RADIOIMMUNOASSAY FOR RAT GROWTH HORMONE; FURTHER STUDIES ON THE CONTROL OF GROWTH HORMONE SECRETION IN THE RAT

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

ELIAS DICKERMAN

1971

This is to certify that the

thesis entitled

RADIOIMMUNOASSAY FOR RAT GROWTH HORMONE;
FURTHER STUDIES ON THE CONTROL OF GROWTH
HORMONE SECRETION IN THE RAT

presented by

Elias Dickerman

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

Joseph Meites
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Date February 24, 1971

O-7639

ABSTRACT

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By

Elias Glickerman

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intact rats and a purified preparation of RGH. No inhibition was observed when using serum from hypophysectomized rats or purified preparations of other rat anterior pituitary hormones, indicating that the anti-RGH serum used was specific, in the rat, for GH. RGH levels of several pituitary preparations were lower when determined by radioimmunoassay than by bioassay; the mean radioimmunoassay levels were, however, within the 95% confidence limits of the bioassay results. The assay reported here has a sensitivity of 0.25 mug of RGH.

2. The anti-rat growth hormone serum was tested for cross-reactivity with purified preparations of GH, pituitary homogenates and plasma of other animal species. Cross-reactions were observed with pituitary homogenates and plasma of hamster, guinea pig, gerbil and mouse, as well as with cat and dog plasma. Partial cross-reation occurred with purified preparations of ovine and bovine growth hormone, while no cross-reaction was observed with purified human growth hormone, monkey pituitary homogenate and rabbit plasma. These results reinforce the concept of a common antigenic structure in the growth hormone of several mammalian species, as well as the difference in antigenicity of primate growth hormones. Furthermore, they suggest that the radioimmunoassay may be employed to measure GH in the hamster, guinea pig, gerbil, mouse, cat and dog, and perhaps other species as well.

3. Growth hormone levels were measured by radioimmunoassay in plasma and serum samples of the same origin varying

the length of incubation time for the second antibody. In all cases plasma GH concentrations were significantly higher than serum GH concentrations. The differences observed were not the result of differential influences on the radioimmunoassay. Recovery of exogenous RGH in plasma or serum of hypophysectomized rats was about 100%. Comparison of intact rat plasma and serum centrifuged at the time of collection and incubated for 0, 12, 24 or 48 hours at 4°C, or incubated for the indicated time and then centrifuged, showed that the former resulted in almost no disappearance of GH, while significant decreases in GH concentration were observed with time in the latter group; the longer the interval the greater the decrease. For any given time period less GH was recovered in the serum than in the plasma group; serum GH at 0 hour centrifugation time was 74-81% of plasma GH. These data suggest that immunological inactivation of RGH takes place during, and perhaps as a result of, the process of coagulation.

4. Plasma and pituitary GH concentrations were determined in male and female rats from age 21 days until maturity or old age. Pituitary and plasma concentration increased steadily during the first 8-12 weeks of age in male and female rats, with the sharpest increase taking place after vaginal opening in females. These hormone levels remained elevated to 120 days with decreases in plasma observed at 180 and 240 days in females and males respectively. In female rats of two different ages with normal estrous cycles, mean plasma GH concentration

in estrus was significantly higher than found in proestrus, metestrus or diestrus, when no differences were observed. No changes in pituitary GH occurred during the cycle. Bilateral ovariectomy significantly decreased plasma GH while elevating pituitary GH levels; daily injections of estradiol benzoate, on the other hand, significantly increased plasma GH with a concomitant decrease in pituitary GH. Since the most rapid rate of body growth takes place when pituitary and plasma GH are lowest, these results suggest: a) that GH may not play as important a role in the life of the young rat as it does in the mature animal, or b) that the increased utilization of GH combined with a low rate of synthesis prevents the building up of pituitary and plasma levels of GH. It would appear that the elevation in plasma GH during estrus is due to estrogen secretion..

5. The effects of castration, thyroidectomy, testosterone propionate (TP)and Na-thyroxine (T_4) on pituitary and plasma GH levels, metabolic clearance rate (MCR) and secretion rate (SR) of GH were determined in male rats. Castration and thyroidectomy significantly decreased pituitary and plasma GH levels. Testosterone propionate and Na-thyroxine, on the other hand, significantly elevated pituitary and plasma GH 10 days after treatment. Metabolic clearance rate was not altered by castration, but it was decreased by thyroidectomy and increased by TP and T_4. All of the treatments produced significant changes in SR. Castration and thyroidectomy reduced SR of GH to one half and one third respectively.

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Conversely, TP and T_4 treatment increased SR to 1.8 and 2.2 times higher than that observed for controls. These results indicate that thyroidectomy and castration reduce the synthesis and release of GH from the pituitary while TP and T_4 increase the rate at which GH is synthesized by and released from the pituitary. These results also suggest that proper distinction be made between MCR, which refers only to the volume of plasma cleared of hormone, and SR, which reflects the amount of hormone used per unit time.

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By

Elias Dickerman

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Physiology

1971

Dedicated to my parents,
David and Sivia Dickerman,
to my wife Katherine, and
to my daughter, Beyle.

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ACKNOWLEDGMENTS

During my tenure as a graduate student I have received assistance from a number of persons. Among those who have been particularly helpful, Dr. A. Gjerding Olsen, Assistant Professor of Biology at Brandeis University, Waltham, Massachusetts, was one of the first. I am most grateful to him for giving me the first opportunity to participate in research, for his guidance throughout my first research project, for his encouragement to pursue graduate study and for his continued interest in me and my career. At Michigan State University, Dr. Joseph Meites, Professor of Physiology and my advisor during my study for the M.S. and Ph.D. degrees, provided sound advice and financial assistance in conjuction with Michigan State University. Equally important, he allowed me to conduct research to the best of my abilities and provided the opportunity to meet leading scientists in the field. My sincere appreciation to Dr. Meites cannot be described accurately in words.

The members of my Ph.D. committee gave much time to the reading and correcting of the thesis manuscript. In addition, their thought -provoking questions on examinations provided new learning situations for me. Including Dr. Meites, these committee members are: Dr. W. Doyne Collings, Dr. Harold D. Hafs, Dr. H. Allen Tucker, Dr. Thomas W. Jenkins, Dr. Allan

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J. Morris and Dr. Edward M. Convay. Their help is gratefully acknowledged.

Finally, I would like to mention the help of a co-worker, Dr. Ytschak Koch. Working with him in the laboratory and discussing my research with him, I was stimulated to many new insights and ideas which helped greatly in the formulation of my thesis. His incisive contributions represent an integral part of my work. Along with Dr. Koch, my brother Dr. Samuel Dickerman, assisted me in many hours of laboratory experimentation and consulted with me at length throughout the course of many conversations.

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INTRODUCTION

It is now generally accepted that the hypothalamus exerts a controlling influence on anterior pituitary function. Green and Harris (1949) had suggested that humoral agents secreted from the hypothalamus into the hypophyseal portal veins traveled to the anterior pituitary to exert a regulatory influence on the secretion of anterior pituitary hormones. Some of these agents, now known as hypothalamic releasing and inhibiting factors or hormones, have recently been detected in hypophyseal portal blood plasma, re-inforcing the suggestion of Green and Harris (1949).

Perhaps the most important contribution in neuroendocrinology has been the recent reports on the elucidation of the structure and synthesis of thyrotropin releasing hormone by the laboratories of Guillemin and Schally working independently. Apparently, Schally's laboratory has also determined the structure and synthesized growth hormone releasing hormone. Needless to say, the availability of synthetic hypothalamic releasing hormones should permit studies heretofore not possible.

The physiological role of GH-RF has been studied by measuring hypothalamic content of GH-RF by in vivo depletion of pituitary GH or in vitro release of GH from

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incubated pituitaries. A few reports on plasma or serum GH in the rat have been published. In general, however, the methods available for the assay of GH have not been sensitive enough to permit the measurement of GH in reasonable volumes of blood plasma or serum of normal animals or have been poorly validated and characterized. Obviously, conclusions regarding synthesis and release of GH based on pituitary levels of GH are subject to errors of interpretation.

This thesis was concerned with the development of a sensitive radioimmunoassay for rat GH. Using this assay method it has been possible to determine some of the natural changes in GH which take place with age, sex, estrous cycles and as a result of other hormonal influences. Perhaps more importantly, it was possible to study the metabolic clearance and secretion rates of GH in animals under different physiological conditions. These observations are also reported in this thesis.

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REVIEW OF LITERATURE

I. Assay of Growth Hormone

The bioassay of a hormone generally reflects a quantitative definition of its biological activity. This definition becomes more complex for a hormone which does not have a specific target organ, as is the case for growth hormone. The numerous biological and metabolic effects of growth hormone have given rise to several assays to measure its activity. They include assays based on a) increase in body size, b) increase in width of the epiphyseal cartilage plate of the tibia, c) changes in organ weights, d) changes in metabolism of nitrogen, phosphorous, sulfur, carbohydrates and others. Only those that have been used with regularity to measure growth hormone in the rat will be discussed here.

The acceleration of body growth observed as a result of injecting an animal with growth hormone has been accepted as a suitable assay procedure for pituitary growth hormone. Several types of animals have been used in this assay: a) "plateaued" mature female rats, b) immature hypophysectomized rats, c) genetically dwarf mice and d) hypophysectomized mice.

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The use of "plateaued" female rats was first suggested by Evans and Simpson (1931). The assay involves the use of 5-6 month old female rats which fail to gain more than 10 grams in a 20 day period. An injection period of 15-20 days is required for the assay. Marx et al. (1942) found that a straight line relationship existed between the logarithm of the daily dose and the response as measured in grams of body weight increase. The assay is not a very sensitive one as it requires a minimum of 50 ug of bovine growth hormone per rat per day in order to elicit an average weight increase of about 8 grams in 15 days. A daily dose of 1 mg causes an average body weight gain of 42 grams.

The observations by Smith (1926, 1927, 1930), that hypophysectomy in the rat resulted in cessation of body growth and that growth resumed following implantation of whole pituitary glands, were first used by Van Dyke and Wallen-Lawrence (1930) for the assay of growth promoting extracts of the anterior hypophysis. Later, Walker et al. (1950) observed that when young rats are hypophysectomized, growth does not cease immediately but continues at a slower rate until the rats reach about 30 days of age. The assay, as it was used later, is essentially that described by Marx et al. (1942) and Li et al. (1945). It consists of immature female rats hypophysectomized at 26-28 days of age. The animals are used 12-14 days after surgery and

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injected daily intraperitoneally for 10-14 days thereafter. The increase in weight is recorded at the end of the injection period. The assay requires 5 times less hormone than the "plateaued" rat bioassay to elicit a response. However, Russell (1955) showed that when the dose is correlated with the body weight of the animals, there is little difference in sensitivity between the two assays.

Hypophysectomized mice were first used to assay growth hormone by Lostroh and Li (1958). The animals were hypophysectomized at 35 days of age and used 12-14 days later. The injection period lasted 14 days. An initial dose of 5 ug of bovine growth hormone daily produced a body weight gain of 33% in 2 weeks. This method, as well as that of Fønss-Bech (1947) using genetic dwarf mice, was never widely used because of the difficulty of the operation and the lack of availability of the animals respectively.

The most widely used assay for pituitary growth hormone is the tibia test. It is based on the observation of Dott and Fraser (1923) that cessation of growth of the epiphysis follows hypophysectomy in dogs and cats. Later, Kibrick et al. (1941) showed that the epiphyseal cartilage response in young hypophysectomized rats to injections of increasing amounts of growth hormone over a 4-day period showed a straight line dose-response curve when plotted on a semi-logarithmic scale. On the basis of these studies Evans et al. (1943) proposed the tibia test

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for the bioassay of growth hormone. The test was later re-standardized and firmly established as a method for the estimation of growth-promoting activity by Greenspan et al. (1949). The assay makes use of immature female rats hypophysectomized at 26-28 days of age. Twelve to fourteen days later the animals are injected intraperitoneally once daily for 4 days. Twenty-four hours after the last injection the animals are killed and their tibias removed, cut and stained for histological examination. An average of 8-10 readings of the cartilage width is taken; 4-6 animals are used per dose, with two or three dose levels used per standard or unknown. This assay is 10-20 times more sensitive than those described above, with a total dose of 5 ug/rat enough to elicit a significant response.

Although the tibia test represents a large improvement in sensitivity it is not sensitive enough to detect growth hormone in reasonable quantities of the body fluids. Contopoulos and Simpson (1957b, 1959), Srebnik et al. (1959), and Dickerman et al. (1969a) have used the tibia test to measure the levels of growth hormone in the plasma of rats. Large doses of plasma were used, 8-32 ml/assay rat/4 days and problems were observed when using the larger doses (Srebnik et al., 1959). Furthermore, the use of the tibia test in measuring growth hormone in such large quantities of plasma is complicated by the fact that the assay is influenced by hormones other than growth hormone. Thus, corticotrophin and hydrocortisone inhibit cartilage

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growth (Geschwind and Li, 1955; Cargill Thompson and Crean, 1963), and estradiol partially inhibits the effect of growth hormone on body weight gain and on cartilage growth (Zondek, 1937; Geschwind and Li, 1955). Thyroxine, prolactin, testosterone and insulin stimulate cartilage growth in hypophysectomized animals (Salter and Best, 1953; Geschwind and Li, 1955; Cargill Thompson and Crean, 1963). Diet (Simpson et al., 1950) and antibiotics (Geschwind and Li, 1955) also influence the tibia test.

Other methods proposed have included the decrease in blood urea (Russell and Cappiello, 1949; Russell, 1951, 1955), the level of non-esterified fatty acids in the plasma (Raben, 1959), and the uptake of radioactive sulfate by the tibial epiphysis (Murphy et al., 1956) or by the cosral cartilage of hypophysectomized rats (Collins and Baker, 1960). These methods have not gained much acceptance to date perhaps because information concerning their specificity is lacking and because of the difficulty in applying certain biochemical procedures to routine laboratory use.

The separation of anterior pituitary hormones by gel electrophoresis (Lewis and Clark, 1963; Kragt and Meites, 1966) led to the observation that the width and depth of the growth hormone band of rats and mice was related to their biological activity (Jones et al., 1965; Lewis et al., 1965a; Lewis et al., 1965b). More recently, the amount of growth hormone in the stained bands has been

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measured using a densitometric technique (Yanai et al., 1968; MacLeod and Abad, 1968; Nicoll et al., 1969; Birge et al., 1970, or by using a colorimetric method (Lewis et al., 1969). The latter method differs from those above in that the stained band is dissolved and the optical density of the solution is then measured. In these studies it was shown that the optical density of the stained band, or of the solution of the stained band, is proportional to the amount of protein employed for electrophoresis. This method offers the advantages of being able to detect very small quantities of hormone and also permits the assay of individual pituitaries. Although the sensitivity of this assay approaches that required to assay plasma or serum growth hormone, quantitation may be affected by masking of the growth hormone band by serum proteins (Orstein, 1964) which have a velocity of migration similar to that of growth hormone (K.H. Kortright, personal communication, 1970).

The development of radioimmunological assays for protein hormones has opened up the possibility of measuring growth hormone levels in the blood. Although several radioimmunoassays for rat growth hormone have been reported (Parker et al., 1965; Schalch and Reichlin, 1966; Frohman and Bernardis, 1968; Birge et al., 1967a; Garcia and Geschwind, 1968; and Trenkle, 1970), few of these have been sensitive enough to detect the levels of growth hormone in the blood of normal rats. In general, the assays have

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differed mainly in the tracer used for iodination, the species of growth hormone used for labeling, and the animals used for the generation of antiserum.

II. Hypothalamic Control of Anterior Pituitary (AP) Secretion of Growth Hormone (GH)

The release of growth hormone from the anterior pituitary is now generally accepted to be under hypothalamic control. For many years, however, the relationship and mechanism of interaction between the central nervous system (CNS) and the anterior pituitary remained virtually unsolved. Several reasons accounted for this, not the least of which were the lack of a sensitive assay for GH as well as the related problem of the lack of a specific target organ.

Perhaps the first observations which linked the CNS with the control of GH release from the pituitary were the clinical observations of Armstrong and Durh (1922) and Frazier (1936). They observed that tumors of the infundibulum and pituitary stalk resulted in slow growth and retarded skeletal age in patients. In 1938, Cahane and Cahane injured the hypothalamic area in rats and observed a reduction of body growth. These authors suggested a possible role for the nervous system in controlling GH secretion.

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or by its removal and retransplantation away from its original location was an obvious approach to study the relationship between the CNS and AP. In this light, Uotila (1939) observed a temporary growth delay in rats following stalk section. Westman and Jacobsohn (1940) transected the pituitary stalk in rabbits but failed to inhibit growth. These authors, however, reported marked gonadal atrophy. Greep (1936) observed some maintenance of growth in hypophysectomized rats which had received pituitary transplants.

The above observations and the description by Popa and Fielding (1933) of the hypophyseal portal system, whose flow was later correctly described to be from the hypothalamus to the pituitary by Houssay et al. (1935), Wislocki and co-workers (1936, 1937, 1938) and by Green and Harris (1949), led Green and Harris (1949) to suggest that humoral agents are secreted into these hypophyseal portal veins and travel to the anterior pituitary and there exert a regulatory influence on the secretion of anterior pituitary hormones. These humoral agents were later to be known as hypothalamic "releasing factors" (Saffran et al., 1955).

In reviewing the post-Green and Harris (1949) literature concerning the existence of a growth hormone releasing factor (GH-RF or GRF), it is of interest to note the criteria established for the definition of a hypophyseal releasing factor and try to relate it with

the work done on GH-RF. These criteria have been adapted from the classical criteria for defining hormones (McCann and Dhariwal, 1966), and include the following:

(a) The existence of a hypothalamic site or sites responsible for the control of anterior pituitary growth hormone. This site would presumably be concerned with the production of GH-RF and its damage or stimulation should result in detectable alterations in the secretion of the hormone from the anterior pituitary.

(b) The GH-RF should be extractable from the area or areas mentioned above.

(c) The GH-RF must be able to alter the secretion of GH when given to an animal whose responses to non-specific stimuli have been blocked. This stimulation must be dose related.

(d) The factor should be effective when applied directly to the pituitary gland in vivo.

(e) The factor in question should be detectable in hypophyseal portal vessel blood.

The dependence of the anterior pituitary on the CNS for the control of growth hormone release and the "chemotransmitter hypothesis" have been substantiated using a number of techniques, among them , pituitary stalk section, pituitary transplantation, in vitro explantation of pituitary, brain lesions, brain stimulation

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The stalk section studies mentioned previously, as well as those of Greep and Barrnett (1951) and Daniel and Prichard (1964) yielded inconclusive evidence. This could be explained by the observation of Harris (1950) concerning the regeneration of the portal vessels and the possibility of incomplete sectioning of the stalk.

Pituitary transplants to hypophysectomized rats have been able to maintain some degree of growth (Greep, 1936; Martini and de Poli, 1956; Goldberg and Knobil, 1957). The transplants have been made in different locations with lesser or greater success. Hertz (1959) reported body growth in young hypophysectomized rats implanted with 4 pituitaries underneath the kidney capsule to be 2/3 of that of intact controls. Swelheim and Wolthuis (1962) also observed significant growth in young hypophysectomized rats given a single implant underneath the kidney capsule. Meites and Kragt (1964) reported growth averaging 46.6% of that of intact control in young hypophysectomized rats bearing a single subcutaneous implant.

The differences in degree of success in part may be related to the site of transplantation. Halasz et al. (1962, 1963) observed no significant body weight gains in hypophysectomized rats bearing implants under the kidney capsule or the temporal lobe of the brain. Significant weight increases were observed when the implant was

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located in the hypophysiotrophic area of the brain. Nikitovitch-Winer and Everett (1958) observed that functional restitution and revascularization of the pituitary took place after retransplantation of the gland from the kidney capsule to the median eminence, after a prolonged sojourn away from the sella turcica. Other factors which account for these differences in success may be related to the age of the pituitary donor (Meites et al., 1962), the delay in transplantation after hypophysectomy (Smith, 1961), the amount of viable anterior pituitary tissue present in the grafts, and to post-graft immunological factors.

Studies involving brain lesions or stimulations and their effect on growth are perhaps better substantiated. The growth disturbances observed by Armstrong and Durh (1922), Frazier (1936), and Cahane and Cahane (1938) are difficult to interpret because of the extent of the lesions. Bogdanove and Lipner (1952), Hinton and Stevenson (1962) and Bernardis et al. (1963) also reported reduced body growth in rats bearing hypothalamic lesions. It should be stressed, however, that extensive cerebral lesions may also interfere with food intake and temperature regulation (Bogdanove and Lipner, 1952; Bernardis et al., 1963) and thereby impair growth. Extensive lesions could also involve areas controlling the secretion of other AP hormones which may affect growth.

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Reichlin (1960a) showed that lesions of the median eminence and primary portal plexus of the stalk significantly reduced the growth rate of rats. Taking into account the possibility of altered secretions of other hormones, Reichlin (1960b) in a subsequent study injected vasopressin, testosterone and thyroxine into the injured animals. Pair-fed controls were used to exclude the effects of differences in food intake. Body growth was not restored to normal following this treatment. In 1961, Reichlin studied the effects of massive ventral lesions of the hypothalamus in rats on body growth and pituitary growth hormone content as measured by bioassay. Together with the decrease in body growth he showed that GH content in injured animals was reduced to 15% of that found in non-injured animals. Bach et al. (1964) found that bilateral lesions of the paraventricular nuclei in weanling kittens caused a reduction in growth rate and pituitary acidophilic degranulation. On the other hand, electrical stimulation of the paraventricular nuclei of weanling kittens by O'Brien et al. (1964) caused acceleration of growth as measured by body weight and tibial length. Median eminence or pituitary stalk lesions in monkeys have been reported to block the release of GH which follows insulin-induced hypoglycemia (Abrams et al., 1966).

The development of radioimmunological methods to assay rat GH has allowed the measurement of GH in the

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plasma of rats after stimulation or destruction of certain hypothalamic areas. Frohman and Bernardis (1968) showed that rats with growth retardation subsequent to ventromedial hypothalamic nucleus destruction have decreased levels of growth hormone in the pituitary as well as in the plasma. In the same year, Frohman et al. (1968) showed that electrical stimulation of the above mentioned area resulted in significant increases in plasma GH levels. Stimulation of the cerebral cortex had no effect on plasma GH.

In a more elegant study, Frohman and Bernardis (1970) studied the secretion rates of GH in rats subjected to ventromedial hypothalamic nucleus stimulation. These authors measured the plasma GH levels and metabolic clearance rate (MCR) of GH to arrive at the secretory rates. The rats subjected to hypothalamic stimulation showed a 5-fold increase in plasma GH within 10 minutes. No change was observed in rats subjected to cerebral cortex stimulation. A significant increase in secretion rate was observed in the hypothalamic stimulated group. Bernardis and Frohman (1970) studied the effects of electrical stimulation of several areas of the hypothalamus in rats on plasma growth hormone concentration. They found that only stimulation of areas within and at the border zones of the ventromedial hypothalamic nuclei resulted in significant increases in plasma growth hormone. Inconsistent changes were found when anterior

or posterior hypothalamic areas were stimulated. The authors attributed the latter observations to be the result of the stimulating current spreading via intrahypothalamic fiber systems to reach the ventromedial nucleus. The work of these authors (Frohman and Bernardis, 1968, 1970; Frohman et al., 1968; Bernardis and Frohman, 1970) is the most convincing on the existence of a site concerned with the production of GH-RF. It suggests that the ventromedial hypothalamic nucleus is responsible for the control of anterior pituitary GH synthesis and release and is therefore a strong candidate for the site of GH-RF production. Their evidence, however, does not rule out the possibility that the site may be concerned with the production of other releasing factors as well.

The measurement of the GH-RF in the hypothalamus has been carried out in three ways: a) direct measurement of pituitary GH content in animals injected with median eminence extracts b) measurement of the pituitary content and release of GH into medium after incubation with median eminence extracts, and more recently c) determination of plasma GH levels following the administration of median eminence extracts or purified preparations of GH-RF into recipient animals.

The first claim of detection of a factor in neural tissue responsible for controlling the release of anterior pituitary GH was that of Franz et al. (1962) in the hypothalamus of swine. Their work and conclusions, however,

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have been criticized since standard assay procedures were not followed and large variations in response were obtained. Deuben and Meites (1964, 1965) were the first to present conclusive evidence for GH-RF. They reported that neutralized acid extracts of rat hypothalamus produced a 4 to 6-fold increase in GH release by rat anterior pituitary after a 6 day culture. Cerebral extract failed to increase GH release upon culture. Deuben and Meites (1965) also reported reinitiation of pituitary GH release in vitro by a neutralized acid extract of rat hypothalamus after release had ceased.

The first in vivo demonstration of GH-RF activity was reported by Pecile et al. (1965). These authors reported that hypothalamic extracts were able to deplete the anterior pituitary of recipient rats of bioassayable GH. Both the in vitro and in vivo observations have been confirmed by several investigators using bioassay procedures either with crude hypothalamic extracts or purified preparations of GH-RF (Schally et al., 1965; Muller et al., 1965; Muller and Pecile, 1965; Schally et al., 1966; Dhariwal et al., 1966; Krulich et al., 1965; Machlin et al., 1967; Schally et al., 1968a). An in vitro method for the quantitation of GH-RF was reported by Dickerman et al. (1969b). These authors were the first to show a dose-response between hypothalamic tissue and the amount of GH released into the medium. The GH-RF activity has been reported to be present in

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several species of mammals as well as in some birds and amphibians (see Schally et al., 1968b).

The large depletion of bioassayable pituitary GH observed following administration of hypothalamic extracts was interpreted to be the result of release of large quantities of GH into the circulation. Sawano et al. (1968) administered a highly purified preparation of pig GH-RF into rats and measured, by bioassay, the pituitary content and plasma concentration of "growth hormone-like activity". They reported extremely high levels of growth hormone in the peripheral blood. These levels were 30-50 times as high as the highest levels observed using radioimmunological assay techniques.

More important, however, has been the failure to confirm the observations obtained by bioassay when using radioimmunological procedures. Neither the decrease in rat pituitary GH nor the increase of GH in blood have been established by radioimmunoassay (Daughaday et al., 1968; Garcia and Geschwind, 1968; Schalch and Reichlin, 1968). It should be stressed, however, that plasma GH measurements by radioimmunoassay in the sheep and rhesus monkey (Machlin et al., 1967; Garcia and Geschwind, 1966; Meyer and Knobil, 1968; Garcia and Geschwind, 1968) have shown a significant increase subsequent to the administration of crude preparations of GH-RF. Highly purified preparations have yielded inconclusive evidence. Garcia and Geschwind (1966, 1968) further showed that the increase

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in the plasma GH of rhesus monkeys was not due to hypoglycemia since a glucose infusion which elevated the plasma glucose concentration to 300 mg % was unable to block the response to the sheep hypothalamic extracts injected. These authors also reported the inability to elicit an increase in plasma growth hormone in rabbits injected with sheep hypothalamic extract.

To our knowledge, confirmation of the action of hypothalamic extracts on rat anterior pituitary GH release by radioimmunoassay has come only from the work of Daughaday et al. (1970) and Wilber and Porter (1970). The former authors were able to show a two-fold increase in the amount of GH released into the medium upon the addition of 1.25 crude hypothalamic equivalents per incubated pituitary. The amount of GH released was not impressive when compared to that evaluated by bioassay methods (Dickerman et al., 1969b), but this difference may be accounted for by the different standards used. Specificity tests, however, indicated that lysine vasopressin at a concentration of 1.5 U/ml was capable of eliciting a response of similar magnitude to that obtained with the hypothalamic extract. Wilber and Porter (1970) collected hypophyseal portal blood and tested the plasma for GH-RF activity in vitro. These authors found that addition of hypophyseal portal plasma to the incubated pituitaries resulted in an increased release of GH into the medium when compared to the release

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observed in incubations with peripheral plasma (149% vs 100% for controls). This constitutes the first evidence for the existence of the GH-RF in the hypophyseal portal circulation. However, neither Daughaday et al. (1970) nor Wilber and Porter (1970) tried to establish a dose-response relationship between the amount of hypothalamic extract and/or hypophyseal portal plasma and the amount of GH released into the medium. Furthermore, it is difficult to assess the work of Wilber and Porter since they give no indication either of the actual amounts of GH secreted or of the validity of the radioimmunoassay in their hands. In contrast to their work, Buse et al. (1970) reported a failure to observe a decrease in pituitary GH, as measured by radioimmunoassay, in rats following administration of hypophyseal portal plasma.

It appears, therefore, that substantial differences exist between biological and immunological data concerning the existence of a GH-RF in the rat. One cannot rule out the existence of a GH-RF in view of the strong biological evidence. On the other hand, it would be premature to assume that the radioimmunological assays available for rat GH are not measuring GH. There is a definite need to establish the relationship between the immunological and biological activities of the rat GH molecule, as this would enable us to better understand these seemingly disparate findings.

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It seems appropriate to close this section with a word on the chemistry of the GH-RF. The factor has been purified from ovine (Krulich et al., 1965; Dhariwal et al., 1966) and porcine or bovine hypothalami (Schally et al., 1965; Schally et al., 1966; Schally et al., 1967; Schally et al., 1968b; Schally et al., 1969; and Ishida et al., 1965). A growth hormone inhibiting factor (GIF) has been reported by Krulich et al. (1968), and by Dhariwal et al. (1969) in purified fractions of sheep and rat hypothalami. Schally et al. (1969), however, using a method of assay similar to that of Krulich et al. (1968) have so far found no evidence for GIF activity in extracts of porcine hypothalamus. Most recent information (A. Schally, personal communication, 1970) indicates that pig GH-RF is a small polypeptide consisting of 10 amino acids. Apparently elucidation of the structure and synthesis of this polypeptide have been accomplished by Schally's laboratory. At present our laboratory is in the process of evaluating the effect of natural and synthetic GH-RF from Dr. A. Schally on the release of GH in vitro as measured by biological and radioimmunological techniques.

III. Secretion of Growth Hormone During Different Physiological States

The advent of radioimmunological techniques for assaying human growth hormone (HGH) has contributed greatly to the understanding of the factors involved in controlling, modifying or affecting its secretion in humans.

It is not the purpose of this review, however, to emphasize the human condition, as this will be mentioned only where it may help to understand what takes place in the rat and other mammals. The reader is referred to the excellent reviews by Glick et al. (1965), Glick and Goldsmith (1968) and Glick (1968, 1969), in which the regulation of GH in humans is treated in depth.

A. Age and GH

Growth hormone is first detectable in the fetal rat pituitary at day 19 of gestation (Contopoulos and Simpson, 1957a). Baker et al. (1956) also reported the ability to detect growth hormone in the pituitaries of fetal pigs. It was later shown that the total amount of GH present in the rat pituitary increased with age, but that the concentration of the hormone per mg of tissue remained constant in rats between 10 and 630 days of age (Solomon and Greep, 1958; Bowman, 1961). Pecile et al. (1965) reported that the hypothalamic content of GH-RF was greater in 30 day old rats than in 2 year old rats.

The above results, all of which were obtained using biological assay methods, do not agree with those reported by Birge et al. (1967a) using a radioimmunoassay for rat GH. These authors reported increases both in content and concentration of GH with age, with male rats having higher concentrations than females after puberty. In males this increase continued into old age, whereas in female rats it plateaued at maturity. It is of interest to note,

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however, that the authors found little or no increase in the weight of the pituitary gland in male rats after 49 days of age, and reported an average weight of 6.70 mg per pituitary for male Sprague-Dawley rats 147 days old and weighing 312 grams. Garcia and Geschwind (1968), also using radioimmunological procedures, reported increases in concentration of pituitary GH with age but no significant difference in concentration between male and female rats.

Burek and Frohman (1970) have recently reported that pituitaries from adult male rats (165 days old) were able to synthesize more GH than pituitaries from young male adult rats (70 days old), and the latter synthesized more GH than pituitaries from weanling male rats (23 days old) in vitro. Comparison of growth hormone synthesis between adult male and female pituitaries of the same body weight showed that the incorporation of ^{3}H-leucine into GH was 2- to 3-fold greater in the male pituitaries whether expressed as dpm/mg pituitary or GH specific activity (dpm/ug growth hormone). Pituitary concentration of GH was similar for both sexes, and no significant difference was found in the ability of male and female weanling pituitaries to synthesize GH. As yet, no information is available on the levels of plasma GH with respect to age in the rat.

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difference in concentration among fetal, adolescent and mature male pituitaries. An increase in total content was explained as a result of the increase in size of the pituitary. Plasma HGH is very high in the fetus and at parturition (Greenwood et al., 1964a), subsequently declining but remaining higher in children than in adults (Greenwood et al., 1964b).

B. Hormonal Influence on GH Secretion

The effect on insulin induced hypoglycemia in humans was first reported by Roth et al. (1963a). They showed that the administration of insulin in doses large enough to lower blood glucose resulted in increased HGH in the plasma. Later Roth et al. (1963b) reported that falling blood glucose levels, as well as interference with the metabolism of glucose by administration of 2 deoxy-D-glucose, results in increased plasma HGH. These results were later confirmed by Hunter and Greenwood (1964), Frantz and Rabkin (1964), and others. Luft et al. (1966), however, reported that small insulin doses which did not cause symptomatic levels of hypoglycemia, elicited a release of GH into the circulation. These latter results were subsequently confirmed by Greenwood et al. (1966) using graded doses of insulin.

Katz et al. (1967) studied the effects of insulin induced hypoglycemia in the rat. They reported a significant depletion of pituitary GH and of hypothalamic GH-RF. It had been previously shown by Krulich and McCann (1966a)

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that insulin induced hypoglycemia in hypophysectomized rats resulted in increased GH-RF activity in the peripheral blood as well as in a decreased content of GH in intact rats (Krulich and McCann, 1966b). Katz et al. (1967) concluded that hypoglycemia stimulated the release of GH-RF from the hypothalamus which in turn stimulated the release of GH from the pituitary. These results were confirmed by Muller et al. (1967a). The above results in the rat have not been confirmed using radioimmunoassay techniques. Daughaday et al. (1968), Garcia and Geschwind (1968), and Schalch and Reichlin (1968) all failed to observe pituitary GH depletion or rise in plasma GH following insulin administration to rats.

Thyroidectomy in the rat results in a degranulation of pituitary acidophiles (Purves and Griesbach, 1946; Schooley et al., 1966), a decrease in growth rate (Koneff et al., 1949; Schooley et al., 1966) and a decrease in pituitary GH content (Contopoulos et al., 1958; Knigge, 1958; Solomon and Greep, 1959; Schooley et al., 1966; Meites and Fiel, 1967). Meites and Fiel (1967) showed that thyroidectomy resulted in decreased content of hypothalamic GH-RF. They also showed that thyroxine therapy resulted in increased GH-RF and pituitary GH. These results have been confirmed by Daughaday et al. (1968) using radioimmunological assays. Hypothyroidism as a result of propylthiouracil or radioiodine ablation was reported to decrease pituitary GH content.

The interference with body growth by estrogens has been suspected for some time (Gaarenstrom and Levie, 1939; Reece and Leonard, 1939), although its mechanism of action remains uncertain. Meites (1949) showed that growth of stilbesterol-treated rats was the same as that of pair-fed controls, attributing the decreased growth in large part to the decrease in food intake elicited by stilbesterol administration. Sullivan and Smith (1957) confirmed those findings using estradiol. Josimovich et al. (1967) and Roth et al. (1968) reported evidence suggesting the existence of peripheral antagonism between estrogen and GH in the rat and humans respectively. Birge et al. (1967b) reported that pituitaries incubated with diethylbesterol caused suppression of GH release but had no effect on the amount stored in the pituitary. Birge et al. (1967a) further showed that males treated with diethylbesterol or estradiol benzoate had a decreased pituitary concentration of GH, while gonadectomy of female rats resulted in a slight, but not significant, increase in GH concentration. Plasma GH levels have been reported to be higher in female than in male rats (Schalch and Reichlin, 1966).

The levels of GH in plasma and their relation to estrogen are better illustrated in the human. No difference in plasma HGH was found between males and females in the basal state, although in the ambulatory state the females had a higher plasma concentration (Frantz and Rabkin, 1965; Garcia et al., 1967). Increase in HGH levels have been

observed following ovulation and during the pre-menstrual phase. Lower levels have been found in post-menopausal women (Frantz and Rabkin, 1965). The ingestion of oral contraceptives is associated with elevation of plasma HGH levels (Garcia et al., 1967). These elevated levels in the female are perhaps related to the need of the body to compensate for the peripheral antagonism of estrogen which may reduce the ability of the body to use GH.

Androgens stimulate growth when given in small doses (Rubinstein and Solomon, 1941). Injections of testosterone propionate to adult female rats for 14 days resulted in increased concentration of pituitary GH when compared to sesame oil injected controls. Castrated male rats treated with testosterone propionate also showed increased concentrations when compared to castrated male rats given estradiol benzoate or sesame oil injections (Birge et al., 1967a; Daughaday et al., 1968).

Adrenal corticoids may have an effect similar to that observed with high doses of androgens (Evans et al., 1943; Marx et al., 1943; and Geschwind and Li, 1955), in which growth of long bones ceases by the closing of the epiphysis. The effect of corticosteroids on pituitary and plasma GH is less substantiated. Reichlin and Brown (1960) studied the effects of adrenalectomy on growth and pituitary GH in the rat. Although growth is impaired they found this to be more closely related to a reduced food intake. No change in pituitary GH concentration was observed following

adrenalectomy. The administration of cortisol to thyroidectomized rats has been claimed to produce acidophilic regranulation and a reaccumulation of GH in the pituitary gland (Meyer and Evans, 1964), as measured by the tibia test bioassay. Lewis et al. (1965b), however, failed to show by acrylamide gel electrophoresis, a reaccumulation of GH following the administration of cortisol to propylthiouracil treated rats. The findings of Lewis et al. (1965b) were later confirmed by Daughaday et al. (1968) using a radioimmunoassay for rat GH. These authors also failed to observe a reaccumulation of GH after cortisol treatment to propylthiouracil treated rats.

The decreased pituitary GH in rats as a result of insulin induced hypoglycemia (Krulich and McCann, 1966b; Katz et al., 1967; Muller et al., 1967a) has been blocked by the administration of cortisol in large doses (Muller et al., 1967b). A decrease in hypothalamic GH-RF was observed, and it was interpreted as indicating a hypothalamic action for cortisol. The in vitro work of Birge et al. (1967a), however, has demonstrated a direct effect of cortisol on hemipituitaries in tissue culture. No data is yet available on the plasma levels of GH after cortisol treatment in rats.

The levels of pituitary and plasma growth hormone activity during pregnancy have been measured in the rat. Contopoulos and Simpson (1956) found no change in pituitary GH concentration. Later, Contopoulos and Simpson (1957b)

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showed a 2- to 3-fold increase in growth hormone activity in the plasma of pregnant rats. This activity did not disappear after hypophysectomy on day 12 of pregnancy (Contopoulos and Simpson, 1959) indicating an extra-pituitary source. These results were not confirmed by Schalch and Reichlin (1966) using a radioimmunoassay technique. They reported no change in plasma GH with pregnancy. These data need to be confirmed by others.

C. Nutrition and Stress

A reduction in pituitary and plasma GH activity was found by Srebnik et al. (1959) in rats fed a protein free diet for prolonged periods of time. Starvation in the rat has been reported to decrease hypothalamic content of GH-RF (Meites and Fiel, 1965; Dickerman et al., 1969a),and pituitary concentration of GH (Meites and Fiel, 1965; Friedman and Reichlin, 1965; Dickerman et al., 1969a). Dickerman et al. (1969a) further showed that starvation resulted in decreased plasma growth hormone activity. These results have been recently confirmed by Trenkle (1970) using a radioimmunoassay technique.

It is of interest to note, however, that other related species do not respond in the same manner to starvation. Thus, Garcia and Geschwind (1968) reported elevations of plasma growth hormone in mice and rabbits after starvation. Machlin et al. (1968a) reported that plasma GH levels in pigs increased during the first 48 hours of starvation and subsequently fell to lower levels. No significant changes

were observed in the plasma GH levels of young sheep fasted for 7 days (Machlin et al., 1968b). This may indicate increased GH secretion during fasting may not take place in all species.

The effects of a variety of stresses on rat pituitary GH have been reported. Muller et al. (1967a) noted that cold, high doses of vasopressin, epinephrine and urecholine depleted pituitary GH. Muller et al. (1967b) later reported cold depletion of hypothalamic GH-RF and increase in plasma GH-RF. It was previously indicated by Krulich and McCann (1966c) that stresses could alter the content of pituitary GH in the rat. Recently, Parkhie and Johnson (1969) found that heat stress would also deplete pituitary GH but elevate the hypothalamic stores of GH-RF. As was the case before, radioimmunological data failed to confirm the above reports. Schalch and Reichlin (1967, 1968) found no increase in plasma GH in rats subsequent to exercise, moderate or severe hypoglycemia, and cold. Daughaday et al. (1968) and Garcia and Geschwind (1968) also failed to observe the effect of cold or hypoglycemia on the content of pituitary growth hormone.

EXPERIMENTAL METHODS AND MATERIALS

I. Animals

Experimental animals were Sprague-Dawley rats obtained from Spartan Research Animals (Haslett, Michigan). Animals for GH bioassays were immature female rats (Charles River Breeding Laboratories, Wilmington, Massachusetts) hypophysectomized at 26 days of age. They were used for GH bioassay 14-16 days after operation.

Experimental animals were maintained on a diet of Wayne Lab Blox pellets (Allied Mills, Chicago, Ill.) and fed ad libitum. The diet of the bioassay animals was supplemented every other day with orange slices, carrots and sugar cubes. All bioassay and experimental animals were housed in a temperature controlled room (75± 1° F) with automatically controlled lighting (14 hours light daily).

II. Preparation of Pituitaries, Plasma or Serum

The rats were killed by guillotine, and the pituitaries were removed, weighed individually, and homogenized in 0.01M phosphate buffer in 0.14M NaCl, (phospho-saline buffer, PBS), pH 7.2, with a Sonifier cell disruptor. The

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individual homogenates were stored at -20°C until the day before assay. On the day before the assay, the homogenates were thawed and diluted to the working concentration (see specific experiments).

Plasma was collected from blood drawn by cardiac puncture in heparinized syringes and immediately centrifuged for 20 minutes at 2200 rpm in an International Centrifuge. The plasma was pipetted off and the individual samples were immediately frozen and kept at -20°C until assayed, unless otherwise indicated in the specific experiments. Serum was obtained in the same manner except that non-heparinized syringes were used. The blood was allowed to remain 24 hours at 4°C to allow the clot to form and the serum to separate. The blood was then centrifuged as described above and the serum frozen at -20°C until assayed.

III. Assay of Growth Hormone

A. Bioassay

Growth hormone activity was measured by the standard tibia test of Greenspan *et al.* (1949). Aqueous solutions of pituitary homogenates were injected intraperitoneally once a day for 4 days. Each assay included two doses of the control and experimental solutions, as well as two doses of NIH-GH-S8 and NIAMD-RGH-RP-1 or two doses of a purified rat GH preparation supplied by Dr. Stanley Ellis (NASA Research Center, Ames, California) designated as

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HVII-38-C. NIH-GH-S8 was kindly supplied by the Endocrinology Study Section, NIH. All NIAMD preparations were supplied by Dr. Albert Parlow (Department of Obstetrics and Gynecology, School of Medicine, Harbor General Hospital, 1000 West Carson Street, Torrance, California). Four assay animals were used per dose of rat pituitary or standard preparation.

B. Radioimmunoassay

Assay of rat serum or plasma and pituitary growth hormone levels was performed by radioimmunoassay. The radioimmunoassay utilizes a double antibody method developed in this laboratory in collaboration with Drs. Ytschak Koch and Samuel Dickerman.

Monkey antiserum to rat GH. Two preparations of monkey anti-rat GH serum have been used throughout this study, a NIAMD-A-Rat GHS-1 from Dr. Parlow, and a monkey anti-rat GH from Dr. W.H. Daughaday (Washington University School of Medicine, St. Louis, Missouri) henceforth called DMD-1. The anti-serums were diluted to 1:500, 1:2500, 1:5000, 1:10,000, 1:20,000, 1:30,000, 1:40,000, 1:50,000 and 1:60,000 in 1:600 normal monkey serum (NMS) previously diluted in 0.05M EDTA-PBS (etheylenedinitrilo tetraacetic acid, disodium salt) pH 7.2. The dilutions were tested for antibody titer to determine a working concentration, 200 ul of which would bind 50% of the radioiodinated growth hormone added to the incubation tubes. It was found that a 1:50,000 dilution of the NIAMD-A-Rat GHS-1 bound 45-58% of the

radioiodinated GH, whereas a 1:16,000 dilution of the DMD-1 was required to bind as much.

Production of goat antiserum against monkey gamma globulin. (Anti-MGG). A mature female goat was obtained from the Endocrine Research Unit through the courtesy of Dr. Nellor and Dr. Riegle. The goat was immunized by weekly subcutaneous injections of monkey gamma globulin (Immunology Inc., Glen Ellyn, Illinois, U.S.A.) emulsified in complete Freund's adjuvant. Four immunizations, once weekly, were given in the following amounts: 30, 35, 40, and 80 mg of monkey gamma globulin. Two weeks later a second 80 mg immunization was given and the goat was bled through the jugular vein. A volume of 500-650 ml of blood was collected. The goat was injected with booster injections of 80 mg every four months. Periodic bleedings at 3-4 month intervals were made. The goat antiserum against monkey gamma globulin was diluted with PBS to 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:10 and 1:15 and titrated for the optimal dilution to precipitate the monkey gamma globulin. Originally the combining dilutions of 200 ul of anti-monkey gamma globulin and NMS (200 ul of 1:600 NMS in EDTA-PBS) were determined empirically by observing the dilution of anti-monkey gamma globulin which gave the greatest amount of precipitate. The proper dilution was later verified in an experiment in which a series of tubes at 2 different radioiodinated growth hormone concentrations were set up as for an assay, the amount of NMS remaining constant while dilutions

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of anti-MGG were varied as per above description. It can be seen in Figure 1 that a peak in precipitation occurred when using a 1:7 dilution of anti-MGG. This procedure for determining the best dilution has been followed for each subsequent bleeding of the goat.

Radioiodination of rat growth hormone. Two highly purified rat GH preparations were used for iodination: Dr. Parlow's NIAMD-RGH-I-1 and Dr. Ellis' HVII-38-C. The following procedures apply to both, although the results presented later in the text have been obtained using the HVII-38-C preparation. Subsequently, the term iodination quality is used to refer to highly purified hormone preparations.

Purified rat growth hormone was first dissolved in double distilled water (pH adjusted to 7.5) to a concentration of 1 ug/ul. Twenty ul (20 ug) of rat GH were pipetted into 1 ml serum bottles. These bottles were kept at -20°C until iodinated. Prior to iodination they were thawed and 100 ul of 0.5M phosphate buffer at pH 7.5 added. The solution was mixed well and 1 millicurrie (mc) of I^{125} with high specific activity was added and mixed (I^{125} with high specific activity and carrier free was obtained from Cambridge Nuclear, Cambridge, Massachusetts). Twenty-five ul (87.5 ug) of Chloramine-T (35 mg/10 ml) were added to the serum bottle, the bottle sealed with parafilm and mixed, and the reaction allowed to proceed for 45 seconds. At the end of the reaction time 125 ug of Sodium Metabisulfate ($Na_2S_2O_5$) (50 ul of 25 mg/10 ml solution) were added and mixed thoroughly.

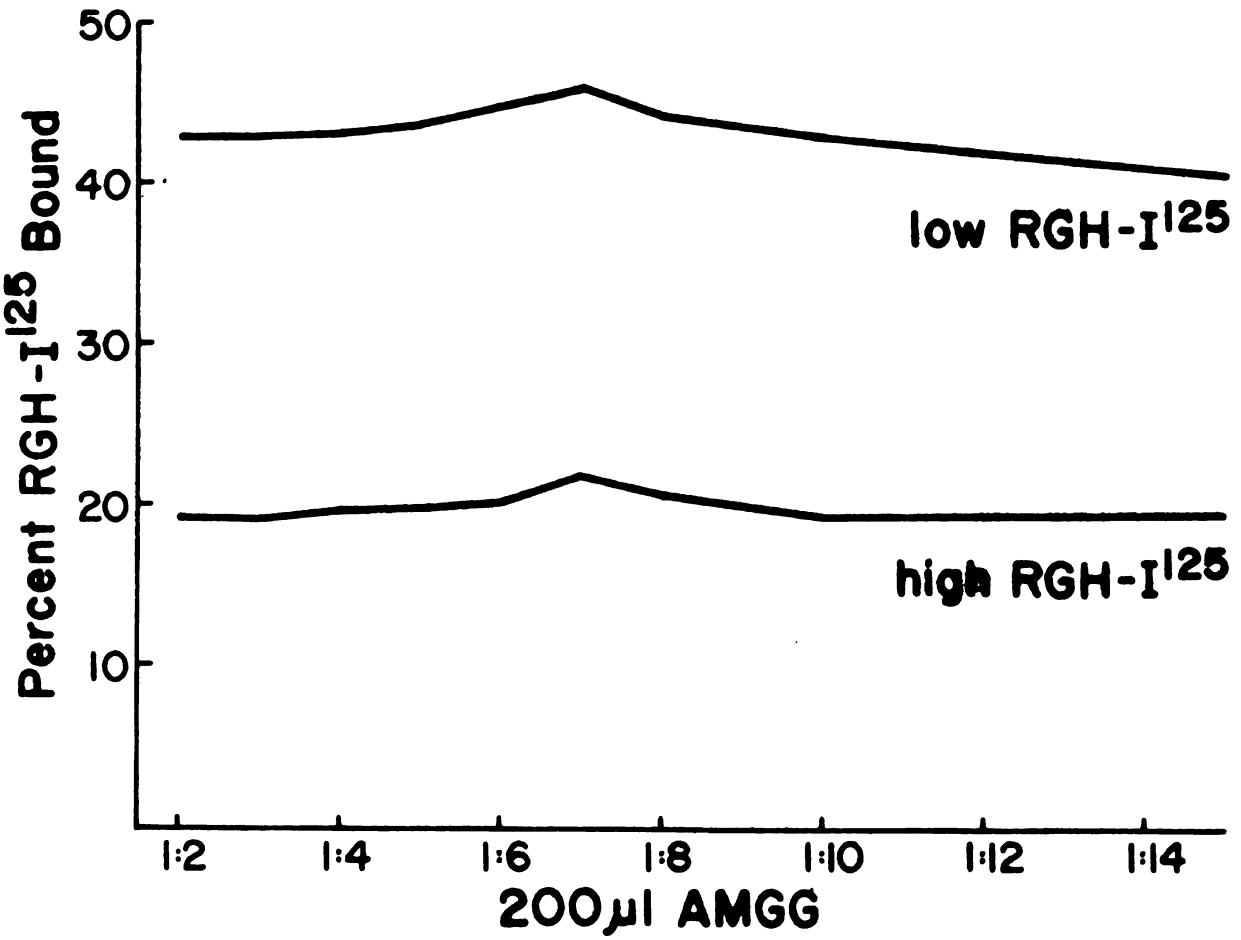

Figure 1. Precipitation of RGH-antibody complex with varying dilutions of anti-monkey gamma globulin serum. RGH-I^{125} bound is expressed in terms of total radioactivity.

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One hundred ul of transfer solution containing 100 mg KI/10 ml in 16% sucrose was added, the solution mixed thoroughly by withdrawing and expelling it from a plastic syringe, and transferred to a 0.9 x 20 cm column of Sephadex G-50 expanded in 0.01M barbital buffer. The serum vial was rinsed with the same syringe used in the previous step using 100 ul of a solution containing 100 mg KI/10 ml and 1 mg bromphenol blue in 8% sucrose. The bromphenol solution was placed in the column to mark the layer of material transferred in the previous step. The outlet of the column was opened and 1 ml aliquots were collected in tubes containing 1-2 drops of 30% bovine serum albumin, or the equivalent, in 0.05M barbital buffer pH 8.6. The tubes were counted individually in a manual counter using a splash shield to prevent contamination. The peak tubes were selected and aliquots of 0.25-0.40 ml (25-35 x 10^6 cpm) were transferred, with 0.15 ml of transfer solution, to a 0.9 x 50 cm column of Sephadex G-100 in barbital buffer pH 8.6 for repurification. Two ml aliquots were collected in 1% BSA-PBS previously coated tubes. The peaks were again selected and 1% BSA-PBS at pH 7.2 added until 100 ul gave 19-21,000 cpm (on the day of iodination). The final dilutions were counted inside a Nuclear Chicago gamma counter under optimal counting conditions. The concentrated tubes were kept frozen at -20°C and aliquots repurified as needed. The diluted solutions were kept at 4°C. We have found that the diluted solution may be kept for as long as two months at 4°C in 1% BSA-PBS without significant alterations in the immunological activity of the

molecule. No attempt has been made to keep the solutions for a longer length of time.

Procedures for radioimmunoassay. The assay was carried out in disposable culture tubes, 12 x 75 mm (Kimble Owens, Toledo, Ohio). The tubes were labeled in the following manner:

1. Tubes A1-5: received 500 ul of 1% BSA-PBS, 200 ul of 1:600 normal monkey serum (NMS) and 100 ul of labeled rat growth hormone. These tubes measured the amount of non-specific binding and were used as background while counting the assay tubes.

2. Tubes A6-10: received 100 ul of labeled hormone (total count tubes).

3. Tubes B1-5, C1-5: received 500 ul of 1% BSA-PBS, 200 ul of NIAMD-A-RatGHS-1 or DMD-1 at the appropriate dilution to bind approximately 50% of the total radioactivity added, and 100 ul of labeled hormone. The radioactivities of these tubes was considered as 100% cpm.

4. Tubes B6-17, C6-17: received the standard solutions of NIAMD-RGH-RP-1 diluted in 1% BSA-PBS to a concentration of 1 mug/ul. In all cases, the standards or unknowns + "x" volume of 1% BSA-PBS was such that it equaled 500 ul. The following volumes (ul) of the standard solution were placed in the tubes which already contained the complementary volume of 1% BSA-PBS: 250, 125, 62.5, 31, 16, 8, 4. A second solution diluted 10 times was used to pipette the smaller amounts: 20, 10, 5, 2.5, 1 ul which corresponded to 2, 1, 0.5, 0.25, and 0.1 mug respectively.

The sample tubes were labeled with consecutive numbers

from #1 through the number that was planned for the assay. Three or more dilutions of unknown serum, plasma or pituitary homogenate were used per sample. These will be indicated in the specific experiments.

The order in which the above reagents were placed in the tubes follows:

1. All tubes, except A6-10, received the 1% BSA-PBS.

2. The standards and unknowns, 100 ul of labeled hormone and 200 ul of anti-rat GH followed. All tubes were mixed with a Vortex mixer following the completion of each rack. All tubes were stored at 4°C for 72 hours.

3. At the end of 72 hours, 200 ul of the anti-MGG solution was added to each tube. The tubes were mixed and stored at 4°C for an additional 24 hours.

4. Upon completion of the 24 hours, the tubes were centrifuged in an International Centrifuge PR-2 at 2,200 rpm for 20 minutes. At the end of 20 minutes each tube received 3 ml of PBS and was centrifuged again for an additional 20 minutes as described above.

5. The supernatant of all tubes was decanted and the precipitate was placed in a disposable plastic jacket and counted in a Nuclear Chicago gamma counter with an automatic changer.

Tubes A1-10 were counted for 60 seconds each. Tubes B1-5, C1-5 were also counted for 60 seconds and then recounted to record the time it took to reach 10,000 counts. (The non-specific binding was subtracted automatically).

The machine was then set up to count with respect to the time recorded above and in this way gave a direct percentage reading. The standard curve was extrapolated on semi-logarithmic paper and the unknowns were read out and expressed as mug of GH per tube. Multiplication by the appropriate factor gave the concentration of GH in mug/ml of serum or plasma or per mg of anterior pituitary.

To minimize variations between assays several procedures were adopted.

1. A large volume of NIAMD-RGH-RP-1 was prepared and diluted to a 1 mug/ul concentration. Aliquots of 1.2 1.4 ml were then placed in disposable culture tubes and kept at -20° C. One tube was used/assay.

2. A stock solution of 1:500 anti-rat GH was prepared. Large amounts of the working concentration (1:50,000 for the NIAMD-A-Rat GHS-1) were prepared and aliquoted into glass bottles. Each bottle contained enough for 300 tubes.

3. Aliquots of the 1:600 NMS to dilute #2 above were stored at 4°C to be used for the non-specific binding determinations.

4. The anti-MGG was diluted to its working concentration and divided so as to have enough for 300 tubes/bottle. Following the above precedures we have been able to maintain our level of non-specific binding constant throughout the course of this study. All racks were kept in an ice bath while pipetting the solutions.

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IV. Metabolic Clearance Rate (MCR)

The metabolic clearance rate of growth hormone under several physiological states was studied using a single injection of radioactive hormone. The animals used for this study were anesthetized with Na-pentobarbital (30 mg/kg) and injected through the tail vein with 1 ml of PBS solution containing approximately 2.4×10^6 cpm of rat GH-I^{125}. Blood samples of 750 ul were obtained at 1, 5, 10, 15, 20, 30, 45, 60 and 90 minutes following the injection and placed in tubes containing heparin solution. All tubes were kept in an ice bath during the collection and centrifuged immediately afterward in an International Centrifuge PR-2 at 2200 rpm for 20 minutes. The plasma was frozen and kept at -20°C until assayed 5 days later.

Two doses of plasma (100 and 50 ul) were used to react with the anti-rat GH (DMD-1) and two doses (100 and 50 ul) to count total radioactivity per sample per time. A high concentration of DMD-1 (1:1000) was used to insure the precipitation of most of the antibody precipitable radioactivity in each sample.

The metabolic clearance rate of GH was determined using the following formula, described by Tait (1963) and Kohler et al. (1968a):

$$MCR = \frac{\text{total immunoprecipitable } I^{125} \text{ RGH injected}}{\int_0^{\infty} x' \cdot dt}$$

in which x' represents the plasma concentration of immunoprecipitable I^{125} - RGH. A disappearance curve of

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immunoprecipitable radioactivity/ml and total radioactivity/ml of plasma was plotted for each animal using linear coordinates. The integral $\int_0^\infty x' \cdot dt$ was determined by numerically measuring the area under the disappearance curve for the immunoreactive RGH-I^{125} for each animal. The value obtained was divided into the total immunoprecipitable RGH-I^{125} injected to obtain MCR. The individual metabolic clearance rates thus obtained were then pooled per group and analyzed statistically. The secretion rate of growth hormone was arrived at by multiplying the individual MCR times the plasma growth hormone concentration of each animal.

V. Methods of Statistical Analysis

Serum, plasma or pituitary samples were assayed at 2 or more different dilutions to insure accurate measurement of growth hormone levels. The average of these dilutions was used as the growth hormone value of that sample. Mean and standard error of the mean were calculated using the average values of all samples in a group. Significance of differences between groups was determined by Student's t test or analysis of variance, followed by Duncan's new multiple range test (Bliss, 1967).

EXPERIMENTS

I. Radioimmunoassay for Rat Growth Hormone

A. Objectives

The purpose of these experiments was to develop a radioimmunological assay for rat growth hormone capable of measuring GH in the body fluids. In developing the assay, several steps have been carried out to test its validity and specificity. These have not been grouped in one specific section; instead, they have been placed in those sections of this thesis which the author feels are most closely related to the topics in question. The steps followed in establishing the assay included: a) iodination of RGH of high specific activity, b) evaluation of the damage caused by the iodination, c) steps to minimize damage after iodination, d) selection of the more immunoreactive portion of the labeled hormone, e) specific activity determination, f) competition curves with rat GH reference preparations, plasma and pituitary homogenates, g) competition curves with serum from hypophysectomized animals, h) cross-reactivity of the anti-rat GH serum with other pure rat hormones, i) measurement of pituitary GH in different physiological conditions by bioassay and radioimmunoassay, and j) cross-reactivity of the anti-rat

GH with preparations from other species. Steps a-i are included in this section.

B. Procedures

The iodination of RGH (NIAMD-RGH-I-1 or HVII-38-C) was accomplished using essentially the method of Greenwood et al. (1963). Initially 2.5 ug of RGH was labeled with 1 mc of I^{131}, but it was later changed to I^{125} because of its longer half-life. Since the number of atoms in a mc of I^{125} is seven times greater than in a mc of I^{131}, the amount of RGH was increased 7 to 8 times greater than the original amount used. As indicated in "Materials and Methods", 20 ug of RGH are now labeled with 1 mc of I^{125}. The reader is referred to "Materials and Methods" for details of this reaction.

C. Results

1. Iodination of RGH. Separation from the free I^{125}.

Separation of the RGH-I^{125} from the free I^{125} was accomplished by placing the reaction mixture in a 0.9 x 20 cm column of Sephadex G-50 expanded in 0.01M barbital buffer at pH 8.6. The column was previously coated with a 5% solution of BSA-barbital. Figure 2 shows a typical elution pattern obtained when 1 ml aliquots are collected in tubes containing 1-2 drops of 30% BSA-barbital or their equivalent. The small volume of BSA added to the tubes is intended to coat the glassware without producing dilution of the collected material. In this way large amounts of hormone can be repurified by transferring small volumes of the

material to a second column, facilitating the formation of a small packed layer. As can be seen in Figure 2, about 50% of the recovered radioactivity is present in the hormone peak, while the remainder is in the form of free I^{125}.

2. Iodination damage.

a. Repurification of RGH-I^{125} in Sephadex G-100.

To establish whether or not damage had taken place during iodination, an aliquot of the RGH-I^{125} peak of the G-50 elution was repurified in a 0.9 x 50 cm column of Sephadex G-100 expanded in 0.05M barbital buffer at pH 8.6. A volume of 0.25-0.40 ml was transferred with 0.15 ml of transfer solution to aid in the packing of the layer. Two ml aliquots were then collected in 1% BSA-PBS coated tubes. Figure 3 shows the elution of the RGH-I^{125} aliquot through the Sephadex G-100 column. As can be seen, three peaks are discernible. The first peak is an aggregated form of RGH-I^{125}; the second peak corresponds to the molecular weight of growth hormone; while the third peak represents the degraded fraction and iodine. It was later learned that the relative proportion of these peaks varied with the source of the aliquot for repurification. Aliquots from tubes of the ascending limb of the G-50 column contained proportionately greater amounts of the aggregated form of RGH-I^{125} (as much as 50-60% of the total radioactivity recovered) than aliquots of the descending limb (generally between 5-10% of the total radioactivity). Furthermore, it was later observed that the interval between iodination

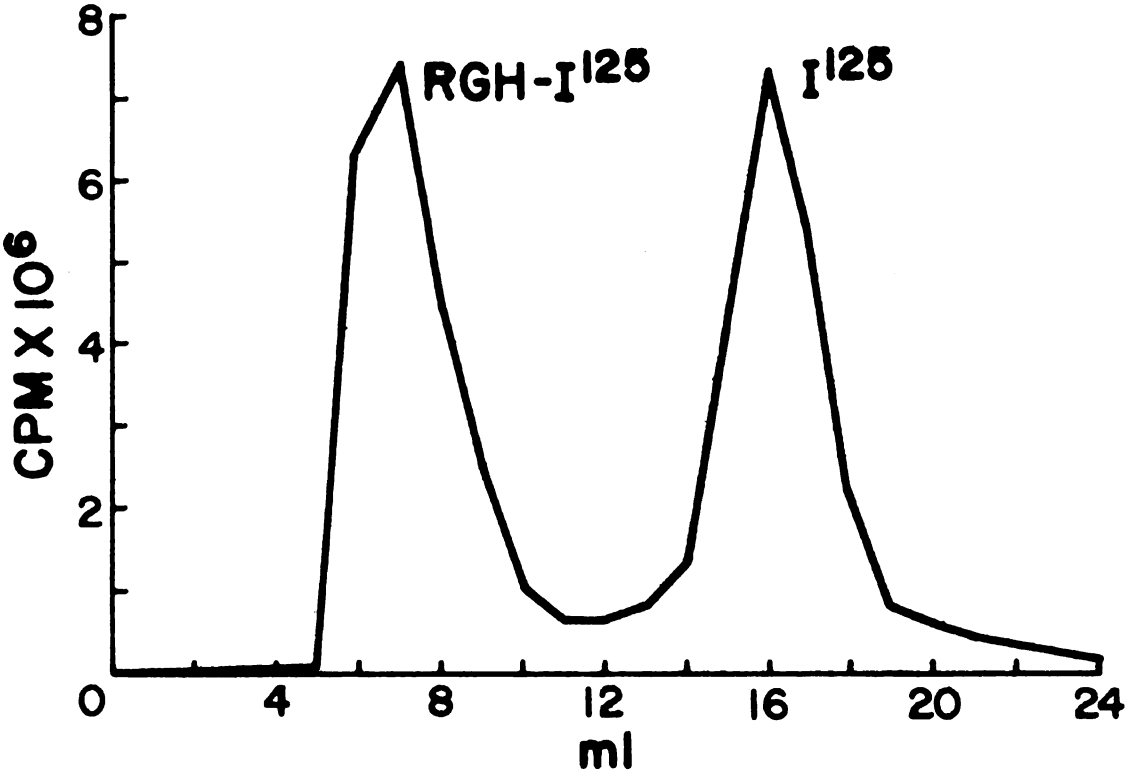

Figure 2. Elution pattern of RGH-I^{125} through a 0.9 x 20 cm column of Sephadex G-50 in 0.01M barbital buffer at pH 8.6.

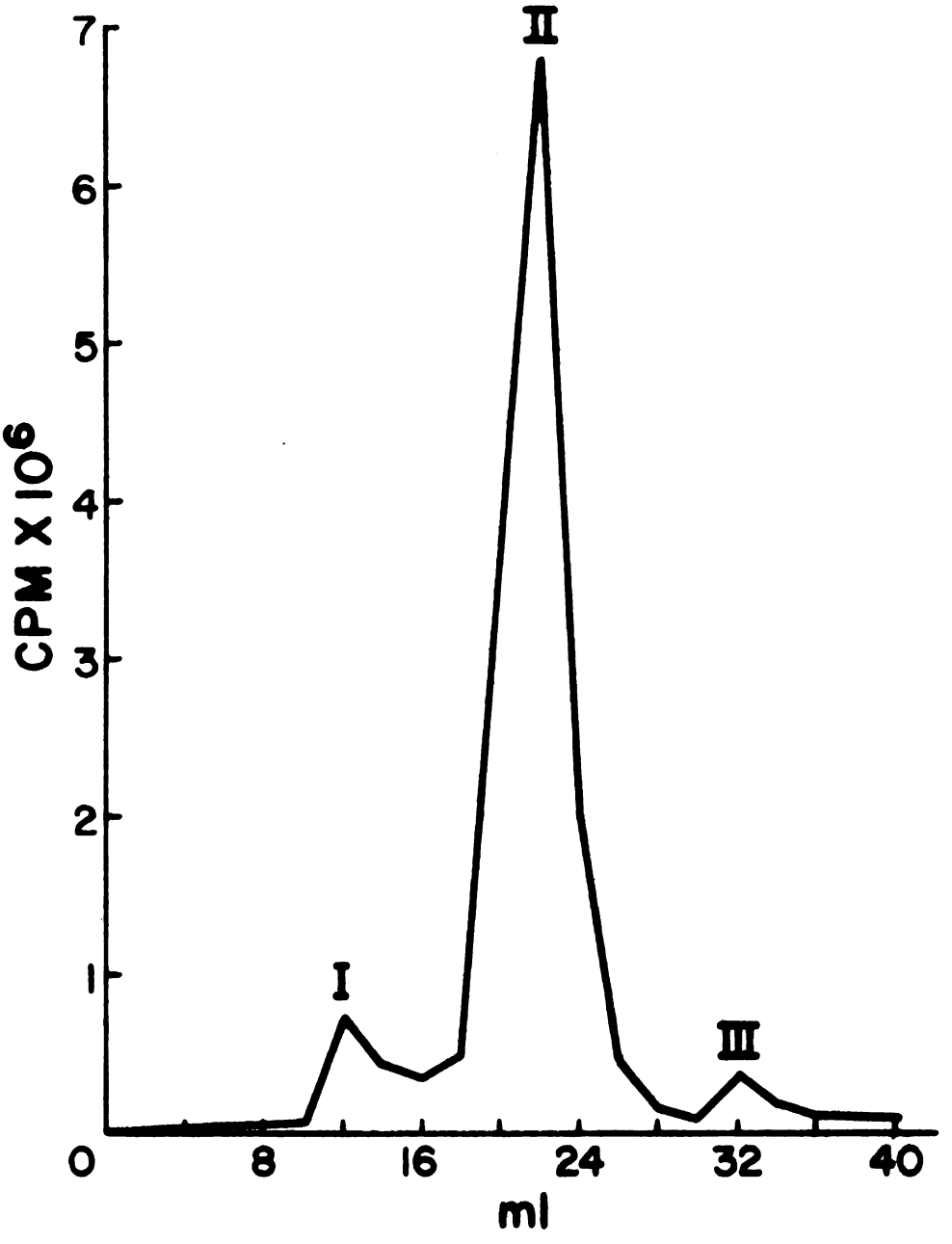

Figure 3. Elution pattern of RGH-I^{125} upon repurification through a 0.9 x 50 cm column of Sephadex G-100 in 0.05M barbital buffer at pH 8.6.
Peak I represents aggregated hormone, peak II undamaged hormone and peak III degraded hormone.

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and repurification, as well as the method of freezing the labeled hormone, affected the proportion of the peaks.

b. Effect of time on repurification.

Figure 4 shows the repurification of aliquots from the same tube of the descending limb of the G-50 separation at different times after iodination. It can be seen that the longer the interval between iodination and repurification the greater the proportions of polymerized and degraded RGH-I^{125}, with a concomitant decrease in the amount of undamaged RGH-I^{125} recovered. The increase in aggregated and degraded RGH-I^{125} with time has been observed with all aliquots repurified regardless of their point of origin.

c. Effect of rapid freezing on repurification.

The effect of rapid freezing versus slow freezing on the amount of damaged hormone can be seen in Figure 5. Aliquots from the same tube of the G-50 collection were subjected to an alcohol-dry ice bath fast freezing or were placed in the freezer (-20°C) to slow freeze them. The samples were defrosted within a week and repurified in Sephadex G-100. It can be seen that fast freezing (solid line) results in an increase in the amount of aggregated RGH-I^{125} recovered. No change was observed in the quantity of degraded RGH-I^{125}.

3. Relative immunoreactivity of the different RGH-I^{125} fractions.

a. Percentage binding.

Figure

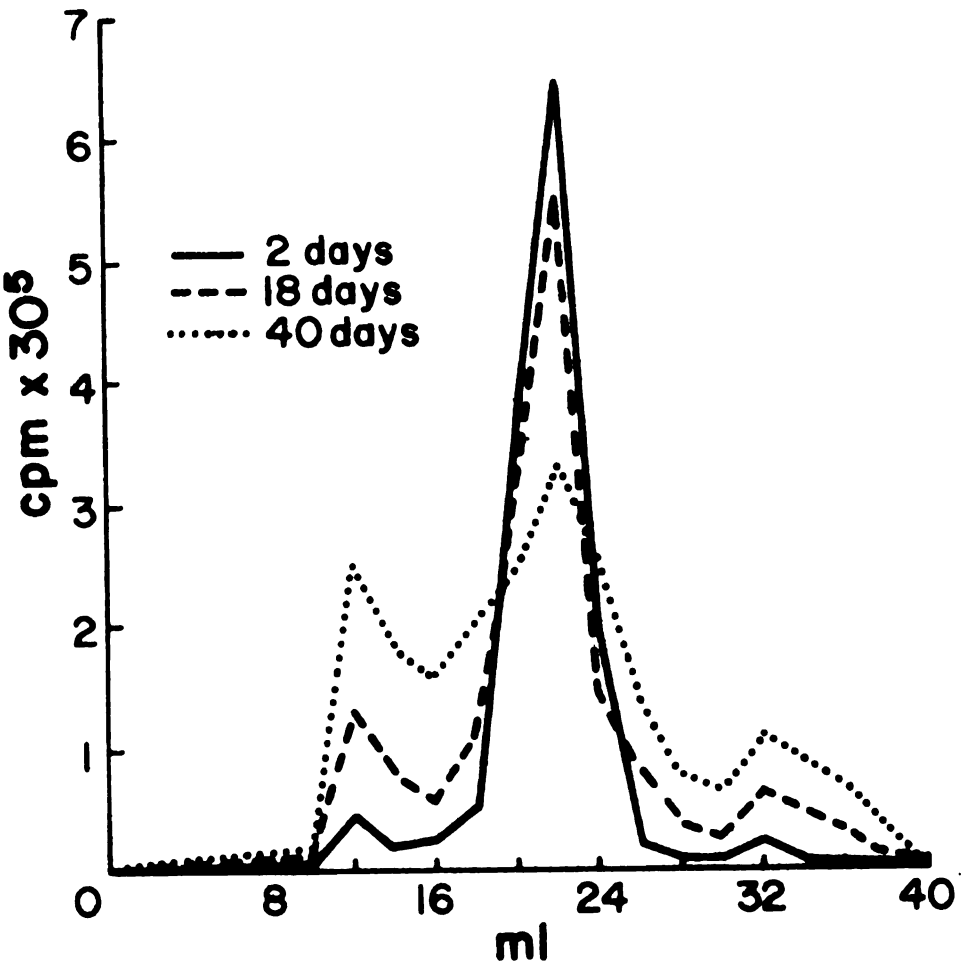

Figure 4. Effect of time on the elution pattern of RGH-I^{125} repurification through Sephadex G-100.
Note the progressive increases in aggregated and degraded labeled hormone with increasing intervals between time of iodination and repurification of the labeled rat growth hormone.

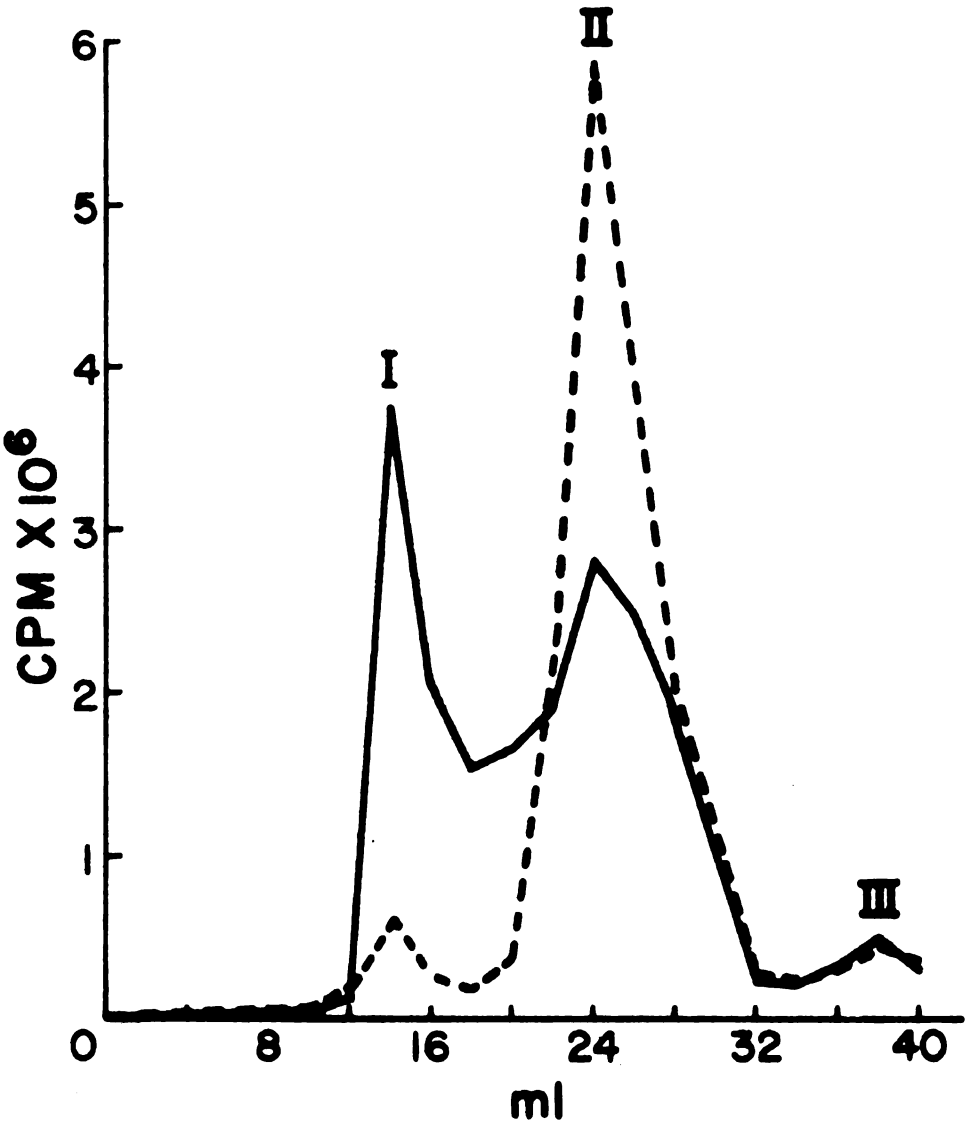

Figure 5. Effect of rapid freezing (——→) on the elution pattern of RGH-I^{125} repurification through Sephadex G-100.
Note the increase in aggregated labeled hormone with rapid freezing.

The immunoreactivity of the three fractions from the G-100 column was tested by diluting the tubes of each fraction in 1% BSA-PBS pH 7.2 such that 100 ul of solution contained approximately 20,000 cpm. Tubes were then set up for each fraction to measure: a) non-specific binding, b) total counts, and c) the percentage of immunoreactivity bound by 200 ul of a 1:50,000 dilution of anti-rat GH serum. The details in setting up these tubes are given in "Materials and Methods; Procedure for radioimmunoassay". As can be seen in the left hand portion of Figure 6, the anti-rat GH serum bound 38% of the aggregated RGH-I^{125}, 51.5% of the undamaged RGH-I^{125}, and only 6.5% of the degraded RGH-I^{125}. It is of interest to note that the non-specific binding of the aggregated RGH-I^{125} was 3-4 times higher than that of the undamaged protein. The right hand portion of Figure 6 shows a comparison of immunoreactivity between a G-50 first peak aliquot and a G-100 second peak aliquot. For any given anti-rat GH serum dilution less of the G-50 first peak hormone was bound. This difference is accounted for by the contamination with aggregated and degraded RGH-I^{125}.

b. Competition curves.

Figure 7 shows the competition between G-100 first peak or G-100 second peak RGH-I^{125} and different quantities of a rat GH reference standard (NIAMD-RGH-RP-1) for the anti-rat GH serum. The set up of this test followed that described in "Materials and Methods" regarding standards

Figure

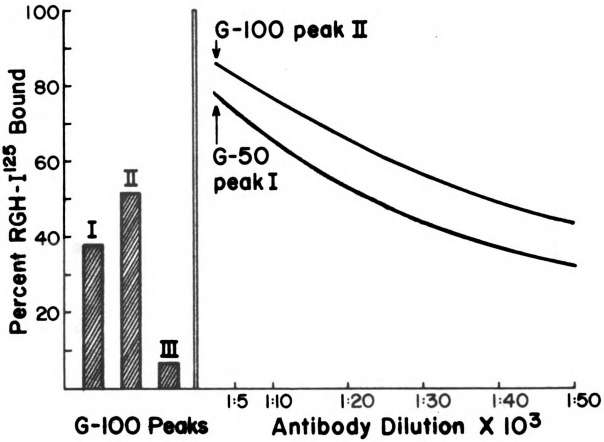

Figure 6. Left side: relative radioimmunoreactivity of the G-100 fractions. The most immunoreactive fraction corresponded to the undamaged labeled hormone (peakII). Right side: comparison between G-50 and G-100 peak II immunoreactivity. For any given antibody dilution less of the singly purified labeled hormone (G-50 peak I) was bound than of the doubly purified labeled hormone correspoding to the undamaged fraction (G-100 peak II).

Percent RGH-I^{125} Bound

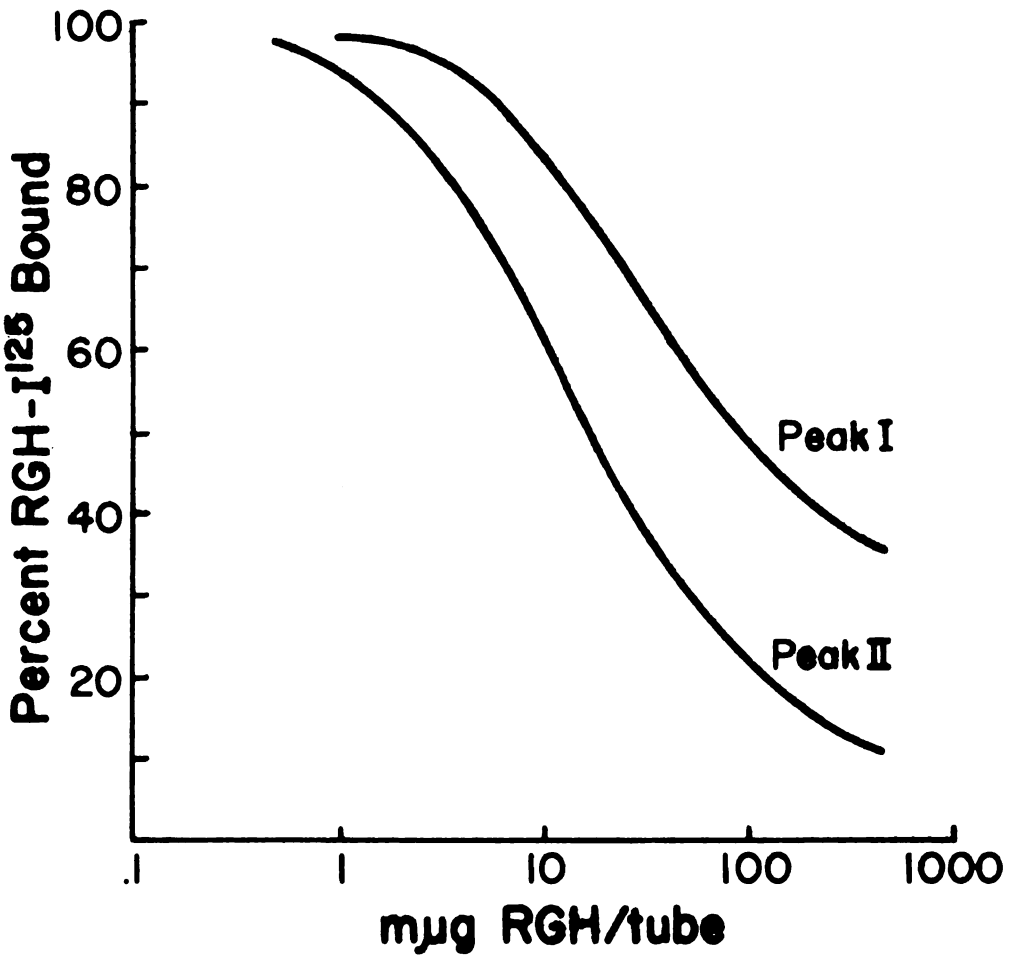

Figure 7. Standard curves obtained with G-100 peak I and G-100 peak II RGH-I^{125}.
One hundred percent binding in abscissa represents binding obtained in the absence of cold hormone. Ordinate shows the amount of cold hormone added per tube. It can be seen that when aggregated hormone was used as tracer less RGH-I^{125} was displaced from the antibody-antigen complex by the addition of cold hormone.

in a radioimmunoassay for each of the RGH-I^{125} G-100 peaks. The competition curves are expressed with respect to their individual 100% tubes (50% binding). As can be seen in Figure 7, any given amount of NIAMD-RGH-RP-1 displaced less of the aggregated RGH-I^{125} from the antibody-antigen complex. It was concluded, therefore, that the second G-100 peak was the most immunoreactive, and that the aggregated labeled hormone is not only more difficult to bind, but once bound, is also harder to displace.

In view of the above observations the following steps have been adopted in the assay: a) slow freezing of the tubes collected from the G-50 column, b) repurification of the G-50 RGH-I^{125} in G-100 columns prior to use and, c) selection of the second fraction of the G-100 repurification, pooling of the tubes and dilution with 1% BSA-PBS pH 7.2 to the desired number of cpm. The diluted solution is kept at 4°C, at which temperature the diluted hormone remains stable for periods of at least 2 months. If more hormone is needed, an aliquot of the frozen G-50 RGH-I^{125} is thawed, repurified, and the second fraction diluted. Henceforth all results have been obtained using labeled hormone from the second fraction of the G-100 repurification step.

4. Competition curves with the G-100 RGH-I^{125} second fraction.

a. Against NIAMD-RGH-RP-1 or NIAMD-RGH-I-1.

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accomplished it was necessary to establish inhibition (competition) curves with rat GH preparations to ascertain the sensitivity and reproducibility of the assay. For this purpose two preparations of rat GH were used: a) NIAMD-RGH-RP-1 which is a reference preparation, and b) NIAMD-RGH-I-1 which is an iodination quality material. This was done also to have a measure of the equivalency between these two preparations since our results would be based on the reference preparation and not on the pure RGH material. Both were prepared as described for standards in "Materials and Methods". Figure 8 shows the results. The lowest limit of detection of the assay was 0.25 mug of the NIAMD-RGH-I-1 material. In repeated assays of the same amounts of hormone reproducibility was found to be within 5.5%. In addition we have found that the NIAMD-RGH-I-1 is, on the average, 3.2 times more potent than the NIAMD-RGH-RP-1.

b. Against rat plasma or pituitary homogenate.

Figure 9 shows the inhibition curves obtained when using different amounts of intact rat plasma or different volumes of a 0.1 mg/ml solution of rat anterior pituitary tissue. The slope of the two curves was the same as that of the NIAMD-RGH-RP-1 standard.

c. Against other pure rat hormones.

The specificity of the anti-rat growth hormone sera for rat GH was tested using purified preparations of other rat hormones available to us, namely

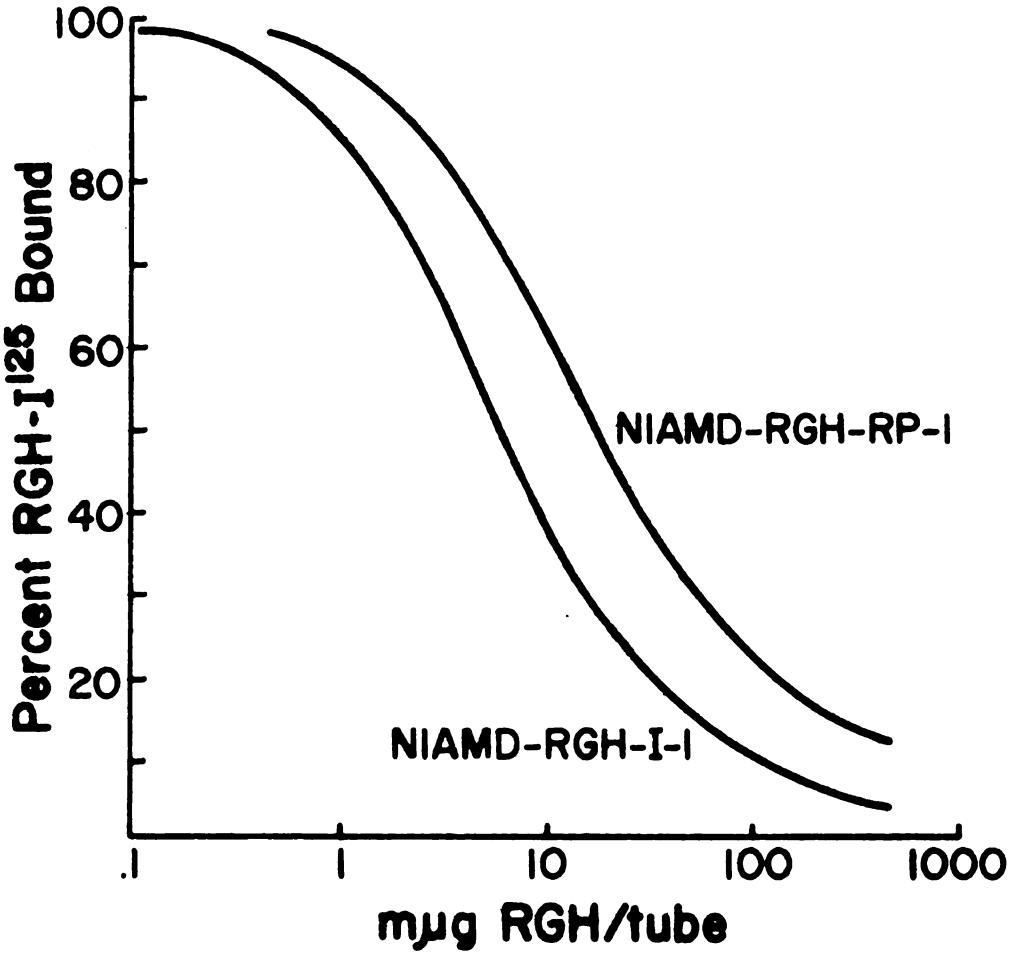


Figure 8. Rat growth hormone standards.
One hundred percent binding in abscissa represents binding of RGH-I^{125} in the absence of cold hormone. Ordinate shows the amount of cold hormone of iodination purity (NIAMD-RGH-I-1) or standard reference preparation (NIAMD-RGH-RP-1) added per tube.

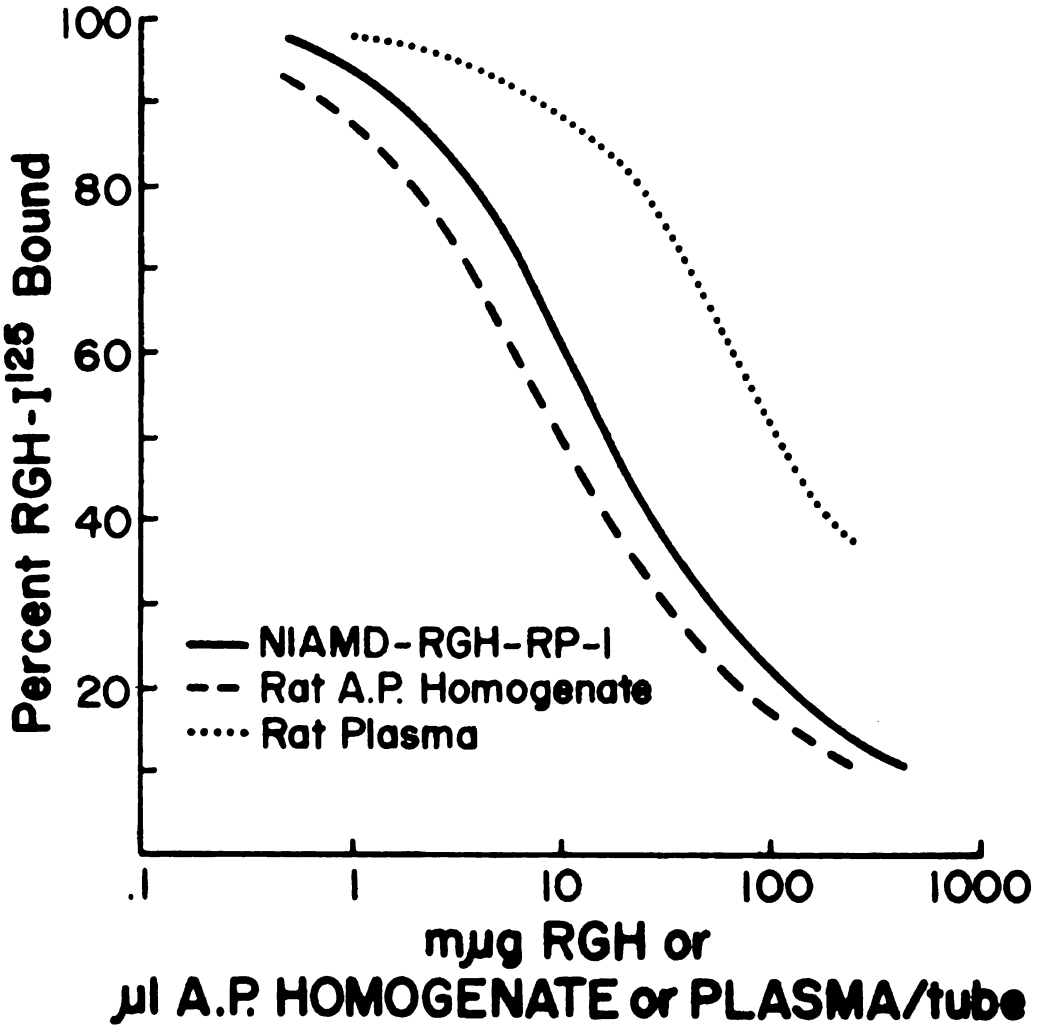


Figure 9. Inhibition curves with rat pituitary homogenate or plasma from intact rats.
One hundred percent binding in abscissa represents binding of RGH-I^{125} in the absence of cold hormone. Note parallel inhibition curves obtained with the addition of plasma and pituitary homogenate, and a standard rat GH reference preparation.

prolactin, follicle stimulating hormone (FSH) and luteinizing hormone (LH), all of iodination quality. These hormones were prepared in the same manner as the rat growth hormone standards. The results in Figure 10 show that none of these hormones is able to significantly inhibit the reaction between the RGH-I^{125} molecule and its anti-serum throughout the range of concentrations used. The very slight inhibition observed with the larger doses of these hormones may indicate a small degree of contamination of these preparations with rat GH.

d. Against hypophysectomized rat serum.

To ascertain that the assay was indeed reacting with pituitary GH in the plasma of rats and not with a non-specific plasma protein, competition was set up against different amounts of hypophysectomized rat serum. Since no inhibition took place the results have been plotted in Figure 11 as the percentage binding of the first antibody. The solid bar on the right side represents the mean percentage binding of the several dilutions of hypophysectomized serum used. There was no significant difference between the mean or any individual percentage binding and that obtained when no hypophysectomized serum was used.

5. RGH-I^{125} specific radioactivity.

In order to determine the specific radioactivity of the labeled hormone, the recovery of free I^{125} was first determined. This was accomplished by carrying out a sham iodination in which all of the reagents were placed

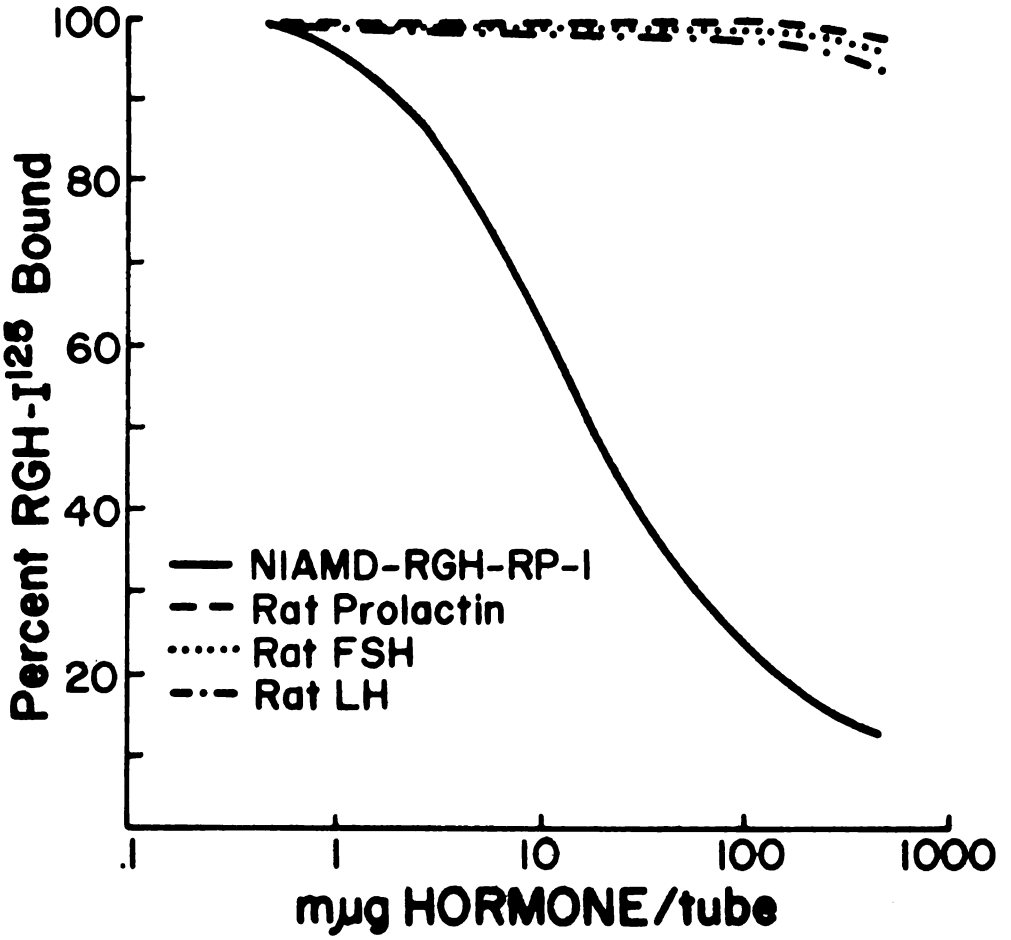


Figure 10. Cross-reaction between monkey anti-rat growth hormone serum and other rat anterior pituitary hormones.
One hundred percent binding in abscissa represents binding of RGH-I^{125} in the absence of cold hormone. No inhibition was obtained upon the addition of pure preparations of rat prolactin, LH and FSH.

Percent RCH I^{125} Bound

Figure 1

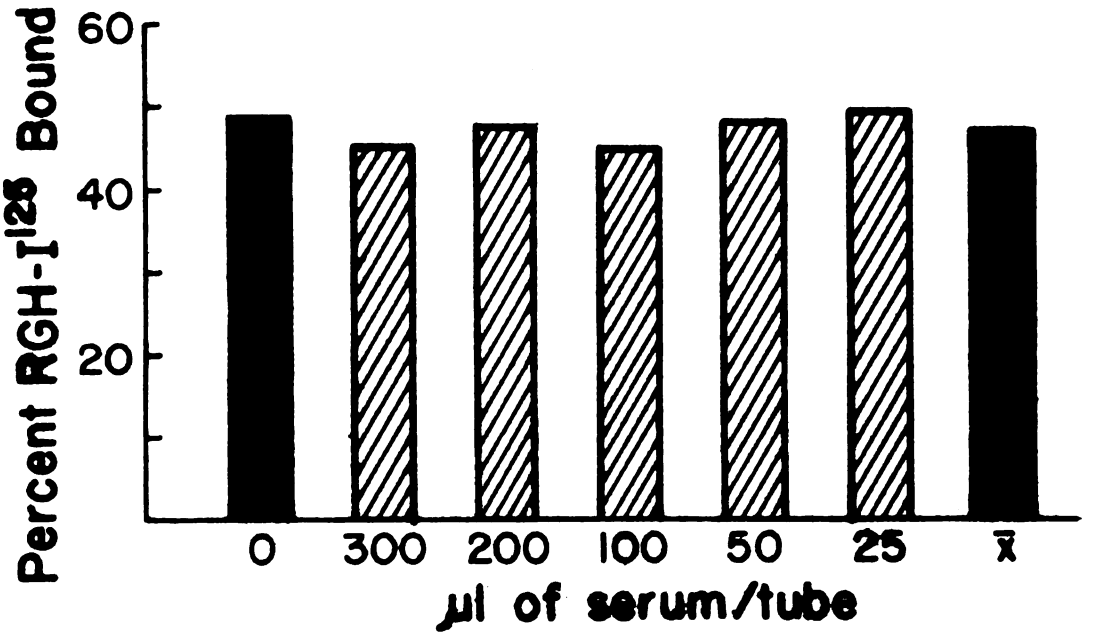


Figure 11. Effect of hypophysectomized male rat serum on percent binding.
The RGH-I^{125} bound is expressed in terms of percent of total radioactivity. Solid bar at left shows binding obtained in the absence of serum; solid bar at right shows the average binding obtained upon the addition of different volumes of hypophysectomized rat serum/ tube. Note that hypophysectomized rat serum did not alter percent binding.

in an iodination vial in their regular sequence, except that buffer alone was used with no hormone. Table 1 shows the counting schedule of a typical preparation. It can be seen that 77.8% of the total iodide is recovered in the salt fraction (10 aliquots of 1 ml each), while 14.7% is lost through the column. The actual recovery of iodide through the column was 84%. The calculation of the yield and specific radioactivity of RGH-I^{125} is based on the 77.8% recovery of the total iodide.

In the RGH-I^{125} preparation shown in the lower half of Table 1 the total radioactivity of the salt peak represents 77.8% of the unchanged I^{125}, since 77.8% of the total I^{125} is recovered in the absence of protein. By simple calculation it is seen that 100% of the unchanged iodide is represented by 3470 cpm. The remaining radioactivity (12860-3470=9390 cpm) represents I^{125} transferred to 20 ug of rat growth hormone. The counts associated with protein represent 73% of the total iodide, or 730 uc. The specific radioactivity is thus 730 uc I^{125}/20 ug RGH or 36.5 uc I^{125}/ug RGH, a percentage transfer of 73% of the original I^{125} iodide. Only 30.7% of the total protein is recovered through the G-50 column and this represents 6.04 ug of protein. The I^{125} labeled growth hormone, of specific radioactivity 36.5 uc/ug, contains 0.75 atom of I^{125}/molecule of growth hormone (molecular weight 45,000; Ellis et al., 1968a).

TABLE 1.--Determination of specific radioactivity of RGH-I^{125}

Iodine loss determination	cpm	% total I^{125}	% column load
Sodium (I^{125}) iodide in vial	8456	100.0	
Reaction vial after KI wash	484	5.7	
Reactivity in syringe wash	148	1.7	
Column Load (calc.)	7824	92.5	100.0
Sodium (I^{125}) iodide recovered (10 ml)	6579	77.8	84.0
Sodium (I^{125}) iodide lost in column (calc.)	1245	14.7	15.9

RGH-I^{125} preparation	cpm	mcI^{125}	ugRGH
Sodium (I^{125}) iodide (1 mc) in vial	12860	1.0	
Unchanged (I^{125}) iodide recovered	2700	0.209	
Actual unchanged (I^{125}) iodide	3470	0.269	
RGH-I^{125} recovered	2840	0.220	6.04
Counts associated with GH = total cpm - actual free I^{125} cpm	9390	0.730	20

Therefore, specific radioactivity = uc I^{125} associated with GH ÷ ug of GH
= 36.5 uc/ug

The above calculations assumed that the specific radioactivities of the RGH-I^{125} adsorbed to the reaction vial, syringe and to the Sephadex G-50 are the same as that eluted from the column. Furthermore, it assumed that the specific radioactivities of the different RGH-I^{125} fractions obtained from the Sephadex G-100 repurification are the same as that eluted from the first column. These assumptions have not been verified experimentally.

6. Potency estimates by RIA or bioassay.

In order to determine the correlation between radioimmunological and biological activity three pituitary preparations were measured using both methods. A group of male rats was gonadectomized, a second group was surgically thyroidectomized while a third group served as controls. Two weeks after surgery the animals were decapitated and their pituitaries removed, weighed, pooled and homogenized in phosphosaline buffer. Pituitary homogenates of control and experimental animals were bioassayed at two dose levels (1 and 4 mg/rat/4 days or 2 and 8 mg/rat/4 days) by the tibia test of Greenspan et al. (1949). Two doses of NIAMD-RGH-RP-1 (10 and 40 ug/rat/4 days) and 2 doses of NIH-GH-S8 (25 and 100 ug/rat/4 days) were also used as reference standards. Four rats were injected per dose. Radioimmunoassays of the pituitary homogenates were done at 4 dilutions (20, 40, 80 and 160 ul) of a solution containing 0.0125 mg/ml (control and gonadectomized) or 0.25 mg/ml (thyroidectomized). Five replications

of the four dilutions were done for each homogenate. The statistical methods of Bliss (1952) were used to compute the bioassay potencies of control and experimental groups, 95% confidence limits and any departures from parallelism.

The results are shown in Table 2. In all cases the radioimmunoassay values were below those obtained by bioassay, with indices of discrimination ranging from 0.63 to 0.74. Over the range of concentrations tested the potency estimates by bioassay and radioimmunoassay were not significantly different since the radioimmunoassay values were within the 95% confidence limits of the bioassay determinations. It is of interest to note that gonadectomy does not result in a significant decrease in pituitary GH concentration when measured by bioassay. This decrease is significant by radioimmunoassay. The decrease in pituitary GH concentration due to thyroidectomy is significant by both assays. The curves obtained with the preparations were indistinguishable from the NIAMD-RGH-RP-1 standards in the bioassay and radioimmunoassay in shape and slope.

D. Discussion

A radioimmunological assay for rat growth hormone is reported in which 20 ug of a purified preparation is iodinated with 1 mc of iodide I^{125}. Under the conditions described there is a net transfer of 73% of the original I^{125} iodide to the protein, 30.2% of which is recovered through the Sephadex G-50 column for a total yield of 6.04 ug of RGH-I^{125}. On the assumption that the transfer of iodide

TABLE 2.--Biological and immunological estimates of potency for male rat pituitary homogenates

Exp. #	Treatment and # of rats	GH by RIA[1,2] ug/mg	GH by tibia[1,3] bioassay ug/mg	RIA-GH[4] / Tibia-GH	λ[5]
I	Intact (20)	26.8±1.3	42.26(23.42-76.19)	0.63	0.188
	Gonadectomized (20)	17.7±1.5	23.88(14.14-39.44)	0.74	
II	Intact (20)	29.4±2.1	39.90(23.55-65.59)	0.74	0.160
	Thyroidectomized (20)	1.9±0.3	2.82(1.61-4.93)	0.67	

[1]Expressed as ug equivalents of NIAMD-RGH-RP-1.

[2]Mean±standard error.

[3]Mean and 95% confidence limits.

[4]Index of discrimination, GH concentration by radioimmunoassay as ug/mg ÷ GH concentration by tibia test at ug/mg.

[5]Index of precision of bioassay.

I^{125} is equally distributed among the 20 ug of hormone a specific radioactivity of 36.5 uc I^{125}/ug of rat hormone can be calculated. Although we have been able to obtain higher specific radioactivities by decreasing the amount of hormone in the reaction, this has resulted in lower yields as well as in greater amounts of damaged fractions. The specific radioactivity of the RGH-I^{125} may be shown to consist of 0.75 atom of I^{125}/molecule of rat growth hormone. In our hands this is adequate to measure pituitary and plasma rat growth hormone.

The behavior of the iodinated rat growth hormone molecule is similar to that reported for human growth hormone. Our observation that subsequent to iodination it is possible to separate the iodinated hormone into three fractions, which correspond to an aggregated component, an undamaged portion and a degraded component, has been reported for human growth hormone by Berson and Yalow (1968) using paper chromatoelectrophoresis and by Giustina *et al.* (1968 a,b) using gel filtration on Sephadex G-100 or G-150. Giustina *et al.* (1968 a,b) also reported an increase in the polymerized form of HGH-I^{125} following rapid freezing and subsequent thawing prior to repurification. These authors tested the radioimmunoreactivity of the three fractions and found that the peak corresponding to the molecular weight of the hormone (second peak) was practically all immunoreactive. The aggregated peak was about 20% immunoreactive while no immunoreactivity was found in the

degraded fraction. Our results are in basic agreement with those reported above.

We have further found that the proportion of polymerized and degraded RGH-I^{125} increases with time. This may be due in part to the formation of "free radicals" in the solution as a result of the low energy radiation of the I^{125}. To decrease this damage we have stored the diluted hormone at 4°C in 1% BSA-PBS so that the excess protein in the system serves as a buffer. Thus, we have been able to utilize the diluted labeled hormone for up to 2 months without noticing any change in the radioimmunological reactivity of the molecule, which is interpreted as a lack of damage. Though it is certain that growth hormone preparations undergo deamidation in solution, it has been found that the immunoreactivity is not affected by this change (Berson and Yalow, 1968). Enzymatic degradation may also play a part in the formation of the degraded form.

In testing the radioimmunoreactivity of the several fractions it was found that the polymerized form was not only less immunoreactive, but once bound more difficult to displace from the antibody-RGH-I^{125} complex. It can be envisioned that the aggregation of molecules can hinder immunologically active sites from competing for the antibody sites; once bound, however, the same aggregation of molecules may hinder a competing molecule from displacing it from the antibody site. If one assumes that the specific radioactivity of the polymerized fraction is the same as that

of the undamaged protein, then it must be concluded that the actual immunoreactivity of the aggregated form is less than that recorded experimentally since the ratio of cpm/ actual bound molecule would be greater for the aggregated molecule. Although this has not been verified experimentally, it is suggested by the observation that the non-specific binding of the RGH-I^{125} polymer was 3-4 times greater than that observed for the undamaged RGH-I^{125}.

It can be calculated that the addition of 19-21,000 cpm of freshly labeled hormone per radioimmunoassay tube represents approximately 0.25-0.35 mug of hormone when the specific radioactivity is 36.5 uc/ug of growth hormone. This amount of tracer permits a sensitivity of 0.25 mug of pure cold hormone in an assay where 50% of the labeled hormone is bound by a 1:50,000 dilution of anti-rat growth hormone serum.

The radioimmunoassay described above is specific for GH in the rat since it is not affected by other rat hormones or by non-specific plasma proteins. Furthermore, parallel inhibition curves were obtained between different rat pituitary homogenates or plasma and a purified preparation of rat growth hormone. Although there is no evidence to show that the immunological and biologically active sites are the same in the GH molecule, no significant difference was found in the estimated potencies of pituitary preparations by radioimmunoassay or bioassay. This suggests that throughout a range of GH concentrations brought

about by physiological manipulations the relationship between these two active sites may be of little consequence in establishing an accurate estimate of potency.

II. Study on the Cross-Reactivity of Monkey Anti-Rat Growth Hormone Serum

A. Objectives

This study was undertaken to determine the immunological cross-reactivity between the NIAMD-A-RatGHS-1 and growth hormones, pituitary homogenates or plasma from other animal species, in an attempt to assess the feasibility of using the previously described radioimmunoassay in measuring the growth hormone of different species.

B. Procedures

Purified preparations of ovine (NIH-GH-S8), bovine (NIH-GH-B12) and human (NIH-GH-HS 1216C) growth hormone; pituitary homogenates of mouse, gerbil, guinea pig, hamster and monkey; and plasma of mouse, gerbil, guinea pig, hamster, rabbit, cat and dog were tested for their ability to compete with RGH-I^{125} for the immunologically active sites of the monkey anti-rat growth hormone antibody.

Pituitary tissue from the mouse, gerbil, guinea pig, hamster and monkey was obtained by decapitation. The pituitaries were weighed and homogenized separately with a Sonifier cell disruptor in phosphosaline buffer. The pituitary homogenates were diluted to a concentration of 0.1 mg per ml. Blood was collected by cardiac or venipuncture in heparinized syringes, centrifuged immediately and the plasma separated and frozen at -20°C until assayed. All preparations were assayed for growth hormone in duplicate volumes of 0.5, 1, 2, 4, 8, 16, 31, 62.5, 125, and 250

or 300 ul per radioimmunoassay tube. The volume in each tube was complemented with 1% BSA-PBS to a total volume of 500 ul. The assay was carried out as described in the preceding section.

C. Results

The results of this experiment can be seen in Figures 12, 13 and 14. A typical standard curve for rat growth hormone is shown in each figure as the basis for comparison.

Figure 12 shows the immunological relatedness between ovine, bovine and human growth hormone, as well as monkey pituitary homogenate, with rat growth hormone. No cross-reactivity was observed with either the purified human growth hormone or the monkey pituitary homogenate within the range assayed. Partial cross-reactivity was seen with the ovine and bovine growth hormone preparations. Neither, however, showed a parallel relationship with rat GH, and very flat slopes were obtained.

Figures 13 and 14 show the dilution response curves for pituitary extracts and/or plasma of gerbil, mouse, rabbit, guinea pig, hamster, cat and dog. All of these preparations, with the exception of rabbit plasma, cross-reacted significantly with the antibody to rat GH.

D. Discussion

The immunological relatedness between growth hormones, pituitary homogenates or plasma from several animal species and rat growth hormone was studied using a double antibody radioimmunoassay for rat growth hormone. The monkey

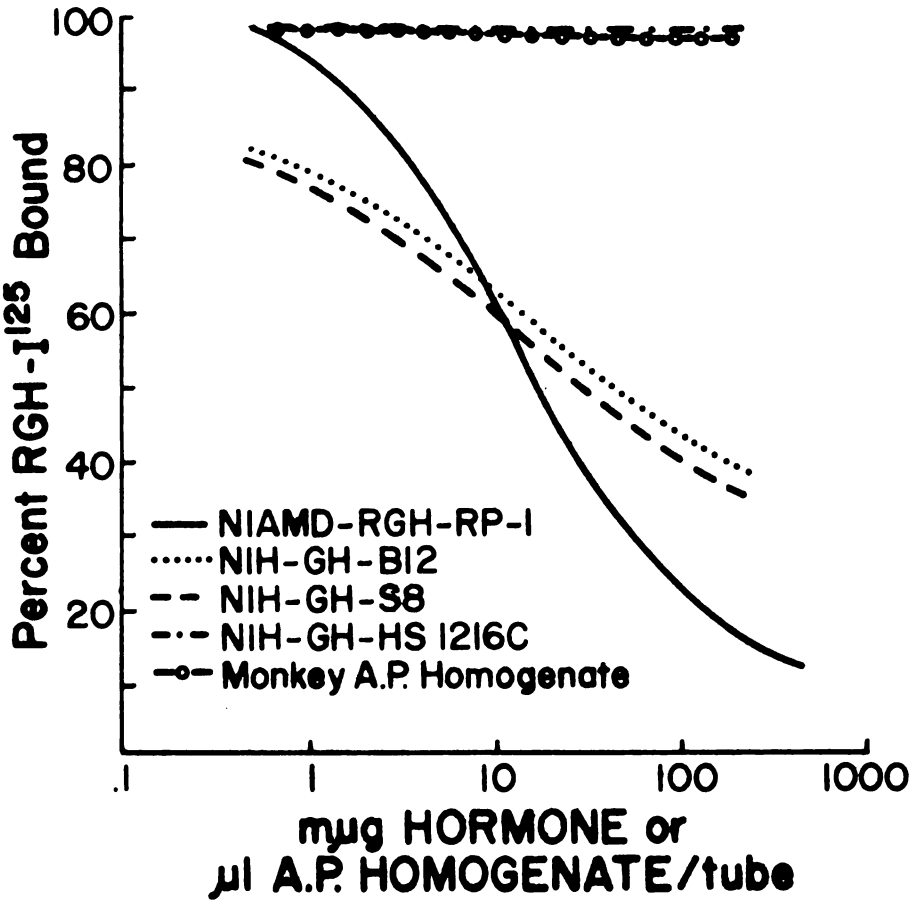


Figure 12. Immunological relatedness of growth hormones. One hundred percent binding in abscissa represents binding of RGH-I^{125} in the absence of cold hormone. No cross-reaction was obtained upon adding human GH (NIH-GH-HS 1216C) or monkey A.P. homogenate to the tubes. Partial cross-reaction was observed with purified ovine (NIH-GH-S8) and bovine (NIH-GH-B12) GH's.

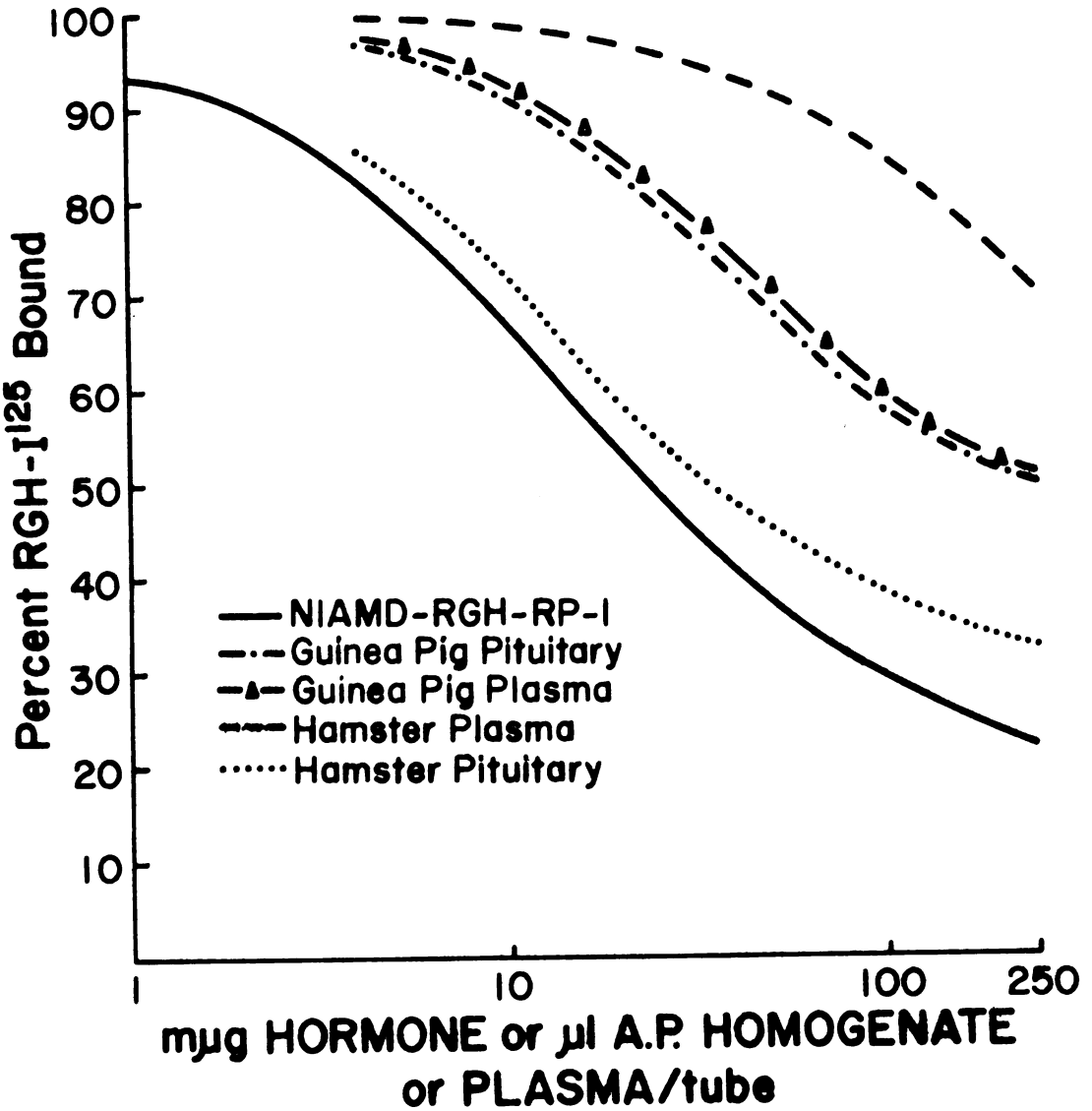


Figure 13. Dilution-response curves for pituitary extracts and plasma of guinea pig and hamster. One hundred percent binding in abscissa represents binding of RGH-I^{125} in the absence of cold hormone. Note parallel cross-reactions obtained upon the addition of plasma or A.P. homogenates.

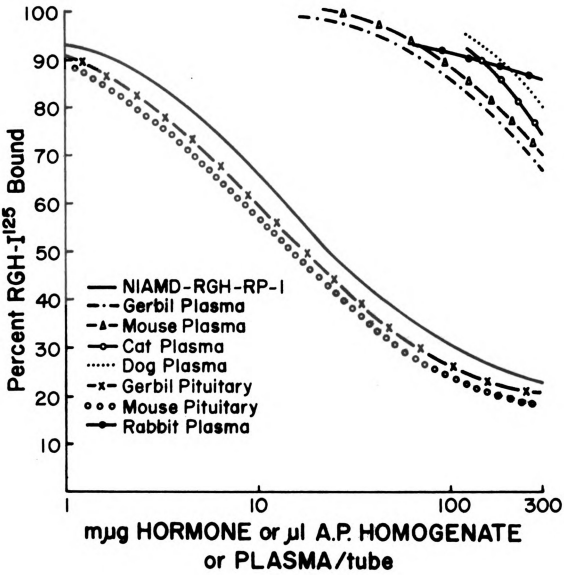


Figure 14. Dilution-response curves for gerbil, mouse, cat, dog and rabbit preparations.
One hundred percent binding in abscissa represents binding of RGH-I^{125} in the absence of cold hormone. Note parallel cross-reactions with gerbil, mouse, cat and dog plasma or A.P. preparations. No cross-reaction was observed with rabbit plasma.

anti-rat growth hormone utilized was shown to cross-react with pituitary homogenates and plasma of hamster, guinea pig, gerbil and mouse. Furthermore, it cross-reacted with cat and dog plasma. Partial cross-reaction occurred with purified preparations of ovine and bovine growth hormone, but no cross-reaction took place when a purified human growth hormone or a monkey pituitary homogenate were used. In this study, rabbit plasma also failed to inhibit the reaction between RGH-I^{125} and its antibody.

The data presented here reinforce the concept of a common antigenic structure in pituitary and plasma growth hormone of several mammalian species, as well as the difference in antigenicity of primate growth hormones. These data agree with those reported by Hayashida and Contopoulos (1967) using double diffusion or immunoelectrophoresis, Ellis et al. (1968a) using micro-complement fixation or immunoelectrophoresis, Tashjian et al. (1968) by complement fixation, and Garcia and Geschwind (1968) by radioimmunoassay. A discordant note is found in that we were unable to show cross-reactivity with rabbit plasma. This could be the result of handling and age of the sample assayed as well as a characteristic of the antiserum used since it has been shown that complete or partial cross-reaction are functions of the antiserum and method employed (Hayashida and Contopoulos, 1967). To our knowledge, this is the first report of cross-reactivity between hamster or gerbil growth hormone and rat pituitary GH using a double antibody

radioimmunoassay.

These cross-reactions should make it possible to measure GH in guinea pigs, hamsters, mice, gerbils, dogs and cats utilizing the monkey antibody to rat growth hormone. However, since neither purified GH preparations of these species nor pure preparations of other hormones were available to test specificity, biological correspondence should be assessed before using the assay. The latter was not within the scope of this study.

III. Difference Between Plasma and Serum RGH Levels

A. Objectives

Preliminary results obtained while establishing dose-response curves for plasma suggested possible differences in the levels of GH between plasma and serum samples. It was the purpose of this experiment to verify the preliminary observations and ascertain the degree of this difference in serum and plasma samples of the same origin.

B. Procedures

Male Sprague-Dawley rats were anesthetized with ether and bled from the abdominal aorta. The blood collected from each animal was divided equally into two large centrifuge tubes one of which contained 0.1 ml of a 10mg% solution of Na-heparin per ml of blood, while the other contained 0.1 ml of physiological saline per ml of blood. The blood from several animals was pooled. The heparinized tubes were immediately centrifuged and the plasma separated from the red blood cells by pipette. Serum was obtained by allowing the clot to form and retract for 24 hours at $4^{o}C$ at the end of which the tubes were also centrifuged and the serum decanted. The level of GH was determined in triplicate volumes of 12, 25, 50, 100, 150, 200, 300, 400 and 500 ul of serum or plasma.

C. Results

Since the possibility existed that the second antibody did not react at the same rate in plasma and serum

the second antibody was incubated for 1, 2, 3 or 5 days. The results are shown in Table 3. For any given length of second antibody incubation the levels of GH measured in plasma were significantly higher than those measured in serum. No difference was found within the plasma samples or within the serum samples with the various lengths of incubation.

From the above results it was concluded that the rate of reaction of the second antibody was not affected by time in serum or plasma. In attempting to explain the large differences observed several questions were posed: a) is the percent binding affected in different ways by serum and plasma, b) does Na-heparin cross-react with the anti-rat GH serum, c) does Na-heparin synergize the levels of GH in plasma, d) when does the loss of activity take place, e) is this loss due to aggregation or degradation of the GH molecule, and f) what causes it.

To answer some of these questions several tests were done. First, the effect of serum or plasma on the percent binding was determined by placing 100 ul of serum or plasma of hypophysectomized male rats into RIA tubes. Both the non-specific binding and the percent binding were determined at 1, 2, 3 or 5 days of second antibody incubation. Control tubes had no plasma or serum. The results can be seen in Figure 15. Neither serum nor plasma altered the percent binding of the first antibody at any time of incubation. The controls are represented by the solid

TABLE 3.--Difference between serum and plasma GH levels

Days of Incubation[1]	Male Serum mug GH/ml (Mean±S.E.)	Male Plasma mug GH/ml (Mean±S.E.)
1	35.5±1.4	111.5±6.4*
2	36.1±2.3	111.3±7.9*
3	36.5±1.2	114.1±8.9*
5	36.9±0.8	112.5±8.8*

*$p < 0.01$

[1]Length of 2nd antibody in days

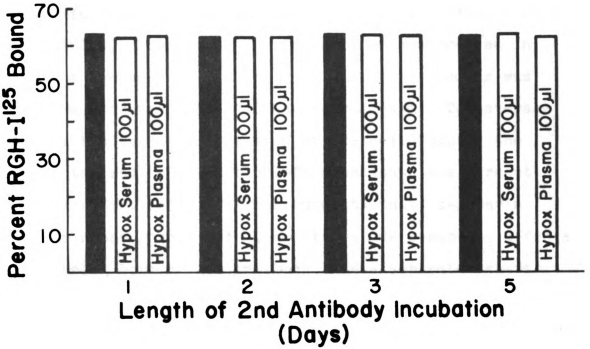


Figure 15. Effect of hypophysectomized male rat serum or plasma on percent binding. RGH-I^{125} bound is expressed in terms of percent of total radioactivity. Binding obtained in the absence of hypophysectomized plasma or serum is shown by the solid bars. The addition of hypophysectomized plasma or serum did not alter percent binding.

bars. In all cases the non-specific binding was the same.

Next, the possible cross-reaction between Na-heparin and the anti-rat GH serum, as well as its effect on the antibody and the labeled hormone was tested by placing different volumes (0.1, 0.25, 0.5, 1, 2, 4, 8, 16, 31, 62.5, 125, 200 and 250 ul) of a 10 mg% Na-heparin solution into RIA tubes. Triplicates of each volume were used. The results, shown in Figure 16, have been expressed in terms of the percent binding. No change in binding was observed with any amount of Na-heparin used. The average percent binding is represented by the solid bar on the right; the solid bar on the left represents the percent binding of the control. This suggests that Na-heparin neither cross-reacts with the anti-rat GH serum nor affects the labeled hormone and antibody. In a separate experiment where 100 ul of the Na-heparin solution were placed in tubes containing the RGH standards, no difference was found between the control and heparinized standards.

To determine whether Na-heparin synergized the levels of GH in plasma and whether a decrease in GH activity occurred with time, a rate of recovery test was done in hypophysectomized plasma and serum to which known amounts of RGH had been added. The radioimmunoassay tubes were set up as described for standards except that 100 ul of either serum or plasma from hypophysectomized male rats were added per tube. Four, 8, 16, 31, 62.5 mug of RGH were added per tube. Each amount was determined in

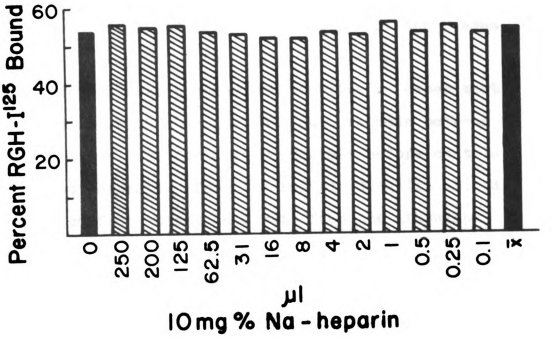


Figure 16. Effect of Na-heparin on percent binding. RGH-I^{125} bound is expressed in terms of percent of total radioactivity. The solid bar on the left shows binding obtained in the absence of Na-heparin; the solid bar on the right shows the average binding obtained upon the addition of different volumes of a 10 mg% Na-heparin solution/tube. Na-heparin did not alter percent binding.

duplicate. The second antibody was incubated for 1, 3 and 5 days. It can be seen in Table 4 that essentially all of the added RGH is recovered. Furthermore, Na-heparin does not synergize the level of GH in the plasma. No decrease in GH activity was observed with increasing length of time in the incubation of the second antibody. This suggested, therefore, that the disappearance of GH took place primarily before the separation of the plasma and serum from the red blood cells.

In order to test the above assumption an experiment was devised in which a comparison could be made between plasma and serum left for different times before centrifugation and separation from the red blood cells. Blood was collected from intact male rats from the abdominal aorta into two syringes: one containing 0.6 ml of a 10 mg% Na-heparin solution, the other containing physiological saline in the same volume. A volume of 5.4 ml of blood was withdrawn into each syringe to make a total volume of 6 ml. The 6 ml of heparinized or non-heparinized blood were divided into 4 groups of 3 tubes each (0.5 ml/tube). The 4 groups corresponded to 4 different times (0, 12, 24 and 48 hours). In each group one tube was for plasma or serum, a second tube for blood to which 20 mug of RGH had been added, and a third tube for plasma or serum to which 80 mug of RGH per ml were added after centrifugation. Blood was collected from 20 animals and pooled for each tube within each time group. All tubes were kept in

TABLE 4.--Recovery rates of RGH from hypophysectomized plasma or serum

mug RGH Added	mug RGH Recovered					
	1 day incubation		3 day incubation		5 day incubation	
	Serum*	Plasma*	Serum*	Plasma*	Serum*	Plasma*
125.0	130.0	125.0	128.0	126.5	125.0	120.0
62.5	68.0	60.0	64.3	61.8	63.2	62.3
31.0	33.0	35.0	32.5	33.0	31.0	33.0
16.0	15.0	17.0	15.7	16.0	15.8	17.0
8.0	7.6	9.3	7.7	8.3	8.2	8.5
4.0	4.1	3.3	3.9	3.9	4.1	3.8
Mean % recovered	101.7	102.3	100.3	102.9	100.8	101.6

*Average of 2 determinations

an ice bath during collection. The level of GH in the plasma or serum was determined in triplicates of three dilutions of 200, 100 and 50 ul per RIA tube. The results have been grouped as follows: IA: plasma or serum centrifuged at 0 time and incubated for 0, 12, 24 or 48 hours at 4°C; IB: plasma or serum centrifuged at the end of 0, 12, 24 or 48 hours of incubation; II: plasma or serum centrifuged at 0 time, 80 mug RGH per ml added, incubated for 0, 12, 24 or 48 hours; and III: heparinized or non-heparinized blood to which 40 mug RGH per ml were added, centrifuged at the end of 0, 12, 24 or 48 hours of incubation. For each group of serum or plasma the level at 0 time was regarded as 100%. The results are shown in Figure 17.

It can be seen that neither plasma nor serum centrifuged at 0 time (groups IA and II) produces significant decreases in GH after 12, 24 or 48 hours of incubation. On the other hand, centrifugation of heparinized and non-heparinized blood after 12, 24 or 48 hours of incubation at 4°C results in significant decreases in GH (groups IB and III) which increase with time. For any given time period less GH is recovered from the serum groups. No definite relationship could be established between the initial level of GH and the degree of inactivation. The actual level of GH in the serum centrifuged at 0 time was 74-81% of that found in the plasma centrifuged at 0 time. Fibrin formation was observed in the plasma samples centrifuged at 24 and 48 hours.

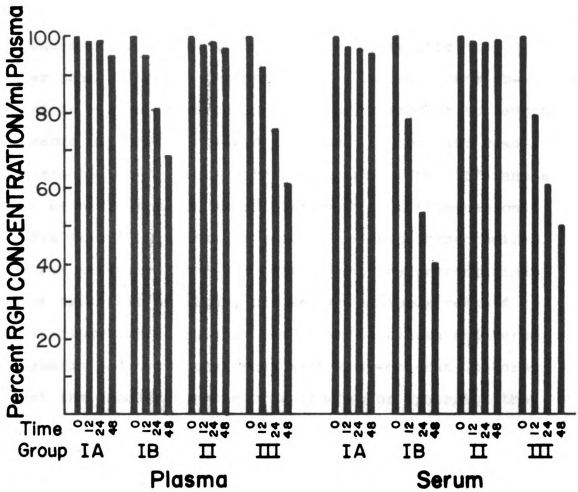


Figure 17. Effect of time on plasma and serum RGH levels. RGH concentration/ml plasma is expressed as percent of control, where control concentration is regarded as 100%. Groups IA= plasma or serum centrifuged at time 0 and incubated for 0,12,24 or 48 hours. Groups IB= plasma or serum centrifuged at 0,12,24 or 48 hours. Groups II and III received same treatment as IA and IB respectively, except that exogenous RGH was added.

D. Discussion

The levels of GH in serum and plasma samples of the same origin were measured by radioimmunoassay, varying the length of incubation time of the second antibody. In all cases plasma GH concentrations were significantly higher than serum GH concentrations. No change was observed in plasma or serum GH levels with respect to length of second antibody incubation, suggesting that the reaction reached equilibrium within 24 hours. The differences in GH were not the result of differential influences on the RIA itself by plasma or serum, i.e. hypophysectomized male rat serum or plasma did not affect the percent binding of the first antibody; the second antibody reached equilibrium within the same time span in plasma and serum; Na-heparin did not cross-react with the anti-rat GH serum, affect the labeled hormone or antibody, or synergize the levels of GH in plasma.

The recovery of exogenous RGH in plasma or serum from hypophysectomized male rats was about 100%, suggesting that the disappearance of immunologically active GH took place before the separation of the plasma or serum from the red blood cells and other blood components. A direct comparison of intact rat plasma and serum, with and without the addition of exogenous GH, centrifuged at the time of collection and incubated for 0, 12, 24 or 48 hours at $4^{o}C$, or incubated for the indicated times and then centrifuged, showed that the former resulted in almost no disappearance

of GH, while the latter resulted in significant decreases in GH concentration with time. The longer the interval, the greater the decrease. It was also observed that for any given time period less GH was recovered in the serum than in the plasma group; at 0 hour centrifugation time serum GH was 74-81% of plasma GH.

This experiment indicates a significant loss of immunologically active GH in serum and plasma when centrifugation takes place after a period of time. It does not indicate whether the loss in activity is due to aggregation or degradation of the GH molecule, nor the cause of the loss.

Somatotropin inactivation by streptokinase activated blood plasma or plasminogen has been reported by Mirsky et al. (1959 a,b), who observed that human plasmin solubilized about 30% of an I^{131} labeled bovine GH. Streptokinase activated human plasminogen or bovine plasmin have also been reported to result in partial hydrolysis of bovine GH (Ellis et al., 1968b). These authors suggested that a tissue peptidase present in crude preparations of pituitary somatotropin was identical with plasmin. Thus, incubation of a crude extract with traces of urokinase eliminated an autocatalytic lag observed in crude preparations which had been extracted for only ½ hour. Incubation of bovine or rat GH with plasmin resulted in fractions of 20,000 and 18,400 m.w. respectively. The conversion of these hormones was shown to be proportional to the plasmin concentration.

It has been shown that the immunological activity of the native and degraded forms of rat GH differ greatly although their biological activities remain about the same (Ellis et al., 1968c). Using micro-complement fixation, the degraded hormone fixed 15% as much complement as the native GH when an antibody to the native GH was used. On the other hand, the native hormone fixed less than 10% as much as the degraded GH when an anti-serum to the degraded moeity was used.

In view of these reports, and our observations concerning the differences in the degree of inactivation between plasma and serum as well as the time lag in inactivation observed in the plasma, it is concluded that immunological inactivation took place and that this inactivation was probably due to degradation of the hormone by factors involved in the process of coagulation. It is recommended, therefore, that plasma instead of serum samples be used to measure GH. The samples should be collected in an ice bath and centrifuged without delay.

Keeping in mind that the radioimmunoassay does not distinguish between degradation or aggregation of a molecule, it is not possible to rule out the loss of GH as a consequence of aggregation. Obviously, simple adsorption of GH to the fibrin strands will also produce similar results. Further studies must be done to clarify the nature of the GH loss observed.

IV. GH as a Function of Age in Male and Female Rats: the Estrous Cycle

A. Objectives

The relationship between age and pituitary GH in the rat has been studied using biological (Contopoulos and Simpson, 1957a; Solomon and Greep, 1958; Bowman, 1961) and radioimmunological (Birge et al., 1967a; Daughaday et al., 1968; Garcia and Geschwind, 1968; Burek and Frohman, 1970) assay methods, yielding differing results (see Review of Literature). In none of the above reports was the estrous cycle considered when relating GH and age in female rats. Furthermore, no report has dealt with the levels of GH in plasma or serum with respect to age in the rat. It was the purpose of this experiment to measure the plasma and pituitary levels of GH in male and female rats of different ages, and in the different stages of the estrous cycle.

B. Procedures

Male and female Sprague-Dawley rats of different ages were obtained from Spartan Research Animals (Haslett, Michigan) and housed in a temperature controlled room (75 $\pm$ 1°F) with automatically controlled lighting (14 hours light daily). A group of female rats 180 days old was subjected to constant illumination (C.L.) for three weeks.

Individual blood samples were taken under ether anesthesia via heart puncture into syringes containing 0.1 ml of a 100 mg% solution of Na-heparin/ml of blood withdrawn. The blood samples were kept in an ice bath during the

collection and centrifuged immediately after the collection at 2200 rpm for 20 minutes. The plasma was separated by pipette and stored at -20°C until assayed. Within an hour after the blood was collected they were killed by guillotine and their pituitaries removed, weighed individually and homogenized in PBS. The individual homogenates were also stored at -20°C until assayed.

Blood samples from male rats were collected between 10:00 and 12:00 A.M. Vaginal smears were taken daily between 8:00 and 10:00 A.M. and the female rats bled between 12:00 and 2:00 P.M. of the same day. Plasma and pituitary GH was assayed by radioimmunoassay (RIA) at 3 different concentrations for each individual sample. Ten animals were used per age group or stage of cycle within a particular age group. The results were analyzed by one way analysis of variance followed by the multiple range test of Duncan (Bliss, 1967). All results are expressed in terms of the NIAMD-RGH-RP-1 standard.

C. Results

Pituitary and plasma GH was measured in male rats of 23, 33, 43, 64, 84, 104 and 120 days of age. Plasma GH was also measured in a group of male rats of about 240 days of age. Pituitary GH as a function of age in the male rat is shown in Table 5; Figure 18 shows the levels of plasma GH in males of different ages.

The concentration and content of pituitary GH in the male rat increased significantly with age to about 84 days.

TABLE 5.--Pituitary GH as a function of age in the male rat

Age (Days)	Body wt. (g)	A.P. wt. (mg)	ug RGH/ A.P.	ug RGH/ mg A.P.
23	62.9± 1.4	1.87±0.05	37.6± 3.1	20.1±2.1
33	113.6± 0.7	3.28±0.15	84.9± 2.9	25.9±1.7
43	169.3± 1.7	4.63±0.18	155.6±12.1	33.6±2.4
64	315.5± 6.1	7.58±0.31	372.9±19.2	49.2±3.3
84	394.7± 4.6	9.07±0.28	542.8±13.4	59.8±4.0
104	434.8± 9.0	8.81±0.21	569.1±20.2	64.6±3.9
120	501.2±22.4	10.18±0.38	637.3±27.4	62.6±6.3

All values expressed as mean ± S.E.

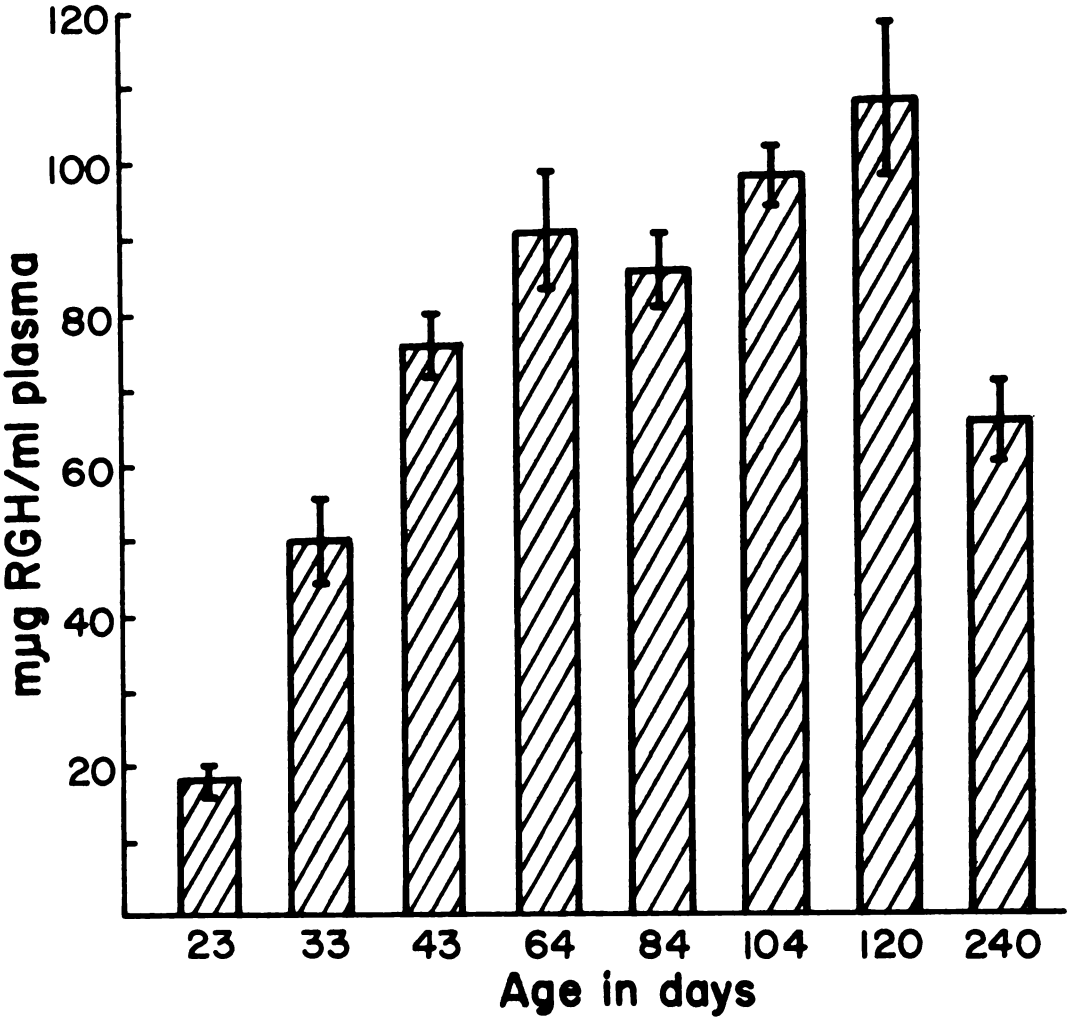


Figure 18. Plasma GH as a function of age in the male rat.

No further increase in concentration was observed with advancing age, although a significant increase in content was further observed at 120 days. Equally significant increases were found in plasma GH from 23 to 64 day old rats. No significant difference was found in plasma GH of rats 64, 84, 104 and 120 days of age. At 240 days the concentration of plasma GH was significantly reduced to levels similar to those found in rats of about 33 to 43 days of age.

Pituitary and plasma GH was also determined in female rats ranging in age from 21 to 560 days old. The rats were divided into the following age groups: 21, 28, 34 in which the vaginal canal was closed (vg. canal cld.), 36 and 43 with the vaginal canal open. Rats 60 and 120 days old were used in determining the levels of GH in the different stages of the estrous cycle; a 180 day old group was subjected to constant illumination to elicit constant estrous (c. estrous, C.L.), while a 560 day old group was found to have estrous-proestrus types of smears exclusively. The results of this experiment are shown in Table 6 and Figure 19.

A significant increase in pituitary content and concentration of GH took place from 21 to 60 days of age. The concentration of GH did not change significantly from 60 to 180 days, while a significant decrease was observed in the 560 day old group. Although a small increase in concentration was observed during proestrus, the levels of GH did not differ significantly from those found in estrus, metestrus, or diestrus. In contrast, the pituitary content of GH

TABLE 6.--Pituitary GH as a function of age in the female rat: the estrous cycle

Age (Days)	Stage of Cycle	Body wt. (g)	A.P. wt. (mg)	ug RGH/ A.P.	ug RGH/ mg A.P.
21	---	60.0± 0.6	2.31±0.07	53.6± 4.7	23.2±1.7
28	---	85.9± 0.6	2.93±0.52	85.8± 7.4	29.3±2.3
34	vg. canal cld.	108.3± 0.8	3.21±0.12	89.2± 9.3	27.8±2.1
36	vg. canal open	122.1± 1.8	6.83±0.27	206.3±14.5	30.2±2.9
43	vg. canal open	146.2± 2.9	6.19±0.35	237.7±17.0	38.4±3.3
60	Proestrus	238.7± 7.2	10.66±0.40	625.7±29.3	58.7±5.2
	Estrus	224.4± 5.6	10.91±0.47	558.6±35.2	51.2±4.8
	Metestrus	230.6± 3.8	10.53±0.28	563.4±32.1	53.5±4.7
	Diestrus	224.4± 4.3	10.65±0.52	535.7±27.3	50.3±5.0
120	Proestrus	264.0± 4.0	11.03±0.25	662.9±35.3	60.1±5.4
	Estrus	259.0± 2.7	12.68±0.37	680.9±31.0	53.7±4.8
	Metestrus	260.0± 5.3	12.25±0.42	674.9±37.1	55.1±3.9
	Diestrus	255.0± 6.1	11.83±0.35	618.7±30.2	52.3±4.9
180	C. Estrus (C.L.)	274.5± 9.0	16.27±0.85	863.9±42.3	53.1±6.3
560	Est-Proestrus	340.5±13.3	18.2 ±0.87	697.1±45.2	38.3±9.3

All values expressed as mean ± S.E.

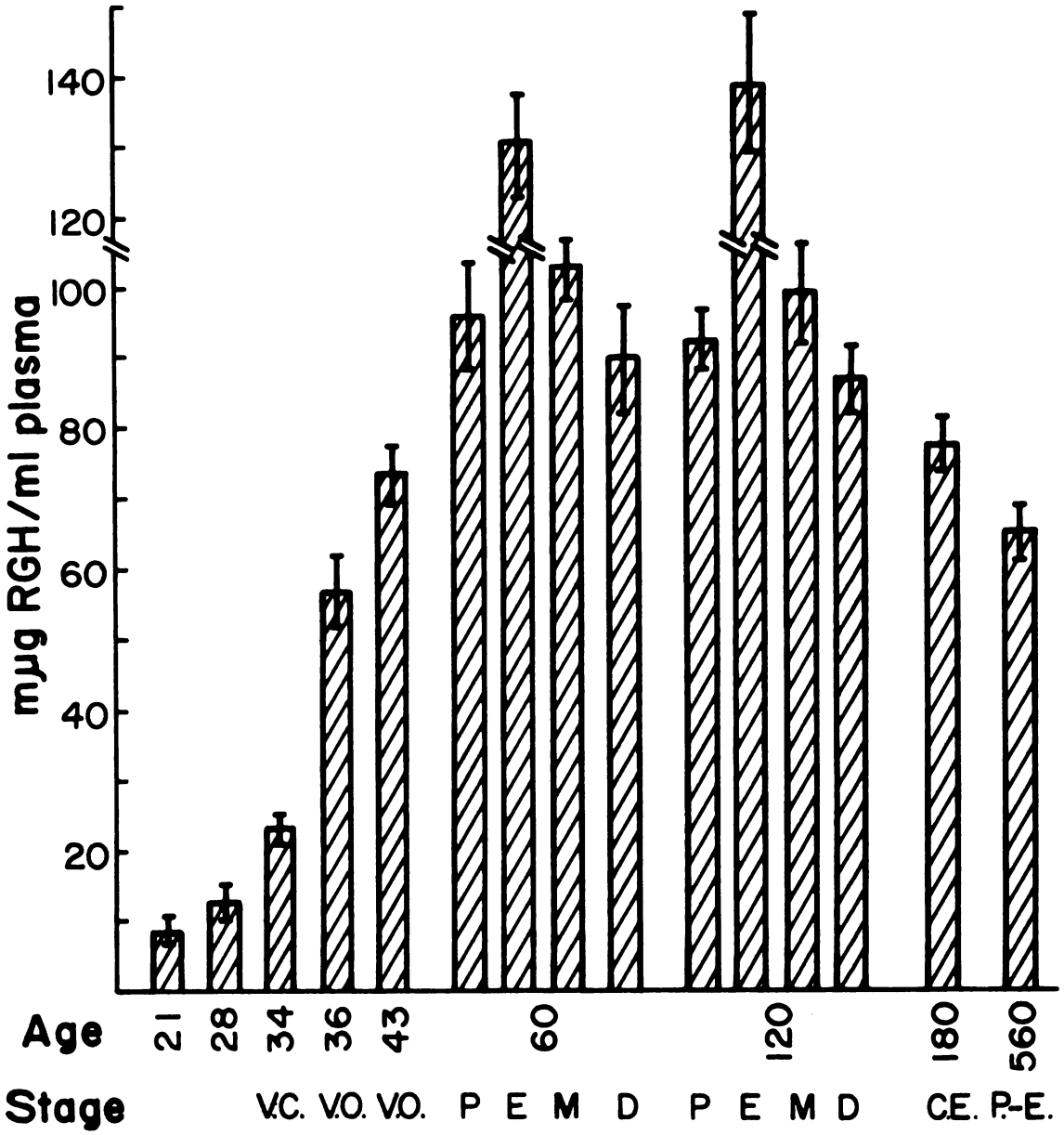


Figure 19. Plasma GH as a function of age in the female rat: the estrous cycle.
Age is expressed in days. Stages are: V.C.= vaginal canal closed, V.O.= vaginal canal opened, P= proestrus, E= estrus, M= metestrus, D= diestrus, C.E.= constant estrus and P.-E.= proestrous-estrus.

increased to about 180 days, with a significant decrease occurring at 560 days of age. No change in content was observed with the different stages of the cycle.

Plasma concentration of GH rose significantly from 21 to 60 days of age, with the sharpest increase taking place from 34 days with a closed vaginal canal to 36 days with the vaginal canal opened. In normally cycling female rats the mean plasma GH concentration in estrus was significantly higher than found in proestrus, metestrus or diestrus, when no differences were observed. The levels of GH decreased significantly in constant estrous rats of 180 days and more so at 560 days of age.

A direct comparison of GH levels in the pituitary and plasma between male and female rats of approximately the same ages failed to show any significant differences, except for the peak in plasma GH observed during estrus.

In view of the results obtained in female rats an experiment was designed to test the possible influence of estrogen on the increased levels of plasma GH observed after canalization and during estrus of each cycle. Female rats 180-200g were divided into the following groups: a) intact control, b) unilateral ovariectomy, c) bilateral ovariectomy, d) bilateral ovariectomy plus 0.2 ml corn oil daily, and e) bilateral ovariectomy plus 5 ug of estradiol benzoate (E.B.) in 0.2 ml corn oil daily. The animals were treated for 2 weeks, at the end of which time plasma and pituitaries were collected and assayed as described in "Procedures".

It can be seen in Figure 20 that unilateral ovariectomy did not alter anterior pituitary concentration. On the other hand, bilateral ovariectomy with or without corn oil resulted in increased pituitary concentration as well as in a decrease in plasma GH. Conversely, the administration of estradiol benzoate to bilateral ovariectomized rats resulted in a decrease in pituitary GH with a concomitant increase in plasma GH levels.

D. Discussion

The relationship between age and GH was studied in male and female rats ranging in age from 21 to 560 days old. In addition, plasma and pituitary GH was measured in the different stages of the estrous cycle in rats of two different ages. The concentration of pituitary GH showed progressive increases from 21 to 60 or 23 to 84 days in female and male rats respectively. Concomitant with this increase in concentration was an increase in content which continued to 180 days in the female and 120 days in the male. The increase in content, in view of the plateau in concentration, can be attributed to the further increase in pituitary size. A decrease in both content and concentration was found in female rats 560 days old. No significant change in pituitary content or concentration was observed with the different stages of the cycle, although a small rise in concentration was measured during proestrus.

Together with the increase in pituitary concentration observed during the first 8-12 weeks of age, a similar

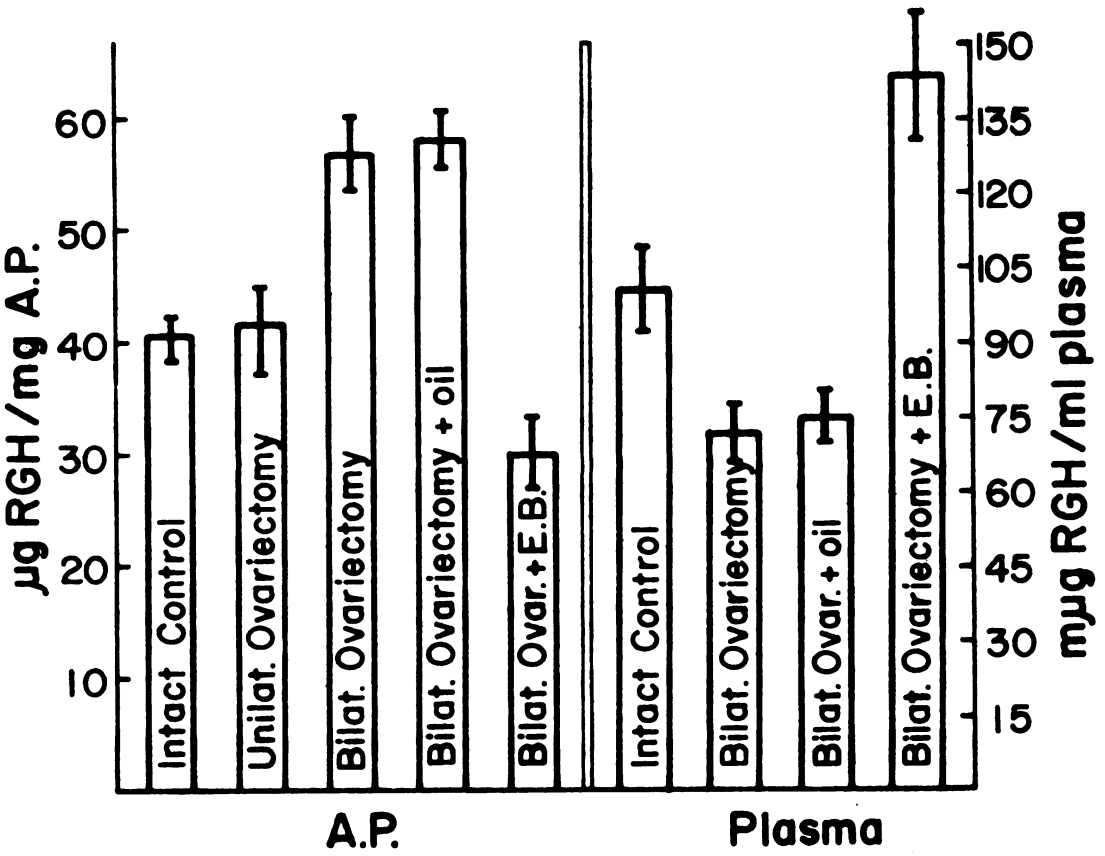


Figure 20. Pituitary and plasma RGH after ovariectomy and estradiol benzoate treatment.

increase was observed for plasma GH. The level of GH in plasma rose significantly in both male and females to 60-64 days of age. Plasma GH remained elevated up to 120 days, with a decline in concentration observed at 180 days in females and at 240 days in males. A further decline was observed at 560 days of age in female rats showing estrous-proestrus types of vaginal cytology. In female rats with normal estrous cycles plasma GH was significantly elevated in estrus. No difference was found in proestrus, metestrus or diestrus. For each stage of the cycle, the levels found at 60 days of age did not differ from those found at 120 days of age.

The data presented here regarding pituitary GH is in basic agreement with that of Garcia and Geschwind (1968), who measured pituitary GH concentration in male and female rats from 5 to 75 days of age, and Burek and Frohman (1970), who used rats of different weights ranging from 61 to 485 grams. Our results seem to differ from those of Birge et al.(1967a) who reported that male pituitary GH concentration continues to increase to old age, whereas female pituitary concentration plateaued at maturity. No difference in content and concentration between males and females was found up to eight weeks of age, after which pituitary content and concentration in males were significantly higher than in females. It is of interest to note, however, that these authors found little or no increase in the weight of the pituitary gland in male rats after 49 days of age. Upon substituting their reported pituitary weights with those

reported by others and our own for animals of the same age or weight, little or no difference in concentration is found between male and female rats. Furthermore, when the substitution is carried out it appears that male pituitary concentration also plateaus between 63 and 77 days of age. If their results had been given by these criteria, we would be in total agreement.

The reports cited above have all utilized radioimmunological assays. With biological assay methods, Solomon and Greep (1958) and Bowman (1961) reported increases in content but not concentration of GH in the pituitary of female and male rats 10 to 630 days old respectively. Upon observation of their data, however, it appears that increases in concentration were observed up to about 6-9 weeks of age, but their significance was discounted.

This is the first study dealing with plasma or serum GH levels with respect to age in rats, and during the estrous cycle in females. Our results show a steady increase in plasma GH from 21 to about 64 days of age in both male and female rats. In addition, female rats show a significant elevation during estrus. If one starts with the premise that GH is indeed required for growth, and since the most rapid rate of body growth takes place during that time when both pituitary and plasma GH are lowest, then consideration must be given to the ability of young rat pituitaries to synthesize and release GH and to the rate at which the body utilizes it. Of course, there is also the possibility

that during the most rapid growth phase of life in rats, other factors may be more important for body growth than GH. It only needs to be mentioned that removal of fetal pituitaries (Jost, 1947) in rabbits does not reduce birth weight of the young. Also, rats hypophysectomized early in life continue to grow up to about 30 days of age (Walker et al., 1952).

Burek and Frohman (1970) have recently reported that pituitaries from adult male rats were able to synthesize more GH than pituitaries from young male adult rats, and the latter synthesize more GH than pituitaries from weanling rats in vitro. If one assumes that rate of body growth can be used as an index of utilization rate, then these results could be interpreted as reflecting a low synthesis rate with almost all of the GH released and utilized by the body, thus maintaining the relatively low levels in plasma and pituitary GH observed during this period of rapid growth. It should be pointed out that these results differ from those reported in the human by Greenwood et al. (1946a,b) in whom the highest plasma HGH levels were found in the fetus and at parturition, subsequently declining but remaining higher in children than in adults. In view of the report by Gershberg (1957) in which he found no difference in pituitary HGH concentration among fetal, adolescent and mature male pituitaries, these plasma concentrations may reflect differences between human and rat pituitaries in their ability to synthesize

GH at different ages.

Our observation that ovariectomy increased while estradiol benzoate decreased pituitary GH concentration confirms the previous report of Jones et al. (1965) and Birge et al. (1967a). We have extended these experiments to include the levels of plasma GH and found that ovariectomy decreases while estradiol benzoate administration increases the levels of plasma GH. Similar results have been reported after the administration of estrogen-progestin to humans (Garcia et al., 1967). Is estrogen, therefore, responsible for the elevation of GH after canalization of the vaginal opening and at estrus of each cycle, or does GH simply follow the increased levels of prolactin, FSH and LH observed during the afternoon of proestrus? If estrogen is responsible, how does it produce this elevation and what is its significance?

Birge et al. (1967b) reported that diethylstilbesterol caused suppression of GH release from pituitaries in vitro. It remains to be demonstrated that this is a physiological and not a pharmacological effect. Does estradiol benzoate also interfere with the synthesis and release of GH in vivo? If such were the case, and in view of the peripheral antagonism between estrogen and GH (Josimovich et al., 1967; Roth et al., 1968) it could be argued that the increase in plasma GH is the result of peripheral inhibition by estrogens which reduce the amount of GH utilized by the body per unit time. However, it is also possible that estrogen increases hypothalamic GH-RF or directly stimulates pituitary release.

The data presented here strongly indicate the need for caution in the interpretation of results based on content or concentration of hormones in plasma or pituitary tissue. The need for such caution assumes special importance when dealing with GH since there is no one tissue which can be called a "target organ" for GH to provide an indirect parameter for utilization rates. The results suggest that there may be differences in the rate of utilization and secretion of the hormone between very young, adult and old animals. The physiological significance of the increased plasma GH at estrus or after estradiol benzoate administration may be clarified in a future study on a) the metabolic clearance and secretion rates of GH in rats as influenced by estrogen and the different stages of the estrous cycle, and b) the effects of estrogen on hypothalamic GH-RF and directly on pituitary GH release.

V. Plasma and Pituitary Concentration, Metabolic Clearance Rate (MCR) and Secretion Rate (SR) of GH in the Male Rat as Influenced by Castration, Testosterone Propionate (TP), Thyroidectomy and Na-thyroxine (T_4).

A. Objectives

It has been known for some time that hypothyroidism in children results in a condition known as cretinism, when growth is greatly impaired. In rats, thyroidectomy results in degranulation of pituitary acidophiles, a decrease in growth rate, and a decrease in pituitary GH and hypothalamic growth hormone releasing factor (GH-RF). Thyroxine therapy, on the other hand, increases pituitary GH (Purves and Griesbach, 1946; Koneff et al., 1949; Contopoulos et al., 1958; Knigge et al., 1958; Solomon and Greep, 1959; Schooley et al., 1966; Daughaday et al., 1968) and hypothalamic GH-RF (Meites and Fiel, 1967).

Androgens in small doses stimulate growth, increase pituitary GH in intact or castrated rats and increase pituitary GH in female rats. Castration, conversely, reduces the amount of GH in the pituitary of males (Rubinstein and Solomon, 1941; Birge et al., 1967a; Daughaday et al., 1968; Kurcz et al., 1969).

In view of the marked influence of thyroxine and to a lesser extent androgen, on growth and pituitary concentration of GH it was of interest to study the effects of these hormones on the production and metabolic clearance rates of GH in the rat.

B. Procedures

Male Sprague-Dawley rats weighing 280-320 g were obtained from Spartan Research Animals (Haslett, Michigan). The animals were divided into five groups: a) surgically thyroidectomized, b) Na-thyroxine treated, c) castrated, d) testosterone propionate treated, e) intact controls. All animals were fed and watered ad libitum. The drinking water of the thyroidectomized group was supplemented with a solution of 2% Ca-gluconate. L-Na-thyroxine (T_4) and testosterone propionate (TP) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Thyroxine was dissolved in saline and injected subcutaneously at a dose of 10 ug/100 g body weight/day. Testosterone propionate (TP) was dissolved in corn oil and injected subcutaneously at a dose of 200 ug/100 g body weight/day. Since we were unaware at the time of the inactivation of GH in serum (see III. Difference Between Serum and Plasma RGH Levels) the animals were bled every other day (non-heparinized blood), and killed at the end of ten days of treatment. Twenty animals were used per group. GH in pituitary homogenates and serum was measured at 4 dilutions. In light of the inactivation of GH in serum only the pituitary values of this group have been kept and are presented in Figure 21.

The experiment was repeated with the following changes incorporated: a) 10 animals were used per group, b) blood was collected at 0, 5 and 10 days of treatment between 8:00

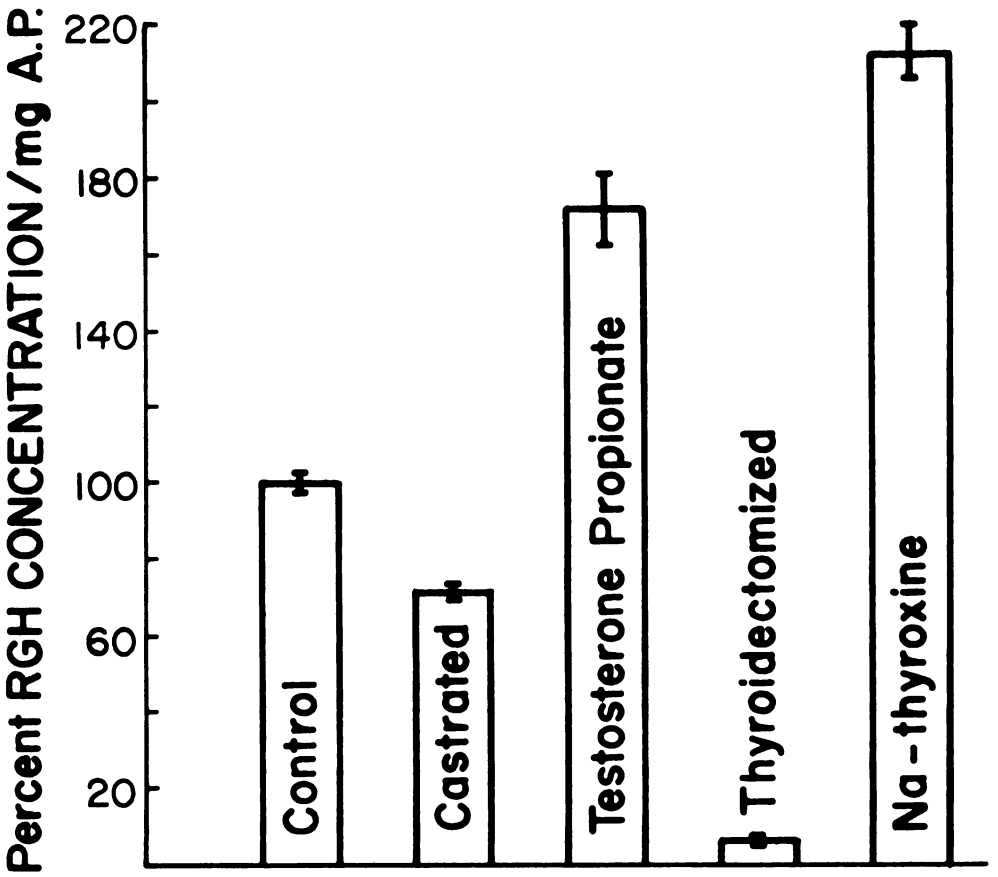


Figure 21. Effects of different treatments on anterior pituitary GH concentration in the male rat. RGH concentration/ mg A.P. is expressed as percent of control, where control concentration is regarded as 100%.

and 10:00 A.M., c) the blood was collected in syringes containing 0.1 ml of a 100 mg% Na-heparin solution/ml blood collected: a total of 1.5 ml was collected each time, d) all collections were done under light ether anesthesia via heart puncture and kept in an ice bath, e) one hour after the last bleeding, the animals were anesthetized with Na-pentobarbital (30 mg/kg) in preparation for the metabolic clearance rate study described below.

Metabolic clearance rate of a hormone may be studied by either constant infusion or single injection of hormone. Since it had been previously shown that the results were similar with either method (Tait, 1963; Kohler et al., 1968a; Coble et al., 1969; Frohman and Bernardis, 1970), and taking into consideration the number of animals used in this experiment, the single injection method was adopted. Following Na-pentobarbital anesthesia the animals were injected through the tail vein with 2.4×10^6 cpm of RGH-I^{125} in 1 ml of PBS. Seven-hundred and fifty ul volumes of blood were collected at 1, 5, 10, 15, 20, 30, 45, 60 and 90 minutes after the injection. The blood samples were placed in heparinized tubes and centrifuged as described previously.

For each time sample/animal two doses of plasma were allowed to react with a 1:1000 dilution of the DMD-1 anti-rat growth hormone serum, and two doses to count total radioactivity. The reaction was allowed to proceed for 72 hours and then the second antibody was added as in

a normal radioimmunoassay. The samples were centrifuged and counted 24 hours later. The remainder of the plasma aliquots were frozen and assayed 5 months later for endogenous growth hormone. The levels found at 5 months did not differ with respect to time sample/animal.

Since the disappearance curves for RGH-I^{125} appeared multiexponential, individual MCR's were calculated using the formula

$$MCR = \frac{\text{total immunoprecipitable RGH-}I^{125}\text{ injected}}{\int_0^\infty x' \cdot dt}$$

as described by Tait (1963) for a system of pools. The metabolic clearance rates obtained were pooled/group and plotted in semi-logarithmic paper as shown in Figure 22. Since in the steady state the amount of GH cleared is equal to the amount of GH secreted, secretion rate (SR) was arrived at by multiplying the mean group MCR times the plasma concentration of GH of the individual animals in each group. The plasma GH concentration on day 10 was used in this calculation.

The data were analyzed by "t" test for paired observations or analysis of variance followed by Duncan's multiple range test (Bliss, 1967).

C. Results

Figure 21 shows the effect of the various treatments on anterior pituitary concentration of GH. The results have been expressed as percent change from the intact

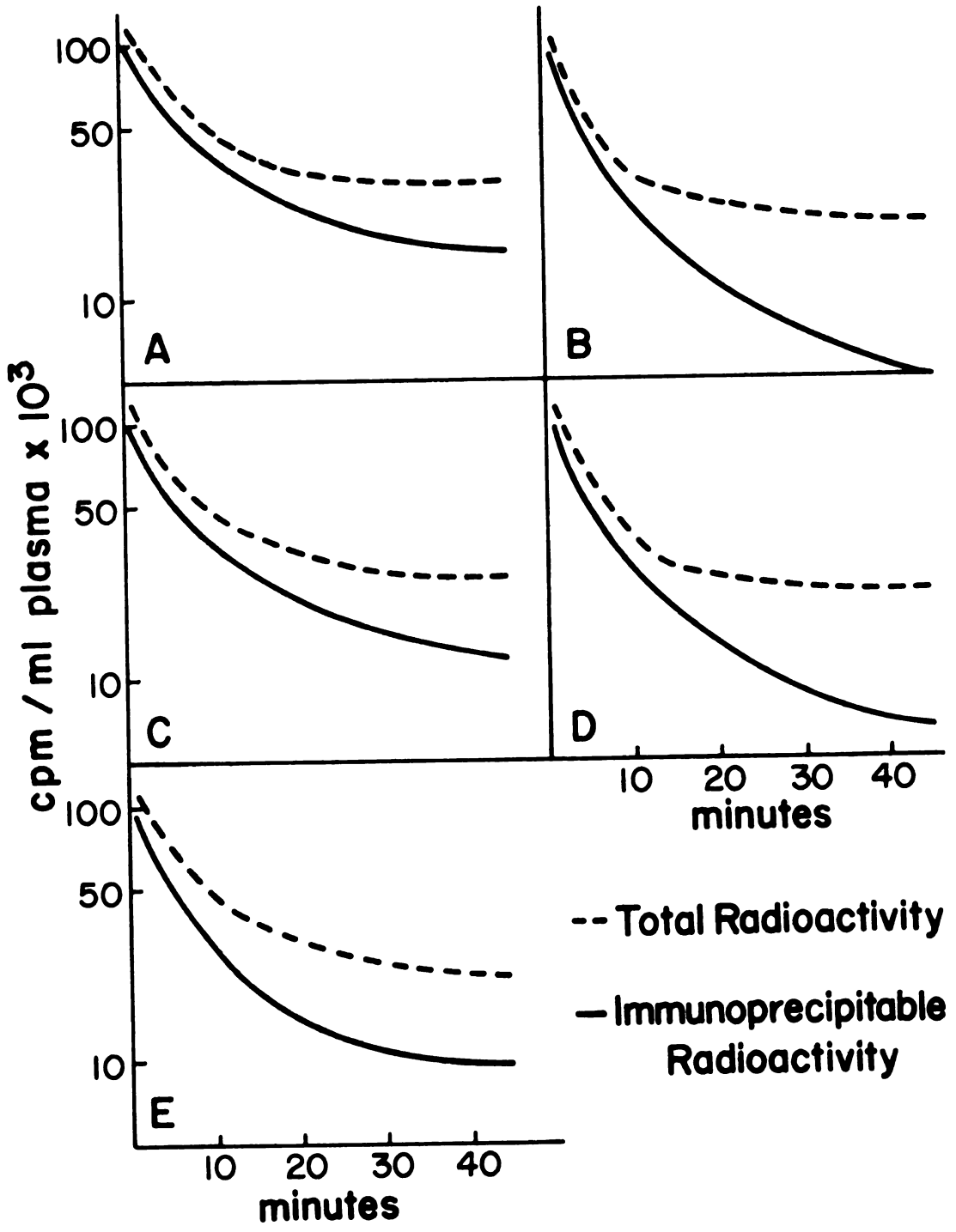


Figure 22. Disappearance of total radioactivity and immunoprecipitable radioactivity (RGH-I^{125}) from plasma following a single iv injection in: A) thyroidectomized, B) Na-thyroxine, C) castrated, D) testosterone propionate, E) intact rats.

control group. At the end of 10 days, castration produced a significant reduction in pituitary GH concentration, whereas daily subcutaneous injections of TP, at a dose of 200 ug/100 g of body weight, caused a significant increase. The castrated group had 72.3% while the TP treated group contained 172.1% of control values. The concentration of GH was very significantly reduced in thyroidectomized animals to only 6.4% of control levels. Daily subcutaneous injections of T_4 at a dose of 10 ug/100 g of body weight produced a significant rise in pituitary GH concentration, to 213.2% of control values. The magnitude of the changes produced by thyroidectomy and T_4 was significantly different from that elicited by castration and TP respectively.

Plasma GH was measured at 0, 5 and 10 days after treatment. The results are presented in Table 7 and expressed in terms of the NIAMD-RGH-RP-1 standard reference preparation. It can be seen that removal of 1.5 ml of blood at 0, 5 and 10 days did not affect the level of plasma GH. In contrast, plasma GH was significantly reduced 5 days after castration, remaining at this low level through day 10. No alteration in plasma GH was observed on day 5 in the TP treated group. On day 10, however, plasma GH was significantly elevated. Thyroidectomy caused a significant decrease in plasma GH. This decrease was already evident on day 5; no further change had taken place by day 10. The administration of T_4 produced a significant increase in plasma GH by day 5. In addition, a small increase was further

TABLE 7.--Plasma GH levels after castration, testosterone propionate, thyroidectomy and Na-thyroxine

Treatment and # of animals	Time of Collection (days) mug/ml±S.E.		
	0	5	10
Intact Controls (10)	109.1± 4.6	118.8± 3.3	116.0±13.2
Castrated (10)	107.6±11.7	71.4± 8.7*	64.2±13.7*
Testosterone Propionate (10)	107.4± 5.1	108.6± 5.3	166.0± 9.6*
Thyroidectomized (10)	118.1±13.2	49.3± 8.2*	49.6± 3.7*
Na-thyroxine (10)	112.8±17.2	156.7±10.3*	181.5± 9.9*

*Significant difference $p < 0.05$

observed on day 10, although the level was not significantly higher than that observed on day 5. The magnitude of the changes observed on day 10 produced by thyroidectomy and T_4 was not significantly different from that brought about by castration and TP respectively.

The metabolic clearance rate of GH was determined using the formula described previously. The plasma concentration of immunoprecipitable and total radioactivity was plotted versus time in linear coordinates for each animal in a group. The integral $\int x' \cdot dt$ was determined by extrapolating the curve to the visual intercept, which occurred generally between 100 to 140 minutes after the injection, and numerically measuring the area under the disappearance curve for the immunoreactive RGH-I^{125} for each animal. In order to do this it was assumed that there were no components of the curve slower than those already observed at 90 minutes. It may be noted that doubling the time for the visual intercept to 200-280 minutes resulted in a 6-8% decrease in the calculated MCR's.

The mean disappearance curves of total and immunoprecipitable radioactivity/group are shown on Figure 22 plotted to 45 minutes on semi-logarithmic paper. The use of a 1:1000 dilution of DMD-1 anti-rat growth hormone serum with 50 and 100 ul of plasma showed 80-90% of immunoprecipitable radioactivity present at 1 minute after the injection. At the end of 45 minutes, only 6-17% of the immunoprecipitable radioactivity remained in the plasma. In ascending order of

immunoprecipitable radioactivity remaining at 45 minutes were: T_4, TP, control, castrated and thyroidectomized. The disappearance of RGH-I^{125} injected appeared nearly linear for the first 10-15 minutes after which it assumed the characteristics of a multiexponential curve. In all cases the disappearance curve of total radioactivity followed that of immunoprecipitable radioactivity for most of the linear part, after which it deviated significantly, suggesting the addition and recirculation of metabolic degradation products of RGH-I^{125} in the plasma which were not immunoreactive.

The individual MCR's obtained as described above were pooled/group and analyzed statistically. The results are shown in Table 8. All half-lives ($t_{\frac{1}{2}}$) were calculated from the linear portion of the immunoprecipitable disappearance curve of each animal and pooled. The $t_{\frac{1}{2}}$ of intact controls was similar to that observed in castrated, TP treated and thyroidectomized rats ranging from 5.6 to 6.7 minutes. Na-thyroxine, however, produced a significant decrease in the $t_{\frac{1}{2}}$ of GH, averaging 4.8 minutes. The mean MCR calculated for intact control rats was 1.29 $\pm$ 0.08 ml/min and was not significantly different from that observed in castrated rats (1.19 $\pm$ 0.04 ml/min). Metabolic clearance rate of GH, however, was significantly altered in animals after thyroidectomy, TP or T_4. Both TP and T_4 raised MCR to 1.70 $\pm$ 0.09 and 1.86 $\pm$ 0.07 ml/min respectively. These levels were not significantly different

TABLE 8.--Metabolic clearance rate (MCR) and secretion rate (SR) of GH in rats after castration, testosterone propionate, thyroidectomy and Na-thyroxine.

Treatment and # of animals	$t_{\frac{1}{2}}$ min.	Plasma GH mug/ml	MCR ml/min	SR mug/min
Intact Controls (10)	6.7±0.9	116.0±13.2	1.29±0.08	149.6±17.1
Castrated (10)	6.2±0.3	64.2±13.7*	1.19±0.04	76.3±16.4*
Testosterone Propionate (10)	5.6±0.2	166.0± 9.6*	1.70±0.09*	282.3±16.3*
Thyroidectomized (10)	6.6±0.5	49.6± 3.7*	1.05±0.08*	52.1± 3.9*
Na-thyroxine (10)	4.8±0.3*	181.5± 9.9*	1.86±0.07*	337.6±18.5*

All results expressed as mean ± S.E.

*Significant difference $p < 0.05$

from each other. Thyroidectomy, on the other hand, produced a significant reduction in MCR (1.05 ± 0.08 ml/min).

Calculation of the secretory rate of GH was performed by multiplying mean group MCR x individual plasma GH concentration observed on day 10. The underlying assumption for this is that a steady state condition exists, where the amount of GH being irreversibly cleared is equal to the amount of GH entering the circulation per unit time. To ascertain that this condition existed during the MCR study the endogenous plasma GH levels were measured 5 months later. They were found not to differ with respect to time sample in individual animals. It may be calculated that the addition of 2.4×10^6 cpm of RGH-I^{125}, of specific activity 36.5 uc/ug RGH, represents an addition of 30-42 mug of rat growth hormone. In animals weighing an average of 300g, and assuming that blood volume represents 8-10% of body weight, it may be shown that such addition represents an increase of 1-1.5 mug/ml blood. This increase is insignificant with respect to endogenous plasma GH concentration. Then, by applying the Fick principle, MCR (ml/min) x plasma concentration (mug/ml) equals the amount of GH in mug cleared per unit time. In the steady state, this is equal to the rate at which GH enters the circulation from the pituitary: this is referred to as secretion rate (SR). The results may be seen on the right hand column in Table 8.

All of the treatments used produced significant changes in SR. Castration and thyroidectomy reduced SR to

approximately one half and one third respectively. Conversely, TP or T_4 treatment increased SR to 1.8 and 2.2 times higher than that observed for controls.

D. Discussion

The data presented here on the changes in pituitary GH as a result of thyroidectomy, thyroxine, castration or testosterone propionate corroborate previous observations by others (see A. Objectives). We have extended these observations to include plasma GH levels at 0, 5 and 10 days of treatment. Since these levels, however, may lead to erroneous conclusions regarding the utilization and secretion of GH, a study of the metabolic clearance and secretion rate was also included.

Two basic assumptions must be met to carry out a valid MCR study: a) the endogenous and tracer hormone must be cleared from the plasma in like manner, and b) all exponential components of a disappearance curve for a single injection must be taken into consideration in order to arrive at the proper integration of the plasma concentration of the tracer hormone with respect to time. A third assumption is necessary if secretion rates are calculated: the system must be in a steady state during the course of the MCR determination.

The first assumption was not tested directly in this study. Frohman and Bernardis (1970) showed, however, that the disappearance of RGH-I^{131} was not significantly different from that of rat pituitary extract following a single

intravenous injection. Similar observations have been reported for labeled and unlabeled human LH in monkeys (Kohler et al., 1968b), human LH-I^{131} and endogenous human LH after hypophysectomy in humans (Kohler et al., 1968a), and endogenous and exogenous HGH in man follwoing hypophysectomy (Refetoff and Sönksen, 1970). It would appear, therefore, that under most conditions endogenous and exogenous tracer hormone are cleared in identical manner.

Since at 90 minutes after the injection of RGH-I^{125} only 1-7.3% of the original immunoprecipitable radioactivity remained in the plasma, it was assumed that all components of the exponential disappearance curve had been included. Had this not been the case, however, it should be noted that doubling the time for the proposed intercept produced only a small change (6-8%) in the calculated MCR. Evidence in support of the third assumption was obtained from measurement of the levels of endogenous GH in all time samples after allowing 5 months ($2\frac{1}{2}$ half-lives) of radioactive decay to take place. No difference in plasma GH levels was found with respect to time in individual samples. As mentioned previously, the addition of 2.4×10^6 cpm of RGH-I^{125} with specific activity 36.5 uc/ug RGH did not significantly alter the concentration of endogenous plasma GH.

The multiexponential disappearance curve for RGH-I^{125} reported here agrees with the previous report of Frohman and Bernardis (1970) for RGH-I^{125}. This curvilinear disappearance has been reported for other pituitary hormones

(Kohler et al., 1968a,b; Coble et al., 1969; Yen et al., 1970), and it is believed to represent the distribution of hormone in more than one compartment (Kohler et al., 1968a). At least two well defined components are measurable: a rapid component constituting an almost linear decay and lasting about 15-20 minutes, and a slow component which lasts to about 100 minutes. The $t_{\frac{1}{2}}$ for GH calculated from the rapid component agrees with that reported by Schalch and Reichlin (1966) and Frohman and Bernardis (1970). Furthermore, the MCR for the control group reported in this paper agrees very closely with that reported by Frohman and Bernardis (1970) using a single intravenous injection of RGH-I^{131} or rat pituitary extract, or a constant infusion of RGH-I^{131}.

If one considers that rate of synthesis equals the amount stored plus the amount released per unit time, and that in a steady state secretion per unit time is equal to the amount of hormone cleared per unit time, then it follows that any simultaneous change in storage and secretion in the same direction of change must, therefore, represent alterations in the rate at wnich the hormone is being synthesized. In light of this, then, our results suggest that the lack of thyroxine produces a dramatic decrease in the rate at which GH is synthesized as well as released. Thyroxine therapy, on the other hand, greatly increases the rate at which GH is synthesized and released. It is obvious, in the context of our steady state assumption, that

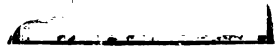
the presence or lack of thyroxin significantly modifies the amount of GH metabolized by the body. The report of Meites and Fiel (1967) makes it likely that these parameters are being affected through changes in the synthesis and release of GH-RF from the hypothalamus, which in turn modifies the synthesis and release of GH from the pituitary. These changes are then reflected, in this particular case, in the concentrations of plasma GH observed. Castration and testosterone propionate appear to produce changes similar to those mentioned for thyroidectomy and thyroxine therapy respectively. These changes, however, would seem to be lesser in magnitude than those involving thyroxine.

This is the first report showing differences in the rate of clearance of GH in different physiological conditions. The authors cited previously (Kohler et al., 1968a, b; Coble et al., 1969) did not find alterations in MCR of HLH and HFSH in pre- and post-menopausal women. Changes in MCR for steroids, however, have been previously reported (Tait, 1963). Does this reflect the lack of a specific target organ for GH action, or put in a different way, does it reflect the fact that GH is utilized by a larger number of cells than those hormones which have a specific target organ? This is unknown. It should be stressed, however, that clearance rate refers to the virtual volume of plasma from which a substance is cleared per unit time. It does not indicate the amount of that substance which is being cleared in that time. It seems logical to assume that the

total amount utilized reflects actual metabolism, while the volume from which that amount is extracted may or may not be more indicative of changes in circulation time, perfusion pressure, or other hydrostatic characteristics which may influence the amount extracted per ml of perfusate.

Given two groups of subjects with similar MCR's but whose plasma concentrations of a particular hormone differed 10-15 fold, and assuming a steady state during the study, would it be correct to conclude that their hormone metabolism did not differ? We think not. However, such a conclution was reached by Kohler et al. (1968a) and Coble et al. (1969) concerning the metabolism of LH and FSH in pre- and post-menopausal women. Our own results suggest that the lack of difference in MCR does not indicate a similarity in the amount of GH metabolized. Thus, although no difference in MCR's was found between control and castrated rats, the castrated animals were utilizing approximately half as much hormone as that used by the control animals. Similar logic may be used to argue the lack of metabolic difference in animals whose clearance differs significantly but whose total amount of hormone used per unit time is the same. This brings out another important point.

There is little doubt that the lack of thyroxine has a more profound influence on growth than the lack of testosterone propionate. Yet, our results indicate that an insignificant difference exists in the amount of GH utilized



by thyroidectomized and castrated animals, or by TP and T_4 treated animals. One must consider therefore, if there is a change in the efficiency of utilization, a change in the amount excreted by the kidney or differences in the amount of hormone degraded in the liver between these two groups.

Finally, the secretion rates reported in this experiment differ significantly from those reported by Frohman and Bernardis (1970). Since we are in agreement concerning MCR, it can be seen that the difference is due to the levels of GH measured in plasma. It is not possible to compare the absolute levels because different standard preparations were used for which no equivalency has been reported. Our pituitary and plasma measurements, however, are in agreement with those reported by Grindeland et al. (1970) and Takahashi and Kipnis (1970) who used the same standards as reported in this experiment.

GENERAL DISCUSSION

This thesis describes, among other things, the development of a radioimmunological assay for rat GH. This assay cannot be considered unique; others have been reported previously. It is, however, an assay that has been carefully characterized. More important, perhaps, is that it makes use of components that are now becoming widely available, and hopefully will help set a standard for the results obtained in different laboratories.

The advantages of a radioimmunoassay over previously available bioassay methods are obvious: it increases sensitivity by 2,000-4,000 times; it permits the measurement of hormone in small volumes of blood plasma or serum; it allows these measurements to be made in individual animals in a repetitive manner; and it has other advantages.

This thesis reinforces the concept of a common antigenic structure in the GH molecule of several mammalian species. It suggests that the radioimmunoassay for rat GH may be utilized in measuring pituitary and plasma GH in other animals including the guinea pig, hamster, mouse, gerbil, dog and cat. Evidence is also presented regarding the lability of GH and certain precautions are suggested to minimize alteration of the antigenic properties of the

molecule in plasma and serum.

Plasma and pituitary GH was measured in male and female rats of different ages, during the estrous cycle in females, and in male rats influenced by the lack or excess of thyroxine and testosterone propionate. The results indicate that in most conditions plasma concentration of GH is a good reflection of pituitary GH concentration. Thus, it was found that during early life (up to 60 days) progressive increases occurred in both pituitary and plasma concentration in male and female rats; castration and thyroidectomy decreased pituitary and plasma GH while testosterone propionate and thyroxine administration produced opposite results. An exception was found in that ovariectomy or estradiol benzoate treatment produced changes in pituitary and plasma GH which were opposite to each other.

As a consequence of these results several questions remain to be answered: is GH necessary for growth in young animals? If not, what is responsible for growth in the developing animal? When does GH become necessary? If it is necessary, is the rate at which young animals synthesize and utilize GH different from that in adult animals? What is the function of the elevated plasma GH level observed at estrus? How is this rise brought about? Perhaps most important, does plasma concentration of GH reflect the actual amounts utilized by the body?

The results obtained in the metabolic clearance and secretion rate study shed some light on the last question.

In this small sample of groups of mature animals it would appear that plasma GH concentration is a good reflection of the rate of GH secretion into the circulation. However, in view of the lack of difference between the groups affected by thyroxine and testosterone propionate respectively, and keeping in mind that the effects of thyroxine are more prominent on growth and general metabolism, caution should be exercised in equating the amount of GH secreted per minute to that actually utilized. Of course, it may be argued that factors such as efficiency of utilization, tissue sensitivity, and others may account for this lack of difference.

A final question may be asked with regard to the MCR and SR results. It has been demonstrated that high GH levels in the blood or implants of GH in the hypothalamus can inhibit GH secretion by the <u>in situ</u> pituitary. What are the effects of increased utilization of GH by body tissues on GH secretion? Is body utilization of GH a controlling influence on the synthesis and release of GH from the pituitary?

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APPENDIX

CURRICULUM VITAE

CURRICULUM VITAE

NAME: Elias Dickerman

DATE OF BIRTH: July 9, 1945

PLACE OF BIRTH: Concepcion, Chile

NATIONALITY: Chilean (permanent resident of U.S.A.)

MARITAL STATUS: Married

PRESENT ADDRESS: Department of Physiology, Michigan State University, East Lansing, Michigan 48823

EDUCATION:

Degree	Year	Institution	Major Field of Study
B.A.	1966	Brandeis University	Biology
M.S.	1968	Michigan State University	Physiology
Ph.D.	1971	Michigan State University	Physiology

HONORS, COMMITTEE AND SOCIETY MEMBERSHIPS:

(a) Wien International Scholar, Brandeis University, 1962-1966
(b) Graduated with Honors in Biology from Brandeis
(c) Ayerst-Squibb Travel Fellow of the Endocrine Society to the Third International Congress of Endocrinology, Mexico City, Mexico, June, 1968
(d) Elected Associate Member of Sigma Xi, 1968
(e) Elected as Full Member of Sigma Xi, 1969
(f) Member of the American Association of University Professors
(g) Chairman, Physiology Graduate Student Committee, Michigan State University, East Lansing, Michigan, 1968-1969
(h) Member, Research and Curriculum Committee of the College of Veterinary Medicine, Michigan State University, East Lansing, Michigan, 1968-1969

POSITIONS HELD:

(a) Peace Corps Language Instructor (Spanish), Brandeis University, Waltham, Massachusetts, June-Sept. 1966
(b) Adult Basic Education Teacher, Lansing, Michigan Public School System, Sept. 1968-Nov. 1969
(c) Teaching and Research Assistant, Department of Physiology, Michigan State University, East Lansing, Michigan, Sept. 1966-Dec. 1970

TALKS PRESENTED AT SCIENTIFIC MEETINGS:

Meeting	Date	Topic
Third International Congress of Endocrinology, Mexico City	1968	Effects of Starvation on Plasma GH in the Rat
54th Meeting of Federation of American Societies for Experimental Biology	1970	Radioimmunoassay of Rat Growth Hormone (GH)

RESEARCH PUBLICATIONS:

(a) Negro-Vilar, A., Dickerman, E., and Meites, J. Effects of Starvation on Pituitary FSH and Hypothalamic FSH-Releasing Factor (FSH-RF) in Male Rats. Federation Proceedings 27: 269, 1968.

(b) Negro-Vilar, A., Dickerman, E. and Meites, J. FSH-Releasing Factor Activity in Plasma of Rats After Hypophysectomy and Continuous Light. Endocrinology 82: 939-944, 1968.

(c) Negro-Vilar, A., Dickerman, E. and Meites, J. Effects of Continuous Light on Hypothalamic FSH-Releasing Factor and Pituitary FSH levels in Rats. Proc. Soc. Exp. Biol. Med. 127: 751-755, 1968.

(d) Dickerman, E., Negro-Vilar, A. and Meites, J. Effects of Starvation on Plasma GH in the Rat. Excerpta Medica 157: 73, 1968.

(e) Dickerman, E., Negro-Vilar, A. and Meites, J. Plasma FSH-RF in Hypophysectomized Rats after Treatment with Testosterone Propionate or Reserpine. Proceedings Internat. Union Physiological Sciences VII: 112, 1968.

(f) Negro-Vilar, A., Dickerman, E. and Meites, J. Removal of Plasma FSH-RF Activity in Hypophysectomized Rats by Testosterone Propionate or Reserpine. Endocrinology 83: 1349-1352, 1968.

(g) Dickerman, E., Negro-Vilar, A. and Meites, J. In Vitro Assay for Growth Hormone Releasing Factor (GH-RF). Neuroendocrinology 4:75-82, 1969.

(h) Dickerman, E., Negro-Vilar, A. and Meites, J. Effects of Starvation on Plasma GH Activity, Pituitary GH and GH-RF Levels in the Rat. Endocrinology 84:814-819, 1969.

(i) Dickerman, E. and Mack, W. N. Radioimmunoassay of Rat Growth Hormone (GH). Federation Proceedings 29:509,1970.

(j) Negro-Vilar, A., Dickerman, E. and Meites, J. Effects of Starvation on Hypothalamic FSH-RF and Pituitary FSH in Male Rats. Submitted for publication , Endocrinology.

(k) Dickerman, E., Koch, Y., Dickerman, S. and Meites, J. Radioimmunoassay of Rat Growth Hormone; Effects of Castration, Testosterone Propionate, Thyroidectomy and Thyroxine on pituitary GH Concentration. In preparation.

(l) Dickerman, E., Koch, Y., Dickerman, S. and Meites, J. Metabolic Clearance Rate of GH in the Rat: Effect of Different Physiological States. In preparation.

(m) Dickerman, E. and Meites, J. Cross-Reactivity of the NIAMD-A-RatGHS-1 Monkey Anti-Rat Growth Hormone Serum. In preparation.

(n) Dickerman, E., Koch, Y., Dickerman, S. and Meites, J. Studies on the Inactivation of GH by Plasma or Serum. In preparation.

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