Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 103 MARCH, 1960 No. 3

SECTION MEETINGS

CLEVELAND, O.	
Western Reserve University	January 18, 1960
ILLINOIS	
University of Illinois Medical School	January 12, 1960
WISCONSIN	
University of Wisconsin	January 7, 1960

Purification of Hypothalamic Corticotropin Releasing Factor.* (25553)

PAUL C. ROYCE† AND GEORGE SAYERS
Dept. of Physiology, Western Reserve University School of Medicine, Cleveland, O.

Appropriate stimuli promptly increase rate of release of corticotropin from the adenohypophysis(1). The response is abolished by destruction of an area at the base of the hypothalamus which includes median eminence. The defect in an animal with such a lesion has been ascribed to loss of a chemical mediator, corticotropin releasing factor (CRF), which originates in the median eminence and is transported in the hypophyseal portal venous system to the adenohypophysis where it acts to accelerate release of corticotropin(2). The presence of corticotropin releasing activity in crude extracts of median eminence tissue lends support to this hypothesis(3). The present communication describes column chromatography of crude extracts which led to the preparation of fractions exhibiting neither pressor nor corticotropic activity at a dose which induces substantial adrenal ascorbic acid depletion in the median eminence lesioned rat.

Methods. Calf brains were dissected at the slaughterhouse.‡ The pituitary stalk was transected immediately dorsal to the diaphragm sella. A portion of tissue including both median eminence and pituitary stalk (SME) was separated from the brain and dropped into acetone (100 to 150 SMEs/2 liters of acetone). The acetone was decanted and replaced daily for 3 days. The dehydrated, defatted tissue was air dried and stored in tightly covered containers at 4°C. Crude extracts were prepared by refluxing the acetone dried tissue for 10 to 15 minutes in glacial acetic acid at 100°C. One ml of glacial acetic acid was used for each SME. The mixture was filtered and the filtrate (crude extract) stored at room temperature in glass stoppered flasks. CRF activity of crude ex-

* Aided by grant from Nat. Inst. of Health, U.S.P.H.S.

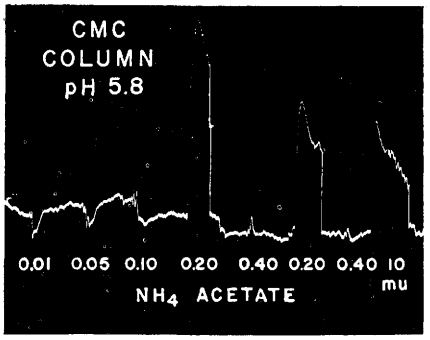
† Upjohn Postdoctoral Fellow 1958-1959.

‡ Drs. C. Graham and E. A. Lazo-Wasem (Wilson Labs., Chicago) arranged for and supervised tissue collections.

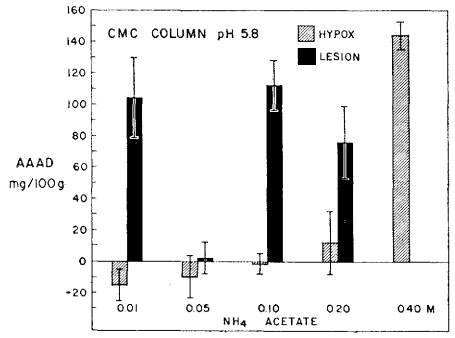
tracts was undiminished after 3 months of storage. As a prelude to chromatography, 1 volume of acetone and 2 volumes of ether were added to the crude extract. One to 12 hours later the precipitate was separated by low speed centrifugation and washed 4 times with a volume of ether equal to original volume of crude extract. The ether washed, acetone-ether precipitate was extracted with 0.01 M ammonium acetate buffer (pH 5.8) (see below for volume). The buffer extract centrifuged at 10,000 x gravity for 10 minutes, and the clear supernatant applied to a carboxymethylcellulose (CMC) column. CMC was prepared by the method of Peterson and Sober(4) as modified by Ellis and Simpson (5). It was equilibrated with 0.01 M ammonium acetate buffer (pH 5.8) and the fines removed by repeated decantation. The CMC was loaded into a 0.9 x 30 cm chromatography tube to a height of 10 cm and washed extensively with 0.01 M ammonium acetate buffer (pH 5.8). In Exp. W-3, 100 ml of crude extract, prepared from 100 SMEs, were processed for chromatography. Ether washed precipitate was extracted 4 times with 1 ml of buffer and 3 ml of buffer extract were applied to the column. In Exp. W-4, 60 ml of crude extract, prepared from 60 SMEs, were processed for chromatography. The ether washed precipitate was extracted 4 times with 3 ml of buffer and 9 ml of buffer extract were applied to the column. In both experiments, effluents were collected at 8 to 10 ml/hour. When the level of buffer extract reached the top surface of CMC, 10 ml of 0.01 M buffer were added. The effluent from this buffer wash was combined with the effluent from the buffer extract. Discontinuous gradient elution was then begun. The column was washed successively with 15 ml each of 0.05, 0.1, 0.2, and 0.4 M ammonium acetate buffer and the effluent of each successive buffer was collected separately. pH of all buffers was 5.8. As a precaution against deterioration of CRF, collection flasks contained sufficient glacial acetic acid so that final concentration of acetic acid in each was 3%. Acetone-ether precipitation and subsequent steps were carried out at 4°C. Assays for CRF activity were performed in median eminence lesioned rats (250 to 300 g) (2). Rats with urine volumes greater than 75 ml in the first 24 hours after lesioning were used for assay. Forty-eight hours post-operatively the left adrenal was removed and material to be assayed infused intravenously over 30 seconds. Thirty minutes later the right adrenal was removed. The difference between concentrations of adrenal ascorbic acid in left and right adrenals is an index of corticotropic action during period between removal of the 2 glands. The response may represent corticotropin release and/or corticotropin injected. Corticotropic activity of extracts was determined by assay in 24-hour hypophysectomized rats (110 to 135 g)(6). Vasopressin content was estimated by pressor response of rats pretreated with phenoxybenzamine (U.S.P. XV method). The dose of each fraction used in the 3 assay systems was equivalent to 0.3 SME.

Results. Fig. 1 illustrates blood pressure response to the fractions of Exp. W-4. No detectable pressor activity was found in material emerging with 0.01, 0.05 or 0.1 M buffer. The bulk of pressor activity emerged with the 0.2 M buffer but a small pressor response was elicited by the 0.4 M fraction. Blood pressure response to 10 milliunits Pitressin (Parke, Davis) is included for comparative purposes. Distribution of pressor activity was the same in Exp. W-3.

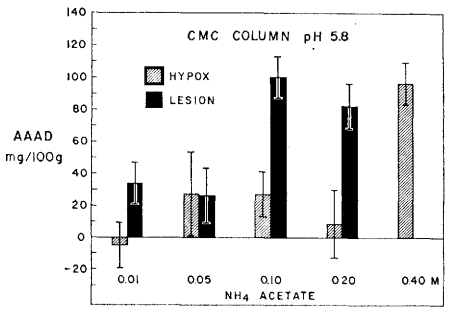
In both experiments W-3 (Fig. 2) and W-4 (Fig. 3), corticotropic activity as measured in the hypophysectomized rat was found exclusively in the 0.4 M fraction. Because of high corticotropic activity of the buffer extract and of the 0.4 M fraction, they were not assayed for CRF activity in the lesioned rat. The 0.1 M fraction which had no detectable pressor or corticotropic activity at a dose of 0.3 SME displayed CRF activity at this dose in the lesioned rat. The protein concentration of the 0.1 M fraction was 15 μg/SME for W-3 and 13 μg SME for W-4. In both experiments the 0.05 M fraction was inactive in the lesioned rat, but significant CRF activity was found in 0.2 and 0.01 M fractions. The presence of CRF activity in the 0.2 M fraction probably results in part from the vasopressin in this fraction and in part from an



1



2



3

FIG. 1. Kymograph record of blood pressure response of rat after administration of fractions of median eminence (SME) extract W-4 eluted from carboxymethylcellulose column with ammonium acetate buffers of increasing molarity. Dose of each fraction equivalent to 0.3 SME. Response to 10 milliunits of Pitressin is shown at extreme right.

FIG. 2. Adrenal ascorbic acid depletion in median eminence lesioned and in hypophysectomized rats after administration of various fractions of hypothalamic (SME) extract W-3. Fractions are designated by molarity of ammonium acetate buffer with which they were eluted from carboxymethylcellulose column. Height of bar indicates avg response of 6 animals to 0.3 SME. Stand. error shown by vertical lines.

FIG. 3. Responses to extract W-4. Same conditions as for W-3 (Fig. 2) except height of bar indicates avg response to 5 or more animals.

overlap of CRF of the 0.1 M fraction into the 0.2 M fraction.

One explanation for presence of corticotropin-releasing activity in the 0.01 M and in the 0.10 M fractions is the existence of 2 chemically distinct CRFs in SME tissue. An alternate explanation is that the fractionation technic is imperfect and the CRF in the 0.01 M fraction is identical with that in the 0.1 M fraction. The much greater CRF activity of the 0.01 M fraction of Exp. W-3 as compared to that of the 0.01 M fraction of Exp. W-4 may be related to differences in concentration of the ether washed precipitate in the buffer extract (W-3, 25 SMEs/ml; W-4, 5 SMEs/ml). No signs of toxicity were observed in the lesioned rat following rapid infusion (30 seconds) of 0.3 SME of the 0.1 M fraction.

Discussion. Our previous report indicated that calf SME tissue contains at least 3 components capable of activating the adrenals of the median eminence lesioned rat(3). One appeared to be corticotropin, another, vasopressin. Presence of an additional active factor was inferred from the following evidence. (1) At appropriate dilutions, activity of crude SME extracts in the lesioned rat was greater than could be accounted for by their corticotropic and/or by their pressor activities. (2) Pepsin treatment of crude SME extracts reduced adrenal stimulating activity in the lesioned rat but did not diminish corticotropic or pressor activity. It was postulated that the pepsin-labile component acted directly on the adenohypophysis to accelerate the release of corticotropin. Biological characterization of this CRF was limited by presence of corticotropin and vasopressin, as well as by its toxicity. The corticotropin activity most likely is due to contamination with adenohypophyseal tissue since it is possible with careful dissection to obtain material high in corticotropin releasing activity but with negligible corticotropic activity. Preparation of CRF by the method described in this report provides non-toxic material which contains non-detectable quantities of pressor and corticotropic activities and which is suitable for further biological investigation. Elucidation of the relationship between the CRF of SME origin

and that reported in portal vein blood(7,8) and in neurohypophyseal tissue(9) must await isolation of active material in homogeneous form from each of these three sources.

Summary. Fractionation of a crude extract of calf median eminence on carboxymethylcellulose columns yields a preparation which induces adrenal ascorbic acid depletion in the median eminence lesioned rat. This partially purified corticotropin releasing factor (CRF) is substantially free from corticotropic and pressor activities.

1. Royce, P. C., Sayers, G., *Endocrinology,* 1958, v63, 794.

2. McCann, S. M., *ibid.,* 1957, v60, 664.

3. Royce, P. C., Sayers, G., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 677.

4. Peterson, E. A., Sober, H. A., *J. Am. Chem. Soc.,* 1956, v78, 751.

5. Ellis, S., Simpson, M. E., *J. Biol. Chem.,* 1956, v220, 939.

6. Sayers, M. A., Sayers, G., Woodbury, L. A., *Endocrinology,* 1948, v42, 379.

7. Rumsfeld, H. W., Porter, J. C., *ibid.,* 1959, v64, 942.

8. Porter, J. C., Rumsfeld, H. W., *ibid.,* 1959, v64, 948.

9. Schally, A. V., Guillemin, R., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 138.

Received December 28, 1959. P.S.E.B.M., 1960, v103.

Evaluation of Type and Degree of Change in Postexercise Electrocardiogram in Detecting Coronary Artery Disease. (25554)

GEORGE P. ROBB AND HERBERT H. MARKS (Introduced by E. F. Beach)
Metropolitan Life Insurance Co., N. Y. City

The value of electrocardiographic double standard 2-step exercise test(1) in detection of occult coronary disease has been proved (2,3,4). The criteria for abnormal response to this exercise test indicative of coronary insufficiency, however, have not been adequately tested or defined. Investigation of validity of the original criteria of a positive or abnormal test by a clinical-statistical follow-up study was started in 1952 and completed in 1956 (4). Incidence of coronary occlusion and coronary death subsequent to the test were determined in a large series of military personnel and insurance applicants suspected of having coronary disease who had been given the double exercise test. The results indicated that the only valid evidence of coronary insufficiency in postexercise electrocardiogram was ischemic ST segment depression. The significance of ST junction depression remained undetermined because, through technical fault in the series on military personnel, unrecognized ST segment depression cases were included in ST junction category. Isolated T-wave change after exercise appeared normal. The present report gives new data regarding significance of (1) various types of ST junction depression after exercise; (2) various types and degrees of ischemic ST segment depression; (3) additional data regarding negative reactors and (4) QS/QT ratio in various types of response to the test(5).

Materials and methods. This is a follow-up study of 922 insurance applicants, most of whom were suspected of having coronary disease and who were given the double standard 2-step exercise test between 1949 and March 1958, and traced to March 1959. Indications for the test, its performance, and electro-cardiographic technic, were described previously(4). The post-exercise electro-cardiograms were analyzed with the P-R segment as the point of reference for measuring ST junction and ST segment depression(1). The term "ischemic" denotes horizontal or sagging depression of the ST segment(6). The changes induced by exercise were classified as in Chart 1: 1. *ST junction depression, any degree.* Types: (a) transitory, during tachycardia; (b) prolonged; (c) "near-ischemic" configuration; *i.e.,* ST segment straight and nearly horizon-