STUDIES ON THE HYPOTHALAMIC CONTROL OF GROWTH HORMONE RELEASE

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ABSTRACT

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OF
GROWTH HORMONE RELEASE

by Roger Raymond Deuben

The ability of a crude acid extract of rat hypothalamus to stimulate release of somatotropin (STH) from rat anterior pituitary was tested *in vitro* and *in vivo*. STH was assayed by the standard tibia test in young hypophysectomized rats. The results of these experiments are as follows:

1. Rat anterior pituitaries were cultured for 18 days on medium 199 at 37° C under constant gassing with 95% O_2 - 5% CO_2. During the first 6 days, 81% as much growth hormone was recovered from the medium as was present in fresh anterior pituitary. During the second and third 6 day periods, 19 and 17% as much STH, respectively, was recovered from the medium as was present in the fresh rat anterior pituitary. When these amounts are combined with the amount of STH remaining in the pituitary explants, 50%, a total of 167% was recovered from the culture. This demonstrates that more STH is released by the rat pituitary *in vitro* than was originally present in the fresh tissue. Also, the rate of release drops off significantly after the first few days of culture.

2. Rat anterior pituitaries were cultured for 6 days with an acid extract of rat hypothalamus, with an identical extract which had been boiled and without an extract (control). Growth hormone release from anterior pituitaries cultured with extract of rat hypothalamus was 6 times greater than that of control anterior pituitaries, whereas boiling the hypothalamic extract reduced its STH releasing activity by about 1/3. This experiment provides evidence for the existence of a growth hormone releasing factor (SRF) in the rat hypothalamus which is relatively heat stable.
3. The medium from anterior pituitaries cultured with acid extracts of rat hypothalamus or rat cerebral cortex were compared to medium from pituitaries cultured without an extract. The acid extract of hypothalamus stimulated the release of 4 times more STH from the cultured pituitaries than was released by the control pituitary tissue. The cerebral cortical extract did not increase STH release by the cultured rat pituitary. This experiment demonstrated that SRF is present in rat hypothalamus but not in an equivalent amount of rat cerebral cortex.
4. Rat anterior pituitaries were cultured for 9 days. Only the medium from the first 3-day period contained significant amounts of STH. However, when an acid

extract of rat hypothalamus was added at the end of a 6-day culture period, STH release was increased to 78% of that released during the first 3 days. The medium from the second 3-day period contained insignificant amounts of STH. These results provide further evidence to the existence of an active principle(s) in the hypothalamus which stimulates growth hormone release.

5. Standard prolactin was injected into hypophysectomized rats and the epiphysial cartilage width was measured. Since the increase in width was not greater than 40μ, it was concluded that prolactin could not have appreciably influenced the previous assay results. In addition, hypothalamic extracts have been shown to inhibit rather than stimulate prolactin release.
6. An acid extract of rat hypothalamus was injected into hypophysectomized rats to test for growth hormone activity by the standard tibia test. It did not significantly increase tibial epiphyseal width, demonstrating an absence of STH in the extract.
7. An intracarotid injection of a neutralized acid extract of rat hypothalamus into intact rats, followed one half hour later by killing the rats, resulted in a considerable decrease in growth hormone content of the pituitary. This indicates that acid extracts of hypothalamus can release growth hormone <u>in vivo</u> as well as <u>in vitro</u>.

STUDIES ON THE HYPOTHALAMIC CONTROL OF GROWTH HORMONE RELEASE

By

Roger Raymond Deuben

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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1964

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Dedicated

to

my Mother

and

my Father

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Many workers have entered the field of neuroendocrinology in the last 5-10 years. This has been due to a large degree to the stimulus of the book written by G. W. Harris entitled Neural Control of the Pituitary Gland (1955). This review brought together many isolated observations and expressed a concept of brain-pituitary interrelationships which opened the door to much fruitful research.

The hormones of the anterior pituitary are primarily tropic in action, acting on target organs or tissues. By measuring the responses of these target organs to pituitary hormones, a quantitative measure of the secretory rate of these hormones can be established and be used to study the release of these hormones into the vascular system. Somatotropin (STH, growth hormone), however, poses a somewhat unique problem. It does not produce an easily measured response on a target organ or stimulate a gland to secrete hormones. It acts on the general growth process of body tissues and the skeletal system. It also exerts an important influence on protein, fat and carbohydrate metabolism. These responses are both difficult and expensive to measure. For these reasons, studies of changes in growth hormone secretion

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during different physiological states has received relatively little attention in the field of endocrinology.

Evidence suggesting that somatotropin secretion may be dependent upon association with the hypothalamus has appeared in recent years. Thus it has been observed that hypothalamic lesions or section of the pituitary stalk can result in depressed growth. However, only few experiments have been designed to demonstrate the existence of a substance in the hypothalamus which directly stimulates release of growth hormone from the hypophysis. Because of the in vitro techniques developed in this laboratory to study anterior pituitary function, it was deemed feasible to test the effects of crude extracts of rat hypothalamus on the release of growth hormone by the isolated rat anterior pituitary gland. By these methods, rat hypothalamus was shown to contain a growth hormone releasing factor (SRF).

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REVIEW OF LITERATURE

There have been many indications that the nervous system is linked to the endocrine system. Such phenomena as induced ovulation in rabbits as a result of coitus, the increase in glucocorticoids secreted by the adrenal gland during stressful situations and release of prolactin by suckling, to mention only a few, leave little doubt that the nervous system influences the functions of the anterior pituitary.

The search for direct innervation of the anterior lobe by the brain has yielded negative results. It was found that the only nervous fibers entering the adenohypophysis were vasomotor in origin (Rasmussen, 1938). In 1933, Hinsey and Markee postulated the possibility of humoral agents passing from the afferent neurons of the posterior lobe to the anterior lobe; however, this was regarded by most investigators as idle speculation. In 1930, Popa and Fielding reported the existence of a portal system connecting the base of the brain and the anterior lobe of the pituitary. This was a milestone in neuroendocrine research. However, they drew one erroneous conclusion from their work (Popa and Fielding, 1933). It was stated that the direction of blood flow was from the anterior pituitary toward the brain. A

series of papers by Wislocki and co-workers (1936a, 1936b, 1937, 1938) clarified this problem by showing that the direction of flow was actually just the reverse. This provided a route by which substances produced in the brain could be transported to the adenohypophysis.

It then remained to be shown that substances produced by the brain were capable of stimulating anterior pituitary activity. Many papers have appeared recently which leave little doubt that the hypothalamus is responsible for the production and release of neurohormones, which in turn control the release of anterior pituitary hormones. For excellent reviews of the anatomical and physiological interractions between the brain and anterior pituitary, see Harris (1955), Nalbandov (1963), Greep (1963), Szentágothai, Flerkó, Mess and Halász (1962) and Reichlin (1963).

Evidence Linking the Nervous System to Somatotropin Secretion

Essentially six methods are available by which the neural influence on growth hormone secretion can be studied. Relatively little has yet been done with these methods; however we shall still consider each one separately.

Electrolytic Lesions of Neural Tissue

Stereotaxic placement of electrolytic lesions in the brain stem produced a great deal of confusion in regard to regulation of growth hormone secretion. In 1938, Cahane and

Cahane placed electrolytic lesions in the hypothalamic portion of the diencephalon of the rat and observed a reduction in body growth. This observation led them to be the first to suggest that the nervous system may have some control over the secretion of growth hormone. This work was long overlooked and it was not until recently that they were given credit for this interpretation.

The major reason for the lack of reports involving STH release after hypothalamic lesions was that these animals normally gained weight. Yet Hetherington and Ranson (1942) mentioned one animal in a group of hypothalamic lesioned, obese rats which actually showed a reduction in body growth. However, they did not speculate on a possible mechanism for this observation. It has since been shown that hypothalamic obesity is caused by hyperphagia, which is not true body growth (Bogdanove and Lipner, 1952).

A major breakthrough came when Bogdanove and Lipner (1952) reported a striking degranulation of pituitary acidophils after massive basal hypothalamic lesions were induced. Since the growth rate, as contrasted to hypothalamic obesity, was markedly impaired in these rats, and growth hormone is generally associated with acidophillic cells, they suggested that growth hormone secretion might be severely disturbed. These results were confirmed by an independent group in both rats and dogs (Endroczi, Kovacs and Szalai, 1957). Reichlin (1960a) produced both unilateral and bilateral massive

lesions in the median eminence region of the hypothalamus of rats. The rats with the bilateral lesions showed a greater reduction in body growth than the ones with the unilateral lesions. However, the bilaterally lesioned rats only lost 50% as much of their growth rate compared to the hypophysectomized controls, indicating that growth hormone release is not completely inhibited (Reichlin, 1960b).

All of this evidence still does not establish that the hypothalamic lesions were interfering with growth hormone release. A disturbance in the secretion rates of other hormones can also influence growth rate (Geschwind and Li, 1958). However Reichlin (1960b) gave physiological replacement doses of thyroxine, testosterone, and Pitressin to his lesioned rats and still could not restore growth to normal. Marescotti, Carnicelli and Maltinti (1961) placed lesions in both the anterior and posterior hypothalamus and observed no reduction in somatic growth. To explain the contrary evidence present in the literature, they postulated that interference with ACTH secretion could account for the reported decrease in growth. It has since been shown, however, that reduced growth rate from ACTH deficiency results from hypophagia, and that control animals actually lose more weight than the deficient ones when limited to the same quantity of food intake (Reichlin, 1960a). Therefore this explanation cannot be valid since in Reichlin's (1960a) experiment, food consumption between groups was controlled.

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Either the lesions reported by Marescotti et al. (1961) did not destroy the areas of the hypothalamus involving growth, or his lesioned animals were hyperphagic and the reduction in rate of growth was not detectable.

In an attempt to be more specific, small discrete lesions were placed in minute areas of the hypothalamus (Hinton and Stevenson, 1962). It was found that a lesion bounded by the supraoptic nucleus, by the dorsal aspect of the optic tract, medially as far as the suprachiasmatic tract and anteriorly to the preoptic nucleus, caused a marked reduction in growth. There was however, histological impairment to the thyroid gland which leaves some doubt as to the significance of the results with respect to release of growth hormone. A single small lesion of the supraoptic nucleus was claimed to impair growth hormone release in weanling rats (Bernardis, Box and Stevenson, 1963). In contrast to this O'Brien, Nobile, Happel and Bach (1962) claimed that destruction of the paraventricular nucleus resulted in degranulation of pituitary acidophils and reduced growth rate in kittens.

Still other indirect evidence for lesion-induced growth hormone deficiency is that such rats are extremely sensitive to the hypoglycemic effects of insulin. This defect is corrected by administration of STH, but not by adrenal corticoids (Spirtos, Ingram, Bogdanove and Halmi, 1954; Spirtos and Halmi, 1959). It should be mentioned that

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a specific lesion of the amygdaloid region of the neocortex was reported to result in a marked reduction of growth (Karkegami, Fuse, Hiroki, Kazami and Kageyama, 1958). Whether this has any connection with growth hormone regulation needs to be determined.

The most convincing evidence for a neural connection to STH release was presented by Reichlin (1961). He found that the content of anterior pituitary growth hormone in rats with massive ventral lesions was only 15% of that found in non-lesioned controls, when measured by the standard tibia test. This is the only experiment in which anterior pituitary STH content from lesioned animals was actually measured.

Transplantation and Stalk Section.

Another approach to the study of growth hormone secretion was begun by May (1935) and Greep (1936). This consisted of hypophysectomizing young rats and transplanting the pituitary. Greep found that if one to five hypophyses were transplanted immediately back into the sella turcica that the growth curves of these animals ascended much more gently and flattened out at 1/2 to 2/3 the height of similar curves for normal non-hypophysectomized controls.

For many years the effects of anterior pituitary grafts on body growth received little attention. Only occasional comments turned up claiming that transplants could

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slightly maintain body growth in the hypophysectomized rat (Martinovitch and Vidovic, 1953; Martini and de Poli, 1956; Goldberg and Knobil, 1957). Greer (1957) mentioned that hypophysectomized mice implanted with up to four pituitaries in the anterior chamber of the eye lost weight to the same extent as the hypopophysectomized controls. This was also found to be true with intraocular transplants in rats (Martini, de Poli, Pecile, Saito and Tani, 1959). It was later demonstrated that if four anterior pituitaries are transplanted beneath the kidney capsule of hypophysectomized rats, growth rate could be maintained at about 2/3 of that of intact controls (Hertz, 1959).

Nikitovitch-Winer and Everett (1958) went one step further by retransplanting the anterior pituitary from the kidney capsule back into the sella turcica. Secretion of ACTH, TSH, FSH and LH was reestablished. Growth was also slightly stimulated. From this, Reichlin (1963) concluded that, "a demonstrable growth rate demands a certain quantity of functional pituitary, even when the gland is in a privileged site--that is, in the sella turcica or under the median eminence." Those transplants which were retransplanted under the temporal lobe remained functionally the same as when under the kidney capsule.

The next step was to determine if a homotransplant could reinitiate growth in a rat which had been hypophysectomized for a period of time. Smith (1961, 1963) found that

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if a pituitary was placed under the median eminence either 60 or 150 days after hypophysectomy, growth was reestablished. However growth by the 150-day group proved to be substantially slower than by the 60-day group.

Ahrén (1961) used a different parameter for measuring the ability of a transplant to secrete growth hormone. When a hypophysectomized rat was injected with estrone and progesterone, lobulo-aveolar development in the mammary gland was not stimulated. When prolactin was given in conjunction with these steroids, moderate development occurred. When growth hormone was given, development was even greater. Rats were hypophysectomized, autotransplanted with a pituitary beneath the kidney capsule, and compared with rats given the three treatments outlined above. Development was noted to be similar to that found in rats receiving the estrone, progesterone and prolactin. From these observations he concluded that the transplanted pituitary gland secreted considerable amounts of prolactin but very small amounts of growth hormone, if any. Oddly enough these same workers at a later date found that if rats were hypophysectomized and transplanted with one anterior pituitary the growth rate was six to twenty per cent above that of hypophysectomized controls (Ahrén and Rubinstein, 1963). If four anterior pituitaries were placed under the kidney capsule the body weight increase was of the order of 26-45% above the hypophysectomized controls while the intact controls were

132-200% above the hypophysectomized controls. Eleven weeks later, the mammary gland response to testosterone stimulation was studied. The treatment produced very few aveoli in rats with one pituitary transplant, but more in rats with four transplants. Aveolar development was scarce compared to the normal controls. It was concluded that there is a deficiency of growth hormone in hypophysectomized rats even when these rats have four anterior pituitary grafts, indicating that a normal connection between the hypothalamus and the anterior pituitary is essential for the secretion of somatotropin (Ahrén and Rubinstein, 1963).

Khazin and Reichlin (1961) found that if the anterior pituitary was grafted to the anterior chamber of the eye, TSH secretion was ample to maintain the thyroid at a normal state but there was no testicular or body growth. These findings are at variance with those of Goldberg and Knobil (1957) and Hertz (1959). Differences in technique could account for these variances. The site of transplantation may have some significance (Reichlin, 1963) since intraocular transplants (Martini et al., 1959) seem to produce much less somatotropin than if the site of implantation is subcutaneous (Meites and Kragt, 1964), intraperitoneal (Swelheim and Wolthuis, 1962), or under the kidney capsule (Hertz, 1959). This could make these results compatible with those of Martini et al. (1959); however the results would still disagree with those of Goldberg and Knobil (1957).

These differences in results may also be related in part to the volume of viable anterior pituitary tissue present in the grafts, the age of the donor, age of the host or to host-graft immunological factors.

Swelheim and Wolthuis (1962) treated hypophysectomized rats with methylthiouracil and a constant dose of thyroxine to keep all of the animals at an equal plane of circulating thyroxine. Some of the animals received kidney capsule pituitary transplants, while other got anterior pituitary tissue in the peritoneal cavity. They found that growth rate, as measured by both increase in body weight and tail length, was significantly higher in the animals with the transplants. They concluded that these grafts produced somatotropin and that the increase in growth was not the result of thyrotropin release.

Halász, Pupp and Uhlarik (1962) discovered that when they transplanted anterior pituitaries into the small neurons of the arcuate, anterior periventricular and retro-chiasmatic region of the hypothalamus, pituitary cytology and normal target tissue morphology were maintained. They termed this location the "hypophysiotrophic area of the hypothalamus." They interpreted this to mean that the neurohormones are produced in this hypophysiotrophic area and are carried by the fine fiber system of Spatz and Nowakowski to the zone in contact with the pituitary circulation. In 1963 they measured body weight gains in animals in which anterior pituitary

transplants were placed in different areas, such as the kidney capsule and under the temporal lobe of the brain. The transplant into the hypophysiotrophic area of the hypothalamus induced body growth significantly above hypophysectomized controls, while transplants in the kidney capsule or temporal lobe of the brain did not elicit a significant weight gain. They also had groups of rats with the same grafts, to which thyroxine was administered. The pituitary graft in the hypophysiotrophic area was the only one to respond to thyroxine by the reestablishment of acidophils. It was concluded from these experiments that the hypophysiotrophic areas of the hypothalamus produces a substance which plays an important role in the maintenance of growth hormone secretion (Halasz, Pupp, Uhlarik and Tima, 1963).

The reports on stalk sections suggest that certain animals (ferrets, rats, rabbits and humans) show an increased sensitivity to the hypoglycemic effect of insulin. Growth hormone replacement was not attempted (Reichlin, 1963).

Direct Electrical Stimulation and Pharmacological Agents

There have been few reports of growth responses by using these techniques. Gaunt has reported that chronic treatment with reserpine causes a decrease in growth (Nalbandov, 1963), however negligible work has been done with these methods insofar as STH is concerned.

Injection of Neural Extracts into Intact Animals

The first apparent evidence for neurohormonal control of growth hormone secretion was presented by DelVecchio, Genovese and Martini in 1958. They showed that chronic treatment of male and female intact rats with posterior pituitary preparations containing ADH, significantly increased the tibial epiphyseal cartilage width. Oxytocin did not elicit this response, and neither ADH nor oxytocin modified the cartilage width in hypophysectomized rats. These results were confirmed by Hiroshige and Itoh, (1961). Both groups concluded that antidiuretic hormone was the growth hormone releasing factor. However other investigators were unable to confirm the results of DelVecchio et al. (1961), even though the procedure was followed exactly (Reichlin and Brown, 1961; Franz, Haselbach and Libert, 1962). The hypothesis that ADH is the STH releasing factor has not been confirmed and is not accepted by most investigators.

In Vitro Techniques

Controlling factors for many of the other anterior pituitary hormones have been described (Guillemin, 1956; Schreiber, et al., 1962; Talwalker, Ratner and Meites, 1963; McCann, 1962; Igarashi and McCann, 1964). Franz et al., (1962), using the incubation technique of Saffaran and Schally (1955) and of Guillemin, Hearn, Cheek and Householder (1957),

attempted to demonstrate the existence of a growth hormone releasing factor in hog hypothalamus. However, since standard assay procedures and statistical analyses were not used, it is very difficult to evaluate the validity of their results. They claimed that systemic injections of a partially purified peptide fraction of hog hypothalamus into intact rats increased the width of the epiphysial cartilage. It did not stimulate the epiphysis in hypophysectomized animals. They concluded that this fraction contained a substance which acted on the pituitary to stimulate the release of growth hormone. However, only a few animals were used and their data can be considered to be of questionable significance.

EXPERIMENTAL METHODS AND MATERIALS

Animals

Mature virgin female Carworth CFN rats (Carworth Farms, New City) 180-210 grams in body weight, were used as donors for pituitary, hypothalamic and cerebral cortical tissues. They were housed under similar conditions for at least 2 weeks prior to use.

Immature female Sprague-Dawley rats (Hormone Assay Labs, Chicago) were used for assay of growth hormone. Some of these animals were hypophysectomized in this laboratory at 26-28 days of age by the parapharyngeal approach. Other rats of the same strain were hypophysectomized by Hormone Assay Laboratories and shipped here. The regular diet of Wayne Lab Blox pellets (Allied Mills, Inc., Chicago) was supplemented daily with fresh oranges, 5% sucrose solution and canned dog food. Survival rate, however, was very poor. Autopsy showed that death was caused by murine pneumonia. Antibiotic therapy proved useless. New stainless steel shoe-box cages were purchased, and the litter of wood shavings was supplemented with unsterile cotton to help preserve body heat. The diet was again revised so that each cage had a container of bread soaked with water, cracked corn mixed with dog food, fresh milk, 1/4 of a fresh orange and a piece of

carrot. The food was replaced daily and the litter was changed every 3 to 5 days depending on the number of animals in the cage. This increased the survival rate to almost 100%.

All animals were kept in a temperature controlled room at 75 $\pm$ 1° F and under illumination for 14 hours per day. All rats had free access to water.

Preparation of Extracts

The median eminence and some adjacent portions of 2 rat hypothalami were removed immediately after decapitation, and frozen on dry ice. They were then homogenized with 1 ml of 0.1 N HCl in a ground glass homogenizer. An equivalent amount, by weight, of rat cerebral cortical tissue was treated in the same manner. The homogenates were centrifuged at 30,000 x g for 30 minutes at 4° C. The supernatent was then neutralized with 1 N NaOH and brought to volume with sterile water, so that 1 ml of solution contained the acid-soluble fraction of 2 rat hypothalami. These extracts were prepared under sterile conditions and were incorporated directly into the medium in place of water, so that each culture dish contained 1 rat anterior pituitary and the extract from 2 rat hypothalami. This acid fraction presumably contains all the active peptides in the hypothalamus.

Preparation of Cultures

The culture method used was the method of Fell and Robison (1929) as modified by Trowell (1959) and again by Nicoll and Meites (1963). This procedure entailed the use of sterile plastic Petri dishes (Falcon Plastics, Inc., Los Angeles), 3.5 x 1.0 cm in size. Stainless steel rafts, 1.0 x 2.6 cm were prepared from #46 grid stainless steel mesh (United Surgical Supply Co., Port Chester). These rectangles were bent so that when placed in the Petri dishes with 3 ml of media, the top of the raft was exactly at the liquid-air interface. Washed, sterile lens paper strips, on which the pituitary explants rested, were draped over the rafts so that the ends dipped into the medium. The lens paper acted as a wick and conducted the nutrients to the explants by capillarity; however the tissue was still exposed to the atmosphere, permitting optimal gaseous exchange. All materials and instruments used during the culture were cleaned and sterilized according to the methods described by Merchant, Kahn and Murphy (1960) except that the sterile plastic Petri dishes were discarded upon termination of the culture.

Labile materials such as antibiotics were passed through a bacteriological filter (Millipore Filter Corp., Bedford) with a pore size of 0.45μ. Water used in preparation of all aqueous solutions was distilled, deionized and deorganified by resin columns, and redistilled from glass.

The culture dishes were placed in a plastic chamber (Nicoll, 1962) which was gassed with humidified 95% 0_2 and 5% $C0_2$ at 10 mm Hg above atmospheric pressure. This chamber was kept in a dark walk-in incubator at 37 $\pm$ 1° C.

The medium was always prepared the same morning of the culture under a plastic hood in which a 15 watt germicidal ultraviolet lamp had been turned on for at least 24 hours previously. Medium 199 (Difco Corp., Detroit, or Microbiological Associates, Inc., Bethesda) in 10 fold concentrate was used. This medium contains all of the known ingredients necessary for maintaining tissue but is not considered optimal for supporting growth. To prepare 100 ml of Medium 199 at pH 7.4, 7 ml of 5.6% $NaHCO_3$ and 1 ml of antibiotic stock solution were added to 10 ml of concentrated medium 199 and brought to volume with distilled water. Extracts were added, when appropriate, in place of an equal volume of water. The final antibiotic content was 0.1 mg streptomycin sulfate and 50 U penicillin G (Nutritional Biochemicals Corp., Cleveland) per ml of medium. Three ml of prepared medium was pipetted into each Petri dish into which a stainless steel raft had been placed.

Culture Procedure

The donor animals, stunned by a blow at the back of the head, were decapitated by a guillotine (Harvard Apparatus Co., Dover). The heads were then quickly dipped in 70%

ethanol. This procedure wets the hair and prevents it from contaminating the area when the anterior pituitaries are removed. The heads were carried to the plexiglass hood, where the pituitary was exposed and the adenohypophysis was separated from the neurohypophysis. The anterior portion was then carefully placed in a sterile plastic Petri dish and cut in half with a scalpel equipped with a #11 disposable blade (Crescent Mfg. Co., Fremont, Ohio). These halves were then quartered to give a total of 8 pieces of tissue. The explants were placed on lens papers which had been moistened with medium 199. The paper was then placed over the raft and the Petri dish was covered.

In some of the experiments 2 explants from each pituitary were placed in 4 dishes. This means that each dish was homogeneous since each received an equal amount of tissue from the same rat anterior pituitary. The dishes were placed in the gassing chamber inside the incubator. The medium was subsequently changed every 3 days until termination of the culture.

At termination of the culture the medium from all the dishes in each group was pooled and frozen for future assay. Representative samples of explants from each dish were fixed in Dietrichs solution. They were embedded in paraffin, sectioned at 6 μ and stained with hemotoxylin-eosin for a subjective estimate of cell viability. Any obvious differences in cell viability between groups invalidated the

experiment, and the hormone assays were not performed. If the medium was to be assayed, it was lyophilized in a Vir Tis lyophilizing apparatus (Vir Tis Co., Inc., Gardiner) and stored in the freezer. Any variations from the above procedures are included in the description of each individual experiment.

Assay of Growth Hormone

The lyophilized medium was diluted to 3 dose levels with distilled water and the growth hormone content was determined by the standard tibia assay of Greenspan et al., (1950). Female Sprague-Dawley rats were hypophysectomized at 26 to 28 days of age. Following a post-operative interval of 12 to 14 days the medium was injected in a 0.5 ml volume once daily for 4 days. Twenty-four hours after the last injection the animals were sacrificed and the right tibia was dissected free of the accompanying soft tissue. They were split with a razor blade at the proximal end in the midsagittal plane, and fixed in neutral buffered formalin. The tibia halves were prepared for reading as follows:

1. Wash in water for 1/2 hour.
2. Immerse in acetone for at least 1 hour.
3. Wash again in water for 1/2 hour.
4. Place in freshly prepared 2% silver nitrate for 1-1/2 to 2 minutes.

5. Rinse in water and concurrently expose to strong light until calcified portions appear dark brown.
6. Immerse in 10% sodium thiosulfate for 25-30 seconds. (This is only necessary if the stained tibias are to be saved.)
7. Read immediately or store in 80% ethanol in the dark.
8. Take a minimum of 8 to 10 readings of the tibial epiphyseal cartilage width under low power magnification, using a micrometer eyepiece.

The lyopilized medium from each group was diluted to 3 dose levels with distilled water. Fifteen assay animals for each experimental group were therefore needed. Five to ten animals were injected with unclutured medium 199 to establish a control tibia epiphyseal width. Ten random readings were taken across each epiphysis, averaged and converted to microns. The average width in microns of the animals in response to a particular dose level was then calculated. To be indicative of growth hormone stimulation, the average width of the group had to be at least 40μ above the hypophysectomized control level (Srebnik, Nelson and Simpson, 1959).

Statistical Evaluation of the Data

Regression lines for each of the experimental groups were calculated by the method of least squares. Both the slopes and elevations of these lines were tested by analysis

of covariance. If the slopes of the regression lines were significantly different, the assay was completely invalidated and the analysis of elevations could not be obtained.

Irwin's method as described by Pugsley (1946) of determining relative potencies, was used to estimate the degree of difference between groups. Standard errors were calculated for all means by the formula:

$$S.E. = \sqrt{\frac{N\Sigma x^2 - (\Sigma x)^2}{N^2(N-1)}}$$

RESULTS AND DISCUSSION

Experiment 1. Relative Amounts of STH Released by the Rat Anterior Pituitary During a Culture Period of 18 Days

To gain some insight into the relative quantities of growth hormone secreted by a cultured anterior pituitary over an 18-day period, the following experiment was designed. Anterior pituitaries from 15 rats were removed and cultured on synthetic medium 199. Three of these pituitaries were cut into 24 explants and placed into one culture dish. A total of 5 such dishes were cultured. The medium was changed each 3 days and frozen. The medium from the first and second 3 days was pooled to give a sample of 6 days duration. Also the medium from the second 6 days and third 6 days was similarly pooled, resulting in a total of 3 groups of medium from successive 6-day periods. At termination of the 18-day culture period, 2 explants from each pituitary were saved for histological examination. The remaining explants were frozen for subsequent assay.

Prior to assay for growth hormone activity, the medium from each 6-day period was lyophilized and diluted to 3 concentrations. The remaining cultured pituitary explants and an equivalent amount of fresh uncultured rat pituitary

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were each homogenized separately in medium 199, lyophilized and likewise diluted to 3 dose levels.

A summary of the results is presented in Table 1 and a graphic representation of the regression lines is shown in Fig. 1. The total growth hormone activity recovered from the medium and the explants was 167% of that found in an equivalent amount of fresh anterior pituitary. During the first 6 days of culture, 81% as much growth hormone activity was released into the medium as was found in fresh anterior pituitary. During the following two 6-day culture periods, growth hormone was only 19% and 17% as much, respectively. These findings are comparable to those reported by Guillemin (1956) for ACTH *in vitro*. The release of this latter hormone also markedly drops off after the first few days of culture. This reduction in release is attributed in part to progressive necrosis which was observed in the pituitary explants, and also to an absence of hypothalamic stimulation. Only in the peripheral areas of the explants did viable cells remain.

Table 1. STH release into the medium from rat anterior pituitary cultured for 18 days.

Group	No. of Assay Animals	Dose AP Equivalents/ Assay Animal/ 4 days	Response μ ± SE	Per Cent of Control* (P = 0.05)
A. Fresh AP (Control)	5	0.2	199 ± 3	
	5	0.6	226 ± 2	100
	5	1.2	251 ± 5	
B. Explants	5	0.2	175 ± 4	
	5	0.6	207 ± 3	49.8
	5	1.2	232 ± 4	
C. Medium (Days 1-6)	5	0.2	190 ± 10	
	5	0.6	220 ± 6	80.9
	4	1.2	246 ± 7	
D. Medium (Days 7-12)	5	0.2	155 ± 8	
	5	0.6	189 ± 5	18.9
	5	1.2	200 ± 6	
E. Medium (Days 13-18)	5	0.6	176 ± 7	
	5	1.2	200 ± 5	17.1
F. Hypophysectomized Assay Controls	18	0	125 ± 3	--

Total STH recovered from culture = 167% of that contained in fresh AP.

*Calculation of relative potencies as described by Pugsley (1946).

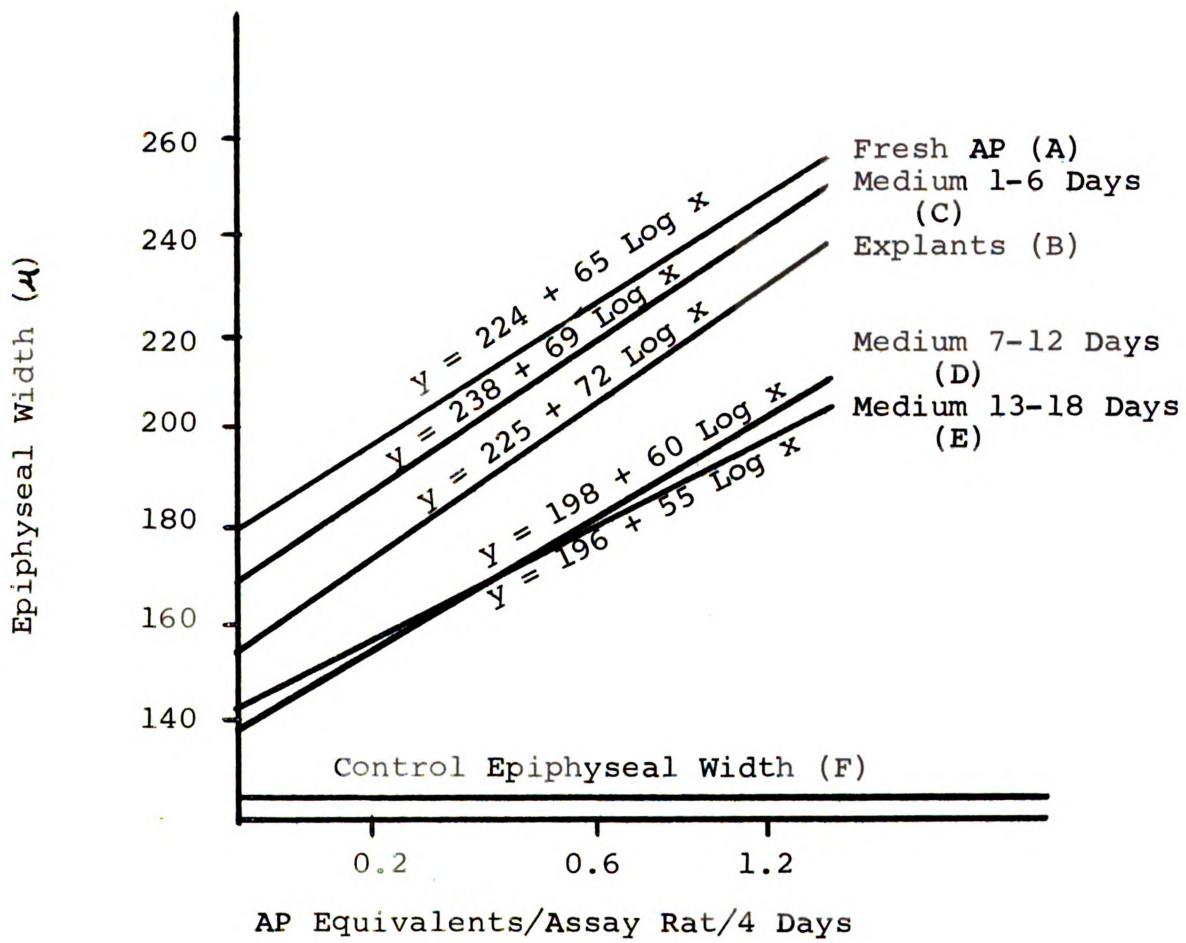

Fig. 1. Regression lines demonstrating growth hormone release by rat pituitary during an 18-day culture period (AP = anterior pituitary).

Experiment 2. Stimulation of STH Release in Vitro by an Acid Extract of Rat Hypothalamus; the Effect of Boiling on this Extract

If the hypothalamus contains a substance which stimulates growth hormone release, incorporation of hypothalamic extract into a culture with rat anterior pituitary should result in an increase in growth hormone released. To investigate this possibility, the present experiment was designed.

Neurohumoral factors from the hypothalamus have been found to be acid soluble. It was therefore decided to make a 0.1 N HCl acid extract of primarily the median-eminence portion of rat hypothalamus and incorporate it directly into culture medium 199. Growth hormone release from these pituitaries was compared with that from pituitaries cultured on medium 199 without hypothalamic extract.

Thirty-four rat anterior pituitaries were cultured on medium 199 for 6 days. Each pituitary was cultured separately on 3 ml of medium. Ten anterior pituitaries were used as controls and cultured without hypothalamic extract; 12 were cultured with an acid extract of rat hypothalamus and 12 with a similar extract of rat hypothalamus which had been boiled for 15 minutes at pH 7. At the end of the culture period the medium from the two 3-day periods for each group was pooled and dialyzed for 20 hours at 4° C. Dialysis was found necessary to remove the high salt concentration of the medium in order to facilitate lyophilization.

It was then lyophilized and subsequently diluted with distilled water for injection into the assay rats. Only 2 dose levels were assayed for the control anterior pituitaries because of an outbreak of murine pneumonia in the assay rats.

The results are given in Table 2 and Fig. 2. Statistical analysis of the regression lines showed that there was approximately 6 times as much growth hormone activity in the medium containing the acid extract of hypothalamus (Group B) as in the medium without the extract (Group A). Boiling the extract resulted in a significant loss of potency; however it still elicited release of 4 times as much growth hormone (Group C) as found in the control medium (Group B). Since growth hormone is virtually destroyed by such treatment (Geshwind and Li, 1955) it is concluded that the stimulation of epiphyseal width was not the result of the presence of growth hormone *per se* present in the extract of hypothalamus. It must have resulted from an increased amount of growth hormone released into the medium from the pituitary explants.

It is concluded that a substance(s) in the extract of hypothalamus is capable of stimulating the isolated anterior pituitary to release greater amounts of growth hormone and that it is relatively heat stable. The loss in releasing potency by the boiling process can be attributed to the extra handling and/or transferring which was involved in this process. The growth hormone releasing factor from

the hypothalamus will tentatively be referred to as SRF (somatotropin releasing factor).

Table 2. Effect of acid extracts of rat hypothalamus on STH release by rat anterior pituitary in vitro.

Group	No. of Assay Animals	Dose AP Equivalents /Assay Animal /4 days	Response μ ± S.E.	Per Cent of Control (P = 0.05)
A. AP Control (Cultured)	4	0.6	176 ± 10	
	5	1.4	209 ± 7	100
B. AP + acid extract of rat hypothalamus	4	0.2	198 ± 4	
	5	0.6	242 ± 2	570
	5	1.6	268 ± 2	
C. AP + boiled acid extract of rat hypothalamus	4	0.2	187 ± 2	
	5	0.6	221 ± 3	370
	5	1.6	263 ± 3	
D. Hypophysectomized Controls	15		139 ± 2	---

Slopes of the regression lines were not significantly different.

Elevations of the following:
A vs B : $P < .01$
A vs C : $P < .01$
B vs C : $P < .01$

AP = anterior pituitary.

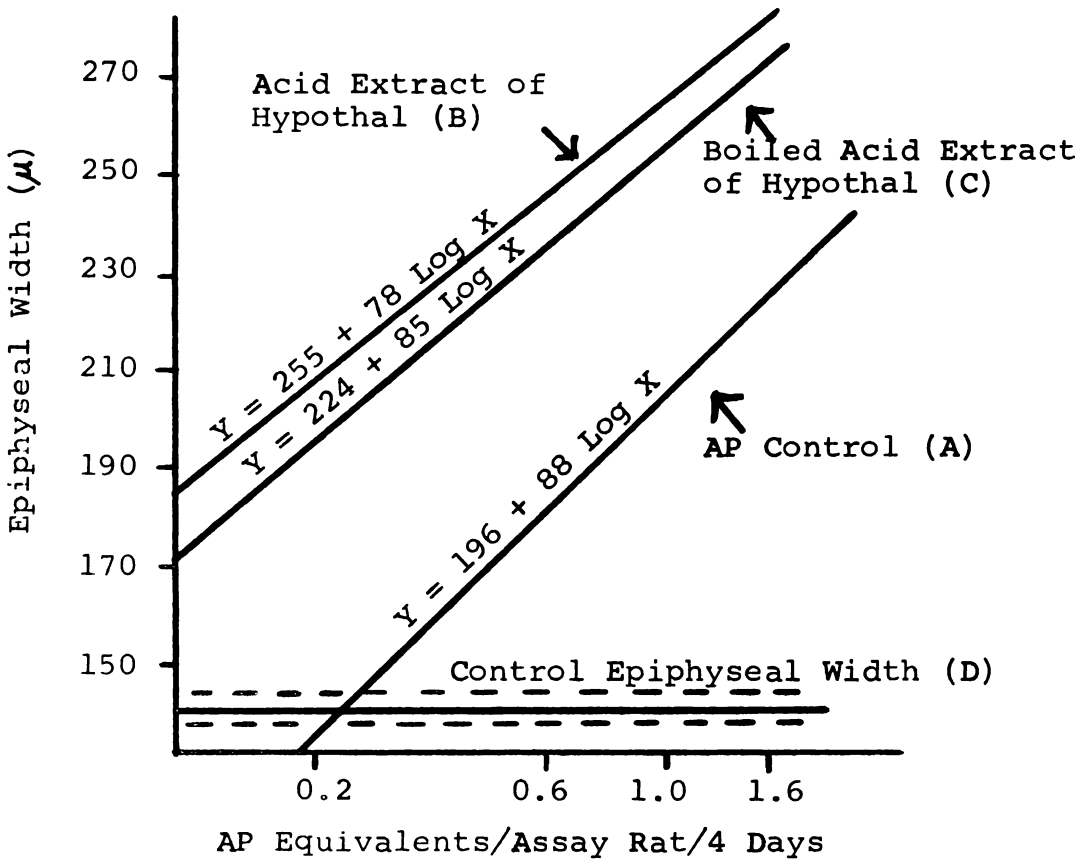

Fig. 2. Regression lines demonstrating the effect of an acid extract of hypothalamus and boiled hypothalamus on growth hormone release (AP = anterior pituitory).

Experiment 3. Comparison of an Acid Extract of Rat Hypothalamus with an Acid Extract of Rat Brain Neocortex with Regard to STH-Releasing Activity

It was necessary to determine if the hypothalamic growth hormone releasing factor was unique to the hypothalamic area or if it was a non-specific property of acid extracts of other brain tissue. Hypothalamic tissue from rats was excised and extracted in the manner already described. An equivalent amount by weight of brain neocortical tissue was similarly prepared from the same donors.

Table 3 and Fig. 3 gives the results of this experiment. Calculation of the relative potencies of the regression lines showed that release of growth hormone from the anterior pituitaries cultured with brain neocortical extract (Group C) did not differ significantly from those pituitaries cultured without an extract (Group A). However the acid extract of rat hypothalamus induced a 4-fold increase in STH release (Group B).

A point of interest is that the cultures with the cerebral cortical extract released the same amount of growth hormone as the cultures without an extract, therefore negating the possibility of an osmotic difference as the cause of the increased release of STH by the hypothalamic extracts.

The above results demonstrate that the STH-releasing factor is present in the hypothalamus but not in cerebral cortical tissue, or at least not in sufficient concentration

to elicit a measurable response by the standard assay technique employed. These results are therefore in accord with the findings of others concerning the hypothalamus as the source of neurohumoral production (Nalbandov, 1963).

Table 3. Effects of acid extracts of rat cerebral cortex and hypothalamus on STH release by rat pituitary *in vitro*.

Group	No. of Assay Animals	Dose AP Equivalent /Assay Animal /4 day	Response μ ± S.E.	Per Cent of Control (P = 0.05)
A. AP Control (Control)	5	0.2	184 ± 11	
	5	0.6	206 ± 8	100
	4	1.6	234 ± 8	
B. AP + acid extract of rat hypothalamus	5	0.2	217 ± 6	
	5	0.6	239 ± 9	395
	5	1.6	255 ± 3	
C. AP + acid extract of rat cortex	4	0.2	186 ± 7	
	3	0.6	205 ± 9	100
	5	1.6	222 ± 6	
D. Hypophysectomized Assay Controls	15	0	116 ± 3	---

Slopes of the regression lines were not significantly different.

Elevations of the following:

A *vs* B : $P < 0.01$
A *vs* C : $P > 0.05$

AP = anterior pituitary

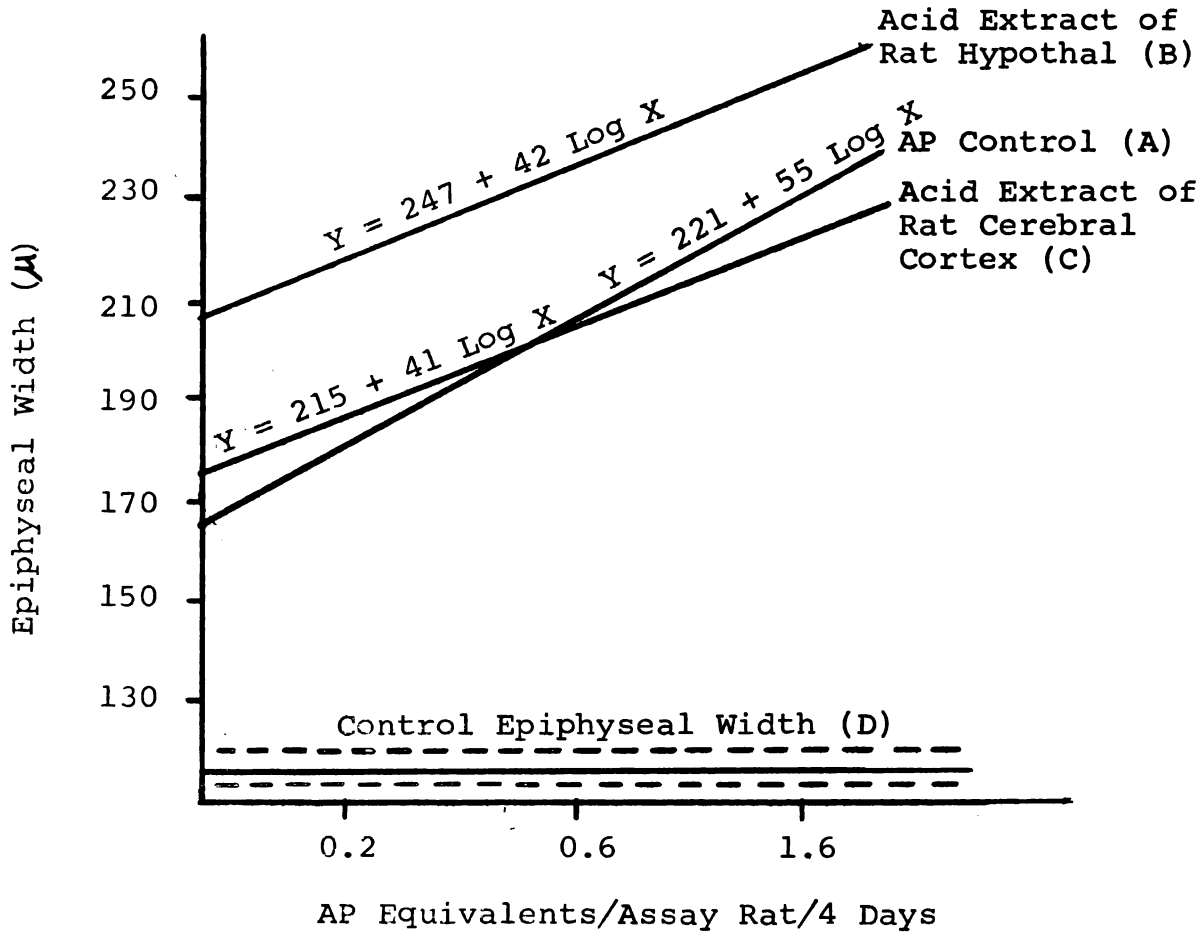

Fig. 3. Regression lines demonstrating the effect of acid extracts of rat cerebral cortex and hypothalamus on growth hormone release.

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Experiment 4. Reinitiation of STH Release from Rat Anterior Pituitary by an Acid Extract of Rat Hypothalamus in Vitro

The question was posed as to whether an acid extract of rat hypothalamus could reinitiate STH secretion from the cultured hypophysis after it had fallen to the low level observed in experiment 1. If it could do this, it would disprove the possibility that the progressively reduced rate of release from unstimulated pituitary resulted entirely from progressive necrosis, and would further substantiate the existence of hypothalamic SRF.

Two groups of 12 anterior pituitaries were cultured. Both groups contained an equal number of explants from the same rat pituitaries. The medium from each group was collected separately each 3 days and frozen. The culture was maintained for 9 full days. On day 6, Group A received an acid extract of 2 rat hypothalami per anterior pituitary. Group B received an equivalent amount by volume of NaCl preparation (12 ml 0.1 N HCl plus 1.2 ml 1 N NaOH). The medium was treated as described previously and assayed for growth hormone release.

The results are presented in Table 4 and Fig. 4. In group B-1 it can be seen that most of the growth hormone was released during the first 3 days of culture. In groups B-2 and B-3, during the second and third days of culture, there is essentially no detectable growth hormone activity.

However when the acid extract of rat hypothalamus was incorporated into the medium at the beginning of the third 3 days of culture, growth hormone release was reinitiated (Group A-3). In fact 78% as much growth hormone activity was found in the medium during the last 3 days of culture (A-3) as during the first 3 days (A-1), even though release had essentially ceased during the second 3-day period (A-2).

This experiment demonstrates that the cultured rat anterior pituitary cannot secrete growth hormone at a detectable rate after the first 3 days of culture without the stimulation provided by an acid soluble substance(s) in the hypothalamus. This is believed to provide cogent evidence for the existence of SRF in rat hypothalamus.

Table 4. Effect of adding extracts of rat hypothalamus on the third 3-day period of a 9-day culture on STH released by rat anterior pituitary.

Group	No. of Assay Animals	Dose AP Equivalent/ Assay Animal/ 4 day	Response μ ± S.E.	Per Cent of Control (P = 0.05)
A-1. AP				
(Cultured)	5	0.2	150 ± 8	100
1st 3 days	5	0.6	170 ± 15	
(Control)	5	1.6	228 ± 5	
A-2. AP				
(Cultured)	5	0.2	145 ± 5	
2nd 3 days	5	0.6	157 ± 12	NS
	4	1.6	164 ± 3	
A-3. AP				
(Cultured)	4	0.2	149 ± 5	
3rd 3 days +	3	0.6	177 ± 7	78
acid extract of rat hypothalamus	5	1.6	199 ± 6	
B-1. AP				
(Cultured)	5	0.2	158 ± 8	
1st 3 days	5	0.6	186 ± 12	---
	5	1.6	210 ± 11	
B-2. AP				
(Cultured)	5	0.2	153 ± 6	
2nd 3 days	4	0.6	138 ± 13	NS
	4	1.6	151 ± 7	
B-3. AP				
(Cultured)	4	0.2	132 ± 2	
3rd 3 days	5	0.6	159 ± 5	NS
	5	1.6	154 ± 4	
C. Hypophysectomized Controls	5	---	131 ± 4	---

Slopes of A-1, A-3, and B-1 are not significantly different, P = 0.01.

Slopes of A-2, B-2 and B-3 are significantly different from the above, P = 0.05.

AP = anterior pituitary

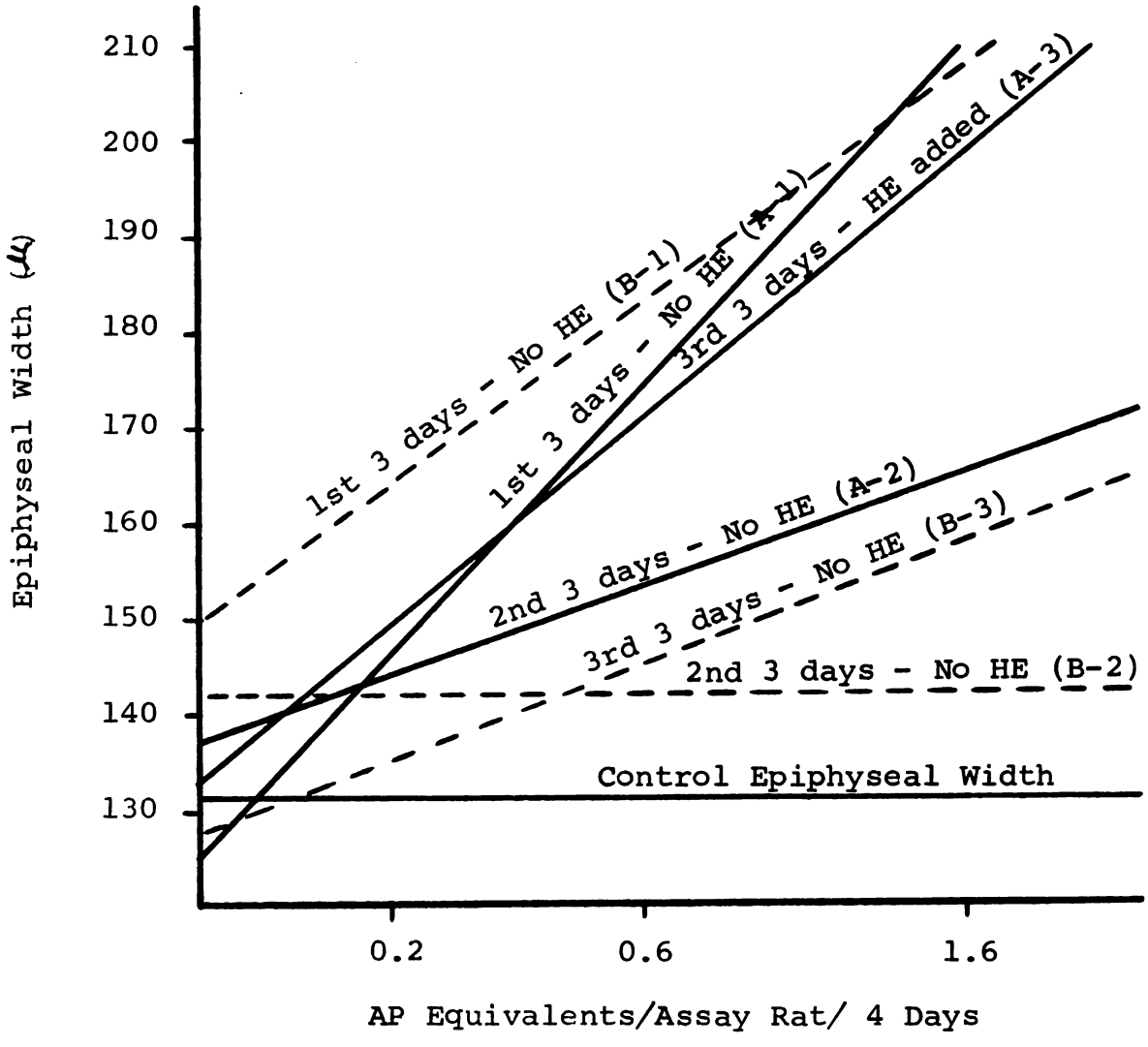

Fig. 4. Regression lines demonstrating the reinitiation of growth hormone secretion in vitro by rat anterior pituitary upon addition of an acid extract of rat hypothalamus (HE = hypothalamic extract).

Experiment 5. Effect of NIH Prolactin on the Tibia Assay

It is known that other anterior pituitary hormones may interfere with the tibia assay for growth hormone. Since prolactin has been shown to be released in large quantities in this culture system (Nicoll, 1962) it was considered relevant to determine the possible magnitude of this interference.

A total of 50 IU of NIH ovine prolactin was dissolved in 60 ml of synthetic medium 199. This corresponds to the amount of prolactin released into the medium by 10 anterior pituitaries from mature cycling rats cultured for 6 days in our system (2.5 IU/ 3 days/ anterior pituitary). The solution was dialyzed against running tap water for 20 hours at 4° C, lyophilized and diluted with distilled water to 3 dose levels for injection into the assay rats.

The results are given in Table 5. The 2 higher dose levels of prolactin stimulated epiphysial cartilage width to a small degree; however this increase did not exceed the 40μ limit used to establish growth hormone activity. The results presented here are in excellent agreement with those of Geshwind and Li (1955) and indicate that prolactin does not produce a typical dose-response curve, and can stimulate the epiphyseal width only to a limited extent.

It is also pertinent that acid extracts of rat hypothalamus greatly inhibits prolactin synthesis and release by

the cultured anterior pituitary (Talwalker, Ratner and Meites, 1963) so that probably very little prolactin was present in these cultures. It can be concluded therefore, that prolactin did not contribute to any significant degree to the growth hormone activity recovered in the medium.

Stimulation of gonadotropins and/or adrenocorticotropin release by hypothalamic extracts into the medium would have inhibited the tibia response to growth hormone (Geshwind and Li, 1955). Therefore, the release of STH from the cultured anterior pituitary tissue may be even greater than reported. Thyrotropin does cause a positive response in the tibia test, but extremely large amounts are required for this effect (Geshwind and Li, 1955). Since it seems likely that cultured anterior pituitaries release only very small amounts of TSH in vitro (Florsheim, Imagawa and Greer, 1957), the effect on the tibia test in these assays was probably negligible. Therefore it is believed that in these assays it is primarily growth hormone that was measured.

Table 5. Effect of prolactin on epiphyseal cartilage width.

Group	IU NIH Prol.* /Rat /4 days	No. of Assay Animals	Response $\mu \pm$ S.E.
A	1	4	171 ± 14
B	3	5	197 ± 13
C	6	5	194 ± 2
D	Hypophysectomized Assay Controls	6	156 ± 10

*Prol. = prolactin

Experiment 6. Effect of an Acid Extract of Rat Hypothalamus on the Tibia Assay

To rule out the possibility that the acid extract itself may be eliciting the widening of the epiphysis in the hypophysectomized rats, the following experiment was designed. Hypothalami from 24 rats were removed and homogenized in synthetic medium 199. This was dialyzed, lyophilized and diluted to the 3 dose levels, as described in the previous experiments, for injection into the assay rats.

Table 6 summarizes the results. It can be observed that the hypothalamic extract slightly stimulated the epiphyseal width. However, as in the last experiment, the increases observed (Group A) did not exceed the controls (Group B) by the 40 microns required for establishment of significance (Srebnik, Nelson and Simpson, 1959). Therefore the hypothalamic extract *per se* which was incorporated into the medium in the previous experiments is not believed to have appreciably influenced the assay results.

Table 6. Effect of acid extract of hypothalamus on epiphyseal cartilage width in hypophysectomized STH assay rats.

Group	Dose Total Hypothalamus / Assay Animal / 4 days	No. of Assay Animals	Response $\mu \pm$ S.E.
A. Acid extract of Hypothalamus	0.4	5	145 ± 5
	1.2	5	137 ± 1
	2.4	5	156 ± 4
B. Hypophysectomized Assay Controls	None	5	124 ± 2

Experiment 7. Effect of an Intracarotid Injection of an Acid Extract of Rat Hypothalamus on Anterior Pituitary Growth Hormone Content

Martini and co-workers (personal communication to Dr. J. Meites) injected an acid extract of rat hypothalamus directly into the carotid artery of rats and measured the growth hormone activity remaining in the anterior pituitary after 15 minutes. They found this reduced the STH content of the pituitary. To check the potency of our extracts *in vivo* this procedure was repeated.

Acid extracts were made of 6 rat hypothalami in 3 ml of 0.1 N HCl. The solution was neutralized with 1 N NaOH by titration with 1 drop of neutral red as the indicator. The left carotid artery of 2 rats was surgically exposed and injected with 0.5 ml of extract. Two similar rats by weight were handled identically and received a sham injection (all substances present except hypothalamus). About 1/2 hour after the injections the animals were decapitated and the anterior pituitaries were removed. They were quickly frozen on dry ice and placed in the freezer until ready for assay.

The pituitaries were homogenized in physiological saline so that each assay rat received 1/4 anterior pituitary during the 4 day period. Only a one point assay was run for this experiment; regression analysis was therefore impossible (Table 7). However, Student's t test showed that the pituitaries from the rats receiving the extract of hypothalamus (Group A) via the carotid artery had significantly less

growth hormone present than the pituitaries from the rats receiving the saline injection (Group B). The cartilage width of groups A and B were significantly greater than in the hypophysectomized controls (Group C).

Table 7. Effect of an intracarotid injection of an acid extract of hypothalamus on anterior pituitary growth hormone content of the intact rat.

Group	No. of Assay Animals	Response $\mu \pm$ S.E.
A. Sham Injected Controls	6	212 $\pm$ 7
B. Acid Extract of Hypothalamus	6	173 $\pm$ 7
C. Hypophysectomized Controls	8	130 $\pm$ 3

GENERAL DISCUSSION

The results of the present experiments provided evidence for the existence of a growth hormone releasing factor in the hypothalamus. Most of the previous reports have been complicated by the involvement of the animal. Indirect methods such as the measurement of growth rates or pituitary growth hormone content under varied experimental conditions were used in an attempt to understand the CNS relationship to growth hormone release by the anterior pituitary. Although the results suggested a positive role for the central nervous system in the secretion of growth hormone by the pituitary, no direct evidence for the existance of SRF was demonstrated *in vivo*. The *in vitro* techniques used in the present experiments made it possible to test the effectiveness of an acid extract of hypothalamus directly on the rat anterior pituitary and to measure the actual amount of growth hormone released.

In the first experiment it was demonstrated that the anterior pituitary is capable of secreting some growth hormone *in vitro*. In fact 167% more was recovered from the medium than was originally placed into it. This is much

less than prolactin, since 10 times more prolactin could be recovered daily from the medium than was originally placed into the culture system. However it has been shown that prolactin release is chronically inhibited by the hypothalamus (Talwalker, Ratner and Meites, 1963), and therefore it can be expected that more prolactin would be released by the pituitary when removed from hypothalamic inhibition.

It was apparent from these experiments that the rate of growth hormone release dropped off markedly after the pituitary was cultured for a period of a few days. If an acid extract of rat hypothalamus was incorporated into the medium after the release rate had fallen to insignificant levels, growth hormone release into the medium was reestablished to almost the original _in vitro_ level.

These _in vitro_ results demonstrate that acid extracts of rat hypothalamus can produce a 4-to 6-fold increase in STH release by cultured rat anterior pituitary. This activity could not be found in an acid extract of an equivalent amount of rat cerebral cortical tissue, indicating that this area of the rat brain does not contain STH-releasing activity.

When an acid extract of rat hypothalamus was boiled, the releasing activity was reduced by about one third. One might be tempted to attribute this to an actual loss of STH present in the extract since boiling does destroy growth hormone activity (Geshwind and Li, 1955); however, injection

of this acid extract directly into hypophysectomized rats did not cause a significant increase in epiphyseal width. This indicates that no STH was present in the extract *per se*. Furthermore, work by Courrier *et al*. (1963) has demonstrated that acid extraction of sheep hypothalamus removes ACTH, TSH and LH activity which may be present. More work on the effect of temperature on the activity of the releasing factor should be done. However, its relative resistance to boiling suggests that it may be a small molecule, perhaps a polypeptide similar to the other hypothalamic factors isolated thus far (Guillemin, 1956; McCann, 1962; Schreiber *et al*., 1963; Schally, Lipscomb and Guillemin, 1962.). This factor is also active *in vivo* when injected via the carotid artery demonstrating that it is not an artifact of the culture system.

The evidence presented here indicates that an acid soluble substance(s) in the hypothalamus can evoke the release of anterior pituitary STH both *in vitro* and *in vivo*. The existence of a discrete molecule in the hypothalamus controlling the release of STH remains to be established. Only chemical isolation and synthesis will prove its identity and specificity.

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