Enzymatic Synthesis of Growth Hormone Releasing Factor (GH-RF) by Rat Incubates and by Extracts of Rat and Porcine Hypothalamic Tissue[1] (37054)

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In previous papers from this laboratory we have described the enzymatic formation of thyrotropin releasing hormone (TRH) from labeled precursor amino acids by rat hypothalamic fragments *in vitro* (1–4). Using bioassay methods it has also been shown that extracts of rat and porcine hypothalamic tissue synthesize TRH (3, 4), luteinizing hormone releasing factor (LRF) (4, 5) and prolactin releasing factor (PRF) (6) from amino acids and cofactors. Based on these findings, it has seemed reasonable to postulate that growth hormone releasing factor (GH-RF) is also formed enzymatically and that its biosynthesis might also be demonstrated by bioassay.

In this paper, we report our findings on the biosynthesis of growth hormone releasing factor (GH-RF). A large body of evidence supports the existence of a growth hormone releasing factor of hypothalamic origin (7–11). In work carried out in our laboratory, a bioassay system has been developed for GH-RF which is demonstrated by assay in estrogen–progesterone–reserpinized male rats utilizing as measure of activity an increment in plasma radioimmunoassayable growth hormone levels (12). By means of this bioassay we have been able to demonstrate that rat hypothalamic fragments synthesize GH-RF as do extracts of rat and porcine hypothalamic tissue in the presence of amino acids and cofactors. This synthesis is not blocked by high concentrations of puromycin.

Methods. Whole tissue incubation. Hypothalamic fragments from male rats were removed in three sections and were incubated in Krebs–Ringer bicarbonate buffer under an atmosphere of 95% oxygen and 5% carbon dioxide as previously reported (1). Hypothalami were incubated in pairs. At the end of the incubation, the tissues were homogenized in the media, and bioassayed in a dosage equivalent to five hypothalamic fragments per assay animal. In other incubations, carried out simultaneously, puromycin was added to the media in a concentration of 200 μg/ml and, for control purposes, hypothalamic tissue (similarly dissected) was frozen until assay.

Extract incubation. Freeze-dried rat hypothalamic tissue (National Pituitary Distribution Committee) weighing 4.65 mg/rat hypothalamic fragment or freeze-dried porcine hypothalamic fragments (Oscar Mayer, Madison, WI) weighing 25–30 mg/fragment were homogenized in 0.01 *M* phosphate buffer, pH 7.4. The amount of tissue and buffer was chosen to give a final concentration of 15–20 fragments/ml for rat and 2–5 fragments/ml for porcine tissue. The volume of the incubation mixture was chosen so as to provide a sufficient amount of material for bioassay.

The preparation of the extracts for incubation was reported previously in detail (5). Extract incubated in the presence of 20 amino acids, each in a final concentration of 10^{-3} *M*, and ATP and Mg^{2+}, each in a final concentration of 10^{-4} *M*, was compared to control incubations which contained the hypothalamic tissue but in which the amino acids and cofactors were replaced by buffer. The following amino acids were added: alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, iso-

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TABLE I. Biosynthesis of Growth Hormone Releasing Factor by Rat Hypothalamic Fragments Incubated *in Vitro*.[a]

Group	No. of assay animals	Change in plasma GH[b] (ng/ml ± SE)	Significance of difference vs frozen control (p value)
Frozen (control)[c]	4	0.12 ± 0.18	
Incubated	4	7.5 ± 4.4	<.05
Incubated + puromycin[d]	4	12.17 ± 7.84	<.05

[a] Male rat hypothalami, dissected in three sections, incubated in Krebs-Ringer bicarbonate buffer for 1.5 hr.

[b] Change in plasma radioimmunoassayable growth hormone 10 min after injection of test substance. Assay rats are EP-reserpinized male rats assayed under pentobarbital anesthesia. Each assay animal received the equivalent of 5 hypothalamic fragments.

[c] Hypothalamus was removed and frozen immediately.

[d] 200 μg/ml of puromycin.

leucine, leucine, lysine, methionine, phenylalanine, proline, pyroglutamic acid, serine, threonine, tryptophan, tyrosine, and valine. Incubations were carried out for 1 hr unless otherwise specified with shaking under a controlled atmosphere (3, 5). The reaction was stopped by the addition of methanol as previously reported (5). Extracts were bioassayed in concentrations of 2 porcine fragments/0.5 ml of saline or 20 rat fragments/0.5 ml saline in assay animals.

Bioassay of GH-RF. Test specimens were injected into the external jugular vein of male rats (250–300 g) prepared for assay by sc treatment daily for 3 days with estradiol benzoate, 50 μg, and progesterone, 25 mg. One day prior to assay, animals received 1 mg/kg of reserpine ip and on the day of as-

TABLE II. Enzymatic Synthesis of GH-RF by Porcine Hypothalamic Extract Incubated in the Presence of Amino Acids and Cofactors for 1 hr.

Group	No. of assay animals	Change in plasma GH[a] (ng/ml ± SE)	Significance of difference vs extract control (p value)
Expt I[b]			
Extract control[c]	4	4.4 ± 1.40	
Extract + amino acids[d] + cofactors[e]	4	21.3 ± 6.3	<.001
Expt II[f]			
Extract control[c]	5	−2.9 ± 2.9	
Extract + amino acids[d] + cofactors[e]	5	5.9 ± 2.4	<.05
Buffer + amino acids[d] + cofactors[e]	5	−0.9 ± 0.4	NS

[a] Increment in plasma GH in EP-reserpinized male rats 10 min after iv injection of test substance under pentobarbital anesthesia.

[b] Extract consisted of 16 porcine hypothalami in 8 ml of buffer. Each assay animal received the equivalent of 2 porcine hypothalami.

[c] Extract incubated alone.

[d] 10^{-3} M each of: alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, pyroglutamic acid, serine, threonine, tryptophan, tyrosine, and valine.

[e] 10^{-4} M of ATP and 10^{-4} M of Mg^{2+}.

[f] Extract consisted of 30 porcine hypothalami in 16 ml of buffer. Each assay animal received the equivalent of 2 porcine hypothalami.

TABLE III. Enzymatic Synthesis of GH-RF by Rat Hypothalamic Extract Incubated in the Presence of Amino Acids and Cofactors for 0.5 and 1.5 hr.[a]

Group	No. of assay animals	Increment in Plasma GH[b] (ng ± SE)	Significance of difference vs extract control (p value)
Incubation, 0.5 hr			
Extract control[c]	6	−0.2 ± 0.98	
Extract + amino acids[d] + cofactors[e]	6	6.0 ± 1.12	<.001
Incubation, 1.5 hr			
Extract control[c]	6	0.4 ± 0.66	
Extract + amino acids[d] + cofactors[e]	6	9.1 ± 3.37	<.001

[a] Extract consisted of 480 freeze-dried rat hypothalami in 24 ml of buffer. Equivalent of 20 hypothalami injected into assay animals.

[b] Increment in plasma radioimmunoassayable GH in EP-reserpinized male rats under pentobarbital anesthesia 10 min after injection of test substance.

[c] Dialyzed extract incubated alone.

[d] 10^{-3} M each of: alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, pyroglutamic acid, serine, threonine, tryptophan, tyrosine, and valine.

[e] 10^{-4} M of ATP and 10^{-4} M of Mg^{2+}.

say, 50 mg/kg of pentobarbital. Plasma samples were removed before and 10 min after injection of hypothalamic incubate extract, were centrifuged in the cold and kept frozen for subsequent radioimmunoassay of GH by the method of Schalch and Reichlin (13) using the National Pituitary Agency Kit modified by the use of the coated charcoal method of Gottlieb *et al.* (14) to separate bound from free hormone.

Results. Biosynthesis of growth hormone releasing factor by rat hypothalamic fragments incubated in vitro. GH-RF activity of hypothalami incubated in buffer was significantly greater when compared to the GH-RF activity of frozen tissue used as controls. Even in the presence of puromycin, the tissues were capable of forming GH-RF in amounts significantly different from controls (Table I).

Synthesis of growth hormone releasing factor by extracts of pig and rat hypothalamic tissue. Dialyzed porcine hypothalamic extract incubated in the absence of amino acids and cofactors for 1 hr had no effect on plasma growth hormone levels in assay rats. In the presence of amino acids, ATP and Mg^{2+}, however, significant amounts of bioassayable GH-RF were formed. Amino acids and cofactors in the absence of hypothalamic extract had no effect on GH release (Table II).

Rat hypothalamic extract incubated for 0.5 and 1.5 hr also yielded GH-RF activity when incubated in the presence of amino acids, ATP and Mg^{2+} (Table III).

Formation of growth hormone releasing factor by ribosome-free extract of rat hypothalamic tissue. When incubated with 20 amino acids and ATP, rat hypothalamic extracts prepared by centrifugation for 20 hr at 100,000g evolved significant amounts of GH-RF as indicated by a significant increase in plasma GH in recipient assay animals (Table IV). Omission of ATP did not prevent the formation of significant amounts of biologically active material. Although the mean effect is less than the group incubated with ATP, the difference is not significant. On the other hand, hypothalamic extract incubated in the absence of amino acids (with or without ATP) had no effect on plasma GH levels.

Discussion. Previous work from our laboratory has demonstrated the synthesis of thyrotropin releasing hormone (TRH) (3), luteinizing hormone releasing factor (LRF) (5) and prolactin releasing factor (PRF) (6) by rat hypothalamic fragments and by rat and porcine hypothalamic extracts when incubat-

TABLE IV. Enzymatic Synthesis of GH-RF by a Ribosome-Free Extract of Rat Hypothalamic Tissue Incubated for 1 hr in the Presence of Amino Acids and Cofactors.[a]

Group	No. of assay animals	Change in plasma GH[b] (ng/ml ± SE)	Significance of difference vs extract control (p value)
Extract control[c]	4	1.6 ± 1.1	
Extract + amino acids[d] + ATP[e]	4	13.8 ± 7.3	$<.02$
Extract + amino acids[d] — ATP	4	9.3 ± 2.0	$<.01$
Extract — amino acids[d] + ATP[e]	4	−4.9 ± 1.7	$<.01$

[a] Ribosome-free extract consisted of 320 rat hypothalami in 16 ml of buffer centrifuged for 20 hr at 100,000g; 20 rat hypothalami/0.5 ml injected into assay animals.

[b] Increment in plasma radioimmunoassayable GH, 10 min after iv injection in EP-reserpinized male rats under pentobarbital anesthesia.

[c] Dialyzed extract incubated alone.

[d] 10^{-3} M each of: alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, pyroglutamic acid, serine, threonine, tryptophan, tyrosine, and valine.

[e] 10^{-4} M of ATP and 10^{-4} M of Mg^{2+}.

ed in the presence of amino acids and cofactors.

In this experiment we have demonstrated that isolated rat hypothalamic fragments form GH-RF and that puromycin has no effect on the synthesis. The inference that ribosomes are not responsible for synthesis is further borne out by the finding that extracts of rat and porcine hypothalamic tissue form GH-RF *de novo* in the presence of amino acids and cofactors as determined by bioassay responses. Particulate-free extracts of rat hypothalamic tissue also are capable of forming GH-RF. Although it is known that amino acids and certain buffers (11) induce growth hormone release, these factors may be excluded on the basis of the ineffectiveness of amino acids dissolved in buffer to stimulate growth hormone release in this assay preparation. Vasopressin may also be excluded as a possibility on the basis of the work of Sachs *et al.* (15), who have demonstrated the ribosomal formation of vasopressin. From the above observations it is concluded that GH-RF, like the other hypophysiotropic hormones, is synthesized enzymatically by a non-ribosomal mechanism. Our results thus far do not demonstrate ATP dependence unequivocally, possibly because of the inherently greater variability of the bioassay used to detect GH-RF activity than the bioassays used previously to measure TRH, CRF and PRF. In the latter systems strict ATP dependence has been shown which has led us to conclude that specific ATP–dependent synthetases are involved in the biosynthesis of these releasing factors.

Summary. Incubations of rat hypothalamic fragments and of extracts of rat and porcine hypothalamic tissue found previously to synthesize thyrotropin releasing hormone (TRH), luteinizing hormone releasing factor (LRF) and prolactin releasing factor (PRF) have been used to study the biosynthesis of growth hormone releasing factor (GH-RF). To demonstrate synthesis, GH-RF was measured by the elevation of radioimmunoassayable growth hormone in the estrogen–progesterone–reserpinized male rat. GH-RF is synthesized by rat hypothalamic fragments incubated in Krebs–Ringer bicarbonate buffer under an atmosphere of 95% oxygen and 5% carbon dioxide and by extracts of rat and porcine hypothalamic tissue in the presence of amino acids, ATP and Mg^{2+}. This synthesis is not blocked by puromycin, indicat-

ing that the synthesizing system may not be ribosomal dependent. Further proof of a non-ribosomal mechanism comes from the fact that a ribosomal-free extract also synthesizes GH-RF. From these observations, it may be concluded that GH-RF is also formed enzymatically by a non-ribosomal mechanism.

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