STUDIES ON THE CONTROL OF GROWTH HORMONE SECRETION IN THE RAT

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ABSTRACT

By Elias Dickerman

I. IN VITRO ASSAY FOR GROWTH HORMONE RELEASING FACTOR (GH-RF)

A four hour incubation method was developed for assaying rat hypothalamic growth hormone releasing factor (GH-RF). Anterior pituitaries from donor rats were hemisected and distributed randomly into flasks containing 4 ml of protein-free medium 199. Six pituitaries (12 halves) were used per flask. The pituitaries were pre-incubated for 1 hour, the medium was discarded and fresh medium containing neutralized acid extract of rat hypothalamus or cerebrum was added. These were incubated for 4 hours under constant gassing with 95% O_2-5% CO_2, and the medium was analyzed for GH by the standard tibia test.

A log-dose relationship was demonstrated when increasing amounts of hypothalamic extract were incubated with rat pituitary. Graded doses of cerebral extract failed to demonstrate such a relationship, indicating that GH-RF exists as a specific hormone in the hypothalamus. Analysis of pituitary

incubation medium at two dose levels showed log-dose relationships in all cases, indicating that the hormone assayed in the medium was GH. This method is suitable for measuring quantitative changes in hypothalamic content of GH-RF.

II. EFFECTS OF STARVATION ON PLASMA GH ACTIVITY, PITUITARY GH, AND HYPOTHALAMIC GH-RF LEVELS IN THE RAT

The effects of complete food removal for 7 days were measured on plasma GH activity, hypothalamic content of growth hormone releasing factor (GH-RF) and pituitary concentration of growth hormone (GH) in male rats. Control male rats were starved or fed ad libitum for 7 days. In another experiment, the starved rats received replacement doses of L-Na-thyroxine (2.5 ug/100 g body weight/day) beginning on day 2 of starvation. On day 8 all rats were weighed, anesthetized with ether and bled from the abdominal aorta. The pituitaries were removed and individually weighed; the hypothalami were placed in ice cold 0.1N HCl (0.1 ml/hypothalamus). Hypothalamic GH-RF was assayed by the in vitro incubation method described above. Lyophylized plasma, anterior pituitaries, and incubation medium were assayed for GH by the standard tibia test, using a 4 point assay procedure. Statistical analysis of the data showed a significantly lower plasma GH content in starved or

thyroxine-treated starved rats ($P<0.01$). Pituitary GH content and hypothalamic content of GH-RF were also significantly reduced in the starved rats. These results indicate that starvation in the rat not only lowers the levels of GH-RF in the hypothalamus and of GH in the pituitary, as shown previously, but also leads to a decrease in plasma GH activity.

STUDIES ON THE CONTROL OF GROWTH HORMONE SECRETION IN THE RAT

By

Elias Dickerman

A THESIS

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Dedicated

to

my Family

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INTRODUCTION

The nervous and endocrine systems are responsible for the integration and coordination of the remaining systems of the body. The relationship between these two controlling systems is close and intricate, and only in the last 10-15 years has information clarified this relationship.

It is now known that neuroendocrine mechanisms regulate a wide variety of body functions. The brain is responsible for control of secretions from the anterior and posterior pituitary as well as from the intermediate lobe of the pituitary. Through hypothalamic control of anterior pituitary secretion, the brain regulates thyroid, adrenocortical and gonadal secretions as well as body growth. The control of anterior pituitary secretions is exerted through releasing and inhibiting factors (hormones) in the hypothalamus: corticotropin releasing factor (CRF), thyrotropin releasing factor (TRF), follicle stimulating hormone releasing factor (FSH-RF), luteinizing hormone releasing factor (LRF), prolactin inhibiting factor (PIF), growth hormone releasing factor (GH-RF) and possibly a GH inhibiting factor (GH-IF). Thus, the classical concept of the pituitary as the master gland of the body needs to be revised to include a brain-pituitary relationship.

Evidence for a physiological role for GH-RF has been slow to accumulate. This reflects, primarily, the lack of good and non-cumbersome methods for the measure of the hypothalamic releasing factor (GH-RF) as well as a lack of a sensitive method for the estimation of GH in the body fluids.

It was of interest, therefore, to attempt to develop a short term incubation method for assaying rat hypothalamic GH-RF which would show log-dose response characteristics and would be sensitive enough to detect quantitative changes in hypothalamic content of GH-RF. This study was also concerned with showing the effects of starvation on plasma GH activity, pituitary GH and GH-RF levels in the rat, in an attempt to correlate the previously reported observations of decreased pituitary content of GH and hypothalamic GH-RF with plasma GH levels.

REVIEW OF THE LITERATURE

I. Nervous Control of Anterior Pituitary (AP) GH Secretion

Although the release of GH from the anterior pituitary is generally thought to be under hypothalamic influence, the dependence of GH secretion on the central nervous system (CNS) has been difficult to elucidate because of a unique characteristic of GH. Growth hormone does not have a specific target organ nor does it stimulate a gland to secrete hormones, as is the case with FSH, LH, TSH and ACTH. Growth hormone acts on general growth processes of the body, and these include important influence on protein, fat and carbohydrate metabolism. However, despite this difficulty, several methods have been used to study the influence of the CNS on AP secretion of GH. These methods include one of two categories: (a) those that measure body growth, (b) those that measure pituitary GH content or release of GH from the pituitary. Several methods in each category are considered below.

A. Body Growth as a Parameter of CNS Control of AP Secretion of GH

The description of the hypophyseal portal system by Popa and Fielding (1933), and the observations by Houssay *et. al.* (1935), Wislocki and co-workers

(1936, 1937, 1938) and by Green and Harris (1949) of the correct direction of blood flow, from the hypothalamus down to the pituitary, led to the suggestion by Green and Harris (1949) that humoral agents are secreted into these hypophyseal portal veins and travel to the pituitary and there exert a regulatory influence on the secretion of anterior pituitary hormones. The indirect approaches to answer this problem consisted in the elimination of hypothalamic influence on the AP by pituitary stalk section or pituitary transplants, and by electrical lesions or stimulation of brain tissue.

Pituitary stalk section in rabbits did not inhibit growth according to Westman and Jacobsohn (1940), although it induced marked gonadal atrophy. A temporary growth delay was observed by Uotila (1939) in rats following stalk section. Pituitary stalk section in goats by Daniel and Prichard (1964) produced severe metabolic disturbances as well as blunted growth of body and pituitary target organs during the first few months after operation. These observations, rather inconclusive, were explained by Harris (1950) to be the result of partial sectioning of the stalk or as a consequence of portal system regeneration.

The observations on brain lesions are perhaps better substantiated. Clinically, growth disturbances

due to brain tumors have been known for many years (Armstrong and Durh, 1922). Cahane and Cahane (1938) injured the hypothalamic area in rats and observed a reduction in body growth. They suggested a possible role for the nervous system in controlling GH secretion. However, difficulties arise in the interpretation of the results obtained. Where extensive lesions are involved, the possibility of involving the areas controlling the secretion of the other AP hormones cannot be excluded. Cerebral lesions may also interfere with food intake and temperature regulation (Bogdanove and Lipner, 1952; Bernardis et. al., 1963). Reichlin (1960 a) showed that lesions of the median eminence and primary portal plexus of the stalk significantly reduced the growth rate of rats. Taking into account the possibility of altered secretions of the remaining AP hormones, Reichlin (1960 b) in a subsequent study injected vasopressin, testosterone and thyroxine to the injured animals. He also used pair-fed controls to exclude the effect of differences in food intake. He found that body growth was not restored to normal following this treatment.

In a more elegant study, where pituitary GH concentration was measured in injured animals, Reichlin (1961) showed that GH content in injured animals was reduced to 15% of that found in non-injured

animals, when measured by the standard tibia test of Greenspan et. al. (1949). These results (Reichlin 1960 a, 1960 b, 1961) and those of Hinton and Stevenson (1962), Bach et. al. (1964) and Endroczi et. al. (1956) suggest that lesions involving the anterior hypothalamus were most effective in retarding growth. This region comprises the area bounded by the supraoptic nucleus, the anterior half of the median eminence and the arcuate nuclei. O'Brien et. al. (1964) found that electrical stimulation of the paraventricular nuclei of weanling kittens caused acceleration of growth as measured by body weight and tibial length.

In general, pituitary transplants to hypophysectomized rats have been able to maintain some degree of growth (Greep, 1936; Martini and de Poli, 1956; Goldberg and Knobil, 1957). Pituitary transplants were made in different areas with lesser or greater success: the anterior chamber of the eye (Goldberg and Knobil, 1957), the kidney capsule (Hertz, 1959), the abdominal cavity (Swelheim and Wolthius, 1962), or the subcutaneous tissues (Meites and Kragt, 1964). The differences in degree of success in part may be related to the site of transplantation (Halasz et. al., 1962, 1963; Nikitovitch-Winer and Everett, 1958) as well as to the age of the pituitary donor (Meites et. al., 1962), the delay in transplantation after hypophysectomy

(Smith, 1961), the amount of viable anterior pituitary tissue present in the grafts, and to post-graft immunological factors.

B. Anterior Pituitary Content or Release of GH as a Function of CNS Control

The direct measurement of pituitary GH content in animals injected with median eminence extracts, as well as the measurement of pituitary content and release of GH into medium after incubation of pituitary tissue with median eminence extracts, constitutes the best evidence for the control of AP synthesis and release of GH by neural tissue.

The first attempt to show the existence of a factor in neural tissue responsible for controlling the release of anterior pituitary GH was that of Franz et. al. (1962). Although the authors concluded that there was growth hormone releasing activity in the hypothalamus of swine, their work and conclusions have been criticized since standard assay procedures were not followed and large variations in response were obtained. Deuben and Meites were the first to show conclusive evidence in this regard (1963, 1964). They reported that neutralized acid extracts of rat hypothalamus produced a 4 to 6-fold increase in GH release by rat anterior pituitary after a 6 day culture. Cerebral cortex failed to increase GH secretion upon

culture. Deuben and Meites (1965) also reported reinitiation of pituitary GH release *in vitro* by a neutralized acid extract of rat hypothalamus after release had ceased. These findings have been confirmed using *in vitro* (Schally *et. al.* 1965; Schally *et. al.*, 1968; Krulich *et. al.*, 1967) and *in vivo* methods (Pecile *et. al.*, 1965; Muller *et. al.*, 1965; Muller and Pecile 1965; Krulich *et. al.*, 1965; Schally *et. al.* 1966; Dhariwal *et. al.* 1966; Machlin *et. al.* 1967).

The specificity of *in vivo* assay procedures for hypothalamic GH-RF has been questioned by Rodger *et. al.* (1967), although dose-response relationships have been reported by Meites and Fiel (1965) and Katz *et. al.* (1967). Dose-response relationships have not yet been demonstrated for *in vitro* methods of assay for GH-RF (Schally *et. al.* 1965; Schally *et. al.* 1968; Krulich *et. al.* 1967). It was of interest therefore, to attempt to develop a short term incubation method for assaying rat hypothalamic GH-RF which would be sensitive enough to detect quantitative changes in hypothalamic content of GH-RF.

II. Factors Affecting GH Secretion

A. Environmental Factors. The advent of radioimmunological methods for measuring GH in body

fluids greatly advanced our understanding of GH control in humans. Roth et. al. (1963a) showed that moderate exercise stimulated human growth hormone (HGH) secretion. The rise in plasma GH could be blunted by the feeding of glucose prior to the exercise. Abdominal and chest surgery under ether anesthesia was frequently followed by an elevation in HGH (Glick et. al. 1965). Takahashi et. al. (1968) have reported increased HGH associated with the initiation of sleep, not related to changes in glucose, insulin or cortisol. It is of interest to note in this respect, that exercise, moderate or severe hypoglycemia, and cold, all known to elicit GH secretion in humans, failed to do so in rats (Schalch and Reichlin, 1967).

B. Hormonal Factors. Administration of insulin in doses large enough to lower blood glucose resulted in increased HGH (Roth et. al. 1963 b). The increased HGH with insulin induced hypoglycemia has been confirmed by Hunter and Greenwood (1964), Frantz and Rabkin (1964) and others. It is of interest that ethanol induced hypoglycemia does not increase HGH (Arky and Freinkel, 1954).

Thyroidectomy in the rat resulted in a degranulation of pituitary acidophiles (Purves and Griesbach, 1946;

Schooley et. al., 1966), a decrease in growth rate (Koneff et. al. 1949; Schooley et. al. 1966) and a decrease in pituitary GH content (Contopoulos et. al., 1958; Knigge, 1958; and Schooley et. al., 1966). Hyperthyroidism in rats also produced blunting of body growth, degranulation of pituitary acidophiles and a reduction in pituitary content of GH (Solomon and Greep, 1959). The interference to growth by estrogens has been suspected for some time (Gaarenstrom and Levie, 1939; Reece and Leonard, 1939) although the mechanism of action remains uncertain (Meites, 1949a; Sullivan and Smith, 1957; Josimovich et. al. 1967; and Birge et. al., 1967). The study by Birge et. al. (1967) suggests that diethylbesterol incubated with rat anterior pituitary caused suppression of GH release, but had no effect on the amount stored in the pituitary. Androgens, on the other hand, stimulate growth when given in small doses (Rubinstein and Solomon, 1941). The effects of corticosteroids may be similar to those observed with high doses of androgens (Evans et. al., 1943; Marx et. al., 1943; and Geschwind and Li, 1955), in which growth of long bones ceases by the closing of the epiphyses.

C. Food Intake. Reduced food intake or starvation have been reported to decrease pituitary, thyroid, and gonadal weights in the rat (Jackson, 1916;

Jackson, 1917; and Mulinos and Pomerantz, 1941). The resultant decreases in gonadal (Mulinos et. al., 1939) and thyroid functions (Stephens, 1940; Meites 1949 b; and Meites and Wolterink, 1950) apparently are due to a fall in circulating gonadotropins and thyrotropin. The decrease in gonadotropins may be due in part to a direct action of under-nutrition on the hypothalamus, resulting in reduced synthesis and release of hypothalamic releasing factors (Piacksek and Meites, 1967; Negro-Vilar, Dickerman and Meites, 1968). Underfeeding has also been reported to result in reduced absolute adrenal weight in the rat (Quimby, 1948).

The decrease in body growth and skeletal length that occurs during starvation in the rat may be due in part to a reduced level of pituitary GH (Meites and Fiel, 1965; Friedman and Reichlin, 1965) and in hypothalamic growth hormone releasing factor (Meites and Fiel, 1965). Since these observations can be interpreted as indicative of increased as well as of decreased release of GH from the pituitary and of GH-RF from the hypothalamus, it was of interest to determine plasma GH activity in addition to hypothalamic GH-RF content and pituitary GH concentration during starvation in the rat.

EXPERIMENTAL METHODS AND MATERIALS

I. Animals

A. Experimental Animals

Intact male rats of the Sprague-Dawley strain (Holtzman, Madison, Wisc.) weighing about 290-310g each, were used as control and experimental animals.

Control animals were maintained on a diet of Wayne Lab Blox pellets (Allied Mills, Chicago, Ill.) and fed ad libitum. Food, but not water, was removed from the experimental animals for 7 days. The rats were weighed at the beginning and end of each experiment.

B. Donor Animals

Mature male rats of the Sprague-Dawley strain (Holtzman, Madison, Wisc.) weighing 200-220g each, were used as hypothalamic and pituitary donors. All donor animals were fed ad libitum.

C. Bioassay Animals

Animals for GH bioassays were immature female rats (Charles River Breeding Laboratories, Wilmington, Mass.) hypophysectomized at 26 days of age. They were used for GH bioassay 15-16 days after operation. The regular diet of

these animals was supplemented daily with orange slices, carrots and sugar cubes. All bioassay as well as experimental animals were housed in a temperature controlled room (25±1°C) with automatically controlled lighting (14 hr light daily).

II. Preparation of Hypothalamic Extracts (HE), Pituitaries and Plasma

A. Preparation of Hypothalamic Extracts (HE)

Donor rats were killed by decapitation and their hypothalami and/or pituitaries were removed. The hypothalami were placed in ice cold 0.1N HCl (0.1 ml per hypothalamus) and homogenized in a total volume of 0.15 ml per hypothalamus. The homogenate was centrifuged at 12,000g for 40 minutes at 4°C. Just prior to use, the supernatants were placed in protein free medium 199 (Difco Labs., Detroit, Mich.) and the pH was adjusted to 7.4 by adding 1N NaOH a drop at a time and testing with glass electrodes. The same procedure was used in preparing cerebral extracts (CE).

B. Preparation of Pituitary Tissue for Assay

The pituitaries were removed from the control and experimental animals, individually weighed and stored at -20°C. Prior to assay,

the pituitaries were thawed, homogenized in saline and injected as an aqueous suspension into assay rats.

C. Preparation of Plasma for Assay

Blood was collected from the control and experimental animals via the abdominal aorta with a heparinized syringe. The blood from each group was pooled, centrifuged at 4 C and the plasma obtained immediately lyophylized and stored at -20 C. Lyophylized plasma was reconstituted to the desired volume with saline prior to being injected into the assay animals. A volume of 2 ml daily was injected intraperitoneally into each assay rat.

III. Incubation

The posterior lobe was removed from each donor pituitary and the anterior lobe was hemisected. The halves were randomly distributed in the incubation flasks. A total of 6 anterior pituitaries (12 halves) were placed in each flask. In preliminary experiments it was observed that pre-incubation of the pituitaries increased the precision of the assay, suggesting that during this time release of GH occurred as a result of injury to the tissue. After a number of preliminary

trials to determine the optimal pre-incubation and incubation times, the following procedure was adopted. The pituitaries were pre-incubated in 4 ml of medium 199/flask for 1 hour and the medium was discarded. Four ml of fresh medium containing the neutralized acid extract of hypothalamus or cerebrum was rapidly added to the flasks, and 4 hours later the incubation was terminated. The pituitary tissue was weighed and the medium stored at -20 C until assayed. Incubations were carried out in a Dubnoff metabolic shaker (60 cycles per minute) under constant gassing with 95% O_2-5% CO_2 at 37 C.

IV. GH Bioassay and Statistical Treatment

Growth hormone activity was measured by the standard tibia test of Greenspan et. al. (1949). Aqueous solutions of incubation medium, pituitary homogenate, or reconstituted plasma were injected intraperitoneally once a day for 4 days. Each assay included two or more doses of the control and experimental unknown solutions, as well as two doses of NIH-GH-S8 for reference standards. NIH-GH-S8 was kindly supplied by the Endocrinology Study Section, NIH.

The data were analyzed by linear regression or one way analysis of variance. Breakdown of significance was determined by Duncan's Multiple range test (Bliss, 1967).

EXPERIMENTAL

I. IN VITRO ASSAY FOR GROWTH HORMONE RELEASING FACTOR (GH-RF)

A. Objectives

The purpose of these experiments was to develop a short term incubation method for assaying rat hypothalamic GH-RF which would show log-dose response characteristics and be sensitive enough to detect quantitative changes in hypothalamic content of GH-RF.

B. Procedures

Donor rat pituitary tissue was pre-incubated in medium 199 for 1 hour. The medium was discarded. Fresh medium containing the neutralized acid extracts were then added to the flasks and incubated for 4 hours. Extract equivalent to 0.75, 1.50, 3.00 or 6.00 rat hypothalami were added to each flask containing six pituitaries. This corresponds to 0.125, 0.250, 0.500 and 1.000 hypothalamic equivalent per incubated pituitary. Rat cerebral extract was used as a control. The medium was then assayed for GH by the standard tibia test of Greenspan et. al. (1949).

C. Results

1. Dose response relationship between hypothalamic extract and anterior pituitary (AP) GH release in vitro. Hypothalamic extracts were assayed at 4 doses of 0.125, 0.250, 0.500 and 1.000 hypothalamic equivalent per incubated pituitary. Cerebral extract was assayed at a dose corresponding to the highest dose of hypothalamic extract/incubated AP. It can be seen in Table I and Fig. 1 that when progressively greater amounts of hypothalamic extract were incubated with male rat pituitaries, more GH was released into the medium. Regression analysis of the data showed these differences to be highly significant ($p<0.001$). Breakdown of significance by a Duncan's Multiple range test showed each point to be significantly different from the next lower point ($p<0.05$ or $p<0.01$). GH release by the pituitaries incubated with cerebral extract was significantly less ($p<0.01$) than released by the lowest dose of hypothalamic extract (0.125 HE/incubated AP).

2. Comparison of cerebral versus hypothalamic extract on AP release of GH. Cerebral and hypothalamic extracts were assayed at two dose levels (0.25 and 1.00 CE or HE/incubated AP).

Table I. Dose Response Relationship Between Hypothalamic Extract and Anterior Pituitary GH Release *in vitro*

Group	HE[a] or CE[b] equivalents/ incubated AP	No. of assay rats	Average tibial width (u) mean ± SE
1	1.000 CE	4	177.6±6.8
2	0.125 HE	4	194.2±4.5[d,e]
3	0.250 HE	4	214.8±4.5[e]
4	0.500 HE	4	227.9±3.6[f]
5	1.000 HE	4	244.5±1.6[e]
6	Hypox[c] assay controls	4	117.0±3.7

[a]HE: hypothalamic extract
[b]CE: cerebral extract
[c]Hypox: hypophysectomized
[d]Compared against CE
[e]$p<0.01$. Compared against next lower dose.
[f]$p<0.05$. Compared against next lower dose.

Reference Standard: NIH-GH-S8
25 ug = 201.3±2.8
100 ug = 231.8±2.7

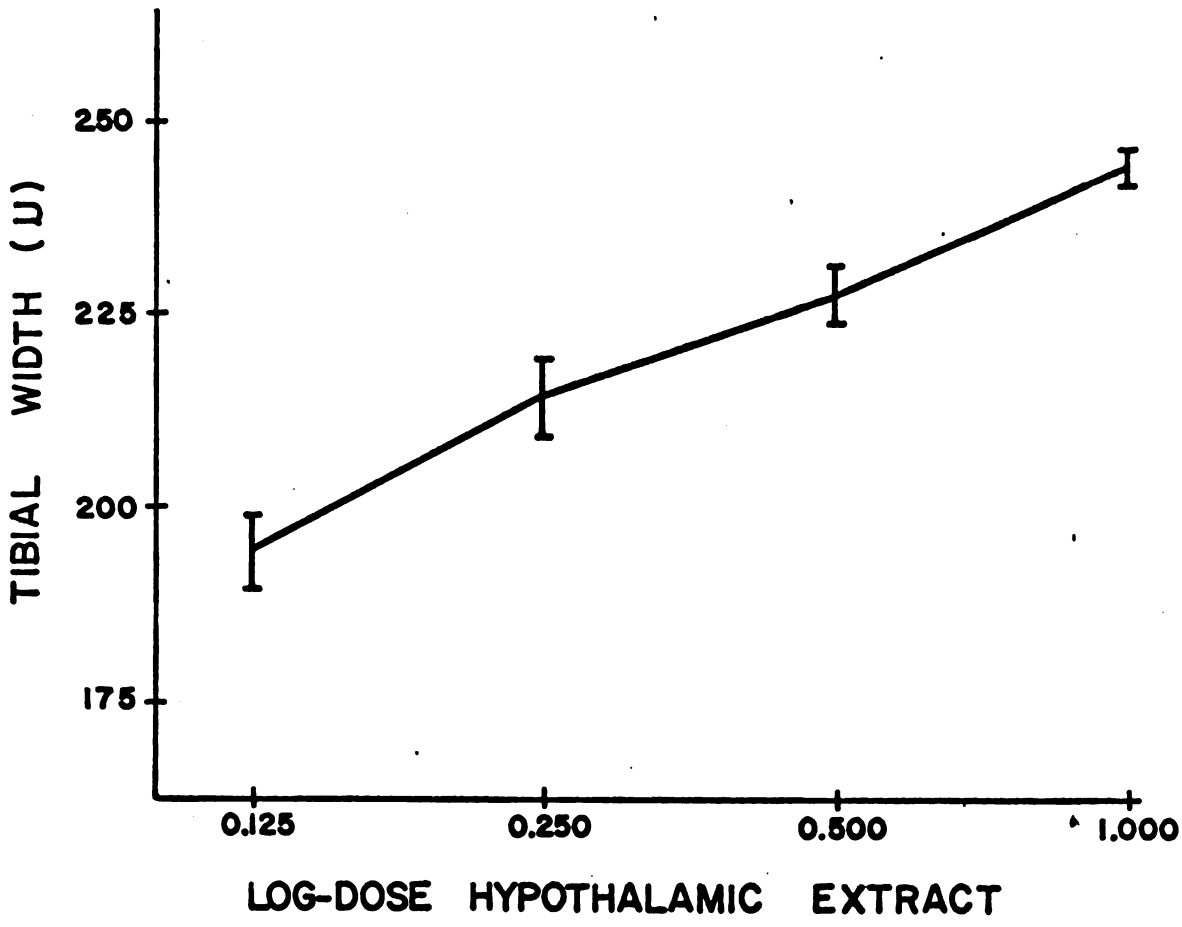

Figure 1. Logarithmic Dose-Response Curve Showing Pituitary GH Release as a Response to Hypothalamic Extract.

It can be seen in Table II and Fig. 2 that when the larger dose of cerebral extract was incubated with male rat pituitary, GH released into the medium was no greater than with the lower dose. On the other hand the larger dose of hypothalamic extract incubated with rat pituitary induced release of significantly more GH into the medium than the lower dose. The amounts of pituitary GH release stimulated by hypothalamic extract were significantly greater ($p<0.01$) than the amounts released by pituitary upon adding cerebral extract at either dose level. These results compare favorably with those obtained in Experiment 1.

3. Dose response to medium from AP incubated with cerebral extract or medium 199 alone. Medium from AP incubated with cerebral extract or medium 199 alone was divided into low and high doses corresponding to 0.25 and 1.00 AP equivalents/assay rat. These were then injected into each assay rat for 4 days. The results in Table III and Fig. 3 show that cerebral extract did not elicit a significantly greater ($p>0.05$) release of GH into the medium than medium 199 alone. The difference in response to the low and high

Table II. Comparison of Cerebral Extract versus Hypothalamic Extract on Anterior Pituitary Release of GH

Group	HE[a] or CE[b] equivalents/ incubated	No.of assay rats	Average tibial width (u) mean ± SE
1	0.25 CE	4	197.7±6.6
2	1.00 CE	4	204.7±3.4
3	0.25 HE	4	253.5±5.1[d]
4	1.00 HE	4	284.1±4.6[d]
5	Hypox[c] assay controls	4	133.2±5.6

[a]HE: hypothalamic extract
[b]CE: cerebral extract
[c]Hypox: hypophysectomized
[d]$p<0.01$. HE versus CE.

Reference Standard: NIH-GH-S8
25 ug = 237.5±1.6
100 ug = 272.5±5.1

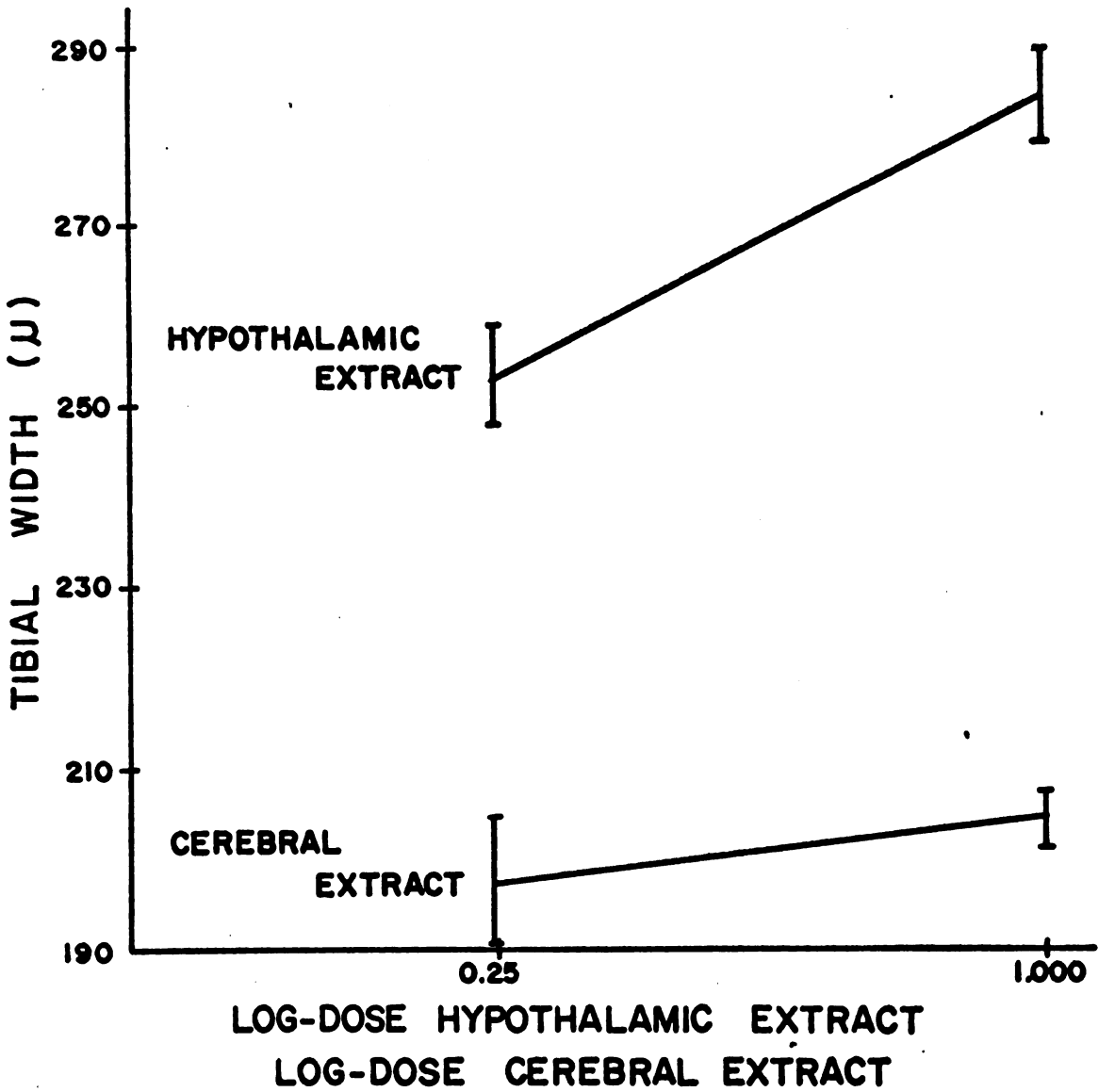

Figure 2. Logarithmic Dose-Response Curves Showing Pituitary GH Release as a Response to Hypothalamic or Cerebral Extract.

dose of medium in each group was significant.

4. Dose response to medium from AP incubated with cerebral or hypothalamic extract. Medium from AP incubated with hypothalamic or cerebral extract was divided into two doses as in 3 above. The results in Table IV and Fig. 4 show that the difference in response to the low and high dose of medium in each group was significant ($p<0.05$ or $p<0.01$). Both doses of hypothalamic extract (0.5 and 1.0 HE/incubated AP) elicited significantly greater release of GH into the medium than cerebral extract. The higher dose of HE elicited significantly greater release of GH than the lower dose. These results are in agreement with those in Tables I, II and III.

D. Discussion

The amount of GH released by male rat pituitary in a 4 hour incubation procedure was found to be directly proportional to the logarithm of the dose of rat hypothalamic extract added to the medium. The log-dose response was linear between the levels of 0.125 and 1.000 hypothalamic equivalents/ incubated AP. Graded doses of hypothalamic extracts elicited significantly greater

Table III. Dose Response to Medium from AP Incubated with Cerebral Extract or Medium 199 Alone

Group	CE[a] equivalents/ incubated AP	Total Dose of AP equivalents/ assay rat/ 4 days	No. of assay rats	Average tibial width (u) mean ± SE
1	Medium 199	0.25	4	161.1±4.2
		1.00	4	191.6±5.8
2	1.00 CE	0.25	4	152.0±4.3[c]
		1.00	4	189.4±4.2[c]
3	Hypox[b] assay controls	----	4	118.8±0.9

[a]CE: cerebral extract
[b]Hypox: hypophysectomized
[c]$p>0.05$. Not significant

Reference Standard: NIH-GH-S8
20 ug = 180.9±5.1
80 ug = 227.6±1.1

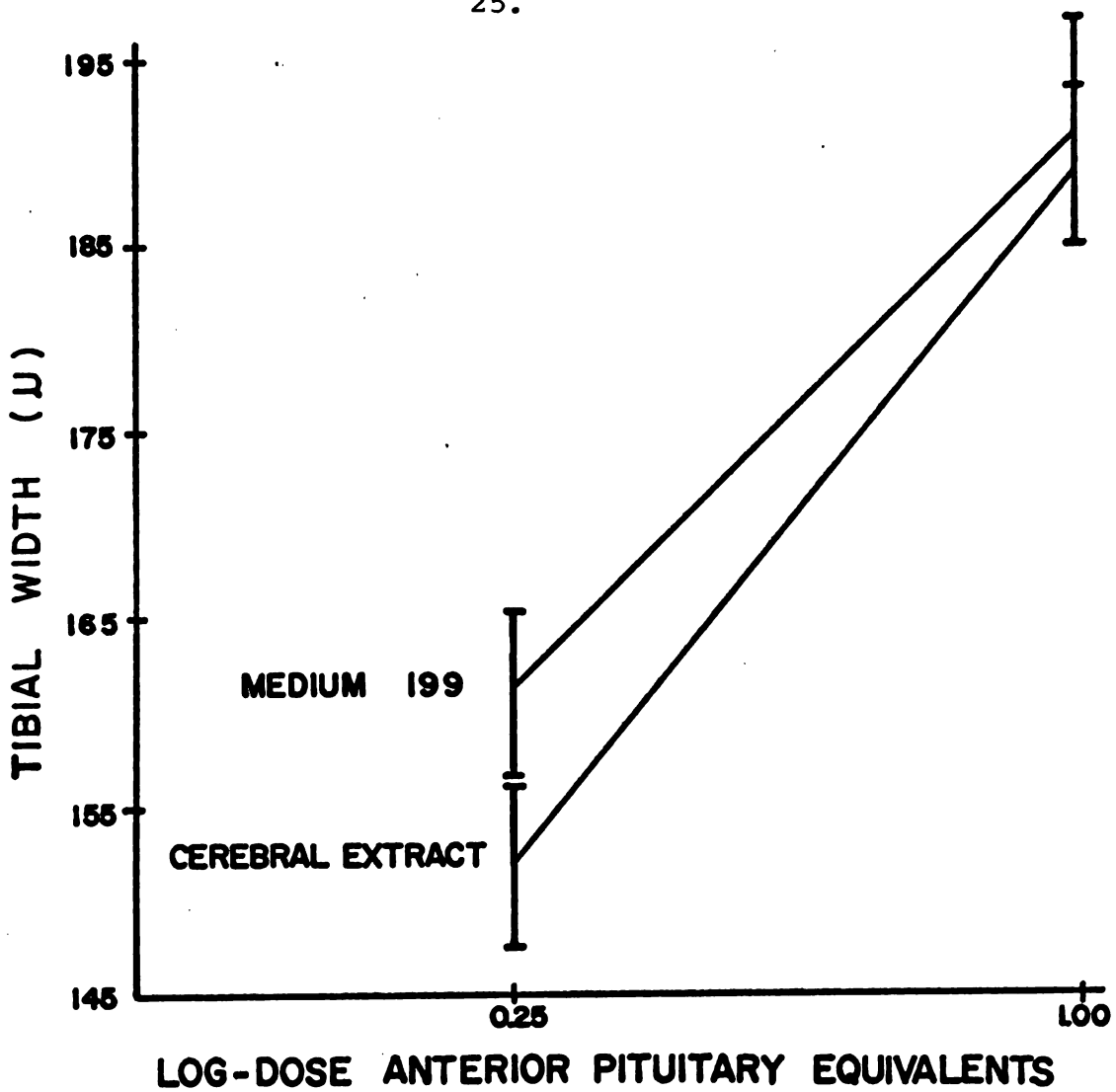

Figure 3. Logarithmic Dose-Response Curves to Medium from AP Incubated with Cerebral Extract or Medium 199 Alone.

Table IV. Dose Response to Medium from AP Incubated with Cerebral or Hypothalamic Extract

Group	HE[a] or CE[b] equivalents/ incubated AP	Total Dose of AP equivalents/ assay rat/ 4 days	No. of assay rats	Average tibial width (u) mean ± SE
1	1.0 CE	0.25	4	129.6±2.5
		1.00	4	168.1±9.7[d]
2	0.5 HE	0.25	4	152.7±2.2
		1.00	4	191.6±9.4[e]
3	1.0 HE	0.25	4	182.0±4.2
		1.00	4	216.4±1.9[e]
4	Hypox[c] assay controls	----	4	121.3±5.3

[a]HE: hypothalamic extract
[b]CE: cerebral extract
[c]Hypox: hypophysectomized.
[d]$p<0.05$
[e]$p<0.01$

Reference Standard: NIH-GH-S8
20 ug = 162.8±2.6
80 ug = 209.7±2.3

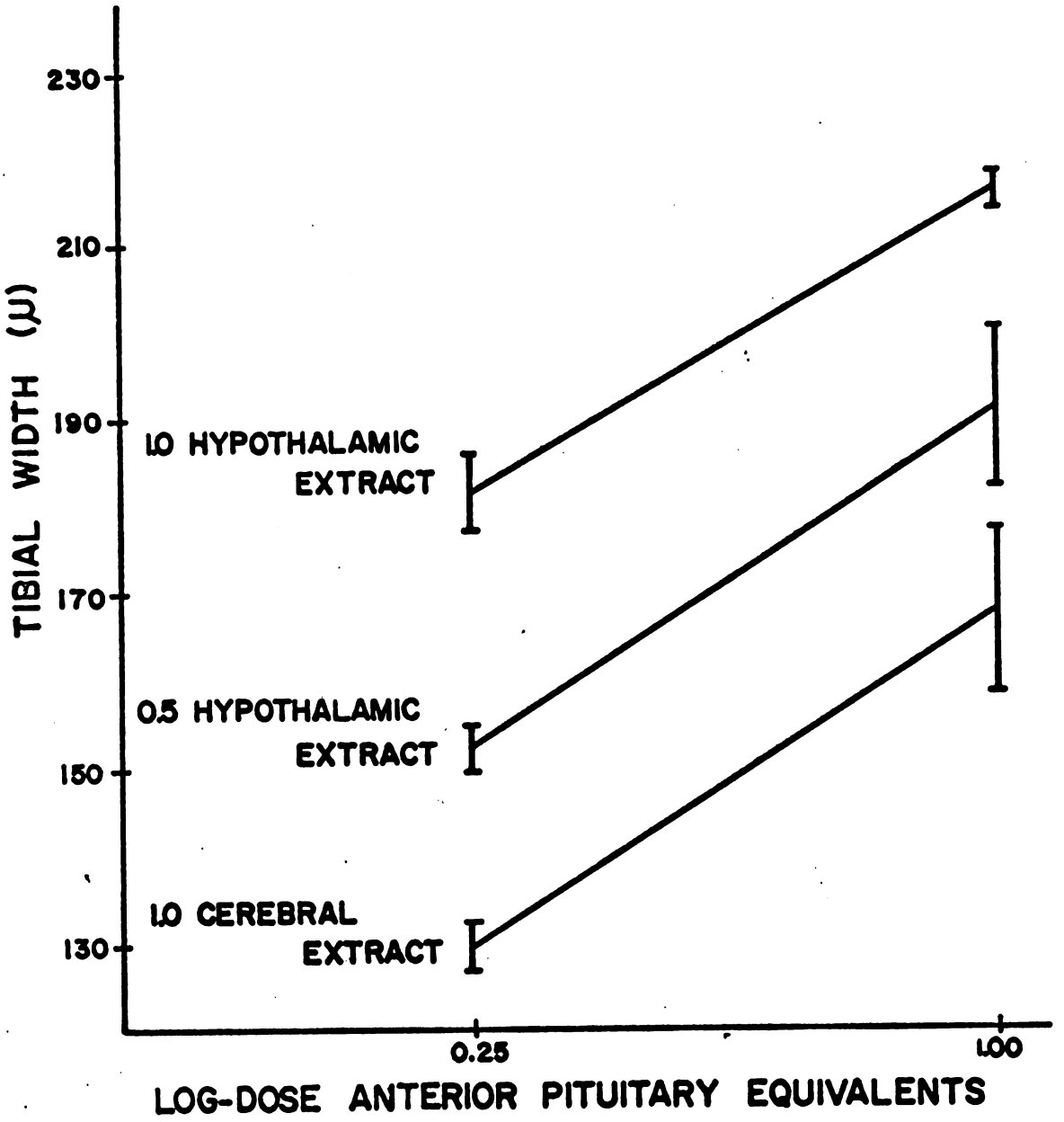

Figure 4. Logarithmic Dose-Response Curves to Medium from AP Incubated with Hypothalamic or Cerebral Extract.

release of GH into the medium in all experiments. However, graded doses of cerebral extract failed to elicit increased release of GH into the medium, indicating that this area of the brain does not contain GH-RF activity. In all experiments, AP incubated with the lowest dose of hypothalamic extract resulted in significantly greater release of GH into the medium than any dose of cerebral extract used. Analysis of the medium at two dose levels showed significant log-dose responses, indicating that the hormone assayed was GH. Some release of GH occurred in incubations of AP with cerebral extract. This is due to spontaneous release of GH since it has been shown that rat pituitary cultures release GH for up to 3 days even in the absence of hypothalamic extract (Meites, Hopkins and Deuben, 1962). There is no evidence that cerebral extracts can induce GH release by the incubated AP (Deuben and Meites, 1964; Deuben and Meites, 1965; Schally et. al., 1965; and Schally et. al., 1968).

Crude hypothalamic extracts can influence the release of anterior pituitary hormones

other than GH (Guillemin, 1956; Schreiber et. al., 1962; McCann, 1962; Talwaker, Ratner and Meites, 1963; Mittler and Meites, 1964). ACTH can reduce epiphyseal cartilage width in young rats (Evans et. al., 1943; Marx et. al., 1943) while TSH can increase it (Fels et. al., 1955). However, intraperitoneal injections of aqueous solutions of ACTH even at high doses have very little effect on the tibial response (Li et. al., 1954), and injections of only very large doses of TSH (500 ug/day/4 days) increase the width of the tibial cartilage plate (Fels et. al., 1955). It appears unlikely, therefore, that either TSH or ACTH in the medium influenced our results. If gonadotropins were present in the medium, they would have reduced rather than enhanced the tibial responses recorded.

Our experimental data support the concept that a specific GH-RF is present in the hypothalamus of the rat, since only such a factor would be expected to release pituitary GH in a log-dose fashion. Our method has been found to be suitable for measuring quantitative changes in hypothalamic GH-RF content produced by different physiological states (see next

section of thesis). The sensitivity of this *in vitro* method for assaying GH-RF should be further increased with the availability of a dependable radioimmunoassay for rat GH.

II. EFFECTS OF STARVATION ON PLASMA GH ACTIVITY, PITUITARY GH, AND HYPOTHALAMIC GH-RF LEVELS IN THE RAT.

A. Objectives

The purpose of these experiments was to observe the effects of starvation on plasma GH activity, pituitary GH and hypothalamic GH-RF levels in the rat. An attempt was made to correlate any changes in pituitary and hypothalamic content with plasma GH levels.

B. Procedures

Control animals were maintained on a diet of Wayne Lab Blox pellets and fed *ad libitum*. Food, but not water, was removed from the experimental animals for 7 days. On day 8, the animals were weighed, anesthetized with ether and bled from the abdominal aorta with a heparinized syringe. The pituitaries and hypothalami were also removed. Blood, pituitaries and hypothalami were prepared as described previously and injected intraperitoneally into assay rats to test for GH by the method of Greenspan *et. al.* (1949).

In a separate experiment, the starved animals received a daily subcutaneous injection of L-Na-thyroxine (T_4) (Nutritional Biochemicals

Corporation, Cleveland, Ohio) at a dose of 2.5 ug/100g body weight/day for 5 days beginning on day 2 of starvation. Only the plasma of the animals was analyzed for GH activity.

C. Results

1. Plasma GH activity of control and starved rats. Table V and Fig. 5 show the effects of 7 days of starvation on plasma GH activity. In two independent experiments plasma GH activity in control and starved rats was assayed at two dose levels with a fourfold difference between doses (8 or 32 ml of plasma equivalent per assay rat). In both experiments, plasma from control animals elicited a significantly greater tibial response than plasma from starved animals at either dose level ($p<0.01$).

The starved rats lost an average 87.7 and 88.6 g per animal, respectively, whereas the ad libitum fed controls gained an average 33.8 and 40.4 g respectively. Pituitary weights were also decreased in the starved rats.

2. Effects of starvation and T_4 on plasma GH activity. Starvation has been shown to reduce thyroid activity in rats (Meites and Wolterink, 1950; Yousef and Johnson, 1968)

Table V. Plasma GH Activity of Control and Starved Rats

Exp.	Treatment & no. of rats	Avg body weight Initial g	Avg body weight Final g	Avg pit wt of rats mg	No. of GH assay rats	Total Dose (ml of plasma/ assay rat/4 days)	Avg tibial width (u) mean ± SE
I	No food (100)	292.6	204.9	7.2	4	8	192.7±5.9
					4	32	214.3±2.8
	Control-fed ad lib. (55)	286.2	320.0	9.8	4	8	232.3±1.5**
					4	32	265.0±5.4**
	Hypox[1] assay controls	-----	-----	---	4	--	132.3±6.2
II	No food (98)	307.1	218.5	6.2	4	8	199.3±6.4
					4	32	235.6±4.6
	Control-fed ad lib. (43)	305.8	346.2	9.6	4	8	235.9±4.2**
					4	32	264.7±7.4**
	Hypox[1] assay controls (4)	-----	-----	---	4	--	125.1±5.3

[1]Hypox = hypophysectomized

**p<0.01. Control vs no food

Reference Standard: NIH-GH-S8
25 ug = 236.4±5.1
100 ug = 266.1±6.5

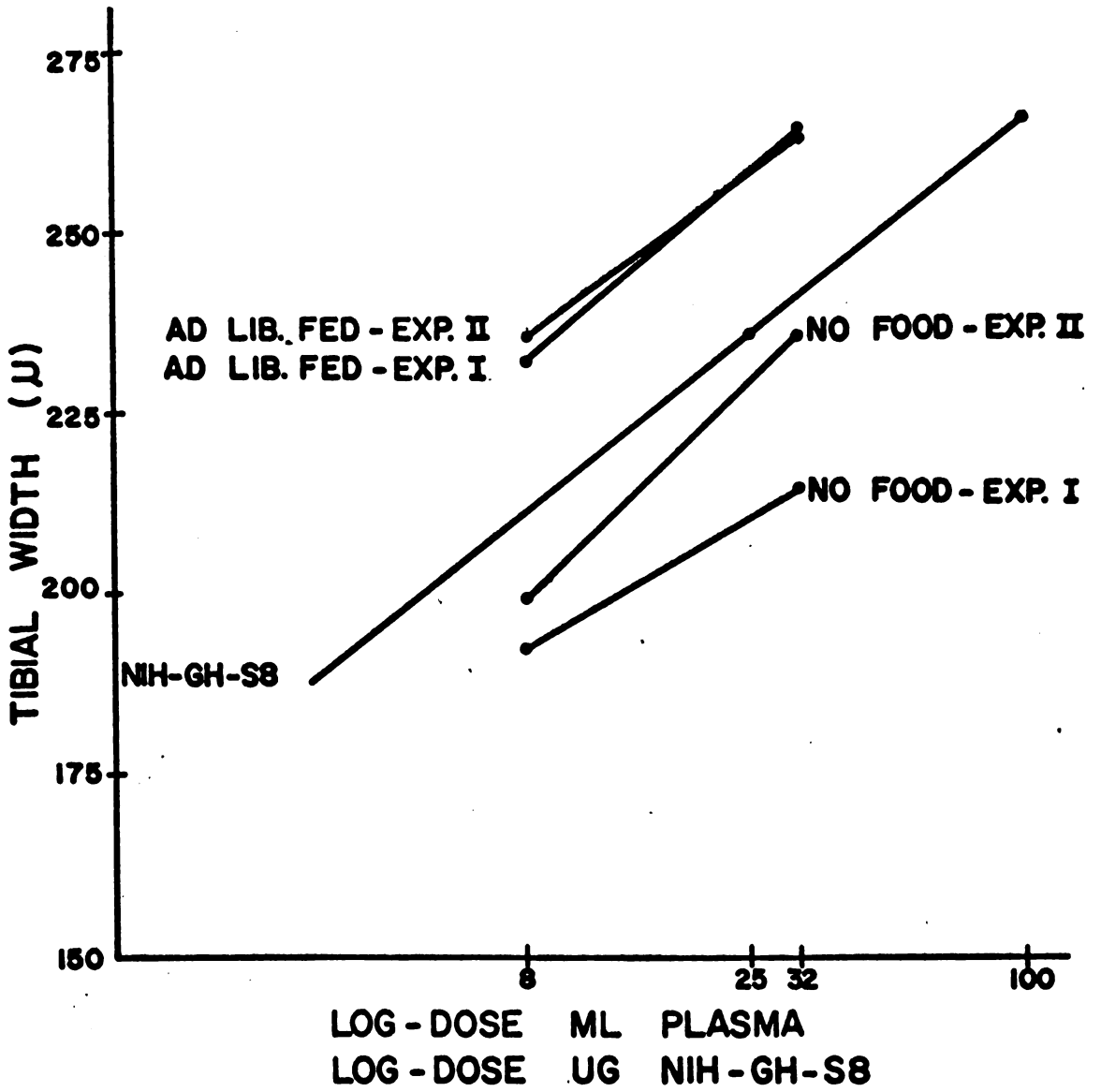

Figure 5. Logarithmic Dose-Response Curves to Plasma from Control or Starved Rats. Standard Curve Extrapolated.

Table VI. Effects of Starvation + T_4 on Plasma GH Activity

Exp.	Treatment & no. of rats	Avg body weight Initial g	Avg body weight Final g	Avg pit wt of rats mg	No. of GH assay rats	Total Dose (ml of plasma/ assay rat/4 days)	Avg tibial width (u) mean ± SE
I	No food + 2.5 ug T_4/100 g body wt (110)	315.9	232.3	6.4	5	8	169.1±4.8
					5	32	196.5±3.9
	Control-fed *ad lib.* (50)	327.1	353.8	9.0	5	8	193.3±2.7**
					5	32	232.3±5.8**
	Hypox[1] assay controls	-----	-----	---	5	---	127.3±4.2

[1]Hypox = hypophysectomized

**p<0.01. Control vs no food

Reference Standard: NIH-GH-S8
25 ug = 181.2±2.9
100 ug = 235.5±5.0

and this probably results in decreased blood levels of thyroxine (Grossie and Turner, 1962). Since the tibial response to GH is decreased in the absence of thyroxine (Schooley et. al., 1966), the starved animals received replacement doses of thyroxine (2.5 ug/100 g body weight/day) during the last five days of starvation. In this way we tried to restore normal levels of circulating thyroxine in the starved rats, thereby reducing the possible effects of thyroid deficiency on GH activity in the plasma.

As can be seen in Table VI, a significant reduction in plasma GH activity was observed in the thyroxine treated starved rats. Body and pituitary weights were also reduced in the starved animals. The reduction in plasma GH activity, body and pituitary weights observed in the thyroxine treated starved rats is comparable to the reductions observed in the previous experiments shown in Table V.

3. Effects of starvation on pituitary GH content. Pituitaries were assayed at two dose levels (2 or 4 mg of anterior pituitary per assay rat). Table VII shows that starvation

Table VII. Effects of Starvation on Relative Pituitary GH Concentration

Exp	Treatment	Avg. body weight Initial g	Avg. body weight Final g	Avg pit wt of rats mg	No. of GH assay rats	Total Dose (mg of AP/assay rat/4 days)	Avg tibial width (u) mean ± SE
I	No food	307.2	218.5	6.2	4	2	203.2±3.3
					4	4	244.3±2.2
	Control-fed ad lib.	305.8	346.2	9.6	4	2	227.9±3.5**
					4	4	280.6±2.1**
	Hypox[1] assay controls	-----	-----	---	4	-	125.1±5.3

[1]Hypox = hypophysectomized

**p<0.01. Control vs no food

Reference Standard: NIH-GH-S8
25 ug = 237.5±1.6
100 ug = 272.5±5.1

significantly reduced the concentration of GH in the pituitary ($p<0.01$). These findings are in agreement with previous reports (Meites and Fiel, 1965; Friedman and Reichlin, 1965). The starved rats lost an average 88.6 g and the ad lib. fed control animals gained an average 40.4 g.

4. Effects of starvation on hypothalamic GH-RF. Hypothalamic content of GH-RF was assayed at two dose levels (.25 or 1 hypothalamic equivalent per incubated pituitary) using the previously described in vitro incubation method. The results in Table VIII show that starvation significantly reduced hypothalamic content of GH-RF ($p<0.01$). Our results confirm those previously obtained by Meites and Fiel (1965) with an in vivo assay method.

D. Discussion

The data presented here indicate that complete food removal for 7 days in the rat significantly reduces plasma GH activity as well as pituitary concentration of GH and hypothalamic content of GH-RF. These results corroborate the previous observations that

Table VIII. Effects of Starvation on Hypothalamic GH-RF Content

Exp.	Treatment	Avg. body weight Initial g	Avg. body weight Final g	Avg pit wt of rats mg	No. of GH assay rats	Dose (H.E./incubated pituitary)	Avg.tibial width (u) mean ± SE
I	No food	307.2	218.5	6.2	4	0.25	191.4±5.7
					4	1.00	201.1±5.6
	Control-fed ad lib.	305.8	346.2	9.6	4	0.25	213.4±3.2**
					4	1.00	241.7±3.6**
	CCE[1] controls	-----	-----	---	4	1.00	177.6±6.8
	Hypox[2] assay controls	-----	-----	---	4	----	125.1±5.3

[1]CCE = Cerebral cortex extract
[2]Hypox = hypophysectomized
**$p<0.01$. Control vs no food

Reference Standard: NIH-GH-S8
25 ug = 237.5±1.6
100 ug = 272.5±5.1

starvation in rats results in lower hypothalamic GH-RF content (Meites and Fiel, 1965) and decreased pituitary concentration of GH (Meites and Fiel, 1965; Friedman and Reichlin, 1965), when compared with ad libitum fed controls, and indicate that these changes are reflected in lower concentration of plasma GH activity. Our observation of reduced plasma GH activity in starved rats agrees with a similar independent study carried out in starved rats by Dr. A. Trenkle (Department of Animal Science, Iowa State University, Ames, Iowa) using a radioimmunological assay for GH (personal communication).

Although we have used the term plasma GH activity rather than plasma GH, we have no evidence that the material measured in the blood differs from that in the NIH-GH-S8. As indicated in Fig. 5, the dose-response curves obtained from the plasma of the control and starved rats were parallel to those obtained with purified NIH ovine GH. This suggests that the tibia test is measuring GH activity.

The GH bioassay values found here in the plasma (Fig. 5) are considerably above those reported by radioimmunoassay in the rat

(Schalch and Reichlin, 1966). However, NIH ovine GH was used in our bioassay of rat GH, whereas a purified rat GH was used as the reference standard for radioimmunoassay of rat GH (Schalch and Reichlin, 1966). The ovine GH used in our experiments has a stated potency of 0.79 USP units/mg, whereas a purified rat GH prepared by Dr. S. Ellis (Ames Research Center, Moffett Field, California, personal communication) has a stated potency of 2.7 to 3.9 USP units/mg. Therefore, purified rat GH may be 3.41 to 4.94 times more potent than ovine GH in the rat.

It has been suggested that the tibia bioassay of plasma GH may measure "sulfation factor" activity. Salmon and Daughaday (see Daughaday and Kipnis, 1966) showed that fasting reduces sulfation factor levels in the plasma of rats. Uptake of sulfate in vitro by cartilage from fasted rats was also decreased when the fast was continued for more than 24 hours. Hypophysectomy in rats decreased serum sulfation factor (Salmon and Daughaday, 1957), but GH injections into hypophysectomized rats restored values to

normal levels. However, direct addition of HGH to *in vitro* incubations containing cartilage from hypophysectomized rats did not stimulate sulfate uptake (Daughaday and Reeder, see Daughaday and Kipnis, 1966). The *in vitro* stimulation of sulfate uptake was found to be a linear function of the logarithm of the dose of normal serum added to the incubation medium containing cartilage from hypophysectomized rats (Daughaday *et. al.*, 1959). Since a relationship was demonstrated between the dose of GH injected and the rise in sulfation factor activity in the plasma of pituitary dwarfs (Almgvist, 1960), it is believed that GH gives rise to a component of plasma capable of stimulating sulfate uptake by cartilage and incorporating this sulfate into chondroitin sulfate (Koumans and Daughaday, 1963). Serum sulfation factor activity therefore may be an index of GH activity in the plasma (Daughaday *et. al.*, 1959).

Our observations on plasma GH levels are in contrast to those reported on human subjects

by radioimmunoassay. Roth et. al. (1963 b) and Cahill et. al. (1966) reported that prolonged fasting in humans resulted in increased serum GH content. Knobil (1966) however, found no increase in serum GH in fasted monkeys. Machlin et. al. (1968) also reported that plasma GH levels in pigs increased during the first 48 hours of starvation and subsequently fell to lower levels. This may indicate that increased GH secretion during fasting may not occur in all species. In a study closely related to ours, Srebnik et. al. (1959) found a significant reduction in pituitary and plasma GH levels as measured by bioassay in rats fed a protein-free diet for prolonged periods of time. The possibility of a different mechanism for control of GH secretion in the rat as compared to humans is indicated by the recent work of Schalch and Reichlin (1967). They found that stimuli such as forced exercise, moderate or severe hypoglycemia, or cold, all known to elicit release of GH in humans, failed to do so in rats.

The interpretation of our results may

be complicated by the presence or lack of other hormones in the plasma of starved rats, particularly ACTH and TSH. Unpublished observations in our laboratory (Dickerman, Negro-Vilar, and Meites, 1967) showed that absolute adrenal weight decreased in starved rats. These observations confirm previous findings by Quimby (1948). Furthermore, Li et. al. (1954) showed that the tibial response to ACTH is dependent upon the mode of injection of ACTH. Thus, a very high dose of α-adrenocorticotropin, of the order of 100 ug, had very little effect on the tibial response when injected alone intraperitoneally or with GH in an aqueous solution. Fels et. al. (1955) reported that injections of a TSH preparation into hypophysectomized rats (200 ug/day/4 days) increased the width of the tibial cartilage plate from 163u to 192u. Administration of the latter dose with GH gave no increment over the values obtained with GH alone. Since all our assay rats were injected intraperitoneally with aqueous solutions of plasma, anterior pituitary or incubation medium, we believe that the changes in tibial width were due primarily to GH.

CONCLUSIONS

The present experiments support the concept of a hypothalamic factor which controls anterior pituitary secretion of GH, GH-RF. The amount of GH released by male rat pituitary upon incubation was directly proportional to the logarithm of the dose of rat hypothalamic extract added. This factor is not present in the cerebrum of rats, since cerebral extract failed to show a log-dose response and was also unable to increase the secretion of GH above that released spontaneously. This method of measuring GH-RF is sensitive enough to detect physiological changes in hypothalamic content of GH-RF. Thus, it was possible to corroborate the decrease reported in hypothalamic GH-RF by an *in vivo* method observed after starvation in rats (Meites and Fiel, 1965).

The decrease in hypothalamic content of GH-RF leads to a decrease in synthesis and release of GH from the pituitary and is reflected in a lower plasma GH activity. Thus the rat appears to differ from the primate in its response to starvation. It is also possible that the GH measured in the serum of humans by radioimmunoassay may measure not only biologically active GH, but also biologically inactive GH which is immunologically active.

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