REGULATION OF BOVINE ANTERIOR PITUITARY HORMONES RELEASE AND SYNTHESIS

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ABSTRACT

REGULATION OF BOVINE ANTERIOR PITUITARY HORMONES RELEASE AND SYNTHESIS

By

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A series of experiments were designed to evaluate the interactions of specific and nonspecific agents which regulate bovine luteinizing hormone (LH) and prolactin release. In *in vivo* experiments the animals were cannulated one day before the experiments began. Gonadotropin releasing hormone (GnRH) was administered in a single injection via a jugular cannula in 10 ml of isotonic saline. Frequent venous blood samples (10 ml) were taken to monitor LH release. The apparatus for *in vitro* studies involved a multiple flow-through system for continuous superfusion of anterior pituitary slices. Incubation chambers and reservoirs containing incubation medium (TC 199) were submerged in a thermoregulated bath at 37°C. Incubation media were kept under continuous gassing (300 m./min) with humidified 95% O_2 : 5% CO_2. Rate of flow of the incubation media varied from 0.2 to 1 ml/min and 2 ml samples were collected at specified intervals. LH and prolactin in blood serum of effluent medium were measured by radioimmunoassays.

LH concentration in serum of heifers averaged 0.5 ng/ml and increased to peaks of 1.9, 7.3, and 11.1 ng/ml after a single iv injection of 5, 20, or 80 µg GnRH, respectively. Similarly, LH concengration in serum from mature bulls average 1.1 ng/ml before GnRH and increased to peaks of 9.2, 19.3, and 39.1 ng/ml after 10, 40, and 160 µg GnRH, respectively. The interval from GnRH to the LH peak and magnitude of the peak were positively correlated.

There was no difference in LH response to GnRH of cows treated during the late luteal phase (day 15) of the estrous cycle and the follicular phase ($x \simeq 20$); day following CL regression to size 1.0-1.2 cm. GnRH significantly enhanced serum LH averaging at the peaks 1.8, 7.7, and 13.9 ng/ml on day 15 and 1.0, 13.6, and 8.8 ng/ml on day 20 following 5, 40, and 320 µg of synthetic GnRH, respectively. Serum estradiol 17 concentration prior to GnRH administration was directly associated with LH response to GnRH. Serum estradiol concentration was increased 4 hours after GnRH injection on day 15, but decreased on day 20. No significant effect on mean estrone and progesterone levels was observed. It was concluded that not only the dose of GnRH administered but also the pretreatment estrogen environment significantly influenced the LH response to GnRH.

LH was released by GnRH *in vitro* indicating a direct action of this decapeptide on pituitary gonadotrophs. LH averaged 0.25 ng/mg/min in the effluent media from continually superfused steer pituitary tissue prior to exposure to GnRH; LH increased ($P < .001$) to 0.70 and 1.2 ng/mg/min after 4 min exposure and to 1 and 4 ng purified porcine GnRH/ml medium, respectively. Comparable values were 0194 and 1.50 ng/mg/min after 20 min. Average LH concentration in the effluent media decreased 8 and 33% ($P > .05$) during the 40 min period following exposure to 4 and 1 ng/ml GnRH, respectively, but these LH concentrations were still 340 and 100% greater than control values at the termination of incubation.

Relative potencies of highly purified porcine and synthetic preparations were comparable. The increases in medium LH concentrations with time during exposure to natural or synthetic GnRH were parallel. When superfusion with TC medium 199 was resumed, LH release decreased linearly ($P < .01$) as the incubation period advanced, but was still more than twice the pre-exposure baseline when the incubation was terminated 4 hr after first exposure to GnRH. These results indicate possible binding of the releasing hormone to the anterior pituitary cell membrane.

Physiological or 1000x physiological concentrations of estradiol 17β (10 pg or 10 ng/ml) did not have any

significant effect on the rate of LH and prolactin release from steer anterior pituitary tissue in vitro. Four hour incubation with 0.10 pg/ml or 10 ng/ml estradiol 17β did not change the rate of LH release from the anterior pituitary tissue of 4 steers. Similarly, anterior pituitary tissue from cows was incubated in a 3 x 3 factorial experiment with progesterone (0, 0.05, and 5 μg/ml) and estradiol 17β (0, 0.05, and 0.50 μg/ml) as the main effects. Neither estradiol 17β nor progesterone significantly altered the rate of LH release from cow anterior pituitary tissue.

Rate of LH release from steer pituitary increased from 0.14 to 1.27 ng/mg/min within first 16 min of exposure to 59mM potassium KRB. The enhanced potassium-induced LH release was completely abolished in calcium-free KRB. The enhanced potassium shortened the time of GnRH-induced LH release in calcium-free KRB. Absence of calcium in KRB decreased ($P < .01$) rate of LH release from base line of 0.18 of 0.18 to 0.08 ng/mg/min. Subsequent addition of 1 or 4 ng/ml GnRH increased the LH release to 0.23 ng/mg/min.

REGULATION OF BOVINE ANTERIOR PITUITARY HORMONES RELEASE AND SYNTHESIS

By

Josef Zolman

A DISSERTATION

Submitted to
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in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

Department of Dairy

1973

To My Parents.

BIOGRAPHICAL SKETCH

Josef Zolman was born in Hradec Kralove, Czechoslovakia, July 8, 1942, and grew up mostly in rural areas of Northeastern Bohemia. He attended public schools in Teplice and Broumov, Czechoslovakia, and graduated from High School in June 1958. The next three years were spent in undergraduate schools and in 1961 he received a Bachelor of Science degree in Animal Husbandry with a minor in Plant Production from the Agricultural Technical College, Caslav, Czechoslovakia. He accepted a graduate assistantship in the Department of Animal Genetics at the University of Brno in September 1961 and completed a thesis entitled "Biometric Analysis of Fertility Indices of Different Breeds of Bulls" in June 1966. Thereafter he spent one year in the Army Officers Training Program.

He was awarded a predoctoral research fellowship by the Czechoslovak Academic of Sciences and spent one year in the Laboratory for Animal Physiology and Genetics, Libechov, Czechoslovakia, in preparation for Ph.D. thesis work related to <u>in vitro</u> fertilization and sexing of embryos. The Russian invasian forced him to leave Czechoslovakia and so interrupted these studies after one year. He was accepted by the Dairy Department,

Michigan State University, and Dr. Edward M. Convey in September 1969 and has been engaged in completing requirements for the Doctor of Philosophy degree at that institution since then.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AP	anterior pituitary
°C	Centigrade
cAMP	adenosine-3', 5'-monophosphate
CL	corpus luteum
CO_2	carbon dioxide
GnRH	gonadotropin releasing hormone
KRB	Krebs-Ringer buffer
LH	luteinizing hormone
LH-RH	luteinizing hormone releasing hormone
$NaHCO_3$	sodium bicarbonate
PG	prostaglandin
O_2	oxygen
TC	tissue culture
TRH	thyrotropin releasing hormone

INTRODUCTION

The ultimate goal of modern agriculture is efficient production of food and fiber. Progress in animal genetics, nutrition, and management has contributed significantly to improvements in livestock production. Research in animal physiology has been directed toward the same goal of exploration of potential methods to increase reproductive efficiency, lactational performance, and growth rate.

Since mammalian metabolism can be specifically altered by changing the endocrine environment it is conceivable that endocrine manipulation may provide the means to control metabolism for economic gain. Technological developments during the last decade provided practical ways of measuring blood hormone concentrations. Accordingly, basic research in endocrinology and neuroendocrinology were intensified. As a result the hypothalamic hormones were identified as components of several endocrine regulatory systems, and four releasing hormones were synthesized.

Recently, the first synthetic hypothalamic hormones became commercially available, thus providing another method to control the endocrine system with a

greater degree of specificity than previously available. Being polypeptides, these hormones had the advantage of easy synthesis and they are not antigenic.

Experiments depicted in detail here were designed to evaluate the interactions of specific and nonspecific agents which regulate bovine luteinizing hormone synthesis and release. The two hormones were selected since the luteinizing hormone release is stimulated and that of prolactin inhibited by the hypothalamus.

It is hoped that the research described herein will mark the beginning of systematic exploration of neuroendocrine concepts with a goal of attaining economic benefits to animal agriculture.

REVIEW OF LITERATURE

A typical feature of mammalian endocrine systems is the coordinated interplay of participating factors. Thus, the importance of individual elements is difficult to estimate. Although the following discussion considers the role of individual factors in control of anterior pituitary hormone synthesis and release, the reader should keep in mind that functions of all factors are integrated.

The hypothalamic releasing and inhibiting hormones represent the principal factors influencing *in vivo* release of anterior pituitary hormones. The neurohormones pass via the hypothalamo-hypophysial portal system to the pituitary wherein they act on the membrane of anterior pituitary cells instantly modifying hormone release.

Hormones of peripheral endocrine glands also control pituitary hormone synthesis and release. This control may be directly on the anterior pituitary, indirectly at the brain, or at both sites. For example, estrogen is thought to influence lutenizing hormone by directly affecting pituitary gonadotrophs but also by modifying releasing hormone release from the hypothalamus. The effect of peripheral hormones is not limited to the

homologous tropin. For example, estrogen not only influence release of luteinizing hormone and follicle stimulating hormone but also prolactin and growth hormone.

Other factors that control pituitary hormone release but are not specific in their action may be grouped as "nonspecific regulators." Included in this group are prostaglandins, adenosine 3', 5'-monophosphate (cAMP), amino acids, catecholamines, and ions. Under physiological conditions nonspecific regulators play a secondary role in pituitary control systems. However, nonspecific factors represent intermediates in expression of pituitary regulation via releasing hormones and target hormone feedback. As such, understanding these factors is important to elucidating the biochemistry of pituitary hormone synthesis and release.

Thus, hypothalamic neurohumors, hormones of target organs, and "nonspecific regulators" all play a role in control of pituitary hormone synthesis and release. The subsequent review will include discussion concerning: (1) the chemistry and biochemistry of hypothalamic hormones, (2) the interaction of tropic hormones in pituitary control, and (3) the role of nonspecific regulators in facilitating hormone release. This review will be limited to a consideration of factors influencing secretion of luteinizing hormone and prolactin.

Secretory Process

Although prolactin and luteinizing hormone are produced by different anterior pituitary cell types, the processes of hormone secretion are similar for each hormone. Thus, the following description applies equally to secretion of both hormones. Protein hormone synthesis is associated with rough endoplasmic reticulum (Farqouhar, 1971). Here in the ribosomes, which are located externally to the membrane of the endoplasmic reticulum, protein is assembled. When the primary protein structure (zymogen) is completed, it is released from the ribosome and penetrates the membrane of the endoplasmic reticulum entering its lumen. All excretory proteins, including hormones, pass through the lumen of the endoplasmic reticulum, and, therefore, the lumen may contain many different proteins at the same time. Here is the primary site where nonprotein macromoleculas are attached to the protein chains. For example, here the carbohydrate portion of glycoproteins is put on. The endoplasmic reticulum appears flat and wrinkled when the rate of protein synthesis is low and is increased in volume when stimulated (Farqouhar, 196la). The primary structure of the zymogen is usually complete upon its departure from the endoplasmic reticulum. The protein is now transported to the Golgi complex in the form of a small vesicles.

The Golgi complex is a membranous organelle consisting of flattened, membrane delimited vesicles. In active protein secreting cells, the Golgi apparatus is swollen with numerous stacked cisternae (Jamieson and Palade, 1967). The main fuction of the Golgi is to pack proteins into secretory granules. Associated with the Golgi cisternae and located nearby are structures with the appearance of accumulated membrane debris. These structures are believed to be membranes which previously had encased protein granules and which were returned for reuse subsequent to secretion of the protein.

Depending on the functional state of the pituitary cell, there are two types of secretory granules (McShan, 1971). Mature granules are always present although their number may vary. These granules are the storage form of protein within the cell. Their membranes are well developed, and the granule surface is sharply delimited and smooth. When the demand for secretory protein exceeds availability of stored hormone, the number of mature secretory granules is reduced and many granules are smaller. These small granules are characterized by a wrinkled, poorly developed membrane (Smith, Farqouhar, 1964). Once formed the secretory granule must move within the cell to the plasma membrane where it can be released. The transport process in the anterior pituitary probably involves microtubules (Kraicer and Milligan, 1971).

The microtubulous or microfilamentous system of the living cell has tentatively been assigned several functions (Lacy et al., 1968). For example, these tubules may function as a cytoskeleton transporting isolated materials via cytoplasmic streaming in the cell. In addition, evidence obtained by the electron microscopy suggests that the microtubules serve as a cytoskeleton on which secretory granules are organized in tandem (Lacy et al., 1968). According to these authors, secretory granules move along the cell membrane toward the location of fenestrae or openings in the capillary endothelium. At these fenestrae the membrane of secretory granule fuses with the cell membrane and the contents of the granule are released from the cell into the capillary lumen. This expulsion process is called emeiocytosis as opposed to pinocytosis which involves penetration of protein from the capillary lumen into the cell. Following expulsion of the granule contents, membrane remnants internalize. These remnants are gathered in the area of the Golgi apparatus and are reutilized as delimiting membranes for new secretory granules (Farqouhar, 1961b).

Mechanism of the Anterior Pituitary Hormone Release

The mechanism by which hormones are released from anterior pituitary cells is common for all pituitary hormones (Geschwind, 1969). Although several theories

explain the mechanism of hormone release, available evidence best fits the theory known as "stimulus-secretion coupling" (Douglas, 1967). "Stimulus-secretion coupling" was originally proposed as the mechanism by which vasopressin is released from the neurohypophysis and catecholamines are released from the adrenal medulla (Douglas and Poisner, 1964; Douglas and Rubin, 1961).

This theory involves an initial decrease in transmembrane potential of the pituitary cell. The result is cell membrane depolarization which increases cell membrane permeability allowing a net flux of calcium ions into the cell. Increased intracellular calcium ion concentration then activates a process leading to hormone release. Evidence supports the view that adenosine-3', 5'-monophosphate serves as a second messenger in this chain of events. The concept of second messenger first advanced by Sutherland and coworkers (Robinson, Butcher, and Sutherland, 1971) proposed that expression of hormone action on cellular processes was mediated via cyclic AMP. This concept as it applies to the action of adrenocorticotropic hormone on the adrenals (Shima, Mitsunaga, and Nakao, 1971; Pearlmutter, Rapino, and Saffran, 1971) and the action of melanocyte stimulating hormone on melanophores (Vesely, 1971) has been well defined. Preliminary evidence that this theory may also apply to secretion by anterior pituitary cells has been reported by Zor et al.

(1970) and Labrie et al. (1971), who showed increased release of pituitary hormones in vitro when incubated in medium containing cAMP. Although still undefined, the hypothalamic hormones may be the first messengers in causing hormone release from the anterior pituitary cells.

Chemistry and Physiology of Hypothalamic Hormones

Pituitary active neurohormones have been demonstrated in experiments utilizing crude hypothalamic extracts. These experiments defined, in part, the important role of neurohumors in regulation of reproductive functions (see Convey, 1973, for references). The presence in the hypothalamus of at least one substance with specific regulatory action on secretion of each anterior pituitary hormone has been confirmed experimentally in rats (for review see McCann and Porter, 1969). Four important hypothalamic hormones are peptides.

A substance isolated from porcine hypothalamus (Schally et al., 1971) exerting luteinizing and follicle-stimulating hormone releasing activities has been identified (Matsuo et al., 1971) as the decapeptide shown in Figure 1. The synthetic hormone of the same amino acid sequence does not have characteristics different from the natural hormone either in potency or in the specificity (Arimura et al., 1972). The decapeptide has also stimulated gonadotropin synthesis during long

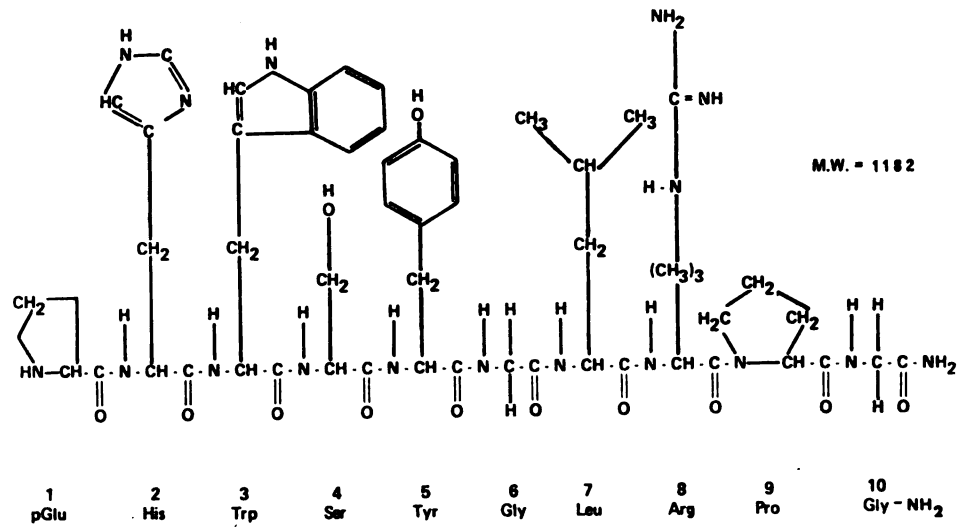

Figure 1.--Amino acid sequence of gonadotropin releasing hormone (GnRH).

incubation *in vitro* similar to the natural releasing hormone (Redding *et al.*, 1972). The FSH response of male rat pituitaries *in vitro* requires a prolonged exposure to GnRH which is not the case with LH release (Arimura, Debeljuk, and Schally, 1970). Purified ovine GnRH has to have the same amino acid sequence as porcine GnRH (Amoss *et al.*, 1972).

The chemical formula of thyrotropin releasing hormone is shown in Figure 2 (Burgus *et al.*, 1969). This (tripeptide) causes release of thyroid stimulating hormone from the anterior pituitary and also release of prolactin and growth hormone in human (Jacobs *et al.*, 1971) and other species. TRH increases serum prolactin, growth hormone, and thyroxine (Convey *et al.*, 1973) in the bovine.

Participation of Ovarian Steroids

The normal sequence of steroid changes during the bovine estrous cycle could be described as a decrease in progesterone to a low level accompanied by increasing serum estrogen concentration which reaches a peak several hours prior to release of LH. Thus, increasing serum estrogen concentration may be responsible for LH release (Henricks, Dickey, and Hill, 1971; Wetteman *et al.*, 1972).

Estrogen administration is essential for LH release in ovariectomized ewes while progesterone could

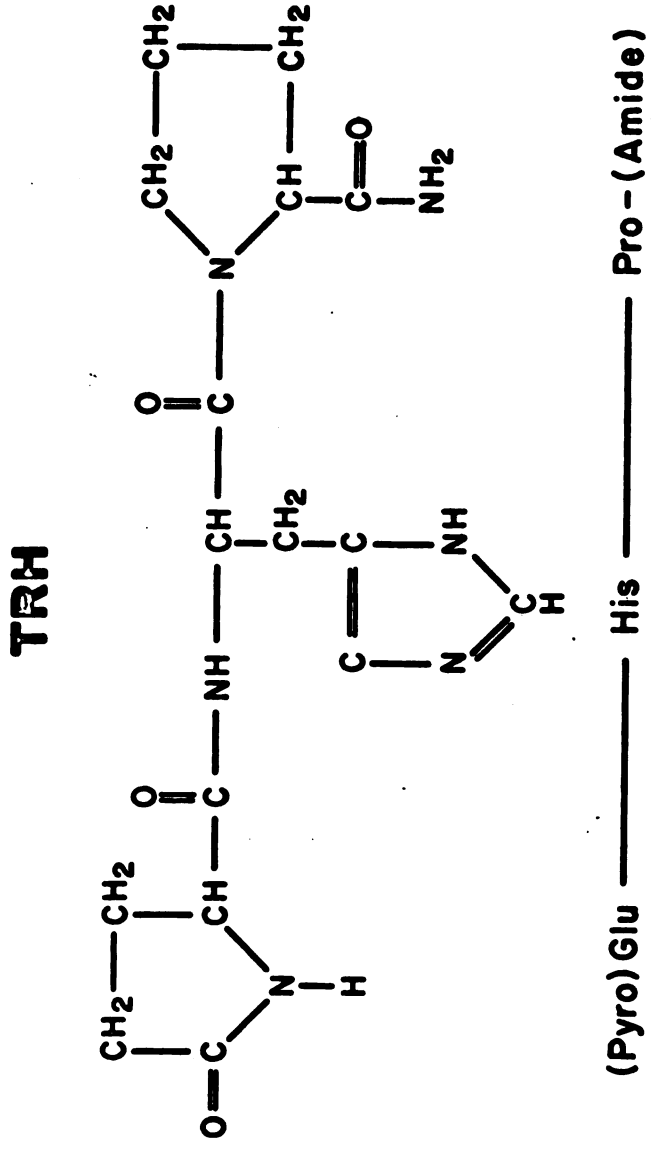

Figure 2.--Amino acid sequence of thyrotropin releasing hormone (TRH).

inhibit the effect of estrogen (Scaramuzzi et al., 1970). Studying the effect of estrogen on GnRH-stimulated LH release, Reeves et al. (1971a) demonstreated increased serum LH concentration when GnRH was administered during eight hours centered on estrus. This would suggest a permissive role of estrogens in potentiation of LH release in response to hypothalamic stimulation.

Recently, Hobson and Hansel (1972) confirmed that estrogen can induce LH release in heifers and cows and suggested that progesterone can inhibit this response. They pointed out that estrogen is a major factor indicating the preovulatory LH peak in cattle but that it does not exert this effect at mid cycle when a functional corpus luteum is present. In addition, Spies and Niswender (1972) demonstrated that injections of progesterone can prevent the normal preovulatory LH surge at least in some species.

Piacsek and Meites (1966) reported that estradiol benzoate of 0.025 or 0.1 μg/ml increased release of rat LH 15 fold during one hour in vitro incubation. In a similar experiment, Schneider and McCann (1970) observed that estradiol (0.5 or 1.0 μg/ml) significantly increased release of LH during 6 hours incubation. But 50 ng/ml estradiol significantly inhibited release of LH. The direct effect on pituitary gonadotropin was explained by a regulatory protein since the estrogen effect was inhibited by puromycin and cycloheximide.

Estrogens are referred to frequently as "potent regulators of prolactin production" (Meites, 1966) and several hypotheses have appeared at various times to explain the effect of estrogen on prolactin synthesis and release. As early as in 1937 exogenous estrogen was found to increase pituitary prolactin content (Reece and Turner, 1937) and more recently estrogen has been shown to increase blood prolactin in rats (Chen and Meites, 1969). Presently, it seems to be generally accepted that the estrogens influence release of prolactin both in hypothalamus and pituitary even though the experimental results *in vitro* are often contradictory. While Nicoll and Meites (1962) and Ben David *et al.* (1964) reported prolactin enhanced release by rat pituitaries exposed to estrogen *in vitro*, Gala and Reece (1964) and Pasteels (1970) were not able to show the same results under similar experimental conditions. Kanematsu and Sawyer (1963) and Ramirez and McCann (1970) established that intrahypophyseal implants of estrogen promoted prolactin release in rats. Therefore, Meites (1970) concluded that direct action of estrogen on the anterior pituitary is one of the mechanisms by which estrogen influences prolactin secretion.

Nonspecific Regulators

Samli and Geschwind (1968) were first to present evidence concerning the mechanism of release of

luteinizing hormone (LH) in rats. They observed that stimulation of LH release *in vitro* by crude hypothalamic extracts or elevated potassium concentration in the culture medium was dependent upon the presence of calcium ions. For example, they reported that potassium at 10x normal concentration (59mM) resulted in LH release 4x greater than controls only when calcium was present. These authors suggested that potassium may mimic the natural stimulus for release. Wakabayshi, Kamberi, and McCann (1969) presented results quantitatively similar to those of Samli and Geschwind (1968). In addition, they demonstrated that the effect of high potassium ion concentration on LH release is reversible and additive to the effect of releasing factors. A possible difference in mechanism of LH release by releasing factors and potassium ion is suggested by their differential dependence on calcium; LH release by releasing factors was partially inhibited in calcium-free media while potassium-induced LH release was completely inhibited.

Wakabayshi and Savano (1970) demonstrated that rat prolactin release *in vitro* was unaffected by high potassium concentration in the culture medium. In an attempt to quantitatively estimate incorporation of leucine $-H^3$ into prolactin during incubation of rat anterior pituitary explants *in vitro*, MacLeod and Fonthan (1970) observed that a five fold increase in potassium

ion concentration had no effect on protein synthesis. But potassium-free media significantly reduced synthesis and release of labeled prolactin. Additionally, these authors also reported that increasing calcium ion concentration caused a significant increase in prolactin synthesis and release while growth hormone synthesis and release was unaffected. When both potassium and calcium concentration in the media were increased, both prolactin and growth hormone synthesis and release were enhanced. Parsons (1969) found that release of prolactin during the first hour of incubation was delayed by preincubation in low calcium medium.

Prostagladins, in particular PGE_1, have been shown to increase cAMP concentration (Zor et al., 1969) and growth hormone release from anterior pituitary explants (Hertelendy, 1971); however, their regulatory function in the hypothalamus cannot be excluded. In the living cell, prostagladins control formation of cAMP, perhaps by regulating the function of the adenyl cyclase system (Savin, 1969).

Rationale for in Vitro Studies

Releasing hormones, steroid hormones, and nonspecific regulators affect pituitary hormone synthesis and release. It is difficult to evaluate participation of individual factors in the regulation of hormone

production because of interaction between factors. *In vitro* techniques attempt to isolate the effects of individual factors or a combination of factors in an effort to explain direct effects free of interaction.

In Vitro Techniques

The classical flask *in vitro* technique was introduced into neuroendocrinology by Saffran and Schally in 1955. They incubated rat hemipituitaries and measured ACTH release into incubation medium at 37°C. Many of the original features of this arrangement are still characteristic for currently used static *in vitro* incubations. Major changes from the original technique include preincubation of tissue slices (Guillemin and Vale, 1970) and replacement of Krebs-Ringer buffer by TC medium 199 as an incubation milieu (Nicoll and Meites, 1963).

Cultures of anterior pituitary tissue provide many experimental advantages over *in vitro* experiments. Rivera and Kahn (1969) recently discussed the merits of anterior pituitary culture relative to *in vivo* experiments. Advantages include: (1) *In vitro* systems allow complete isolation of the anterior pituitary from all systemic influences, (2) hormone release can be measured as a function of time, (3) a variety of substances can be added to the cultures and their influence on hormone secretion compared with controls, and (4) relationship

between release, synthesis, storage, and activation can be studied.

The advantages of cell culture lie mainly in uniformity of cultured cells and easier manipulation of timed experiments (Vale et al., 1972).

In Vitro Superfusion

The first detailed description of continuous flow incubation with well outlined possibilities for superfusion of endocrine tissue has been reported in studies of steroid biosynthetic pathways (Tait et al., 1967) and response of rat pituitary to crude hypothalamic extract (Serra and Midgley, 1970).

Although a detailed discussion comparing static and continuous perfusion of cultures in vitro is beyond the scope of this review, major advantages of the latter are to allow continuous precursor repletion, product removal, and addition or deletion of regulators. Treatment effects are distinct since the rapid change in the culture medium prevents accumulation of nonspecific factors. In vitro superfusion allows the experimentator to follow the time course of response during culture.

MATERIAL AND METHODS

Experimental Animals--General

Holstein heifers from the Michigan State University dairy herd were 12 to 16 months old in these experiments. Only heifers which had at least 2 regular consecutive estrous cycles were included. Heifers were maintained in a loose housing arrangement and fed hay and corn silage ad libitum with 1 to 1.5 kg of grain daily. Heifers were on pasture when it was available. Heifers were palpated per rectum daily beginning 4 days before expected estrus to detect ovulation. This was the procedure during the experimental cycle and the preceding cycle. Each heifer assigned to be treated following luteal regression was palpated per rectum twice daily beginning 4 days before expected estrus and treatment was administered within 12 hours after the corpus luteum regressed (1.0 to 1.2 cm).

Heifers were observed for heat twice daily between 0750 and 0850 and 1700 and 1800 hr. Signs of estrus included: (1) standing heat, (2) being ridden, (3) bloody vaginal discharge, (4) riding others, (5) mucous dishcarge, (6), red, swollen vulva, and (7) alert, nervous, and bawling. Criteria (4) through

(7) were only marginal signs while a combination of the first three was the leading indicator.

Four mature dairy bulls, ranging in age from 6 to 11 years, were housed in individual pens at Select Sires, Inc. (East Lansing, Michigan) and received 5 kg grain and 8 kg hay daily. These bulls weighed on the average 783 kg at the time of these experiments (January to March 1972).

Pituitary donors for *in vitro* experiments were cows and steers slaughtered at a local abattoir. Animals were distinguished with regard to sex but not with regard to reproductive or lactational history.

Blood Sample Collection

One day preceding each *in vivo* experiment a cannula (Vinyl IV Tubing, Clay Admas, Inc., New York City) was inserted into each animal. Approximately 45 cm of the 240 cm cannula were placed in a jugular vein and affixed to the neck and withers with tag cement (Nasco, Forst Atkinson, Wisconsin) on 7.6 x 12.7 cm adhesive tape. Each cannula was flushed with 3.5% sodium citrate and sealed until used for blood collection. The blood sampling procedure included the following steps: (1) 5 ml of blood and citrate were withdrawn and discarded, (2) a 10 ml blood sample was withdrawn and placed into a 12 ml polypropylene centrifuge tube (Sorval, Inc.,

Newtown, Conn., and (3) the cannula was flushed with 3.5% sodium citrate and resealed.

The Method of Continuous Superfusion of the Anterior Pituitary Tissue in Vitro

Heads of cattle, excluding the skin and tongue, were transported to the laboratory within 45 minutes of slaughter. The pituitary was removed, the posterior pituitary was discarded, and the anterior pituitary was cut midsagitally. Each anterior pituitary half was further divided into central and peripheral anterior pituitary regions. Tissue from each region was cut into 8 ribbons of approximately equal size (1 x 1 x 10 mm). Depending on the experimental design, ribbons from the peripheral or central areas or both areas were incubated.

The incubation apparatus is illustrated in Figure 3. Pituitary explants were retained in glass chambers (A) of approximately 0.5 ml volume by perforated polythylene discs (B). A dekastaltic pump continuously moved incubation medium through Tygon tubing (1.52 mm i.d., Technicon Instrument Corp., Chauncey, N.Y.) from the reservoir (C), through the efferent tube (D), through the culture chamber (A) and out the efferent tube (E). The effluent was fraction collected as it left the outlet tubing. Incubation chambers and reservoirs containing incubation medium were submerged in a thermoregulated bath at 37°C. Incubation media were kept under continuous

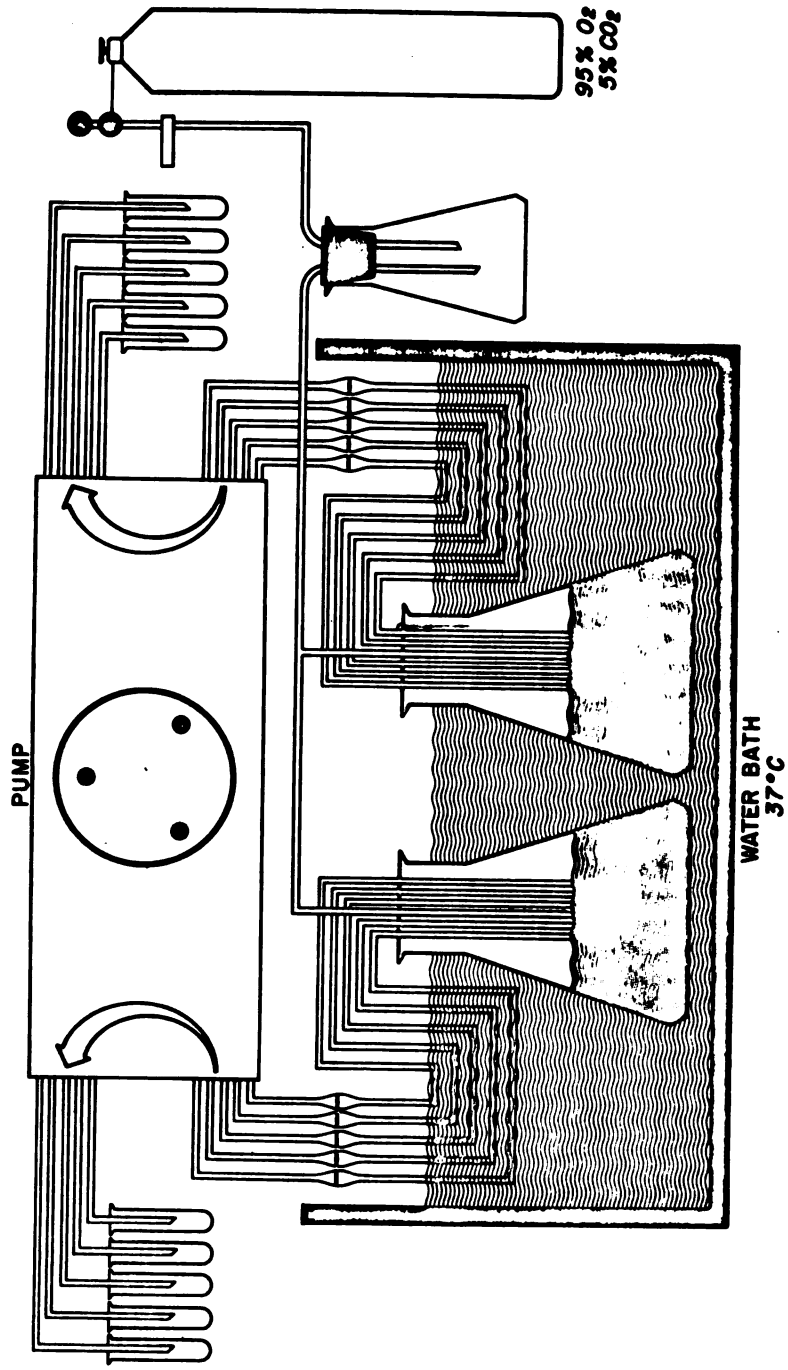

Figure 3.--Schematic representation of incubation apparatus used for <u>in vitro</u> superfusion of bovine pituitary pieces.

gassing (300 ml/min) with humidified 95% O_2 : 5% CO_2. Rate of flow of incubation media varied from 0.2 to 1 ml/min depending on experimental design, and 2 ml samples were collected at specified intervals.

Experiment 1

Objective: To develop an in vitro superfusion system for bovine anterior pituitary explants and to evaluate several incubation variables on prolactin and luteinizing hormone release.

Experimental design: To determine whether explant surface area influenced hormone release, 4 ribbons from each region were incubated without further division (1 x 1 x 10 mm) and 4 were cut into a total of 20 pieces of approximately equal size (1 x 1 x 2 mm). Two ribbons or 10 pieces were superfused in each incubation. Incubation media was TC 199 (Appendix 1) containing 68 µg/ml penicillin-g-phosphate and either 0.224 or .035% $NaHCO_3$. Following 2 hr of culture, one-half of the pituitary tissue in each chamber was removed and incubation of the remaining tissue was continued for an additional 4 hr. The experimental design is illustrated in Figure 4.

Experiment 2

Objective: To evaluate the effect of ovarian steroids on release of prolactin and luteinizing hormone from bovine pituitary tissue in vitro.

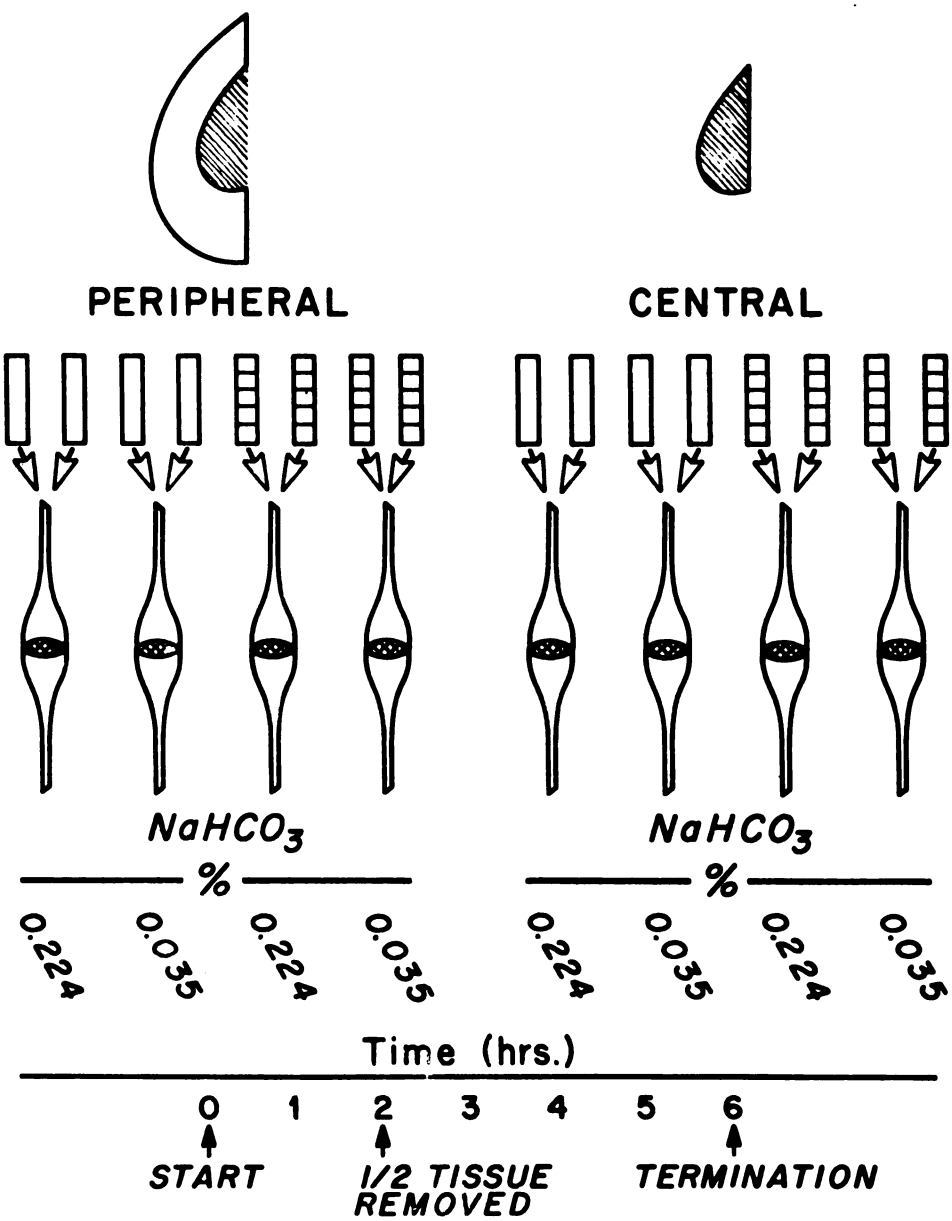

Figure 4.--Schematic representation of the design of Experiment 1.

Experimental design: Initially, concentrations of estradiol 17β which were approximately physiological (10 pg/ml) or 1000x physiological (10 ng/ml) were tested in a randomized block design. Anterior pituitary slices from each of 4 steers were incubated in triplicate with either 0, 10 pg/ml or 10 ng/ml estradiol 17β for 4 hr. To evaluate the effect of pharmacological levels of estrogen and the interaction of estrogen and progesterone, anterior pituitary tissue from cows was incubated in a 3 x 3 factorial design with progesterone (0, 0.05, and 5 μg/ml) and estradiol 17β (0, 0.05, 0.50 μg/ml) concentration as the main effects. This experiment was replicated with anterior pituitary from each of 3 cows.

Experiment 3

Objective: To evaluate the effect of purified porcine gonadotropin releasing hormone (GnRH; Abbott Laboratories, North Chicago, Ill.) on luteinizing hormone release from bovine anterior pituitary tissue *in vitro* and to compare the effect of synthetic (Abbott Laboratories, North Chicago, Ill.) and purified porcine GnRH.

Experimental design: The superfusion system described in Experiment 1 was used to evaluate the direct effect of GnRH on LH release from bovine pituitary tissue in this experiment. Tissue (≈ 75 mg) from peripheral area of the anterior pituitary of individual steers was placed in each of 9 incubation chambers, then continuously

superfused with medium 199 (0.224% sodium bicarbonate) at 1.0 ml/min for 2 hr, exposed to 0, 1, or 4 ng/ml GnRH in medium 199 for 20 min, and then superfused in control TC medium 199 for an additional 40 min. Pituitary tissue was from 4 steers and treatments were assigned randomly to explants from each pituitary with 3 explant replicates per treatment. Effluent media were fraction-collected at 1-min intervals and frozen until assayed for LH. A similar experiment was conducted to compare the efficacy of purified porcine GnRH versus synthetic GnRH in causing LH release *in vitro* using pituitary tissue from a single steer. Peripheral pituitary tissue (3 explant replicates per treatment) was exposed to 4 ng/ml synthetic or purified porcine GnRH for 30 min. Medium was fraction-collected and frozen until assayed for LH (Appendix 3).

Experiment 4

Objective: To compare and evaluate the interaction between luteinizing hormone release induced by GnRH and enhanced potassium concentrations.

Experimental design: Schematic representation of the experimental design is depicted in Figure 5. Anterior pituitary tissue was preincubated in superfusion for 2 hr in TC medium 199. Preincubation was continued in Krebs-Ringer bicarbonate-glucose buffer (KRB, Appendix 4) with or without calcium for 1 hr. A calcium-free KRB was obtained by substituting NaCl for $CaCl_2$. After 1 hr

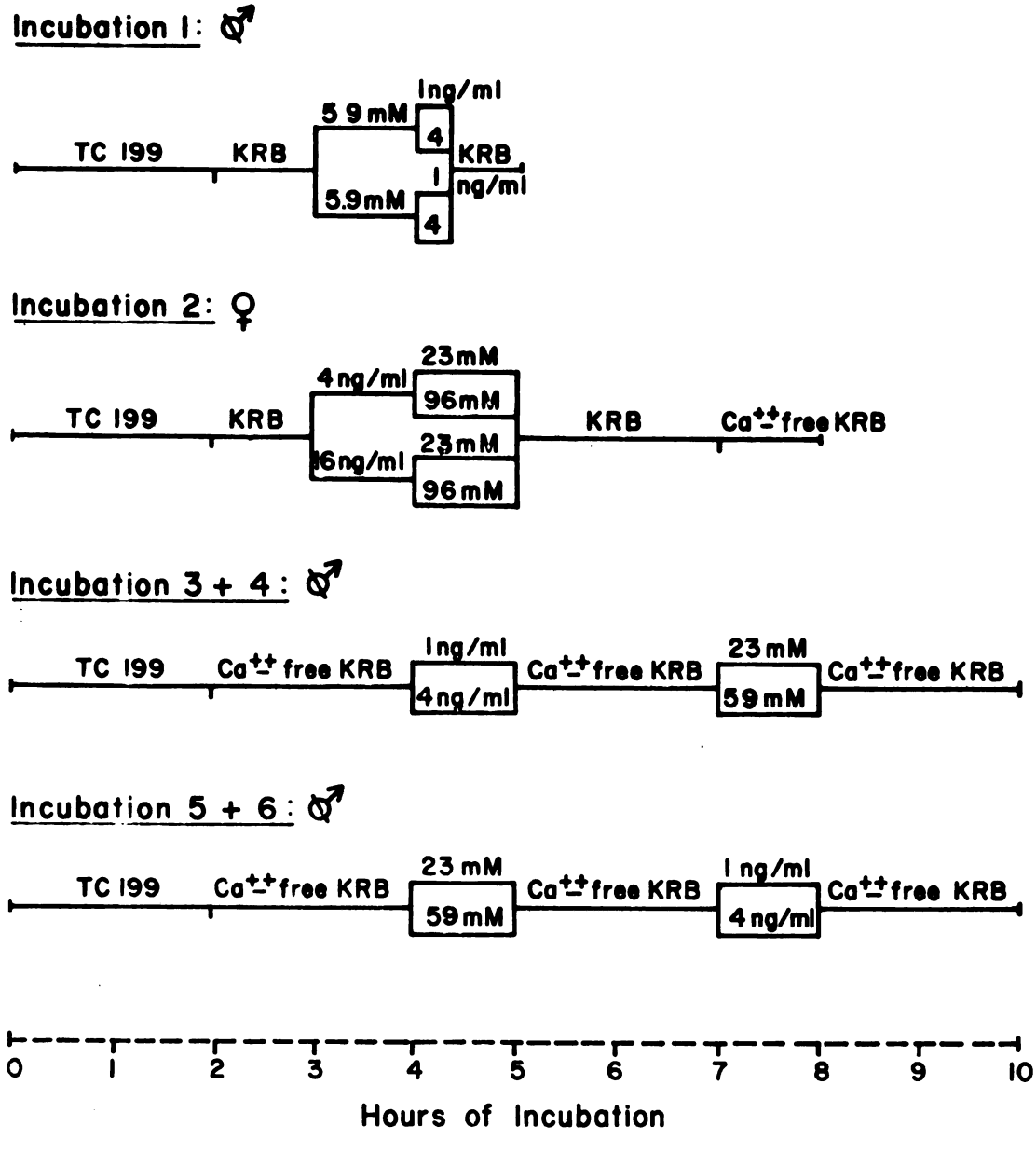

Figure 5.--Schematic representation of design of Experiment 4. Steer (♂) or cow (♀) anterior pituitary tissue was preincubated in TC medium 199 (TC199) for 2 hr. Then, the tissue was in Krebs-Ringer bicarbonate buffer with glucose (KRB), or in KRB where $CaCl_2$ was substituted by equivalent amount of NaCl (Ca^{++} free KRB). GnRH (1, 4, or 16 ng/ml) and enhanced potassium (23, 56, and 96 mM) treatments were administered in this or the reversed order during intervals indicated on the graph.

preincubation in KRB (normal or calcium free) the pituitary tissue was subsequently incubated in potassium-enhanced KRB (23, 56, or 96 mM) and GnRH-enhanced KRB (1, 4, or 16 ng/ml) in this or the reversed order for 1 hr. Potassium-enhanced KRB was prepared by increasing the concentration of potassium and removing an equivalent amount of sodium (Appendix 4). Effluent media were fraction-collected at 1-min intervals and frozen until assayed for LH.

Treatments were randomly assigned to channels in a 2 x 2 factorial design with 2 replicates. In the first 2 incubations (normal calcium KRB) the treatments were replicated within 1 steer and 1 cow while in the remaining 4 incubations (calcium-free KRB) the treatments were replicated within each of 2 steers.

Experiment 5

Objective: To investigate the effect of synthetic GnRH on release of LH in heifers and bulls.

Experimental design: Synthetic GnRH, in 10 ml isotonic NaCl, was administered in a single injection via jugular cannula to each of 4 yearling Holstein heifers and 4 mature dairy bulls. Heifers, in the early luteal phase (days 5-10) of the estrous cycle at the time of treatment, received 5, 20, or 80 μg and bulls 10, 40, or 160 μg GnRH on 3 consecutive days in a sequence determined at random for each animal. Sera prepared from jugular

blood collected at intervals before and after treatment were stored at -20°C until assayed for LH.

Experiment 6

Objective: To investigate the influence of different ovarian steroid environment on GnRH-stimulated LH release in vivo.

Experimental design: Twenty-four yearling heifers, exhibiting regular estrous cycles, were assigned at random to groups of 4 and received 5, 40, or 320 μg of synthetic GnRH via jugular cannula on day 15 of the estrous cycle or on day following corpus luteum regression as determined by rectal palpation ($\bar{x} \simeq 20$ days). Estradiol, estrone, and progesterone concentrations in serum from each heifer collected at the time GnRH was administered and at 4 hr after GnRH were determined by radioimmunoassays (Wettemann et al., 1972; Kittok, Britt, and Convey, 1973) LH was measured in sera prepared from jugular blood collected at frequent intervals before and after treatment.

Statistical Procedures

Analysis of variance (ANOVA) of various degree of complexity was used to analyze data. Partition of the error term in split-plot ANOVA was used as a principal method for quantitative evaluation of dynamic aspects of hormone release in vitro (Gill and Hafs, 1971). Correlation

analysis (Snedecor, 1956) was utilized to study the functional relationships of different dependent variables within homogenous groups and for partial analysis. Dose response relationships were characterized by regression curves and correlation coefficients. Selection of serum steroids covariates pertinent to the model in Experiment 6 was accomplished utilizing the stepwise addition method (Draper and Smith, 1966) and covariance analysis was applied to evaluate the relative importance of variables and covariates influencing LH release following treatment.

RESULTS AND DISCUSSION

Experiment 1. Bovine Pituitary LH and Prolactin during Superfusion in Vitro

Prolactin release from steer pituitary explants averaged 20.5 ng/mg/min (Table 1), 187% greater ($P < .03$) than the comparable average for explants from cows. Although average prolactin concentration in explants after 2 and 6 hr superfusion was 46% greater in steers than in cows, the difference was not significant ($P > .25$).

Explants cultured in media containing 0.224% $NaHCO_3$ released 59% more prolactin than explants cultured in similar media containing 0.035% $NaHCO_3$ ($P < .01$). Greater prolactin release by explants incubated in 0.224% bicarbonate is reflected in a 35% lower average prolactin concentration in the tissue postculure ($P < .01$, Table 2). These responses in superfused bovine pituitaries to composition and buffering capacity of the incubation media are similar to responses in prolactin release by rat pituitaries *in vitro* (Gala and Reece, 1964).

Jubb and McEntee (1954) demonstrated tinctoral zonation of cell types in the bovine pituitary which have been associated with secretion of different hormones. Thus, selection of explants from given regions of the pituitary may reduce variation within animal in hormone

TABLE 1.--Anterior Pituitary (AP) Superfusion--Prolactin Release.

Source of Variation	Prolactin ng/mg/min	DF	F	Probability Level
AP source		1	1.71	0.032
Steer	20.5			
Cow	7.1			
Media		1	14.08	0.001
Low $NaHCO_3$	10.7			
High $NaHCO_3$	17.0			
Area		1	13.45	0.010
Peripheral	17.7			
Central	10.0			
Number of pieces		1	3.10	0.087
1	12.3			
5	15.3			
Time		5	4.91	0.005
60	13.0			
120	10.5			
180	12.1			
240	13.0			
300	14.1			
360	20.2			

TABLE 2.--Anterior Pituitary (AP) Superfusion--Prolactin Concentration.

Source of Variation	Prolactin µg/mg	DF	F	Probability Level
AP source		1	1.62	0.251
Steer	16.3			
Cow	11.2			
Media		1	14.27	0.021
Low $NaHCO_3$	15.8			
High $NaHCO_3$	11.6			
Area		1	32.25	0.001
Peripheral	18.6			
Central	8.9			
Number of pieces		1	0.24	0.628
1	13.5			
5	14.0			
Time		1	2.25	0.142
120	13.3			
360	14.2			

release. In our studies, explants cut from the peripheral area of the pituitary released 77% more prolactin ($P < .01$) and contained 110% more prolactin postculture ($P < .01$) than explants from the central pituitary area. These results are consistent with the greater density of prolactin producing acidophils in the peripheral area of the bovine pituitary (Jubb and McEntee, 1954).

Explant size influenced hormone production by rat pituitary explants in vitro (Gala, 1970). However, cutting our explants into 5 pieces increased prolactin release

only about 25% ($P < .08$), and explant size did not influence prolactin concentration postculture ($P > .05$).

Average prolactin release measured at the end of each hour of incubation remained unchanged through 5 hr but was increased ($P < .001$) after 6 hr. The interaction between sex of the donor and incubation was significant ($P < .01$); it resulted from a linear increase ($P < .01$) in prolactin release with time from explants from steers but not cows. Similarly, the interaction between level of bicarbonate and incubation interval was significant ($P < .01$); it resulted from a linear increase ($P < .001$) in prolactin release from explants incubated in medium 199 buffered with 0.035 but not 0.224% $NaHCO_3$.

Explants from cow pituitaries released 137% more LH during culture ($P < .05$; Table 3) and contained 49% more LH postculture ($P < .06$) than explants from steer pituitaries (Table 4). It is not surprising that explants from the central pituitary area released 17% more LH ($P < .05$) and contained 72% more LH postculture ($P < .01$) than did explants cut from the periphery of the gland. These results provide additional evidence for hormonal zonation in the bovine pituitary.

Neither explant dimension nor bicarbonate concentration in the media influenced LH release or tissue concentration postculture. LH release into the media (ng/mg/min) decreased from 0.50 at 60 min to 0.29 at 120 min

TABLE 3.--Anterior Pituitary (AP) Superfusion--LH Release.

Source of Variation	LH ng/mg/min	DF	F	Probability Level
AP source		1	6.36	0.045
Steer	0.22			
Cow	0.51			
Media		1	0.01	0.934
Low $NaHCO_3$	0.36			
High $NaHCO_3$	0.37			
Area		1	6.61	0.042
Peripheral	0.34			
Central	0.39			
Number of pieces		1	1.03	0.316
1	0.34			
5	0.38			
Time		5	4.09	0.001
60	0.50			
120	0.29			
180	0.29			
240	0.33			
300	0.35			
360	0.42			

TABLE 4.--Anterior Pituitary (AP) Superfusion--LH Concentration in Tissue.

Source of Variation	LH µg/mg	DF	F	Probability Level
AP source		1	5.18	0.063
Steer	0.87			
Cow	1.30			
Media		1	2.89	0.098
Low $NaHCO_3$	1.14			
High $NaHCO_3$	1.02			
Area		1	40.72	0.001
Peripheral	0.80			
Central	1.37			
Number of pieces		1	0.01	0.967
1	1.08			
5	1.08			
Time		1	6.84	0.012
120	1.01			
360	1.16			

and increased thereafter to 0.42 ng/mg/min at 360 min ($P < .01$). LH release from explants from cows showed no consistent pattern with time while LH release from steer explants was relatively stable. Average LH concentration in tissue after 6 hr of incubation was 15% greater than at 2 hr of incubation ($P < .01$). This fact, coupled with increased rate of release with increased incubation interval, suggests active LH synthesis in vitro.

Net hormone synthesis during the last 4 hr of culture was estimated; average postincubation pituitary hormone concentration (6 hr) plus average media hormone

concentration minus hormone concentration of explants after 2 hr incubation. In this regard, explants synthesized on the average 1.98 and 0.23 μg prolactin and LH/mg pituitary tissue, respectively, during 4 hr of culture under conditions of constant superfusion. It is also interesting to note that the ratio of prolactin to LH release (approx 40:1) observed here compared favorably with the same ratio for bovine plasma as measured in our laboratory.

The variables tested in this study accounted for 90% of the overall variability associated with LH release. We found no serious obstacles for use of this superfusion system to study the bovine anterior pituitary *in vitro*.

Experiment 2. The Effect of Ovarian Steroids on Release of Anterior Pituitary Hormones in Vitro

Basal rate of prolactin release from steer anterior pituitary tissue was constant, averaging 8.33 ng/mg/min. Prolactin release did not change ($P > .05$, Table 5) when estradiol 17β was added to the incubation media in concentrations of 0, 10 pg/ml or 10 ng/ml. Only steers and interaction of steer x time were significant suggesting a differential release of prolactin from tissue from different steers over time but no effect of estradiol 17β. These results support those of Gala and Reece (1964) and Pasteels (1970) who reported no effect of estradiol 17β on prolactin release *in vitro* from rat and human

TABLE 5.--Effect of Estradiol 17β on Luteinizing Hormone and Prolactin Release from Steer Anterior Pituitary Tissue in Vitro.[a]

Hormone	Estradiol 17β (ng/ml medium)		
	0	0.01	10
Luteinizing hormone	0.21 ± 0.01	0.20 ± 0.02	0.22 ± 0.03
Prolactin	7.2 ± 1.1	9.3 ± 2.0	8.2 ± 1.0

[a]Values represent the means ± S.E.; expressed as ng/m./min.

pituitaries, respectively. These results are, however, in contrast to results reported by Nicoll and Meites (1962) and Ben-David (1964), who found that estrogen in suitable concentrations stimulated prolactin release from rat pituitary tissue in vitro.

Addition of physiological or 1000x physiological concentrations of estradiol 17β to the culture media did not affect ($P > .05$) rate of the LH release from steer anterior pituitary tissue under our experimental conditions (Table 5). The rate of LH release declined steadily over the 4 hr incubation period from 0.28 to 0.18 ng/mg/min in accord with results observed in the previous experiment. A significant interaction between steer and incubation time indicates at least partial differences between tissue of different origin, but as with prolactin there was no effect of estradiol 17β.

These results differ from similar studies involving rat anterior pituitaries. Schneider and McCann (1970) reported a significant increase (up to 100%) in release of LH from rat AP during a 6 hour culture period. In these experiments pituitary tissues from several animals were pooled and, therefore, it is likely that variation among animal was reduced. But at the same time the resulting concentration of hormones was 10^3 or 10^4 higher than is physiological for rats. The underlying assumption is that in these experiments the rate of release was normal; i.e., not influenced by autofeedback. Metabolities and tissue or blood components which are constantly removed by the superfusion system here, presumably build up to larger concentrations in the static system which may directly influence pituitary hormone release or interact with estradiol 17β. Obviously, the quantitative discrepancies between results reported for Schneider and McCann (1970) and those reported by Piacsek and Meites (1966) for rats necessitate further study and clarification. The latter report a 15 fold increase of LH in incubation medium at the end of 1 hour incubation with estradiol benzoate.

In subsequent incubation, basal rate of luteinizing hormone release from cow anterior pituitary (Table 6) differed slightly from that observed in Experiment 1 and could have reflected differences in the reproductive status

TABLE 6.--Effect of Ovarian Steroids on Release of Selected Hormones from Cow Anterior Pituitary Tissue in Vitro.[a]

Hormone	Estradiol 17β (µg/ml medium)	Progesterone (µg/ml medium) 0	0.05	5
Luteinizing hormone	0	0.81 ± 0.28	1.40 ± 0.35	0.76 ± 0.70
	0.05	1.24 ± 0.57	1.22 ± 0.25	1.40 ± 0.50
	0.50	1.41 ± 0.39	0.83 ± 0.23	0.96 ± 0.21
Prolactin	0	9.2 ± 2.5	9.6 ± 3.0	8.7 ± 2.6
	0.05	9.8 ± 3.6	11.4 ± 2.8	11.4 ± 4.6
	0.50	7.5 ± 3.0	9.1 ± 2.1	10.1 ± 2.0
Growth hormone	0	1.76 ± 0.28	3.69 ± 1.40	2.08 ± 0.16
	0.05	2.97 ± 1.08	3.29 ± 1.50	3.46 ± 1.82
	0.50	3.34 ± 1.54	2.53 ± 1.01	1.93 ± 0.49

[a]Values represent the means ± S.E.; expressed as ng/ml/min.

of donor cows. However, the rate of LH release did not change with time confirming results of Experiment 1 with regard to anterior pituitary tissue from cows. Estradiol alone in concentrations 0, 0.05, and 0.50 ng/ml caused an average increase of 80% in the rate of LH release (Table 6); however, this increase was not consistent which is apparent from the high standard errors. Progesterone did not influence LH release nor was the interaction for either of the two steroids significant.

Thus, although the basal rate of LH release was similar in these incubations, the responsiveness to various treatment combinations differed widely among animals. Small differences in the release of LH due to treatment can be hidden in the considerable among animal variation. However, since the previous essentially negative results with estradiol alone necessitated a test of a wide range of steroid concentrations, and also for technical difficulties (only 10 channel pump), it was impossible to design experiment to control within animal variation.

An interaction between estrogens and progestins has been postulated as part of physiological feedback control of luteinizing hormone release. For example, estradiol administration was essential for the episodic LH release following ovariectomy in heifers (Hobson and Hansel, 1972) and ewes (Scaramuzzi, Caldwell, and Moore, 1970) while progesterone inhibited the estrogen-induced release of LH in sheep (Scaramuzzi, Caldwell, and Moore, 1970). In the rabbit, progesterone inhibited LH discharge by acting directly on the cells of the adenohypophysis (Spies et al, 1969).

Basal rate of prolactin release was in the range previously described (7.5-11.4 ng/mg/min; Table 6). The presence of ovarian steroids in the incubation media did not change the rate of prolactin release but minor changes might not be detected. A number of investigators earlier

suggested that the increase in serum prolactin is estrogen dependent (Neil, Freeman, and Tillson, 1971), while progesterone probably participates in the regulation of prolactin release only indirectly (Chen and Meites, 1970).

Basal rate of growth hormone release during 4 hr incubation was 1.8 ng/mg/min (Table 6). This rate is approximately 300% faster than that of LH release but only about 25% the rate of prolactin release. Thus, the relative basal release of the 3 hormones from cow anterior pituitary *in vitro* resembles their relative serum concentrations. Basal growth hormone and prolactin concentrations in cows were reported to be 4.2 and 4.0 ng/ml, respectively (Tucker and Oxender, 1973). Serum LH concentration in adult cow during the nonestrus period is below 1 ng/ml (Swanson and Hafs, 1970). The similarity in the relative release of the 3 selected anterior pituitary hormones *in vivo* and under our experimental conditions *in vitro* represents further evidence for suitability of the *in vitro* superfusion as a technique to study mechanisms of anterior pituitary hormone release.

Introduction of estradiol and/or progesterone into the incubation media did not significantly change rate of growth hormone release. Large standard errors again indicate considerable variation among animal, which may reflect differences in reproductive state of donor cows and presumably also differences in quantities of pituitary

bound steroids. Detail studies of steroid receptors may be instrumental in helping to explain these differences and eliminate undesirable variation.

Experiment 3. Release of Bovine Luteinizing Hormone by Purified Porcine and Synthetic Gonadotropin Releasing Hormone (GnRH) in Vitro

Luteinizing hormone averaged 0.25 ng/mg/min in the effluent media from continually superfused steer pituitary tissue prior to exposure to GnRH; LH increased ($P < .001$) to 0.70 and 1.12 ng/mg/min after 4 min exposure to 1 and 4 ng purified porcine GnRH/ml medium, respectively (Figure 6). Comparable values were 0.94 and 1.50 ng/mg/min after 20 min. LH in the effluent media did not change significantly without GnRH addition to the medium. Average LH concentration in the effluent media decreased 8 and 33% ($P < .05$) during the 40 min period following exposure to 4 and 1 ng/mg GnRH, respectively, but these LH concentrations were still 340 and 100% greater than control values at the termination of incubation.

Luteinizing hormone in the effluent media of steer pituitary exposed to 4 ng/ml synthetic or purified porcine GnRH was 0.46 and 0.52 ng/mg/min immediately prior to exposure and increased linearly ($P < 0.1$) to 1.72 and 2.48 ng/mg/min, respectively, after 30 min exposure. The increases in media LH concentration with time during exposure to natural or synthetic GnRH were parallel; the

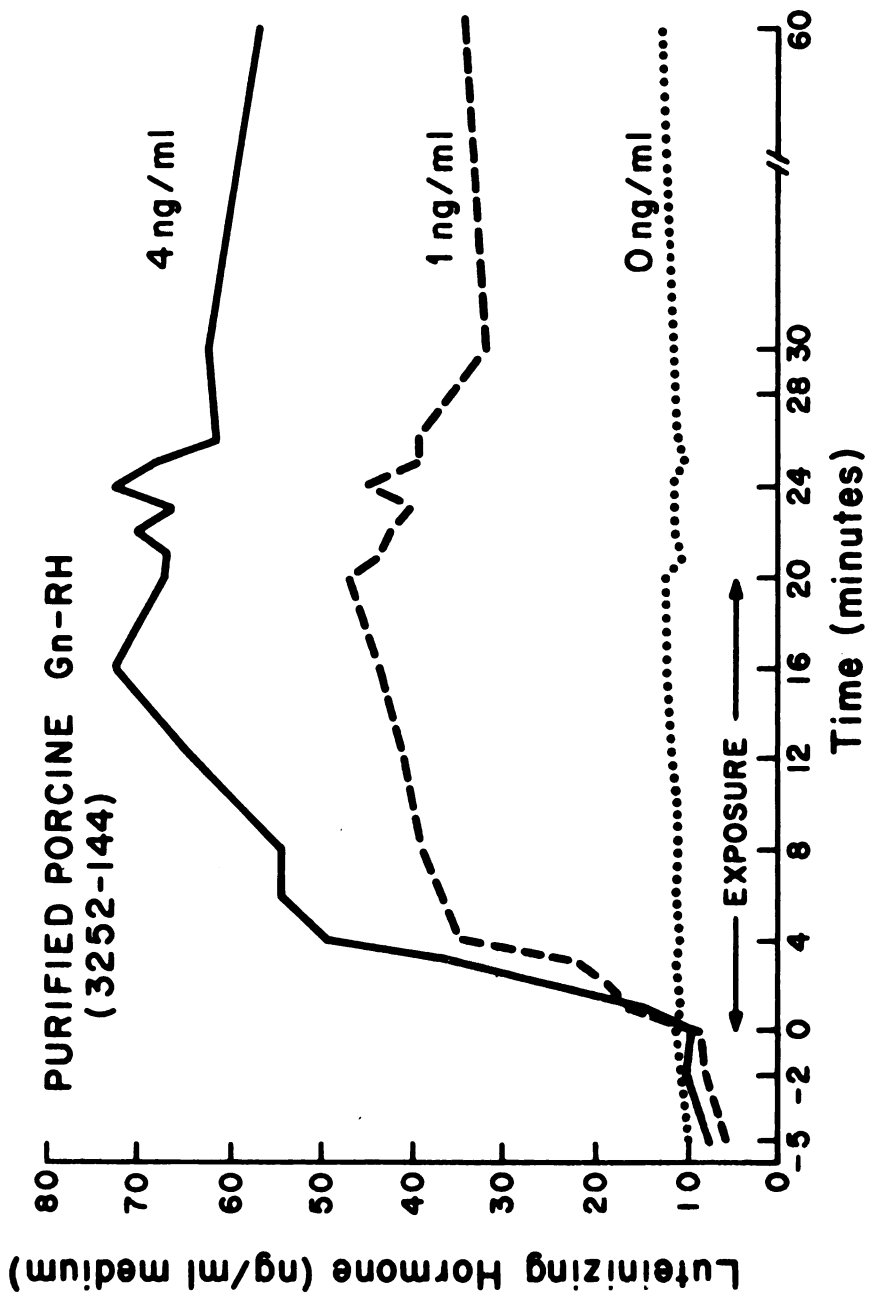

Figure 6.--Luteinizing hormone response of steer pituitary explants to purified porcine releasing hormone in continuous superfusion.

difference between response slopes was not significant ($P > .05$). When superfusion with control media was resumed, LH release decreased linearly ($P < .01$) as the incubation period advanced, but was still more than twice the preexposure base line when the incubation was terminated 4 hr after first exposure to GnRH.

Demonstration of a dose-related LH release *in vitro* is evidence that GnRH acts directly on the anterior pituitary. Prolonged release of LH after GnRH was removed from the superfusing medium suggests that only a brief exposure of the pituitary to GnRH is required to induce events which are not immediately reversible. Interpretation of this phenomena will require additional study.

Experiment 4. Interactions of GnRH and Enhanced Potassium Concentration in Stimulation of LH Release in Vitro

In the first incubation of this experiment, bovine anterior pituitary tissue was incubated in TC 199 for 2 hr, KRB for 1 hr, then exposed to either 4 or 16 ng/ml GnRH for 1 hr. Thereafter, the pituitary was incubated in KRB containing either 23 or 96 mM potassium for 1 hr followed by consecutive incubations in KRB for 1 hr and calcium-free KRB for 2 hr. The basal rate of LH release averaged 0.53 ng/mg/min 20 min before the end of preincubation in TC 199 and 0.51 ng/mg/min during 1 hr preincubation in normal KRB and the difference between means was not significant ($P > .05$). That these values

are comparable indicates that LH release is not changed by incubation in normal KRB following preincubation in TC 199, at least for 1 hr. It was important to establish this fact since treatments were administered in KRB. The rate of LH release from bovine anterior pituitary tissue is approximately half that reported for bovine anterior pituitary tissue (Jutisz et al., 1971).

LH release increased stepwise during 1 hr exposure to GnRH. It was increased ($P < .01$) 3 and 5 fold within the first 10 min following 4 and 16 ng/ml GnRH, respectively (Figure 7a and b). Furthermore, during the following 50 min, LH release increased an additional 60 or 10% from tissue exposed to 4 or 16 ng/ml GnRH. Differences in the magnitude and pattern of LH release following 1 hr exposure to 4 or 16 ng/ml GnRH were significant ($P > .01$). These results indicate that after an initial increase, depending on the time of exposure and concentration of GnRH, the rate of LH release either remains constant or increases again after a short period of time (up to 30 min). It appears that both concentration of GnRH and time of exposure importantly influence rate of LH release and may explain fluctuations in the rate of LH release from rat (Serra and Midgley, 1970) and ovine (de la Llosa and Jutisz, 1971) anterior pituitary tissues exposed to hypothalamic extracts in vitro.

Using continuous superfusion of rat anterior pituitaries, Serra and Midgley (1970) observed a rapid

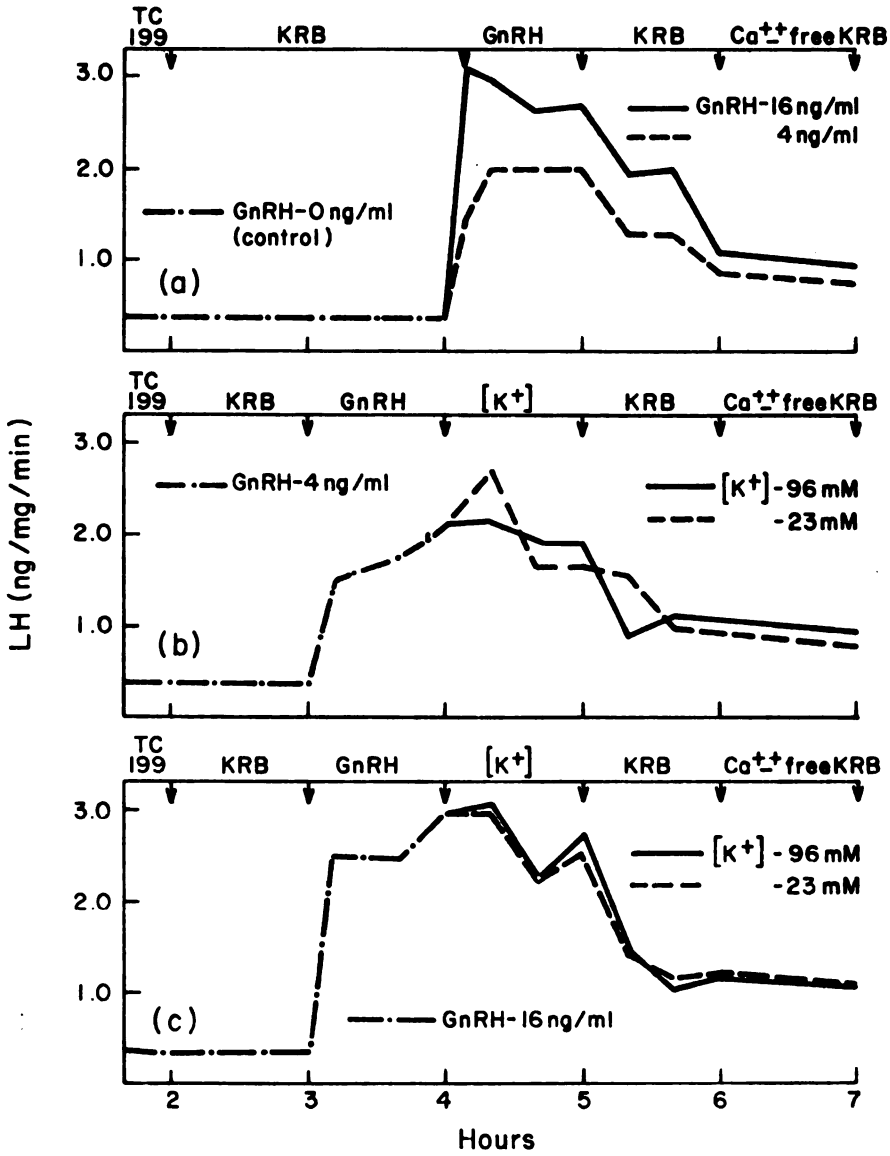

Figure 7.--Luteinizing hormone response of cow pituitary explants to synthetic gonadotropin releasing hormone and enhanced potassium in vitro. Arrows (↓) indicate start and termination of the anterior pituitary tissue to specified media: (TC 199) TC medium 199; (KRB) Krebs-Ringer bicarbonate buffer with glucose as depicted on the graph; (GnRH 0,4 or 16 ng/ml synthetic gonadotropin releasing hormone in KRB; (K^+) 23 or 96 mM potassium in KRB as depicted in the graph; (Ca^{++}-free KRB) Krebs-Ringer bicarbonate buffer with glucose where calcium chloride was substituted by equivalent amount of sodim chloride.

increase in rate of LH release following addition of crude hypothalamic extracts to the superfusion medium. The decrease in LH release was always closely associated with treatment withdrawal and the quantity of LH released was correlated with time of exposure to the extract, therefore, dose related. Since peak LH release also increased with time of exposure to hypothalamic extract LH release rate was not constant. In contrast, de la Llosa and Jutisz (1971) reported that rate of LH release as determined by frequent sampling from a static in vitro system from ovine anterior pituitary tissue was constant during 5 hr exposure to purified ovine luteinizing hormone releasing factor. In another experiment, these authors prolonged the constant rate of LH release beyond 7 hr. They also found that autofeedback did not decrease rate of LH release.

In the present experiment exposure of the anterior pituitary tissue to either low (23 mM)--or high (96 mM)--potassium KRB did not increase ($P > .05$) rate of LH release beyond that caused by GnRH. Exposure of control tissue (Figure 7c) to GnRH during this same period demonstrated that the magnitude of LH release was comparable to that previously ascribed to GnRH in the treatment channels. This result demonstrated that failure to alter the rate of LH release with changes in potassium in the medium was not due to prolonged incubation in normal KRB since

the ability of GnRH to stimulate LH release *in vitro* was unaffected. Enhanced potassium concentration caused release of thyrotropin (Value, Burgus, and Guillemin, 1967), luteinizing hormone (Samli and Geschwind, 1968), and adrenocorticotropin (Kraicer *et al.*, 1969). When simultaneously added to the incubation medium, 56 mM potassium and hypothalamic extracts caused LH release, and the effects were additive (Wakabayshi, Kamberi, and McCann, 1969; Jutisz and de la Llosa, 1970). Augmentation of GnRH stimulated LH release by 96 mM potassium in the medium was not observed in this study because of GnRH pretreatment.

Rate of LH release decreased markedly during the first 40 min following exposure to potassium treatments and return to KRB (Figure 7a, b, and c; Table 7). This decrease may reflect a GnRH x potassium interaction during the time enhanced potassium was in the incubation media and concurrent release of LH at the expense of synthesis during the same time. The effect is most apparent ($P < .05$) in high potassium KRB (Figure 7a, b, and c). Subsequent incubation in calcium free did not affect ($P > .05$) the rate of LH release from either experimental or control tissues.

In the second incubation of this experiment, steer anterior pituitary tissue was preincubated in TC 199 for 2 hr then in KRB for 1 hr. Thereafter the tissues were

TABLE 7.--Interaction of GnRH and Potassium in Stimulation of LH Release from Cow Anterior Pituitary.[a]

GnRH	Potassium	Time (min)[b]				
		-10	16-20	60	76-80	120
(ng/ml)	(mM)	ng/mg/ml				
4	23	0.43 ± .03	1.59 ± .39	1.59 ± .39	2.65 ± .10	1.69 ± .06
	96	0.47 ± .04	1.93 ± .42	2.54 ± .40	2.06 ± .28	1.90 ± .01
16	23	0.67 ± .04	2.86 ± .77	3.11 ± .36	3.25 ± .81	2.61 ± .53
	96	0.49 ± .01	2.46 ± .49	2.88 ± .50	3.08 ± .33	2.75 ± .11

[a]Mean ± S.E.

[b]Explants exposed to GnRH during 0 to 60 min and to enhanced concentrations of potassium during 60 to 120 min of incubation.

exposed to either 5.9 or 59 mM potassium KRB for 1 hr, after which GnRH 1 or 4 ng/ml was added to the medium. The rate of luteinizing hormone release from steer anterior pituitary tissue averaged 0.14 ng/mg/min during the last 10 min of preincubation in the normal KRB (Table 8). LH release increased 10 fold relative to base line within the first 16 min of exposure to 59 mM potassium KRB. Thus, the initial increase in the rate of LH release due to potassium-enhanced KRB closely resembles that of GnRH-induced LH release (Figure 8 a and b). However, in contrast to LH release following GnRH stimulation the rate of LH release remained constant during the period 20 to 60 min of exposure to 59mM potassium KRB. Release of LH from pituitary tissue exposed to the 5.9 mM KRB was unchanged throughout the treatment period.

Gonadotropin releasing hormone increased ($P < .01$) LH release from tissue previously treated with 5.9 mM potassium KRB from 0.14 ng/mg/min to 1.09 and 1.86 ng/mg/min within 16 min following 1 and 4 ng GnRH. GnRH also increased LH release ($P < .05$) from pituitaries incubated in 59 mM potassium KRB but the difference in response due to 1 and 4 ng GnRH was not significant ($P > .05$). The interaction between potassium and GnRH was not significant indicating that the LH increase after GnRH in 5.9 mM and the 59 mM potassium KRB were similar (Figure 10 a and b). Rate of LH release at 1 hr after 4 ng/ml GnRH was not

TABLE 8.--Interaction of Potassium and GnRH in Stimulation of LH Release from Steer Anterior Pituitary.[a]

Potassium	GnRH	Time (min)[b]				
		-10	16-20	60	76-80	120
(mM)	(ng/ml)	ng/mg/ml				
5.9	1	0.15 ± .03	0.13 ± .02	0.12 ± .02	1.09 ± .04	1.10 ± .04
	4	0.15 ± .01	0.14 ± .01	0.14 ± .02	1.86 ± .04	1.45 ± .10
59	1	0.13 ± .10	1.25 ± .14	1.33 ± .10	1.51 ± .20	1.50 ± .13
	4	0.14 ± .01	1.29 ± .16	1.40 ± .12	1.38 ± .12	1.93 ± .62

[a]Mean ± S.E.

[b]Explants exposed to different potassium concentrations during 0 to 60 min and GnRH during 60 to 80 min of incubation.

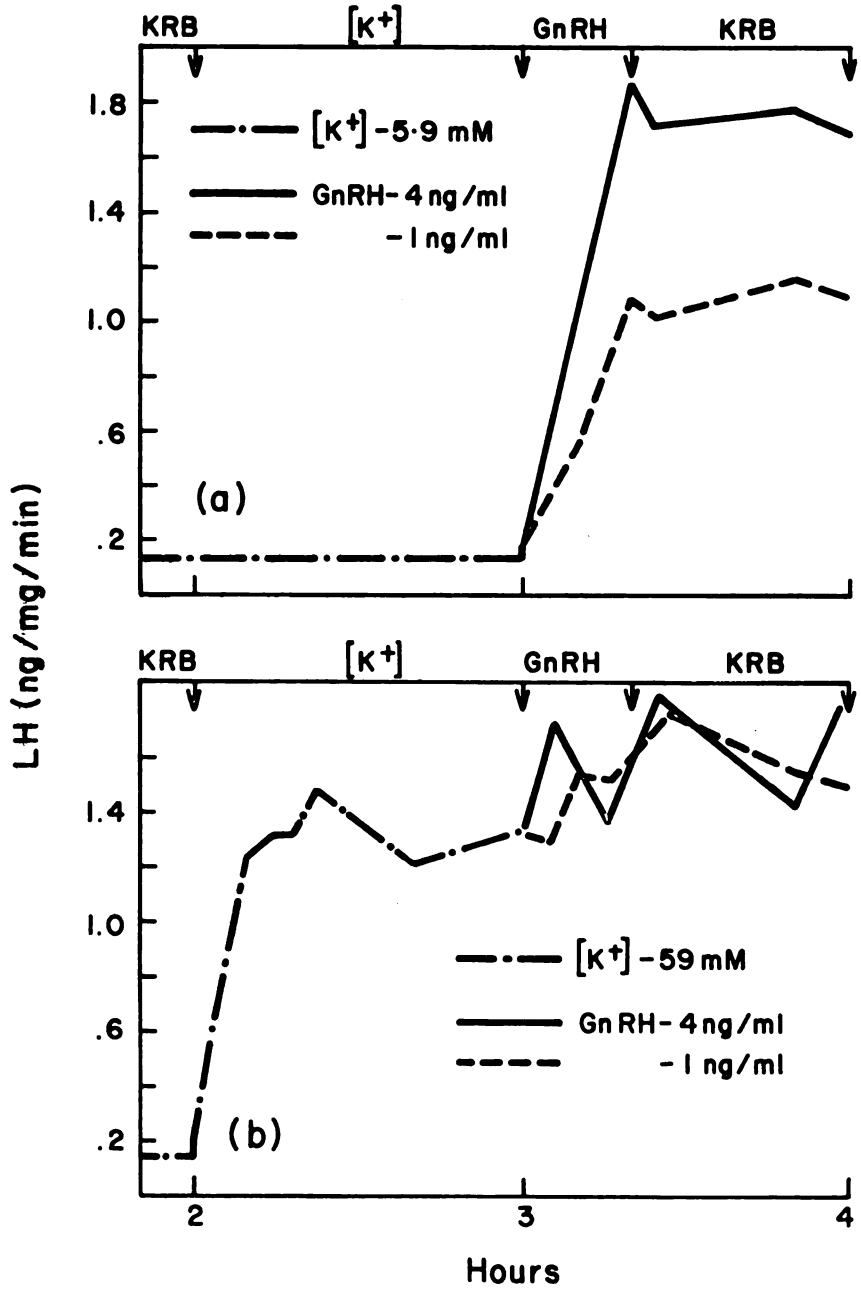

Figure 8.--Luteinizing hormone response of steer pituitary explants to enhanced potassium and synthetic gonadotropin releasing hormone in vitro. Arrows (↓) indicate start and termination of the exposure of the anterior pituitary tissue to specified media: (KRB) Krebs-Ringer bicarbonate buffer with glucose; (K^+) 5.9 or 59 mM potassium in KRB; as depicted on the graph; (GnRH) 1 or 4 ng/ml gonadotropin releasing hormone in KRB, as depicted on the graph.

different whether AP tissue was pretreated with potassium-enhanced KRB or not. The final rate of LH release after 1 ng/ml GnRH was lower from tissue pretreated with 5.9 mM KRB than from tissue pretreated with 59 mM potassium KRB. This indicates that the LH release in vitro cannot be stimulated beyond a maximal rate which is characteristic of the pituitary tissue being used and presumable dependent on the pituitary donor.

In the next two incubations of this experiment, steer anterior pituitary tissues were preincubated in TC 199 for 2 hr then in calcium-free KRB for 2 hr challenged with either 1 or 4 ng GnRH/ml medium 1 hr and exposed to KRB for 2 hr. Then the tissue was incubated with 23 or 56 mM potassium in calcium-free KRB for 1 hr which was replaced by calcium free for last 2 hr. Preincubation in calcium-free KRB caused a decrease ($P < .01$) in the rate of LH release from steer AP from a baseline of 0.18 ng/mg/min to 0.08 ng/mg/min (Figures 9 and 10). Since LH release is not impaired in KRB, a partial decrease in the stimulatory effect of potassium-free KRB (5.9 mM-k+) may account for this decline, but an inhibitory effect due to absence of calcium and independent of potassium cannot be eliminated. Thus, in this investigation enhanced potassium caused LH release and also normal LH release is calcium dependent. These results are in contrast to those of others (Samli and Geschwind, 1968; Wakabayshi,

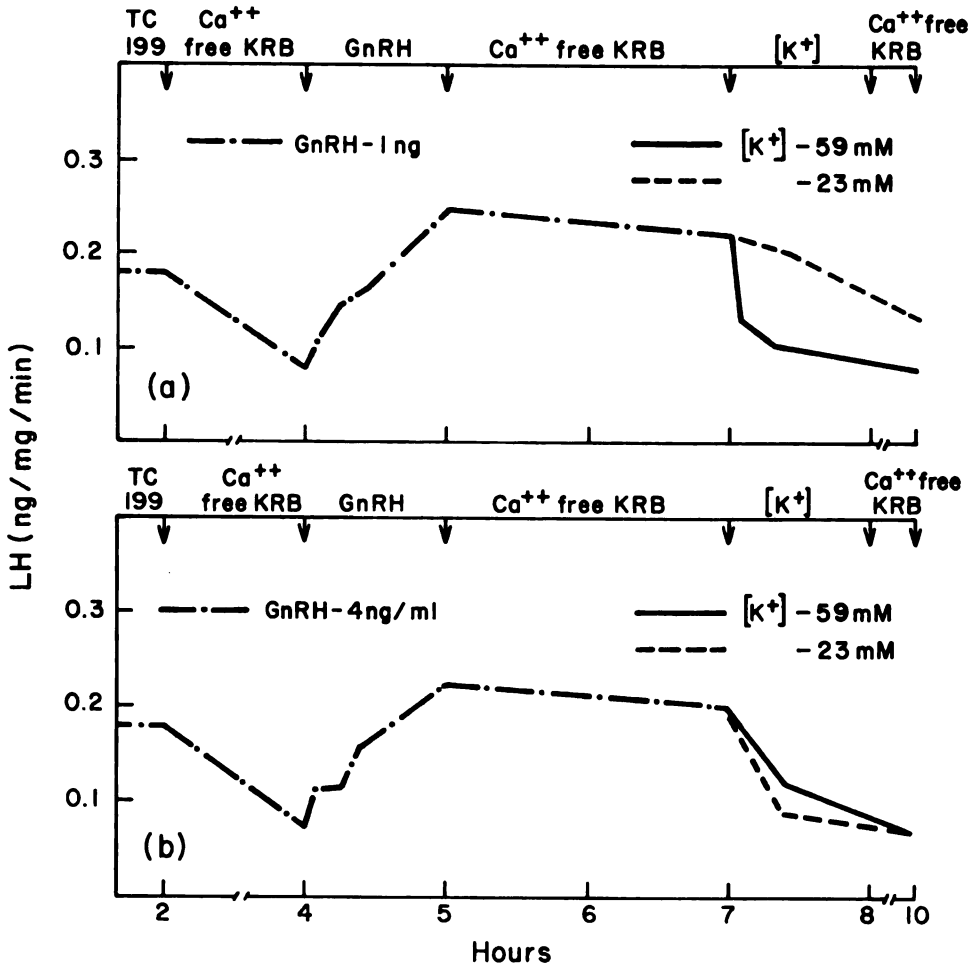

Figure 9.--Luteinizing hormone response of steer pituitary explants to synthetic gonadotropin releasing hormone and enhanced potassium in calcium-free medium. Arrows (↓) indicate start and termination of the exposure of the anterior pituitary tissue to various media: (TC 199) medium 199, (Ca^{++}-free KRB) Krebs-Ringer bicarbonate buffer with glucose where calcium chloride was substituted by equivalent amount of sodium chloride, (GnRH) 1 or 4 ng/ml synthetic gonadotropin releasing hormone in calcium-free KRB, as depicted on the graph; (K^{+}) 23 or 56 mM potassium in calcium-free KRB, as depicted on the graph.

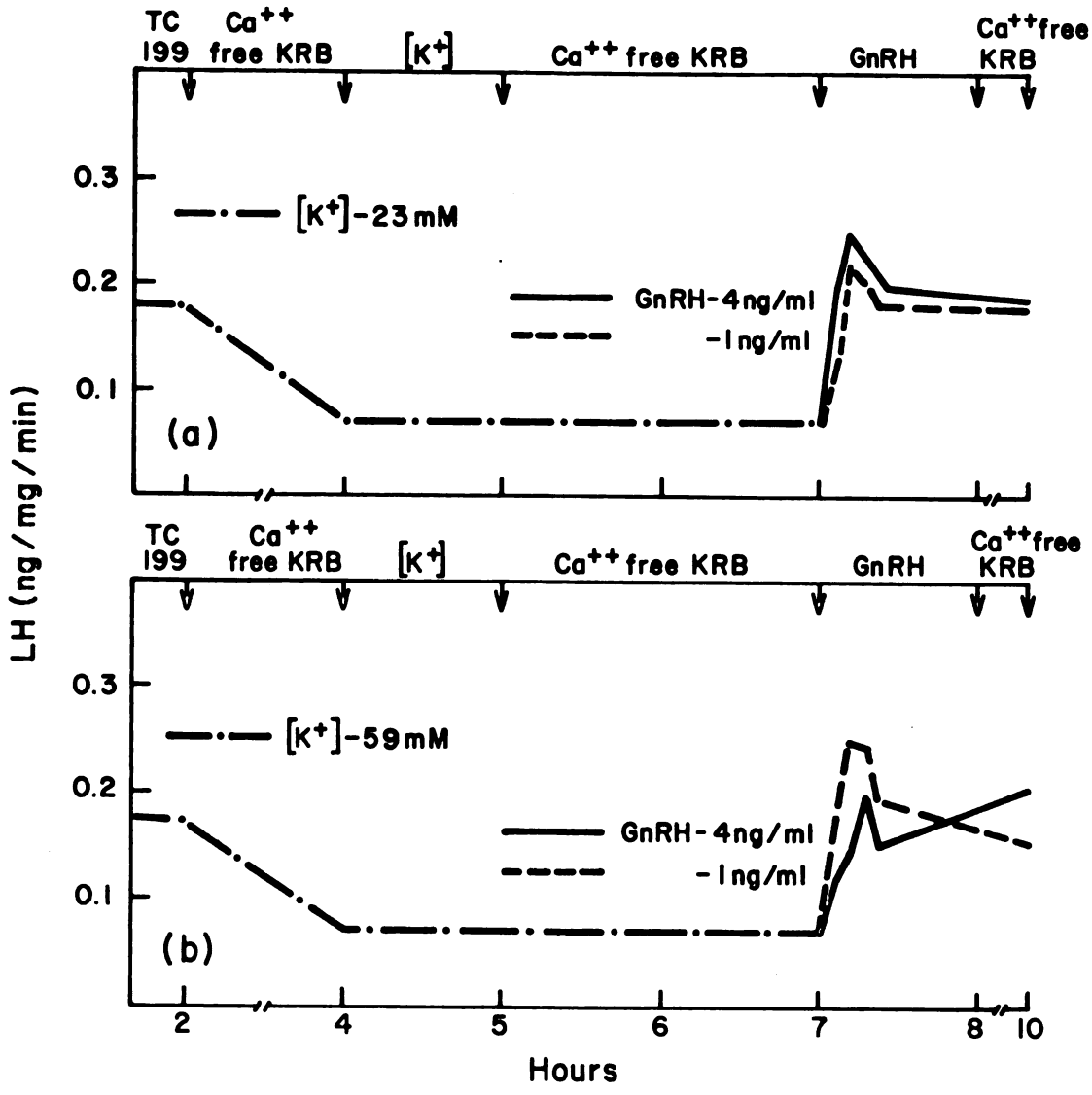

Figure 10.--Luteinizing hormone response of steer pituitary explants to enhanced potassium and synthetic gonadotropin releasing hormone in calcium-free medium. Arrows (↓) indicate start and termination of the exposure of the anterior pituitary tissue to specified media: (TC 199) TC medium 199; (Ca^{++}-free) Krebs-Ringer bicarbonate buffer with glucose where calcium chloride was substituted by equivalent amount of sodium chloride; (K^{+}) 23 or 56 mM potassium in calcium-free KRB, as depicted on the graph; (GnRH) 1 or 4 ng/ml synthetic gonadotropin releasing hormone in calcium-free KRB, as depicted on the graph.

Kamberi and McCann, 1969; Jutisz and de la Llosa, 1970) who observed that LH release caused by potassium-enhanced medium was completely blocked in calcium-free medium. Earlier, Rasmussen and Tennenhouse (1968) postulated a key role for calcium ions in mechanisms associated with cellular secretory activity. Increased intake of ^{45}Ca was associated only with ACTH release induced by a potassium-enriched medium (Kraicer and Milligan, 1971). Crude acid extract of rat stalk-median-eminence, synthetic lysine vasopressin, dibutyryl cyclic AMP or theophylline all stimulate ACTH release but did not increase ^{45}Ca intake by pituitary tissues in vitro. Therefore, the authors concluded that "perhaps only the presence of calcium and not an increased intake of calcium is required for release of preformed hormones from the adenohypophysis."

Gonadotropin releasing hormone in concentration 1 and 4 ng/ml increased ($P < .05$) LH release from 0.08 to 0.23 ng/mg/min and there was no difference ($P > .05$) between treatment means (Table 9). The increase was steady but relatively slow (Figure 9) compared to the stimulation in normal KRB (Figure 8). Others have emphasized an absolute calcium dependency of LRF-induced LH release (Samli and Geschwind, 1968; Justisz and de la Losa, 1970); medium was supplemented with ethylene-diamino-tetraacetic acid (EDTA) in order to remove all

TABLE 9.--Interaction of GnRH and Potassium[a] in Stimulation of LH Release from Steer Anterior Pituitary in Calcium-Free Medium.

GnRH	Potassium	Time (min)[b]				
		-10	16-20	60	196-200	240
(ng/ml)	(mM)	ng/mg/min				
1	23	0.08 ± .02	0.17 ± .07	0.32 ± .10	0.20 ± .08	0.13 ± .06
	56	0.07 ± .02	0.11 ± .01	0.17 ± .02	0.11 ± .03	0.08 ± .03
4	23	0.08 ± .01	0.13 ± .01	0.26 ± .02	0.10 ± .02	0.08 ± .02
	56	0.12 ± .01	0.12 ± .01	0.23 ± .03	0.12 ± .01	0.08 ± .02

[a]Mean ± S.E.

[b]Explants exposed to GnRH during 0 to 60 min and to enhanced potassium concentrations during 180 to 240 min of incubation.

calcium ions. However, Wakabayshi, Kamberi, and McCann (1969) demonstrated that at least Na-EDTA affects LH releasing mechanism independent of calcium removal and concluded that LRF-induced LH release was only partially inhibited in calcium-free medium.

As with normal KRB medium (Table 7), calcium-free medium with enhanced potassium concentration did not cause any additional increase in rate of LH release but rather a significant decrease (Table 9). These declines for high (56 mM) and low (23mM) potassium were not parallel (Figure 9) indicating an interaction when the enhanced potassium was present in KRB following GnRH exposure. For possible explanation the reader is referred to the appropriate section of General Discussion.

In the final two incubations of this experiment, steer anterior pituitary tissue was preincubated for 2 hr in TC 199, ror 2 hr in calcium-free KRB, for 1 hr in either 23 or 56 mM potassium KRB, for 2 hr in calcium-free KRB; then the tissue was challenged for 1 hr with either 1 or 4 ng/ml GnRH and further incubated for 2 hr in calcium-free medium. Incubation for 1 hr in potassium-enhanced KRB (23 or 56 mM) did not change ($P > .05$) the rate of LH release from tissue preincubated in calcium-free KRB (Table 10). Subsequent exposure to GnRH (1 or 4 ng/ml) increased the rate of LH release on the average 100% within 16 min but not more than previously due to

TABLE 10.--Interaction of Potassium and GnRH in Stimulation of LH Release from Steer Anterior Pituitary in Calcium-Free Medium.[a]

Potassium	GnRH	Time (min)[b]				
		-10	16-20	60	196-200	240
(mM)	(ng/ml)	ng/mg/ml				
23	1	0.07 ± .01	0.08 ± .01	0.09 ± .01	0.18 ± .05	0.18 ± .03
	4	0.05 ± .01	0.06 ± .01	0.07 ± .01	0.20 ± .02	0.18 ± .02
56	1	0.07 ± .01	0.08 ± .01	0.10 ± .01	0.19 ± .07	0.16 ± .10
	4	0.05 ± .01	0.08 ± .01	0.10 ± .01	0.16 ± .07	0.22 ± .06

[a]Mean ± S.E.

[b]Explants were exposed to enhanced potassium concentrations during 0 to 60 min and to GnRH during 180 to 240 min of incubation.

GnRH alone. The rate of LH release remained markedly elevated even 2 hr after the treatment withdrawal (Figure 10), possibly due to the potentiation of the anterior pituitary tissue to GnRH stimulation by elevated potassium.

It is concluded:

(1) Both the GnRH and the enhanced potassium stimulated LH release from bovine AP if present in KRB as separate entities.

(2) The enhanced potassium-induced LH release was completely dependent on extracellular calcium.

(3) The effects of the two are not additive and only when the treatment with enhanced potassium precedes that with GnRH, or perhaps if both are present at once, increase in LH release is observed. However, the stimulatory action of enhanced potassium was completely abolished when GnRH, in concentration causing only submaximal release of LH, was present in the medium prior to enhanced potassium.

(4) In calcium-free KRB, the enhanced potassium inhibited GnRH-induced LH release.

Experiment 5. Release of Bovine Luteinizing Hormone by Synthetic Gonadotropin Releasing Hormone (GnRH) in Vivo

Serum LH concentration of bulls prior to GnRH treatment averaged 1.1 ng/ml and increased to peaks of 9.2, 19.3, and 39.1 ng/ml after 10, 40, and 160 μg GnRH. The average interval after GnRH to attain these peaks was

20, 53, and 113 min ($P < 0.01$). The relationship between dose of GnRH and magnitude of the LH peak is described by the linear function in Figure 11. The interval from GnRH administration and the magnitude of LH peak were significantly correlated 0.88 ($P < .01$). Neither individual bulls nor days of treatment affect ($P > .05$) the magnitude of the LH peak.

Average serum LH (in heifers) before GnRH treatment was 0.5 ng/ml; LH increased ($P < .001$) to peaks of 1.9, 7.3, and 11.1 ng/ml after 5, 20, and 80 µg GnRH. In contrast to results for bulls, the relationship between dose of GnRH administered and LH peak response was described for heifers by a quadratic function (Figure 11). The LH peaks occurred 13, 16, and 26 min ($P < .01$) after 5, 20, and 80 µg GnRH, respectively, and magnitude of the LH peak and the interval to the peak were significantly correlated 0.92 ($P < .01$). Differences in the magnitude of the LH response attributed to day of treatment or to individual heifers were not significant.

The response curves shown in Figure 12 for an individual bull and early luteal phase heifer can be considered typical of each group since between animal variation was not significant. These curves show the time course of LH response to GnRH; serum LH in all animals was increased within 5 min after GnRH injection, increased to a peak at a time that was related to the magnitude of

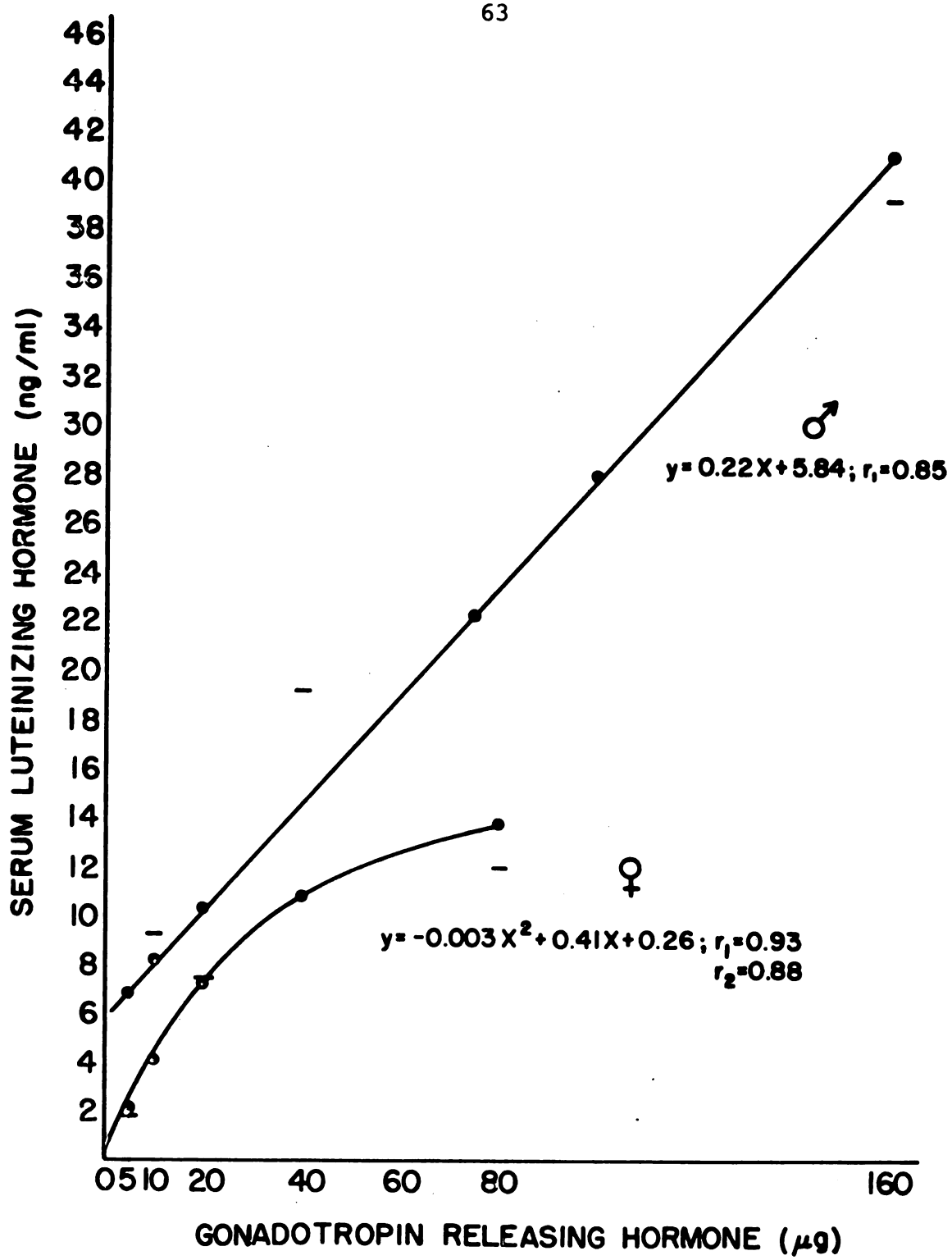

Figure 11.--Peak serum luteinizing hormone response in bulls and heifers after synthetic gonadotropin releasing hormone.

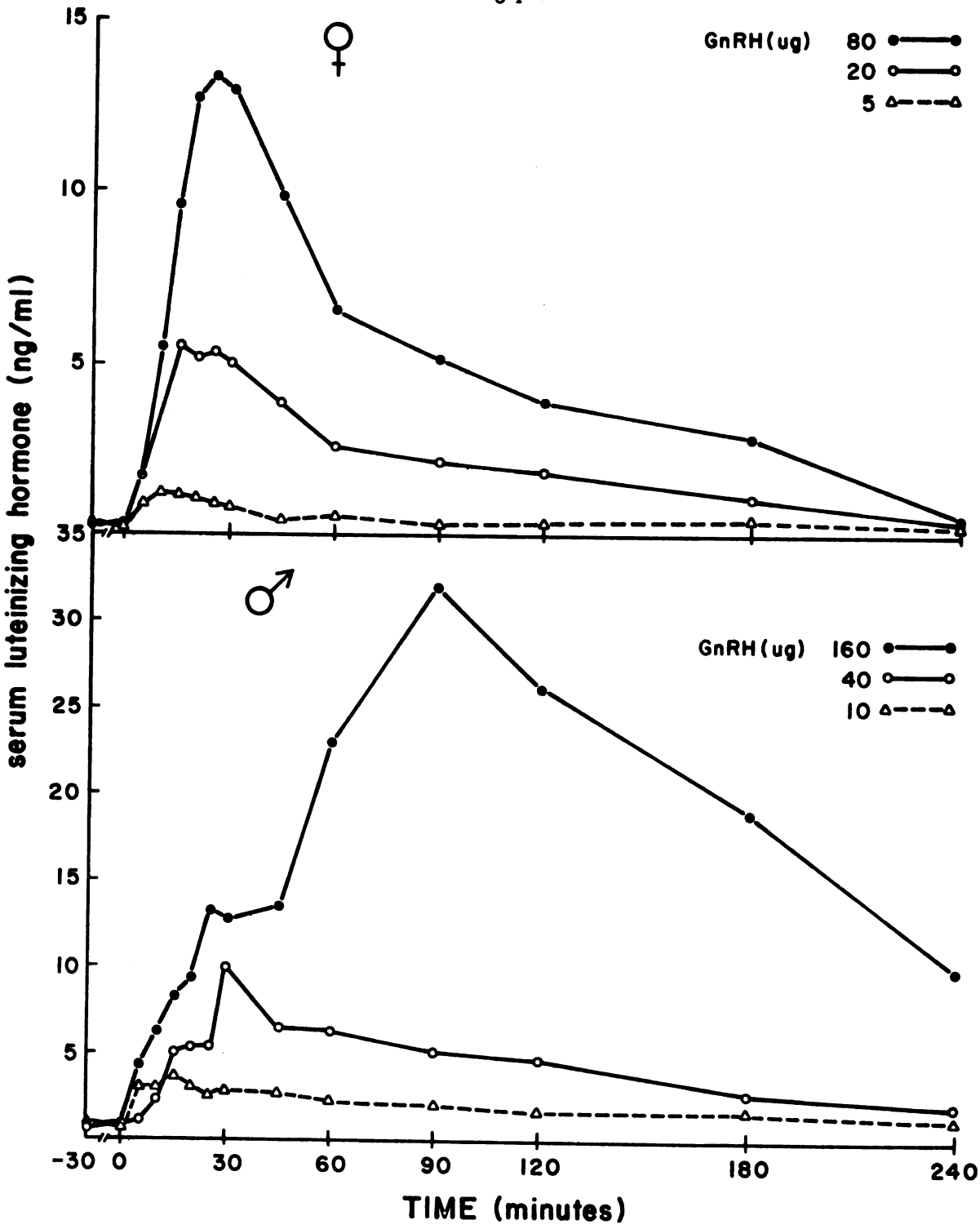

Figure 12.--Time course of the luteinizing hormone response to synthetic gonadotropin releasing hormone for one bull and one heifer.

the LH peak, and then LH declined progressively. In addition, the LH response in bulls was characterized by an early rapid increase followed by a plateau then further surge. This was the response observed for each bull tested.

To my knowledge, this is the first report to demonstrate that synthetic GnRH induces release of LH in heifers and bulls. The magnitude of the LH peak increased linearly with increasing dose up to 160 µg of GnRH in bulls while peak LH response in heifers appeared to plateau at 80 µg GnRH, indicating that the male bovine pituitary is more responsive to GnRH than that of females subject to the restriction that these early luteal phase heifers are characteristic of all ages and all stages of the estrous cycle. This is in contrast to reports by others (Reeves, Arimura, and Schally, 1971; Arimura et al., 1972) who observed no difference in LH response of diestrus ewes and rams to purified porcine or synthetic GnRH, nor did they observe a dose response relationship. However, Reeves, Arimura, and Schally (1970) demonstrated a linear increase in LH response with increasing GnRH in wethers, not unlike the response we observed in bulls. Differences in pituitary responsiveness to GnRH may reflect changes in steroid hormone environment since increased serum estrogens precede and may trigger the spontaneous LH surge in cattle (Wetteman et al., 1972),

and pituitary sensitivity to GnRH is maximum during an 84 hr period around estrus in ewes (Reeves, Arimula, and Schally, 1971).

The increase in LH which followed the maximum level of GnRH for heifers in this study was approximately half that which was recently reported from this laboratory (Swanson and Hafs, 1971) to characterize the ovulatory surge of LH (i.e., mean, 25.9; range, 19-35 ng/ml). This comparison suggests that under the conditions of this study, GnRH did not release LH in quantities characteristic of the ovulatory surge. Possibly a single surge of releasing hormone from the hypothalamus is not the exclusive event which mediates the preovulatory surge of LH.

Although the time periods involved are much shorter, the plateau in LH response for bulls in this experiment is reminiscent of result reported for anestrous ewes. Thus, Arimura et al. (1972) observed that large doses of GnRH (250 μg) caused an initial increase in ovine serum LH followed by a plateau that was sustained for 25 min or longer, then a further LH surge with the second peak occurring at 1.5 hr after GnRH. Perhaps the earlier occurrence and shorter duration of the plateau in serum LH in bulls relative to anestrous ewes (Arimura et al., 1972) is the result of the lower dose of GnRH used; our highest dose of GnRH in bulls (av 78.3 kg body wt) was only 60% of that used in anestrous ewes for the

biphasic response. Failure to detect a similar two-component response in heifers in the early luteal phase may suggest that this type of response is a condition of the prevailing endocrine environment.

That the interval to the LH peak after GnRH is directly related to the magnitude of LH peak is new. This result suggests that LH in samples taken at a few preset intervals after GnRH (Debeljuk, Arimura, and Schally, 1972) may not describe accurately the LH response; it also indicates that the effect of GnRH is prolonged beyond the time when GnRH is presumably removed from circulation, possibly reflecting binding of GnRH to pituitary cells and continued release for some time thereafter. This hypothesis is supported by the continued LH release after withdrawal of GnRH in vitro (Figure 6).

Others have demonstrated large variation between animals in response to synthetic GnRH although it was not verified statistically (Debeljuk, Arimura, and Schally, 1972). In contrast, we observed no significant variation among heifers or among bulls, or the day of GnRH treatment. This result suggests that GnRH may be used, at least within a homegeneous population, with high predictability in treatment of cattle.

Experiment 6. Relationship between the LH Response to GnRH and Endogenous Ovarian Steroids

Estradiol averaged 10.8 pg/ml in serum collected from heifers on day 20 of the estrous cycle, which was greater ($P < 0.05$) than the comparable average on day 15 (5.1 pg/ml; Table 11). Mean estrone concentrations were 6.9 and 7.4 pg/ml in serum collected on day 15 and 20 of the estrous cycle, and differences between means were not significant ($P > .05$). However, serum progesterone concentration decreased ($P < .01$) 10 fold from 3.9 ng/ml on day 15 of the estrous cycle to 0.39 on the day following the demise of the corpus luteum.

Serum LH concentrations prior to GnRH treatment were 0.6 and 0.8 ng/ml on day 15 and 20 of the estrous cycle and differences between means were not significant ($P > .05$). GnRH at doses of 5, 40, and 320 µg increased ($P < .01$) serum LH concentration to peaks 1.8, 7.7, and 13.9 in heifers treated on day 15 of the estrous cycle, and to peaks of 1.0, 13.6, and 8.8 ng/ml in serum of heifers treated on day 20 of the cycle (Table 12). The effect of day of estrous cycle when treatments were administered was not significant ($P > .05$). However, the interaction of the main effects (dose of GnRH x day of estrous cycle) was significant ($P < .05$) indicating a failure of each dose GnRH to cause a similar release of LH on both days. This interaction can be explained on the

TABLE 11.--Serum Estradiol, Estrone, and Progesterone on Day 15 and 20 of the Estrous Cycle in Holstein Heifers.[a]

Hormone		Day of the Estrous Cycle	
		15	20
Estradiol	(pg/ml)	5.05 ± 0.70	10.79 ± 2.25
Estrone	(pg/ml)	6.87 ± 1.25	7.45 ± 0.89
Progesterone	(ng/ml)	3.90 ± 0.51	0.39 ± 0.12

[a]Values are means ± standard error, n = 5.

TABLE 12.--Peak Concentration of Luteinizing Hormone in Serum of Heifers Administered Gonadotropin Releasing Hormone (GnRH) on Days 15 and 20 of the Estrous Cycle.[a,b]

GnRH[c]	Day of the Estrous Cycle	
	15	20
(µg)	(ng/ml)	
5	1.8 ± 0.5	1.0 ± 0.1
40	7.7 ± 1.4	13.6 ± 4.3
320	13.9 ± 1.8	8.8 ± 1.2

[a]Values are means ± standard error.

[b]Five heifers per group.

[c]Administered iv.

basis of differences in serum ovarian steroids prior to GnRH treatment and will be discussed below.

Baseline LH values in this study are comparable to those reported by Swanson and Hafs (1971). In contrast, stimulation of LH release by GnRH did not yield LH peak values comparable to those characteristic of the preovulatory LH peak reported by these authors. In only 1 of 24 observations did concentrations of LH at the peak exceed 19 ng/ml, the lowest value previously reported for the preovulatory peak using comparable standards (Swanson and Halfs, 1971). Estimates of the nagnitude of the LH peaks in this experiment should be accurate since blood was collected on a 2-minute sampling schedule at the time when LH peaks occurred. We conclude that the in vivo preovulatory LH surge is probably not facilitated by a single surge of releasing hormone of short duration. But we do not exclude the possibility that the LH release characteristic of the preovulatory surge can be imitated by repeat administration or infusion of GnRH.

The estradiol concentration measured in serum collected 4 hours after GnRH treatment was increased relative to the comparable pretreatment average on day 15 but decreased on day 20 of the cycle ($P < .02$, Table 13). The average increase on day 15 was 0.87, 8.39, and 12.51 ng/ml following 5, 40, and 320 µg GnRH, respectively, and might reflect stimulation of follicular growth since GnRH

TABLE 13.--Change in Serum Estradiol 17β Concentration Following GnRH Administration in Heifers.[a,b]

GnRH[c] (μg)	Day of the Estrous Cycle	
	15	20
	(pg/ml)	
5	0.87 ± 1.11	-6.36 ± 4.58
40	8.39 ± 7.53	-7.27 ± 3.84
320	12.51 ± 7.66	4.44 ± 1.91

[a]Values are means ± standard error.

[b]Five heifers per group.

[c]Administered iv.

also causes release of follicle stimulating hormone in the bovine (Zolman and Convey, 1973). Progesterone was increased only when LH release was sufficiently stimulated (Table 14) and when functional luteal tissue was present. This increase averaged 3.8 and 2.7 ng/ml following 40 and 320 μg GnRH, respectively, and represents stimulation of progesterone release from functional corpora lutea. These results point out the luteotropic function of LH in the bovine (Snook et al., 1969). After luteal regression, presumably complete on day 20, no change in serum progesterone was observed. Serum estrone was not changed ($P > .05$) by GnRH treatment (Table 15).

In an attempt to clarify the relationship between the day of the estrous cycle and serum LH response to

TABLE 14.--Change in Serum Estrone Concentration Following GnRH Administration in Heifers.[a,b]

GnRH[c] (µg)	Day of the Estrous Cycle 15 (pg/ml)	20 (pg/ml)
5	-0.11 ± 0.38	11.35 ± 6.97
40	13.31 ± 15.63	-0.14 ± 2.05
320	4.31 ± 6.57	3.60 ± 3.56

[a]Values are means ± standard error.

[b]Five heifers per group.

[c]Administered iv.

TABLE 15.--Change in Serum Progesterone Concentration Following GnRH Administration in Heifers.[a,b]

GnRH[c] (µg)	Day of the Estrous Cycle 15 (ng/ml)	20 (ng/ml)
5	-0.83 ± 0.75	0.24 ± 0.05
40	3.86 ± 2.08	0.45 ± 0.36
320	2.68 ± 1.18	0.79 ± 0.46

[a]Values are means ± standard error.

[b]Five heifers per group.

[c]Administered iv.

GnRH administration, analysis of covariance was utilized. Pretreatment, post-treatment, or increment (calculated by subtraction of pretreatment from post-treatment) for each steroid hormone were entered into the analysis as groups of covariates. The significance level associated with the day of administration did not change following introduction of all covariates into the analysis. This indicates that the effect of day cannot be explained simply by any of the covariates. The interaction between the two main factors GnRH x day could be at least in part explained by the covariates. The final analysis of covariance revealed that the day effect, the day x GnRH interaction, and all the progesterone covariates did not influence ($P > .05$) the LH response to GnRH. Therefore, these variables and covariates were not included in the final analysis covariance table (Table 16). Failure of the endogenous progesterone to influence the LH response to GnRH is of particular interest. Cummings et al. (1972) also reported no effect of injected progesterone on GnRH-induced LH release in the ewe. Differences in dose of GnRH was the most important individual factor in the covariance analysis (Table 16). However, the LH response to GnRH was also influenced significantly by pretreatment levels of estradiol and estrone. Estrone post-treatment and estradiol increments were related also to the response to GnRH, suggesting direct action of

TABLE 16.--Analysis of Covariance of Peak Serum Luteinizing Hormone in Response to Gonadotropin Releasing Hormone (GnRH) Administered on Days 15 and 20 of the Estrous Cycle.

Variable	DF	S.S.	M.S.	F	SIG
Totals	23	0.356			
GnRH	2	0.082	0.041	13.59	0.0005
Estradiol-pre	1	0.020	0.020	6.72	0.019
Estrone-pre	1	0.016	0.016	5.29	0.034
Estrone-post	1	0.022	0.022	7.36	0.015
Estradiol Δ	1	0.011	0.011	3.49	0.079
Error	17	0.052	0.003		

luteinizing hormone (or perhaps also follicle stimulating hormone) on stimulation of estrogen release from the ovary.

In conclusion, LH release after GnRH in heifers not only was affected by the dose of GnRH but also by the pretreatment estrogen concentration. Our observations confirm findings of others in rats and sheep (Arimura and Schally, 1971; Reeves, Arimura, and Schally, 1971b) that estrogen can modify the effect of GnRH on LH release. The mechanism by which estrogens affect pituitary sensitivity to GnRH is still obscure and so far all attempts *in vitro* (Schneider and McCann, 1970) have failed to confirm *in vivo* observations. The effect of estrogen seems to be long term, mediated through more complicated

mechanisms like RNA synthesis (Convey and Reece, 1969) or thyroxine bindings (Schneider, Pribyl, and Rohacova, 1971), related perhaps rather to the synthesis than release of LH (MacLeod, Abad, and Eidson, 1969); or perhaps even associated with growth of the anterior pituitary gland mass (Convey and Reece, 1969; Lisk, 1969).

GENERAL DISCUSSION

For purposes of these investigations, luteinizing hormone and prolactin were considered typical examples of anterior pituitary hormones since the former is under stimulatory, and the latter, inhibitory influences of the hypothalamus (McCann, 1962; Talwalker, Ratner, and Meites, 1963). Numerous experiments of Everett and Nikitovitch-Winder (for review, see Everett, 1964) showed conclusively that rat anterior pituitary tissue transplanted under the kidney capsule will eventually lose most of its ability to secrete LH while prolactin secretion will increase several fold over that characteristic of the intact animal. Gala (1970) and Pasteels (1970) demonstrated persistent prolactin release for 3 weeks or longer in cultures of rat anterior pituitary tissue. The change in release of individual hormones is very likely dependent on time since in this investigation the relative concentration of luteinizing hromone and prolactin in the incubation medium and in explants did not change during 6 hr of incubation in the superfusion system. The relative release of growth hormone (Tucker, Koprowski, and Oxender, 1973), prolactin, and luteinizing hormine into the medium was comparable to that of serum concentrations.

It is conceivable that if the anterior pituitary was under a chronic influence of the hypothalamus which regulated minute-to-minute release of hormones, the relative release rate would not hold during 6 hr incubation *in vitro*. The rate of prolactin release would be expected gradually to rise and that of LH to decline. Since no such change occurred and steady base line was observed throughout our 6 hr experiments explants were either fully functional or, if necrosis occurred, all types of cells were strickened equally. On the other hand, it has been demonstrated that the secretory activity of rat anterior pituitary tissue transplanted under the kidney capsule can be restored to normal by transfer back to sella turcica (Nikitovitch-Winer and Everett, 1958). This indicates a selective stimulation of prolactin release and inhibition of LH release from transplants under the kidney capsule. The apparent discrepancy between *in vivo* and *in vitro* results may suggest that

(1) the influence of the hypothalamus is not one of minute-to-minute regulation, and

(2) the hypothalamus produces a growth promoting (inhibiting) substance for at least certain types of anterior pituitary cells and this substance may be the hypothalamic releasing or inhibiting hormone but with a distinct long-term effect.

Unfortunately, histological examinations did not accompany our *in vitro* experiments and, therefore, the second hypothesis cannot be stated more firmly. Earlier reports (Jutisz, *et al.*, 1971; Tixier-Vidal *et al.*, 1971) indicated partial necrosis of anterior pituitary tissue as early as after 4 hr of incubation in a static *in vitro* system. In the experiments involving pituitary transplants the change in the relative secretion rate of the anterior pituitary hormones proceeded relatively slowly.

Surprisingly, we have observed repeatedly that the basal rate of LH release *in vitro* from steer anterior pituitary is less than 40% that from cow anterior pituitary tissue. Cow and bull anterior pituitary tissues released LH in a similar rate (Zolman and Convey, unpublished). However, Hobson and Hansel (1970) earlier reported that basal serum LH concentration increased following ovariectomy in heifers. Regarding castrates, the difference between *in vivo* and *in vitro* LH release suggests involvement of a releasing hormone. Our data show that the gonadotropin releasing hormone in concentration of 1 ng/ml increased the rate of LH release *in vitro* from steer AP 6 fold over base line (Figure 6), while the same relative increase from cow AP tissue was observed with 16 ng/ml GnRH (Figure 7).

Arimura and Schally (1970, 1971) observed that estradiol increased and progesterone decreased LH response to GnRH when synthetic GnRH was administered into rats

24 or 48 hr after injection of estradiol or progesterone. Therefore, these authors reintroduced the concept of Bogdanove (1964) about potentiation of the anterior pituitary to hypothalamic stimulation by estrogen. Since then this hypothesis has been tested with mostly positive results in the human (Kastin et al., 1970) and ewe (Reeves, Arimura, and Schally, 1971a).

Recently, the responsiveness to GnRH has been shown to fluctuate with serum estrogen during menstrual cycle (Yen et al., 1972). However, in other species, the physiological role of endogenous estrogen in potentiation of the anterior pituitary to GnRH is uncertain. For instance, during the estrous cycle in cattle there are profound changes in concentration of ovarian steroids. Estradiol 17 is elevated over base line for at least 4 days during late luteal and follicular phases of the cycle. Thus, estrogens may act at both pituitary and hypothalamic level and their effect does not have to depend necessarily on any particular concentration in serum at any particular time. In this investigation, the stimulatory effect of endogenous estrogen on LH response to GnRH became apparent only when excessive variation among animals was removed by means of analysis of covariance technique. This indicates that changes in serum estrogen which are smaller than normal differences among animals exert a significant influence. Although this effect was minor in comparison

with the effect of GnRH in our experiment, under normal conditions, the physiological importance of estrogen will depend also on physiological concentration of GnRH "equivalent."

An expression "equivalent" is used here to indicate the releasing activity which may be mediated by unknown quantities of a decapeptide of hypothalamic origin, or by some other LH releasing agents of hypothalamic origin with varying potencies in relation to GnRH. Our data indicate that GnRH is at least one of the luteinizing hormone releasing hormones in the bovine. Dose response relationship between quantity of this peptide used and LH response in vivo and in vitro was established. Moreover, the same relative potency of this synthetic preparation and the purified porcine preparation indicated a high degree of similarity. However, the search for pituitary active substance of hypothalamic origin still continues (Coy, Coy, and Schally, 1973) even though GnRH in portal blood has already been measured (Ben-Jonathan and Porter, 1973).

Profiles of FSH and LH release after GnRH in bulls have many similar features (Zolman and Convey, 1973). Under the assumption that FSH and LH serum concentration after GnRH follow a similar distribution, interpretation of steroid changes after GnRH treatment is rather difficult. Our data showed that estradiol 17 and estrone were increased ($P < .08$) in serum of heifers 240 min after GnRH

treatment. Furthermore, covariance analysis indicated direct cause-effect relationship between LH response to GnRH and these increases. Similarly, both androstenedione and testosterone in serum were significantly increased after GnRH in bulls (Zolman and Convey, 1973). However, whether both gonadotropins are responsible for steroid increases or only one of them has not been established. Nevertheless, participation of all three components controlling bovine reproduction--the releasing hormone, gonadotropins, and steroids--has been experimentally confirmed here.

Undoubtedly, higher nerve centers above the hypothalamus also make input into the neuroendocrine scheme. The feedback from the endocrine system is mediated mostly by nonproteins such as steroids which modify some physiological components of the nerve system; for example, the electrical activity of forebrain (Lincoln, 1969) and limbic system (Innes and Michal, 1970). If the synthetic decapeptide is the gonadotropin releasing hormone, the influence of the brain on LH release was presumably passed by in our experiments. However, in some instances this presumption might not be valid. For example, it might not be taken into account if brain exerted long-term effect on LH release. Thus, interpretation of sex differences in LH response to GnRH on the basis of different steroid environment might not be

unequivocal. The main site of the regulatory action can be the anterior pituitary itself, as well as hypothalamus, other parts of brain or stalk-medium eminence, which all have been shown to bind steroids selectively (Stumpf, 1969; Anderson and Greenwald, 1969; Eisenfeld, 1970).

Largely, through the mechanism which is initiated by this binding (Clark, Campbell, and Peck, 1972; Leavitt, Kimmel, and Friend, 1973), steroids have been implicated in regulation of gonadotropin and prolactin secretion in many different ways. Experiments with steroid implants in the hypothalamus or anterior pituitary were not conclusive as far as the site of action of the steroids, and usually both sites were at least partially implicated. Results of in vitro studies testing the effect of steroids on gonadotropin (Piacsek and Meites, 1966; Schneider and McCann, 1970) and prolactin (Nicoll and Meites, 1962; Gala and Reece, 1964) varied among laboratories while the effects on release and synthesis of hypothalamic hormones was investigated only indirectly (for review, see McCann and Porter, 1968) because of lack of sufficiently sensitive and specific methods for their measurements. The effects of steroids on the brain was examined almost exclusively in vivo with a wide variety of results (Lincoln, 1969; Innes and Michal, 1970). Thus, despite considerable effort, the mechanisms and site of steroid action in endocrine tissue remain as one of many unresolved problems.

Although some steroids (glucocorticoids) have been implicated in interference with binding of releasing factor (corticotropin releasing factor; Kraicer et al., 1969) at the anterior pituitary cell membrane the regulatory action of steroids is probably mediated via effects on transcription or translation.

Recent efforts to answer questions concerning mechanisms that are involved in minute-to-minute regulation of anterior pituitary hormone release did not yield expected results (McCann, 1971). In particular, the comparison of enhanced potassium- and releasing factor-induced, anterior pituitary hormone release and emphasizing additivity of their effects without any distinction (Samli and Geschwind, 1968) was misleading. Furthermore, the role of extracellular calcium in regulation of anterior pituitary hormone release was apparently overestimated (for review, see McCann, 1971). It is getting more and more clear that the releasing hormone induced anterior pituitary hormone release has two distinct features: (1) it is mediated via cAMP (Zor et al., 1969), and (2) it is not completely dependent on extracellular calcium.

Luteinizing hormone release can be stimulated in calcium-free medium. Since this stimulation is only partial and slow, it is a possibility that intracellular stores may be involved. Recent results from other laboratories suggested no participation of extracellular calcium

when ACTH (Milligan and Kraicer, 1971) and GH (Wilson, Dhariwal, and Peake, 1973) release was stimulated by hypothalamic extract, but the enhanced potassium-induced ACTH and GH release *in vitro* were always associated with calcium influx. Our data also indicate a complete dependency of potassium-induced LH release on presence of calcium in the incubation media. It has also been shown recently (Fleischer *et al.*, 1973) that oubain stimulated ACTH and GH release *in vitro* is calcium dependent. Oubain, a digitalis glycoside, is known to bind to and inhibit membrane active Na^+, K^+/ATPase. Because of this analogy, it is conceivable that potassium-induced LH release is also mediated by this enzyme.

Both enhanced potassium and GnRH, in our study, stimulated LH release *in vitro* in a similar manner, but there was no evidence that the two effects were additive. Furthermore, the rate of LH release was increased when tissue previously exposed to enhanced potassium (causing submaximal release) was further stimulated by GnRH, but not vice versa. This indicates that the two mechanisms are not completely independent, but the nature of relationship between the two has not been elucidated. Adenosine-3', 5'-monophosphate is not involved in potassium-induced LH release (Zor *et al.*, 1970). Since the cell membrane is likely to be involved in initial steps of stimulation by the releasing hormone, the enhanced

potassium activates the releasing process probably somewhere down toward the end of the pathway. In the case of GnRH, the calcium necessary for completion of hormone release may be translocated from the membrane during initiation step and utilized later. This may cause conformational changes resulting in a decreasing in permeability or availability of potassium which, in the case of involvement of Na^+, K^+/ATPase, might be of critical importance. This could explain the absence of stimulation of LH release from tissue exposed previously to GnRH when potassium was added to the incubation medium. Under physiological conditions this kind of interaction may determine quantitative relationship between tonic and acute release of LH.

Hypothalamus, pituitary gland, and target glands of anterior pituitary hormones comprise the skeleton of the mammalian endocrine system. Hormones of the pituitary gland have been characterized and anterior pituitary hormones appear to play a key role in overall regulation of mammalian metabolism. Detail understanding of mechanisms of hormone release from the anterior pituitary and its regulation may lead to new possibilities in the effort to manipulate specifically endocrines in medicine and for purposes of animal agriculture.

SUMMARY AND CONCLUSIONS

Hormone release from bovine anterior pituitary was studied by both *in vivo* and *in vitro* methods.

Gonadotropin releasing hormone (GnRH) stimulated ($P < .01$) LH release from bovine anterior pituitary. Serum LH of yearling heifers tested during the early luteal phase (day 5-10) of the estrous cycle increased from a base line of 0.5 ng/ml to peaks of 1.9, 7.3, and 11.1 ng/ml after a single iv injection of 5, 20, or 80 µg GnRH. Peak serum concentration occurred invariably within 30 min of GnRH administration. In a similar experiment, GnRH elevated serum LH to 1.89, 7.7 and 13.9 ng/ml in doses of 5, 40, and 320 µg during the late luteal phase (day 15) of the estrous cycle. The same dose of RH increases serum LH on the average to 1.0, 13.6, and 8.8 ng/ml on day 20. In the latter experiment, high serum estradiol 17 and estrone concentrations prior to GnRH were associated ($P < .01$) with greater LH response to GnRH. However, our data strongly indicate that the LH response to GnRH is similar throughout the entire estrous cycle.

Following GnRH treatment, mean serum concentration of estradiol 17 measured at 240 min post-treatment increased on day 15, but decreased on day 20. This

difference was significant. The increment in estradiol 17 concentration and LH concentration at peaks were correlated with each other ($r = 65$; $P < .01$). Covariance analysis revealed direct cause-effect relationship between LH response to GnRH and estrogen (estradiol and estrone) increases.

Luteinizing hormone concentration in serum from mature bulls averaged 1.1 ng/nl before GnRH and increased to peaks of 9.2, 19.3, and 39.1 ng/ml after 10, 40 and 160 µg GnRH, respectively. The interval from GnRH injection to the LH peak and magnitude of the peak were positively correlated ($r = 0.92$; $P < .01$). The relationship between the dose of GnRH and magnitude of the LH peak was best described by linear function, $y = 0.22x + 5.84$. The regression equation calculated for the same relationship in heifers during the early luteal phase had a singificant quadratic component ($y = -0.003x^2 + 0.41x + 0.26$). It is concluded that (1) there is a dose response relationship between GnRH and serum LH in both bulls and heifers, but the bulls responded in a broader dose range, and (2) in terms of LH response, mature bulls are much more sensitive to GnRH treatment than heifers.

It was demonstrated that synthetic decapeptide (GnRH) is active directly in AP tissue causing LH release in vitro in proportion to the dose. Basal rates of LH release into incubation medium of 0.25 ng/mg/min increased

to 0.70 and 1.12 ng/mg/min after 4 min exposure to 1 and 4 ng purified porcine gnRH/ml medium, respectively. Comparable values were 0.94 and 1.50 ng/mg/min after 20 min. Relative potencies of highly purified porcine and synthetic GnRH preparations were comparable in respect to both the magnitude of stimulated LH release in vitro and its profile. Failure of LH release in vitro to return to base line after releasing hormone withdrawal was interpreted as indicative of association of luteinizing hormone releasing hormone with the anterior pituitary cell membrane.

Under our experimental conditions, ovarian steroids were found totally ineffective in any changing in the rate of luteinizing hormone, prolactin, and growth hormone release in vitro.

On the other hand, enhanced potassium concentration consistently stimulated LH release in a manner similar to that of GnRH. On the average, rate of LH release from steer pituitary increased from 0.14 to 1.27 ng/mg/min within first 16 min of exposure to 59 mM potassium KRB. The enhanced potassium-induced LH release was completely abolished in calcium-free KRB. Both stimulators, GnRH and enhanced potassium probably stimulate the same biochemical pathway since (1) the effects of the two are not additive and (2) the stimulatory action of the enhanced potassium was completely abolished when GnRH in concentration which causes only submaximal LH release was in medium prior to

enhanced potassium. In addition, the enhanced potassium shortened the time of GnRH-induced LH release in calcium-free KRB. Absence of calcium in KRB decreased ($P < .01$) rate of LH release from base line of 0.18 to 0.08 ng/mg/min. Subsequent addition of 1 or 4 ng/ml GnRH increased the LH release to 0.23 ng/mg/min.

In the course of this thesis work, an in vitro superfusion system for bovine anterior pituitary has been developed and effect of several incubation variables evaluated. Explants from cows released 137% more LH than those from steers but 65% less prolactin. Average LH concentration 2 and 6 hr postculture was 49% greater in explants from cows. Prolactin release but not LH release was greater into medium containing 0.224% than 0.035% $NaHCO_3$ and postculture concentration in the pituitary tissue inversely reflected this pattern. Release and postculture tissue concentration of LH and prolactin was greatest for explants from the central and peripheral pituitary regions, respectively. Explant size did not influence release of prolactin and LH but release of both hormones was increased significantly during the incubation period.

APPENDICES

APPENDIX A

PREPARATION OF TC MEDIUM 199

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PREPARATION OF TC MEDIUM 199

Sodium bicarbonate solution (2.8% $NaHCO_3$) . . .	80 ml
Antibiotic solution (170 mg penicillin g/100 ml) .	40 ml
TC 199, 10x concentrate	100 ml
(for composition, see Appendix B)	
Sterile, deionized water	780 ml
Total	1,000 ml

APPENDIX B

COMPOSITION OF TC

MEDIUM 199

APPENDIX B

COMPOSITION OF TC MEDIUM 199

TABLE 17.--Composition of TC Medium 199.

Ingredients Per Liter		
l-Arginine HCl	70	mg
l-Histidine HCl H_2O	21.88	mg
l-Lysine HCl	70	mg
l-Tyrosine	40	mg
l-Tryptrophan	10	mg
l-Phenylalanine	25	mg
l-Cystine	20	mg
l-Methionine	15	mg
l-Serine	25	mg
l-Threonine	30	mg
l-Leucine	60	mg
l-Isoleucine	20	mg
l-Valine	25	mg
l-Glutamic Acid H_2O	75	mg
l-Aspartic Acid	30	mg
l-Alanine	25	mg
l-Proline	40	mg
l-Hydroxyproline	10	mg
Glycine	50	mg
Ca-D-Pantothenate	0.01	mg
Biotin	0.01	mg
Folic Acid	0.01	mg
Choline Chloride	0.5	mg
Inositol	0.05	mg
para-Aminibenzoic Acid	0.05	mg
Vitamin A Acetate	0.1147	mg
Calciferol	0.1	mg
Menadione	0.01	mg
Alpha Tocopherol Phosphate Na_2	0.01	mg
Ascorbic Acid	0.05	mg
Glutathione	0.05	mg
Cholesterol	0.2	mg
Sodium Acetate	50	mg
l-Glutamine	100	mg
Adenosinetriphosphate Na_2	10	mg

Table 17.--Continued.

Ingredients Per Liter		
Adenylic Acid	0.2	mg
Ferric Nitrate $9H_2O$	0.1	mg
Ribose	0.5	mg
l-Cysteine HCl H_2O	0.1	mg
Adenine Sulfate	10	mg
Guanine	0.3	mg
Xanthine	0.3	mg
Hypoxanthine	0.3	mg
Thymine	0.3	mg
Uracil	0.3	mg
Thiamine HCl	0.01	mg
Riboflavin	0.01	mg
Pyridoxine HCl	0.025	mg
Pyridoxal HCl	0.025	mg
Niacin	0.025	mg
Niacinamide	0.025	mg
Deoxyribose	0.5	mg
Tween 80	5	mg

	Earle's Base		Hank's Base	
Sodium Chloride	6.8	g	8	g
Potassium Chloride	0.4	g	0.4	g
Calcium Chloride	0.2	g	0.14	g
Magnesium Sulfate	0.0977	g	0.108	g
Monosodium Phosphate	0.125	g	0.06	g
Dextrose	1	g	1	g
Sodium Bicarbonate	(2.2)	g	(0.35)	g

APPENDIX C

PREPARATION OF MEDIUM AND TISSUE SAMPLES FOR HORMONE ASSAYS

APPENDIX C

PREPARATION OF MEDIUM AND TISSUE SAMPLES FOR HORMONE ASSAYS

Neither TC medium 199 nor Krebs-Ringer bicarbonate solution crossreacted with LH as validated in this laboratory, while the prolactin assay slightly crossreacted with undiluted medium but not with medium diluted with Bovine Serum Albumin Phosphate Buffer Saline (see below). Since concentration of hormones in the effluent depends primarily on the size of explant and flow rate, the dilution of medium samples has to be considered a variable. Generally, with explant size up to 50 mg and flow rate 1 ml/min, medium samples were assayed undiluted for LH the aliquots per tube being 100 µl and 200 µl. For the prolactin assay in the first experiment, samples were diluted in the range 1:100 to 1:1,000 dependent on the source of anterior pituitary tissue. Pituitary tissue samples were prepared for assays by sonification in Experiment 1 (Sonifier Cell Disruptor, Model W 1850, Heat Systems-Ultrasonics, Inc., Plainview, New York) and by grinding since later technique has been found to be satisfactory and easier to perform. For sonification the pituitary

slices were placed in plastic vial 10-20 ml of volume containing 1 ml of bovine serum albumin phosphate buffer saline per 3 mg of tissue. Homogenates were further diluted to 1:900 and 1:9,000 with either 1% egg white or 1% BSA (see below) for LH and prolactin assays, respectively. Sonifications or grindings were performed in an ice bath to prevent overheating. Grinding was done in teflon covered glass tubes with teflon grinder for about 3 min. Sixty to 80 watts were applied intermittently for a total of 3 min. Homogenates, dilutions, and medium samples were kept at -20°C until assayed by double antibody radioimmunoassays (Tucker, 1971; Swanson et al., 1971).

APPENDIX D

PREPARATION OF KREBS-RINGER BICARBONATE-GLUCOSE BUFFERS

APPENDIX D

PREPARATION OF KREBS-RINGER BICARBONATE-GLUCOSE BUFFERS

Solutions:
1. 0.90% NaCl
2. 1.15% KCl
3. 1.22% $CaCl_2$ (5 ml equivalent to 11 ml 0.1 $AgNO_3$)
4. 2.11% KH_2PO_4
5. 3.82% $MgSO_4$ 7 H_2O
6. 1.30% $NaHCO_3$ (gas with CO_2 for 1 hr)

To prepare various modifications of the Krebs-Ringer bicarbonate-glucose buffer, the following amounts parts of the above are mixed:

KRB-Modification	Solutions					
	1	2	3	4	5	6
Normal KRB K^+ 5.9 mM	100	4	3	1	1	21
Enhanced K^+KRB K^+ 23 mM	85	19	3	1	1	21
59 mM	55	49	3	1	1	21
96 mM	25	79	3	1	1	21
Calcium-free KRB	103	4	-	1	1	21

As soon as mixed, the solution is gassed for 10 min with 5% CO_2 - 95 O_2. Glucose is then added to the media in the amount of 0.4 ml of 5% solution to 19.6 ml of medium.

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