REGULATION OF THYROTROPIN, PROLACTIN, AND GROWTH HORMONE SECRETION BY THYROTROPIN-RELEASING HORMONE AND THYROXINE IN CATTLE

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ABSTRACT

REGULATION OF THYROTROPIN, PROLACTIN, AND
GROWTH HORMONE SECRETION BY THYROTROPIN-RELEASING
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In vivo and *in vitro* studies were conducted to examine the effects of thyrotropin releasing hormone (TRH) and thyroxine on thyrotropin (TSH), prolactin and growth hormone (GH) secretion in cattle.

Twenty-four Holstein heifers were assigned to one of four treatment groups to be given: 1) thyroprotein for 6 days; 2) casein for 6 days; 3) thyroprotein for 27 days; or 4) casein for 27 days. Thyrotropin releasing hormone (33.3 μg/100 kg body weight) was injected at 1030 and 1330 hr on day 6 or 33 to heifers treated for 6 or 27 days, respectively.

Prior to TRH treatment, serum thyroxine averaged 133.8 and 82.3 ng/ml in heifers given thyroprotein or casein for 6 days and 24.0 and 82.2 ng/ml at 6 days after feeding thyroprotein or casein for 27 days. Six hr following TRH, average serum thyroxine concentration had increased 35 ng/ml in heifers given casein for 6 or 27 days, and 4.8 ng/ml in heifers given thyroprotein for 27 days, but remained unchanged in heifers fed thyroprotein for 6 days.

Basal serum thyrotropin (TSH), prolactin and growth hormone (GH) concentrations were not affected by thyroprotein treatments. Following

TRH, TSH increased to 26 ng/ml in heifers given casein for 6 or 27 days and to 20 ng/ml in heifers given thyroprotein for 27 days. However, TSH concentration was unchanged after TRH given on the sixth day of thyroprotein feeding. Serum prolactin and GH increased by 36 and 100 ng/ml, respectively, following injection of TRH and responses were independent of thyroprotein treatments.

Bovine anterior pituitaries were enzymatically dispersed then incubated for 5 days to establish primary cell cultures. Thyroxine and TRH were added to the media to examine their influence on pituitary hormone release. Cell cultures were incubated for 2 or 24 hr with .1 or 5 μg thyroxine/ml media prior to addition of TRH to the culture media. Chronic (24 hr) exposure of pituitary cells to 0.1 or 5 μg thyroxine/ml media reduced TSH 28 and 31% and prolactin secretion 20 and 42% relative to controls. In addition, acute (2 hr) exposure of cells to 5 μg thyroxine/ml media reduced basal secretion of prolactin 37%. Acute or chronic exposure of these cells to thyroxine did not alter basal GH release.

Concentration of TSH, prolactin and GH in media after 2 hr incubation with 10 ng TRH/ml averaged 248, 769 and 198 ng/ml; greater than the comparable averages for cultures not given TRH (149, 426 and 130 ng/ml). Acute (2 hr) exposure of cultures to 0.1 μg thyroxine/ml media increased ($P<0.01$) TRH-induced TSH release an average of 20% whereas 5 μg thyroxine/ml media for 24 hrs decreased ($P<0.01$) TSH release 35%. Acute or chronic exposure of cultures to 0.1 μg thyroxine/ml media increased TRH mediated prolactin release to 115 and 928 ng/ml whereas 5 μg thyroxine/ml media decreased TRH mediated prolactin release to 542 and 479 ng/ml. Growth hormone release after TRH was reduced 25 to 40% if cells were incubated acutely in .1 or 5 μg thyroxine/ml media or chronically in 5 μg thyroxine/ml media.

Effects of thyroxine on hormone release were not consistent between *in vivo* and *in vitro* experiments and are discussed in the text.

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INTRODUCTION

The endocrine system is vital for maintenance of proper growth, production and reproduction in animals. Increasing the efficiency of these traits is economically desirable in animals used for food production. Therefore, endocrinologists are attempting to find ways to manipulate hormone balance in animals in such a way as to economically attain these goals. Use of prostaglandin $F_{2\alpha}$ to synchronize ovulation and diethylstilbestrol to improve efficiency of feed utilization examplify benefits gained from this type of research.

The anterior pituitary gland secretes a number of hormones. The three pituitary hormones investigated in this thesis have been demonstrated to importantly influence animal growth and metabolism. Thyrotropin (TSH) stimulates the thyroid to secrete thyroxine and triiodo-thyronine which in turn alters systemic protein synthesis and basal metabolic rate. Growth hormone (GH) acts synergistically with thyroxine to stimulate body growth and maturation. Prolactin, although most frequently mentioned in association with initiation of lactation, also influences general body metabolism.

Recently scientists discovered that TSH-releasing hormone (TRH), which is secreted by the hypothalamus, is able to increase anterior pituitary secretion of TSH, prolactin and GH in many species including cattle. In addition, several studies indicate that TSH, prolactin and GH secretions are also modified by the thyroid hormones.

The purpose of this thesis is to describe the extent to which TRH and thyroxine influence secretion of TSH, prolactin and growth hormone *in vivo* and *in vitro*. Hopefully, the information provided by this thesis will contribute to understanding control of these important metabolic hormones and may lead to practical applications of this knowledge to problems of animal agriculture.

REVIEW OF LITERATURE

A. A General Description of the Cytology and Secretions of the Hypothalamo-Thyroidal Axis.

1. The Hypothalamus:

The hypothalamus consists of the medial-basal portion of the diencephalon. It is made up of a number of nuclear areas, both distinct and diffuse which receive and project nerve tracts to virtually every major segment of the central nervous system (reviewed by Knigge and Silverman, 1974). The hypothalamic vascular system is also extensive and includes a rich network of sinusoids in the median eminence - infundibular region. These capillary beds empty into the hypophysial portal vessels which transverse the infundibulum (Popa and Fielding, 1930) and transport venous blood from the hypothalamus to the anterior pituitary (Green and Harris, 1947).

Because of its extensive neural integration, the hypothalamus is capable of exerting major influences on the endocrine and autonomic nervous system. Only hypothalamic regulation of the endocrine system will be discussed here, with emphasis on control of the anterior pituitary and thyroid gland.

The hypothalamus secretes hormones via neurons that can be classified into two groups according to size and organization in the hypothalamus, i.e., magnocellular and parvicellular (Knigge and Silverman, 1974). Neurons comprising the magnocellular system are characterized by large cell bodies. These cell bodies or perikarya are located in the supraoptic

and paraventricular nuclei and synthesize antidiuretic hormone and oxytocin. Bound to neurophysins (carrier substances), antidiuretic hormone and oxytocin are transported from the perikarya along the neural axons to the posterior pituitary where they are stored until released. Neurons of the parvicellular neurosecretory system have small cell bodies that are diffusely located throughout the hypothalamus. These cells synthesize specific releasing or inhibiting hormones (Blackwell and Guillemin, 1973). These hormones are transported to and released into the hypophysial portal vessels where they have been measured using specific radioimmunoassays (Eskay et al., 1975).

Extracts of hypothalamic tissue and peptides of hypothalamic origin have been demonstrated to influence thyrotropin (TSH), prolactin, and growth hormone (GH) secretion. Thyrotropin releasing hormone is a tripeptide of hypothalamic origin (Burgus et al., 1969; Boler et al., 1969) that stimulates not only TSH release, but also prolactin and GH release in many species. In mammals, prolactin inhibiting (PIF) and releasing activity (PRF) have been demonstrated in hypothalamic extracts. While structural identification of these factors has not been completed, PIF may be norepinephrine or dopamine (Schally et al., 1976) and PRF activity may be due to the presence of TRH in hypothalamic extracts. Growth hormone is also regulated by an inhibiting (Somatostatin; SRIF) and releasing hormone (GH-RH). Somatostatin is a tetradecapeptide (Brazeau et al., 1972) and may influence prolactin and TSH secretion as well as GH secretion (Weeke et al., 1975; Drouin et al., 1976). Porcine GH-RH has also been isolated and identified as a decapeptide (Schally et al., 1971). Note that the initial concept that each hypophysiotropic hormone influences the secretion of only a single pituitary hormone appears outmoded.

2. The Anterior Pituitary:

The anterior pituitary, or adenohypophysis, is an endocrine gland attached to the ventral surface of the brain (hypothalamus) via the infundibular stalk. The hypophysial portal vessels tranverse the infundibulum from the hypothalamus to the anterior pituitary. The anterior pituitary is made up of three distinct areas. The pars tuberalis circumvents the infundibular stalk proximally, the pars intermedia is abutted against the distal portion of the infundibular stalk (posterior pituitary), and the pars distalis is ajoined to the pars intermedia rostrally. In most mammals, the pars distalis comprises the largest portion of the anterior pituitary. A residual cleft of Rathke's pouch, which is the embryological origin of the anterior pituitary, exists to varying degrees between the partes intermedia and distalis. In some mammalian species, the pars intermedia is absent, i.e., man, ape.

The cells of the anterior pituitary are arranged in loose cords surrounded by a rich network of sinusoids. The cellular population is heterogeneous within any tissue section as discerned by tinctorial stains. However, certain cell types predominate in different subsections of the bovine pituitary (Jubb and McEntee, 1955). By experimentally altering the secretion rate of individual hormones, attempts have been made to classify the various cell types. Until recently tinctorial stains were employed to differentiate cells. More recently, immunological stains have been used, lending functional significance to cell classifications. From these studies has evolved the hypothesis that only one or two hormones are secreted by a single cell type.

The anterior pituitary secretes a number of hormones. Those relevant to this thesis are TSH, prolactin and growth hormone. Thyrotropin, a

glycoprotein, is an example of a pituitary hormone which stimulates the activity of a specific target tissue, i.e., the thyroid gland. After TSH stimulation, the thyroid secretes thyroid hormones that exert a negative feedback on TSH secretion (Reichlin, 1966). Conversely, prolactin and GH which are proteins, effect many tissues and are not known to be regulated by any specific target tissue hormone feedback. Instead, the rate of hormone secretion is probably controlled by release stimulating and inhibiting factors. Both prolactin and GH appear capable of inhiting their own secretion via negative feedback on the hypothalamus (Clemens and Meites, 1968; MacLeod, 1966).

3. The Thyroid Gland:

The thyroid gland consists of two lateral lobes on either side of the upper trachea joined on the ventral surface by an isthmus. The main structural and functional unit of the thyroid is the follicle, which consists of cuboidal epithelial cells surrounding a central lumen. Stored within the lumen is thyroglobulin which is an iodinated glycoprotein used for thyroid hormone synthesis. Thyrotropin stimulates increased synthesis and secretion of the thyroid hormones, 3, 5, 3', 5'-tetraiodo-L-thyronine (thyroxine) and 3, 5, 3'-triiodo-L-thyronine (triiodo-thyronine). Peripheral to the follicles are the parafollicular cells which secrete thyrocalcitonin. Thyrocalcitonin functions in the regulation of serum calcium concentration.

Thyrotropin stimulation of the thyroid is associated with a number of cellular changes and has been extensively reviewed by Dumont (1971). A brief description of these changes follows. Immediately following administration of TSH, increases are noted in cellular cyclic 3', 5'-adenosine monophosphate (cyclic AMP) concentration, prostaglandin synthesis,

Na^+/K^+-dependent-adenosine triphosphatase (ATPase) activity and cell membrane depolarization. Each of these changes probably aid in transmitting the TSH signal into the cell.

After TSH stimulation, the rate at which iodide is taken up by thyroid cells is initially reduced. However, within a few hours iodide is concentrated by the thyroid at a rate greater than before TSH stimulation.

Thyrotropin increases the rate at which cellular iodide is incorporated into thyroglobulin (organification). This effect is probably secondary to an increase in the rate of glucose metabolism via the pentose monophosphate pathway which supplies NADPH needed for the organification process. Finally TSH stimulates endocytosis of thyroglobulin from the follicular lumen. The thyroglobulin is enzymatically degraded, liberating the thyroid hormones, which are discharged into the intracellular fluid and then pass to the blood.

Duration of TSH stimulation determines to what extent thyroid histology is altered. Acute TSH exposure increases thyroid blood flow rate, while chronic TSH induces thyroidal hypertrophy and hyperplasia. Many of the TSH-induced effects are independent of each other and some appear independent of increased cellular cyclic AMP concentration. This suggests that the TSH induces a second messenger(s) that has multiple, independent actions. It also implies that other factors along with cyclic AMP are required to initiate all the changes associated with the TSH stimulation of the thyroid.

The thyroid hormones exist in blood in free and protein bound forms. Thyroxine has a high affinity for thyroxine binding globulin and a lesser affinity for albumin and prealbumin. In contrast, triiodo-thyronine has a low affinity for all three proteins. This may partially explain the

shorter 1/2-life of triiodo-thyronine (Sterling et al., 1954), as well as its greater biological activity (Robbins and Rall, 1967).

The actions of the thyroid hormones, which have been reviewed by Turner and Bognora (1971), are both metabolic and growth promoting in nature. Many excised tissues from hyperthyroid animals respire at accelerated rates. These tissues include skeletal and cardiac muscle, liver, kidney, diaphragm and gastric mucosa. Increased respiration is reflected in an increase of the tissue enzyme activity, particularly enzymes associated with the glucose oxidation and oxidative phosphorylation systems. Consequently, an increase in serum thyroid hormone concentrations result in an acceleration in basal metabolic rate in homeothermic animals. Respiration apparently does not increase in brain, spleen or testis upon thyroid hormone administration.

Hypothyroidism is associated with decreased primary and secondary sex organ size in both sexes and irregular estrual cycling, small litter sizes, and susceptibility to ovarian cyst formation in females. It is not known whether these effects are primary or act through altered gonadotropin secretion.

B. A General Description of the Interrelationships of the Hypothalamo-Hypophysial-Thyroidal Axis.

1. The Anterior Pituitary and Thyroid Glands:

Studies that demonstrated a functional link between the anterior pituitary and thyroid glands were first conducted by P. E. Smith. Ablation of tadpole pituitaries retarded metamorphosis and induced thyroid atrophy (Smith, 1916), and treatment with beef pituitary extracts reversed these effects (Smith and Smith, 1922). In 1933, Kuschinsky observed that exogenous injections of thyroxine induced atrophy of the thyroid gland and reduced pituitary TSH content in rats. Based on these data,

Kuschinsky (1933) set forth the present view that anterior pituitary secretions stimulate thyroid activity and thyroid hormone secretion. The thyroid hormones in turn exert a negative feedback to regulate TSH secretion.

The thyroid hormones inhibit TSH secretion via a direct effect on the anterior pituitary. Infundibular stalk sections (Uotila, 1940; Brown-Grant et al., 1957), pituitary autotransplants (von Euler and Holmgren, 1956; Khazin and Reichlin, 1961), and massive destructions of the hypothalamic "thyrotropic area" (Florsheim, 1959; Reichlin, 1960b) all fail to prevent thyroid hormone mediated inhibition of TSH secretion.

Severinghaus et al. (1934a,b) reported that the acidophils of the anterior pituitary became degranulated after thyroidectomy and became more granulated after giving thyroxine or thyroid powder. Since acidophils are believed to secrete prolactin and GH, these reports constitute an early demonstration that the thyroid hormones modify prolactin and GH secretion.

2. The Hypothalamus and Anterior Pituitary:

Anterior pituitary function is controlled at least in part by the central nervous system. For example, Grafe and Grunthal (1929) observed that dogs with diencephalic damage had low basal metabolic rates. Houssay and co-workers (1935) noted that hypothalamic lesions in the infundibulotuberal region in toads reduced cell height in thyroid follicular epithelium. Long and Evans (1922) discovered that cervical stimulation via sterile mating induced pseudopregnancy in rats, while Selye (1934) noted that suckling stimulus induced prolactin release.

Experiments in which either the infundibular stalk was severed, the pituitary was autotransplanted to a site distal from the hypothalamus, or hypothalamic areas were destroyed by lesions demonstrated decreased

TSH and GH secretion and increased prolactin release relative to controls. In contrast anterior pituitary tissue incubated with hypothalamic extracts resulted in increased TSH and GH release and decreased prolactin release relative to that of pituitary tissue not exposed to hypothalamic extracts.

Three major hypothesis have been proposed to explain brain regulation of anterior pituitary hormone secretion. The first hypothesis was based upon blood flow studies from which Worthington (1960) suggested that an anatomical basis existed for local vasomotor control in the anterior pituitary. Thus, it was postulated that hormonal negative feedback to the pituitary could be regulated by modifying blood flow rate. Secondly, Brown-Grant (1960) proposed that the ability of the hypothalamus to concentrate thyroid hormones from the hypophysial portal vessels reflected an intrinsic mechanism to 'filter' humoral regulators. Neither of these proposals have gained significant acceptance.

The third hypothesis, and the one currently accepted, was set forth following the demonstration that the hypothalamus was capable of secreting hormones (Bargmann, 1949; Scharrer and Scharrer, 1954) and that a vascular portal system flowed from the hypothalamus to the pituitary gland (Popa and Fielding, 1930; Green and Harris, 1947). It was proposed that the hypothalamus secreted humoral factors which acted on the pituitary cells to alter hormone secretion. TSH-releasing activity (TRF) was first detected in dog hypothalamic and urine extracts by Shibusawa and co-workers. Thus, crude hypothalamic extracts containing TRF were reported to increase protein bound iodine, blood TSH, thyroid size (Shibusawa et al., 1956b) and prevent thyroid atrophy that followed infundibular stalk section (Shibusawa et al., 1956a).

C. Regulation of TSH Secretion.

1. Effects of TRH on TSH Secretion:

Shibusawa's original observations that extracts of the hypothalamus and urine contained substances that released TSH were not well described and thus subjected to criticism. However, these reports prompted others to examine hypothalamic extracts for TRF activity (Schreiber et al., 1961, 1963; Guillemin et al., 1963). As a result, Guillemin et al. (1963) demonstrated that crude ovine hypothalamic extracts caused TSH to be released from rat pituitary explants and the quantity of TSH released was directly related to the dose of extract used.

Following elucidation of the structure of TRH (Burgus et al., 1969; Boler et al., 1969), it became apparent that TRH stimulated TSH release in most mammalian species (Mittler et al., 1969; Fleischer et al., 1970; Redding et al., 1970; LaBella and Vivian, 1971; Kelly et al., 1973; May and Donabedian, 1973). That TRH was the physiological releaser of TSH was supported by the finding that TRH activity could be demonstrated in the hypophysial portal vessels of rats (Wilber and Porter, 1970; Eskay et al., 1975) and that TRH infused into the hypophysial portal vessels increased serum TSH concentration in rats (Porter et al., 1971).

Addition of TRH to anterior pituitary tissue in vitro stimulates TSH release and causes enhanced TSH synthesis. Thus, total TSH in anterior pituitary cells and culture medium (Mittler et al., 1969; Vale et al., 1972; Labrie et al., 1973) was increased by TRH, as was incorporation of radiolabeled glucosamine or alanine into TSH (Wilber, 1971). Mittler and co-workers (1969) reported that there was no difference in the amount of TSH released from anterior pituitary explants incubated for 24 hr with either thyroxine alone or thyroxine and highly purified hypothalamic

extracts; suggesting that thyroxine inhibits TRH-induced TSH release. However, in these same cultures there was a significant increase in cellular TSH after exposure of anterior pituitary explants to thyroxine and purified hypothalamic extracts as compared with explants exposed to thyroxine only. These results suggest that TRH can stimulate TSH synthesis independent of TSH release.

2. Effect of Thyroid Hormones on TSH Secretion:

Following thyroidectomy or propylthiouracil feeding, the change in TSH content of rat pituitaries is bi-phasic. During the first few weeks of hypothyroidism, pituitary TSH content is less than that of intact controls, whereas, after approximately 12 to 32 weeks of hypothyroidism, pituitary TSH content is greater in treated rats than controls (Contopoulus and Koneff, 1963; Bakke and Lawrence, 1964; Wilber and Utiger, 1967). Conversely, pituitary TSH content is initially increased when rats are given exogenous thyroxine, but after 14 days of thyroxine treatment, pituitary TSH content is less than in pituitaries of untreated controls (Contopoulus and Koneff, 1963; Bakke and Lawrence, 1964; Wilber and Utiger, 1967). Serum TSH concentration decreases when rats are given exogenous thyroxine and increases following thyroidectomy or propylthiouracil (Bakke and Lawrence, 1967; Wilber and Utiger, 1967; Reichlin et al., 1970). These data may be explained as follows: removal of negative feedback by thyroid hormones quickly follows thyroidectomy or feeding propylthiouracil and TSH release increases markedly, whereas TSH synthesis is not immediately effected. Eventually, rate of TSH synthesis surpasses rate of release and pituitary TSH content increases. The converse explanation can be made for rats receiving exogenous thyroid hormones.

Sinha and Meites (1965) reported that pituitary TSH content was increased when rats were thyroidectomized and decreased when given

thyroxine after 30 to 45 days. However, pituitary contents in this report were based on TSH release by anterior pituitary explants in vitro. Based on the above hypothesis, pituitary TSH content should be low 30 to 45 days after thyroidectomy despite increased TSH release.

Serum TSH concentration was increased in thyroidectomized sheep (Davis and Borger, 1973; Hopkins et al., 1975) and hypothyroid humans (Fleischer et al., 1972; Wartofsky et al., 1976). Davis and Anfinson (1976) reported that basal serum TSH concentration was suppressed 6 hr after the final injection of 50 μg triiodo-thyronine given twice daily for 2 wk, but TSH concentration was not different from controls 1 hr after a single injection (50 μg) of triiodo-thyronine. Hopkins et al. (1975) noted no change in ovine serum TSH concentration after giving thyroxine and/or triiodo-thyronine.

3. Interactions of TRH and Thyroid Hormones on TSH Secretion:

There is general agreement that serum thyroid hormone concentrations are inversely related to magnitude of TRH-induced TSH release (reviewed by Reichlin et al., 1966). This inverse relationship has been convincingly demonstrated in a number of species, both in vivo (Bowers et al., 1967; Averill, 1969; Fleischer et al., 1970; Davis and Anfinson, 1976) and in vitro (Guillemin et al., 1963; Schally and Redding, 1967; Vale et al., 1972; May and Donabedian, 1973).

D. Regulation of Prolactin Secretion.

Control of prolactin secretion differs from that of TSH secretion in at least four major ways. 1) In mammals prolactin secretion is chronically inhibited by hypothalamic secretion (Everett, 1954; Haun and Sawyer, 1960), i.e., prolactin inhibiting activity (Talwalker et al., 1963; Schally et al., 1976) predominates over prolactin releasing activity (Nicoll et al., 1970;

Valverde et al., 1972; Milmore and Reece, 1975). 2) No end target negative feedback control system has as yet been demonstrated. 3) Prolactin is capable of inhibiting its own secretion via a feedback at the level of the hypothalamus (Clemens and Meites, 1968). 4) Serum prolactin concentrations are altered markedly in response to a number of stimuli, both endogenous (estrogens, biogenic amines, prostaglandins) and exogenous (temperature, light, suckling, stress).

1. Effects of TRH on Prolactin Secretion:

Initial experimentation with purified or synthetic TRH led to the belief that TRH released TSH specifically. However, Tashjian et al. (1971) demonstrated that cloned pituitary tumor cells exposed to TRH released and synthesized more prolactin than cells incubated in the absence of TRH. Subsequently, TRH was shown to be a potent releaser of prolactin and TSH in humans (Jacobs et al., 1971, 1973), cattle (Convey et al., 1973; Kelly et al., 1973) and sheep (Fell et al., 1973; Mosely et al., 1973). Bowers (1971) and Lu et al. (1972) were unable to demonstrate increase prolactin release in male rats injected with TRH. However, subsequent studies indicate that TRH-induced prolactin release occurs in proestrus (Mueller et al., 1973; Deis and Alonso et al., 1973; Blake, 1974), lactating (Blake, 1974) and male rats with (Mueller et al., 1973) or without prior estrogen treatment (Mueller et al., 1973; Takahara et al., 1974).

In vitro studies have revealed that TRH acts upon the anterior pituitary to release prolactin (Machlin et al., 1974; Vale et al., 1973; Smith and Convey, 1975). Infusion of TRH into the hypophysial portal vessels of rats increased serum prolactin concentrations (Takahara et al., 1974). However, the minimum effective TRH dose was 100 ng/30 minutes.

Since rat hypothalami are estimated to contain approximately 3.5 ng TRH (Oliver et al., 1974), one must question whether sufficient endogenous TRH is released to affect prolactin secretion.

Thus, TRH may represent at least a portion of the prolactin releasing activity noted in hypothalamic extracts.

2. Effects of Thyroid Hormones on Prolactin Secretion:

Exogenous thyroid hormones given to rats increased acidophil granulation (Severinghaus et al., 1934a; Solomon and Greep, 1959) pituitary prolactin content (Chen and Meites, 1969) and prolactin release from anterior pituitary explants (Nicoll and Meites, 1963). Goitrogens or thyroidectomy induce acidophil degranulation (Severinghaus et al., 1934b; Solomon and Greep, 1959), low pituitary prolactin content (McQueen-Williams, 1933; Chen and Meites, 1969; Peake et al., 1973), but no change in serum prolactin concentration (Peake et al., 1973). Suzuki and Shibasaki (1970) demonstrated that the rate of radiolabeled amino acid incorporation into newly synthesized prolactin was positively correlated with serum thyroid hormone concentrations. These observations indicate that the thyroid hormones stimulate prolactin synthesis and subsequently cause increased prolactin release. However, Ieiri (1971) reported that thyroidectomy stimulated the pituitaries of rats to synthesize more prolactin with no change in the amount of prolactin released when compared with intact controls. If triiodo-thyronine was then injected, the rate of prolactin synthesis was decreased within 3 hours. We are unable to explain the discrepancy between these reports.

The day following thyroidectomy in sheep, when serum thyroxine concentration had declined to 66% of pre-thyroidectomy concentrations, Davis and Borger (1973) noted that serum prolactin concentration was

increased. This increase was attributed to a significant reduction in the prolactin metabolic clearance rate and a 5-fold increase in prolactin secretion rate. These same investigators also reported that serum prolactin concentration was unaltered within 1 hr after a single 50 μg triiodo-thyronine injection or after 2 weeks of 5 μg triiodo-thyronine injected twice daily (Davis and Anfinson, 1976). Hopkins et al. (1975) observed no change in serum prolactin concentration in thyroidectomized ewes or ewes given thyroxine and triiodo-thyronine.

Basal serum prolactin concentration was unchanged by increasing or decreasing serum thyroid hormone concentration in cattle (Shaw et al., 1975).

Some clinical studies illustrate that serum prolactin concentration is not altered in humans given exogenous thyroid hormones or in those afflicted with primary hypothyroidism or hyperthyroidism (Snyder et al., 1973; Katoaka et al., 1973; Marlarkey and Beck, 1975). Others report that thyrotoxic patients exhibit decreased serum prolactin concentration while patients with primary hypothyroidism have increased serum prolactin concentrations (Bowers et al., 1973; Wartofsky et al., 1976).

3. Interactions of TRH and Thyroid Hormones on Prolactin Secretion:

Relative to euthyroid patients, prolactin release after TRH is greater in patients with primary hypothyroidism and less in hyperthyroid subjects (Bowers et al., 1973; Katoaka et al., 1973; Snyder et al., 1973; Yamaji, 1974; Wartofsky et al., 1976). When euthyroid humans are given triiodo-thyronine, the increase in serum prolactin concentration that normally follows TRH injections was suppressed (Katoaka et al., 1973; Yamaji, 1974). However, these investigators gave large doses of triiodo-thyronine (100 μg triiodo-thyronine given singly and 75 μg triiodo-

thyronine/day for 2 weeks, respectively) and did not measure the thyroid hormone concentrations resulting from these treatments. Snyder et al. (1973) gave humans 120 μg thyroxine and 30 μg triiodo-thyronine/day for 4 weeks and noted significant increases in serum thyroxine and triiodo-thyronine concentrations which remained within the normal ranges for these hormones. No change in the TRH-induced prolactin release was noted.

Giving triiodo-thyronine (50 μg) to sheep increased serum triiodo-thyronine concentration 16-fold within 1 hour. In addition, relative to sheep not given triiodo-thyronine, TRH-induced increases in serum prolactin concentration were reduced when TRH was given 1 hr after giving triiodo-thyronine (Davis and Anfinson, 1976). This reduction of TRH-induced prolactin release did not occur if TRH was given 6 hr after the final injection of triiodo-thyronine (50 μg) given twice daily for 2 weeks (2-fold increase in serum triiodo-thyronine existed at the time of TRH injection).

Propylthiouracil fed rats yielded anterior pituitary cells that, when cultured in vitro, released less prolactin after TRH if incubated in the presence of the thyroid hormones (Vale et al., 1973).

These studies indicate that the thyroid hormones are capable of influencing TRH-induced prolactin release. However, this control mechanism is apparently not as sensitive as it is for thyrotropin.

E. Regulation of GH Secretion

Control of GH secretion closely parallels that of prolactin in that 1) both stimulatory and inhibitory hypothalamic controls are operative (Schally et al., 1971; Brazeau et al., 1972), 2) there is no one specific end target feedback inhibitory hormone, 3) the ability to autoregulate

via the hypothalamus is operative (MacLeod, 1966), and 4) serum concentrations are easily altered by many stimuli (glucose, amino acids, corticoids, suckling, sleep, stress).

1. Effects of TRH on GH Secretion:

While TRH is as potent a stimulator for prolactin release as TSH, this does not appear to be the case for GH release. The action of TRH upon the somatotrophs, if physiological, may not be of the same nature as for thyrotrophs and lactotrophs. Tashjian et al. (1971) observed that while TRH induced prolactin release and synthesis from cloned anterior pituitary cells, GH production was reduced.

Intravenous administration of TRH causes increased serum GH concentrations in cattle (Convey et al., 1973; Tucker et al., 1975), although some animals are unresponsive to TRH injections. Attempts to demonstrate TRH-induced GH release from bovine anterior pituitary explants (LaBella and Vivian, 1971) or cell monolayers (Machlin et al., 1974; Smith and Convey, 1975; Queen et al., 1976) cultured in vitro have been successful, but not consistently repeatable. Usually, the TRH dose required to induce increased GH release was greater than that needed to increase TSH and prolactin release. Serum GH concentration did not change when TRH (50 μg) was given to sheep, but was increased 6 hr after a 1 mg TRH injection (Davis et al., 1976).

Not all humans respond to TRH with increased serum GH concentration (Fleischer et al., 1970; Bowers et al., 1971; Karlberg and Almqvist, 1972). Compared to healthy humans, increased serum GH concentration induced by TRH was noted in a greater percentage of subjects with acromeglia or gigantism (Schalch et al., 1972; Irie and Isushima, 1972), renal failure (Gonzalez-Barcena et al., 1973), mental depression (Maeda et al., 1975) or hypothyroidism (Hamada et al., 1976).

Thyrotropin releasing hormone will increase serum GH concentrations if rats are anesthetized with urethane (Takahara et al., 1974; Kato et al., 1975; Chihara et al., 1976) or if pituitaries are removed and exposed to TRH in vitro (Carlson et al., 1974).

2. Effects of Thyroid Hormones on GH Secretions:

Thyroidectomized or propylthiouracil fed rats are characterized by a reduction in the granulation of anterior pituitary acidophils (Severinghaus et al., 1934b; Purves and Griesbach, 1946; Knigge, 1958), anterior pituitary GH content as measured by bioassay (Contopoulus and Koneff, 1963; Schooley et al., 1966) and radioimmunoassay (Peake et al., 1973; Hervas et al., 1975), GH synthesis as determined by radiolabeled leucine or amino acid incorporation (Suzaki and Shibasaki, 1970; Ieiri, 1971; Figurova et al., 1975) and serum GH concentration (Eisenberg et al., 1972; Peake et al., 1973; Hervas et al., 1975). Conversely, giving thyroid hormones increase acidophil granulation (Severinghaus et al., 1934a; Purves and Griesback, 1946; Solomon and Greep, 1959) anterior pituitary GH content (Contopoulos and Koneff, 1963; Peake et al., 1973; Hervas et al., 1975), GH synthesis (Suzuki and Shibasaki, 1970; Ieiri, 1971; Figurora et al., 1975) and serum GH concentration (Eisenberg et al., 1972; Peake et al., 1973; Hervas et al., 1975).

Neither increasing serum thyroxine concentration by feeding thyroprotein to cattle (Shaw et al., 1975) nor decreasing serum thyroxine by withdrawing thyroprotein fed to cattle (Shaw et al., 1975) or thyroidectomizing sheep (Davis and Borger, 1973) are effective in changing serum GH concentration. Katz et al. (1969) noted that children with primary hypothyroidism or thyrotoxicosis had lower serum GH concentration than

that did healthy children. Hamada and co-workers (1976) reported that adult humans with hypothyroidism had higher serum GH concentrations than those with hyperthyroidism.

3. Interactions of TRH and Thyroid Hormones on GH Secretion:

Investigators successful in inducing GH release with TRH in rats have also demonstrated that thyroidectomy or propylthiouracil treatment enhance the TRH-induced GH release (Chihara et al., 1976) and that thyroid hormone pretreatment inhibits this response (Carlson et al., 1974; Kato et al., 1975).

F. Mechanism of Action: TRH.

The stimulation of TSH, prolactin and GH release by TRH is due to a direct action upon the anterior pituitary (see Literature Review, Section C, D, E). Thyrotropin and prolactin synthesis is also promoted by TRH although this effect may be secondary to release. Actinomycin-D, puromycin, and cycloheximide do not alter TSH release in vitro after acute TRH exposure (Schally and Redding, 1967; Bowers et al., 1968), indicating that new ribonucleic acid (RNA) or protein synthesis is not required for this process. Anterior pituitary explants incubated in vitro with both TRH and puromycin or puromycin alone for 24 hr released less TSH than explants incubated without either compound (Mittler et al., 1969). The process of TRH-induced TSH release is energy dependent, i.e., blocked by the oxidative phosphorylation inhibitors 2,4-dinitrophenol and oligomycin (Wilber and Utiger, 1968).

Specific TRH receptors have been demonstrated to be present in cells from normal (Labrie et al., 1972; Wilber and Seibel, 1973) and tumorous anterior pituitaries (Grant et al., 1971; Hinkle and Tashjian, 1973). The number of available TRH receptors in anterior pituitary tissue from

cattle and rats increase after feeding propylthiouracil and decrease following thyroxine treatment (Wilber and Seibel, 1973; Labrie et al., 1975). Tixier-Vidal and co-workers (1975) reviewed evidence indicating that significant amounts of radiolabeled TRH are found within the cytoplasmic and nuclear fractions of anterior pituitary cells within 30 min of adding ^{3}H-TRH to the incubation medium. On the other hand, Gourdji et al. (1976) have convincingly shown that after TRH binds to pituitary tumor cells and stimulates prolactin release, it is released unaltered into the media.

Cyclic AMP is increased in cells following interaction of protein hormones with their specific receptors (Robinson et al., 1968). Similarly, cellular cyclic AMP increased when TRH was added to the incubation medium of rat anterior pituitary tissues in culture (Labrie et al., 1975) or cloned pituitary tumor cells (Dannies et al., 1976). Furthermore, the dose of TRH required to induce 1/2-maximal prolactin release and cyclic AMP accumulation was the same (.3nM TRH). Sundberg et al. (1976) failed to detect an increase in cyclic AMP after incubation of rat hemi-pituitaries with either TRH, high concentrations of K^{+}, or ovine hypothalamic extracts. These results may be due to the fact that Sundberg et al. (1976) did not add theophylline to the incubation medium. Theophylline blocks the cyclic AMP degrading enzyme, phosphodiesterase. Adenyl cyclase activity was increased when crude sheep, rat, or pig hypothalamic extracts were added to whole (Zor et al., 1969) or halved rat anterior pituitaries (Steiner et al., 1970) or dispersed pig anterior pituitary cells (Kudo et al., 1972), respectively. However, when dealing with crude hypothalamic extracts, the possibility exists that high K^{+} concentration or other non-specific factors are the active components (Steiner et al., 1970; Vale and Guillemin, 1967).

High concentrations of extracellular K^+ mimic many characteristics of the TRH stimulus. These include increased TSH, prolactin and GH secretion (Vale and Guillemin, 1967; Wakabayashi et al., 1973; Labrie et al., 1973) and increased TSH incorporation of radiolabeled glucosamine (Wilber and Utiger, 1969). In addition, release of hormones induced by high concentrations of extracellular K^+ is blocked by thyroxine, low concentrations of extracellular Ca^{++} (Vale and Guillemin, 1967) and a prostaglandin antagonist (Vale et al., 1971). High concentrations of extracellular K^+ reverse the plasma membrane potential (Milligan and Kracier, 1970) and increase the $^{45}Ca^{++}$ space (Milligan and Kracier, 1971) in anterior pituitary tissue. Since high extracellular K^+ mimics TRH, the TRH stimulus may also require a change in plasma membrane permeability and/or polarity.

Possibly cell membrane depolarization permits Ca^{++} flux into the cell, as extracellular Ca^{++} is a necessary component for TRH-induced hormone release (Vale et al., 1967b). Rat hemi-pituitaries incubated with theophylline secreted less prolactin in a Ca^{++} free medium than did those in which Ca^{++} was present in the medium (Wakabayashi et al., 1973). This result suggests that Ca^{++} either inhibits cyclic AMP degradation or acts distal to cyclic AMP accumulation. The latter possibility is supported by studies demonstrating that GH and TSH release induced by crude hypothalamic extracts or a phosphodiesterase inhibitor, aminophylline, is blocked when rat hemi-pituitaries are incubated in Ca^{++} free medium despite increased cellular cyclic AMP concentrations (Steiner et al., 1970).

Prostaglandins may also play a role in TRH-induced hormone release. When prostaglandin E_1 was incubated with dispersed porcine anterior

pituitary cells, adenyl cyclase activity increased as did TSH and GH release (Kudo et al., 1972). In addition, prostaglandin E_1 increased cellular cyclic AMP content when added to the incubation medium of whole rat anterior pituitaries (Zor et al., 1969). Prostaglandins may also act synergistically with TRH to release pituitary hormones as higher serum prolactin and GH concentrations were elicited by giving cattle both prostaglandin $F_{2\alpha}$ and TRH than TRH alone (Tucker et al., 1975). The prostaglandin antagonist, 7-oxa-13-prostynoic acid, prevented the TRH-induced TSH release in rat hemi-pituitary cultures (Vale et al., 1971). However, neither this prostaglandin analogue nor prostaglandin E_1 appear to alter TRH receptor binding (Wilber and Seibel, 1973).

G. Mechanism of Action: Thyroid Hormones.

The influence of thyroid hormones on TSH, prolactin and GH secretion apparently is hormone specific. To our knowledge, studies pertaining to the mechanism of action of the thyroid hormones have been limited to effects on TSH secretion.

Actinomycin-D, puromycin, and cycloheximide inhibit thyroid hormone reduction of TRH-induced TSH release (Schally and Redding, 1967; Bowers et al., 1968). This suggests that the thyroid hormones stimulate transcription of RNA, which subsequently promotes translation of protein(s) capable of altering TSH secretion. Characterization of this protein(s) has not been undertaken. However, reduction in the number of TRH receptors associated with increased thyroid hormone concentrations may represent one possible action of these proteins (Wilber and Seibel, 1973; Labrie et al., 1975).

Although it is generally accepted that the thyroid hormones act directly upon the pituitary to reduce TSH secretion, circumstantial

evidence supports the possibility that the thyroid hormones also alter anterior pituitary hormone secretion via influences on the hypothalamus. Silverman and Knigge (1972) reported that rat median eminence tissues cultured *in vitro* sequestered thyroxine via an energy mediated mechanism; the sequestering was blocked by adding ouabain, iodoacetate, sodium fluoride, potassium cyanide or reducing the temperature. The thyroxine sequestering was promoted by adding .2 or 2mU TSH/ml to the incubation medium. Reichlin *et al*. (1972) demonstrated that hypothalamic TRH synthetase activity was increased after cold stress or addition of thyroxine to the incubation medium. The increase in TRH synthetase activity by thyroxine may be interpreted as maturation of the enzyme system or a form of positive feedback. Thyrotropin releasing activity in hypothalamic extracts from thyroidectomized rats was 2 to 3 times greater than those from euthyroid rats and exogenous thyroxine prevented this increase (Sinha and Meites, 1965). However, Bassiri and Utiger (1974) noted no difference in hypothalamic content of radioimmunoassayable TRH when rats were thyroidectomized or given thyroxine for as long as 4 weeks. Surprisingly, hypophysectomized rats, whether receiving thyroxine replacement or saline, has less hypothalamic TRH than did intact controls. Two explanations of these results are possible. First, in the absence of negative feedback from the pituitary (possibly TSH), TRH may be released as fact as it is synthesized. Secondly, a TRH-stimulating factor from the pituitary may have been eliminated due to hypophysectomy (positive feedback). Thus, due to the lack of convincing evidence, the question as to whether thyroid hormones influence TSH release via the hypothalamus remains unsolved.

Is one thyroid hormone more important than the other in the physiological scheme? Blood triiodo-thyronine concentration is

approximately 3% that of thyroxine. However, triiodo-thyronine has greater biological activity (Robbins and Rall, 1967) and a larger extrathyroidal pool volume than thyroxine (Woeber et al., 1970). The latter observation can be interpreted to mean that triiodo-thyronine has a greater intracellular pool volume than thyroxine.

Possibly thyroxine must be converted to triiodo-thyronine in order to effectively inhibit TSH secretion. The rate of monodeiodination of thyroxine to triiodo-thyronine in the blood is substantial. For example, Pittman et al. (1971) estimated that humans derive approximately 30% of their extrathyroidal triiodo-thyronine pool from thyroxine deiodinated in the blood. Rats obtain 20% of their total body triiodo-thyronine pool via this source (Schwartz et al., 1971). Conversion of thyroxine to triiodo-thyronine in the blood is greatly reduced by feeding propylthiouracil. Based on this premise, Escobar Del Rey et al. (1974) demonstrated that thyroidectomized rats given thyroxine and propylthiouracil for 2 weeks exhibited a greater TRH-induced TSH release than those given thyroxine and saline. When radiolabeled thyroxine was injected into rats, the anterior pituitaries contained less radiolabeled triiodothyronine if rats were also given propylthiouracil rather than saline. The total amount of thyroxine in these pituitaries was not different between the two groups.

Within 2 to 4 hr after humans ingested 50 μg triiodo-thyronine, serum triiodo-thyronine concentration increased and the TRH-induced TSH released decreased. However, after humans ingested 1000 μg thyroxine, TRH-induced TSH release was not reduced until serum triiodothyronine concentration increased 48 to 72 hr later (Wenzel et al.,

1975). In addition, Shadlow et al. (1972) observed specific and saturable binding for triiodo-thyronine, but not thyroxine, in rat anterior pituitary homogenates.

These data suggest, but do not prove, that triiodo-thyroxine may play a more important role than thyroxine in the regulation of TSH secretion.

MATERIALS AND METHODS

A. Design of Experiment One: Bovine, TSH, Prolactin and GH Concentrations after Thyroprotein and TRH in Vivo.

Twenty-four Holstein heifers from the Michigan State University dairy herd were used. Heifers ranged in age from 5 to 11 months and were assigned on the basis of body weight (BW) to one of six blocks of four heifers each. Body weight ranged from 115 to 290 kilograms. One heifer from each block was assigned to one of four treatment groups. Twelve heifers were fed thyroprotein for either 6 or 27 days. Thyroprotein was fed at 6.6 g/100 kg BW/day on days 0 to 3 to quickly establish high serum thyroxine concentrations, then at 3.3 g/100 kg BW/day thereafter. The remaining 12 heifers served as controls, receiving casein (Nutritional Biochemicals Corp., Cleveland, OH) in amounts and according to schedules described for thyroprotein above. Dates when thyroprotein or casein treatments were begun were designated day 0. Casein and thyroprotein were given orally via capsule at approximately 1600 hr, daily.

Thyrotropin releasing hormone (33.3 μg/100 kg BW) was given via jugular cannula at 1030 and 1330 hr either on day 6 or 33 to heifers treated for either 6 or 27 days, respectively. Heifers were started on thyroprotein or casein such that TRH was given to all heifers on the same calendar date. This experiment took place during November and December, 1974. Heifers were housed in an enclosed pole barn without supplemental heat or light and were fed grain and silage twice daily.

Blood was collected via jugular puncture on days -3, 0, 2 and 4 from all heifers and on days 6, 8, 12, 16, 28, 29, 30, 31 and 32 from heifers given thyroprotein or casein for 27 days. These samples were assayed for serum thyroxine concentration. In addition, serum was collected via jugular cannulae at -30, -15, 0, 4, 8, 12, 16, 20, 30, 45, 60, 90, 120 and 180 min relative to each TRH injection for determination of serum thyroxine, TSH, prolactin and GH concentrations.

B. In Vitro Procedures.

1. Culture Media:

Media used for the preparation of dispersed cells, and for the 2 hr incubations on the day of treatment consisted of a 1:1 (v/v) mixture of TC Medium 199: Eagle's TC Minimal Medium, Hanks Balanced Salt Solution (Difco Labs., Detroit, MI). The medium was supplemented with .5% essential and 1% nonessential amino acids (GIBCO, Grand Island, NY), Fungizone (Amphotericin B, 250 mcg/l; GIBCO), penicillin-G (68 mg/l - 1599U/mg; Sigma Chemical Co.), and streptomycin sulfate (100 mg/l; Sigma Chemical Co.). The pH of the medium (7.4) and buffering capacity were adjusted by adding 2.8% $NaHCO_3$ and 10mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES, Sigma Chemical Co.).

Growth medium, used for incubation of cells on days 0-5 consisted of the aforementioned medium with 10% serum from a postpartum cow.

2. Preparation and Incubation of Dispersed Cells:

Culturing procedures are based on earlier studies (Vale et al., 1972; Smith et al., 1974; Smith and Convey, 1975).

Bovine pituitaries were obtained at a local abbatoir and transported to the laboratory within 1 hr of the animals' deaths. After discarding the posterior pituitaries, the anterior pituitaries were sagittally

sliced with a Stadie-Riggs microtome. Anterior pituitary slices were diced with a scalpel into approximately 1 mm^3 pieces and washed thrice with medium. Diced pituitary tissue was enzymatically dispersed by vigorously stirring the tissue in medium containing 0.3% collagenase (Type I - 150 μ/mg; Sigma Chemical Co.) for 45 minutes. The resulting suspension of unit cells and cell clumps was strained through cheese cloth to eliminate pieces of undispersed tissue. The dispersed cells were then stirred in medium containing 0.25% Viokase (GIBCO) for 15 min to further separate cell clumps. Both incubations were conducted at 37°C. Following collagenase and Viokase incubations, dispersed cells were precipitated and then washed with culture media two and four times, respectively, using a Sorvall RC-3 centrifuge (160 xg for 5 min).

Washed cells were resuspended in growth media to a concentration of ≈ 3.5×10^5 cells/milliliter. Four ml of cell suspension were dispensed into each culture flasks (30 ml, Cat. No. 10-126-9, Fischer Scientific Co.) and incubated for 5 days at 37°C with a 95% O_2 -5% CO_2 gas environment. The growth media were changed at daily intervals beginning 48 hr after cell plating.

3. Treatment of Cells:

On the day of TRH treatment (day 5), the growth media were discarded and the pituitary cell monolayers were washed with media (without serum) five times at 10-min intervals to stabilize hormone release. These washes were performed because preliminary studies had indicated that without repeat washing, hormone secretions were much greater during the first 2 hr treatment incubations, than during subsequent periods. Also, large variations in hormone release existed between vials within periods and treatments.

Following these repeat washes, cells were incubated with the appropriate treatments in media (without serum) for either two (experiment #2) or three (experiment #3) consecutive 2 hr periods. Media collected after each period were assayed for TSH, prolactin and growth hormone. Upon completing the incubations, cells were lysed with .5% lauryl sulfate (4 ml) for subsequent determination of cellular TSH, prolactin and GH content.

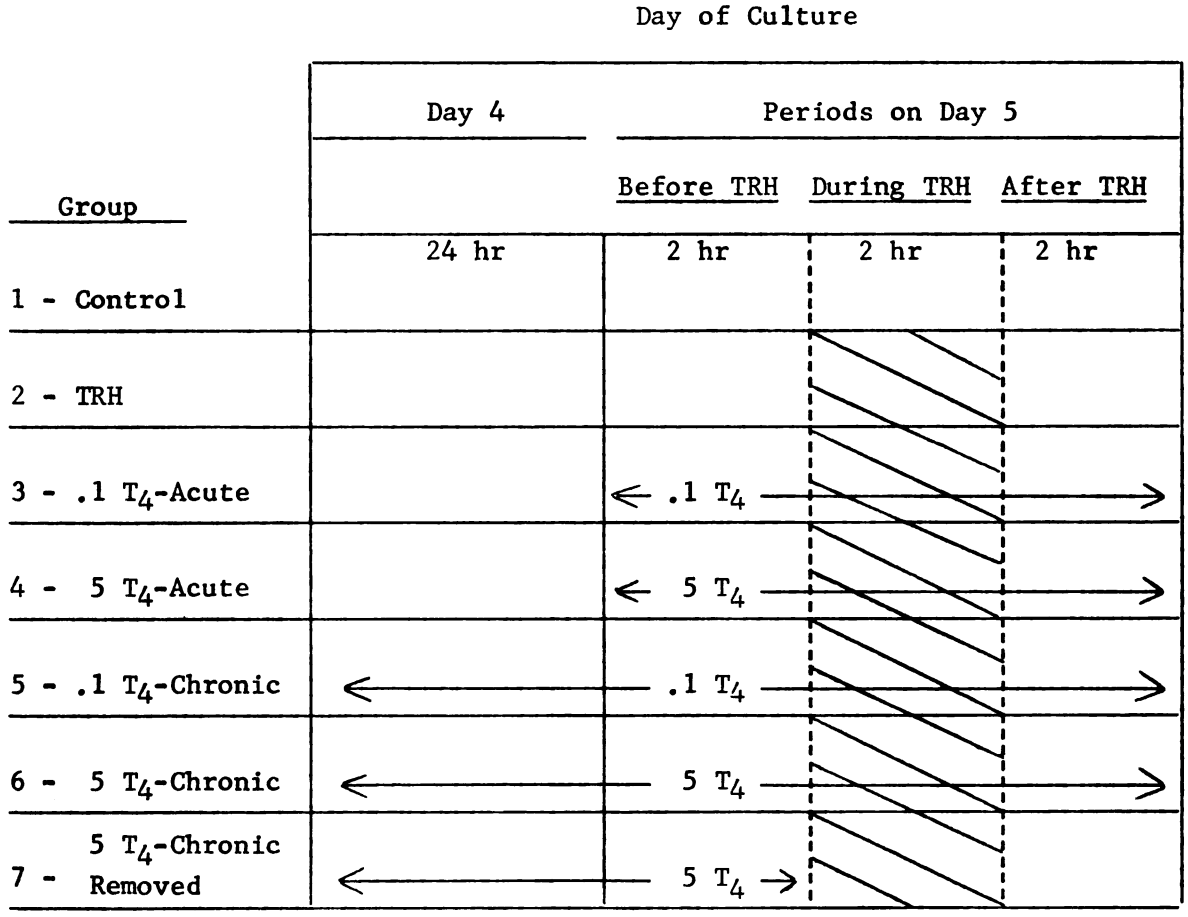
C. Design of Experiment Two: TRH-Induced TSH, Prolactin and GH Release: Dose Related Responses.

Following repeat washes on day 5 of incubation, cells were incubated in media for two consecutive 2 hr periods. During the first period cells were incubated in media (without serum) to establish basal hormone secretion rates. During the second period TRH (0, 1, 10, 10^2, 10^3 or 10^4 pg/ml) was added to the culture media to determine the TSH, prolactin and GH response. The TRH used in this and the following experiment was prepared in 1% bovine serum albumin. Ten µl of the appropriate TRH stock solution was added per ml culture medium.

D. Design of Experiment Three: Effects of Thyroxine and TRH on TSH, Prolactin and GH Secretion.

The objectives of this experiment were to examine basal and TRH-induced hormone release by pituitary cells exposed to different thyroxine regimes, i.e., low vs high concentration; acute vs chronic exposure. Table I depicts the experimental design. Pituitary cell cultures were incubated in 0, .1 or 5 µg thyroxine/ml media. Thyroxine treatment began either 2 or 24 hr prior to and continued until 2 hr after a 2 hr exposure of 0 or 10 ng TRH/ml media on day 5 of cell culture (Table I). In an additional group, thyroxine was present in the media (5 µg/ml) for 24 hr then omitted from the culture media at the time TRH was added.

Table I. Experimental Design for Testing the Effects of Thyroxine and TRH on TSH, Prolactin and GH Secretion from Bovine Anterior Pituitary Cell Cultures.



= 10 ng TRH/ml medium

On days 1 - 4 cell cultures were incubated in growth medium with 10% serum

On day 5 cell cultures were incubated in medium without serum

N = 18 for each treatment group

Media were collected from the three cultures on day 5

.1 or 5 T_4 refers to μg T_4/ml media

The thyroxine stock solution was prepared in slightly basic double distilled water. Five µl of the appropriate thyroxine stock solution was added per ml media.

Media was collected following the 2 hr incubations prior to, during and after TRH exposure (Table I). This experiment was repeated three times with each consisting of these seven treatment groups and six replicates (culture flasks) per treatment group.

E. Assays.

Blood sera, culture media and pituitary cell extracts were assayed for TSH, prolactin and GH using double antibody radioimmunoassays previously described by Kesner et al. (1977), Tucker (1971) and Purchas et al. (1970), respectively. Serum thyroxine and progesterone concentrations were also determined in selected samples as described by Shaw et al. (1975) and Louis et al. (1973), respectively.

Thyrotropin was assayed using antisera previously described for measurement of ovine TSH (Borger and Davis, 1974). Before use in immunoassay, antisera was absorbed with bovine luteinizing hormone (NIH-LH-B_8) and follicle stimulating hormone (NIH-FSH-B_1) to remove non-specific antibodies which reacted with these gonadotropins. Procedures for antisera absorption were described previously (Borger and Davis, 1974). Highly purified bovine TSH (Dr. J. G. Pierce) was used for iodination and NIH-TSH-B_4 (National Institutes of Health) was used for the standard.

The useful range of assay sensitivity for measuring bovine TSH was between .3 and 10 ng per tube. Dilution curves for two pools of bovine sera, containing low or high concentrations of TSH, were parallel to the TSH standard curve. Known quantities of NIH-TSH-B_4 added to bovine sera

were quantitatively recovered (98.7%). Linear regression of the amount of NIH-TSH-B_4 added against amount of TSH recovered gave a line with a slope of 1.20 and an intercept of -.16. Neither the slope nor the intercept were different ($P>.4$) from the ideal of 1.0 and 0, respectively. Bovine GH (NIH-GH-B_{11}), follicle stimulating hormone (NIH-FSH-B_1), prolactin (NIH-PRL-B_3) and luteinizing hormone (NIH-LH-B_8) at concentrations up to 500 μg/500 μl reaction volume caused negligible reductions in binding of labeled TSH. The minor degree of displacement that did occur could be accounted for on the basis of known TSH contamination of the NIH preparations used.

F. Statistical Analysis.

The analysis of variance for repeat measurement data from all experiments were patterned after that described by Gill and Hafs (1971). In experiment one, data from serum samples obtained prior to TRH (-30, -15 and 0 min samples) were pooled according to individual animals and entered into the analysis as single values. In addition, all data following TRH injections were inspected to determine when average hormone concentrations for heifers in each treatment group had returned to pre-TRH concentrations, and only points prior to that time were entered into the analyses of variation. Data from experiment two and three were expressed as repeat measurements, denoting the pre-TRH, TRH and post-TRH periods as points in time. The three sets of cultures comprising experiment three were analysed both individually and combined, designating culture set as a main effect.

Significance of differences between main effects in experiments one and three were determined by using Bonferroni's t-test (Miller, 1966) and Dunnett's t-test (Kirk, 1968). Linear regressions was performed to test the hormone responses to increasing TRH doses in experiment two.

RESULTS AND DISCUSSION

A. Experiment One: Bovine TSH, Prolactin, and GH Concentrations after Thyroprotein and TRH in Vivo.

Serum thyroxine concentrations averaged 87 and 70 ng/ml over the treatment period in heifers fed casein for 6 or 27 days (figure 1). On the other hand, serum thyroxine concentrations in heifers given thyroprotein for 6 or 27 days averaged 79 and 60.5 ng/ml, respectively, before thyroprotein feeding (days -3 and 0) and increased ($P<.05$) to 133.8 and 116.2 ng/ml, respectively, by day 6 of thyroprotein treatment. In heifers given thyroprotein for 27 days, serum thyroxine concentrations on days 8, 12 and 16 were greater ($P<.05$) than corresponding values for controls. Cessation of thyroprotein feeding on day 28 resulted in a marked decline in serum thyroxine concentrations comparable in duration and magnitude to that seen under similar circumstances in lactating cows (Shaw et al., 1975). On day 33, serum thyroxine averaged 24.0 ng/ml in heifers fed thyroprotein for 27 days, which was lower ($P<.05$) than corresponding values for controls (82.2 ng/ml). The marked decrease in serum thyroxine concentrations observed in lactating cows (Shaw et al., 1975) and heifers following cessation of thyroprotein feeding may result from increased thyroxine metabolic clearance rate (Premachandra and Turner, 1961). A decrease in endogenous thyroxine secretion may also have resulted from latent suppression of TSH secretion due to chronic high serum thyroxine concentrations (Krugman et al., 1975). The alterations in serum thyroxine concentration created by thyroprotein feeding were utilized to determine

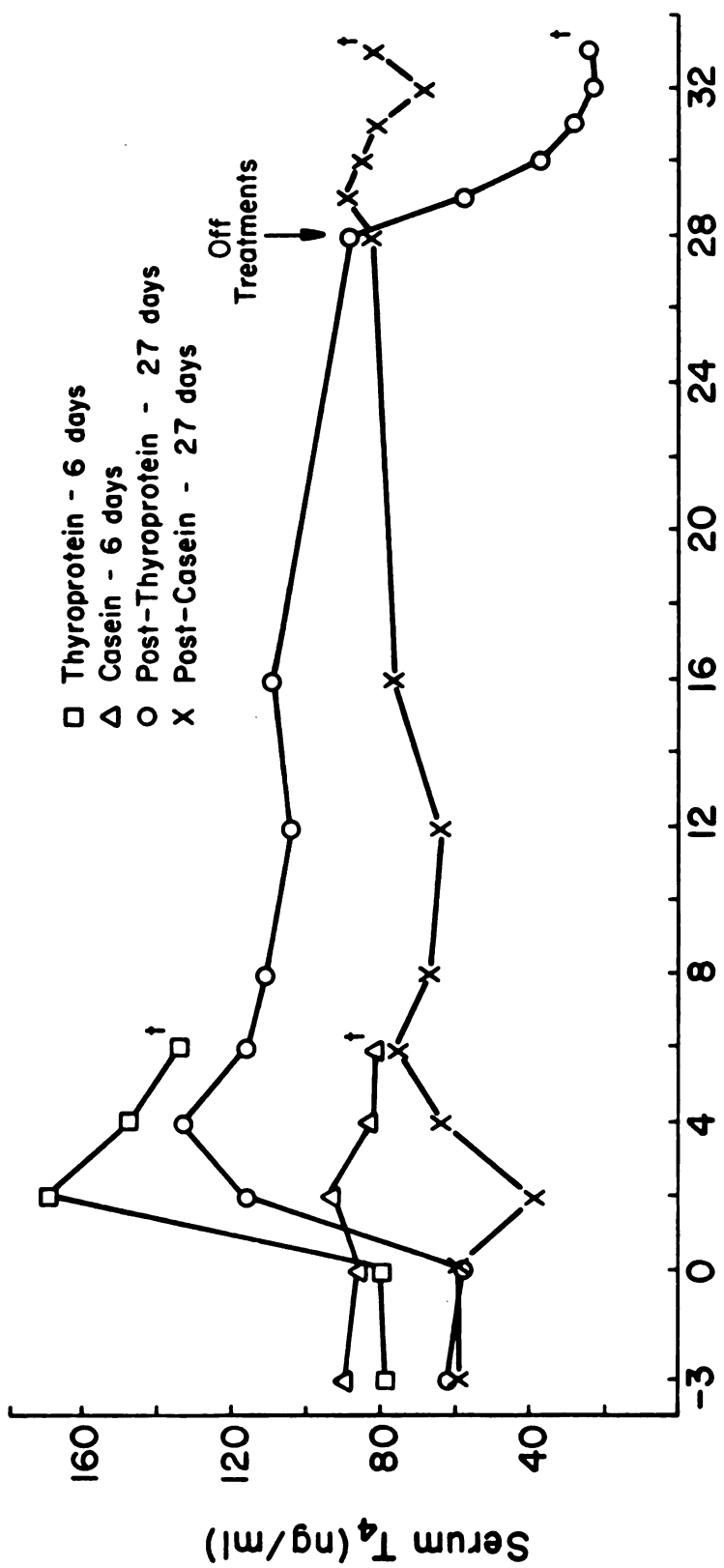


Figure 1. Serum thyroxine concentration prior to, during and after feeding thyroprotein (6 □-□ or 27 o-o days) or casein (6 Δ-Δ or 27 x-x days) to Holstein heifers. TRH was injected on day 6 or 33 (†).

what effect serum thyroxine concentration had on basal and TRH-induced changes in serum TSH, thyroxine, prolactin and GH concentrations. Accordingly, TRH was given when thyroxine concentrations were increased (day 6 or thyroprotein feeding) or reduced (6 days after thyroprotein feeding was ended), relative to corresponding controls.

1. Thyrotropin:

Differences in the magnitude of TSH release after the first or second TRH injection were not significant and therefore only the change in TSH after the first TRH injection is presented (figure 2). Basal serum TSH concentrations (-30, -15 and 0 min) were not different among the four treatment groups; the overall average was 6.7 ng TSH/milliliter. Hopkins et al. (1975) observed no change in basal serum TSH concentrations of sheep in which serum thyroxine concentration was increased but noted an increase in serum TSH after thyroidectomy. Similarly, Davis and Borger (1973) observed increased serum TSH concentrations in thyroidectomized sheep. Failure in the present study to detect a change in basal serum TSH concentrations in heifers with low concentrations of thyroxine 6 days after 27 days thyroprotein feeding may have resulted from an insufficient decrease in thyroxine concentrations. Hopkins et al. (1975) did not observe a change in TSH concentration in sheep until serum thyroxine decreased below 20 ng/ml serum. The lowest thyroxine concentration attained in the present experiment was 24 ng thyroxine/ml serum. Alternatively, chronic high concentrations of serum thyroxine may have suppressed TSH synthesis by the thyrotrophs to the extent that they could not increase the secretion rate of TSH in response to the low concentrations of thyroxine that follows thyroprotein withdrawal.

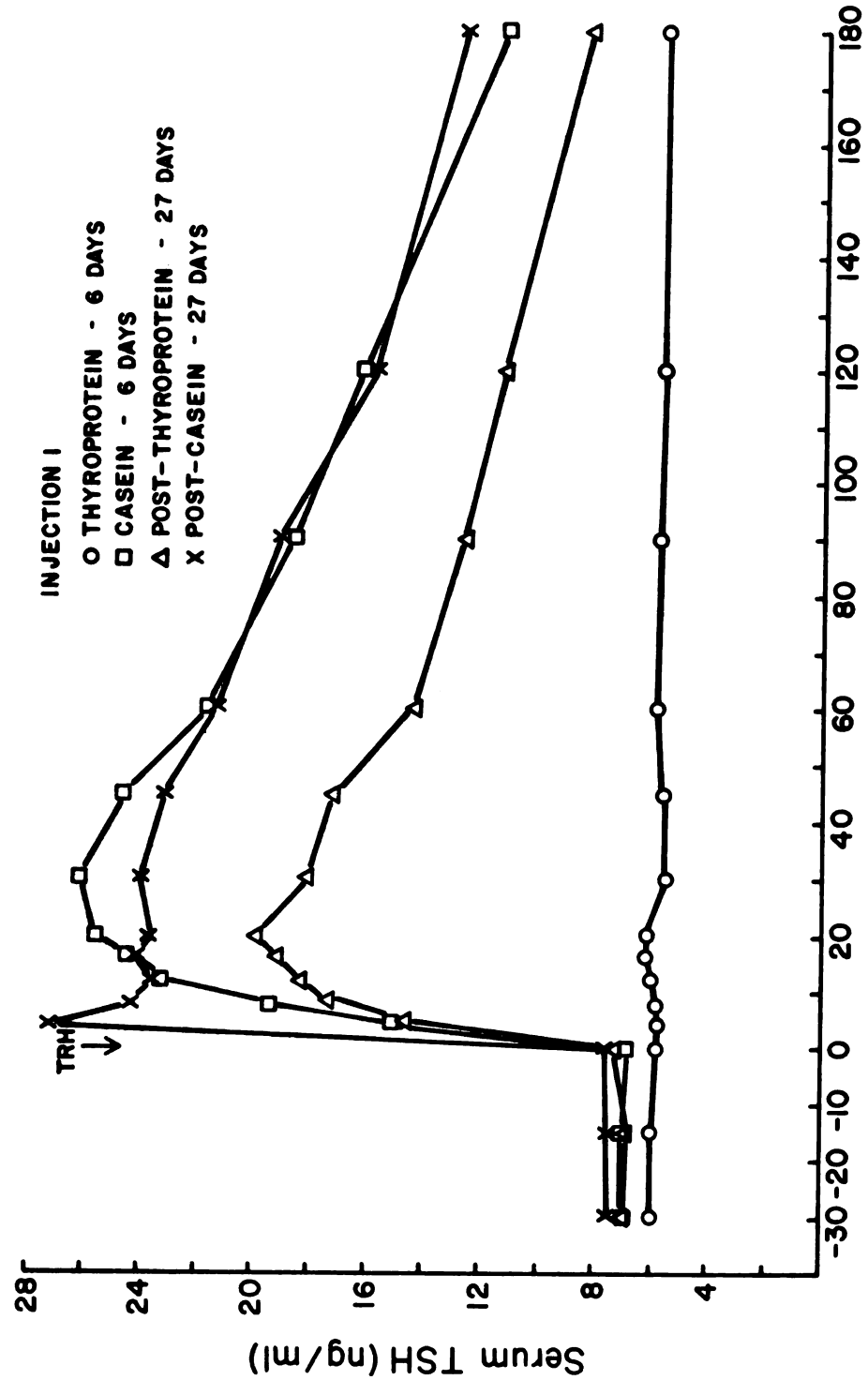


Figure 2. Serum TSH before and after the first iv injection of TRH (33.3 μg/100 kg BW) to thyroprotein (6 o-o or 27 Δ-Δ days) or casein (6 □-□ or 27 x-x days) treated Holstein heifers.

Serum TSH concentrations in heifers given casein for 6 or 27 days were increased ($P<.05$) within 4 min after TRH injection relative to the average TSH concentration of pretreatment samples and both reached peaks of 26 ng/ml within 30 min after TRH (figure 2). In contrast, serum TSH concentration was unchanged by TRH given on day 6 of thyroprotein feeding. These results suggest that increased serum thyroxine concentrations (6 days of thyroprotein feeding) exerted a negative feedback on the pituitary, inhibiting TRH-induced TSH release, similar to the thyroxine inhibition of TSH release described for rats (Schally and Redding, 1967; Vale et al., 1967a). However, TRH given when serum thyroxine concentrations were decreased (6 days after 27 days of thyroprotein feeding) did not cause an exaggerated TSH release. In these animals the maximum serum TSH concentration attained was 20 ng/ml, which occurred 20 min after the TRH injection. The average TSH concentration over the 3-hr sampling period was less than that of the appropriate casein treated control, although the differences only approached significance ($P<.10$). Humans given prolonged thyroid hormone therapy required approximately 2 weeks to regain normal TSH release in response to TRH challenges (Krugman et al., 1975). Thus, negative feedback effects of thyroxine on pituitary thyrotrophs for 27 days possibly were not totally reversed by 6 days after cessation of thyroprotein feeding. Although serum thyroxine is low after thyroprotein feeding has ceased in cattle, these animals probably are not functionally hypothyroid and should not be equated with hypothyroid or thyroidectomized animals.

2. Thyroxine:

Serum thyroxine concentrations in heifers given casein for 6 or 27 days averaged 82.8 and 82.2 ng/ml, respectively, prior to TRH (0 min)

and increased to 119.2 and 117.6 ng/ml, respectively, 3 hr after the second TRH injection (figure 3). This increase in thyroxine was probably in response to TSH released by TRH, because when serum TSH concentration was not increased by TRH, thyroxine concentration did not increase in serum. Thus, in heifers given TRH on day 6 of thyroprotein feeding, serum thyroxine averaged 133.8 ng/ml at the time TRH was given and was not increased by the TRH injections. Although the increase in serum TSH was significant ($P<.05$) in heifers given TRH 6 days after feeding thyroprotein for 27 days, serum thyroxine increased only 4.8 ng/ml in these heifers. These data indicate that thyroid gland inhibition by chronic thyroxine treatment causes a decreased ability of the thyroid to respond to TSH and this inhibition persists for at least 6 days after cessation of thyroprotein treatment.

3. Prolactin:

In agreement with previous results from this laboratory (Shaw et al., 1975), thyroprotein feeding did not effect basal serum prolactin concentrations (-30, -15 and 0 min), which averaged 7.0 ng/ml (figure 4). However, Davis and Borger (1973) reported that the marked reduction in thyroid hormone availability that occurs after thyroidectomy in lambs is accompanied by an increase in serum prolactin concentration.

Changes in serum prolactin concentration after the first TRH injection are shown in figure 4. Following TRH injections, prolactin increased from basal concentrations to maximum values of 60 to 140 ng/ml at 4 minutes. Although there was a tendency for low serum thyroxine concentrations to augment and high serum thyroxine to reduce the amount of prolactin release by TRH in these heifers, treatments did not significantly alter the prolactin release ($P\simeq.10$). In contrast, magnitude

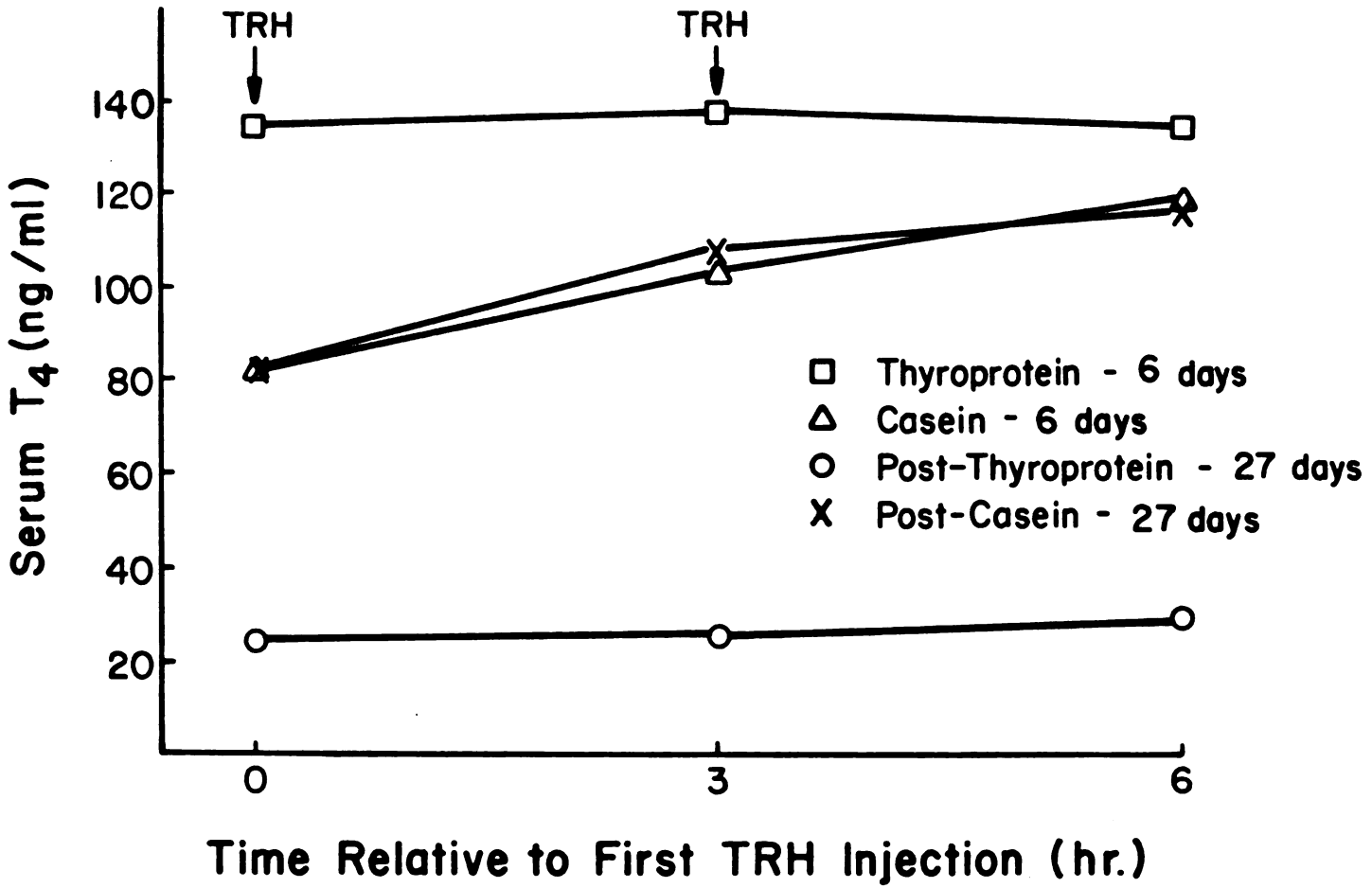


Figure 3. Serum thyroxine before and after iv injections of TRH (33.3 µg/100 kg BW) to thyroprotein (6 □-□ or 27 o-o days) or casein (6 Δ-Δ or 27 x-x days) treated Holstein heifers.

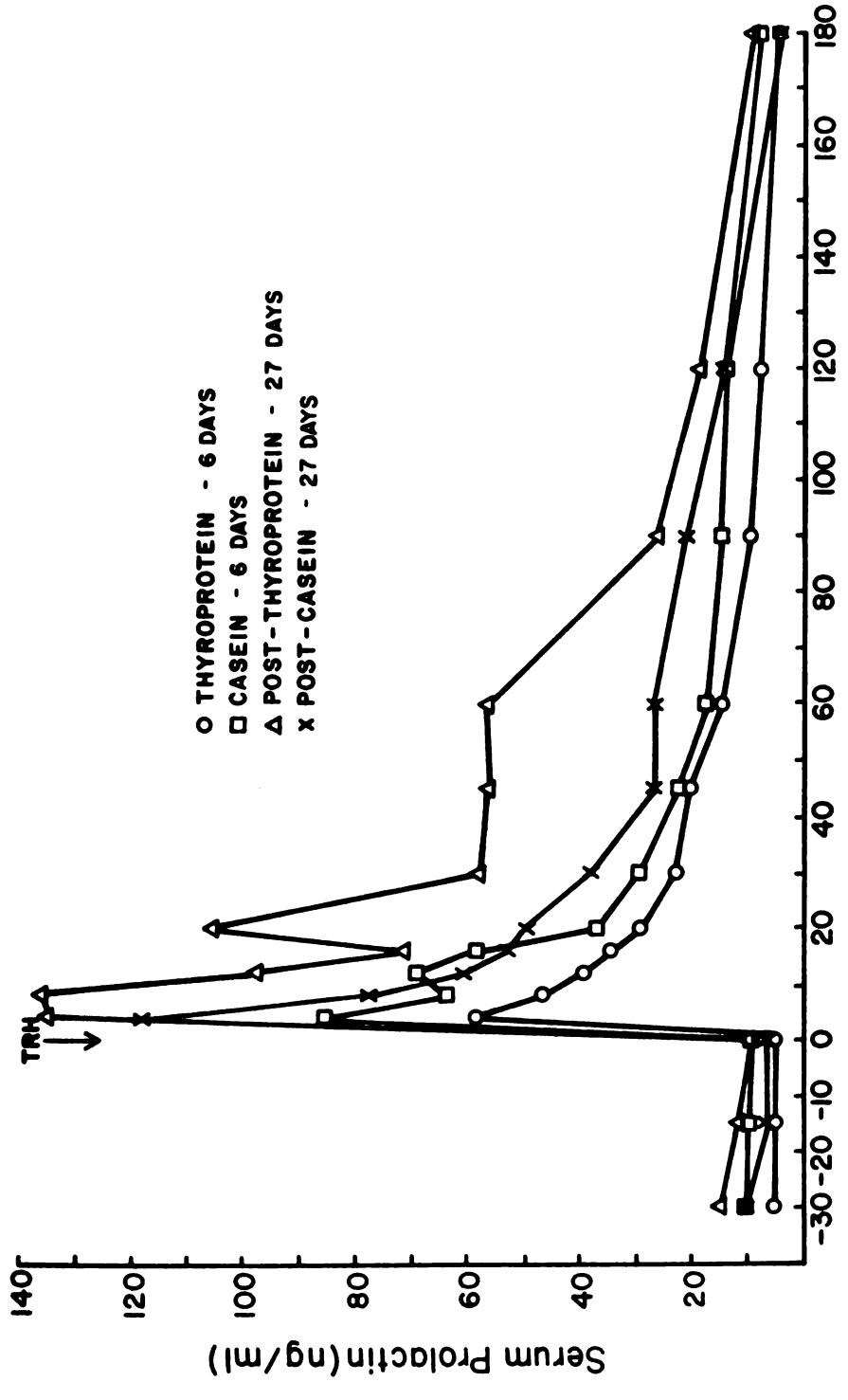


Figure 4. Serum prolactin before and after iv injections of TRH (33.3 μg/100 kg BW) to thyroprotein (6 o-o or 27 Δ-Δ days) or casein (6 □-□ or 27 x-x days) treated Holstein heifers. Data pooled from two TRH injections three hr apart.

of increase in serum prolactin following TRH is inversely related to serum thyroxine concentrations in humans (Snyder et al., 1973; Yamji et al., 1974) and rats (Vale et al., 1973).

Although prolactin release after TRH was not different between treatment groups, the analysis of variance did reveal a difference ($P<.05$) in prolactin response from the first to second TRH injection, regardless of treatment (figure 5). Thus, the magnitude of increase in serum prolactin after the first TRH injection (Δ=132.6 ng/ml) was greater ($P<.05$) than the response after the second injection (Δ=51.7 ng/ml). Similarly, the increase in serum GH concentration induced by the first TRH challenge (Δ=36.8 ng/ml) was greater ($P<.05$) than the increase after a second injection (Δ=9.6 ng/ml) regardless of treatment (figure 6). In view of the fact that changes in serum thyroxine concentrations induced by treatments did not alter magnitude of TRH-induced prolactin or GH release (see below for GH results) in these heifers, reduced prolactin or GH release after the second TRH injection probably was not due to increased serum thyroxine resulting from the first TRH injection. Rippel et al. (1974) and Mongkonpunya et al. (1975) observed a similar phenomenon with gonadotropin releasing hormone (GnRH)-induced luteinizing hormone release; increased serum luteinizing hormone was greatest after the first GnRH injection. Tucker et al. (1975) showed that, following an initial increase, serum prolactin and GH concentrations declined in the presence of a chronic TRH infusion. However, a subsequent injection of prostaglandin $F_{2\alpha}$ induced a further significant increase in serum prolactin and GH content, but to a "refractoriness" of the pituitary to the TRH stimulus. This "refractoriness" may represent altered TRH receptors and

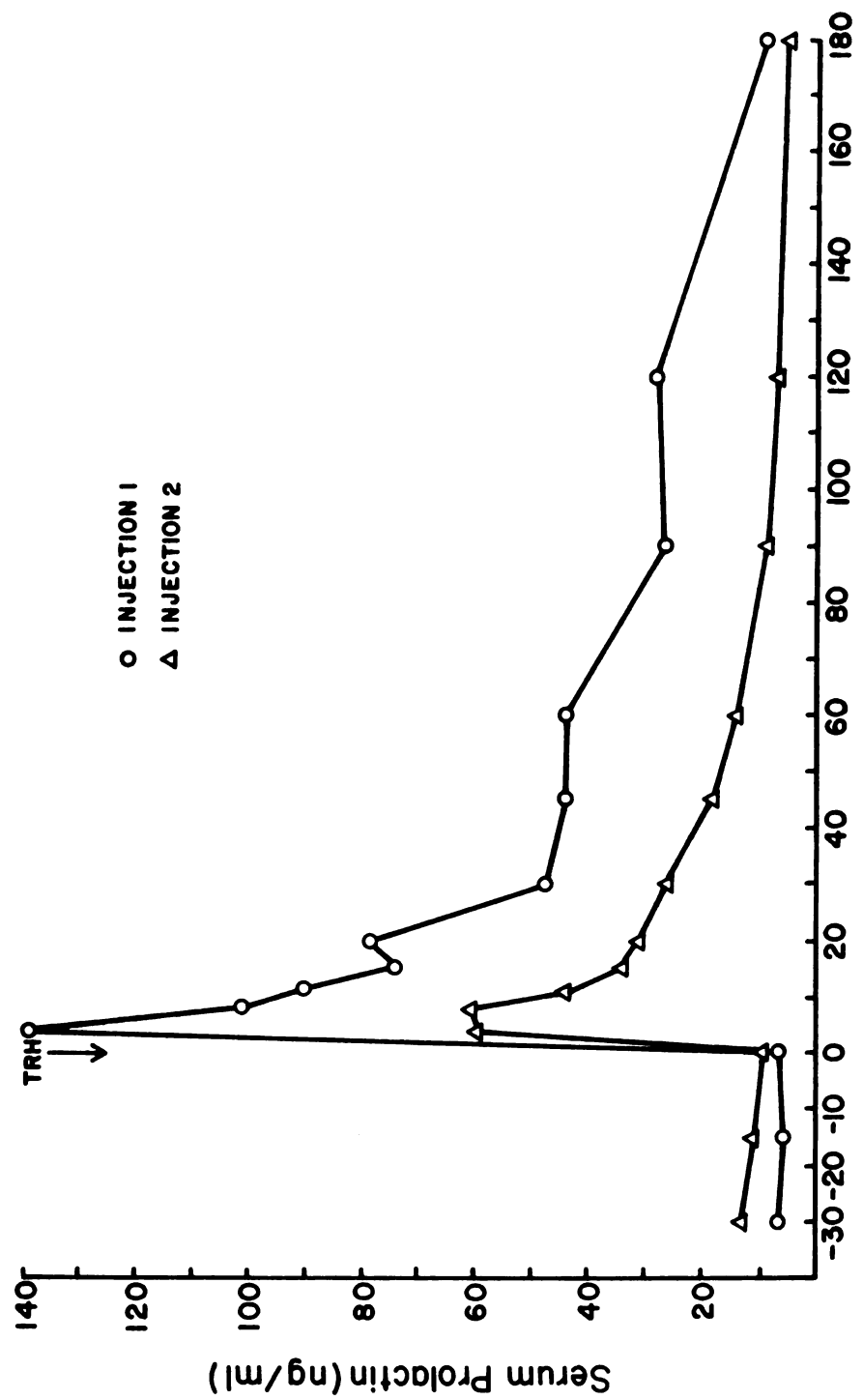


Figure 5. Serum prolactin before and after consecutive iv injections of TRH (33.3 μg/100 kg BW/injection) to Holstein heifers.

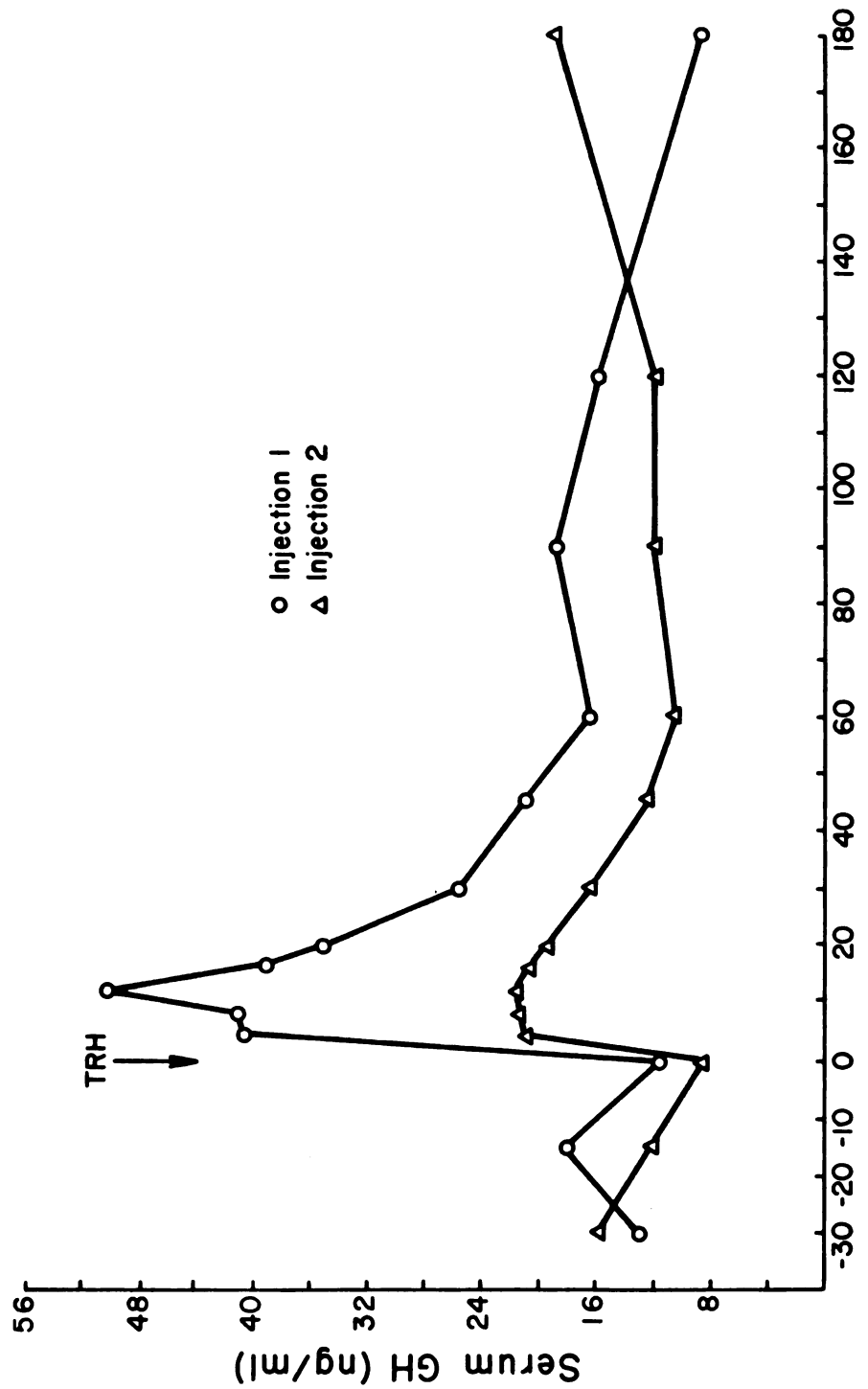


Figure 6. Serum GH before and after consecutive iv injections of TRH (33.3 μg/100 kg BW/injection) into Holstein heifers.

cell membrane properties, or an induced negative feedback upon prolactin and GH due to the increased serum hormone concentrations elicited by the first TRH injection.

Heifers in this experiment ranged in age from 5 to 11 months, and were therefore blocked by weight. Body weight significantly ($P<.05$) affected TRH-induced release of prolactin (figure 7), but not TSH or growth hormone. Furthermore, virtually identical positive correlations ($r = +.49$, $P<.05$) exist between heifer weight or age and average TRH induced prolactin response. Thus, the magnitude of prolactin release after TRH was less in lighter-younger heifers (blocks 1, 2 and 3) than in heavier-older heifers (blocks 4, 5 and 6) irrespective of treatment group or injection. Why the increase in prolactin caused by TRH should be grouped so distinctly into age-weight groups, particularly since there was no distinct break in age or weight between blocks 1 to 3 and 4 to 6, is not known. Average heifer weights for blocks 1 to 6 were 129, 139, 153, 176, 216 and 256 kg and average ages were 186, 178, 214, 248, 284 and 345 days. Some of these heifers were near puberty at the time of this experiment. However, progesterone determinations in serum collected on alternate days for 17 days, centered for all heifers on the day of TRH challenge, suggested that only the three heaviest heifers had ovulated. These three heifers did not exhibit unusually high TRH-induced prolactin responses.

4. Growth Hormone:

Average basal serum GH (-30, -15 and 0 min) concentrations were not altered by thyroprotein or casein feeding (figure 8), although there was a tendency for GH in serum of heifers given thyroprotein for days to be less than the respective values for the other groups ($P\simeq.10$). Similarly,

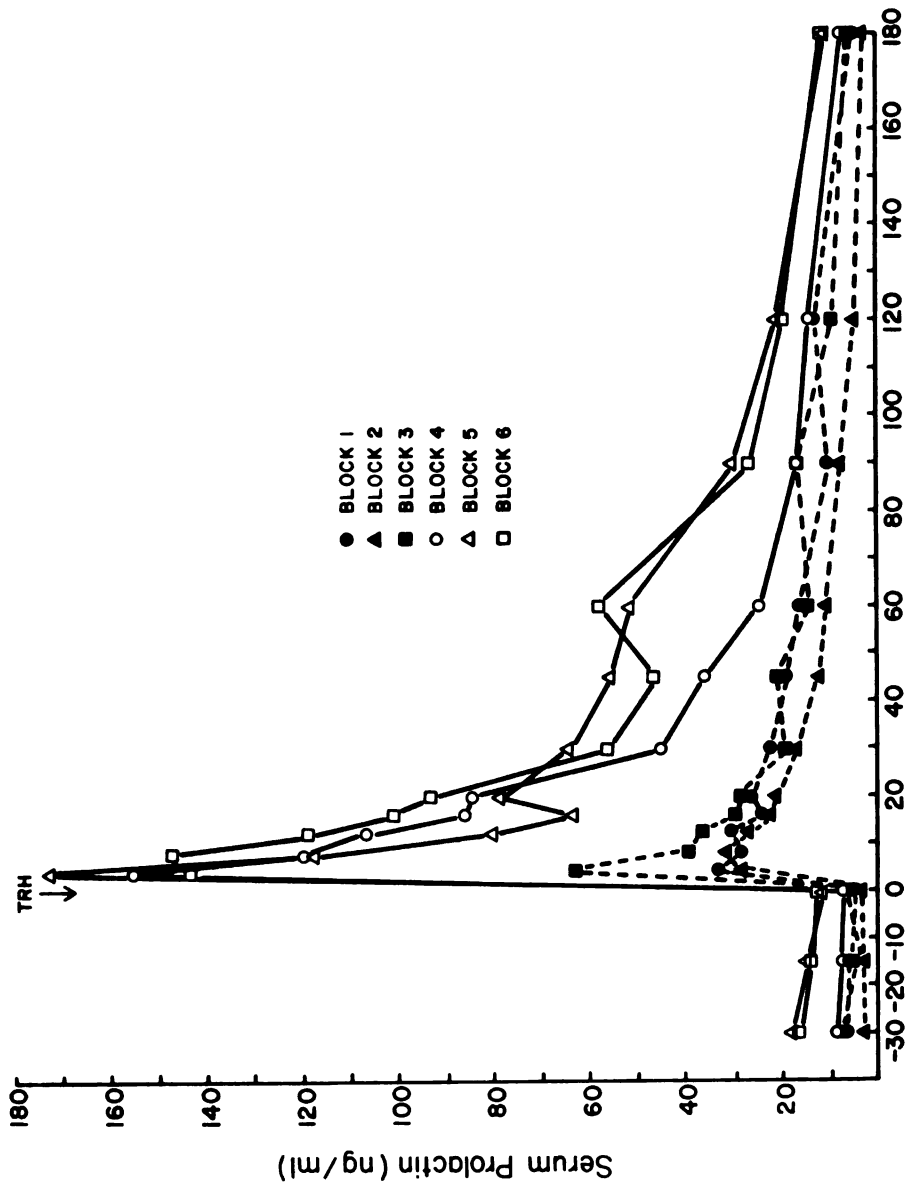


Figure 7. Serum prolactin before and after iv injections of TRH (33.3 μg/100 kg BW) into Holstein heifers blocked according to weight, lightest (block 1) to heaviest (block 6).

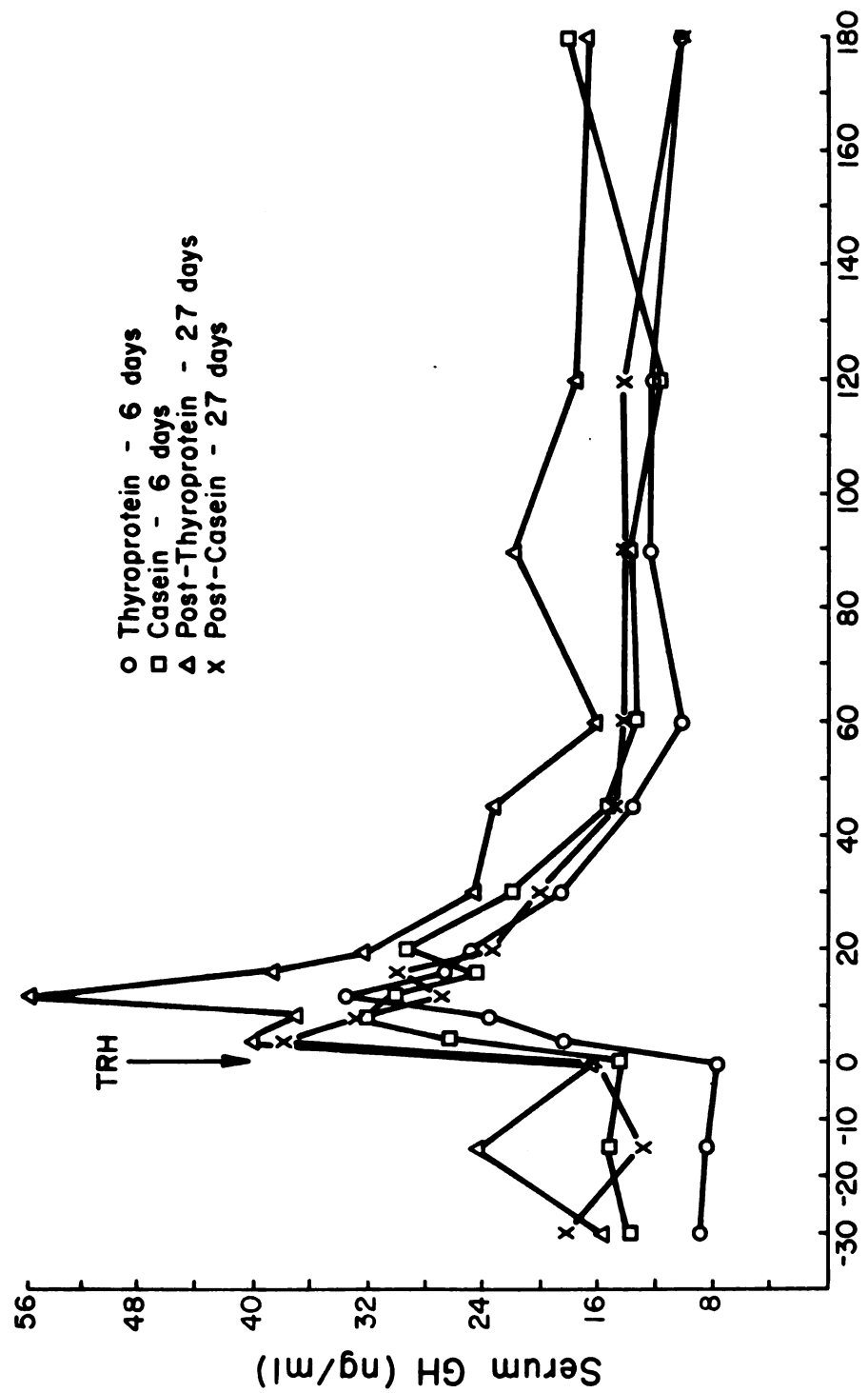


Figure 8. Serum GH before and after iv injections of TRH (33.3 μg/100 kg BW) to thyroprotein (6 o-o or 27 Δ-Δ days) or casein (6 □-□ or 27 x-x days) treated Holstein heifers. Data pooled from two TRH injections three hr apart.

feeding thyroprotein to lactating cows for 13 weeks (Shaw et al., 1975) or thyroidectomizing lambs (Davis and Borger, 1973) did not change serum GH concentrations. Adult humans with hypothyroidism exhibited higher serum GH concentrations than those with hyperthyroidism (Hamada et al., 1976), while Katz et al. (1969) noted that primary hypothyroid and thyrotoxic children had reduced serum GH compared with healthy children. However, in rats, thyroid insufficiency reduced basal serum GH (Eisenberg et al., 1972; Peake et al., 1973).

Following TRH, serum GH consistently increased in all groups of heifers from an overall average basal concentration (-30, -15 and 0 min) of 13.8 ng/ml to an average of 36.5 ng/ml at 12 min following TRH (figure 8), and differences between treatment means after TRH were not significant ($P>.05$). This is in contrast to rats in which the TRH-induced GH release is inversely related to serum thyroid hormone concentrations (Carlson et al., 1974; Kato et al., 1975; Chihara et al., 1976).

B. Experiment Two: TRH-Induced TSH, Prolactin and GH Release: Dose-Related Responses in Vitro.

Thyrotropin releasing hormone added to bovine anterior pituitary cell cultures on day 5 caused TSH and prolactin to be released in quantities that were directly related to dose of TRH administered. In contrast, growth hormone release was not related to the dose of TRH used, but was increased ($P<0.05$) at relatively high doses of TRH.

Increasing amounts of TSH were released from bovine pituitary cells when TRH was added to the incubation media in doses ranging from 1 to 10^4 pg TRH/ml as illustrated in figure 9A. Based on linear regression analysis, basal TSH release during the 2-hr incubation period averaged 117.4 ng/ml and increased by 60.5 ng/ml increments with every 10-fold

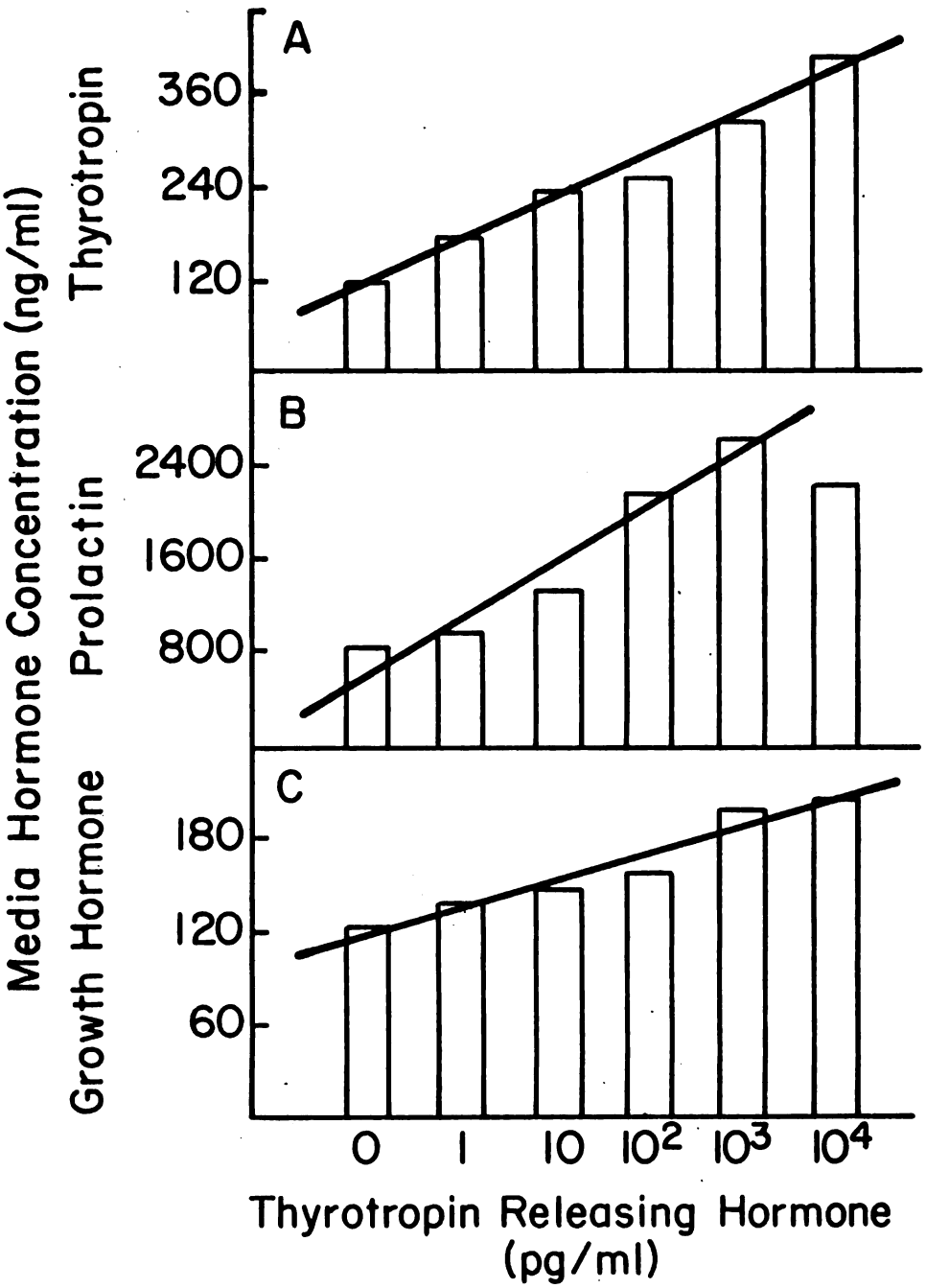


Figure 9. Thyrotropin (A), prolactin (B) and GH (C) concentration of medium from bovine anterior pituitary cell cultures incubated in media containing TRH (1 - 10^4 pg) for 2 hours. Slope of linear regressions and correlation coefficients are described in the text. n = 7

increase in dose of thyrotropin releasing hormone. The correlation coefficient between TRH dose and the amount of TSH released was r = .87 ($P<.05$).

Prolactin release also increased in a stepwise fashion when the culture media was supplemented with 1 to 10^3 pg TRH/ml ($P<.05$). These pituitary cell cultures secreted prolactin at a rate of 817.9 ng/ml media during a 2-hr period when TRH was not added to the media and increased an additional 550.4 ng/ml media with every 10-fold increase in thyrotropin releasing hormone. Increasing the concentration of TRH to 10^4 pg/ml media resulted in an increase in prolactin release which was less than that caused by 10^3 pg TRH/ml (figure 1B). Smith and Convey (1975) also noted decreased prolactin release by 100 ng TRH/ml media compared to lower doses.

Thus, the minimum dose of TRH required to increase TSH and prolactin release was the same; that is, 1 pg TRH/ml media. Noel et al. (1974) came to similar conclusions after infusing small doses of TRH into humans. These results support the premise that TRH may have a physiological role in regulating prolactin as well as TSH secretion.

Quantities of GH released into the media after adding increasing concentrations of TRH are shown in figure 9C. The regression of media GH concentration on dose of TRH is described by a significant slope (16.9; $P<.05$) and correlation coefficient (.54; $P<.001$). Adding 10^3 or 10^4 pg TRH/ml media increased ($P<.05$) GH release 53% relative to control values. However, media GH of cells exposed to 0, 1, 10 or 100 pg TRH/ml were not different from one another; nor were response to 10^3 or 10^4 pg TRH/ml different from each other. We interpret these data to indicate that only higher doses of TRH increase GH release and that the magnitude

of this additional release is not dependent upon the TRH dose (up to 10^4 pg/ml media in our system). This result supports the view that TRH does not play a physiological role in GH regulation although large doses of exogenous TRH can increase GH release. However, one should not discount the possibility that the somatotroph population in this particular experiment was such that the TRH doses were below the point of sensitivity of this bioassay.

C. Experiment Three: Thyrotropin, Prolactin and Growth Hormone Secretion by Bovine Pituitary Cells Exposed to Thyroxine and TRH.

1. Thyrotropin:

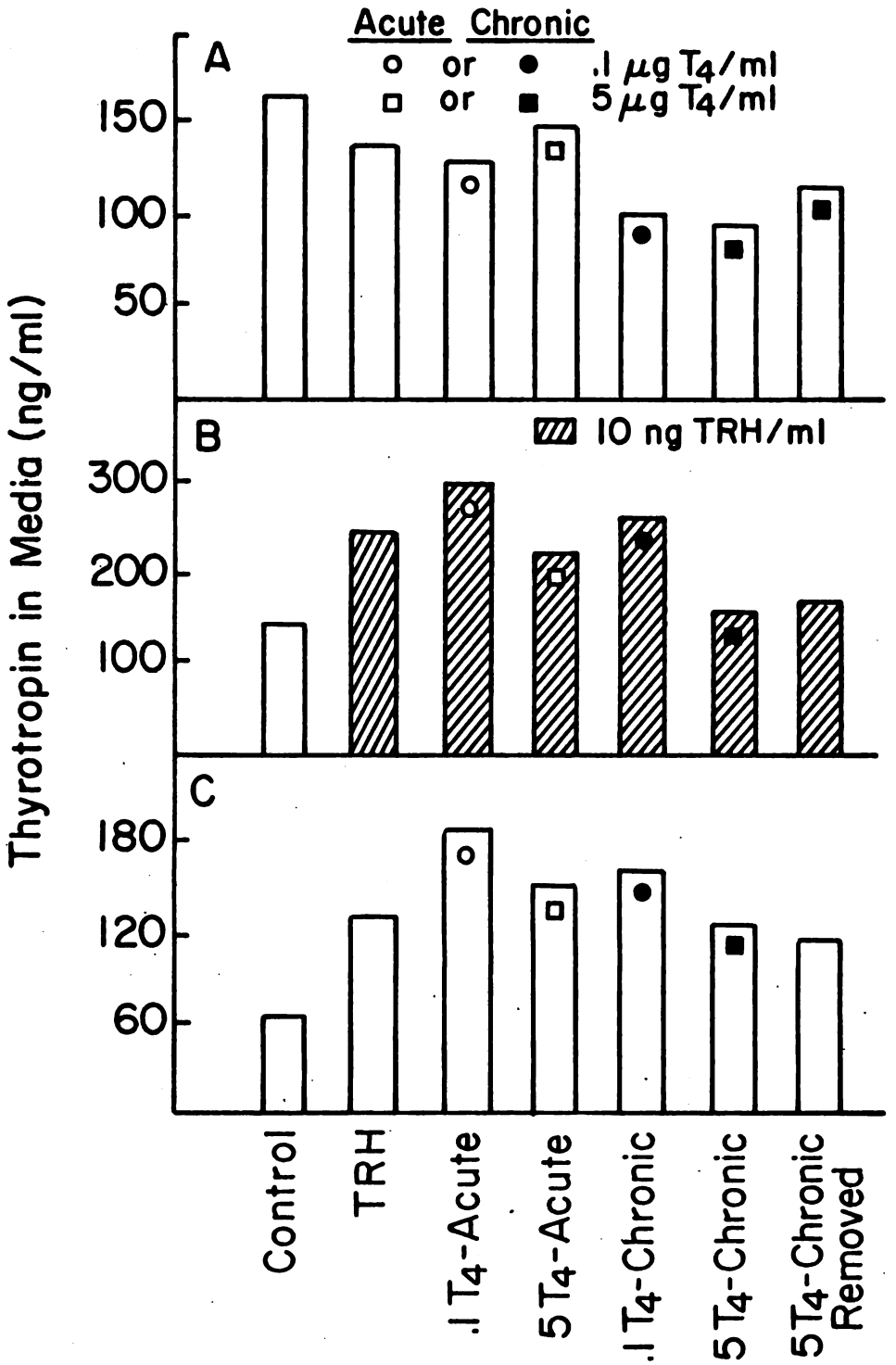
Effects of including thyroxine in the culture media at concentrations of .1 or 5 μg/ml for 2 (acute) or 24 (chronic) hr on TSH secretion by pituitary cells is shown in figure 10. Chronic exposure of these cells to .1 or 5 μg thyroxine decreased ($P<0.05$) basal TSH secretion to 103 and 94 ng/ml, respectively, relative to cultures not given thyroxine which averaged 154 ng/ml (figure 10A). Acute thyroxine treatments did not alter the amount of TSH release. However, analysis of variance revealed a significant ($P<0.01$) treatment by experiment interaction resulted from a failure of thyroxine to alter basal TSH release in 2 of 3 experiments.

Media TSH concentration in cultures not exposed to TRH averaged 148 ng/ml and was increased ($P<0.01$) to 248 ng/ml by TRH (10 ng/ml) (figure 10B). Acute, but not chronic, exposure of pituitary cells to .1 μg thyroxine/ml media augmented ($P<0.01$) TRH-induced TSH release to 301 ng/ml media. In contrast, chronic but not acute exposure of pituitary cells to 5 μg thyroxine/ml media completely inhibited ($P<0.01$) TRH-induced TSH release. Removal of thyroxine (5 μg/ml) prior to addition

of TRH did not prevent the inhibitory effect of chronic thyroxine exposure on TRH-induced TSH release. The pattern of TSH release during the 2-hr period following TRH was similar to that of the preceding period (figure 10C).

These results support the view that thyroxine has the potential to feedback directly on the pituitary to reduce basal TSH secretion in cattle. Davis and Borger (1973) and Hopkins et al. (1975) reported increased serum TSH concentration following thyroidectomy in ewes. Hopkins et al. (1975) reported no change, while Davis and Anfinson (1976) observed decreased TSH concentration in intact ewes given prolonged infusions of thyroid hormones. The increases in TSH release by TRH observed here is in agreement with other reports. Thus, bovine pituitary primary cell cultures released more TSH if TRH was included in the culture media (LaBella and Vivian, 1971). Likewise, the inhibitory effect of 5 μg thyroxine/ml media on TRH-induced TSH release in these bovine pituitary cells appears similar to that reported for rats (Vale et al., 1972) and humans (May and Donabedian, 1973). Vale et al. (1972) demonstrated that the degree to which thyroxine inhibited TRH-induced TSH release was directly related to dose of thyroxine used. In the present experiment adding .1 μg thyroxine/ml media for only 2 hr increased the magnitude of TRH-induced TSH release, while 5 μg thyroxine/ml media present for 24 hr reduced it. On the basis of this observation, one could speculate that thyrotrophs, like other somatic cells have a thyroxine requirement for maximum function. Alternatively, .1 μg thyroxine/ml media may have reduced basal TSH release, thereby increasing cellular TSH reserve, but was not at a concentration high enough to block TRH-induced TSH release.

Figure 10. Thyrotropin concentration of medium from bovine anterior pituitary cell cultures incubated with thyroxine (T_4) for 2 hr (.1 μg/ml o or 5 μg/ml □) or 24 hr (.1 μg/ml ● or 5 μg/ml ■) before adding 10 ng TRH/ml (slashed bars). Medium was collected from all flasks prior to (A), during (B) and after (C) TRH. Periods were 2 hr in duration. Standard error of mean calculated from error mean square was 12.3 for n = 18.


Acute Chronic
A
o or ● .1 μg T4/ml
□ or ■ 5 μg T4/ml
150
100
50
B
10 ng TRH/ml
300
200
100
C
180
120
60
Thyrotropin in Media (ng/ml)
Control
TRH
.1 T4-Acute
5 T4-Acute
.1 T4-Chronic
5 T4-Chronic
5 T4-Chronic Removed

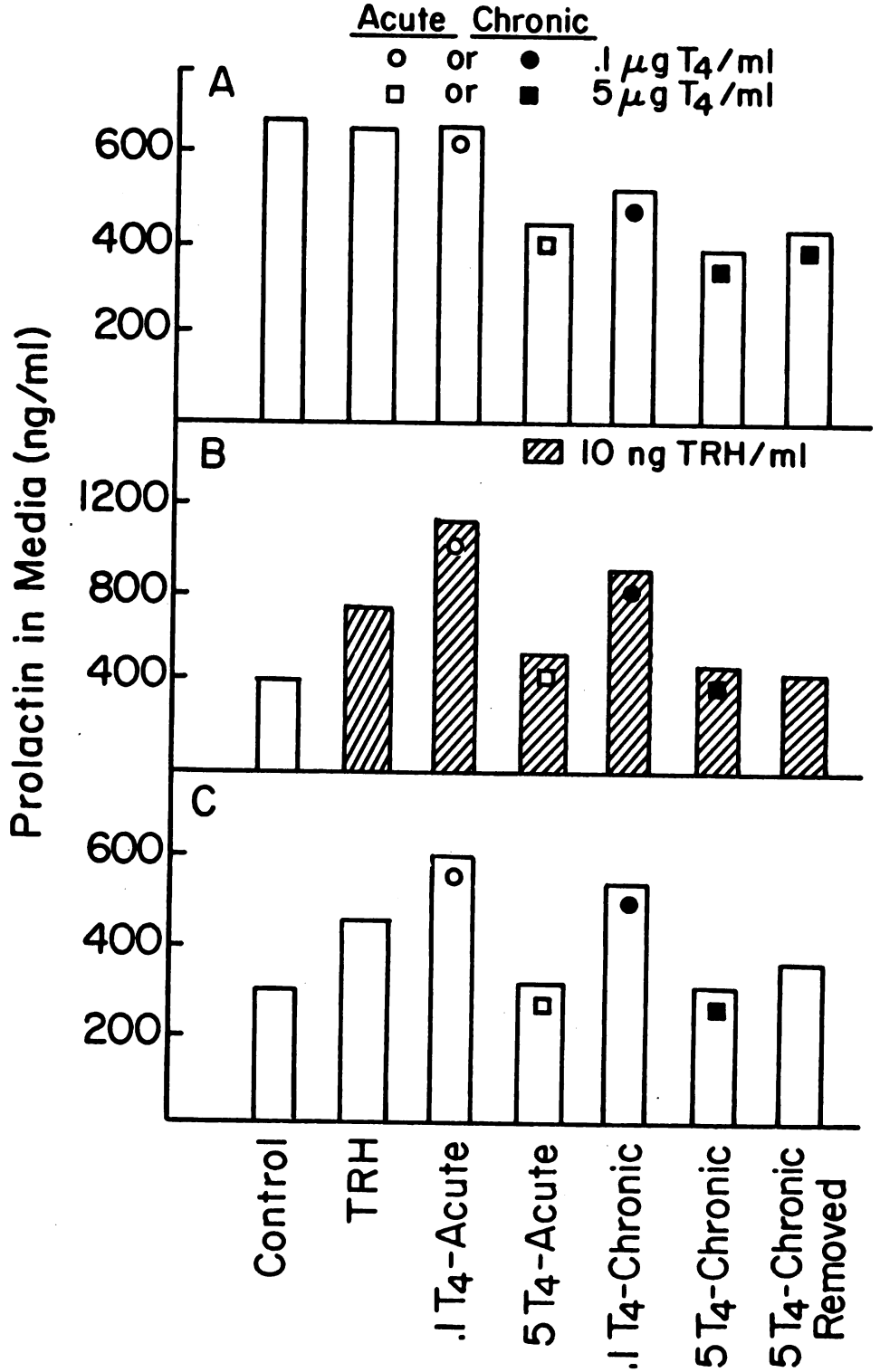
2. Prolactin:

The effect of thyroxine and TRH on prolactin release by bovine anterior pituitary cells is shown in figure 11. Basal prolactin release averaged 677 ng/ml in cultures not supplemented with thyroxine and was reduced ($P<0.05$) to 449 and 387 ng/ml by chronic inclusion of .1 or 5 μg thyroxine in the culture media (figure 11A). In addition, acute (2 hr) exposure of these cells to 5 but not .1 μg thyroxine/ml media decreased ($P<0.05$) prolactin release relative to controls.

In cells not treated with TRH, prolactin release averaged 426 ng/ml media and increased ($P<.05$) to 769 ng/ml when TRH (10 ng/ml) was added to the media (figure 11B). The degree to which TRH increased prolactin release was augmented ($P<.05$) by inclusion of .1 μg thyroxine/ml media, and the increase was greater ($P<.05$) when this dose of thyroxine was present for 2 hr (1155 ng/ml) rather than for 24 hr (929 ng/ml) (figure 11B). Conversely, TRH-induced prolactin release by pituitary cells incubated in 5 μg thyroxine/ml media for 2 hr (543 ng/ml) or 24 hr (480 ng/ml was inhibited ($P<0.05$); prolactin release was not different from that of cells given no TRH challenge. Chronically adding thyroxine (5 μg/ml) reduced TRH-induced prolactin release irrespective of whether thyroxine was present in the media at the time TRH was added.

The relative pattern of prolactin release between treatment groups for the 2-hr period following removal of TRH from the media was similar to that of the previous period when TRH was present in the media (figure 11C). This prolonged stimulatory effect of TRH on prolactin release *in vitro* relative to that resulting from TRH injection *in vivo* may reflect the absence of a negative feedback which is operative in the living animal. For example, a "shoot-loop" negative feedback of prolactin on

Figure 11. Prolactin concentration of medium from bovine anterior pituitary cell cultures incubated with thyroxine (T_4) for 2 hr (.1 μg/ml o or 5 μg/ml □) or 24 hr (.1 μg/ml ● or 5 μg/ml ■) before adding 10 ng TRH/ml (slashed bars). Medium was collected from all flasks prior to (A), during (B) and after (C) TRH. Periods were 2 hr in duration. Standard error of mean, calculated from error mean square, was 55.6 for n = 18.


Acute Chronic
o or ● .1 μg T4/ml
□ or ■ 5 μg T4/ml
A
600
400
200
B
10 ng TRH/ml
1200
800
400
C
600
400
200
Prolactin in Media (ng/ml)
Control
TRH
.1 T4-Acute
5 T4-Acute
.1 T4-Chronic
5 T4-Chronic
5 T4-Chronic Removed

the hypothalamic centers controlling prolactin release has been demonstrated in rats (Clemens and Meites, 1968). Thus, the marked increase in prolactin concentration that occurs in serum of cattle given TRH may feedback to truncate duration of prolactin release.

These results suggest that thyroxine inhibits basal prolactin release. This is consistent with the observation that prolactin secretion increases in thyroidectomized sheep (Davis and Borger, 1973). However, relative to controls no change in serum prolactin concentration was observed after serum thyroid hormone concentrations were increased in cattle (Shaw et al., 1975) and sheep (Hopkins et al., 1975; Davis and Anfinson, 1976) or decreased in cattle (Shaw et al., 1975). However, serum hormone concentrations should not be assumed to parallel hormone release as Davis and Borger (1973) demonstrated that thyroidectomy reduced prolactin metabolic clearance rate in sheep. In contrast to our results, prolactin secretion by rat anterior pituitaries increased when incubated with the thyroid hormones (Nicoll and Meites, 1963). This discrepancy may be explained by species difference, culturing methods or assays. In addition, Nicoll and Meites (1963) included insulin in the culture media.

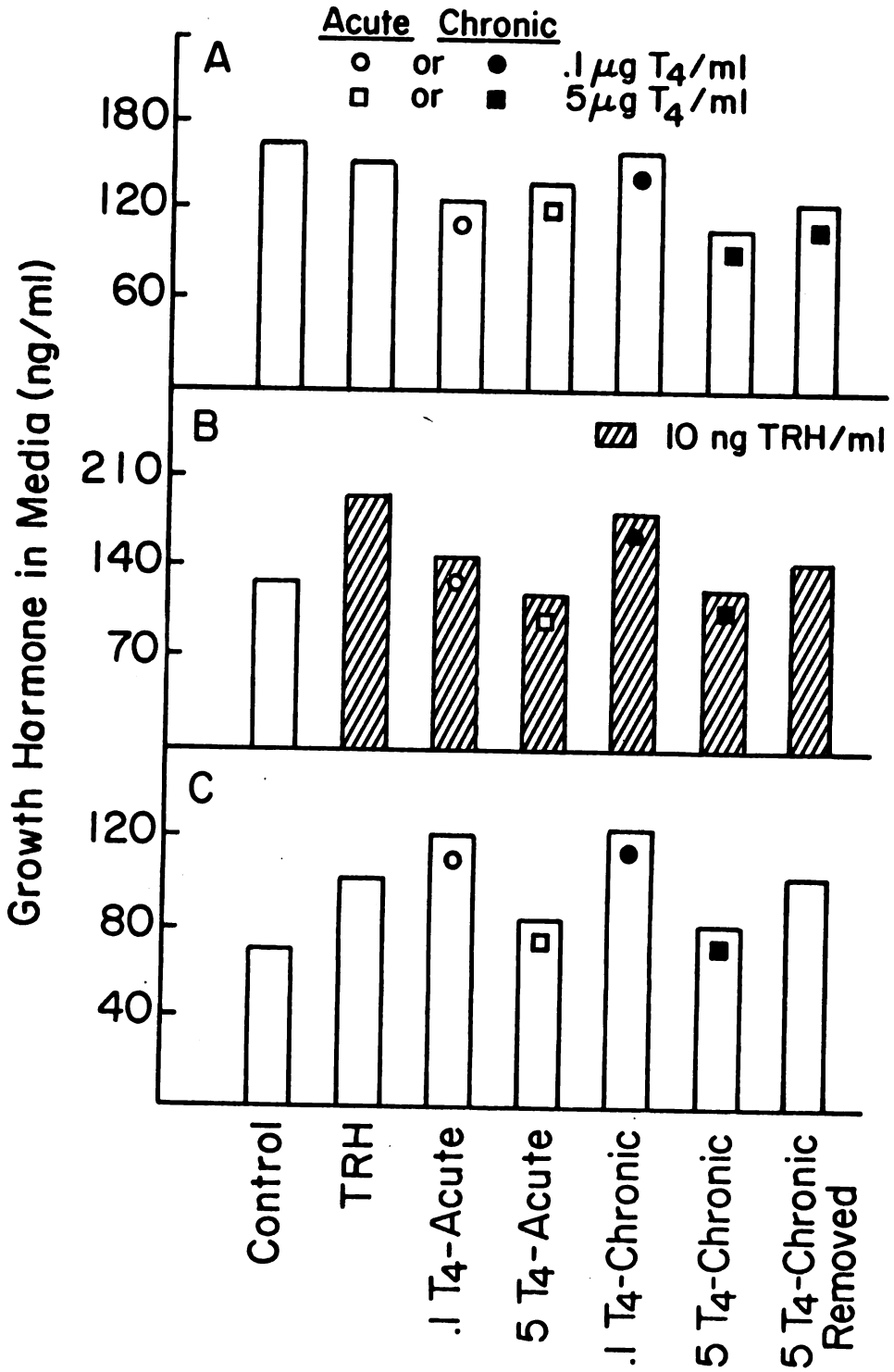
Our demonstration of increased prolactin release by pituitary cells incubated with TRH is in agreement with other studies in which bovine anterior pituitaries were cultured in vitro (Machlin et al., 1974; Smith and Convey, 1975). Thyroid hormones inhibit TRH-induced prolactin release in humans (Bowers et al., 1973; Snyder et al., 1973; Wartofsky et al., 1976) and rats (Vale et al., 1973). High serum concentrations of triiodothyronine (24 ng/ml) were effective in decreasing the magnitude of the normal TRH-induced prolactin releases in vivo in sheep (Davis and Anfinson, 1976).

3. Growth Hormone:

Addition of thyroxine to media for 2 or 24 hr at doses of .1 or 5 μg/ml did not alter the quantity of GH released from bovine anterior pituitary cell cultures in the absence of TRH (figure 12A). This supports other studies in which serum GH concentration was unchanged in cattle (Shaw et al., 1975) and sheep (Davis and Borger, 1973) after marked alterations of serum thyroid hormone concentrations.

Growth hormone release from bovine pituitary cells increased ($P<.05$) from 130 ng/ml in controls to 198 ng/ml when cells were incubated in media containing 10 ng TRH/ml; a 52% increase (figure 12B). Others have previously shown that exogenous TRH causes GH release from bovine pituitaries in vivo (Convey et al., 1973) and in vitro (LaBella and Vivian, 1971; Machlin et al., 1974). Magnitude of TRH-induced GH released by cells incubated in the absence of thyroxine (198 ng/ml) was greater ($P<.05$) than the overall average GH release of cells incubated with thyroxine (146 ng/ml) as determined by Bonferroni's t-test. Inhibition of TRH-induced GH release by chronically adding 5 ng thyroxine/ml was observed even when thyroxine was omitted during the 2 hr TRH incubation. Furthermore, 5 μg thyroxine/ml media was more effective ($P<.05$) in reducing TRH-induced GH release than .1 μg thyroxine/ml, whether present for 2 (121 vs 150 ng/ml) or 24 hr (126 vs 185 ng/ml). Thyroid hormones also reduce growth hormone release from rat pituitary tissue cultured in vitro (Carlson et al., 1974; Kato et al., 1975). TRH-induced GH release was not altered if cells were incubated in .1 μg thyroxine/ml media for 24 hours. This low level of thyroxine stimulates somatotroph growth (Venter et al., 1976). During the 2-hr period following exposure of these cells to TRH, magnitude of GH release into the media was not different ($P>.05$) between treatment groups (figure 11C).

Figure 12. Growth hormone concentration of medium from bovine anterior pituitary cell cultures incubated with thyroxine (T_4) for 2 hr (.1 µg/ml o or 5 µg/ml □) or 24 hr (.1 µg/ml ● or 5 µg/ml ■) before adding 10 ng TRH/ml (slashed bars). Medium was collected from all flasks prior to (A), during (B) and after (C) TRH. Periods were 2 hr in duration. Standard error of mean, calculated from error mean square, was 15.1 for n = 18.


Acute Chronic
A
o or ● .1 μg T4/ml
□ or ■ 5μg T4/ml
180
120
60
B
10 ng TRH/ml
210
140
70
C
120
80
40
Growth Hormone in Media (ng/ml)
Control
TRH
.1 T4-Acute
5 T4-Acute
.1 T4-Chronic
5 T4-Chronic
5 T4-Chronic Removed

4. Total Hormone in Culture System:

Cell, media and total hormone content for cultures incubated with and without TRH are presented in Table II. Media hormone content represents the sum of the 2-hr periods during and subsequent to TRH (or control) treatment. Inclusion of TRH in the culture media increased ($P<.001$) TSH, prolactin and GH release during the 4-hr period 80, 70 and 50%, respectively, compared with controls. However, in the presence of TRH, lactotrophs and somatotrophs maintained intracellular hormone (prolactin or GH) content while intracellular TSH was reduced 18% ($P<.05$) as compared with controls. Thus, of the three hormones measured, only total prolactin content changed ($P<.05$). Within 4 hr of the initial TRH exposure, total prolactin increased from 8,248 ng/flask in controls to 11,440 ng/flask in TRH treated cultures; a 38% increase. Therefore, TRH may be either increasing synthesis or decreasing degradation of prolactin. Prolactin synthesis was noted to increase within 4 hr after TRH was added in rat pituitary tumor cultures (Tashjian et al., 1971). Others have noted increased total TSH in rat pituitary culture systems (Wilber, 1971; Vale et al., 1972; Labrie et al., 1975), but in these cases TRH was present for 1-5 days.

Table II. The Effect of TRH on Media and Cellular TSH, Prolactin and GH Content in Bovine Anterior Pituitary Cell Cultures.[a]

		TSH	PROLACTIN	GH
			ng/flask	
Media[b]	control	848	2,952	800
	TRH	1,528[d]	4,960[d]	1,200[d]
Cells	control	2,608	5,296	15,224
	TRH	2,200[c]	6,480	17,024
Total	control	3,456	8,248	16,024
	TRH	3,728	11,440[c]	18,224

a = n = 18 per treatment group.

b = Media represents sum of TRH (or control) and post-TRH incubations; 2 hr each.

c = Different ($P<.05$) from control.

d = Different ($P<.001$) from control.

GENERAL DISCUSSION

The data presented here indicate that TRH stimulates release of TRH, prolactin and GH when infused into a jugular vein of heifers or incubated with bovine anterior pituitary cell cultures *in vitro*. However, these results should not necessarily be interpreted as proof that TRH plays a significant physiological role in regulating the secretion of these three pituitary hormones.

Schally *et al.* (1969) investigated activity of fractions of porcine hypothalamic extracts and noted that only one component contained TSH-releasing activity. Furthermore, all mammals examined thus far exhibit increased TSH secretion following TRH administration. Thus, TRH is portrayed as a very potent, if not the sole stimulator of TSH secretion in mammals. Thyrotropin-releasing hormone is also a potent, dose-dependent stimulator of prolactin release as has been demonstrated here and elsewhere (Noel *et al.*, 1974). However, unlike TSH, increased prolactin release can also be elicited with fractions of bovine hypothalamic extracts that can be differentiated from TRH on the basis of 1) elution pattern after chromatographic filtration, 2) insusceptibility to degradation after incubation with blood containing TRH-degrading-enzyme and 3) ability to consistently stimulate prolactin release in male rats not treated with estrogen (Dular *et al.*, 1974; Machlin *et al.*, 1974; Kokubu *et al.*, 1975). Furthermore, to the extent that it has been explored (principally in rats) there appears to be few occasions when serum TSH

and prolactin concentrations change in a parallel fashion. However, the secretion of pituitary hormones is most likely determined by several humoral factors. Therefore, any effect that TRH may exert upon prolactin release may be hidden by the effects of opposing agents.

On the other hand, TRH probably plays only a minor role in regulating GH secretion. Firstly, hypothalamic GH-releasing activity can be distinguished from TRH by the same parameters as were described for differentiating prolactin releasing activity (Wilber et al., 1971; Machlin et al., 1974; Szabo and Frohman, 1975). Secondly, doses of TRH capable of eliciting submaximal TSH and prolactin release in our bovine pituitary cell cultures, were ineffective stimulants of GH release. Doses of TRH sufficient to evoke a maximum prolactin response in cattle only induced increased serum GH concentration in a fraction of the cattle tested (Convey et al., 1973). Finally, when added to bovine pituitary cell cultures, TRH did not induce dose-dependent GH responses. This represents a major deviation from the apparent mechanism of action of other hypophysiotropic hormones. Another significant finding was that 10 ng TRH/ml media stimulated approximately 40% of the total cellular TSH and prolactin in the bovine pituitary cell cultures to be released, but less than 7% of the growth hormone.

Several studies indicate an inverse relationship exists between serum thyroid hormone concentrations and basal serum TSH concentrations in ruminants (Davis and Borger, 1973; Hopkins et al., 1975; Davis and Anfinson, 1976). This was observed in our in vitro, but not in vivo studies. Similarly, Davis and Anfinson (1976) reported that increased serum TSH concentration normally occurring in ewes after given TRH was inhibited by injecting triiodo-thyronine. We have shown that high thyroxine concentration eliminates TRH-induced TSH release in vivo (134 ng thyroxine/ml)

and reduces TRH-induced TSH release *in vitro* (5 μg thyroxine/ml). In contrast, lower concentration of thyroxine (.1 μg/ml) incubated with pituitary cells for 2 hr increased TRH-induced TSH release. Venter *et al.* (1976) obtained superior pituitary cell growth *in vitro* following addition of triiodo-thyronine. We have also noticed increased pituitary cell growth after adding triiodo-thyronine (unpublished observation). Thus, pituitary cells, including thyrotrophs, may require small quantities of thyroid hormone for growth.

Heifers, in which serum thyroxine concentrations were reduced to low levels (24 ng/ml), did not display altered basal or TRH-induced TSH release when compared with casein controls. However, these animals had been exposed to high serum thyroxine (greater than 90 ng/ml) for 27 days and showed evidence of impaired secretory responsiveness by thyrotrophs and thyroid cells to TRH and TSH, respectively. Thus, had hypothyroidism been induced differently in these animals, basal and TRH-induced TSH release might have been increased as reported in other species (Hopkins *et al.*, 1975, Shenkman *et al.*, 1973). One should keep in mind that the same argument can be made for prolactin and GH secretion, although there is less evidence to support this.

Basal prolactin release by pituitary cells cultured *in vitro* was reduced by thyroxine, but serum prolactin concentration in heifers was not changed by altering serum thyroxine concentration. Davis and Borger (1973) have demonstrated in sheep that thyroidectomy reduces prolactin secretion while high serum concentrations of triiodo-thyronine were ineffective at changing basal serum prolactin concentration (Davis and Anfinson, 1976). This is additional evidence that thyroxine can alter the rate of prolactin secretion. However, unless extreme serum thyroxine

concentrations are attained *in vivo* serum prolactin concentrations are not often altered. As was the case for TSH, TRH-induced release of prolactin by pituitary cells cultured *in vitro* was increased by .1 μg thyroxine/ml media and decreased by 5 μg thyroxine/ml media. Magnitude of serum prolactin concentration increase in heifers after TRH was not significantly changed by altering serum thyroxine concentration.

Basal GH release was not changed by thyroxine *in vivo* or *in vitro* which is consistent with results of studies done with sheep (Davis and Borger, 1973). Thyroxine reduced GH release after TRH, *in vitro*, but not *in vivo*. Magnitude of TRH-induced GH release is inversely related to serum thyroid hormone concentrations in rats (Carlson *et al.*, 1974; Kato *et al.*, 1975; Chihara *et al.*, 1976).

These results emphasize that thyroxine exerts various effects on hormone release; effects which are dependent upon experimental conditions and pituitary hormone. That is, results were dependent upon 1) whether the experiment was conducted *in vivo* or *in vitro*, 2) the individual *in vitro* culture, and 3) dose and duration of thyroxine treatment.

A number of possibilities exist to explain the difference between *in vivo* and *in vitro* results. Firstly, in the experiment conducted *in vivo*, the secretory responsiveness of thyroid and thyrotroph cells may have been reduced in heifers in which serum thyroxine concentrations were reduced due to previous chronic exposure (27 days) to high serum thyroxine concentration. A second possibility is that serum hormone concentrations were not representative of hormone secretion rates due to altered metabolic clearance rates of hormones. Altering thyroxine concentration in serum has been shown to change the metabolic clearance rate for thyroxine (Premachandra and Turner, 1961) and prolactin (Davis and Borger, 1973).

The difference in cell response *in vivo* and *in vitro* may result from differences between the complex hormonal milieu found in blood and the comparatively simple culture media. Blood contains triiodo-thyronine, TRH and probably other factors that may alter TSH, prolactin and GH release. Blood also contains other hormones (i.e., insulin, estrogens) that may influence cell growth or secretion in some way. In addition, blood contains enzymes capable of forming triiodo-thyronine via mono-deiodination of thyroxine. Finally, one should not lose sight of the possibility that differences in secretory patterns *in vivo* and *in vitro* may be a consequence of enzyme dispersion of cells.

Between-culture variation for effects of thyroxine on basal hormone release may result from uncontrolled variations in the culturing techniques. However, a more likely possibility is that the reproductive or thyroidal status or sex of pituitary donors affected the degree of responsiveness of the various pituitary cells. The possibility that sex of the donor may determine *in vitro* results appears reasonable in that: 1) basal serum TSH and thyroxine concentrations are higher in male than in female rats (Rapp and Tyun, 1974; Fukuda *et al.*, 1975), 2) basal serum prolactin concentration and TRH-induced TSH and prolactin release is greater in women than in men (Bowers *et al.*, 1971; Noel *et al.*, 1974), 3) prolactin release by TRH occurs in female or estrogen treated male rats but not in untreated male rats (Mueller *et al.*, 1973), and 4) basal serum prolactin and TSH concentrations fluctuate according to the day of the rat estrous cycle (Sar and Meites, 1967; Keiffer *et al.*, 1975) or after use of oral contraceptives by women (Lemarchand-Beraud *et al.*, 1974; Weeke and Prange, 1975).

Vale *et al.* (1972) demonstrated that the thyroxine inhibition of TSH release *in vitro* was dose dependent. However, we have demonstrated bi-phasic effects of thyroxine on TRH-induced TSH and prolactin release *in vitro*. That is, .1 μg thyroxine/ml media stimulated while 5 μg thyroxine/ml media inhibited TRH-induced release of TSH and prolactin. This bi-phasic effect of thyroxine on TRH-induced TSH and prolactin release may reflect two actions of thyroxine at different loci (i.e., release and synthesis) or single action demonstrating a dose dependency.

SUMMARY AND CONCLUSIONS

In vivo and *in vitro* studies were conducted to examine the effects of thyrotropin-releasing hormone (TRH) and thyroxine on thyrotropin (TSH), prolactin and growth hormone (GH) secretion in cattle.

When Holstein heifers, 5-11 months of age, were fed thyroprotein for 6 days, serum thyroxine concentration increased to 134 ng/ml compared with 82 ng thyroxine/ml in heifers fed casein. Despite this increase in serum thyroxine, neither basal serum concentrations of TSH, prolactin and GH, nor the magnitude of the TRH-induced prolactin and GH release were altered. However, the TSH release normally seen after TRH administration was eliminated when serum thyroxine concentrations were increased.

On day 33, serum thyroxine concentration averaged 24 ng/ml in heifers fed thyroprotein for 27 days which was lower than corresponding values for heifers fed casein during the same period (82 ng/ml). Neither basal nor TRH-induced TSH, prolactin or GH release were significantly altered on day 33 in these heifers. However, while serum thyroxine was at concentrations characteristic of hypothyroidism on day 33 in heifers fed thyroprotein for 27 days, there were indications suggesting that secretory mechanisms of thyrotrophs and thyroid cells were impaired due to 27 days of high serum thyroxine concentrations.

The magnitude of increases in serum prolactin, GH, but not TSH concentration were greater after the first TRH injection than after the second. Based on previous data from our laboratory, we conclude that this probably is not due to a depletion of pituitary hormone.

Within the scope of this experiment, we can conclude that thyroxine inhibits TRH-induced TSH release, but does not significantly change serum concentrations of prolactin and GH in peripubertal heifers.

Incubation in a bovine anterior pituitary cell culture in .1 or 5 μg thyroxine/ml media for 24 hr reduced TSH release to 103 and 94 ng/ml and prolactin release to 526 and 387 ng/ml, respectively, compared with 154 ng TSH/ml and 677 ng prolactin/ml in cultures not treated with thyroxine. Incubating cells in 5 μg thyroxine/ml media also effectively reduced prolactin release to 449 ng/ml media. Basal GH release was not changed by these treatments. Thyrotropin, prolactin and GH release were increased 67, 80 and 52% upon addition of 10 ng TRH/ml media to cell cultures. Adding TRH to cell cultures previously incubated for 2 hr in .1 μg thyroxine/ml media increased TSH (301 ng/ml) and prolactin release (1155 ng/ml) and reduced GH release (150 ng/ml) relative to TRH-induced TSH (248 ng/ml), prolactin (769 ng/ml) and GH release (198 ng/ml) in the absence of thyroxine. On the other hand, TRH-induced hormone release was reduced by adding 5 μg thyroxine/ml media for either 2 hr (543 ng prolactin/ml and 121 ng GH/ml) or 24 hr incubations (156 ng TSH/ml, 480 ng prolactin/ml and 126 ng GH/ml) compared to the aforementioned controls. Inasmuch as TRH increased TSH, prolactin and GH release, only lactotrophs and somatotrophs maintained intracellular hormone (prolactin and GH) content. Thus, TRH either increased synthesis or decreased degradation of prolactin within 4 hr as there was 38% more total prolactin in TRH-treated cultures than in controls.

We conclude that TRH stimulates TSH, prolactin and GH release *in vitro* and *in vivo*. Further, TRH increases prolactin synthesis in cell cultures within 4 hr of its addition. Thyroxine in the media reduces

basal release of TSH and prolactin and appears to exert a dose-dependent bi-phasic affect on TRH-induced TSH and prolactin release while inhibiting TRH-induced GH release only at the higher dose.

We have reported some seemingly discrepant results between our in vivo and in vitro experiments. That is, high serum thyroxine concentrations (134 ng/ml) in vivo only reduced TRH-induced TSH release, while adding thyroxine (.1 or 5 μg/ml) to cell cultures in vitro decreased basal release of TSH and prolactin and increased or decreased TRH-induced TSH, prolactin and GH release, depending on the dose of thyroxine. In addition, we observed experiment x treatment interactions within the in vitro studies. Namely, basal TSH and prolactin release were not reduced in all experiments and the magnitude of thyroxine effect on TRH-induced hormone release varied significantly among experiments. We feel these discrepancies and interactions can best be explained on the basis of 1) differences in culturing procedures from one set of cultures to the next, 2) reproductive and thyroidal status and sex of the experimental animals or pituitary donors, 3) dose of thyroxine administered, 4) differences between culture media and blood serum, and 5) alteration in the integrity of pituitary cells after 5 days of culture in vitro.

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