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Somatostatin and growth hormone releasing factor differentially regulate the release of immunoassayable and bioassayable growth hormone secretion from two populations of rat pituitary somatotrophs

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somatotrophs**

Tietjen, Glenn Howard, M.S.

San Jose State University, 1990

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SOMATOSTATIN AND GROWTH HORMONE RELEASING FACTOR
DIFFERENTIALLY REGULATE THE RELEASE OF IMMUNOASSAYABLE AND
BIOASSAYABLE GROWTH HORMONE SECRETION FROM
TWO POPULATIONS OF RAT PITUITARY SOMATOTROPHS

A Thesis

Presented to

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San Jose State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts

By

Glenn H. Tietjen

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Abstract

The rat pituitary contains at least 2 distinct populations of somatotrophs. These cells secrete growth hormone (GH) with different activities as measured by radioimmunoassay (RIA), and as measured by tibial bioassay (BA). Type 1 somatotrophs (density 1.050-1.070 g/cc) secrete GH with a low BA/RIA ratio. By contrast, type 2 somatotrophs (density 1.071-1.084 g/cc) secrete GH with a high BA/RIA ratio. Rat plasma bioassayable GH (BGH) is elevated and immunoassayable GH (IGH) relatively unchanged in response to stresses, suggesting a differential regulation of these two cell types. The main purpose of this study was to determine if rat GRF and SRIF selectively regulate secretion of IGH and BGH from these two cell types. In order to test that hypothesis, pituitary cells were separated into 2 populations on density gradients. Mixed, type 1, and type 2 cells were then cultured (4 h) in the presence of GRF, SRIF, or GRF + SRIF. Media were assayed by both RIA and BA and data were expressed as ng GH per 1000 somatotrophs. The experiment was reproduced with essentially identical results. Data verified that in untreated cells BGH is the major form of hormone secreted by type 2 cells, which secrete 3-5 times more BGH than type 1 cells. GRF stimulated secretion of both BGH and IGH by both cell types. Treatment with SRIF inhibited BGH and IGH secretion 41-68% in type 1 cells and IGH secretion in type 2 cells, whereas SRIF doses as high as 10 nM did not inhibit secretion of BGH by type 2 cells. Most importantly, combined treatments with GRF and SRIF produced a

shift in the BGH/IGH ratio for secreted GH. The shift differed depending upon cell type. In mixed and type 2 cells the combined treatments favored release of BGH, while in type 1 cells BGH was undetectable during these treatments. The fact that BGH and IGH secretion from type 1 and type 2 somatotrophs was reflected in the mixed cell data was the apparent result of differences in regulation of the two cell types. Since there is evidence that both SRIF and GRF are released during environmental stress in rats, these results could explain the relatively greater release of BGH vs. IGH observed during stress in rats.

I. INTRODUCTION AND LITERATURE REVIEW

Over the past seven decades researchers have made considerable progress toward understanding the chemistry and physiology of growth hormone (GH). Since the reports of the first reliable assays for GH (all bioassays) in the late 1930's, a major form of GH has been purified from pituitaries, sequenced, and used in the production of antisera for specific immunoassays. The availability of purified GH has made possible the careful study of GH variants and fragments enriched or depleted of growth-promoting activity. Purified GH has also been used extensively to study the direct and indirect effects of GH on various tissues. The availability of specific antisera has reduced the amount of GH required for assay by about a thousand-times, and made it possible to rapidly identify GH or GH-like molecules in nitrocellulose trans-blot of electrophoretic gels and in chromatographic eluents. More recently, GH antisera has been used for definitive identification of pituitary somatotrophs. The success in GH research since the advent of the immunoassay in the early 1960's has been quite rapid and profitable, but two important, and as yet unresolved problems appear to have been overlooked: 1) the ratio of GH activity measured by tibial bioassay to that measured by radioimmunoassay (RIA) ranges from 1 to >400 depending upon sampling site, and 2) under certain physiological conditions there is selective release of GH with activity in the tibial bioassay and relatively little activity in the immunoassay. This literature review presents evidence that there

are at least 2 forms of GH that can be independently released. The evidence that the variants are released from 2 different somatotroph cell types is also discussed. Finally, the experimental hypothesis that the 2 somatotroph types are differentially controlled by the hypothalamic regulatory peptides, somatostatin (SRIF) and growth hormone releasing factor (GRF) is presented.

A Note on Nomenclature

There is much inconsistency in terminology found in published literature relating to this thesis. The major growth-promoting protein of the pituitary has been variously called somatotrophin (STH), somatotropin (STH), and growth hormone (GH). In this thesis this protein will be referred to as GH. Similarly, the regulatory protein which stimulates GH release has been called somatoliberin, growth hormone releasing hormone (GRH), growth hormone releasing factor (GRF), somatotropin releasing hormone (SRH), somatotropin releasing factor (SRF), and somatocrinin. This protein will be referred to as GRF since this term is most frequently used in recent reports. The GH release inhibiting peptide, somatostatin, has been called growth hormone release inhibiting hormone (GH-RIH), and somatotropin release inhibiting factor (SRIF). The latter term will be used in this thesis.

Measurement of GH

The presence of GH or a growth-promoting substance was originally surmised from the existence of giants, acromegalics, and dwarfs [34]. Hypophysectomy of rats and other animals produced failure of skeletal growth and weight gain [34]. Experiments involving injection of alkaline pituitary extracts into hypophysectomized rats to restore growth indicated that the pituitary is the source of GH [34]. In several early studies, injection of pituitary homogenates restored growth following hypophysectomy [34]. In 1935 bovine GH was partially purified [59]. The availability of partially purified GH made it possible to establish bioassays based on various physiological effects of GH such as body weight increase, tail-length increase, widening of tibial epiphyseal cartilage, organ weight increase (e.g. thymus), change in nitrogen balance, mobilization of fatty acids from adipose tissue, and diabetogenic effects [58]. The tibial assay method of Greenspan, *et al.* [33], using purified GH, has been the most widely used bioassay procedure to date. It involves the administration of standard amounts of GH (typically ranging from 5 to 45 μg of a highly purified hormone) to 35-44 day old hypophysectomized female rats over 4 days. Typically the rats are hypophysectomized at 26 days of age. When the tibia is removed, longitudinally sectioned, stained, and measured via ocular micrometry the log of the total dose plotted versus the mean tibial epiphyseal cartilage width gives a linear dose-response relationship over a broad range.

The assay is generally considered to be specific for GH, though thyroxine exerts a synergistic effect, and adrenocorticotropin has an inhibitory effect on tibial growth [58]. Simultaneous daily doses of 0.25 µg of thyroxine and 6.25 µg of bovine GH caused a significant increase in tibial width from the GH alone controls [58]. The inhibitory effects of adrenocorticotropin are not apparent with doses as high as 25 µg per day [58] and for this reason should be considered insignificant. Moreover, adrenocorticotropin is rapidly destroyed in blood and media.

Controversy has surrounded the issue of whether the tibial growth response is due to a direct GH effect, or is mediated by circulating somatomedins. Grindeland *et al.* [35] injected somatomedin A over the 4 day assay period at a dose of 450 units¹ and did not observe significant growth relative to saline controls. However, Schoenle *et al.* [94] found significant increases in tibial epiphyseal width with 43 µg/day doses of IGF-I (insulin-like growth factor-I), and 131 µg/day doses of IGF-II (insulin-like growth factor II). These are relatively large "pharmacological" doses of IGF, and probably never occur physiologically. Isaksson *et al.* [46, 47] showed that direct epiphyseal injections of 10 µg of GH on days 14, 16, and 18 post-hypophysectomy produced a highly significant increase in the tibial epiphyseal width as compared to the saline injected control epiphysis on the opposite leg of the same animals.

¹One unit of somatomedin is equal to 1 mL of normal serum activity. The somatomedin used was a Kabi (Stockholm, Sweden) preparation.

This experiment suggests that GH can directly stimulate longitudinal bone growth. It may be that GH initiates local production of IGF-I, as it has been shown that antisera to IGF-I blocks GH-induced collagen synthesis in neonatal calvarial osteoblast-like cells [26]. Despite potential interference from some of the agents mentioned, the tibial bioassay of GH appears highly specific to GH. Indeed, in a 1977 review on GH bioassay, Li [58] concluded by stating: "It appears that procedures involving biological assay will continue to be essential for the detection of GH activity, and it seems certain that they will never be replaced entirely by immunological methods in endocrinological research."

In 1966 the RIA for rat GH was first reported [89]. The antisera used in the immunoassay are raised against GH obtained primarily from the pituitary granule fraction. The availability of specific antisera has reduced the amount of GH required for assay by about a thousand-times, and made it possible to rapidly identify GH or GH-like molecules in nitrocellulose trans-bLOTS of electrophoretic gels and in chromatographic eluents. While the thousands of GH physiology research papers since 1964 attest to the significance of the immunoassay, it has become apparent through diligent use of the tibial bioassay that the immunoassay does not measure all forms of circulating GH. If some GH is modified upon or after secretion, is covalently or non-covalently polymerized, or is released through a pathway which bypasses the secretory granules, then it is possible that variants could exist and it is possible that

the antisera used in current immunoassays does not detect all GH forms. Indeed, there is evidence, to be discussed below, that all of the aforementioned processes occur in the rat. The presence of GH variants would, perhaps, be of little interest if all forms were co-released and had equivalent biopotency. However, as will be described in the section on GH variants, this is not the case.

The late 1960's through the early 1970's was a period of great excitement in biological research. Using the immunoassay it was possible to readily measure numerous biomolecules which could previously be measured only with great difficulty in biological samples. Soon a flood of reports on various aspects of GH physiology appeared in the journals, accelerated in part by the rapidity with which results could be obtained. Perhaps fueled by the great excitement over the new assay procedure, a widespread partiality in favor of the immunoassay emerged. In the face of discrepancies between immunoassay and bioassay results (to be discussed below) most researchers either a) assumed equivalence between GH measured by tibial assay (BGH) and GH measured by immunoassay (IGH), or b) favored the immunoassay results over the bioassay results. Malacara, *et al.* [62] failed to detect depletion of IGH from rat pituitaries following injection of the equivalent of extracts of 25 hypothalami, though significantly lesser amounts had been shown to deplete BGH. They summarized the prevailing attitude when they stated that "...we would interpret the results of the present experiment to mean that when an authentic growth

hormone releasing factor is identified, it will cause a discharge of immunoassayable GH into the blood with or without an associated depletion of immunoassayable GH content of the pituitary." It appears that they implicitly considered bioassay results to be insignificant. The researchers went on to criticize the results of Schally *et al.* [92] who reported a decapeptide with GH releasing activity as measured by bioassay, but inconsistently measurable by immunoassay. More will be said about these discrepancies below; the goal here is to introduce the reader to a prevalent bias within the literature. In spite of this bias, studies of GH using only RIA have provided a tremendous amount of useful information. The GH RIA has been an invaluable tool.

The Hypothalamic-Pituitary Axis In the Control of GH Release

It is now dogma that hormone-secreting cells of the anterior pituitary are regulated directly by polypeptides or monoamines released from the hypothalamus. Numerous experimental observations have led to the view that hypothalamic releasing factors are secreted into the hypothalamic-pituitary portal vessels following integration of neural and metabolic signals, and that cells of the pituitary are subjected to high concentrations of these factors which stimulate or inhibit specific hormone release. Transection of the pituitary stalk or placement of a barrier between the pituitary and hypothalamus causes atrophy of the pituitary [41],

while revascularization restores normal morphology and hormone secretion [41]. Wislocki and King [115] were the first to report that blood in the hypothalamic portal vessel flows from the hypothalamus to the anterior pituitary. Known release-inhibiting, or releasing factors, are found in the highest concentrations in the portal blood, as indicated by studies involving *in vitro* incubation of hemipituitaries with portal or peripheral plasma followed by assay for pituitary hormones [75]. Microcannulation of the portal vessels of rats [76] has made it possible to administer hypothalamic extract (HE) or putative secretagogues *in vivo*, thus bypassing the hypothalamus. These studies have shown that hypothalamic regulatory factors directly regulate hormone release from the anterior pituitary [75]. Fluorescein dye-linked antibodies or enzyme-conjugated antibodies to releasing factors have been used to localize hypothalamic cell bodies responsible for secretion of these factors. Antisera to a recently isolated GRF has been used to localize GRF neurosecretory cell bodies in the arcuate nucleus [11]. Lesions in the arcuate nucleus significantly reduce basal plasma IGH levels [63]. In addition to factors of the hypothalamic-pituitary axis, other substances control pituitary hormone release. With GH it has been shown that somatomedin C (IGF-1), which is primarily derived from the liver, stimulates hypothalamic somatostatin release, and directly (but more slowly) inhibits IGH release from the pituitary [8].

Studies with Hypothalamic Extracts

As with other studies of hypothalamic regulatory peptides, the first studies of GH regulation utilized HE to determine the presence of releasing factor(s). Deuben and Meites first showed that in 6-day cultures of rat pituitary explants, addition of an acid extract of the hypothalamus significantly increased release of BGH into the medium [22]. Other researchers reported similar results using rat HE and tibial assay in *in vitro* studies of various duration [85] and *in vivo* studies with rat HE [70], guinea pig HE [64, 48] and beef HE [48]. *In vitro* studies have used pituitary explants sectioned into halves, quarters, or eighths, or they have used dissociated pituitary cells. *In vivo* studies have typically involved systemic venous or carotid arterial injections of HE. Radioimmunoassay has yielded similar, but sometimes conflicting results [61]. *In vivo* injections of porcine HE in rats caused release of IGH [61, 62]. Porcine, ovine, and human HE have been shown to cause release of IGH when injected *in vivo* into female Rhesus monkeys [50]. Russell, *et al.* [84], using both RIA and tibial assay found that HE from hypophysectomized rats stimulated the release of BGH from rat adenohypophyses 2 to 2.75-times, whereas RIA showed increased GH secretion by HE in only 2 of 4 experiments and to a lesser degree (1.4 to 1.6-times). It should be noted that most *in vivo* studies of BGH have used depletion of pituitary BGH as an index of release. It should also be noted that Malacara *et al.* [62] had to estrogen-progesterone "prime" their rats in order to observe a significant

effect in response to *in vivo* injection of 10 and 25 hypothalamic equivalents (one hypothalamic equivalent equals the extract from one hypothalamus).

These early reports clearly indicate the presence of GRF in HE, but they are important to reexamine for other reasons. The results of Russell, *et al.* [84] demonstrate that HE causes greater release of BGH than IGH, thus suggesting that some species of GH may not be detectable by RIA. The collective *in vivo* and *in vitro* studies of BGH release indicate that the pituitary depletion method can reflect release of GH into culture media. Moreover, Parkhie, *et al.* [70] found a linear log-dose relationship in a 6-point *in vivo* assay using 6 doses of HE, offering further support for the validity of the depletion method, and countering some of the criticisms of the depletion method [78, 81]. There are 2 principal reasons why researchers have relied upon the depletion assay; 1) large volumes of plasma are required in order to obtain enough GH for detection by tibial bioassay, and 2) exposure of plasma to glassware causes some conversion which renders it toxic to recipient animals [35]. The latter problem can be remedied by siliconizing glassware [35]. Indeed, much of the criticism of depletion methods can be explained by not assuming that all GH with bioactivity possesses immunoreactivity with antisera raised against pituitary granule-derived GH, or by assuming that there are potency differences amongst GH variants. These possibilities are discussed below.

Studies with Purified GRF

The early work with hypothalamic extracts inevitably led to attempts at isolating the active GRF. Excellent reviews of this work are available [30, 40, 107]. Some of the earliest notable results were those of Sawano *et al.* [87]. Working with a GRF purified from porcine hypothalami and later shown to be a decapeptide [91], they demonstrated that intracarotid injection of 4 μ g of this GRF in rats caused a significant pituitary depletion and corresponding plasma elevation of BGH [87]. This was later replicated using smaller doses. It was shown that intracarotid injections of as little as 80 ng would cause significant pituitary depletion, while 400 ng would cause a significant plasma elevation of BGH [67]. In other work it was shown that injection of the decapeptide caused a significant lowering of plasma IGH and a significant elevation of plasma BGH 15 minutes after injection in rats. The pituitary depletion was in excellent quantitative agreement with the plasma elevation [109]. In similar *in vitro* work with hemipituitaries using RIA, tibial assay, and incorporation of ^{35}S into costal cartilage (as assays) corresponding *in vivo* results were not consistent. However, increasing doses of the decapeptide, both natural extract-derived and synthetic, generally caused a significant release of BGH with little or no effect on IGH release [91]. Other *in vitro* experiments have demonstrated that the decapeptide stimulates IGH release, though the effect on BGH is greater [110]. Schally has reported that the GRF was highly

effective in promoting the *in vitro* release of BGH but relatively inactive in stimulating the release of IGH, an effect which should cause the secreted GH to have a high BGH/IGH ratio [91]. As already mentioned, a bias that BGH is equivalent to IGH was clearly prevalent at this time. The failure of Schally's decapeptide to consistently release IGH caused most researchers to disregard this "GRF" as insignificant or artifactual and to search for other factors.

In 1982 a human GRF was isolated from a pancreatic tumor in a patient exhibiting symptoms of acromegaly [79]. The major protein obtained was sequenced and shown to consist of 40 amino acid residues, while minor fractions contained 37 and 44 residues. The GRFs were synthesized using solid phase technology and tested in cell culture. Maximal response doses as low as 0.1 nM resulted in an 8-times increase in the release of IGH relative to controls. Antisera to the 40 residue GRF has been used to localize immunoreactive cell bodies in the arcuate nucleus of the rhesus monkey hypothalamus. In the rat, lesions in this area impair [11, 28], and electrical stimulation increases [28] IGH release. In 1983 a rat GRF was isolated from a pool of 80,000 hypothalami [100]. As with human GRF it has been sequenced, synthesized, and tested in cell culture. The rat GRF has 43 amino acid residues, has 70% sequence homology to human GRF-40 (hGRF-40), and is similar in potency to hGRF-40 in cell culture. The GRF has been localized to the arcuate nucleus of the rat hypothalamus using immunocytochemical methods [63]. Since the original discoveries,

numerous studies have verified the specificity of these GRFs for GH release. The experimental animal used has typically been the rat, though clinical trials in humans are now underway. The results of the original research have been verified in numerous *in vitro* studies [2, 9, 13, 12, 15, 18, 95, 100, 108, 111] and *in vivo* studies [16, 112, reviewed in 30]. Clearly hGRF-40 and rGRF-43 are physiologically important peptides along with somatostatin, the GH release inhibiting peptide (14 residues) in control of GH secretion. However, to date there are no publications citing the effects of these GRFs on the release of BGH. This is unfortunate considering the discrepancies between assay systems mentioned above.

Somatostatin

The cyclic tetradecapeptide, somatostatin, was originally isolated from ovine hypothalami during the search for the hypothetical GRF [14]. It was subsequently isolated from porcine hypothalami, and its sequence was shown to be identical to ovine somatostatin [93]. During the process of purification, SRIF was measured in various fractions by *in vitro* bioassay based on the inhibition of release of IGH. It is now synthesized using solid phase methods [80]. Several reviews have summarized the numerous effects of SRIF including inhibition of GH release, inhibition of insulin release, inhibition of thyroid stimulating hormone (TSH) release, as well as numerous effects on the gastrointestinal tract [71, 93]. It is evident that SRIF is physiologically important in the

regulation of GH release since *in vivo* administration of antiserum to SRIF causes an elevation of plasma IGH [1, 105, 106]. Somatostatin suppresses both basal and GRF-stimulated IGH secretion *in vitro* [9, 51, 108]. It has been shown in *in vitro* perfusion studies that SRIF withdrawal causes a short-lived burst of IGH release [51, 101]. The amplitude of the burst is increased with addition of GRF [51] and with increasing SRIF concentrations [101]. The effects of SRIF on the release of BGH either *in vivo* or *in vitro* have not been determined.

In rats, "stress" causes an inhibition of IGH release as will be described in the next section. Since SRIF decreases IGH secretion *in vitro* to levels below control secretion, SRIF secretion is probably increased during stress in rats. More direct evidence in support of the aforementioned comes from studies in which SRIF antiserum was injected prior to stress. Injection of SRIF antiserum caused reversal of stress-induced IGH suppression due to i.p. injection of saline (used as a stressor) [106], 30 minutes forced swimming [106], 72-hour fasting [105], bleeding [1], and electroshock [1]. These findings suggest that increased hypothalamic SRIF release occurs during stress in the rat. Though BGH was not measured in the experiments described above, it seems likely that BGH and IGH release are differentially affected by SRIF alone, or in combination with GRF, since it has been shown indirectly that hypothalamic GRF release is also increased following stress in rats [49, 53; see next section].

Growth Hormone Release During Stress, Hypoglycemia, and Spaceflight

In various *in vivo* "stress" conditions there have been discrepancies between GH immunoassay and bioassay results. This has included both hypothalamic extract and GRF injections (see above). In these studies "stress" is defined as exposure to cold, fasting, and insulin-induced hypoglycemia, spaceflight, and various pathological disease states in humans.

In primates cold exposure, fasting, insulin-induced hypoglycemia, and administration of 2-deoxyglucose produce elevation of plasma IGH [32, 82, 83, 88, 90]. This is not the case with rats, however. Using the pituitary depletion method and the tibial assay, BGH has been shown to be depleted (as much as 35%) under a variety of stresses to rats including 30-60 minutes exposure to cold [54], fasting [54], insulin-induced hypoglycemia [52, 68, 104] and administration of 2-deoxyglucose [104]. Pecile *et al.* [72] provided an excellent review of the numerous studies demonstrating BGH release during stress in rats, and the associated release of GRF from the hypothalamus. Muller, *et al.* [66] reviewed secretion of BGH and IGH. Their review contains an excellent discussion of several studies demonstrating BGH, but not IGH pituitary depletion during stress, and failure to detect elevated plasma IGH during stress. The phenomenon has also been observed in hypoglycemic or cold-stressed guinea pigs, but not in mice [65].

Similar depletion has not been detected by immunoassay [19, 35, 66, 90, 104]. Though fewer investigations of plasma GH have been done, it has been shown that cold-stress or insulin-induced hypoglycemia cause a significant plasma BGH elevation [24] and no elevation (or a decrease) of plasma IGH concentration [19, 24, 29, 35].

Hypoglycemia has been shown to cause release of GRF into the blood of hypophysectomized rats. These studies used injection of plasma of experimental animals into the assay test animals with subsequent pituitary depletion bioassay after 30 minutes [53]. This indicates that the stress (hypoglycemia) effects are probably mediated by releasing factor activity. This interpretation has been confirmed by bioassay of pituitary homogenates of rats treated with HE from hypoglycemic rats [49]. While the pituitary depletion assay has been criticized due to occasional inconsistent results or failure of plasma IGH elevation in the presence of pituitary BGH depletion [20, 77], the aforementioned results are understandable if one does not assume an equivalence of BGH to IGH. Chemical studies, to be discussed below, indicate that the two are different.

An unusual opportunity for study of GH regulation has emerged with the onset of modern spaceflight and its host of associated physiological problems. In light of reports of bone and muscle loss following spaceflight of 7-days duration, the GH system has been the subject of intensive investigation. Seven days of spaceflight produced a significant elevation of intracellular (somatotroph) IGH concentration [37]. When flight pituitary cells were implanted into

the brain ventricles of hypophysectomized recipient rats there was decreased growth over a 2 week period relative to control animals. Most important, however, is the observation that following high pressure liquid chromatography (HPLC) fractionation of 6 day pituitary cell culture media, it was found that the flight cells released significantly less of a large molecular weight ($\approx 100\text{kD}$) GH variant rich in biological activity, but relatively devoid of immunoactivity. Whatever the source of regulation, it appears that release of this large molecular weight variant was preferentially impaired. This could, in part, account for bone loss and muscle atrophy.

Plasma GH Concentrations and Unique Cell Culture Experiments

In a recently completed study by Bauman and Grindeland [5] human plasma BGH and IGH determinations were made under several conditions; normal resting, hypophysectomized, deep sleep (stage 4), GRF-treated, DOPA-treated, and acromegaly. The BGH values (ng/mL) presented in the above respective order were: <100 to 780; undetectable; <333 to 825; 667 to 5200; 500 to 540; and 2020 to 2965. The corresponding IGH values (ng/mL) were: 8.1 to 10.2; undetectable; 15 to 63; 5 to 11; and 28 to 132. No correlation was found between BGH and somatomedin C or somatomedin A. Evaluation of the percentage increase and absolute increase over normal GH levels in the DOPA and GRF-treated subjects (not shown)

revealed that these treatments produced a much larger percentage increase for IGH secretion than for BGH secretion. However, in terms of absolute amounts of GH, the above treatments are more potent for BGH secretion. These data are suggestive of differential regulation of BGH and IGH secretion in humans.

Plasma BGH/IGH ratios in humans have been reported to be about 200:1 in normal subjects [24, 23], while the value ranges from 30-400:1 in rats, depending upon sampling site [23, 25, 109]. Measurements of granular (somatotroph) and pituitary homogenate GH have consistently revealed a BGH/IGH of 1 [23, 35, 110]. Measurements of pituitary cytosol fractions yield a BGH/IGH ratio of 1.7 while measurements in media from dissociated, cultured mixed cells indicate that the ratio increases to 2 or slightly greater [23,110]. The increases in the ratio of BGH/IGH have been interpreted by some researchers as evidence that GH is modified upon secretion [110]. A recent study by Grindeland and Hymer [36] indicated that the highest obtainable BGH/IGH ratio for secreted GH was about 5 in purified somatotrophs. This reported ratio is significantly lower than the plasma ratios mentioned above. It is most likely that GH is not only modified upon secretion, but also after secretion. The work of Grindeland and Ellis [35] has shown that the tibial growth response to injections of GH in hypophysectomized rats could be blocked with trasylol injections (a broad-spectrum proteolytic inhibitor of trypsin like enzymes). Growth responses due to injections of native bovine and native rat

GH could be blocked with trasylol, whereas no inhibitory effect on growth was observed following injection of plasmin-degraded rat GH or plasma from intact rats. Similar results were found by Vodian and Nicoll [110]. These results indicate that rat tibial growth is due to a GH form which differs from native rat GH isolated from the pituitary. The results of Grindeland and Ellis [35] and Vodian and Nicoll [110] also indicate that some transformation of GH occurs at an extrapituitary site, and offer an explanation of why the BGH/IGH ratio for secreted GH is significantly lower than plasma levels.

Chemical Studies of GH Variants

There have been numerous studies on the chemical nature of GH variants. The methods employed are diverse and include HPLC, electrophoretic techniques, affinity and gel chromatography, and immunological techniques. While it is unfortunate that BGH has been neglected, good reviews on human GH are available [56, 57]. A truly comprehensive review of this literature would be extremely lengthy, and is beyond the scope of this thesis. Nevertheless, it is worthwhile to mention select studies which demonstrate the complexity of the GH system.

Recent experiments have indicated that a 22 kD phosphorylated form of GH [60] and a 26-27 kD glycosylated form of GH [97] is synthesized and released by rat pituitary glands. Western blot analysis of 2-dimensional polyacrylamide gels has indicated

the presence of about 20 different immunoreactive forms (or fragments) of GH [43, 117]. The physiological implications of these results are unclear at present. Some immunoreactive species may represent biologically inactive fragments resulting from degradation. Interpretations of plasma studies are now complicated by the recent evidence for a serum binding protein specific for GH [3, 4, 55].

Ellis and Grindeland have shown that plasma human GH with a high BGH/IGH ratio (490-680) elutes in a position indicative of a large molecular weight protein (>100kD) following gel chromatography [23]. This has been independently verified in human pituitary extracts using HPLC [44]. The large M.W. human protein(s) with high BGH/IGH activity is/are essentially devoid of prolactin activity, whereas the classic 22 kD human GH possesses about 4 international units of prolactin activity per milligram [43]. Following affinity chromatography on a column prepared with anti-GH sera, Grindeland *et al.* [35] have shown that considerable growth-promoting activity is still present when the eluate is measured by tibial assay. They have also shown that the growth of young, normal rats cannot be suppressed by antisera to immunoassayable pituitary granule-derived GH [23]. It appears that a significant proportion of the pituitary-derived growth-promoting activity in plasma does not react with GH antisera.

Russell *et al.* [85] processed media and tissue from pituitary explants by polyacrylamide discontinuous gel electrophoresis, and

analyzed eluates for BGH and IGH. They found that in the tissue 80-99% of the GH was found in segments with an Rf range of 0.3-0.6, whereas in the media, both assays recorded a more cathodal distribution of most of the hormone (85-96%) in the Rf region 0.00-0.42. The tissue contained GH that had a high BGH/IGH ratio (11.1-25.7; Rf 0-0.29), whereas the media contained GH with an even higher ratio (32.7-33.8; Rf 0.57-0.68). These results indicate that GH exists in electrophoretically heterogeneous forms which differ in BGH/IGH activity ratios and that the BGH/IGH ratio increases as GH leaves the intracellular compartment.

Stachura *et al.* [102] have shown that at least two pools of GH exist; a storage pool, and an early release pool which is not stored. The results were obtained through use of a double pulse-labeling method in *in vitro* perfusion experiments. The first label, administered for 2 hours prior to the second label, was ^{14}C -Leucine, while the second label (1 hr later) was ^3H -Leucine. Collection of eluate began immediately after the second label. Following gel chromatography (Sephadex G-200) of dialyzed perfusion eluates, they found different results for the 0-40 minute than for the 80-120 minute eluates. At both times ^{14}C -GH eluted at approximately 25 kD, while in the 0-40 minute fraction ^3H -GH eluted at 40-45 kD. In the 80-120 minute fraction there was an emergence of a 25kD ^3H -GH peak. The 40 kD form is not simply a dimer, as some 40kD ^{14}C -GH would have been observed in the 0-40 minute eluate, but was not. The results demonstrate the presence

of more than one path for GH release, each path releasing a different GH form.

While the results of Stachura *et al.* indicate that at least one large molecular weight form of GH is not a dimer, other studies have indicated that some polymerization occurs. Stolar *et al.* [103] found that immunoreactive forms of GH with Mr (relative molecular weight) of 45, 62, 80, and 110 kD in SDS-PAGE (sodium dodecyl-sulfate polyacrylamide gel electrophoresis) could be nearly 100% converted to 22 kD (major) and 20kD (minor) forms with sulfhydryl reduction. Beitins *et al.* [6] found that injection of radiolabeled monomeric 22 kD GH into humans led to a near 100% conversion to larger molecular weight moieties (majority was >100 kD). *In vitro* incubation in plasma produced a similar conversion, though the timecourse was slower. Incubation in serum albumin or gamma-globulin was without effect. Benviste *et al.* [7] have shown that mercaptoethanol treatment of large M.W. GH obtained from 2 human pituitaries resulted in a 60% conversion to 22 kD GH. Further dissociation was obtained by combined treatment with the reducing agent plus urea. It appears that at least some of the larger M.W. GH is a result of interchain disulfide linkages. Whether or not this is a regulated process remains speculative. The interpretation of these results is difficult. The analysis has been complicated by the fact that very seldom does a researcher employ bioassay and immunoassay in the same experiment. Indeed, many researchers use affinity chromatography to purify GH used in their chemical studies.

The probability that non-immunoreactive forms of GH are removed during these procedures is very real in light of the studies of Grindeland *et al.* (40, see above) which indicated that considerable growth promoting activity is not retained by GH affinity columns. The apparent inability of GH antisera to react with some forms of GH should not be surprising in light of the fact that antisera is raised against pituitary GH where the BGH/IGH ratio is approximately one. Further studies and replication of others using both assay procedures is necessary before the chemical nature of GH can be thoroughly understood.

While the biological significance of these GH variants remains unclear, differences in activities of variants at different tissues could, in part, be responsible for the diversity of physiological effects of GH including increased transport of amino acids across the plasma membrane of many cells, increased protein synthesis, mitosis of thymocytes, proliferation of chondrocytes and osteoblasts, and early hypoglycemic and delayed anti-insulin effects (such as hyperglycemia and mobilization of fatty acids from adipocytes). It has been reported recently that there are 2 classes of GH receptors in rat livers with K_d values of 8.0×10^{-12} M and 1.6×10^{-12} M, and corresponding binding capacities of 4.34 and 12.4 femtomoles/mg protein, respectively [86]. The presence of more than one GH receptor type could be a means by which GH variants differentially mediate GH effects as postulated above.

Neurotransmitters and Regulation of GH Release

Direct experimental evidence for differential regulation of the release of GH variants has been provided by Grindeland and his colleagues [39]. They used jugular and lateral brain ventricle cannulation of rats to inject (into ventricle) 5, 10, or 25 μg of serotonin (5HT), 5 μg of dopamine (DA), or saline and collect plasma samples at 5, 15, 30, and 60 minutes via the jugular cannula. Saline had no effect compared to controls, while 5 μg of 5HT depleted 25% of the intrapituitary BGH at 5 minutes, and 50% at 15, 30 and 60 minutes. Dopamine depleted 50% of the hormone at all measured times. Plasma BGH concentration corresponded to the pituitary depletion. The only rise in plasma IGH values occurred 30 minutes after 5HT injection, which followed by 15 minutes the nadir of pituitary GH concentration. In another experiment using similar methodology, the results with 5HT and DA were replicated, but it was additionally found that 5 μg of 5HT at time zero, followed by 5 μg of DA at 15 minutes produced a 90% depletion of pituitary BGH in conscious normal rats. When the same experiment was done with intraventricular injections of noradrenergic agonists, there was approximately equal depletion of both BGH and IGH. These results suggest that two pools of GH exist which may correspond to different cell types (see below). In another experiment it was found that cold exposure or insulin injections had no effect on serum IGH, but significantly increased BGH concentrations with a concomitant decrease in pituitary BGH. This lack of effect on IGH in rats is in

contrast to the observed elevation of IGH in humans during insulin injections or cold-stress. Cold exposure or insulin injections accompanied by intraventricular injections of a 5HT antagonist or DA antagonist produced different results. The DA antagonist completely blocked the rise in plasma BGH and the decrease in pituitary BGH, whereas the 5HT antagonist had only a slight attenuating effect on BGH release. These results, as well as those mentioned earlier, indicate that some differential regulation of the release of BGH and IGH exists in the rat. They also indicate that the release of regulatory peptides governing release of GH variants is under differential neuronal control. It appears that the observed increases in plasma BGH during cold-stress and insulin injections is mediated by a dopaminergic system, even though activation of a serotonergic system increases BGH release in normal rats.

Studies on Two Populations of Somatotrophs

Insight into the mechanism of the presumed differential regulation of GH release was provided by the work of Snyder *et al.* [98, 99], Grindeland *et al.* [36] and Hymer [45]. In these studies dissociated pituitary cells were separated on the basis of density using a bovine serum albumin (BSA) density gradient procedure. In this scheme, 1.5 mL of cells plus media are applied directly on top of an upper layer of 1.5 mL of 1.071 g/mL BSA, which resides above a 1.5 mL layer of 1.085 g/mL BSA solution. The entire system is centrifuged for 30 minutes at 1000 X G at 4°C. Centrifugation

produces a pellet and two bands of cells at the solution interfaces. The bands are separately collected and the pellet discarded. The lower density cells (1.050-1.070 g/mL) are called type 1 somatotrophs, while the higher density cells (1.071-1.084 g/mL) are called type 2 somatotrophs. Numerous *in vitro* and *in vivo* studies have been done. The high density cells represent 55-65% of pituitary somatotrophs, are densely granulated, increase IGH secretion in response to thyroxine and cAMP but not dexamethasone, secrete hormone with a high BGH/IGH ratio (4.5 in 4-day cultures), and are far more synthetically active than type 1 cells. By contrast, the type 1 cells are sparsely granulated but have an extensive endoplasmic reticulum and Golgi apparatus, increase secretion in response to dexamethasone and cAMP but not thyroxine, secrete hormone with a low BGH/IGH ratio (0.50 in 4-day culture), and are far less synthetically active than type 2 cells. Recently, Farrington and Hymer reported that when extracts of type 2 cells were treated with the reducing agent β -mercaptoethanol, there was a three to four-times increase in immunoactivity, whereas the same treatments were without effect on type 1 somatotrophs [27]. Pre- and post-reduction western blot analyses indicated that variants greater than 25 kD were eliminated following reduction. Since reduction of culture media from type 2 cells also caused a 3-5-times increase in immunoactivity, it was suggested that the variant released from type 2 cells is a large M.W. disulfide-linked aggregate of monomeric GH forms. The main differences between cell types

are summarized in Table 1. Since the type 2 somatotroph releases GH with a high BGH/IGH ratio, it would seem that the selective release of BGH is mediated by this cell type. Indeed, when donor rats were subjected to 4°C for 30 minutes followed by dissociation and separation of pituitary cells, it was found that the only difference between control and cold-treated cells in 4-day cultures was that the release of BGH from the type 2 cell was significantly less than controls [38]. The mechanism of hypothalamic control of this phenomenon is unknown.

TABLE 1
Comparison of Two Types of Rat Somatotrophs

	<u>TYPE 1</u>	<u>TYPE 2</u>
Density (g/mL)	1.050-1.070	1.071-1.084
Secretory Granules	Few	Many
Appearance (transmission- electron microscopy)	Abundant Regions of RER (rough endoplasmic reticulum) and Golgi Apparatus	Few Regions of RER and Golgi Apparatus
Dibutyryl cAMP (3.0 mM)	Stimulated IGH Secretion	Stimulated IGH Secretion
Somatostatin (10^{-7} M)	Inhibited IGH Secretion	Inhibited IGH Secretion
Thyroxine (10^{-6} M)	No Effect	Stimulated IGH Secretion
Hydrocortisone	Increased cell IGH	No effect

Adapted from Snyder *et al.* [99]

Summary

Rats secrete two or more structural forms of growth hormone (GH). One class of GH, immunoactive GH (IGH), is detectable by immunoassays with antisera raised against GH purified from pituitary granules. A second class of GH, bioactive GH (BGH), is measurable by the hypophysectomized rat tibial bioassay, and has low or no crossreactivity to the antisera used in the IGH radioimmunoassays. In rats, cold exposure, fasting, insulin-induced hypoglycemia, or administration of 2-deoxyglucose do not produce elevation of plasma IGH. Yet, when rat GH is measured by tibial bioassay it is found that the aforementioned stressors cause a significant depletion of pituitary BGH (as much as 50%) and a corresponding elevation of plasma BGH. The inability to detect increases in plasma IGH suggests that plasma GH in rats differs from pituitary granule GH. The main reason for failure in rats to detect plasma IGH increases under these conditions is probably due to the fact that the antisera used in GH radioimmunoassays is raised against GH derived from the secretory granules of pituitary homogenates. In the pituitary granule fraction of rats the ratio of BGH/IGH activities is about 1.0, whereas in the nongranular cytosol fraction the ratio increases to 1.7, and in pituitary cell culture media the ratio is as high as 5. In rat plasma the BGH/IGH ratio can be as high as 400:1 depending upon sampling site. Clearly there are differences in biological and immunological activities of pituitary and plasma GH in the rat.

In primates, stimuli such as cold exposure, fasting, and insulin-induced hypoglycemia produce an elevation of plasma IGH. Though the physiology of GH secretion in primates differs from rats in this respect, the major form of circulating GH in humans is also BGH. The plasma BGH and IGH values in resting humans are <100 to 780 ng/mL, and ≤ 0.8 ng/mL, respectively, while in GRF-treated humans the corresponding values are 667 to 5200 ng/mL, and 15 to 63 ng/mL. GRF treatments produce a larger percentage increase in IGH secretion, but a larger *absolute amount* increase in BGH secretion relative to controls. It appears, therefore, that in humans there is differential regulation of BGH and IGH secretion, suggesting that the physiology of GH secretion in humans and rats is probably more similar than different.

Growth hormone is now considered by most researchers to be a heterogeneous family of proteins. This subject has been extensively reviewed elsewhere for human GH, but not for rat GH. Rat GH exists as monomeric 22 kD, monomeric 40 kD, 45 kD interchain disulfide dimer forms, and covalent and noncovalent oligomers of up to 100 kD. Recently, glycosylated, and phosphorylated forms have also been detected. Most researchers use antisera raised against pituitary-derived GH at some point in their various approaches to the study of GH variants, without concurrent bioassay. Variants devoid of immunoactivity would not be detected by these assays. While the biological significance of these GH variants remains unclear, it is tempting to speculate that their existence parallels the diversity of

physiological effects of GH including increased transport of amino acids across the plasma membrane of many cells, increased protein synthesis, mitosis of thymocytes, proliferation of chondrocytes and osteoblasts, and early hypoglycemic and delayed anti-insulin effects such as hyperglycemia and mobilization of fatty acids from adipocytes.

The hypophysectomized rat tibial bioassay measures only the growth-promoting activity of GH; specifically, the effects on chondrocyte proliferation. "Classical" GH, derived from the pituitary, possesses growth promoting activity, and is used as the reference standard for the bioassay. Secretion of BGH and IGH may or may not parallel each other, as indicated. Differences between pituitary and plasma BGH/IGH ratios must, therefore, represent either loss of immunoactivity initially present, enhancement of growth promoting activity, selective release of a GH form devoid of immunoactivity, or a combination of these. It is clear that the existing immunoassays alone do not adequately reflect GH physiology in the rat. For this reason it is important that rat GH be measured by both bioassay and immunoassay.

Investigations over the past 15 years have led to the separation of somatotrophs into two functionally distinct populations using bovine serum albumin (BSA) density gradient techniques. The lower density cells (1.050-1.068 g/mL) are called type 1 somatotrophs, while the higher density cells (1.071-1.084 g/mL) are called type 2 somatotrophs. Type 1 cells represent 40-

45% of pituitary somatotrophs, while type 2 cells represent 55-60% of pituitary somatotrophs. Somatostatin (SRIF) has been shown to decrease IGH release from both cell types, while dibutyl cyclic AMP has been shown to increase IGH release. The effects of these agents on BGH release are unknown. Hydrocortisone and thyroxine have differential effects on the two cell types, the former increases intracellular IGH in type one cells only while the latter stimulates IGH secretion in type two cells only. These results suggest a potential heterogeneity in control of these two somatotroph types. Importantly, the type 2 cells secrete significantly more GH (2-6 times) over a 4-day culture period than do the type 1 cells. The BGH/IGH ratio for secreted GH in 4-day cultures is about 5 in type 2 cells and about 0.5 in type 1 cells. The type 2 cells thus release more of a GH variant which is rich in bioactivity but relatively devoid of immunoactivity.

Hypothesis

In 1980 rat hypothalamic GH releasing factor (GRF) was purified to homogeneity and subsequently sequenced and synthesized by solid phase techniques. In *in vitro* and *in vivo* studies the GRF caused release of IGH with associated increases in growth. Therefore, it seems unlikely that hypothalamic release of this particular GRF (43 amino acid residues) can explain the phenomenon of selective BGH release in the stress experiments mentioned above. Yet there is evidence that GRF is released during stress in rats.

SRIF is also released during stress in rats. It may be that in those experiments which have reported selective BGH release, that the type 2 somatotroph is preferentially stimulated by a certain ratio of concentrations of hypothalamic GRF and SRIF, and that when the concentration ratio is appropriate, differential suppression of IGH release from the type 1 somatotroph occurs. This is a reasonable conclusion if the sensitivities to GRF and SRIF differ between type 1 and type 2 cells. Therefore, the purpose of this thesis is to test the hypothesis that the appropriate dose combination of GRF and SRIF stimulates release of BGH from type 2 somatotrophs with little or no increase in release of IGH from type 1 somatotrophs. Alternatively, it may be that another, as yet unspecified releasing factor differentially regulates the two somatotroph populations.

II. MATERIALS AND METHODS

Pituitary Donors.

Male Sprague-Dawley derived rats (Simonsen; Gilroy, CA) were used in all experiments, except where otherwise noted. Rats were typically 240-260 g at the time of termination. All rats were housed in groups of 5 in temperature controlled rooms (24-26°C). Food (Wayne Lab-blox F-6) and water was provided *ad libitum*. Animal care was provided by the Animal Care Facility at NASA-Ames Research Center and all experiments were approved by the Institutional Animal Care and Use Committee, NASA-Ames Research Center. On the day of each experiment, all animals were killed by decapitation within a 1-hour period occurring between 6 and 9 AM. After opening the cranium with poultry shears (to remove the brain), the heads were transferred to an aseptic surgical field, where separate workers removed the pituitaries. The neurohypophysis was separated and discarded, and the adenohypophysis was transferred to Petri dishes containing filter-sterilized media A (see Appendix A). After pituitaries were collected from the donors, the dissociation procedures were carried out under sterile conditions within a laminar flow hood.

Preparation of Adenohypophyseal Cell Suspensions.

The procedures for cell dissociation are described by Wilfinger, *et al.* [114] with modifications by Snyder, *et al.* [99]. All

glassware was siliconized with Sigmacote™ (Cat. no. SL-2, Sigma Chemical Co.; St. Louis, MO) to reduce cell attachment and to eliminate potential toxicity to bioassay rats subsequently injected with cell culture media (see introduction and literature review: studies with hypothalamic extracts). Within a laminar flow hood, adenohipophyses were transferred to a sterile Petri dish and cut into 1.0-1.5 mm³ blocks with a sterile razor blade. The blocks were transferred to a 25 mL Spinner flask (Cat. no. 1967-00025; Bellco Glass, Vineland, NJ) containing 10 mL of filter-sterilized trypsin-containing media B (see appendix A). Not more than 20 adenohipophyses were transferred to each Spinner flask. The flasks were stirred constantly for 45 minutes at ≈100 r.p.m. on a magnetic stirrer unit, then the partially dissociated cells were transferred with media and 100 µL of sterile 2 mg/mL DNase to a 15 mL centrifuge tube and centrifuged using a table-top centrifuge for 10 minutes at ≈ 2500 X G. After centrifugation, the supernatant was discarded and the pellet was resuspended in filter-sterilized media C (see appendix A) by triturating (slowly drawing the suspension into a Pasteur pipette and then ejecting it) 50 times with a sterile Pasteur pipette. The triturating also served to mechanically dissociate the partially dispersed cells. After triturating, any adenohipophysis pieces were allowed to settle for 2-3 minutes. The cell suspension was next collected and transferred to a sterile 15 mL centrifuge tube. Soybean Trypsin Inhibitor (100 µL of 1 mg/mL soybean trypsin inhibitor [Cat. no. T-9003; Sigma Chemical Co., St.

Louis, MO] in media A) was added to the cell suspension to inhibit further proteolysis. The pieces were transferred back to the Spinner flask with 10 mL of fresh trypsin-containing media B for a second 45 minute dissociation. After the second dissociation, the fresh cell suspension was obtained and processed exactly as the first suspension. The cell suspension obtained was pooled with cells from the first dissociation. After the second dissociation few, if any, pituitary pieces remained. In all cases any remaining pieces were discarded. Typical pituitary cell yield was 3 million cells per rat. Consequently, the stock cell suspension obtained after the second dissociation was diluted to a volume equivalent to 1.0 mL per donor rat, giving a final concentration of approximately 3 million cells per mL of media C.

Cell Counting, Dilutions, and Treatments.

The stock cell suspension was triturated several times, and a small aliquot was accurately counted using a hemocytometer. Once the cell concentration in the stock cell suspension was determined, the remaining procedures were performed. For mixed pituitary cell experiments, the cells were diluted to a final cell concentration of 1.5×10^6 cells per 5.0 mL of media C (see appendix A). Five mL aliquots of the mixed cell suspension were transferred to sterile 15 mL centrifuge tubes. The suspension was triturated 5 times between aliquots to assure homogeneity. The tubes were then centrifuged for 10 minutes at 2500 X G, and the supernatant was

discarded. Five mL aliquots of fresh media C alone, or supplemented with various concentrations of GRF, SRIF, or both, were then added to the cell pellets. The pellets and media were triturated 20 times with a Pasteur pipette, and the suspension was quantitatively transferred to 70 mL culture flasks (25 cm² surface area, cat. no. 25100; Corning, Palo Alto, CA). During all experiments, except where otherwise noted, flasks of cells in media were incubated with the cap of the flask turned to the position which enables gas exchange with the atmosphere. The flasks were incubated at 37°C in the presence of humidified 5% CO₂ and 95% air.

For the cell separation procedure, aliquots of the stock cell suspension were centrifuged for 10 minutes at 2500 X G using a table top centrifuge, supernatant was discarded, and replaced with cold media A to produce a final concentration of 1×10^7 cells per 1.5 mL. The cells obtained were pooled and triturated 50 times to produce a homogeneous gradient cell stock suspension for the density gradient procedure.

Density Gradient Cell Separation Procedure.

The bovine serum albumin (BSA) density gradient cell separation procedure has been described in detail by Hymer [45]. The procedure of Shortman [96] was used to prepare the BSA. The BSA stock solution (see appendix A) was gradually diluted with unbalanced salt solution (UBSS; see appendix A) and the refractive index was determined using a Baush and Lomb Abbe refractometer

(Cat. No. 33.45.71). The dilution procedure was repeated until the correct refractive index was obtained. The equation for converting refractive index to density, as described by Shortman [96], was:

$$\text{Density} = (10^4 n_{\text{sample}} - 6263.2) / 7047.2$$

where n_{sample} is the refractive index of the sample solution, as determined by the refractometer. When solutions of the correct density were obtained, the samples were delivered to a laminar flow hood where 1.5 mL aliquots of the 1.085 g/mL BSA solution were transferred to 15 mL sterile plastic centrifuge tubes and stored frozen (-5°C). The 1.071 g/mL BSA solution was also stored frozen (-5°C). On the day before an experiment the solutions were thawed overnight under refrigeration (4°C) and mixed prior to use to ensure homogeneity. All solutions were kept at 4°C to ensure correct density. A 1.5 mL aliquot of 1.071 g/mL BSA solution was carefully layered on top of the 1.085 g/mL layer, and a 1.5 mL aliquot of the gradient cell suspension was carefully layered on top of the 1.071 g/mL layer. The entire system was carefully transferred to a 4°C refrigerated centrifuge, and centrifuged at 1000 X G for 30 minutes. The majority of cells sedimented in 3 locations within the system: at the interface between 1.071 g/mL BSA and media C (type 1 cells); at the interface between 1.085 g/mL and 1.071 g/mL BSA (Type 2 cells); and a pellet at the bottom of the tube. In all cases the pellet was discarded. Type 1 and type 2 cells were carefully removed with a sterile Pasteur pipette in a laminar flow hood. The dense BSA solutions and cells were diluted with fresh media A, triturated 50

times with a Pasteur pipette, transferred to 15 mL sterile plastic centrifuge tubes, centrifuged 10 minutes at 1000 X G (22-24°C), and the supernatant was discarded. The type 1 and type 2 cell pellets were resuspended to an approximate concentration of 3 million cells per mL of media C, triturated 50 times, or until all "clumps" were separated, and counted on a hemocytometer. Once accurate cell concentrations were determined for type 1 and type 2 cell stock suspensions, the concentrations were adjusted to 3.6×10^6 cells per 5.0 mL media C and 0.6×10^6 cells per 5.0 mL media C, respectively¹. At this point the remaining procedures were identical to those mentioned for mixed cells (see above).

Cell Extracts for Intracellular GH Determination.

In many experiments both start and post-treatment extracts of cells were made to determine intracellular GH concentrations. For mixed cell start extracts 3.0×10^6 cells were typically extracted in 10.0 mLs of alkaline solution. For type 1 and 2 cells, 7.2×10^6 and 1.2×10^6 cells, respectively, were extracted in 10.0 mL of alkaline solution. In the early experiments the alkaline solution used was 0.01 M NaHCO₃, but this was later replaced with 0.01 M Na₂CO₃ since GH is more soluble in solutions of higher pH. The cells were incubated overnight (4°C for 12-18 h), followed by centrifugation

¹ Choice of cell concentration was designed to keep the number of somatotrophs constant between cell types. W. Hymer has shown that when mixed somatotrophs are cultured with increasing numbers of 3T3 fibroblasts, secretion of IGH remains constant when expressed on a per somatotroph basis (unpublished).

and, finally, collection of the supernatant which was kept frozen until assay.

Viability Determination.

Viability determinations were made using a neutral red dye uptake procedure. In this method viable cells actively transport neutral red into their interior, thus producing stained cells. Non-viable cells remain unstained. The viability media were prepared on the day of each experiment by mixing 0.3 mL of the neutral red stock solution (see appendix A) with 9.7 mL of media C. Aliquots of cells were incubated for 20-40 minutes in viability media at room temperature. After the incubation, differential cell counts were made on a hemocytometer. Viability estimates were typically 85% or better.

Immunoperoxidase Staining of Somatotrophs

The immunoperoxidase method for staining pituitary cells described by Denef *et al.* [21], was used in these studies with minor modifications. Aliquots of 50,000 cells per 100 μ L media C were placed onto 12 mm round polylysine-coated coverslips in multiwell culture plates (24-well plates, Linbro Cat. #76-033-05; Flow Laboratories, McLean, VA) and allowed to incubate for 1 hour before fixation. The coverslips had been previously prepared by soaking in 95% ethanol for 10 minutes, washing with distilled water, and then spreading the coverslips into a monolayer in a Petri dish containing

0.01% w/v poly-L-lysine (HBr M.W. >300 kD, Cat. # P-1524; Sigma Chemical Co., St. Louis MO) for 30 minutes. The coverslips were then rinsed with distilled water and transferred to the 2 mL wells on the Linbro™ plates. The plates were placed into a laminar flow hood equipped with a U.V. lamp (2 Westinghouse Sterilamp 782L-30 lamps) and sterilized for 24 hours (2.5 feet from light source). The cells were fixed for 30 minutes with 0.5 mL/well of Zamboni's fluid (see appendix A). After the 30 minute fixation, the cells were washed two times quickly, followed by one 10 minute wash with 0.5 mL per well of phosphate buffered saline (PBS; see appendix A). At this point the cells were stored at 4°C for 24-48 hours. After this period, the cells were incubated for one hour in 0.5 mL per well of 0.15% phenylhydrazine HCl (pH 7.2; filtered through Whatman #1 filter paper) in PBS in order to inhibit endogenous peroxidase activity. The cells were then washed twice with 0.5 mL per well of PBS, and incubated at 4°C overnight in 0.5 mL per well of 1% crystalline BSA in PBS in order to decrease non-specific binding. Cells were then incubated for 36-60 hours at 4°C with 0.5 mL per well of primary antibody. The primary antisera used in these experiments was Rhesus monkey anti-rat pituitary GH (provided by R.E. Grindeland) at a dilution of 1/10,000 in 0.4% Triton X-100 (Sigma, St. Louis, MO) in Tris-NaCl buffer (6.005 g/L Tris, 30 g/L NaCl, pH 7.6). Non-immune monkey serum (obtained from Rhesus monkeys housed at the animal care facility; NASA Ames Research Center, Moffett Field, CA) at the same dilution served as a control.

After the incubation period, the cells were washed 3 times, 10 minutes each with 0.5 mL per well Tris-NaCl buffer. The cells were then incubated in 0.5 mL per well secondary antibody for 60 minutes at 37°C. The secondary antibody was goat peroxidase conjugated goat anti-monkey IgG (Cat. #3210-0081; Cappel, Malvern, PA) at a 1/200 dilution in 0.025% Triton X-100 in Tris-NaCl buffer. After the secondary antibody incubation the cells were washed 3 times, 10 minutes each with 0.5 mL per well Tris-NaCl buffer, and incubated for 1 hour at 37°C in 0.5 mL per well of tertiary antibody. The tertiary antibody was goat peroxidase-conjugated antiperoxidase (gPAP Cat. #0100-1221; Cappel, Malvern, PA) and served to amplify the staining activity when substrate was added. The cells were then washed 2 times, 10 minutes each with 0.5 mL per well of 0.025% Triton X-100 in Tris-NaCl buffer, and then washed 2 times, 10 minutes each with 0.5 mL per well of Tris-NaCl buffer. After the final wash step, 0.5 mL per well of substrate was added. The substrate solution was prepared by mixing 10 mg of 3,3'-diaminobenzidine-tetrachloride (Cat no. D-5637; Sigma Chemical Co., St. Louis, MO) in 50 mLs of Tris-NaCl, pH 7.6, for 30 minutes. At the end of the 30 minutes of mixing, 100 µL of 3% H₂O₂ was added and the substrate was delivered to the wells. The system was incubated at 37°C for 30-45 minutes, until the desired degree of staining was obtained. Once the cells were adequately stained, they were washed 3 times with distilled water and mounted onto glass microscope slides with 60°C Kaiser's glycerol (see appendix A) for

differential cell counting. The differential cell counting enabled GH secretion to be expressed on a per somatotrophs-seeded basis rather than a per cells-seeded basis. For each determination, at least 1000 cells were counted. In all cases the normal-monkey serum controls were unstained.

Radioimmunoassay of GH

The procedure for the radioimmunoassay of rat GH and its specificity has been described by Schalch and Reichlin [89]. The assay is a homologous double antibody RIA. The assay used in these experiments differed from that of Schalch and Reichlin in that the primary antibody (1/50,000 in BSA-phosphate buffer pH 7.5) was raised against highly purified rat pituitary granule-derived GH in rhesus monkeys¹. The primary antisera was freed of complement proteins by incubating at 56°C for 30 minutes. The precipitating second antibody was goat anti-monkey (Antibodies, Inc.; Davis, CA) at a 1/25 dilution. Normal monkey serum (obtained from Rhesus monkeys housed at the animal care facility at NASA Ames Research Center, Moffett Field, CA) at a 1/20 dilution in BSA-phosphate buffer pH 7.5 was used as a carrier to increase the mass of precipitate. Tracer GH was radiolabeled (¹²⁵I) by the chloramine T/metabisulfite method [42]. Radioactive counts per minute were quantitated using a Multi-Pras™ 4 gamma counter (Packard). The

¹The antiserum was raised against rGH with 3 U.S.P. units per milligram (preparation XVII-38-38C); supplied by R.E. Grindeland. The U.S.P. unit is defined in the footnote on p.45.

assay has a range of linearity (when the logit transformation is performed) from 1.6 to 50.0 ng/mL, with an ED₅₀ of 10 ng/mL. Interassay coefficient of variation, was 11% for experiments A-G, and < 6% for experiments H-J. The differences were most likely due to use of positive displacement pipettes in the latter assays, whereas positive displacement pipettes were not used in the earlier assays.

Bioassay of GH

The tibial bioassay using hypophysectomized rats was followed unmodified according to the procedures of Greenspan, *et al.* [33]. Twenty-six day old hypophysectomized rats were purchased from either Zivic-Miller (Zelienople, PA), Simonsen Laboratories (Gilroy, CA), or Charles River Laboratories (Portage, MI). The dose-response was statistically identical in rats from all suppliers, as was the epiphyseal width for saline-injected rats. In all assays, sample injections began 2 weeks post-hypophysectomy. The 2-week interval allowed for surgical recovery and allowed time to determine whether hypophysectomy was complete. Typically, if the rat weight was greater than 90 g, or the weight change over the 2-week period was greater than 15 g, the rat was not considered to be hypophysectomized and was not used in the assay. The injections occurred over a period of 4 days, with one 0.5 mL i.p. injection of sample or standard per rat per day. Bovine GH standards (XIV-44C5,

1.5 U.S.P. units per mg¹, supplied by R.E. Grindeland) were 45 µg, 15 µg, and 5 µg total injected over the 4-day period. On the fifth day, rats were terminated by CO₂ asphyxiation, and a single tibia was removed, placed on a wooden block with the dorsal surface down, and longitudinally sectioned with a razor blade to expose the proximal epiphysis. In cases where the right tibial epiphysis broke during sectioning, the left tibia was substituted. The tibias were then rinsed in 0.5 L of 0.85% w/v saline for 30 minutes, followed by distilled water (0.5 L) for 20 minutes to remove chloride ions. The tibias were then defatted in 100% acetone (0.5 L) for 10 minutes, rinsed again with distilled water for 10 minutes, and finally stained by immersion in 100 mL of 2.5% w/v AgNO₃ for 2.5 minutes, and briefly (30 sec.) rinsed in distilled water. The tibias were then placed in Petri dishes containing distilled water, and the cut surfaces were exposed to sunlight for 1-3 minutes to initiate the photostaining reaction. The sunlight exposure occurred at about 4 p.m. on the day of termination of bioassay rats. The duration of exposure depended upon the extent of darkening which had occurred prior to that step in the procedures. This silver staining method stains primarily mineralized collagen. The newly produced (i.e. non-mineralized) cartilage of the epiphysis remains white. Optimal photostaining is somewhat subjective depending on the microscopist,

¹One U.S.P. unit equals the amount of GH which, when injected over a 10-day period, produces a 30-35g weight change in a 100g male rat which has been hypophysectomized for 2 weeks.

but should yield maximal contrast between mineralized and non-mineralized collagen, thereby favoring consistency of measurement. After staining was completed, the tibias were then placed in vials containing 70% ethanol until measurements of the epiphyseal plate could be made via ocular micrometry. Ten equidistant measurements of a single growth plate were averaged to give a single value for each bioassay rat. The log of the total 4-day dose was plotted against the epiphyseal width to give a linear standard curve. In all values reported, readings of epiphyseal width were performed by Dr. R.E. Grindeland. Dr. Grindeland has had over 20 years experience with the assay and a single evaluator resulted in low assay variability. Pituitary-derived rat GH has a potency which is exactly twice that of the bovine standards [35]. This factor was used in the tibial assay to convert the bovine GH value into rat GH equivalents. In many cases, both high and low doses of sample were assayed so that the linearity of the dilution curve could be statistically compared to that of the standards with a multiple regression statistical computer program based on the method of Bliss [10].

Statistical analyses

The design of individual experiments was limited by three factors. Due to the approximately 1000-times lower sensitivity of the tibial bioassay as compared to the RIA, 1000-times more GH was required for assay than would be required in experiments using RIA alone. Secondly, the cell separation procedure required very large

numbers of cells (see Methods section; Density Gradient Cell Separation Procedure). Thirdly, due to the large number of cells required for each experiment and the number of experimental variables, the number of procedural operations per experiment was usually at the maximum possible. The aforementioned factors made it impossible to obtain a large enough sample size for valid analysis of variance (see individual experiments below). Statistical comparisons of triplicate data point determinations would not be valid in experiments in which cells were derived from a common donor pool. For the reasons above, comparisons between treatment groups were normalized and expressed as percent-differences. The most important experiments were replicated to demonstrate the reliability of results (see individual experiments below).

Experiment A: Mixed Cell BGH and IGH Secretion in Response to hpGRF-40

The purpose of this experiment was to examine the effects of human pancreatic tumor-derived hpGRF-40 (from a human tumor extract) on BGH and IGH release in 4-day-old monolayer cultures of mixed pituitary cells. This experiment represented the first investigation of the effects of hpGRF-40 on BGH secretion. It was not expected that differential secretion of BGH could be explained on the basis of GRF alone (as previously described), but this possibility needed to be ruled out before proceeding with further experiments.

To determine the dose response to hpGRF-40, pituitaries were obtained from 8 male Simonsen rats, 242-260 g. Dissociated cells were plated at a concentration of 150,000 cells per mL per well in 24-well plates (Linbro Cat. #76-033-05, Flow Laboratories, McLean, VA) and pre-cultured at 37°C in humidified air with 5% CO₂ for 4 days prior to treatment with GRF. One 3×10^6 cell aliquot of "start" cells was extracted with 0.01 N NaHCO₃ as previously described. Choice of the 4-day preculture period was based both on the frequent use of this procedure in the literature [17, 40, 100]. Choice of the 4-hour treatment period was based on the demonstration by Bilezikjian and Vale [9] that, when sampled at 15, 30, 60, 120 and 240 minutes, basal and 1 nM hpGRF-stimulated IGH secretion is linear with 3, and 17-times increases in media IGH concentration, respectively, over this 4 hour period (relative to control secretion at 15 minutes). After the 4 day preculture, alpha MEM culture media were collected, and replaced with fresh media for a one hour incubation. After the one hour incubation, 4-hour treatments with culture media containing various concentrations of GRF began. The GRF concentrations were 0, 0.39, 1.56, 6.25, 25.0, 100, or 400 nM hpGRF-40 (note each successive dose was 4 times the previous dose). There were 20 culture wells per treatment dose. At the end of the 4-hour treatment, media were collected and 24-hour extracts with 0.01 N NaHCO₃ were made as previously described. Viability determinations were made post-dissociation and at the end of the 4-hour treatment period, as already described. Two groups of 10 wells per treatment dose were pooled,

and 100 μ L samples were used for duplicate RIA determinations for both media and extracts. The two groups of 10 wells per treatment dose were pooled for bioassay. The hypophysectomized bioassay rats were supplied by Zivic-Miller (Zelienople, PA). One sample point (5 rats) was read against a standard curve generated from 3 dose levels (5 rats per point); 45, 15, and 5 μ g, as already described.

Experiment B: Determination of Preculture Optimal Duration

As mentioned above, most researchers preculture cells for 3-5 days prior to treatment with GRF or other test secretagogues. To the best of the writer's knowledge, in no case has experimental validation of this procedure been reported. It should be noted that the handling of cells is easier after attachment to substrate, which occurs following preculture. The purpose of this experiment was to examine the effect of duration of preculture on basal and GRF-stimulated GH secretion.

Fifteen male Simonsen rats, 208-247 g, were used as pituitary donors for separate, duplicate experiments which were run simultaneously. Seven and 1/2 pituitaries per experiment were dissociated to single cell suspensions, precultured for 0, 1, 2, or 4 days, and then treated for 4 hours with media C containing 0, 5, or 20 nM hpGRF44 (supplied to R.E. Grindeland by Jean Rivier, Salk Institute, La Jolla, CA). Cells were cultured in 70 mL flasks (25 cm² surface area, Cat. no. 25100; Corning, Palo Alto, CA) at a concentration of 1.5×10^6 cells per 5.0 mL per flask. The cells were incubated at 37°C

with closed caps (no gas exchange). There was one flask per treatment condition per experiment. Post-dissociation and post-treatment viability determinations were made, with the exception of the time-zero post-treatment groups. Treatment media were collected as previously described. For all treatment groups GH was measured by RIA and by tibial assay. For simplicity, the duplicate experiments will be called experiment B1 and B2, respectively. Experiment B1 hypophysectomized bioassay rats were supplied by Charles River Labs, while experiment B2 hypophysectomized bioassay rats were supplied by Zivic Miller Labs. In the experiment B1 bioassay, one sample point (5 rats) was read against a standard curve generated from 3 dose levels (5 rats per point); 45, 15, and 5 μg , as described above. In the experiment B2 bioassay, one sample point (3 rats) was read against a standard curve generated from 2 dose levels (5 rats per point); 45, and 15, as described above.

Experiment C: Effects of Culture Conditions on Basal and hpGRF-Stimulated IGH Secretion

In experiment B, cells were incubated in 70 mL culture flasks with closed caps, thus preventing gas exchange with the external environment. Not only does this reduce the amount of O_2 available to cells, but CO_2 exchange is also reduced. Both of these conditions could conceivably affect hormone production and release, thus complicating the interpretation of experiment B results. For this reason, in this experiment the effects of a closed culture flask filled

with air only (C/A) were compared with closed flasks filled with 5% CO₂ / 95% air (C/C), and "open" flasks constantly exposed to humidified 5% CO₂ / 95% air (O/C). "Open" flasks simply have the cap loosely fitted so that gas exchange can occur.

Eight male Taconic Farms rats, 329-420 g, served as pituitary donors in this experiment. Cells were precultured in 70 mL culture flasks for durations of 2 or 4 days, media were collected, and cells were then incubated for a 4-hour GRF-treatment period. On each treatment day, 2 flasks per culture condition were exposed to media C containing 5 nM hpGRF-40. Preculture and treatment media from duplicate flasks were collected and assayed by RIA. Start and post-treatment viability determinations were made for all groups as described earlier.

Experiment D: Determination of GH Stability in Culture Media

To the best of the writer's knowledge, there are no reports within the published literature which address the problem of degradation of IGH over time, as it sits in culture media at 37°C in the presence of adenohypophyseal cells. This experiment examined the stability of human pituitary-derived GH (hGH; 3 U.S.P. units per mg; Kabi, Stockholm, Sweden) over a 4-day culture period.

Eight male Taconic Farms rats, 329-420g, served as pituitary donors in this experiment. The mixed, dissociated cells were incubated in multiwell culture plates (24-well plates, Linbro Cat.

#76-033-05, Flow Laboratories, McLean, VA) at a concentration of 1.5×10^5 cells per 1.0 mL culture media per well. Twenty-four wells containing media C spiked with 5 μ g hGH per mL, and 24 wells containing media C only, were incubated in the presence of cells at 37°C in 5% CO₂ and 95% humidified air. Six wells each were collected on days 1, 2, 3, and 4. Similarly, aliquots of the same hGH-spiked media C were incubated without cells and collected at the same times. At time zero, aliquots of media were exposed to cells and then collected for subsequent assay. An aliquot of fresh hGH-spiked media were also saved for assay. Since the rabbit anti-hGH antiserum (Grindeland) does not cross-react with rat GH, and the monkey anti-rat GH antiserum does not cross-react with hGH, media were independently radioimmunoassayed for hGH and rat GH. The hGH results provided estimation of GH degradation over time. When compared with alpha MEM media controls, the rat IGH results for hGH-spiked media provided an estimate of feedback inhibition of rat GH secretion.

Experiment E: Validation of Cell Separation Procedure - Single Phase Method

Initially, it was planned that somatotrophs would be separated using a single 1.071 g/mL layer of BSA, rather than the 2-density system already described. This method was preferred over the 2-density system in light of the increased simplicity and reduced processing steps. This experiment was thus performed to replicate

the earlier work of Hymer (see Introduction), and to gain experience with the cell separation procedure.

In this method 1.5 mL of cells (10×10^6 cells) plus media are layered on top of 1.5 mL of sterile 1.071 g/mL BSA in a plastic centrifuge tube. With the exception of this difference, procedures are identical to those already described (see Density Gradient Cell Separation Procedure). After centrifugation, the type one cells form a layer at the interface between the media and BSA layer, while the type two cells form a pellet at the bottom of the centrifuge tube. In this experiment the aforementioned method was employed to separate mixed cells into type one and type two cell populations. Mixed, type one, and type two cells were cultured for 12 days in 70 mL culture flasks at concentrations of 1.5, 3.6, and 0.6 million cells per 5.0 mL of alpha MEM culture media per flask. Media were collected at days 4, 8, and 12 and replaced with fresh culture media on days 4 and 8.

Ten male Simonsen rats (mean=248.3 g, S.D.=8.4 g) served as pituitary donors. Ten adenohypophyses were pooled and independently dissociated, separated via density gradient procedure, and cultured. Immunocytochemistry and viability procedures were performed as previously described. Mixed and type one cell treatments were performed in duplicate, while single type two treatments were performed. The cells were incubated in closed flasks filled with air only. Four, eight and 12-day media samples were radioimmunoassayed for IGH and bioassayed for BGH. The hypophysectomized bioassay rats were supplied by Charles River

Labs. Pooled media samples were assayed at a single dose level (4 rats per point) and read against a standard curve generated from 3 dose levels, as described earlier (5 rats per point).

Experiment F: Validation of Cell Separation Procedure - 2-Phase Method

While the single phase method of cell separation apparently yields similar results as the 2-phase method in 12-day monolayer cultures, the latter method was recommended for subsequent experiments since many more experiments have been done with this method than the former (W. Hymer, personal communication, 1987). This experiment was thus performed to replicate the results of Hymer (see Introduction) and gain experience with the two-density method before proceeding with more elaborate experiments.

In this experiment, 21 male Simonsen rats (227-250 g) served as pituitary donors. In a single experiment, adenophypophyses were dissociated into single cell suspensions and separated via the 2-phase BSA density gradient procedure already described. Immunocytochemistry and viability procedures were performed as previously described. Mixed, type one, and type two cells were cultured as in experiment E, with the exception that all culture flasks were "open" to 5% CO₂ / 95% humidified air. Mixed cell treatments were performed in triplicate, while type one and type two cell treatments were performed in quadruplicate. Four, 8, and 12-day media samples were radioimmunoassayed and bioassayed for IGH and

BGH, respectively. The hypophysectomized bioassay rats were supplied by Zivic-Miller Labs (Zelienople, PA). Pooled media samples were assayed at a single dose level (4 rats per point) and read against a standard curve generated from 2 doses, as described earlier (5 rats per dose).

Experiment G: Type One and Type Two Somatotroph Response to rGRF-43

The purpose of this experiment was to generate dose-response curves in mixed, type one, and type two cells using rat GRF-43 (rGRF-43; #138-41-15 supplied by Jean Rivier, Salk Institute) in order to assess whether there are differences in responsiveness to GRF in terms of IGH and BGH secretion.

The pituitary donors were Simonsen male rats (n=121, mean body weight = 245.3 g, S.D.=8.4 g). Pituitaries were removed and dissociated as previously described. The 2-phase density gradient centrifugation procedure was used. Mixed, type one, and type two cells were cultured as described in experiment F, with the exception that treatments with media containing various concentrations of GRF began immediately after delivery of cell aliquots to culture flasks. The treatment rGRF-43 concentrations were 0, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25.0, 100, and 400 nM. The treatment duration was 4 hours. Immunocytochemistry and viability procedures were performed as described above. Mixed and type one cell treatments were performed in duplicate, while single type two cell treatments

were performed. All media samples were radioimmunoassayed for GH. Selective samples were bioassayed. The hypophysectomized rats for the bioassay were supplied by Zivic-Miller Labs (Zelienople, PA). Pooled media samples were assayed at a single dose level (4 rats per point) and read against a standard curve generated from 3 dose levels, as described earlier (5 rats per dose).

Experiment H: Effects of BSA Incubation on Cell Viability and GH Secretion

Because of the consistently low viability for type two cells in experiments E, F, and G, it was thought that perhaps the exposure to BSA during the density gradient procedure was preferentially toxic to type two cells or in some way affected viability. This experiment examined the effect of incubation in BSA media on cell viability and both basal and GRF-stimulated GH secretion.

Twenty male Simonsen rats (mean body weight = 269.6 g, S.D.=8.13 g) served as pituitary donors. Pituitaries were removed and dissociated into single cell suspensions as previously described. Dissociated cells in media A were either kept at room temperature (22-24°C), or exposed to 4°C, while a third group of cells were incubated in 1.078 g/mL BSA at 4°C for 45 minutes per group. Choice of 1.078 g/mL BSA was based on the fact that, in the two-phase gradient system, type two cells are exposed to both 1.071 and 1.085 g/mL BSA. Viability determinations were made immediately post-dissociation and after the 45-minute incubation. After the 45 minute

incubations, media and cells were centrifuged at 1000 X G for 10 minutes in a table top centrifuge, media were discarded and replaced with alpha MEM culture media. The cells were diluted to a concentration of 1.5×10^6 cells per 5.0 mL, and 5.0 mL aliquots were delivered to 15 mL plastic centrifuge tubes. After centrifugation the media were discarded, and replaced with fresh alpha MEM culture media containing 0, 0.5, or 1.5 nM hGRF-40 (10235-15, supplied by Jean Rivier, Salk Institute). Media plus cells (in the centrifuge tube) were triturated 20 times with a Pasteur pipette, and delivered to 70 mL plastic culture flasks as in previous experiments. The treatment duration for all 3 groups was 4 hours. At the end of the treatment period, media were collected for subsequent radioimmunoassay and bioassay. The hypophysectomized bioassay rats were provided by Simonsen Labs. For 0 nM GRF controls, media from duplicate flasks were combined and assayed. Whereas for 0.5 nM GRF-treated groups, media from single flasks were assayed. These differences were required to ensure that the resultant tibial width would fall within the range of the standard curve. The 1.5 nM GRF-treated groups and the groups which were cold-exposed only, were not bioassayed. All samples were assayed at a single dose level and read against a standard curve generated from 3 dose levels, as described earlier (5 rats per point).

The experiment was replicated at a later date using identical conditions with one exception; a 0.17 nM hGRF-40 dose level was included so that a more complete dose-response curve would be

obtained. In the repeat experiment pituitary donors were also Simonsen males (mean body weight = 237.5 g, S.D.=8.7 g). For simplicity, the first experiment will be referred to as experiment H1, and the second will be referred to as experiment H2. Only the 0.17 and 0.5 nM samples for experiment H2 were bioassayed. The groups which were cold-exposed only were not bioassayed. Both experiment H1 and H2 samples were assayed in the same bioassay.

Experiment I: Type One and Type Two Somatotroph Response to rGRF-43 and SRIF

The results of experiment G did not demonstrate consistent differences between somatotroph responsiveness to rGRF-43, which could explain the phenomenon of selective release of BGH *in vivo*. One might interpret this as evidence of a second GRF which either differentially affected the two cell types, or preferentially stimulated BGH release from both cell types. While this is possible, it seemed plausible to explain the phenomenon of selective BGH release in terms of known hypothalamic regulatory peptides. The most probable candidates would be GRF and SRIF, since they are specific to the GH system. Therefore, the purpose of this experiment was to test the hypothesis that various dose combinations of GRF and SRIF could stimulate release of BGH from type 2 somatotrophs with relatively little increase in release of IGH from type 1 somatotrophs.

Eighty male rats (Simonsen Labs, mean=236.8 g, S.D.=8.56 g) served as pituitary donors in this experiment. Pituitaries were

removed, dissociated and separated via 2-phase density gradient procedures, as described above. Viability and immunocytochemistry procedures were performed as previously described. Freshly dissociated mixed, and freshly separated type one and type two somatotrophs were each subjected to 7 different 4-hour treatment conditions: 0 nM GRF and 0 nM SRIF (0/0), 0.25 nM GRF and 0 SRIF (0.25/0), 1.0 nM GRF and 0 nM SRIF (1.0/0), 0 nM GRF and 0.5 nM SRIF (0/0.5), 0 nM GRF and 10 nM SRIF (0/10), 1.0 nM GRF and 0.5 nM SRIF (1.0/0.5), and finally 1.0 nM GRF and 10 nM SRIF (1.0/10). In all cases the culture media were alpha MEM, as previously described. Choice of rGRF-43 (lot #138-41-15, supplied by Jean Rivier, Salk Institute) doses were based on the dose-response curves for experiments G, H1, and H2. In those experiments these doses gave approximately 33 and 66% of maximal GRF-stimulated secretion for both BGH and IGH. Choice of SRIF (Cat. #S-0885; Sigma Chemical Corp., St. Louis, MO) doses were based on the work of Bilezikjian and Vale [9]. In their work, 1.0 nM hpGRF-40-stimulated IGH release in monolayer cultures of mixed rat somatotrophs was suppressed 50 and 100 percent (to control levels), respectively, with the SRIF doses listed above. Since the culture procedures employed by these researchers are similar to those of this work, the choice of SRIF dose level seemed reasonable. The culture conditions were as described for experiment G. Mixed and type one cell treatments were performed in triplicate. Type two cell treatments were performed in duplicate. At the end of the 4-hour incubation, media were collected and cells were extracted with 0.01

N Na_2CO_3 , as previously described. All media and cell extracts were radioimmunoassayed as previously described. Pooled media were bioassayed for BGH. The hypophysectomized rats for bioassay were provided by Simonsen Labs (Gilroy, CA). All samples were assayed at two dose levels (4 rats per dose). The standard curve was generated from 3 dose levels, as described earlier (5 rats per dose). The unknowns were statistically compared to the standards in a 4-point multiple regression analysis using a computer program based on the procedure of Bliss ([10]; see Bioassay section in Methods).

Experiment J: Type One and Type Two Somatotroph Response to rGRF-43 and SRIF

This experiment was a replication of experiment I in all respects with the exception that 100 rats served as pituitary donors (mean body weight = 236.8 g, S.D.=2.86 g; only 10 rat weights measured). The increased number of rats provided enough cells so that triplicate type two cell treatments could also be performed.

III. Results

Experiment A: Mixed Cell BGH and IGH Secretion in Response to hpGRF-40

Post-dissociation and post-treatment cell viabilities were 96% and 81%, respectively. The results for BGH and IGH secretion are shown in Figure 1, panels A and B, respectively. A plot of the BGH/IGH (ng / 1000 cells) ratio for secreted hormone is shown in panel C of Figure 1. Numeric values for each treatment in Figure 1 are indicated above each bar (panels A-C). Line graphs of the BGH and IGH dose response data (not shown) were used to calculate the ED50 dose (dose producing 50% of the maximum response). The approximate hpGRF-40 ED50s for BGH and IGH secretion were 6.5nM and 0.25 nM, respectively. While GRF-stimulated IGH secretion was maximal at 1.56 nM (Figure 1B), GRF-stimulated BGH secretion had not yet reached a maximum at 400 nM (Figure 1A). The progressively increasing BGH/IGH ratios (Figure 1C) reflect this phenomenon. The ED50 of 6.5 nM for BGH secretion was approximately 65-times lower than the 400 nM dose, which may or may not represent the dose which produces maximum secretion, since no higher dose was utilized. Consequently, the BGH dose-response curve was biphasic with one linear region in the GRF dose range of 0-6.25 nM, and a second linear region in the GRF dose range of 25-400 nM. The linear regression equation for the low GRF dose range was: Secretion (ng/mL) = 15.61 X Dose + 4.70 ($r=0.943$). The linear regression equation for the high GRF dose range was: Secretion (ng/mL) = 52.8

Figure 1. The effect of hpGRF-40 on BGH release (panel A), on IGH release (panel B), and on the BGH/IGH ratio for secreted GH (ratio of values in panel A and B; panel C). Panel B data points for duplicate determinations are shown above each bar. Numeric values shown above each bar correspond to the value for that bar (calculated value for panels A and C; arithmetic mean for duplicate determinations in panel B).

$X \text{ Dose} + 0.094$ ($r=0.985$). Net hormone production during the total culture period (preculture + 4-hour treatment) was calculated by subtracting the IGH nanogram per 1000 cells for start extracts from the sum of secreted hormone during the 4-day preculture period, the 4 hour treatment period, and the post-treatment extracts. The IGH content (ng / 1000 cells) for the start extract was 6.48 (a single determination). The 4-day preculture IGH secretion was 9.57 (a single determination). The results of the calculations are shown in Table 2. Each value, with the exception of the start extract and the 4-day media, are the result of duplicate determinations. GRF-induced IGH production was calculated by subtracting the GH production for zero controls from hormone production for GRF-treated groups. Those results are also shown in Table 2. It can be seen in the table that the post-treatment intracellular IGH concentration was greatest in the zero GRF media controls, but with increasing GRF-doses the intracellular IGH concentration rapidly decreased and stabilized at the 1.56 nM GRF dose. The net IGH production during the total culture period also increased with increasing GRF dose, and plateaued at about 1.56 nM. At this dose, IGH production was 1.7-times greater than in controls (Table 2; 9.32 vs. 16.13 ng / 1000 cells). At the 1.56 nM GRF dose, GRF-induced IGH production was 6.81 ng / 1000 cells. This value is slightly greater than

TABLE 2.
The Effect of 4-Hour GRF-Treatment on IGH
Production in Mixed Cells
(all values are expressed in ng per 1000 cells seeded)

<u>GRF Dose (nM)</u>	<u>Secreted IGH</u>	<u>Post-Treatment IGH</u>	<u>Net IGH Production*</u>	<u>GRF-Induced IGH Production*</u>
0	2.75	3.48	9.32	0
0.39	7.55	3.26	13.9	4.58
1.56	10.19	2.85	16.13	6.81
6.25	10.15	2.65	15.89	6.57
25	9.51	2.86	15.46	6.14
100	12.13	2.39	17.61	8.29
400	10.55	2.47	16.11	6.79

* Note: Net IGH production = (total secreted IGH + post-culture intracellular IGH) - (initial intracellular IGH); GRF-induced IGH production = (Net IGH production) - (Net IGH production for the 0 nM GRF dose).

the amount of intracellular IGH at the start of the culture (6.48 ng / 1000 cells).

Experiment B: Determination of Preculture Optimal Duration

Post-dissociation and post-treatment cell viability was 88 to 92% for all groups in both experiments. The BGH and IGH secretion for experiment B1 is shown in Figure 2. Growth hormone secretion

for the 4 different durations of preculture is plotted in groups according to GRF dose during the 4-hour treatment period. The results for BGH secretion (panel A, Figure 2) do not reveal any definite pattern with respect to duration of preculture, though it does appear that there is a trend toward decreased BGH secretion with increasing durations of preculture. This is most evident in the 20 nM GRF-treated groups, though it should be noted that the value of 547 ng / 1000 cells for the 1-day preculture group seemed anomalous. It is not clear why the value was so high, but it could be related to some illness in the bioassay rats.¹ There was a dose-related increase in BGH secretion, with the exception of the 1 and 4-day preculture groups for the 5 nM GRF dose. The results for IGH secretion (panel B, Figure 2) indicated that there is a temporally-related decrease in basal and GRF-stimulated IGH secretion. Secretion of IGH for the 4-day preculture group, expressed as a percent of the 0-day preculture group, was 51%, 45%, and 31% for 0, 5, and 20 nM GRF treatments, respectively. With the exception of the 0 nM GRF treatments, IGH secretion for the 2 and 4-day preculture groups was intermediate between 0 and 4-day preculture groups for the appropriate GRF-treatment. There was a dose-related increase in IGH secretion which appeared to have reached maximum at 5 nM (or less), with the exception of the one anomaly (0-day preculture / 20 nM GRF). The BGH and IGH secretion for experiment

¹ Four rats died over the course of the assay. Cause of death was not determined.

B2 is shown in Figure 3. The results for BGH secretion reveal the same basic pattern as those for IGH secretion in experiment B1 (panel B, Figure 2). That is, with increasing duration of preculture, both basal and GRF-stimulated secretion during the 4-hour treatment period declined. Secretion of BGH for the 4-day preculture group, expressed as a percent of the 0-day preculture group, was 49%, 53%, and 74% for 0, 5, and 20 nM GRF treatments, respectively. With the exception of the 0-day preculture / 0 nM GRF group and the 2-day preculture / 20 nM GRF group, all values were intermediate between 0 and 4-day preculture groups for the appropriate treatment condition. There was a dose-related increase in BGH secretion which appeared to have not yet reached maximum at 20 nM GRF. The results for IGH secretion in experiment B2 (panel B, Figure 3) reveal the same basic pattern as the IGH results for experiment B1 and the BGH results for experiment B2. With increasing durations of preculture, both basal and GRF-stimulated IGH secretion during the 4-hour treatment period declined. Secretion of IGH for the 4-day preculture group expressed as a percent of the 0-day preculture group, was 50%, 18%, and 22%, for the 0, 5, and 20 nM GRF treatments, respectively. With only one exception (1-day preculture / 0 nM GRF), IGH secretion for the 2 and 4-day preculture groups was intermediate between the 0 and 4-day groups for the appropriate treatment. There was a dose-related increase in IGH secretion which appeared to have plateaued at about 5 nM.

Figure 2. The effect of increasing durations of preculture of mixed somatotrophs on basal and hpGRF-40 stimulated BGH release (panel A), and IGH release (panel B) during a 4-hour treatment period. All values are the result of a single determination in experiment B1.

Figure 3. The effect of increasing durations of preculture of mixed somatotrophs on basal and hpGRF-40 stimulated BGH release (panel A), and IGH release (panel B) during a 4-hour treatment period. All values are the result of a single determination in experiment B2.

The results indicated that when maximal IGH and BGH secretion are used as an index for optimal cell "performance," then the preculture period of 3 to 5 days used by most researchers compromises secretory function. Indeed the results of the duplicate experiments presented here indicate that both basal and GRF-stimulated BGH and IGH secretion progressively decline with increasing duration of preculture. On the basis of these two experiments, the 3 to 5-day preculture period was eliminated in subsequent experiments using GRF-stimulation of GH secretion. In all subsequent experiments, cells were treated on the same day the rats were terminated (i.e., without a preculture period).

Experiment C: Effects of Culture Conditions on Basal and hpGRF-Stimulated IGH Secretion

Experiment C investigated the effects of closed culture flasks filled with air only (C/A), closed culture flasks filled with 95% air / 5% CO₂ (C/C), and open culture flasks exposed continuously to 95% air / 5% CO₂ in a humidified incubator (O/C) on basal and GRF-stimulated IGH secretion. Post-dissociation cell viability was 91%. Day 2 post-treatment cell viability was 96% among all 3 groups. On day 4, the post-treatment cell viabilities were 89% in the closed system groups (C/C and C/A), and 93% in the open system group (O/C). The results for IGH secretion during preculture are shown in Figure 4 (panel A). All bars represent means for duplicate

determinations, which are indicated as individual data points within the figure. Mean values for IGH concentration for all preculture groups (panel A) ranged between 2.8 and 3.4 $\mu\text{g/mL}$, with no definite pattern between groups for either 2-day or 4-day preculture media. It appears that the IGH concentrations between 2 and 4-day preculture groups did not differ. The results for IGH secretion during the 4-hour GRF-treatment period are shown in Figure 4 (panel B). The mean IGH secretion values for the 2-day preculture GRF-treated groups ranged between 4.7 and 4.8 $\mu\text{g/mL}$ media. It appears that there were no differences between treatment groups for the 2-day preculture GRF-treatment condition. The mean IGH secretion values ranged between 3.7 and 4.8 $\mu\text{g/mL}$ media. The open system group (O/C) had 14% greater GRF-stimulated IGH secretion than the closed-system groups. Mean IGH secretion for all three 2-day preculture/GRF-treated groups was 4.74 $\mu\text{g/mL}$, while the corresponding value for the 4-day preculture group was 3.88 $\mu\text{g/mL}$. The difference between these averaged values was 18%. Experiment C was performed because experiment B cells were incubated in culture flasks with closed caps, thus preventing gas exchange with the external environment. Since this reduces the amount of O_2 available to cells and results in an accumulation of CO_2 , it was thought that these effects might have contributed to the observed

Figure 4. The effect of various culture conditions on basal (panel A) and hpGRF-40 stimulated (panel B) IGH release. Basal IGH secretion was determined at days 2 and 4 of the culture period, as was GRF-stimulated secretion (4-hour treatments). The culture conditions were: closed flasks charged with 5% CO₂ / 95% air (C/C); closed flasks charged with air only (C/O); and open-system culture flasks constantly exposed to 5% CO₂ / 95% humidified air (O/C). The label for each bar is followed by the duration of culture prior to collecting media (panel A; either 2 or 4 days), or prior to GRF-treatment (panel B; either 2 or 4 days). Data points for duplicate determinations are included to show the response variability.

results in experiment B. It should be noted that the choice of the "closed system" in experiment B was based on the demonstration by W. Hymer that rat pituitary cells cultured for 4 days in closed 4.8 mL glass vials with only 0.8 mL air secrete GH at the same rate as control cells cultured in "open system" plastic culture plates (2 mL well volume) exposed to 95% air-5% CO₂ (personal communication). The results of experiment C are consistent with those of Hymer, and

indicate that it is unlikely that the progressively decreasing basal and GRF-stimulated BGH and IGH secretion found with increasing duration of preculture (experiment B) were due to use of the "closed system" culture.

Experiment D: Determination of GH Stability in Culture Media

Post-dissociation cell viability was 91%. Viabilities on days 1, 2, 3, and 4 were 84, 97, 95, and 96%, respectively. The hGH concentrations in media incubated from 0-4 days are shown in Figure 5 (panel A). Media were not collected on day 3. The hGH concentrations in media incubated with cells for 0-4 days are shown in Figure 5 (panel B). The bars for both panels represent mean values. Error bars represent the standard error of the mean for 6 determinations. The maximum deviation from the presumed absolute starting hGH concentration of 5 $\mu\text{g/mL}$ was not more than 10% for either media incubated alone or in the presence of cells. No pattern of degradation with respect to time is suggested by the results shown in Figure 5. The timecourse of secretion of rat IGH over the 4-day culture period is shown in Figure 6. The dark bars represent rat IGH $\mu\text{g/mL}$ media for cells incubated in media C (MEM) alone, while the hatched bars represent the same values for cells incubated in media C containing 5 μg hGH per mL media (MEM+GH). Secretion for both groups was linear over time, and as can be seen in the figure, 5 μg hGH per mL media did not inhibit release of native rat GH into

media. The mean values for IGH secretion for both treatment groups were combined to yield the linear regression equation for IGH secretion over the 4 day period: $\text{Secretion } (\mu\text{g/mL}) = 1.855 \mu\text{g} \times \text{mL}^{-1} \times \text{day}^{-1} \times \text{days} + 0.997$. The Pearson correlation coefficient for the equation above was 0.994.

The results of this experiment indicated that pituitary-derived 191-amino acid human GH is stable for at least four days when cultured in the presence of mixed rat somatotrophs. Additionally, the data indicated that human GH at these concentrations does not exert a negative feedback effect on rat IGH secretion over a four-day culture period. Moreover, the data suggest that rat GH secreted into media does not exert a negative feedback effect on rat IGH secretion, since rat GH secretion was highly linear with respect to time in days as shown by the above linear regression equation and correlation coefficient using data from media alone and media supplemented with human GH. It appears therefore, that with the culture conditions used in these experiments negative feedback of secreted IGH is not an important factor in explaining results.

Figure 5. The effect of culture duration on degradation of human immunoreactive GH in media containing 5µg hGH per mL (panel A) and the same media in the presence of 1.5×10^5 mixed rat adenohypophyseal cells per mL media (panel B). Each bar represents the mean $\pm$ S.E.M. for 6 wells containing 1 mL of solution each.

Figure 6. Secretion of rat immunoreactive GH from mixed rat adenohypophyseal cells incubated in culture media alone (MEM), or media containing 5µg hGH per mL. Each bar represents the mean $\pm$ S.E.M. for 6 wells containing 1 mL of solution each. Numeric values for mean GH secretion are also indicated.

Experiment E: Validation of Cell Separation Procedure

- Single Phase Method

Post-dissociation cell viability was 88%. Post-gradient cell viabilities for type 1 and type 2 cells were 76% and 44%, respectively. At the end of the 12-day culture period, the mixed, type 1, and type 2 cell viabilities were 85%, 89%, and 11%, respectively. The results for secreted GH in experiment E are summarized in Table 3. Type 1 cells released slightly less BGH than mixed cells over the 12-day culture, while type 2 cells released nearly twice as much BGH as mixed cells. In contrast, type 1 cells released about 1.5-times more IGH than mixed cells, while type 2 cells released about 1.5-times less IGH than mixed cells. For mixed and type 1 cells BGH secretion increased and then decreased in successive 4-day culture intervals. Type 2 cell BGH secretion progressively increased in successive 4-day culture intervals. BGH secretion for both mixed and type 1 cells increased and then decreased during days 5-8, and 9-12, respectively. IGH secretion from mixed cells increased and then decreased in successive 4-day culture intervals. IGH secretion from type 1 and type 2 cells progressively decreased during successive 4-day culture intervals. The differences in forms of GH released from the 2 cell types are most dramatically illustrated by the BGH/IGH ratio for the total GH released over the 12-day culture. The ratios for mixed, type 1, and type 2 cells were 3.6, 2.0, and 10.8, respectively. A pattern similar to that for total hormone released can be seen for both absolute

amounts of GH released and BGH/IGH ratios for the 0-4 day secretion.

The results of this experiment, using the single density BSA gradient separation procedure, were in general agreement with published results describing the differences between cell types [98, 99]. The general agreement was interpreted as evidence of successful somatotroph separation, indicating that the procedure could be used in subsequent experiments. However, as mentioned above, it was recommended to the author that the 2-density BSA gradient separation scheme be used in subsequent experiments since that procedure had been utilized in most published experiments, and was thus better established.

TABLE 3

**BGH and IGH Secretion in Mixed, Type 1, and Type 2 Somatotrophs
Cultured for 12 Days**

(Values in ng GH per 1000 cells seeded; three successive
4-day intervals plus cumulative totals)

	Mixed Cells			Type 1 Cells			Type 2 Cells		
	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH
Days 0-4	60.9	22.7	2.7	11.2	52.7	0.21	110	40.4	2.7
Days 5-8	127	32.0	4.0	171	48.2	3.6	185	8.8	20
Days 9-12	104	26.3	3.9	71.8	27.2	2.6	279	3.8	74
12 day total	292	81	3.6	254	128	2.0	574	53	10.8

Experiment F: Validation of Cell Separation Procedure - 2-Phase Method

Post-dissociation cell viability was 89%. Post-gradient cell viabilities were 84% for type 1 and 68% for type 2 cells. Mixed cell viability at recovery on days 4, 8, and 12 were 94%, 94%, and 82%, respectively. Type 1 cell viability at recovery on days 4, 8, and 12 were 83%, 94%, and 86%, respectively. Type 2 cell viability at recovery on days 4, 8, and 12 were 83%, 96%, and 71%, respectively. The percentage somatotrophs in mixed, type 1, and type 2 cell fractions were 44.8, 17.4, and 77.6%, respectively. The results for BGH and IGH secretion expressed on a per 1000 cells seeded basis are summarized in Table 4. The data shown in Table 4 were transformed into the per 1000 somatotroph data shown in Table 5 by using the percentage somatotroph values obtained from the immunocytochemistry procedure. The values shown allow for a more direct comparison between somatotroph types than the data in Table 4. In each of the successive 4-day culture intervals shown in Tables 4 and 5, the type 2 cell BGH/IGH ratio was greatest (7.6-20), followed by mixed cells (1.7-7.7), and finally type 1 cells (0.3-1.5). As shown in Table 5, BGH secretion was greatest in the type 2 cells, followed by type 1 cells, and then mixed cells. IGH secretion was greatest in the type 1 cells, followed by mixed cells and then type 2 cells. The aforementioned patterns in cell response are also reflected in the 4-day secretory data. BGH *production* in type 2 cells (Table 5) was 2.6-times greater than mixed cells, and 1.7-times

greater than type 1 cells over the 12-day culture. BGH *secretion* in type 2 cells was 2.5-times greater than mixed cells, and 1.7-times greater than type 1 cells over the 12-day culture. IGH production in type 1 cells was 6.1-times greater than mixed cells, and 14-times greater than type 2 cells over the 12-day culture. IGH secretion in type 1 cells was 5.4-times greater than mixed cells, and 9.8-times greater than type 2 cells over the 12-day culture. These basic patterns were also reflected in the 4-day secretion data (Table 5).

The results of this experiment, using the two density BSA gradient separation procedure, were in agreement with published results describing the differences between cell types [98, 99]. The agreement was interpreted as evidence of successful somatotroph separation, indicating that the procedure could be used successfully in subsequent experiments.

TABLE 4

BGH and IGH Secretion From 12-Day Cultures of Mixed, Type 1, and Type 2 Somatotrophs Expressed on a Per Cell Basis.

(Values in ng GH per 1000 cells seeded; three successive 4-day intervals plus cumulative totals; start and end extracts used for determination of GH production)

	<u>Mixed Cells</u>			<u>Type 1 Cells</u>			<u>Type 2 Cells</u>		
	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH
Start Extract	11.1	11.3	1.0	5.06	1.55	3.3	63.6	27.1	2.3
Days 0-4	59.7	35.1	1.7	46.3	43.4	1.1	624	31.3	20
Days 5-8	111	14.3	7.8	78.3	51.5	1.5	295	19.4	15
Days 9-12	69.6	20.8	3.3	17.2	52.2	0.3	126	16.6	7.6
12 day total	240	70	3.4	142	147	1.0	1044	67	16
End Extract	12.7	4.22	3.0	7.11	3.74	1.9	101	7.58	13
Production	241	63	3.8	144	149	1.0	1082	47.8	23

TABLE 5

BGH and IGH Secretion From 12-Day Cultures of Mixed, Type 1, and Type 2 Somatotrophs Expressed on a Per Somatotroph Basis.

(Values in ng GH per 1000 somatotrophs seeded; three successive 4-day intervals plus cumulative totals; start and end extracts used for determination of GH production)

	<u>Mixed Somatotrophs</u>			<u>Type 1 Somatotrophs</u>			<u>Type 2 Somatotrophs</u>		
	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH
Start Extract	24.8	25.2	1.0	6.52	2.00	3.3	81.9	34.9	2.4
Days 0-4	133	78.3	1.7	266	249	1.1	804	40.3	20
Days 5-8	248	31.9	7.7	450	296	1.5	380	25.0	15
Days 9-12	155	46.4	3.3	99.0	300	0.3	162	21.4	7.6
12 day total	536	156	3.4	812	843	1.0	1346	86.3	16
End Extract	28.3	9.42	3.0	40.9	21.5	1.9	130	9.77	13
Production	539	140	3.8	825	856	1.0	1394	61.6	23

Experiment G: Type One and Type Two Somatotroph Response to rGRF-43

Post-dissociation cell viability was 92%. Post-gradient cell viabilities were 81% for type 1 and 40% for type 2 cells. No post-treatment viability determinations were made. The percentage somatotrophs in mixed, type 1, and type 2 cells were 48.9, 20.7, and 73.6%, respectively. The complete results, with the exception of BGH results for 12.5, and 400 nM GRF, are shown in Table 6. These same results are graphically represented for IGH only (Figure 7). Of the BGH values presented in Table 6, five of the dilution "curves" for

high and low doses were non-parallel to the standard "curve" when tested at $\alpha=0.05$ in the multiple regression test. Those groups were: mixed cells - 6.25 nM GRF; type 2 cells - 0.39 nM GRF; and type 2 cells - 3.13 nM GRF. The reason for deviation from the standards is not known. However, it was not due to the low dose not differing from saline controls, as the low dose tibial readings in all cases were well beyond saline readings. The values shown for these groups should be considered as an estimate only. In two assay samples, the low dose tibial readings did not differ from saline-injected controls. Consequently, the values obtained were based on the high dose tibial readings alone. Those groups were: mixed cells - 0 nM GRF; and type 1 cells - 0.78 nM GRF. When the results for GRF-stimulated BGH and IGH secretion from the three different groups of cells are examined (Table 6, Figure 7), it appears that maximal secretion was obtained at 1.56 nM GRF in all cases, with the exception of IGH secretion for type 2 cells (which peaked at 3.13 nM). Progressive increases in both BGH and IGH release are seen for both mixed and type 1 cells over the dose range of 0 to 1.56 nM. Progressive increases in IGH release are seen for type 2 cells over the GRF dose range of 0 to 3.13 nM (Figure 6), but similar increases are not seen for BGH (consequently BGH data were not graphically plotted). BGH secretion for type 2 cells started at 168 ng / 1000 somatotrophs at 0 nM GRF, decreased to as low as 63.7 ng / 1000 somatotrophs at 0.78 nM GRF, and finally increased to 528 ng / 1000 somatotrophs at 1.56 nM GRF. The BGH and IGH secretion data from

Table 6 were used to determine the percent increase over control (0 nM) GH secretion at the plateau dose (1.56 nM; 3.13 nM for type 2 IGH). Those results are shown in Table 7. It can be seen that the percent increase in BGH secretion from type 2 cells (310) was considerably lower than the values for either mixed or type 1 cells. The maximal percent increase in IGH secretion was about the same for mixed and type 2 cells, but slightly higher for type 1 cells (Table 7). It can be seen in Table 6 that the supra-maximal (>1.56 nM) GRF doses produced erratic results for BGH secretion in all three cell groups. The results for IGH secretion yielded an interesting biphasic response. It can be seen in Table 6 that for all 3 cell types, GRF-stimulated IGH secretion declined at doses 2- and 4-times greater than the maximal response, and then increased with further increases in dose beyond the nadir. Despite the post-nadir increases, 400 nM GRF-stimulated IGH secretion in no case exceeded that of the maximal dose (Table 6 - 400nM vs 1.56 or 3.13 nM GRF). In general the BGH/IGH ratios for type 2 cells at the various GRF doses were considerably higher than either mixed or type 1 cell BGH/IGH ratios (Table 6). In general, type 1 cell BGH/IGH ratios were not different from the corresponding mixed cell ratios. The results of this experiment demonstrated that treatment of mixed, type 1, or type 2 cells with rGRF-43 causes release of both BGH and IGH from each group of cells. Moreover, with the exception of BGH secretion from type 2 cells, the maximal GRF-stimulated percent-increase over control BGH and IGH secretion were similar

between the 3 groups of cells. The selective release of BGH under conditions of stress thus does not appear to be explainable on the basis of GRF stimulation alone, a conclusion consistent with the hypothesis of this thesis.

TABLE 6
Bioassayable and Immunoassayable Growth Hormone
Secretion in Response to Rat Hypothalamic GRF-43

(values are ng per 1000 somatotrophs seeded)

GRF Dose	<u>BGH</u>			<u>IGH</u>			<u>BGH/IGH</u>		
	Mixed Cells	Type 1 Cells	Type 2 Cells	Mixed Cells	Type 1 Cells	Type 2 Cells	Mixed Cells	Type 1 Cells	Type 2 Cells
0 nM	30.3	41.3	168	14.9	15.7	11.6	2.0	2.6	14.5
0.39 nM	39.8	42.2	111	34.1	29.5	26.6	1.2	1.4	4.2
0.78 nM	43.3	95.7	63.7	41.4	38.3	33.3	1.0	2.5	1.9
1.56 nM	152	229	528	63.5	77.8	48.2	2.4	3.0	10.9
3.13 nM	127	66.8	321	54.3	67.6	58.4	2.3	1.0	5.5
6.25 nM	168	213	262	49.8	54.2	50.3	3.4	3.9	5.2
12.5 nM	n/a	n/a	n/a	49.4	42.2	44.2	n/a	n/a	n/a
25 nM	199	125	429	57.7	52.1	55.9	3.4	2.4	7.7
100 nM	219	250	423	51.0	64.2	64.2	4.3	3.9	6.6
400 nM	n/a	n/a	n/a	44.5	69.3	58.8	n/a	n/a	n/a

Figure 7. Immunoreactive rat GH secretion in response to rGRF-43 in mixed, type 1, and type 2 cell types. Log dose is plotted below the abscissa, while actual GRF dose in nM is indicated above the abscissa.

TABLE 7

	Maximal rGRF-43-Stimulated Percent-Increase Over Control BGH and IGH Secretion		
	<u>Mixed Cells</u>	<u>Type 1 Cells</u>	<u>Type 2 Cells</u>
BGH:	510	554	314
IGH:	436	469	416

Experiment H: Effects of BSA Incubation on Cell Viability and GH Secretion

For experiment H1, post-dissociation cell viability was 89% while the viability immediately after the 45-minute BSA-incubation was 87%. Following the 4-hour GRF treatment period, viabilities for the BSA-incubated group (BSA), and the group incubated at room temperature (RT; 22-24°C) were 80% and 69%, respectively. Viability determinations were not done for the cold-treated group. For experiment H2, post-dissociation cell viability was 92% while the viability immediately after the 45-minute BSA-incubation was 91%. Following the 4-hour GRF treatment period, viabilities for the BSA-incubated group, RT group, and cold-treated group were 82%, 87%, and 88%, respectively. The results for BGH and IGH secretion for experiment H1 are shown in Figure 8, panel A and B, respectively. The results for BGH and IGH secretion for experiment H2 are shown in Figure 9, panel A and B, respectively. In Figure 8A it can be seen that 4-hour treatment with GRF (experiment H1) caused a large increase in BGH secretion. The increase was about 3.4-times for the RT group, and 6.5-times for the BSA group. Samples from the cold-treated group were not bioassayed. GRF-stimulated BGH secretion was 1.4-times greater in the BSA group than in the RT group, while the differences between basal secretion appeared to be insignificant. In Figure 8B it can be seen that, for experiment H1, 4-hour treatment with GRF also caused a large increase in IGH secretion. The 0.5 nM dose produced about 5-, 7.5-, and 4-times

increases over basal IGH secretion in RT, BSA, and cold-treated cell groups, respectively. The 1.5 nM dose produced about 6-, 9-, and 9.5-times increases over basal IGH secretion in RT, BSA, and cold-treated cell groups, respectively. Basal IGH secretion did not differ between groups.

In Figure 9A it can be seen that 4-hour treatment with GRF (experiment H2) also caused a large increase in BGH secretion. The 0.17 nM dose produced increases of about 1.8-times for the RT group, and 2-times for the BSA group. The 0.5 nM dose produced increases of about 2.4-times for the RT group, and 2.1-times for the BSA group. Samples from the cold-treated group, and 1.5 nM GRF-treated groups were not bioassayed. GRF-stimulated BGH secretion was 1.3 to 1.5-times greater in the RT group than in the BSA group at all GRF treatment doses. In Figure 9B it can be seen that 4-hour treatment with GRF (experiment H2) also caused a large increase in IGH secretion. The 0.17 nM dose produced about 2.3-times increases over basal IGH secretion in all treatment groups. The 0.5 nM dose produced about 6-times increases over basal IGH secretion in all treatment groups. The 1.5 nM dose produced about 8-times increases over basal IGH secretion in all treatment groups. Numeric precision between groups was excellent for this experiment.

These two experiments investigated the effect of BSA incubation and exposure of cells to 4°C on cell viability and both basal and GRF-stimulated BGH and IGH secretion. The results showed that neither BSA incubation nor exposure to cold affect

basal or GRF-stimulated BGH or IGH secretion. These experiments were important in order to be certain that differences between cell types in the cell separation experiments are not an artifact of the separation procedure.

Figure 8. The effect of 45-minute pituitary cell incubation in 1.078 g/mL BSA on subsequent basal and rGRF-43-stimulated BGH (panel A) and IGH secretion (panel B) during a 4-hour treatment period in media C alone or supplemented with GRF. The data shown are for experiment H1. Abbreviations in the figure legend are as follows; RT= cells incubated at 22-24°C, BSA= cells incubated in 1.078 g/cc BSA at 4°C, and Cold= cells incubated at 4°C in MEM media. Error bars in panel A represent the 95% confidence interval for pooled media bioassayed at 4-5 rats per point. Error bars in panel B represent the S.E.M. for triplicate determinations.

Figure 9. The effect of 45-minute pituitary cell incubation in 1.078 g/mL BSA on subsequent basal and rGRF-43-stimulated BGH (panel A) and IGH secretion (panel B) during a 4-hour treatment period in media C alone or supplemented with GRF. The data shown are for experiment H2. Abbreviations in the figure legend are as follows; RT= cells incubated at 22-24°C, BSA= cells incubated in 1.078 g/cc BSA at 4°C, and Cold= cells incubated at 4°C in MEM media. Error bars in panel A represent the 95% confidence interval for pooled media bioassayed at 4-5 rats per point. Error bars in panel B represent the S.E.M. for triplicate determinations.

Experiment I: Type One and Type Two Somatotroph Response to rGRF-43 and SRIF, Alone and in Combination

Post-dissociation cell viabilities were 93%, 94%, and 87% for mixed, type 1, and type 2 cells, respectively. The percentage somatotrophs were 41.3, 31.6, and 82.9%, for mixed, type 1, and type 2 cells, respectively. These values were used to express GH secretion on a per 1000 somatotroph basis. The results for BGH and IGH secretion from the 3 cell groups are shown in Table 8 and Figure 10. Table 8 shows the actual values for IGH and BGH secretion, and the associated BGH/IGH ratio for each treatment group. Panels A, B, and C in Figure 10 show the results for mixed, type 1, and type 2 somatotrophs, respectively. The notation for each of the seven treatment groups (Table 8, Figure 10) gives the GRF dose (nM) followed by the SRIF dose (nM) for that treatment group. The control group (no GRF or SRIF) is thus represented by the term "0/0". The raw bioassay data for the SRIF treatments for mixed and type 1 cells, and the combined GRF/SRIF treatments for type 1 cells did not differ from saline-injected controls, indicating that BGH was not detectable. It should be noted that the limit of detection of rat GH is 1 μ g in the bioassay. Using this assay, failure to detect BGH means that there is less than 1 μ g of BGH in the sample. Hence, this value was used to calculate the upper limit of the possible value. The actual values for these groups are thus less than or equal to the values shown. When possible, the BGH value was based on a 4-point

multiple regression analysis using two doses of unknowns (see methods section). This analysis enabled determination of the 95% confidence interval for the actual BGH values. In several cases, however, the low dose did not differ from saline-injected controls. In these cases the high dose was used for calculations for unknowns.

In all 3 cell groups, GRF-treatment produced a dose-related increase in GH secretion relative to controls. The GRF-stimulated percentage increase over control GH secretion is shown in Table 9. It can be seen that in all cases, the percentage increase for BGH and IGH secretion was greatest in mixed cells, followed by type 1 cells, with the exception of IGH secretion for the 1.0 nM treatment in type 2 cells. While the GRF-stimulated percentage increase for BGH was lowest for type 2 cells, the absolute per thousand somatotroph BGH secretion was greatest of all 3 groups for type 2 cells (Table 8). Since only 2 GRF doses were used in this experiment, estimates of ED50 and maximal dose differences between cell types cannot be made. It does appear from the data in Table 9, however, that rGRF-43 has a more potent effect on GH secretion from type 1 somatotrophs than from type 2 somatotrophs in terms of percent increase since, with the exception of IGH secretion from 1.0 nM GRF-treated type 2 cells, the percent increase was greater in type 1 cells than type 2 cells. Examination of the BGH/IGH ratios for GRF-stimulated GH secretion from type 1 and type 2 cells (Table 8) reveals that at 0.25 nM GRF doses, both BGH and IGH release increase in parallel relative to controls, since the associated BGH/IGH ratios

do not differ from controls. However, when the GRF dose is increased to 1.0 nM, type 1 somatotrophs begin to secrete more BGH relative to IGH (Table 8; note increased BGH/IGH ratio relative to control), and type 2 somatotrophs begin to secrete more IGH relative to BGH (note decreased BGH/IGH ratio relative to control). These data suggest that the sensitivity of the 2 somatotroph types to rGRF-43 differs. While reconstruction of mixed cell GH secretion from type 1 and type 2 cell data yields poor agreement with actual mixed cell GH secretion, it can be seen from the data shown in Figure 10 that most of the mixed cell BGH secretion during control and GRF-treatments is derived from the type 2 somatotroph. Mixed cell IGH secretion during control and GRF-treatments is a result of combined IGH secretion from both type 1 and type 2 somatotrophs (Figure 10).

Somatostatin treatments produced a large suppression of both BGH and IGH secretion from mixed and type 1 somatotrophs (Figure 10, Table 8). During these treatments, BGH secretion was actually undetectable in mixed and type 1 somatotrophs. Growth hormone secretion from somatotrophs treated with SRIF, expressed as a percent of control secretion, is shown in Table 10. In no case was BGH or IGH secretion from mixed somatotrophs greater than 41% of the appropriate control secretion. Type 2 somatotrophs responded very differently to SRIF than either mixed or type 1 somatotrophs. In Table 10 it can be seen that 0.5 and 10 nM SRIF treatments inhibited IGH release in a dose-related fashion, to 59 and 42% of

control secretion, respectively. In this respect, and in terms of absolute ng per thousand somatotroph IGH secretion (Table 8), type 2 somatotrophs responded to SRIF in a manner similar to mixed and type 1 somatotrophs. However, BGH secretion from type 2 somatotrophs during SRIF-treatment was 153% and 109% of control secretion for 0.5 and 10 nM treatments, respectively (Table 10). These large values, consistent with a stimulatory effect, were not due to problems with the bioassay. Whether or not the "true" absolute ng per 1000 type 2 somatotroph BGH secretion during SRIF treatment was as reported, it appeared that SRIF does not suppress type 2 somatotroph BGH secretion below control levels. This absence of suppression was not apparent in mixed or type 1 somatotroph BGH secretion, for which BGH was undetectable (Figure 10).

Combined GRF and SRIF treatment produced markedly different effects on the 3 cell groups (Figure 10). Table 11 shows GH secretion from the 3 cell groups in response to combined GRF and SRIF treatment, expressed as a percent of the appropriate controls. Secretion of both BGH and IGH from type 1 somatotrophs in response to 1.0 nM GRF in combination with either 0.5 nM SRIF or 10 nM SRIF did not differ from SRIF treatments alone, with the exception of IGH secretion during the 1.0nM GRF / 0.5 nM SRIF treatment. Secretion of IGH from the aforementioned group was 68% of control (Table 11) versus 40% of control for the SRIF treatment alone (Table 10), thus indicating that the presence of GRF diminished the inhibitory effect

of SRIF. In type 2 somatotrophs combined treatment with GRF and SRIF produced a large decrease in BGH and IGH secretion relative to 1.0 nM GRF treatment alone (Figure 10). However, BGH secretion was 149%, and 139% of control for the 1.0 nM GRF / 0.5 nM SRIF, and 1.0 nM GRF / 10 nM SRIF treatments, respectively (Table 11). Within the range of experimental error, it appears that IGH secretion was suppressed to control levels during the aforementioned treatments (Table 11). In mixed cells, the combined GRF and SRIF treatments produced effects similar to those for type 2 somatotrophs. Combined treatment with GRF and SRIF produced a large decrease in BGH and IGH secretion relative to 1.0 nM GRF treatment alone (Figure 10). BGH secretion was 138%, and 86% of control for the 1.0 nM GRF / 0.5 nM SRIF, and 1.0 nM GRF / 10 nM SRIF treatments, respectively (Table 11). IGH secretion was 116%, and 36% of control for the 1.0 nM GRF / 0.5 nM SRIF, and 1.0 nM GRF / 10 nM SRIF treatments, respectively (Table 11). The effects of the combined treatments were most pronounced when the BGH/IGH ratios for secreted GH during combined treatments were compared to controls (Table 8). It can be seen in the table that in mixed and type 2 somatotrophs, the combined GRF/SRIF treatments cause increases in the BGH/IGH ratios for secreted hormone relative to controls. The shift in forms of GH released during these treatments thus favored BGH. The situation was the opposite for type 1 somatotrophs. The combined GRF/SRIF treatments caused decreases in the BGH/IGH ratio for secreted GH relative to controls (Table 8). Taken collectively, the

data from Tables 8 and 11 indicate that treatment of mixed and type 2 cells with 1.0 nM GRF and 0.5 nM SRIF caused an increase in BGH release relative to controls, with corresponding increases in the BGH/IGH ratio for secreted GH. The same treatment in type 1 cells decreased BGH and IGH release, and caused a decrease in the BGH/IGH ratio for secreted GH relative to controls. Treatment with 1.0 nM GRF and 10 nM SRIF diminished release of both BGH and IGH relative to controls in mixed and type 1 somatotrophs, though the magnitude of effect differed between cell groups (Table 11). The same treatment suppressed IGH secretion from type 2 somatotrophs to control levels, though BGH secretion remained increased relative to controls (Table 11).

The results of this experiment showed mainly that type 2 somatotrophs are less responsive to SRIF than type 1 somatotrophs. This observation, coupled with the high BGH/IGH ratio for GH secreted from type 2 somatotrophs under basal and GRF-stimulated conditions, produced important results during combined GRF/SRIF treatments. The combined GRF/SRIF treatments suppressed IGH secretion to untreated control levels or below in all 3 cell groups, but only suppressed BGH secretion to below controls in type 1 cells. Type 2 cell BGH secretion was stimulated to above untreated controls during the combined GRF/SRIF treatments. The GRF/SRIF treatments produced an increase in the BGH/IGH ratios relative to untreated controls in mixed and type 2 cells. By contrast, type 1 cell BGH/IGH ratios were unchanged. These results showed that

mixed cell secretion is a composite result of type 1 and type 2 cell activities. Most importantly, the results showed that the observed increases in BGH secretion and BGH/IGH ratios for secreted GH during stress in rats could be explained by the differential effects of combinations of SRIF and GRF on the 2 populations of somatotrophs.

TABLE 8
Secretion of BGH and IGH from Mixed, Type 1, Type 2
Somatotrophs in Response to rGRF-43 and SRIF Treatment,
Alone and in Combination

(values are ng per 1000 somatotrophs seeded)

	Mixed			Type 1			Type 2		
Treatment*	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH
0/0	14.1	9.92	1.42	8.07	6.91	1.17	40.4	7.80	5.18
0.25/0	32.7	30.1	1.09	12.6	11.7	1.08	46.0	8.91	5.16
1.0/0	75.4	55.3	1.36	31.3	18.6	1.68	86.3	24.6	3.51
0/0.5	≤4.72	3.94	≤1.20	≤2.57	2.79	≤0.92	62.2	4.62	13.5
0/10	≤4.72	4.03	≤1.17	≤2.57	2.79	≤0.92	44.2	3.26	13.6
1.0/0.5	19.4	11.55	1.68	≤2.57	4.71	≤0.55	60.1	6.71	8.96
1.0/10	12.1	3.54	3.42	≤2.57	2.94	≤0.87	56.3	9.13	6.17

*The notation for treatments is the GRF dose (nM), followed by the SRIF dose (nM); the 0/0 treatment thus represents the untreated control.

Figure 10. The effect of rGRF-43 and SRIF treatment, alone, and in combination, on secretion of BGH and IGH from mixed (panel A), type 1 (panel B), and type 2 (panel C) somatotrophs. BGH secretion is represented by the solid black bars, while IGH secretion is represented by the hatched bars. The notation for treatments gives the rGRF-43 dose (nM) followed by the SRIF dose (nM). Error bars are shown for all groups, except those where BGH was undetectable (see text). Error bars for BGH secretion represent the 95% confidence limits for single determinations in pooled media. Error bars for IGH secretion represent the S.E.M. for triplicate determinations. Several error bars are not visible, since the value was so small.

TABLE 9

**Growth Hormone Secretion from rGRF-43-Treated
Somatotrophs Expressed as a Percent of Control Secretion**

	<u>0.25 nM GRF</u>		<u>1.0 nM GRF</u>	
	<u>BGH</u>	<u>IGH</u>	<u>BGH</u>	<u>IGH</u>
<u>Mixed Cells</u>	232	303	535	557
<u>Type 1 Cells</u>	156	169	388	269
<u>Type 2 Cells</u>	114	114	214	315

TABLE 10

**Growth Hormone Secretion from SRIF-Treated
Somatotrophs Expressed as a Percent of Control Secretion**

	<u>0.5 nM SRIF</u>		<u>10 nM SRIF</u>	
	<u>BGH</u>	<u>IGH</u>	<u>BGH</u>	<u>IGH</u>
<u>Mixed Cells</u>	≤33	40	≤33	41
<u>Type 1 Cells</u>	≤32	40	≤32	40
<u>Type 2 Cells</u>	153	59	109	42

TABLE 11

**Growth Hormone Secretion from Somatotrophs
Treated with Combinations of rGRF-43 and SRIF
Expressed as a Percent of Control Secretion**

	<u>1.0 nM GRF / 0.5 nM SRIF</u>		<u>1.0 nM GRF / 10 nM SRIF</u>	
	<u>BGH</u>	<u>IGH</u>	<u>BGH</u>	<u>IGH</u>
<u>Mixed Cells</u>	138	116	86	36
<u>Type 1 Cells</u>	≤32	68	≤32	43
<u>Type 2 Cells</u>	149	86	139	117

Experiment J: Type One and Type Two Somatotroph Dose-Response to rGRF-43 and SRIF, Alone and in Combination

Post-dissociation cell viabilities were 93%, 94%, and 95% for mixed, type 1, and type 2 cells, respectively. The percentage somatotrophs were 40.0, 31.9, and 73.7%, for mixed, type 1, and type 2 cells, respectively. These values were used to express GH secretion on a per 1000 somatotroph basis. The results for BGH and IGH secretion from the 3 cell groups are shown in Table 12 and Figure 11. Table 12 shows the actual values for IGH and BGH secretion, and the associated BGH/IGH ratio for each treatment group. Panels A, B, and C in Figure 11 show the results for mixed, type 1, and type 2 somatotrophs, respectively. The notation for each of the seven treatment groups (Table 12, Figure 11) gives the GRF dose (nM) followed by the SRIF dose (nM) for that treatment group.

The control group (no GRF or SRIF) is thus represented by the term "0/0." The raw bioassay data for the SRIF treatments for mixed and type 1 cells, and the combined GRF/SRIF treatments for type 1 cells did not differ from saline-injected controls, indicating that BGH was not detectable. Since the limit of detection of rat GH is 1 μg in the bioassay, this value was used to calculate the upper limit of the possible value. The actual values for these groups are thus less than or equal to the values shown. When possible, the BGH value was based on a 4-point multiple regression analysis using two doses of unknowns (see Methods section). This analysis enabled determination of the 95% confidence interval for the actual BGH values. In several cases, however, the low dose did not differ from saline-injected controls. In these cases the high dose was used for calculations for unknowns.

In all 3 cell groups, GRF-treatment produced a dose-related increase in GH secretion relative to controls. The GRF-stimulated percentage increase over control GH secretion is shown in Table 13. The GRF-stimulated percentage increase over control BGH and IGH secretion varied across cell groups, without a definite pattern. However, type 2 somatotrophs appeared to be more responsive to GRF in terms of IGH secretion than type 1 somatotrophs (compare type 2 cell IGH values with type 1 cell IGH values in Table 13). Note that in all cases the mixed cell percent increase over control secretion was intermediate between the type 1 and type 2 cell values. It should be noted that while the 0.25 nM GRF-stimulated

percentage increase for BGH was lowest for type 2 cells, the absolute per thousand somatotroph BGH secretion was greatest of all 3 groups for type 2 cells (Table 12). Since only 2 GRF doses were used in this experiment, estimates of ED50 and maximal dose differences between cell types cannot be made. Examination of the BGH/IGH ratios for GRF-stimulated GH secretion from type 1 cells (Table 12) reveals that at either the 0.25 or 1.0 nM GRF doses, both BGH and IGH release increases in parallel relative to controls, since the associated BGH/IGH ratios do not differ from controls. In type 2 cells, however, the BGH/IGH ratios decreased by over 40% with GRF treatment (Table 12). These data indicate that the sensitivity of the 2 somatotroph types to rGRF-43 differs. While reconstruction of mixed cell GH secretion from type 1 and type 2 cell data yields poor agreement with actual mixed cell GH secretion, it can be seen from the data shown in Figure 11 that most of the mixed cell BGH secretion during control and GRF-treatments is derived from the type 2 somatotroph. Mixed cell IGH secretion during control and GRF-treatments is a result of combined IGH secretion from both type 1 and type 2 somatotrophs (Figure 11).

Growth hormone secretion from somatotrophs treated with SRIF, expressed as a percent of control secretion, is shown in Table 14. Somatostatin treatment suppressed secretion of both BGH and IGH by 52% or greater from mixed and type 1 somatotrophs (Figure 11, Table 12, Table 14). Type 2 somatotrophs responded differently to SRIF than either mixed or type 1 somatotrophs. In Table 14 it can

be seen that 0.5 and 10 nM SRIF treatments inhibited type 2 somatotroph IGH release to 47 and 45% of control secretion, respectively. In this respect, and in terms of absolute ng per thousand somatotroph IGH secretion (Table 12), type 2 somatotrophs responded to SRIF in a manner similar to mixed and type 1 somatotrophs. However, BGH secretion from type 2 somatotrophs during SRIF-treatment was 61% and 64% of control secretion for 0.5 and 10 nM treatments, respectively (Table 14). These values were considerably greater than those for type 1 cells (for which BGH was undetectable), suggesting that type 2 cells are less responsive to SRIF than are type 1 cells. This lower responsiveness of type 2 cells to SRIF seemed apparent in the corresponding values for mixed cells (Table 14; note that, with the exception of IGH secretion from 10 nM SRIF-treated mixed cells, mixed cell values are intermediate between type 1 and type 2 cell values). These conclusions are strengthened by the fact that BGH was undetectable during SRIF treatments of mixed somatotrophs.

Combined GRF and SRIF treatment produced markedly different effects on the 3 cell groups (Figure 11). Table 15 shows GH secretion from the 3 cell groups in response to combined GRF and SRIF treatment, expressed as a percent of the appropriate controls. Secretion of both BGH and IGH from type 1 somatotrophs in response to 1.0 nM GRF in combination with either 0.5 nM SRIF or 10 nM SRIF did not differ from SRIF treatments alone, with the exception of IGH secretion during the 1.0 nM GRF / 0.5 nM SRIF treatment. Secretion

of IGH from the aforementioned group was 230% of control (Table 15) versus 24% of control for the SRIF treatment alone (Table 14), thus indicating that the stimulatory effect of GRF dominated over the inhibitory effect of SRIF. With the higher SRIF dose (1.0 nM GRF / 10 nM SRIF), the GRF stimulatory effect on IGH was almost completely overridden by the SRIF inhibitory effect (compare Table 15 vs Table 14; 46% vs 38%). In type 2 somatotrophs, combined treatment with GRF and SRIF produced a large decrease in BGH and IGH secretion relative to 1.0 nM GRF treatment alone (Figure 11). However, BGH secretion was 107%, and 96% of control for the 1.0 nM GRF / 0.5 nM SRIF, and 1.0 nM GRF / 10 nM SRIF treatments, respectively (Table 15). Type 2 cell IGH secretion was stimulated to 472% and 136% above control levels during the aforementioned respective treatments (Table 15), but to a considerably lesser extent than with 1.0 nM GRF alone (Table 13; 1034% of control for 1.0 nM GRF alone). In mixed cells, the combined GRF and SRIF treatments produced effects similar to those for type 2 somatotrophs. Combined treatment with GRF and SRIF produced a large decrease in BGH and IGH secretion relative to 1.0 nM GRF treatment alone (Figure 11). BGH secretion was 349%, and 93% of control for the 1.0 nM GRF / 0.5 nM SRIF, and 1.0 nM GRF / 10 nM SRIF treatments, respectively (Table 15). IGH secretion was 294%, and 52% of control for the 1.0 nM GRF / 0.5 nM SRIF, and 1.0 nM GRF / 10 nM SRIF treatments, respectively (Table 15). The effects of the combined treatments were most apparent when the BGH/IGH ratios

for secreted GH during combined treatments were compared to controls (Table 12). It can be seen in the table that in mixed somatotrophs, the combined GRF/SRIF treatments caused increases in the BGH/IGH ratios for secreted hormone relative to controls. The shift in forms of GH released during these treatments thus favored BGH. The situation was the opposite for type 1 somatotrophs. The combined GRF/SRIF treatments caused decreases in the BGH/IGH ratio for secreted GH relative to controls (Table 12). In fact BGH was not detectable in type 1 somatotrophs during these treatments. For type 2 cells the combined GRF/SRIF treatments caused decreases in the BGH/IGH ratio for secreted GH relative to controls (Table 12). It is important to note, however, that during these treatments type 2 cells continued to secrete predominantly BGH, yielding BGH/IGH ratios of 1.7 and 5.4 for the 1.0 nM GRF / 0.5 nM SRIF, and 1.0 nM GRF / 10 nM SRIF treatments, respectively (Table 12). During these treatments it is evident that the mixed cell BGH/IGH values are a result of the combined activities of the type 1 and type 2 somatotrophs of which they are composed. Taken collectively, the data from Tables 12 and 15 indicate that treatment of mixed cells with combined 1.0 nM GRF / 0.5 nM SRIF caused an increase in BGH release relative to controls, with corresponding increases in the BGH/IGH ratio for secreted GH. For type 2 cells the aforementioned treatment decreased the BGH/IGH ratio, mainly due to increased IGH secretion in response to the treatment. The same treatment in type 1 cells decreased BGH but not IGH release, and caused a decrease in

the BGH/IGH ratio for secreted GH relative to controls. Treatment with combined 1.0 nM GRF / 10 nM SRIF diminished release of both BGH and IGH relative to controls in mixed and type 1 somatotrophs, though the magnitude of effect differed between cell group (Table 15). The same treatment suppressed BGH secretion from type 2 somatotrophs to control levels, though IGH secretion remained increased relative to controls (Table 15).

The results of this experiment showed mainly that type 2 somatotrophs are less responsive to SRIF than type 1 somatotrophs. This observation, coupled with the high BGH/IGH ratio for GH secreted from type 2 somatotrophs under basal and GRF-stimulated conditions, produced important results during combined GRF/SRIF treatments. Type 2 cell BGH secretion was stimulated to above untreated controls during the combined GRF/SRIF treatments. The GRF/SRIF treatments produced an increase in the BGH/IGH ratios relative to untreated controls in mixed and type 2 cells. By contrast, type 1 cell BGH/IGH ratios were unchanged. These results showed that mixed cell secretion is a composite result of type 1 and type 2 cell activities. Most importantly, the results showed that the observed increases in BGH secretion and BGH/IGH ratios for secreted GH during stress in rats could be explained by the differential effects of combinations of SRIF and GRF on the 2 populations of somatotrophs.

TABLE 12
Secretion of BGH and IGH from Mixed, Type 1, and Type 2
Somatotrophs in Response to rGRF-43 and SRIF
Treatment, Alone and in Combination
(values are ng per 1000 somatotrophs seeded)

	Mixed			Type 1			Type 2		
Treatment*	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH	BGH	IGH	BGH/IGH
0/0	36.6	10.3	3.6	11.5	11.7	1.0	86.0	11.2	7.7
0.25/0	97.0	42.1	2.3	33.5	28.1	1.2	204.6	49.0	4.2
1.0/0	204.9	73.4	2.8	59.9	62.1	1.0	531.9	115.7	4.6
0/0.5	≤17.6	3.3	≤5.3	≤4.3	2.8	≤1.5	52.4	5.3	10.0
0/1.0	≤15.2	2.9	≤5.2	≤4.3	4.5	≤1.0	54.8	5.0	11.0
1.0/0.5	127.6	30.2	4.2	≤4.3	26.8	≤0.2	91.9	52.7	1.7
1.0/1.0	33.9	5.4	6.3	≤4.3	5.3	≤0.8	82.2	15.3	5.4

*The notation for treatments is the GRF dose (nM), followed by the SRIF dose (nM); the 0/0 treatment thus represents the untreated control.

Figure 11. The effect of rGRF-43 and SRIF treatment, alone, and in combination, on secretion of BGH and IGH from mixed (panel A), type 1 (panel B), and type 2 (panel C) somatotrophs. BGH secretion is represented by the solid black bars, while IGH secretion is represented by the hatched bars. The notation for treatments gives the rGRF-43 dose (nM) followed by the SRIF dose (nM). Error bars are shown for all groups, except those where BGH was undetectable (see text). Error bars for BGH secretion represent the 95% confidence limits for single determinations in pooled media. Error bars for IGH secretion represent the S.E.M. for triplicate determinations. Several error bars are not visible, since the value was so small.

TABLE 13

**Growth Hormone Secretion from rGRF-43-Treated
Somatotrophs Expressed as a Percent of Control Secretion**

	<u>0.25 nM GRE</u>		<u>1.0 nM GRE</u>	
	<u>BGH</u>	<u>IGH</u>	<u>BGH</u>	<u>IGH</u>
<u>Mixed Cells</u>	265	409	560	713
<u>Type 1 Cells</u>	291	240	521	531
<u>Type 2 Cells</u>	238	438	618	1034

TABLE 14

**Growth Hormone Secretion from SRIF-Treated
Somatotrophs Expressed as a Percent of Control Secretion**

	<u>0.5 nM SRIF</u>		<u>10 nM SRIF</u>	
	<u>BGH</u>	<u>IGH</u>	<u>BGH</u>	<u>IGH</u>
<u>Mixed Cells</u>	≤48	32	≤42	28
<u>Type 1 Cells</u>	≤37	24	≤37	38
<u>Type 2 Cells</u>	61	47	64	45

TABLE 15

**Growth Hormone Secretion from Somatotrophs
Treated with Combinations of rGRF-43 and SRIF
Expressed as a Percent of Control Secretion**

	<u>1.0 nM GRF / 0.5 nM SRIF</u>		<u>1.0 nM GRF / 10 nM SRIF</u>	
	<u>BGH</u>	<u>IGH</u>	<u>BGH</u>	<u>IGH</u>
<u>Mixed Cells</u>	349	293	93	52
<u>Type 1 Cells</u>	≤37	229	≤37	45
<u>Type 2 Cells</u>	107	470	96	137

IV. Discussion

The results for experiment A suggest that the BGH secretion in response to hpGRF-40 in mixed cells differs from IGH secretion. Maximal GRF-stimulated IGH secretion occurred at 1.56 nM, while BGH secretion appeared to have not yet reached maximum at 400 nM. Moreover, at the full range of GRF doses, from 0 nM to 400 nM, the BGH/IGH ratio ranged from 2.5 to 8.5, indicating that at all doses BGH was the predominant form of GH released into the media. Treatment with hpGRF-40 produced a maximal 9-times increase in BGH secretion at 400 nM, while the maximal increase in IGH release was 3.6-times at 1.56 nM. These results for IGH maximal secretion and ED50 are in agreement with previous reports for hpGRF-40 [13,18, 79]. The maximal BGH increase is difficult to interpret due to the biphasic nature of the BGH dose-response. There were two linear regions in the BGH dose-response curve; one in the dose range of 0 to 6.25 nM, and another in the 25 to 400 nM range. The biphasic curve in the mixed cell population could represent a combination of heterogeneous responses in type 1 and type 2 somatotroph populations. Alternately, it could be due to the presence of some impurity(-ies) in the GRF preparation, since the preparation used was not purified to homogeneity. Whatever the source of the biphasic response, mixed somatotroph populations clearly have different BGH and IGH dose-response curves to hpGRF-40, and at all doses BGH secretion is greater than IGH secretion.

If one interprets the 6.25 nM dose as the maximal dose for BGH secretion then the ED50 can be calculated from the regression equation to be 0.77 nM. This interpretation seems unwarranted, however, given the 2-times increase in BGH secretion at the 400 nM dose relative to the 6.25 nM dose. This is in contrast to the approximate BGH ED50 of 6.25 nM for the complete dose-response curve. Whichever value is considered, both differ considerably from the approximate IGH ED50 of 0.25 nM. The differences in maximal dose and ED50 between BGH and IGH explain the progressive increase in the BGH/IGH ratio with increasing GRF doses. It is difficult to interpret the physiological significance of the heterogeneity in the BGH and IGH response to hpGRF-40. Originally, it was thought that since BGH can be selectively released *in vivo* (see Introduction: GH Release During Stress, Hypoglycemia, and Spaceflight), there might be more than one releasing factor, with each GRF controlling release of each form of GH. While the possibility of an additional GRF cannot be eliminated on the basis of these experiments, its existence seems improbable and is not necessary to explain *in vivo* results. It also seems improbable that hpGRF-40, or presumably rGRF-43 (which bears 70% sequence homology to hGRF40) can alone account for selective BGH release under stress conditions in rats. The effect of GRF could be modulated by SRIF or some other factor(s) acting directly on the somatotrophs to modify their response. For example, the recently discovered 29-amino acid brain/gut peptide, galanin, stimulates IGH secretion *in vivo*, but not *in vitro* [69] suggesting

that GRF/SRIF release can be modulated by galanin. It is possible that varying concentration ratios of hypothalamic factors differentially regulate the two somatotroph populations, resulting in selective BGH versus IGH release. Due to the heterogeneous BGH and IGH dose-response curves to hpGRF-40, it was decided that further investigation of the GH secretory response using purified rGRF-43 and mixed, type 1, and type 2 cells should be performed (experiment G, below). The 4-hour GRF-treatment in experiment A produced a profound dose-related increase in IGH "production" (Table 2). The effect was maximal at the 1.56 nM dose, which parallels the data for IGH secretion. It is noteworthy that the maximal IGH production during the 4-hour treatment (Table 1) was slightly greater than the initial intracellular IGH (6.48 ng / 1000 cells). The increased synthesis is due to increased transcription and translation since it has been shown that hpGRF-40 [17] and hpGRF-44 [31] increase GH messenger RNA in cultures of rat pituitary cells.

One of the main purposes of this thesis research was to determine differences between BGH and IGH secretion in response to GRF exposure in various somatotroph types. As mentioned previously, Bilezikjian and Vale [9] have determined that both basal and GRF-stimulated IGH secretion is linear over the 4-hour treatment period. One aspect of the experimental conditions which was not evaluated critically was use of 3 to 4-day preculture periods prior to treatment with secretagogues (e.g., GRF). Experiment B was designed to make this evaluation. In general, increasing duration of preculture resulted

in a decline in both basal and GRF-stimulated BGH and IGH secretion. As indicated in the results section for experiment B1 (Figure 2) and for experiment B2 (Figure 3), there were some exceptions to the aforementioned statement. However, it can be seen that with the exception of the 20 nM treatment in Figure 2A, secretion of BGH and IGH from the 4-day preculture groups was reduced relative to the 0-day preculture groups. Secretion from 4-day preculture groups expressed as a percent of 0-days preculture groups, ranged from 18 to 53%. Based on these data, preculture was eliminated in all subsequent experiments (i.e., H, I, and J). The discrepancies between BGH secretion in experiment B1 and B2 are difficult to explain, considering the relatively good agreement between IGH results. The bioassay rats for these experiments were supplied by Charles River Labs. This was the first time that this supplier had been used in Dr. R.E. Grindeland's laboratory. Several rats died over the course of the assay. These facts may contribute to the discrepancies. Dose-related increases in BGH and IGH secretion were observed in experiment B. It appears that BGH secretion was not yet maximal at 20 nM GRF and that IGH secretion was maximal by 5 nM. These results are consistent with those of experiment A, which also used hpGRF-40.

One aspect of the culture conditions employed in experiment B left some uncertainty. The cells were incubated in a closed culture system, which prevented gaseous exchange with the atmosphere. Choice of this procedure was based on unpublished observations by W. Hymer. Hymer found that 9-day IGH secretion from dispersed

pituitary cells incubated in sealed 4.8 mL glass vials containing 4.0 mL of media plus cells and 0.8 mL of air, did not differ from secretion from cells incubated under "open-system" conditions, with constant exposure to humidified 95% air / 5% CO₂. No differences between culture systems were found at all cell concentrations tested (1-, 10-, 50-, 100-, and 200-thousand per vial). Saturation of culture media with O₂ prior to incubation in closed vials also produce no measurable differences. Given these results, Dr. Hymer advised the use of "open system" culture conditions for future experiments. It was also advised that the effects of "closed system" incubation under the conditions employed in experiment B be evaluated.

Experiment C investigated the effects of closed culture flasks filled with air only (C/A), closed culture flasks filled with 95% air / 5% CO₂ (C/C), and open culture flasks exposed continuously to 95% air / 5% CO₂ in a humidified incubator (O/C) on basal and GRF-stimulated IGH secretion. As can be seen in Figure 4A, there were no apparent effects of the 3 culture conditions on release of IGH into media after 2 and 4 days culture. Similarly, in Figure 4B, it can be seen that after 2 days culture there were no apparent differences in IGH secretion among the 3 groups in response to 4-hour treatment with 5 nM hpGRF-40. After 4 days of preculture there was an average 18% decline in 5 nM hpGRF-40 stimulated IGH release, the decrease being slightly more than 18% for closed culture conditions, and slightly less for the open culture condition. The difference

between the closed and open culture conditions at 4 days (Figure 4B) suggests that the culture conditions might have some slight effect on GRF-stimulated IGH secretion. The effect, however, does not compare to the large decreases seen with increasing preculture duration in experiment B. Moreover, temporally related decreases in 5 nM GRF-stimulated IGH secretion were observed in the open culture condition as well, further indicating that the results from experiment B were valid. The decrease in 5 nM GRF-stimulated IGH secretion in Figure 4B was interpreted as a partial replication of the results from experiment B.

The results of experiment D indicated that pituitary-derived 191 amino acid human GH is stable for at least four days when cultured in the presence of mixed rat somatotrophs. Additionally, the data indicated that human GH does not exert a negative feedback effect on rat IGH secretion over a four-day culture period. Moreover, the data suggested that rat GH secreted into media does not exert a negative feedback effect on rat IGH secretion, since rat IGH secretion was highly linear with respect to time in cells cultured in media alone or supplemented with human GH (see regression equations and correlation coefficients). One might question whether the observed lack of hGH degradation over the four-day culture period could have been due to species differences in the enzymes involved in degrading GH. While this is a possible explanation for the observed results, it seems more likely that secreted GH is degraded by relatively non-specific proteases, perhaps released into

media from dying cells. Barring the possible aforementioned alternate explanation, it therefore appears that with the culture conditions used in these thesis experiments that negative feedback of secreted IGH is not an important factor in explaining results.

The experiments discussed to this point (A-C) served as controls to validate the procedures employed herein. Before the primary experiments could be conducted, however, validation of the cell separation procedure was required. Hymer has employed two methods for separation of somatotrophs [45]. The method most frequently employed utilizes a 2-density discontinuous gradient system, as previously described. An alternate method uses a single BSA density (concentration 1.071 g/mL), with cells applied directly above this solution prior to centrifugation. Originally it was thought that this was the best method for cell separation. Experiment E investigated the functional differences in type 1 and type 2 somatotrophs separated by the latter method. The results (Table 3) were in agreement with the data reported by Grindeland and Hymer (36). Type 2 cells released about 2.3-times more BGH, and 2.4-times less IGH over the 12-day culture period. Twelve-day BGH and IGH secretion from mixed cells was intermediate between the values for type 1 and type 2 cells. At each of the 4-day culture intervals, the BGH/IGH ratio for GH secreted from type 2 cells was 6 to 28-times greater than the corresponding ratio for type 1 cells. Complete results are shown in Table 3, but emphasis will be placed on secretion during days 0-4, since 1) this period most closely

approximates *in vivo* physiology; 2) numerous biochemical changes (including cell death) can occur over a 12-day culture period; and 3) the main experiments in this thesis (experiments G through I) all involve cultures which end within 16 hours following termination of pituitary donors. During the 0-4 day culture period, type 2 cells secreted 1.8-times greater BGH and IGH than did mixed cells. By contrast, type 1 cells secreted 5.4-times less BGH and 2.3-times greater IGH than mixed cells. From these data it can be deduced that type 1 cells secreted primarily IGH, and type 2 somatotrophs secreted primarily BGH. This relationship holds true even when secretion is estimated on a per somatotroph basis (type 1≈15%, and type 2≈80% somatotrophs). Actual per somatotroph secretion could not be calculated, as the immunocytochemistry procedure failed for this experiment. The results indicate that cell separation was successful, and they essentially replicate the data of Grindeland and Hymer [36].

The single density cell separation system appears to replicate results obtained using the 2-density system, but only a few experiments have been done. For this reason the 2-density separation method was employed in experiment F to validate the cell separation procedure to be used in experiments G, H, and I. Over the course of the twelve-day culture, mixed cell and type 1 cell viabilities were within 71-89% at each determination. While 71% is lower than desired for this system, it was considered acceptable to provide valid data. However, type 2 cell viability was about 43%

post-dissociation and 11% following the 12-day culture. More will be said about the issue of viability below. The results of experiment F, a 12-day culture of separated cells obtained with the 2-phase method, are summarized in Table 4 and 5. Table 4 shows the results expressed on a per thousand cells seeded basis, while Table 5 shows the results on a per thousand somatotrophs basis. By comparing results from Table 4 with those from Table 5 an interesting point can be deduced. If a comparison of secretion rates for BGH and IGH is made between cell types, then the data in Table 4 leads one to believe that, over the 12 day culture period, type 2 cells secrete 7.5-times greater BGH and 3-times less IGH than type 1 cells. However, to describe accurate functional differences between somatotroph types, data must be compared on a per somatotroph basis. The data in Table 4 are included for completeness, and because prior to use of the immunocytochemistry procedure to quantitate numbers of cell types, results were expressed on a per thousand cells "seeded" basis [36]. As with experiment E, the experiment F discussion focuses on GH secretion during days 0-4, for the reasons mentioned above. It should be noted (Table 4) that 12-day type 2 cell BGH secretion was 4.5-times greater, and IGH secretion 1.3-times less than in mixed cells. By contrast, type 1 BGH secretion was 1.7-times less, and IGH secretion was 2.4-times greater than in mixed cells. These results, as well as those for 4-day secretion (Table 4), are in good agreement with the results from experiment E (Table 3) *qualitatively*, if not *quantitatively*. It is a

truism of cell culture that absolute levels of secretion do not reproduce well - even with aliquots of the same cells. However, with replicate experiments, the *pattern* of secretion is usually preserved. When expressed on a per thousand somatotroph basis (Table 5) the differences between cell types seen in Table 4 are repeated, though in all cases the magnitude of the difference is reduced. A reason for the diminution of differences is that type 1 cells were 17.4%, while type 2 cells were 77.6% somatotrophs. When the secretory activity of type 1 and type 2 cells are compared on a per thousand somatotroph basis the "true" functional differences become apparent. Over the 12-day culture, type 2 somatotrophs secreted 1.7-times more BGH than type 1 somatotrophs. It is not clear why mixed cells secreted less BGH than type 1 cells (Table 5), as this has not been observed before. It could be due to an error in cell counting. Over the 12-day culture period, type 1 somatotrophs secreted 9.9-times greater IGH than did type 2 somatotrophs (Table 5). Mixed cell IGH secretion was intermediate between that for type 1 and type 2 cells. The 12-day BGH/IGH activity ratios for media from mixed (BGH/IGH=3.4), type 1 (BGH/IGH=1.0), and type 2 cells (BGH/IGH=16) were in agreement with data from previous work by Grindeland and Hymer [36]. The pattern of BGH and IGH secretion for the 3 groups over the 3 successive 4-day intervals was interesting, but emphasis will be placed on 0 to 4-day secretion since it is this interval which is most relevant to the objectives of this thesis. In the 0 to 4-day

culture interval type 2 somatotrophs secreted 3-times greater BGH and 6-times less IGH than did type 1 somatotrophs. The BGH/IGH activity ratio was 20 for type 2 somatotrophs and 1.1 for type 1 somatotrophs. It can thus be seen that type 2 somatotrophs released primarily BGH. While it cannot be said, on the basis of these data, that type 1 somatotrophs release primarily IGH, it seems probable that most¹ of the circulating IGH comes from type 1 somatotrophs. Ideally, one would expect that the results from type 1 and type 2 somatotrophs could be used to predict mixed somatotroph BGH and IGH secretion, particularly if there were no cell-cell paracrine interactions. If, for the sake of simplicity, one assumes that type 1 and type 2 somatotrophs each represent 50% of the total somatotrophs, then the simple average of BGH and IGH secretion for type 1 and type 2 somatotrophs should yield a value similar to that for mixed somatotrophs. Inspection of the values in Table 5 indicate that in most cases, the averaged values exceed the mixed cell values by several-times. Perhaps the separation procedure disrupts some paracrine communication between pituitary cells. The cause of the discrepancy is unknown, but has also been observed by Hymer (personal communication, 1987). For this reason the BGH/IGH ratio appears to be a better index for comparison between cell groups. As described above, the BGH/IGH ratios for mixed, type 1, and type 2

¹ 86% on the basis of these data, assuming 50% type 1 somatotrophs *in vivo*. While this calculation does not include the effects of GRF or SRIF it demonstrates the differences between somatotroph types.

cells were in agreement with the work of Grindeland and Hymer [36]. BGH production in type 2 somatotrophs over the 12-day culture was 2.6-times greater than mixed somatotrophs, and 1.7-times greater than type 1 somatotrophs. By contrast, IGH production in type 2 somatotrophs was 6.1-times less than mixed somatotrophs and 14-times less than in type 1 somatotrophs. It can be seen from the BGH/IGH ratios in Table 5 that the major form of GH produced in mixed and type 2 somatotrophs is BGH. In type 1 somatotrophs approximately equal amounts of BGH and IGH were produced. If the BGH and IGH values are considered to be independent, then the per thousand somatotroph net GH production for type 1 somatotrophs ($825+856=1681$ ng / 1000 somatotrophs) was slightly more than type 2 somatotrophs ($1394+61.6=1456$ ng / 1000 somatotrophs). In actuality, BGH and IGH values are not independent since classical pituitary-derived GH possesses considerable activity in the tibial assay. Nevertheless, the aforementioned comparison was made to demonstrate that, contrary to the findings of Grindeland and Hymer [36], in this experiment type 2 somatotrophs did not produce significantly more GH than type 1 somatotrophs. It is unknown why this occurred; a probable cause is that separation of somatotrophs was incomplete. Considering the relatively large amounts of BGH produced and secreted from type 1 somatotrophs (compared to the results of Grindeland and Hymer), this explanation seems reasonable. The type 1 cell pool could have contained some type 2 somatotrophs. Overall, based on the percentage somatotrophs, the relative amounts

of BGH and IGH secreted, and the BGH/IGH activity ratios in the 3 different cell groups, it is evident that the cell separation procedure in experiment F was successful, and that the 12-day culture results were in agreement with the results of Grindeland and Hymer [36]. As with experiment E, however, the post-gradient cell viability for type 2 cells (68%) was markedly lower than for type 1 (84%) or mixed cells (80%). The type 2 cell viability on days 4, 8, and 12 was 83, 96, and 71%, respectively. These values are certainly acceptable for valid *in vitro* somatotroph study. However, the repeated observation (experiments E and F) that type 2 cell viability is lower than mixed or type 1 cells raised the question of whether or not incubation with BSA affects cell viability. It was agreed by all involved that the viabilities obtained in experiment F were acceptable for subsequent work.

In experiment G, dose response curves to rGRF-43 were generated in mixed, type 1 and type 2 somatotrophs. Results are not shown in graphic form, as the lower dose data points occurred so close together that display was difficult. As indicated in the results section, the dilution curve for BGH secretion from three treatment groups (mixed cells - 6.25 nM, type 2 cells - 0.39 nM, and type 2 cells - 3.13 nM) differed significantly from the slope of the standard curve, and should be considered as an estimate only. With the exception of IGH secretion from type 2 somatotrophs, BGH and IGH secretion for all groups reached a maximum at 1.56 nM. For type 2 somatotrophs, IGH secretion was maximal at 3.13 nM. At the

maximum dose of 1.56 nM, per thousand somatotrophs, IGH secretion was greatest in type 1 somatotrophs, followed by mixed somatotrophs, and type 2 somatotrophs, as would be expected given the results of previous studies (Grindeland and Hymer [36] and experiment F). The ED50s for mixed, type 1, and type 2 somatotrophs differed, being 1.0, 1.1, and 1.3 nM, respectively, as determined from the dose-response curves shown in Figure 7. The BGH data for the 3 cell groups were too variable to obtain accurate ED50 values. When the data in Table 6 are examined carefully, it can be seen that after BGH and IGH secretion for the 3 groups peaked (about 1.56 nM) there was a considerable decline in secretion reaching a minimum at 12.5 to 25 nM, followed by a second increase in IGH secretion reaching another peak at about 100 nM. This curious biphasic result has not been reported elsewhere, but seems to be valid. In the original report on rGRF-43 by Speiss, Rivier, and Vale [100], IGH dose-response curves up to 12.5 nM were reported. Maximal secretion occurred at 0.5 to 2.5 nM for both native and synthetic rGRF-43, but declined by about 12% at the 2.5 nM dose for native rGRF-43, and declined by about 12% for synthetic rGRF-43 at 12.5 nM. Their results were not as dramatic as those shown in Table 6, but they did not report doses higher than 12.5 nM. Since no other researchers have published results using GRF doses higher than that which produces the maximal GH secretory response (≈ 2 nM), it cannot be determined whether this is a repeatable phenomenon. Yet the results of Speiss *et al.* [100] suggest that this biphasic response

to GRF may be repeatable. Given that this observation is real, the mechanism of the response, and its physiological significance, remain open to speculation.

Table 7 shows the maximal percent increase over control BGH and IGH secretion for the three cell groups. Though the absolute amount of maximal-dose BGH released per thousand type 2 somatotrophs (528 ng / 1000 somatotrophs) was 2.3-times greater than in type 1 somatotrophs (229 ng / 1000 somatotrophs), the increase over type 2 control secretion was only 310% compared to 560% for type 1 somatotrophs. By this analysis the type 1 somatotroph appears to be more sensitive to rGRF-43 than the type 2 somatotroph. A better comparison would be to consider BGH ED50s, but as indicated, this was not possible due to high variability in the data. At most GRF doses, the BGH/IGH ratio was several-times higher in type 2 somatotrophs than in type 1 somatotrophs. In neither type 1 nor type 2 cell groups, were steadily increasing BGH/IGH ratios seen during increasing GRF dose, as were found in experiment A (Figure 1B). In mixed cells, BGH secretion at 100 nM was 1.5-times greater than at 1.56 nM (Table 6), suggesting a response pattern similar to that of experiment A, though diminished. The mixed cell BGH/IGH ratio also increased from 2.4 at 1.56 nM to 4.3 at 100 nM, with intermediate GRF doses yielding intermediate BGH/IGH ratios. Since a similar pattern was not seen in either type 1 or type 2 somatotrophs, the mechanism of the response remains unknown.

The results of experiment G demonstrate: 1) that the BGH and IGH responses to hpGRF-40 in experiment A were a composite result of type 1 and type 2 somatotroph secretion; 2) that both type 1 and type 2 somatotrophs increase release of both BGH and IGH in response to rGRF-43; 3) that type 1 and type 2 somatotrophs have similar dose-responses to rGRF-43 in terms of BGH and IGH maximal doses, and IGH ED50s; 4) that maximal GRF-stimulated BGH secretion from type 2 somatotrophs is 2.3-times greater than for type 1 somatotrophs; and 5) that maximal GRF-stimulated IGH secretion from type 2 somatotrophs is 1.3-times less than for type 1 somatotrophs. The majority of BGH released *in vivo* in rats is apparently derived from type 2 somatotrophs, as indicated by results from experiment G during GRF-stimulation and data obtained from 4-day cultures in the absence of GRF, experiment F. The results of experiment G also demonstrate that it is unlikely that separate GRFs govern the release of BGH and IGH through independent regulation of somatotroph types, since rGRF-43 stimulates release of both forms of GH from both cell types. Thus, it seems reasonable that combinations of GRF and some other factor control the selective release of BGH, presumably through inhibition of type 1 somatotroph secretion with simultaneous stimulation of type 2 somatotroph secretion. The most likely candidate for interaction with GRF is SRIF since this is a well-established regulatory peptide affecting GH release. Experiments I and J were designed to specifically examine the effects of GRF and SRIF, alone, and in

combination, on the release of BGH and IGH from the 3 cell groups. It was once again observed that in experiment G, post-gradient type 2 cell viability was 40% compared to 81% in type 1 cells, and 92% in mixed cells. Since this was the third incidence in 3 cell separation experiments in which type 2 cell viability was low, it was decided that formal investigation of this problem was critical before further work was performed.

The formal investigation of the effects of 45-minute incubation of mixed, dispersed pituitary cells in 1.078 g/mL BSA (experiment H) indicated that the BSA incubation is without measurable effect on cell viability and basal or GRF-stimulated BGH or IGH secretion when compared with cells incubated at room temperature (RT; 22-24°C). In replicate experiments (experiment H1 and H2; Figure 8 and 9, respectively) it was found that there was no pattern in the data which suggested that BSA incubation affected cell function. Pre- and post-BSA incubation viabilities were in good agreement in both experiments. Though some differences were occasionally noted between GH secretion between groups, there was no consistent pattern. In some cases secretion for the BSA group was higher than in the RT group, while in other cases the situation was reversed. Incubation at 5°C for 45 minutes was also without effect, as would be expected given that BSA-incubation at 5°C was also without effect. Since type 2 cell viability was consistently low in experiments E, F, and G, the results of experiment H raise the important question of why this occurred and, secondly, can the

qualitative results of experiments E, F, and G be considered valid with such low viability in type 2 cells? Typically, cells for viability determinations were incubated for 20 minutes in viability media prior to differential cell counting with a hemocytometer. On one occasion the procedures were so time-consuming that cells had to incubate for about 45 minutes prior to cell counting. It was observed that the counting process was considerably easier than with the 20 minute incubation period, since the cells had taken up more neutral red! In procedures with mixed cells that were processed at room temperature, this change in procedure did not affect viability. However, as will be mentioned below for experiments I and J, this change yielded type 2 cell viabilities that did not differ from mixed or type 1 cells. It seems likely that the cell separation procedure might transiently affect metabolic rate in type 2 cells, thus leading to a decreased rate of transport of neutral red across the plasma membrane. Alternately, type 2 cells may simply transport neutral red at a slower rate than type 1 cells and if one waits long enough accurate viability data can be obtained with this technique. It was concluded, therefore, that the low type 2 cell viability in experiments E, F, and G were probably artifactual and conclusions reached were valid.

Experiment I examined the effects of GRF and SRIF alone, and in combination, on both BGH and IGH secretion from mixed, type 1, and type 2 somatotrophs. In all 3 cell groups GRF treatment caused a dose-related increase in both BGH and IGH secretion. In mixed

cells, both BGH and IGH were secreted in parallel during GRF-treatment (no change in BGH/IGH ratios). In type 1 cells, 1.0 nM GRF treatment produced an increase in the BGH/IGH ratio relative to controls or 0.25 nM GRF treatment. In type 2 cells, 1.0 nM GRF treatment produced a decrease in the BGH/IGH ratio relative to controls or 0.25 nM GRF treatment. This indicates that type 1 and type 2 somatotrophs differ in response to rGRF-43. Type 1 and type 2 somatotrophs also differ in their percent increase over control BGH and IGH secretion during GRF-treatment (Table 9). With the exception of IGH secretion in response to 1.0 nM GRF, the percent increase over control secretion was consistently greater for type 1 somatotrophs. This is consistent with BGH, but not IGH secretion in experiment G (Table 7). During control and GRF treatment, BGH secretion was consistently several-times greater in type 2 somatotrophs than in type 1 somatotrophs, findings consistent with the results of experiments E, F, and G, and Grindeland and Hymer [36]. Based on these results, and the fact that each somatotroph type represents about 50% of the total somatotrophs, it seems reasonable to conclude that in mixed cells, the majority of BGH secretion in the absence of secretagogue and during GRF-treatment comes from type 2 somatotrophs, while IGH secretion in mixed cells is about equally from type 1 and type 2 somatotrophs (Table 8, Figure 10). On the basis of these data, it is clear that the results for mixed cell secretion are due to secretion of different GH forms from the 2 somatotroph types.

Treatment with SRIF alone suppressed both BGH and IGH secretion from mixed and type 1 somatotrophs to 40-41% of control secretion, and was maximally suppressed with the 0.5 nM dose (Table 8, Table 10). The SRIF-induced maximal inhibition of IGH release from mixed cells was in agreement with the results of Brazeau *et al.* [14]. In their work, 5.0 nM SRIF inhibited IGH release to 34% of control. In type 2 somatotrophs IGH secretion was suppressed in a dose-related manner to 59% and 42% of control secretion with 0.5 and 10 nM SRIF, respectively. The results for type 2 somatotroph BGH secretion during SRIF treatment (Table 8, Table 10) were quite surprising, suggesting that 0.5 nM SRIF has a stimulatory effect on BGH release (Table 10). It seems unlikely that this is the case, however, since type 2 cell BGH secretion in the presence of 10 nM SRIF was 1.4-times less than in the presence of 0.5 nM SRIF. On the basis of these data it seems reasonable to conclude that SRIF does not suppress (or minimally suppresses) BGH release from type 2 somatotrophs at doses as high as 10 nM. Further evaluation of this statement will be considered below when the results of the repeat experiment J are discussed. Despite the apparently anomalous results for type 2 somatotroph BGH secretion in the presence of 0.5 nM SRIF, it can be seen in Figure 10 that the two somatotroph types respond differently to SRIF. It can also be seen in Figure 10 that the mixed cell BGH and IGH secretion does not mirror the composite results for type 1 and type 2 somatotrophs. This finding is not unusual since it has been observed previously (experiment F, G, and

Hymer and Grindeland [36]). However, most often the composite *pattern* of secretion from type 1 and type 2 cells is reflected in the mixed cell data. It is thus not clear why BGH secretion from type 2 somatotrophs during SRIF treatment deviated so sharply from the expected pattern based on mixed cell secretion (Figure 10). Perhaps some paracrine communication between the two somatotroph types, or other cell types, was disrupted as a result of the cell separation procedure. It is important to note, that approximately 80% of the type 1 cell fraction is comprised of a mixture of corticotrophs, mammotrophs, gonadotrophs, thyrotrophs and other pituicytes. Similarly, about 15% of high density cells is comprised of other pituicytes, though of a lower density than in type 2 somatotrophs.

The effects of combined GRF and SRIF treatments demonstrated the most profound differences between cell types and offered an explanation of the mechanism of selective release of BGH during stress in rats. In mixed cells the combined 1.0 nM GRF / 0.5 nM SRIF treatment produced a large suppression of both BGH and IGH secretion relative to the 1.0 nM GRF treatment alone (Figure 10). However, both BGH and IGH secretion remained stimulated relative to controls (Table 11). The BGH/IGH ratio for secreted GH for the aforementioned treatment was increased to 1.68 relative to 1.42 in controls (Table 8). The combined 1.0 nM GRF / 10 nM SRIF treatment produced an even larger suppression of both BGH and IGH secretion relative to the 1.0 nM GRF treatment alone (Figure 10). In the 1.0 nM GRF / 10 nM SRIF treatment group, both BGH and IGH secretion were suppressed below

control levels (Table 11), though suppression of IGH was greater than BGH, thereby causing an increase in the BGH/IGH ratio to 3.42 (compare with 1.42 for control, Table 8). Since stress in rats causes an inhibition of IGH release [1, 35, 104, 105, 106], release of SRIF [1, 105, 106], and presumptive release of GRF [50, 53], it is reasonable to assume that the selective release of BGH during stress in rats [52, 54, 68, 104] is due to the combined effects of hypothalamic GRF and SRIF on somatotrophs. The *in vitro* mixed cell response to 1.0 nM GRF / 0.5 nM SRIF treatment are in accordance with this reasoning. The results fit well with this view and appear to support the experimental hypothesis that combinations of GRF and SRIF cause selective release of BGH. This view is strengthened by the fact that the effects of only 2 dose combinations were examined. If a more complete range of doses were examined, it seems likely that a combination could be found which would suppress IGH secretion to below control levels while simultaneously stimulating BGH secretion.

Based on the results of experiment I, the cellular origin of the selective release phenomenon is in type 2 somatotrophs. As can be seen in Figure 10, both BGH and IGH secretion from type 1 somatotrophs were suppressed to levels below control secretion with 1.0 nM GRF treatment in combination with either 0.5 nM or 10 nM SRIF. Suppression of IGH release was related to the SRIF dose in combination with GRF (Table 11). In type 2 somatotrophs the combined treatments suppressed IGH secretion to control levels (Figure 10, Table 11), but simultaneously stimulated BGH secretion to

149 and 139 percent of control secretion (1.0 nM GRF in combination with 0.5 nM and 10 nM SRIF, respectively). The BGH/IGH ratios for secreted GH during combined GRF and SRIF treatments decreased for type 1 cells and increased for type 2 cells relative to the appropriate controls (Table 8). This finding is consistent with the associated BGH/IGH ratios for mixed cell treatments, and with *in vivo* results already mentioned. The results are also in agreement with the findings of Grindeland *et al.* [38], which demonstrated that, compared to controls, mixed and type 2 somatotrophs from cold-exposed (4°C, 1h) donor rats release 40 and 75% less BGH during 4-day culture. Secretion of IGH was unaffected in all 3 cell groups, and type 1 cell BGH secretion was also unaffected by cold exposure.

Though the chemical nature and functional significance of the different forms of GH released from the 2 somatotroph populations remains to be determined, these data demonstrate several important points: 1) bioassay of GH is an important tool in the evaluation of GH physiology; 2) secretion of BGH and IGH are under differential regulation by hypothalamic GRF and SRIF; 3) the majority of BGH is derived from type 2 somatotrophs and; 4) there is a functional heterogeneity of pituitary somatotrophs.

Experiment J, an exact replication of experiment I, examined the effects of GRF and SRIF alone, and in combination, on both BGH and IGH secretion from mixed, type 1, and type 2 somatotrophs. As in experiment I, GRF treatment caused a dose-related increase in both BGH and IGH secretion in all 3 cell groups. In mixed cells, both

BGH and IGH were secreted in parallel during GRF-treatment (no change in BGH/IGH ratios), replicating experiment I data. In contrast to experiment I, 1.0 nM GRF treatment of type 1 cells did not produce an increase in the BGH/IGH ratio relative to controls or 0.25 nM GRF treatment. Rather, the ratio was unchanged. In agreement with experiment I, both 0.25 nM GRF and 1.0 nM GRF treatments of type 2 cells produced a decrease in the BGH/IGH ratio relative to controls. Though the values differ between experiments, the results replicate the observation that type 1 and type 2 somatotrophs differ in response to rGRF-43. Type 1 and type 2 somatotrophs also differ in their percent increase over control BGH and IGH secretion during GRF-treatment (Table 13). In contrast to experiment I (where the percent increase over control BGH and IGH secretion was greatest for type 1 somatotrophs) the percent increase over control BGH and IGH secretion was greatest for type 2 somatotrophs. This variability between experiments is likely due to variation in completeness of cell separation between experiments. Since separation of type 1 and 2 somatotrophs is never perfect with the density gradient procedure, some of the more subtle differences between cell types can only be resolved by performing several replicate experiments (recall that for experiments I and J cells were derived from a common "pool" of approximately 100 anterior pituitaries per experiment). During control and GRF treatment, BGH secretion was consistently several times greater in type 2 somatotrophs than in type 1 somatotrophs, findings consistent with the results of

experiments E, F, G and I, and Grindeland and Hymer [36]. As with experiment I, it is apparent that the majority of BGH secretion, in the absence of secretagogue and during GRF-treatment, comes from type 2 somatotrophs, while IGH secretion in mixed cells is contributed about equally from type 1 and type 2 somatotrophs (Table 12, Figure 11).

Treatment with SRIF alone suppressed both BGH and IGH secretion from mixed and type 1 somatotrophs to 24-48% of control secretion, and was maximal with the 0.5 nM dose (Table 12, Table 14). As in experiment I, the SRIF-induced maximal inhibition of IGH release from mixed cells was in agreement with the results of Brazeau *et al.* [14]. In their work, 5.0 nM SRIF inhibited IGH release to 34% of control. In type 2 somatotrophs, IGH secretion was suppressed to 47% and 45% of control secretion with 0.5 and 10 nM SRIF, respectively. BGH secretion from type 2 somatotrophs was suppressed to 61% and 64% of control secretion with 0.5 and 10 nM SRIF, respectively (Table 12, Table 14). It appears that both BGH and IGH secretion were already maximally suppressed with the low SRIF dose of 0.5 nM, a result somewhat different than in experiment I. Though the effect of SRIF in experiment I appeared to be dose-related, note that the 0.5 nM dose seemed to have a stimulatory effect on BGH secretion. Experiment J supports the conclusion that experiment I was in error in this regard. Despite minor variability between experiments, it can be seen in Figure 11 that the two somatotroph types respond differently to SRIF. It can also be seen in

Figure 11 that the composite *pattern* of secretion from type 1 and type 2 cells is reflected in the mixed cell results.

As in experiment I, the effects of combined GRF and SRIF treatments demonstrated the most profound differences between cell types and offered an explanation of the mechanism of selective release of BGH during stress in rats. In mixed cells, the combined 1.0 nM GRF / 0.5 nM SRIF treatment produced a suppression of both BGH and IGH secretion relative to the 1.0 nM GRF treatment alone (Figure 11). However, both BGH and IGH secretion remained stimulated relative to controls (Table 15). This result is in agreement with experiment I. Also in agreement, the BGH/IGH ratio for secreted GH for the 1.0 nM GRF / 0.5 nM SRIF treatment was increased to 4.2 relative to 3.6 in controls (Table 12). The combined 1.0 nM GRF / 10 nM SRIF treatment produced an even larger suppression of both BGH and IGH secretion relative to the 1.0 nM GRF treatment alone (Figure 11). In the 1.0 nM GRF / 10 nM SRIF treatment group, both BGH and IGH secretion were suppressed below control levels (Table 15), though suppression of IGH was greater than BGH, thereby causing an increase in the BGH/IGH ratio to 6.3 (compare with 3.6 for control- Table 12). It has already been mentioned, but is worth repeating that stress in rats causes an inhibition of IGH release [1, 35, 104, 105, 106], release of SRIF [1, 105, 106], and presumptive release of GRF [50, 53]. For these reasons it is reasonable to assume that the selective release of BGH during stress in rats [52, 54, 68, 104] is due to the combined effects of hypothalamic GRF and SRIF on somatotrophs. The

in vitro mixed cell response to 1.0 nM GRF / 0.5 nM SRIF treatment are in accordance with this reasoning. The results fit well with this view. Furthermore, they appear to support the experimental hypothesis that combinations of GRF and SRIF cause selective release of BGH. This view is strengthened by the fact that the effects of only 2 dose combinations were examined. If a more complete range of doses were examined, it seems likely that a combination could be found which would suppress IGH secretion to below control levels while simultaneously stimulating BGH secretion.

Based on the results of experiments I and J, the cellular origin of the selective release phenomenon is in type 2 somatotrophs. This has already been discussed for experiment I. For experiment J, it can be seen (Figure 11) that, with the exception of IGH secretion from 1.0 nM GRF / 0.5 nM SRIF group, both BGH and IGH secretion from type 1 somatotrophs were suppressed to levels below control secretion during both of the combined GRF/SRIF treatments. Suppression of IGH release was related to the SRIF dose in combination with GRF (Table 15). In type 2 somatotrophs the combined treatments suppressed BGH secretion to control levels (Figure 11, Table 15), and simultaneously stimulated IGH secretion to 472% and 136% of control secretion (1.0 nM GRF in combination with 0.5 nM and 10 nM SRIF, respectively). This is in contrast to experiment I, in which BGH secretion was stimulated and IGH secretion was suppressed to control levels during the combined treatments. It is not clear why the 2 experiments differed, though it may be related to differences in the completeness

of cell separation between the two experiments. Whatever the cause of the discrepancy for type 2 cells, the pattern of effects of GRF and SRIF on BGH and IGH secretion from mixed somatotrophs were equivalent in experiment I and J. Moreover, it is apparent from these experiments that the majority of the mixed cell BGH secretion was from type 2 somatotrophs. Therefore, since the mixed cell BGH/IGH ratio increased during combined GRF/SRIF treatments (experiments I and J) it is reasonable to conclude that the type 2 somatotroph is less sensitive to SRIF (in terms of BGH secretion) than is the type 1 somatotroph. The data from the SRIF treatments alone (Table 14) also support the conclusion that type 2 somatotrophs are less sensitive to SRIF in terms of BGH secretion. Note that in Table 14 IGH secretion is suppressed to a greater extent than BGH secretion during SRIF treatment in type 2 cells.

Evidence for multiple molecular forms of growth hormone was presented above (see Introduction and Literature Review). In light of the multiple physiological effects that have been demonstrated for growth hormone (increased transport of amino acids across the plasma membrane, increased protein synthesis, mitosis of many cell types, early insulin-like and delayed anti-insulin effects such as increased lipolysis) it is tempting to speculate that the differential control of BGH and IGH release from type 1 and 2 somatotrophs is of physiological significance. Based on the results of experiments I and J, *in vivo* results indicating that both GRF and SRIF are released during stress in rats (see above), and results showing that cold-

stress in rats depletes only BGH from type 2 cells (and neither BGH or IGH from type 1 cells [38]) it is possible to propose a theory for the control of BGH and IGH secretion from type 1 and 2 somatotrophs. It is proposed that in rats, stressors such as 4°C for 30 minutes, insulin-induced hypoglycemia, or overnight fasting (see Introduction and Literature Review section) cause hypothalamic release of GRF and SRIF in a GRF/SRIF concentration ratio in the approximate range of 0.1 to 2 with absolute concentrations similar to those used in experiments I and J. It is also proposed that the type 2 somatotroph is less sensitive to SRIF in terms of BGH release and that the above combinations of GRF and SRIF produce an increase or no change in plasma concentration of BGH with a concomitant decrease in the plasma concentration of IGH. The resultant increase in the ratio of BGH/IGH mediates as yet undetermined physiological responses that are of benefit to the "stressed" animal. This theoretical framework could provide the basis for numerous future investigations of GRF and SRIF receptor affinities and concentrations, post-receptor differences, differences in secretory granules and enzymes for processing GH, *etcetera*. Additionally, it will be important to determine differences in physiological effects of BGH and IGH on various tissues. Some important questions remaining unanswered are: 1) Are type 1 and 2 somatotrophs discrete cell types or are they interconvertible?; 2) Are there 2 types of secretory granules or are BGH and IGH co-localized in a single type of secretory granule?; 3) Do the quantities and/or affinities of GRF and SRIF receptors differ

between cell types?; and 4) How is the BGH/IGH ratio of GH released from a single cell controlled intracellularly? The answers to some of these important questions will undoubtedly broaden our understanding of one of the most important regulators of cellular metabolism, but will also broaden our understanding of hormone and neurotransmitter release in general.

The GH system is not unique in containing cells which store and release more than one form of hormone. Corticotrophs have been shown to synthesize and release both adrenocorticotropin and β -endorphin [116], while gonadotrophs have been shown to store and release both follicle-stimulating hormone and luteinizing hormone [74]. Some of the adrenergic cells of the adrenal gland contain dopamine, epinephrine, norepinephrine, adenosine triphosphate, dopamine- β -hydroxylase, and phenylethanolamine-N-methyltransferase, all localized in the same secretory granules [113]! The GH system is apparently not alone in possessing cells which contain more than one hormone type. It is also known that insulin complexes with zinc within beta cell secretory granules [73]. Perhaps GH is complexed in some analogous fashion, thereby producing a decrease in immunoactivity with no loss in bioactivity. It is unlikely that the answers to these complex questions will be simple or be found quickly, but the discoveries significantly increase our understanding of the physiology of the growth hormone system.

V. SUMMARY AND CONCLUSIONS

1. This research has shown that rat somatotrophs are functionally heterogeneous in both their secretory activity and regulation by hypothalamic hormones as evidenced by:
 - Differing quantities and ratios of BGH and IGH secreted.
 - Greater sensitivity of type 1 cells to SRIF.
 - Greater effect of SRIF on IGH than on BGH secretion by type 2 cells.
2. During GRF treatment, BGH and IGH are released with similar percent increases over control secretion for both GH forms.
3. Bioassay of GH is an important adjunct to the immunoassay in the evaluation of GH physiology.
4. The combined GRF/SRIF data are consistent with earlier work by Grindeland and Hymer in which cold stress (4° C, 30 min.) of rats depletes BGH but not IGH from type 2 somatotrophs, without effect on IGH or BGH in type 1 somatotrophs.
5. Since there is evidence that both SRIF and GRF are released during environmental stress in rats, the results presented here could explain the unchanged or decreased IGH levels and elevated BGH levels in plasma of "stressed" rats.

Appendix A: Procedures for Preparation of Media and Solutions

Media A

Media A was Spinner's Minimum Essential Medium (SMEM; Gibco Cat. no. 410-1400; Grand Island, NY), 10 mL/L of 10,000 U/mL Penicillin and 10,000 µg/mL streptomycin (Penn-Strep; GIBCO Cat. #600-5140; Grand Island, NY), and 0.1% crystalline bovine serum albumin (BSA; Cat. #A4378; Sigma Chemical Co.; St. Louis, MO) adjusted to pH 7.3 with NaOH.

Media B - Dissociation Media

Media B consisted of media A with 3% w/v BSA (final percentage), 0.3% w/v Trypsin (Difco, 1:250), 0.02 mg/mL deoxyribonuclease (DNase; Cat. #D-5025; Sigma Chemical Co.; St. Louis MO), adjusted to pH 7.6 with NaOH.

Media C - Primary Culture Media

Media C was alpha-modified Eagle's media (Cat. no. 10-311; Flow Laboratories; McLean, VA), 1% w/v crystalline BSA (Cat. #A4378; Sigma Chemical Co.; St. Louis, MO), 5% v/v calf serum (Cat. #200-6170AG; GIBCO; Grand Island, NY), 10 mL/L Penn-Strep, 10 mg/L Gentamycin sulfate (Cat. #G-3632; Sigma Chemical Co.; St. Louis, Missouri), 25 mM hydroxyethylpiperazine-N-2-ethane-sulfonic

acid (HEPES; Cat. no. H-0506; Sigma Chemical Co.; St. Louis, MO), and 0.2% sodium bicarbonate, adjusted to pH 7.4.

BSA Stock Solution and Unbalanced Salt Solution (UBSS)

Fraction V BSA was exhaustively dialyzed (200 mL of BSA solution dialyzed against 17 L of double distilled water for 3 days with water changes twice daily), freeze dried, and reconstituted in an unbalanced salt solution (UBSS) to a stock of 40% (w/w). UBSS is 0.801% w/v NaCl, 0.034% w/v KCl, 0.025% w/v CaCl₂, 0.016% w/v KH₂PO₄, 0.028% w/v MgSO₄ in deionized + distilled water. The 40% BSA stock was filter-sterilized through a series of 8.0 µm, 3.0 µm, 1.2 µm, and 0.45 µm filters. The UBSS was filter sterilized through a 0.22 µm filter. In a 4°C cold room in a sterile work field, solutions of 1.071 g/mL and 1.085 g/mL were prepared using Shortman's procedure [96].

Neutral Red Stock Solution

A 0.1% w/v neutral red stock solution was prepared by dissolving dye (Cat. #NX265; Matheson Coleman & Bell; Norwood, Ohio) in distilled water in a conical flask, mixing well, covering with aluminum foil and autoclaving at 15 p.s.i. for 15 minutes. When the solution was cooled, a rubber stopper was inserted, the top was covered with aluminum foil, and the solution was stored at room temperature for repeated use.

Zamboni's Fluid

Zamboni's fixative was prepared as follows: in a fume hood, 20 g of paraformaldehyde was added to 50 mLs of distilled water and heated to 60°C. Sodium hydroxide (1.0 N) was added dropwise until the solution became clear. The solution was then filtered hot through Whatman #1 filter paper and allowed to cool. Saturated picric acid (75 mL) was added, and the solution was brought to 500 mL with phosphate buffer (3.31 g $\text{Na H}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 33.77 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ per liter H_2O ; pH 7.3). The solution was stored at 4°C and discarded after one month.

Phosphate Buffered Saline (PBS)

PBS consists of 8.00 g/L NaCl, 0.20 g/L KCl, 2.16 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.20 g/L KH_2PO_4 , 0.05 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, in deionized, distilled water, pH 7.2.

Kaiser's Glycerol

Kaiser's glycerol was prepared by soaking 8.0 mLs of gelatin (Cat. #G-2625; Sigma Chemical Corp.; St. Louis, MO) in 52 mLs of water for 1-2 hours, adding 50.0 mLs of glycerol and 0.01 g merthiolate, then heating to 60°C for 15 minutes while stirring to homogeneity.

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