant difference between them. In this experiment, maximum pressor activity injected, contained in the utmost dose of CRF (i.e. 4.3 µg), was 70 mU. In view of the finding (see Fig. 4) that 80 mU of vasopressin caused a negligible rise in the corticosterone concentration in plasma, the response obtained in the present experiment would be essentially due to CRF.

Fig. 5. Dose response curve of Corticotrophin-Releasing Factor in dexamethasone-Nembutal treated rats. Each point and vertical line represent the means of 10–15 animals and the standard errors.
DISCUSSION

It is well known that the level of corticosterone in rat plasma is markedly influenced by many environmental factors (e.g. Table 1) through a wide variety of receptors, especially the central nervous system (Guillemin et al., 1958; Barrett and Stockham, 1963).

Therefore, the following conditions are necessary for the assay method of CRF.

1) Corticotrophic effect due to environmental stress or the effect of various activating drugs on the pituitary-adrenal axis must be completely blocked.

2) Corticotrophic activity of vasopressin must be suppressed as much as possible.

3) A dose-response relationship must be obtained especially for CRF, and pituitary and adrenal responsiveness must not be diminished.

4) The assay method must be easy and simple.

The present paper describes the details of an assay method for CRF in the light of the above-mentioned criteria by the combined use of dexamethasone and Nembutal.

Dexamethasone, as reported by de Wied (1964) or Rerup (1964 a and b) had a strong action on the stress-induced ACTH release when the pharmacological doses were administrated subcutaneously or intraperitoneally. As dexamethasone was crystalline and not soluble in water, it was administered 16 to 24 hrs. before the experiment, suspended in oil. However, in the present study, dexamethasone of ester type, dexamethasone-21-phosphate disodium salt, was used because it is easily dissolved in water, and is about 8,000 times as soluble as the crystalline, and possesses a strong and immediate effect lasting for more than 24 hrs., as reported by Kendall (1962). In addition, the steroid dose not interfere with corticosterone determination in the blood though it is injected intravenously (Martini et al., 1962).

It was observed that the saline injected control level of plasma corticosterone in rats was reduced 3.5±0.3 μg/100 ml by treatment with 0.2 mg of dexamethasone and 5.0 mg Nembutal. This treatment was capable of suppressing the corticotrophic effect of vasopressin in doses less than 80 mU, as well as stressful stimuli, i.e., administration of pharmacological doses of histamine, epinephrine and serotonin or a bilateral fracture of the femur (Table 3). However, it was unsuccessful in the case of laparotomy, which produced a significant rise in plasma corticosterone level (15.4±2.3 μg/100 ml) that was completely suppressed by an additional intraperitoneal injection of 2.0 mg of Morphine 45 min. after Nembutal injection.* At any rate, treatment with dexamethasone and Nembutal seems to be useful enough in the usual assay method for CRF as a stress blocking agent.

A log dose response curve between CRF in μg and Cpd. B in μg/100 ml was obtained with acceptable linearity as shown in Figure 5, though the CRF used in the present study was still contaminated with a minute amount of vasopressin. It was also suggested that the peptide administered in this assay system was by no

* Lipscomb recommended the combined use of dexamethasone, morphine and Nembutal for the assay of CRF (personal communication 1965).
means vasopressin itself, but another peptide, CRF, in view of the results of lysine vasopressin administered in the same assay system as shown in Figure 4.

In the assay procedure for CRF, however, contamination by vasopressin is most troublesome. Since CRF seems to resemble vasopressin closely in the chromatographic separation owing to their similarity in chemical nature, it is difficult to distinguish CRF from vasopressin if a minute amount of vasopressin causes the plasma corticosterone concentration to rise. Therefore, it is desirable to develop a sensitive assay method for CRF which is not responsive to vasopressin. Guillemín et al. (1959a) showed evidence in morphine-Nembutal treated rats of inhibiting the corticotrophic activity of vasopressin at a dose less than 30 mU. Recently, Schally et al. (1965, in press) described a new assay method of CRF in Monase-Nembutal treated rats in which Monase and Nembutal inhibited the increase in corticosterone secretion in response to 5–15 mU of lysine vasopressin but not in response to 30 mU. In dexamethasone-Nembutal blocked rats, however, vasopressin failed, at a dose less than 80 mU pressor activity, to increase the plasma corticosterone concentration. Accordingly, when assaying CRF activity, it is suggested that the assay procedure must be performed after determination of pressor activity on the test materials and finally checked for ACTH in 24-hour hypophysectomized rats.

On the possible significance of vasopressin in the release of ACTH, a clear answer has not been found. Nichols (1961) produced considerable evidence indicating that vasopressin itself is the ACTH-releasing neurohumor. Hearn et al. (1961) reported that synthetic lysine vasopressin has a CRF-like activity. Furthermore, the dissociation between endogenous secretion of vasopressin and secretion of ACTH was demonstrated in hypothalamic lesioned animals with water loading (Nichols and Guillemín, 1959). Although the role of vasopressin in the release of ACTH is still controversial, the present study clearly demonstrates that ACTH release is controlled at least by CRF, which is certainly different from vasopressin.

On the other hand, Leeman et al. (1962) stated that corticosteroid pretreatment could not be safely used in the assay for ACTH-releasing neurohumor because determination of plasma corticosterone after Pitressin showed no difference between normal and corticoid blocked animals, whereas the responses to histamine or laparotomy were prevented by corticosteroid pretreatment. They explained that Pitressin acts at a site in the central nervous system through a mechanism different from that of most other stimuli.

However, the reports of Schally et al. (1958) and of this laboratory (Ishihara and Kakegawa, 1960; Ishihara, 1963; Ishihara and Nagamachi, 1964) indicate that Pitressin is not a pure vasopressin but contains a neurohumor as a contaminant. Pitressin is separated into about three components, each of which shown oxytocic, pressor and CRF activity, by means of paper chromatography in n-buthanol/acetic acid/water system. There is also dissociation of pressor effect on Urethane-Dibenamine treated rats between synthetic lysine-8 vasopressin (Sandoz) and Pitressin (Parke-Davis). That Pitressin is 1.2 to 2.0 times as synthetic vasopressin in pressor activity was recently observed in this laboratory. Therefore, such a comparison between two different preparations, synthetic and crude, seems to be insignificant.
Moreover, still controversial is the site of the blocking action of glucocorticoids on the hypothalamo-hypophysio-adrenal axis, although it is generally accepted that the release of ACTH can be suppressed by high level of corticosteroid in the blood. Some authors suggest the negative feed-back action on the central nervous system (Porter and Jones, 1956; McCann et al., 1958; Leeman et al., 1962; Yates and Urquhart, 1962; Davidson and Feldman, 1963; Fraschini et al., 1964), others present evidence on the anterior pituitary (Ganong and Hume, 1955; de Wied and Mirsky, 1959; Yoshida and Sayers, 1961; de Wied, 1964) and others suggest the adrenal cortex (Birmingham and Kurlents, 1958; Péron et al., 1960; Saffran and Vogt, 1960; Feket and Görgö, 1962). It is reasonable to suppose that the blocking action of dexamethasone is located in the central nervous system, probably in the hypothalamus, although it cannot be denied that the adrenal responsiveness to endogenous or exogenous ACTH is certainly lowered when large amount of dexamethasone is administered. Further studies are needed to solve reming problems.

ACKNOWLEDGEMENTS

The author would like to express his sincere thanks to Prof. K. Ishihara, Director of Surgery, Gunma University School of Medicine, and Prof. N. Ui and Dr. Y. Kondo, Institute of Endocrinology, Gunma University for their kind guidance and encouragement. And the author wishes to thank Miss S. Matsumura for her skilful technical assistance.

Posterior pituitary powder was generously supplied by Dr. S. Matsushima, Research Laboratory, Teikoku Hormone Mfg., Co., Ltd. Synthetic lysine-8 vasopressin was kindly provided by Drs. A. Francamps and W.v. Orelli, Sandoz Ltd., Basel, Switzerland, and Dibenamine HCl was a gift from Dr. F. Grant, Smith Kline & French Laboratory, Philadelphia, U.S.A.

Dexamethasone-21-phosphate disodium salt was supplied by Nippon Merck-Banyu Co., Ltd., Tokyo.

REFERENCES

Martini, L., A. Pecile, G. Giuliani, F. Frasconi and A. Carraro. Gewebs- und Neurohormon-