Purification of Growth Hormone-Releasing Factor from Beef Hypothalamus.* (31260)

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The existence of a hypothalamic growth hormone releasing factor (GRF) has been established(1-6).

In vitro and *in vivo* studies in our laboratory demonstrated GRF in extracts of beef and pork hypothalami(3,5) and indicated that GRF activity appeared to be due to a polypeptide with a molecular weight of 2500. The present study describes the concentration of GRF activity from extracts of beef pituitary stalk-median eminence region of the infundibulum.

Materials and methods. Bioassays. Pressor and oxytocic assays were performed by the Dekanski(7) and Thompson(8) methods, respectively. Melanophoretic activity was measured by the *in vitro* assay of Shizume *et al* (9).

Test for effects of the extracts (GRF activity) on pituitary growth hormone (GH) content. The extracts were injected into carotid arteries of normal male rats and 15 minutes later pituitaries were removed(4,5) for measurement of the depletion of GH content.

Growth hormone assay. GH content of pooled pituitaries from each experimental group (5-7 rats) was measured by the "tibia test" method of Greenspan *et al*(10). Female Sprague-Dawley rats, hypophysectomized when 26 to 28 days of age, were obtained from Hormone Assay Labs., Chicago, Ill. Twelve to 14 days after hypophysectomy they were used for assay. One-half ml of the test material was injected intraperitoneally daily for 4 days. On the 5th day, the rats were sacrificed and their right tibias prepared for measurement of epiphyseal cartilage width. Six to 8 rats were used to assay each sample. The standard preparation of GH used in these experiments was NIH-GH-S5-Ovine. The significance of the differences between groups was determined by Student's t-test or factorial analyses.

Extractions. Fragments of beef hypothalamic tissue made up mainly of the pituitary stalk and the median eminence area (SME), were lyophilized. The details of this extraction procedure have been reported(11). GRF activity in lyophilized acetic acid extracts was concentrated by repeated extraction with glacial acetic acid. The glacial acetic acid extracts were diluted with water and lyophilized.

Gel filtration on Sephadex. Molecular sieving on columns of Sephadex G-25 was performed as described previously(11,12). Peptide concentration was followed by the Folin-Lowry method(13).

Results. Previous tests established that 2 N acetic acid extract of beef hypothalamus was active in depleting rat pituitaries of growth hormone. The extracts were subsequently purified on Sephadex.

For gel filtration on Sephadex, 4 g aliquots of lyophilized glacial acetic acid extract of bovine SME were extracted with 40 ml 0.1 M pyridine acetate buffer, pH 5 and then centrifuged at 15,000 rpm for 1 hour. The residue was reextracted twice with 15 ml buffer. The extracts were applied successively on a column of Sephadex G-25. Fig. 1 shows the chromatographic pattern and biological activities obtained. There was a separation of the extracts into several peaks distinct biologically and chemically. The first peak near the exclusion (void) volume of the column contained substances with molecular weight larger than 5000. Two MSH areas (R_f = 0.63 and R_f = 0.50) corresponded to β- and α-MSH, respectively. Arginine vasopressin gave an R_f of 0.37, in agreement with results reported previously.

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TABLE I. GRF Activity (Effect on Pituitary GH Content) of Fractions from Acetic Acid Extracts of Beef Hypothalamus Purified on Sephadex.

Fraction No.	Dose/test rat, μg (dry wt)	AP* equivalents assay rat/4 days, mg (total dose)	Tibia width, μ ± S.E.†	p *vs* control
Control	—	1	201 ± 6.3	
33–43	1280	1	199 ± 8.5	NS
44–49	1300	1	179 ± 5.5	.05
50–57	1300	1	169 ± 8.1	.02
58–66	1050	1	198 ± 7.4	NS
67–71	1250	1	208 ± 9.3	NS
Saline	—	—	134 ± 1.8	—
GH 20 μg‡	—	—	177 ± 10.0	—
GH 80 μg‡	—	—	206 ± 5.5	—
Control	—	1	186 ± 7.7	
44– 49	460	1	158 ± 6.1	.05
50– 57	400	1	158 ± 8.8	.05
72– 84	1300	1	165 ± 7.3	NS
85– 94	840	1	188 ± 3.7	NS
95–105	840	1	185 ± 7.6	NS
106–134	650	1	192 ± 13.5	NS
Saline	—	—	137 ± 6.3	—
GH 20 μg‡	—	—	157 ± 5.9	—
GH 80 μg‡	—	—	210 ± 12.8	—
Control	—	1	167 ± 8.3	
72–84	600	1	170 ± 11.0	NS
Control	—	1	206 ± 8.6	
50–57	16	1	164 ± 6.5	.05
Control	—	1	216 ± 9.5	
50–57	1	1	175 ± 7.9	.05
Saline	—	—	145 ± 4.1	—
GH 20 μg‡	—	—	185 ± 10.3	—
GH 80 μg‡	—	—	251 ± 15.3	—

* Anterior pituitary. † 5-6 animals per group. ‡ GH = growth hormone standard.

The data in Table I show that only fractions #44-57 were active in depleting the pituitary growth hormone content of rats. This Table also shows that the purified material was active at a dose of 1 μg dry weight. The same fractions were active in releasing growth hormone from anterior pituitaries *in vitro*(3, 14). The weight of material in area 44-57 from 20 columns was 1.2 g as compared to the total weight applied of 80 g.

On the basis of elution from Sephadex the molecular weight of GRF appeared to be about 2500.

Discussion. Though Reichlin(15) and Bogdanove(16) showed that the hypothalamus may exert some control over growth hormone secretion, direct evidence of this control was lacking until Franz *et al*(1) showed that pork hypothalamic extracts stimulated release of growth hormone *in vitro*.

In vitro studies by Deuben and Meites(2) and Schally *et al*(3) provided even more convincing evidence of hypothalamic control of growth hormone secretion.

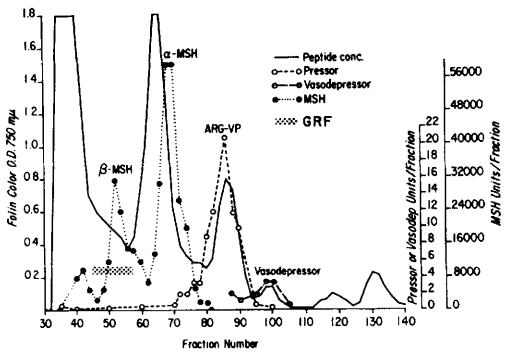

FIG. 1. Gel filtration of beef SME extracts (4 g material corresponding to about 250 beef SME) on a column of Sephadex G-25 (4.3 × 160 cm) in 0.1 M pyridine acetate, pH 5.0. Fraction size 25 ml; 50 μl aliquots taken for Folin-Lowry analyses.

The demonstration by Pecile *et al*(4) that rat hypothalamic extracts deplete rat pituitary of growth hormone provided the necessary *in vivo* test for GRF activity (as well as confirmation of the *in vitro* results). Ishida *et al*(5) showed that in rats, pig and beef hypothalamic extracts also deplete the pituitary of growth hormone. They also showed that brain cortex extract, vasopressin, oxytocin, and α-MSH were inactive in this test.

Both by molecular sieving on Sephadex, as well as by biological tests on purified fractions, our studies show that GRF activity is distinct from follicle-stimulating hormone-releasing factor (FSH-RF), luteinizing hormone-releasing factor (LRF), corticotropin-releasing factor (CRF) and thyrotropin-releasing factor (TRF)(14).

Summary. Potent preparations of growth hormone-releasing factor (GRF) were prepared from bovine hypothalamic extracts, by the glacial acetic acid concentration procedure followed by gel filtration on Sephadex. Purified GRF is distinct from vasopressin, oxytocin, α-MSH and other hypothalamic-releasing factors.

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1. Franz, J., Haselbach, C. H., Libert, O., Acta Endoc. (Kobenhaven), 1962, v41, 336.

2. Deuben, R., Meites, J., Endocrinology, 1964, v74, 408.

3. Schally, A. V., Steelman, S. L., Bowers, C. Y., Proc. Soc. Exp. Biol. & Med., 1965, v119, 208.

4. Pecile, A., Müller, E., Falconi, G., Martini, L., Endocrinology, 1965, v77, 741.

5. Ishida, Y., Kuroshima, A., Bowers, C. Y., Schally, A. V., ibid., 1965, v77, 759.

6. Krulich, L., Dhariwal, A. P. S., McCann, S. M., Proc. Soc. Exp. Biol. & Med., 1965, v120, 180.

7. Dekanski, J., Brit. J. Pharmacol., 1952, v7, 567.

8. Thompson, R. E., J. Pharmacol. Exp. Ther., 1944, v80, 373.

9. Shizume, K., Lerner, A. B., Fitzpatrick, T. B., Endocrinology, 1954, v54, 553.

10. Greenspan, F. S., Li, C. H., Simpson, M. E., Evans, H. H., ibid., 1949, v54, 455.

11. Schally, A. V., Bowers, C. Y., ibid., 1964, v75, 608.

12. Porath, J., Schally, A. V., ibid., 1962, v70, 738.

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.

14. Schally, A. V., Bowers, C. Y., Kuroshima, A., Ishida, Y., Redding, T. W., Kastin, A. J., Proc. 23rd Int. Congress of Physiol. Sciences, Tokyo, Excerpta Med., 1965, Series 87, 183.

15. Reichlin, S., Endocrinology, 1960, v67, 760.

16. Bogdanove, E., Lipner, H. J., Proc. Soc. Exp. Biol. & Med., 1952, v81, 410.

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Chronotropic Action of Glucagon on the Sinus Node.* (31261)

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Glucagon, a polypeptide hormone with a molecular weight of 3500, is secreted by the alpha cells of the islets of Langerhans. The liver is the major site of action, where glucagon accelerates hepatic glycogenolysis to produce an increase in peripheral blood sugar. Unger and co-workers have noted falling levels of blood glucose lead to an increased concentration of glucagon in portal and peripheral venous blood(1,2). Other effects of glucagon include inhibition of gastric motility, an antiphlogistic effect, an increase in urinary nitrogen and plasma free fatty acids, and a decrease in plasma amino acids. Regan *et al* have demonstrated an atrioventricular nodal tachycardia following injection of glucagon into the left coronary artery of dogs(3).

Because the results of Regan suggest a positive chronotropic action, we have studied the effect of glucagon by direct perfusion of the canine sinus node through the nutrient artery *in vivo*.

Materials and methods. Mongrel dogs

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