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Relationship between pituitary GnRH binding sites and pituitary release of gonadotrophins in beef cows.

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RELATIONSHIP BETWEEN PITUITARY GNRH BINDING SITES AND PITUITARY RELEASE OF GONADOTROPHINS IN BEEF COWS

By

Kwanyee Leung

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Animal Science

ABSTRACT

RELATIONSHIP BETWEEN PITUITARY GNRH BINDING SITES AND PITUITARY RELEASE OF GONADOTROPHINS IN BEEF COWS

By

Kwanyee Leung

The relationship between numbers of GnRH binding sites in the pituitary gland and changes in the ability of the gland to release LH and FSH during proestrus and postpartum anestrus in beef cattle was studied.

Estrus in beef cows was synchronized using a progestagen implant and a prostaglandin $F_2 \triangleleft$ injection. Cows were given GnRH at 40-min intervals for 6 h beginning at -24, 0, 18 or 36 h after removal of the progestagen implant. The ability of the pituitary gland to release LH and FSH in response to GnRH increased by 36 h after implant removal. Other groups of cows subjected to the same synchronization regimen were slaughtered at 0 h, 24 h or at various times after onset of estrus. The number of GnRH binding sites in pituitary glands from these cattle remained unchanged until the period of estrus during which a 50% decline was detected. Thirty two primiparous suckled beef cows were slaughtered on either days 7, 14, 28, 42 or 56 from parturition. One of 6, 5 of 8 and 5 of 6 cows resumed estrous cyclicity at time of slaughter on days 28, 42 and 56, respectively. Among acyclic cows between days 7 and 42, basal release of LH from pituitary explants doubled, whereas basal release of FSH increased only by 20%. GnRH-induced release of LH, but not FSH, increased 2-fold during the same period. Concentrations of pituitary LH of acyclic cows doubled whereas concentrations of pituitary FSH increased only 15% between days 7 and 42. During postpartum anestrus, although overall mean concentrations of serum FSH did not change, overall mean concentrations of serum LH increased. Numbers of GnRH binding sites remained constant during the period studied.

I conclude that during proestrus and postpartum anestrus there is not direct relationship between the ability of the anterior pituitary gland to release gonadotrophins and the numbers of GnRH binding sites in a crude membrane preparation of the anterior pituitary gland of beef cattle. Therefore, under these experimental conditions, number of GnRH binding sites in the pituitary gland of a beef cow may not be a limiting factor in the ability of the gland to release gonadotrophins.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophin
CAMP	adenosine 3',5' monophosphate
CGMP	guanosine 3',5' monophosphate
°C	degree Centigrade
fmol	femtomole
FSH	follicle stimulating hormone
g	gram(s)
GnRH	gonadotrophin releasing hormone
h	hour(s)
125 _I	radioactive isotope of iodine
kg	kilogram(s)
LH	luteinizing hormone
M	molar
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
N	normal (solution)
ng	nanogram(s)
pg	picogram(s)
PGE2	prostaglandin E ₂
PGF2a	prostaglandin F2α
SD	standard deviation
SEM	standard error of mean
TRH	thyrotrophin releasing hormone
TSH	thyroid stimulating hormone
μ g	microgram(s)
μ1	microliter(s)

INTRODUCTION

Gonadotrophin releasing hormone (GnRH) stimulates release of gonadotrophins from the anterior pituitary gland. Depending on the reproductive state of an animal, the same amount of GnRH can elicit different quantities of release of gonadotrophins. For example, GnRH-induced release of gonadotrophins increases prior to the preovulatory gonadotrophin surge in cattle. Similar increases have also been observed in rats and hamsters. Furthermore, release of gonadotrophins in response to GnRH decreases during the last trimester of pregnancy but increases gradually during postpartum anestrus. It is believed that increases in the ability of the anterior pituitary gland to release gonadotrophins is a prerequisite to development of the preovulatory surge of gonadotrophins and subsequent ovulation. The exact mechanism that leads to these increases in release is not clear. But with the knowledge that the interaction of GnRH with its binding sites is the initial step leading eventually to the release of gonadotrophins, it is tempting to hypothesize that the number and/or affinity of GnRH binding sites may be increased during these periods of increased ability of the gland to release gonadotrophins. Furthermore, recent advances in the synthesis of non-biodegradable analogs to GnRH make estimations of numbers and affinities of GnRH-binding sites in the pituitary gland feasible.

The aim of this thesis research was to examine the relationship between GnRH binding sites in the anterior pituitary gland and the ability of the gland to release gonadotrophins. Elucidation of this relationship is important in the overall understanding of the mechanism by which the hypothalamus regulates secretion of gonadotrophins from the anterior pituitary gland.

REVIEW OF LITERATURE

I. Gonadotrophin releasing hormone (GnRH) Hypothalamic GnRH

In 1948, Harris suggested that neurohumors originating from the hypothalamus influenced hormonal secretions from the anterior pituitary gland. This marked the beginning of the research for the hypothalamic releasing factors. With regard to GnRH, McCann <u>et al</u>. in 1960 demonstrated the existence of luteinizing hormone (LH)-releasing activity in crude aqueous extracts of hypothalamic tissue from rats. But it took more than 10 years before GnRH was finally isolated from hypothalami of swine (Schally <u>et al</u>., 1971) and sheep (Amoss <u>et al</u>., 1971). Its amino acid sequence was reported by Matsuo <u>et al</u>. (1971), and it was synthesized later that year (Baba <u>et al</u>., 1971).

With the development of antibodies specific for GnRH, localization of GnRH in the hypothalamus using radioimmunoassay or immunohistological techniques was reported in rats (Palkovits <u>et al</u>., 1974; Wheaton <u>et al</u>., 1975), dogs and cats (Barry and Dubois, 1975), monkeys (Barry and Carette, 1975), sheep (Dees <u>et al</u>., 1981) and cattle (Estes <u>et al</u>., 1977). In general, immunoactive GnRH was found in the arcuate nuclei-median eminence (AN-ME) region and preoptic-suprachiasmatic-anterior hypothalamic (PO-SC-AH) region. These areas were thought to be the centers of dual

hypothalamic control of gonadotrophin release (Barraclough, 1966). Barraclough suggested that control of tonic release of gonadotrophins resided in the AN-ME region of the hypothalamus, whereas control of cyclic changes in gonadotropins was in the PO-SC-AH region. This concept remains valid today. The AN-ME region is necessary for normal basal secretion of gonadotrophin in rats (Soper and Weick, 1980), sheep (Jackson <u>et al</u>., 1978) and monkeys (Plant <u>et al</u>., 1978). And the PO-SC-AH region is indispensable for normal reproductive cycles and ovulations in rats (Clemens <u>et al</u>., 1976) and in sheep (Domanski <u>et</u> <u>al</u>., 1980).

Using light and fluorescent microscopic techniques, Naik (1975) observed immunoactive GnRH neurons originated from AN-ME and PO-SC-AH region and terminated around the capillaries of the primary portal plexus in the median eminence of rats. Thus, it is not difficult to visualize that GnRH is synthesized in perikarya in those regions, passes along axons to axon terminals where it is released into fenestrated capillaries of the pituitary portal system, then transported to the anterior pituitary gland to exert its action, i.e. to increase release of LH and FSH.

Localization of Extrahypothalamic GnRH

Immunoactive GnRH is found in the anterior commissure, paraolfactory area and organum vasculosum of the lamina terminalis (Reichlin et al., 1976). In addition, high

concentrations of GnRH are found in ovine, bovine and porcine pineal glands, but not in pineal glands from rats or monkeys. GnRH in these locations may serve as a neural transmitter. Furthermore, these sources of GnRH may also be important in the expression of sexual behavior. Moss and McCann (1973) found that GnRH could induce mating behavior in female rats independent of sex steroids or pituitary gonadotrophins.

Secretion of GnRH

The amount of GnRH released from the hypothalamus is quite small and by the time it reaches beyond the hypophyseal portal system, it is diluted to the point where the rise in peripheral concentration of GnRH in blood is not sufficient to be measured by radioimmunoassay (Nett and Adam, 1977). This hampered early efforts to directly assess hypothalamic control of gonadotrophin releases. In the mid 1970's, surgical techniques for collecting blood samples from a severed hypophyseal stalk were first attempted. To date, such techniques have been performed in rats (Sarkar et al., 1976), Rhesus monkeys (Carmel et al., 1976; Neill et al., 1977) and rabbits (Tsou et al., 1977). GnRH concentrations in the hypophyseal stalk blood varies from <20 to 120 pg/ml and it is released in a pulsatile fashion. In spite of the fact that LH from the pituitary gland is also released in pulsatile manner, no direct temporal

relationship between GnRH and LH secretion has been established until recently. Clarke and Cummins (1982) described a surgical procedure that permits collection of hypophyseal portal blood from ovariectomized ewes with minimal disturbance to pituitary function. They observed a temporal relationship between GnRH and LH secretion. Each LH pulse was synchronous with a GnRH pulse, thus providing direct proof of the chemotransmitter theory first proposed by Harris in 1948. Furthermore, increased GnRH releases were observed at the time of preovulatory surges of gonadotrophins in rats (Sarkar et al., 1976), rabbits (Tsou et al., 1977) and monkeys (Neill et al., 1977). This increase in GnRH secretion may be important for induction of the gonadotrophin surges and subsequent ovulation.

Mechanism of GnRH action

GnRH binding sites in the pituitary gland

GnRH, similar to other peptide hormones, is believed to exert its action by first interacting with specific binding sites on the plasma membrane of gonadotroph in the anterior pituitary gland. Early attempts to study these GnRH binding sites using radiolabelled synthetic GnRH encountered many problems. Instead of observing a single class of binding sites, two classes were detected. The predominant binding site (~ 75% of total number of GnRH binding sites) had an

affinity constant in the order of $10^6 M^{-1}$ and high capacity for GnRH. The remaining binding sites had an affinity of 10^8 to 10^9 M⁻¹ and a saturable capacity for binding GnRH (Spona, 1973; Grant et al., 1973; Marshall et al., 1976). Clayton et al. (1979) suggested that the low-affinity, high-capacity binding sites represented GnRH bound to degradative enzymes. There is evidence that these degradative enzymes cleave GnRH primarily at the peptide bond between glycine and leucine at positions 6 and 7 (Marks and Stern, 1974) and secondarily at the peptide bond between proline and glycine at positions 9 and 10 (Koch et al., 1974). GnRH analogs with amino acid substitutions at position 6 and/or modifications at position 10 were observed to bind selectively to high affinity binding sites. Scatchard analyses of binding assays using these analogs showed a single class of high affinity and saturable binding sites (reviewed by Clayton and Catt, 1981). The GnRH analog, $D-Ala^{6}-des-Gly^{10}-GnRH-ethylamide$, used in this dissertation binds to a single class of binding sites with an affinity constant between 10^9 and $10^{10}M^{-1}$. Because of a much slower rate of degradation, this analog has a longer half life and is more potent than the native GnRH in stimulating release of LH and FSH. It has a LH releasing potency 14 to 21 times greater than native GnRH (Rivier et al., 1976; Perrin et al., 1980). Furthermore, this analog when added to bovine pituitary cell cultures has LH-releasing activity 19 times and FSH-releasing activity 42

times greater than that of native GnRH (Appendix II).

Patch formation and internalization of GnRH-binding site complexes

Hazum <u>et al</u>. (1980) observed movement of GnRH into gonadotrophs using fluorescently labelled GnRH. At the moment when GnRH binds to its binding sites, the binding sites appear to be uniformly distributed on the cell surface of gonadotrophs. Within 5 min, GnRH-binding site complexes aggregate to form patches on the cell surface. By 10 min, these patches are internalized and incorporated into endocytic vesicles. These vesicles are then transported to the Golgi complex and to lysosomes where they accumulate and are degraded (Duello <u>et al</u>., 1983). The process of aggregation and internalization of GnRH-binding site complexes are calcium dependent (Hazum et al., 1980).

Initially, it was believed that this binding site-mediated internalization of GnRH was required for release of LH. However, when GnRH-binding site complexes were immobilized by attachment of GnRH to agarose beads (Conn and Hazum, 1981), GnRH could still stimulate LH release with full efficiency. Furthermore, Blum and Conn (1982) demonstrated that aggregation formed by as few as two GnRH-binding site complexes without subsequent internalization was sufficient to evoke release of LH. Thus, the current concept is that a binding sitebinding site interaction is required for initiation of LH release in response to GnRH and the internalization of GnRH-binding site complexes within the gonadotroph is required for termination of response to GnRH and possibly stimulation of <u>de novo</u> synthesis of gonadotrophins.

Second Messengers

Early studies suggested cAMP was a second messenger required for GnRH-induced release of LH. In 1972, Borgeat et al. first demonstrated parallel stimulation of cAMP accumulation in the pituitary gland and gonadotrophin release of GnRH. Two years later, the same group of investigators showed that both GnRH and GnRH agonists stimulated accumulation of cAMP and release of LH from pituitary halves of male rats; whereas a GnRH antagonist induced neither cAMP accumulation nor LH release (Borgeat et al., 1974). Groom and Boyns (1973) found that dibutyryl cAMP stimulated release of LH and FSH from human fetal pituitary glands. The proposed mode of action of GnRH is as follows (Labrie et al., 1979). Binding of GnRH to its binding sites leads to an increase in adenylate cyclase activity and increased concentrations of intracellular cAMP. Increased cAMP concentrations activate protein kinase which increases the levels of phosphorylation of proteins within gonadotrophs which eventually leads to release of LH and FSH.

On the other hand, there is also evidence to suggest that cAMP is not involved in GnRH action. Naor <u>et al</u>. (1975) failed to induce release of LH from pituitary glands of male rats with PGE₂ and cholera toxin, non-specific stimulants of cAMP production. Secondly, addition of dibutyryl cAMP to dispersed male rat pituitary cells did not alter the dose response curve of GnRH-induced release of LH (Conn <u>et al</u>., 1979b).

Beginning in the late 1970s, attention has been drawn to cGMP which is a potent secretogogue for growth hormone (Peake <u>et al.</u>, 1972). When pituitary glands of either male or female rats were incubated in the presence of GnRH (5 X 10^{-9} M), cGMP contents in the glands increased within 2 to 5 minutes, whereas the contents of cAMP remained unchanged. In an <u>in vitro</u> system, cellular concentrations of cGMP increased before any release of LH from enriched population of gonadotrophs could be detected (Synder <u>et al</u>., 1980). However, when dispersed pituitary cells from female rats were given mycophenolic acid to inhibit formation of cGMP or sodium nitroprusside to stimulate formation of cGMP, GnRH-induced release of LH from these cells was not affected (Naor and Catt, 1980).

Collectively, the results do not support a role of cAMP or cGMP in the stimulation of acute LH release from gonadotrophs, but they do not rule out the possibility that cAMP or cGMP may play a role in synthesis and/or processing of LH and FSH.

Calcium

The hypothesis that calcium is an intracellular mediator in hormone action is not recent. In 1968, Samli and Geschwind reported that hypothalamic extract-stimulated release of LH from pituitary halves of rats was prevented in the absence of calcium. As the dose of GnRH increased in medium, there was a parallel increase in the amount of calcium accumulated in the cytosol of porcine pituitary cells in vitro (Hopkins and Walker, 1978). The initial hypothesis was that increased calcium translocation across cell membranes of gonadotrophs occurred upon GnRH stimulation. This suggestion was supported by the observation that movements of calcium into cells was detected as soon as 1.5 min after GnRH stimulation (Conn et al., 1981b). Furthermore, addition of 0.1 mM of A23187, an ionophore for calcium, to rat pituitary cells to create a calcium channel in plasma membranes of gonadotrophs released LH comparable to the maximal LH release after GnRH (Conn et al., 1979a).

Calcium of extracellular origin, however, cannot be the sole intracellular mediator. Complete inhibition of GnRH-induced release of LH was observed only when sheep pituitary cells were extensively washed with calcium-free medium containing EGTA before administration of GnRH (Adams and Nett, 1979). In addition, GnRH-induced release of LH was only suppressed 50% when the movement of calcium across membrane was inhibited by Ruthenium Red (Marian and Conn,

1979). Schechter (1976) reported that calcium was localized in mitochrondria, endoplasmic reticulum, Golgi and secretory granules and also bound to the inner surface of plasma membrane of rat pituitary glands. Mobilization of intracellular calcium into the cytosol may play an important role in 3nRH-induced release of LH. At present, the proportion of the increase in cytosolic calcium contributed by influx of extracellular calcium and mobilization of intracellular calcium remains to be determined. Nevertheless, these data support a role for calcium as a mediator in GnRH-induced LH release from anterior pituitary glands.

Calcium-Calmodulin Interaction

The present hypothesis is that the action of calcium is mediated by calmodulin, an intracellular binding protein for calcium. It has a molecular weight of 16,700 daltons and each molecule is capable of binding four molecules of calcium (Cheung <u>et al</u>., 1982). When pimozide was given to inhibit calcium binding to calmodulin, GnRH-induced release of LH was inhibited (Conn <u>et al</u>., 1981a). So far, calcium-calmodulin complexes have been shown to stimulate activity of many enzymes including adenylate cyclase, calcium-dependent phosphodiesterase, calcium-dependent protein kinase and phospholipase in brain tissues (Cheung <u>et</u> <u>al</u>., 1982). Stimulation of these enzymes by calcium-calmodulin complexes in gonadotrophs, however, has

not been determined. Calcium-calmodulin complexes also regulate the microtubule-microfilament system (see next section).

Release of LH and FSH

It is not known exactly how the secretory process Current theory is that it may involve the occurs. microtubule-microfilament system. Microtubules provide a structural lattice to orient the directional flow of the secretory granules and microfilaments can provide the motile force for granule movement (Means and Dedman, 1980). Two drugs have been used to study the involvement of microtubules and microfilaments in the release of LH and FSH; colchicine to depolymerize microtubules and cytochalasin B to impair the function of microfilaments. When anterior pituitary cells were preincubated with colchicine. GnRH-induced release of LH was either unaffected (Adams and Nett, 1979) or suppressed by 50% (Khar et al., 1979). Similar studies with cytochalasin B showed consistent suppression of GnRH-induced release of LH (Adam and Nett, 1979; Khar et al., 1979). Therefore, the microtubule-microfilament system may facilitate release of LH.

Calcium-calmodulin complexes affect the function of microtubules and microfilaments. They stimulate depolymerization of microtubules and contraction of microfilaments (Means and Dedman, 1980). Contraction of microfilaments is the result of initial stimulation of the myosin light chain kinase by calcium-calmodulin complexes which in turn activates myosin ATPase to provide energy for the contraction (Korn, 1978). To date, actual involvement of calcium-calmodulin complexes in the function of microtubules and microfilaments in gonadotrophs has not been elucidated.

Regulation of GnRH binding sites

Gonadal steroids affect numbers of GnRH binding sites. After gonadectomy, numbers of GnRH binding sites in pituitary glands of male and female rats increase to about twice that of appropriate intact controls (Clayton and Catt, 1981; Frager <u>et al</u>., 1981). This 2-fold increase in GnRH binding sites could be detected as early as 24 h after castration on male rats and 3 days after ovariectomy in female rats. Subcutaneous injections of testosterone to castrated male rats, and estradiol, progesterone and estradiol and progesterone to ovariectomized female rats prevent the "post-gonadectomy" rise in numbers of GnRH binding sites in pituitary glands. At present, the effect of gonadal steroids on numbers of GnRH binding sites in pituitary glands of intact animals has not been reported.

In an <u>in vitro</u> study, Giguere <u>et al</u>. (1981) demonstrated that dihydrotestosterone added to anterior pituitary cells of intact female rats reduces the number of GnRH binding sites by 40%. These results provide evidence

that gonadal steroids directly reduce numbers of GnRH binding sites on gonadotrophs.

GnRH can also affect its own binding sites in the pituitary gland. Frager et al. (1979) was first to report that thrice daily injections of GnRH or D-Ser⁶-des- Gly^{10} -GnRH-ethylamide, a GnRH agonist, for 4 days increased up to 70% numbers of GnRH binding sites in the pituitary glands of intact male rats. Clayton et al. (1980) also demonstrated that single daily injections of [D-Ser⁶(TBu)⁶]des-Gly¹⁰-GnRH-ethylamide (D-Ser analog), a GnRH agonist, induced a 50% increase in numbers of GnRH binding sites in pituitary glands of intact male rats as early as 2 days after onset of injections of the GnRH agonist. Infusion of the D-Ser analog at 3.4 μ g/day for 5 days increases numbers of GnRH binding sites in pituitary glands of intact male rats up to 70% (Clayton and Catt, 1981). Furthermore, with injections of the D-Ser analog, the "post-castration" rise in numbers of GnRH binding sites in pituitary glands can be restored in castrated male rats receiving subcutaneous testosterone implants (Frager et al., 1981), but not castrated controls. Therefore, an increase in release of GnRH from the hypothalamus can potentially increase in the number of GnRH binding sites in the pituitary gland, except in castrated animals. It is possible that the number of GnRH binding

sites in pituitary glands of castrated rats is maximal, and, therefore, additional GnRH will not induce any further increase in number of GnRH binding sites.

GnRH-induced synthesis of gonadotrophins

Like other glycoproteins, peptide components of LH and FSH are believed to be synthesized in the rough endoplasmic reticulum and transported through the channels of endoplasmic reticulum where membrane bound glycosyltransferases attach one sugar at a time to the growing carbohydrate components of LH and FSH. The newly synthesized gonadotrophins are then concentrated and packaged for secretion in the Golgi apparatus (Spiro, 1970). The rate of synthesis of the carbohydrate moiety of glycoprotein is much slower than that of the polypeptide moiety. Therefore, the synthesis of the carbohydrate portion of glycoprotein is more likely to be rate-limiting and easily subject to physiological regulation by hormones and other factors than the synthesis of the polypeptide components (Sprio, 1970; Liu and Jackson, 1977).

Effects of GnRH on gonadotrophin synthesis have not been investigated as extensively as that of gonadotrophin release, and most of the studies involved incubations of pituitary tissue lasting only 4 h. Although early studies using anterior pituitary halves from rats failed to detect an increase in the incorporation of either radioactively labelled leucine or glucosamine into LH (Samli and Geschwind, 1967; Wakabayashi and McCann, 1970), more recent studies showed that GnRH-stimulated incorporation of ³H-glucosamine into the carbohydrate moiety of LH (Liu <u>et</u> <u>al</u>., 1976; Liu and Jackson, 1977; Azhar <u>et al</u>., 1978). These results suggested that the short-term effect of GnRH on the synthesis of LH and FSH was on glycosylation rather than peptide synthesis, thus supporting the aforementioned concept of acute regulation of glycoprotein synthesis.

An increase in the glycosylation of LH after GnRH did not increase total LH (LH in medium and tissue) in 4 h cultures (Liu <u>et al.</u>, 1976; Liu and Jackson, 1977; Azhar <u>et</u> <u>al.</u>, 1978). But when incubations in the presence of GnRH were extended to 5 days, an increase in total (medium and tissue) immunoactive LH and FSH was detected (Redding <u>et</u> <u>al.</u>, 1972), suggesting an increase in <u>de novo</u> synthesis of gonadotrophins.

The mechanism by which GnRH regulates synthesis of gonadotrophins is not known. An adenylate cyclase - protein kinase system may be a possibility. Calcium-calmodulin complexes stimulate production of cAMP by adenylate cyclase (Lynch <u>et al.</u>, 1976). Increased concentrations of cAMP in the cytosol activate cAMP-dependent protein kinases which then phosphorylate nuclear, ribosomal and/or Golgi proteins (Adams and Nett, 1979). Phosphorylation of these proteins leads ultimately to an increase in glycosylation and later synthesis of gonadotrophins.

GnRH action on gonadotrophs - a model

Based on the studies covered in this review, a schematic representation of the possible mechanisms of action of GnRH that leads to release and subsequent synthesis of LH and FSH is presented in fig. 1 (modified after Adams and Nett, 1979). The major steps in GnRH actions are reiterated in the figure legend. Fig. 1. Diagrammatic representation of a model of GnRH-induced release and synthesis in the gonadotroph. The major steps in GnRH action include:

- GnRH binding to its binding sites (B) in plasma membrane.
- Influx of calcium (Ca⁺⁺) from an extracellular source and/or
- Mobilization of calcium from mitochondria (M), endoplasmic reticulum (ER), Golgi (G), secretory granules (SG) and inner surface of plasma membrane.
- 4. Formation of calcium-calmodulin complexes (Ca⁺⁺·CM).

Synthesis of gonadotrophins

- Calcium-calmodulin complexes stimulate adenylate cyclase
 (AC) to produce cAMP.
- 6. Activation of protein kinase (PK) by cAMP
- 7. Phosphorylation of nuclear, ribosomal and/or Golgi proteins which increases synthesis of LH and FSH.

Release of gonadotrophins

- 8. Stimulation of depolymerization of microtubules (MT) by calcium-calmodulin complexes
- 9. Stimulation of contraction of microfilaments (MF) by calcium-calmodulin complexes
- 10. Exocytotic release of gonadotrophins



Experiment I: Relationship between pituitary sensitivity to GnRH and number of GnRH binding sites in pituitary glands of beef cows during proestrus

INTRODUCTION

The quantities of LH and FSH released from the anterior pituitary gland after a single dose of GnRH vary during different stages of the ovulatory cycle in rats (Aiyer <u>et</u> <u>al.</u>, 1974), hamsters (Arimura <u>et al.</u>, 1972), sheep (Reeves <u>et al.</u>, 1971) and women (Yen <u>et al.</u>, 1972). In each of these reports, GnRH-induced LH and FSH release was greatest just before the preovulatory gonadotrophin surges. This proestrus increase in responsiveness of the pituitary gland to GnRH was also observed in dairy heifers in which estrus was synchronized with PGF_2^{α} (Convey <u>et al.</u>, 1976). This increase in responsiveness may be a prerequisite for the development of gonadotrophin surges.

GnRH is a peptide hormone and therefore is believed to bind to specific binding sites in the plasma membrane of the gonadotrophs, thereby initiating intracellular changes which result in synthesis and release of LH and FSH. Changes in responsiveness of the pituitary gland to GnRH may be due to changes in numbers of GnRH binding sites. So far, during the time of increased responsiveness of the anterior pituitary gland to GnRH either no change or a decline in numbers of GnRH binding sites was observed in rats (Savoy-Moore <u>et al</u>., 1980; Clayton <u>et al</u>., 1980; Reeves <u>et</u> <u>al</u>., 1982) and ewes (Wagner <u>et al</u>., 1979; Crowder and Nett, 1982). The experiments described herein evaluate the relationship between the ability of the pituitary gland to
release gonadotrophins in response to GnRH and total number of GnRH binding sites in pituitary glands of beef cows.

MATERIALS AND METHODS

Hormones

GnRH and its analog, D-Ala⁶-des-Gly¹⁰-GnRHethylamide (GnRH-A) were obtained from Beckman (Palo Alto, CA) and Pennisula Laboratories (San Carlos, Ca), respectively. GnRH-A has LH-releasing activity 14 to 21 times (Rivier <u>et al.</u>, 1976; Perrin <u>et al.</u>, 1980; Appendix II) and FSH-releasing activity 42 times (Appendix II) greater than that of native GnRH.

Preparation of crude membrane fraction from pituitary glands

Pituitary glands were processed as previously described by Savoy-Moore <u>et al</u>. (1980) with modifications. Each anterior pituitary gland was bisected and then homogenized in 30 ml of Tris-HCl buffer (pH 7.7, 10 mM Tris with 1 mM dithiothreitol) using an Omni Mixer (50 ml container, Sorvall, Norwalk, CT) at maximal speed for 2 min. The resulting slurry was further homogenized with a hand driven tissue grinder (glass to glass Pyrex 7726, 0.15 mm clearance, 10 strokes). The homogenate was centrifuged at 300 X g for 5 min., and the supernatant fluid was centrifuged at 10,800 X g for 20 min. The resulting pellet consisted of a membrane-rich upper layer and a lower layer of secretory granules (Clayton <u>et al</u>., 1978). In view of the fact that GnRH binding sites in membranes were of our

primary interest. I selectively recovered the loosely packed membrane portion of the pellet discarding the dense and tightly-packed secretory granule pellet. This procedure was repeated twice after membrane portion of the pellet was washed with buffer and centrifuged. This crude membrane fraction was then resuspended in 6 ml Tris-HCl buffer per g anterior pituitary gland (wet weight) to provide 300-500 μ g of membrane protein in 100 μ l volume which was used in the binding assay.

GnRH-A iodination

¹²⁵I-GnRH-A was prepared by the lactoperoxidaseglucose oxidase method described by Clayton <u>et al.</u>, (1979). The specific activity of the ¹²⁵I-labelled GnRH-A was estimated by plotting the quantity of ¹²⁵I-GnRH-A specifically bound in the presence of various concentrations of unlabelled or labelled GnRH-A. Specific activity, calculated as described in the legend of fig. 2, ranged from 1,000 to 1,350 µCi/µg. The maximal percent of specific binding of ¹²⁵I-GnRH-A to an excess of membrane protein represents that portion of intact ¹²⁵I-GnRH-A that could be recognized by the binding sites. This maximal value varied with iodinations and ranged from 27% to 42%. Total radioactivity added to each assay tube was corrected for variation in maximal specific binding prior to Scatchard analyses.

fig. 2. Estimation of the specific activity of $^{125}I-GnRH-A$ (D-Ala⁶-des-Gly¹⁰-GnRH-ethylamide). x----x percent specific binding of $^{125}I-GnRH-A$ in the presence of increasing amounts of unlabelled GnRH-A. •----• percent specific binding in the presence of increasing amounts of $^{125}I-GnRH-A$.

Specific Activity (S.A.) = [cpm of 125I-GnRH required to achieve 50% of maximal specific binding] ÷ [pg of unlabelled GnRH-A required to displace 50% of the 125I-labelled GnRH-A bound]

% Specific Binding = [total binding (cpm) - nonspecific binding (cpm)] ÷ [125I-GnRH-A added (cpm)] X 100%



OOI× (JATOT÷ GNUOB YLLALICALLY BOUND÷TOTAL) ×100

Validation of GnRH binding assay

Fresh bovine pituitary glands were obtained from a local abattoir and processed as described above, unless otherwise stated. The time course of binding reaction was estimated by incubating the crude membrane preparation with 20,000 cpm (15 pg) of $125_{I-GnRH-A}$ in the presence of 0 or 1 µg of unlabelled GnRH-A for variable periods ranging from 10 to 360 min. By 60 min, maximal binding was achieved. To determine temperature dependency, the binding assay was conducted similarly but incubation temperature was varied, i.e., 4°C, 17°C or 37°C for 60 min. It was found that maximal binding was obtained at an incubation temperature of 4°C. Very little binding was observed at 17 or 37°C. Hence all incubations were at 4°C for 60 min hereafter, unless otherwise stated.

The number of GnRH binding sites in crude membrane preparations was limited as depicted in fig. 3. Amount of ^{125}I -GnRH-A in excess of 10^6 cpm did not increase specific binding to a constant amount of membrane preparation. Thus, the specific binding of ^{125}I -GnRH-A to crude membrane preparation was a saturable phenomenon.

Scatchard analyses of binding assays using crude membrane fractions of pituitary glands from four ovariectomized cows and three intact cows (early luteal phase) gave similar estimates of affinity constant (averaged

fig. 3. Specific binding of 125_{I} -labelled GnRH-A to a crude membrane fraction prepared from bovine anterior pituitary glands, expressed as a function of 125_{I} -labelled GnRH-A concentration. Specific binding is the difference between total binding and non-specific binding.



 $1.0 + 0.1 \times 10^{10} \text{ M}^{-1}$, Table 1). However the numbers of GnRH binding sites in the pituitary glands of these four ovariectomized cows were higher (P=.01) than that of the three intact cows (82.7 vs. 40.0 fmole/mg protein respectively). Affinity constants obtained from Scatchard analyses of binding using 224 and 735 µg of a crude membrane preparation of pituitary glands from the local abattoir were similar (1.2 and 1.4 X 10^{10} M⁻¹, respectively; fig. 4). Furthermore, comparable numbers of GnRH binding sites (121.6 and 125.9 fmol, respectively) per mg protein were found. These values were higher than the aforementioned values in this paragraph. I believe that this difference may be due to the undefined reproductive status of pituitary glands from the local abattoir. In fact, consistently higher numbers of GnRH binding sites were obtained from Scatchard analyses of binding assays performed after each GnRH-A iodination using the abattoir as the source of pituitary glands.

Hormone specificity of binding of ^{125}I -GnRH-A to a crude membrane preparation of anterior pituitary glands was determined by incubating 1 µg bovine TSH, bovine FSH, bovine LH, ovine LH, ovine FSH, somatostatin, TRH, ACTH, oxytocin, synthetic GnRH, and increasing amounts of unlabelled GnRH-A with a constant amount of ^{125}I -GnRH-A (20,000 cpm/tube). Only synthetic GnRH and GnRH-A inhibited the binding of radioactive GnRH-A.

Table 1. Number and affinity of GnRH binding sites in bovine anterior pituitary glands from three intact cows and four ovariectomized cows

		Affinity constant	Binding sites
		(X 1010 M-1)	(fmol/mg protein)
Intact cows	1	0.9	47.0
	2	1.2	37.3
	3	1.0	35.8
Mean <u>+</u> SEM		1.0 <u>+</u> 0.09	40.0 <u>+</u> 3.51
ovariectomized	1		
COWS	1	0.9	89.6
	2	1.3	59.3
	3	1.0	100.7
	4	1.0	81.2
ean <u>+</u> SEM		1.1 <u>+</u> 0.09	82.7 <u>+</u> 8.76*

*Value significantly higher (P<0.01) than that of intact

cows.

fig. 4. Scatchard analysis of ¹²⁵I-labelled GnRH binding to two concentrations of a crude membrane fraction prepared from bovine anterior pituitary glands (224 and 735 mg). Each analysis consisted of three replicates of five concentrations of unlabelled GnRH-A.



To determine if GnRH-A binding is specific for membranes from anterior pituitary glands, binding was tested using crude membrane fraction of various tissues prepared as described for anterior pituitary glands. There was little if any binding of ¹²⁵I-GnRH-A to crude membrane fractions of bovine pancreas, lung, renal cortex, corpus luteum, cerebral cortex and hypothalami from 3 cows (0 to 6% of that observed with crude membrane fraction of pituitary glands on per mg protein basis). We did, however, detect specific GnRH-A binding to liver and the adrenal glands (12% and 11%, respectively).

GnRH binding assay

The assay reaction was carried out in a total volume of 500 µl of assay buffer consisting of 10 mM Tris-HCl with 1 mM dithiothreitol and 0.5% bovine serum albumin. $1^{25}I$ -GnRH-A (15 pg) was incubated with increasing amounts of unlabelled GnRH-A and 300-500 µg of crude membrane preparation in polypropylene tubes at 4°C for 60 min. Nonspecific binding was assessed in tubes containing 1 µg unlabelled GnRH-A. At the end of incubation, 3 ml of assay buffer was added to each tube and all were centrifuged at 27,000 X g for 20 min. The supernatant fluid was decanted and the pellet counted in a gamma spectrometer with a counting efficiency of 86%.

Experiments

Beef cows showing normal estrous cyclicity were used. Estrus was synchronized using the following treatment scheme (Staigmiller <u>et al</u>., 1982). A synthetic progestagen (SC21009, Searle Co., Chicago, IL) was implanted into an ear of each cow and left in place for 7 days. At 24 h before removal of the implant, 25 mg prostaglandin $F_2\alpha$ (Upjohn Co., Kalamazoo, MI) was given intramuscularly to induce regression of any existing corpora lutea. The time when the progestagen implant was removed was designated at time zero. Based on considerable experience with this synchronization regimen, we expected cows so treated to exhibit estrous behavior from about 36 h. Proestrus was therefore the time period from removal of progestagen implants to onset of estrus.

A. Pituitary responsiveness to GnRH during proestrus

Beef cows, six in each of four groups, were randomly assigned to be given nine injections of GnRH (500 ng/injection) via jugular cannula at 40-min intervals beginning -24, 0, 18 or 36 h in relation to implant removal. Blood was obtained via the same cannula at 10-min intervals beginning 10 min before the first GnRH injection and ending 40 min after the ninth injection of GnRH.

B. Pituitary binding of GnRH during proestrus/estrus

Beef cows (n=31) were randomly assigned to be slaughtered at 0 (n=8) or 24 h (n=8) after removal of the

progestagen implant, or at times after first detection of estrus (n=15). Onset of estrus was detected with the aid of a sterile bull fitted with a grease marking harness and was defined as the first time a cow would stand to be mounted by the bull. Blood was collected at 4 and 2 h before, and at the time of slaughter for cows slaughtered at time 0 and 24 hours. Cows in the third group were observed for signs of estrus at 30-min intervals beginning at 30 h and were bled at 6-h intervals beginning at 24 h; then 2-h intervals after estrus was first observed. The times of slaughter were between 0.5 and 11.8 h after first detection of estrus and onset of preovulatory gonadotrophin surges (or 43.0 and 78.8 h after removal of progestagen implant).

Pituitary glands were collected and placed in Tris-HCl buffer within 15 min after the donor was killed. Anterior pituitary glands were dissected, weighed and homogenized as described earlier in Materials and Methods. Portions of this homogenate were stored at -70°C until assayed for LH and FSH content. A crude membrane fraction was prepared from the remaining homogenate as described. Binding assays were carried out for individual animals and Scatchard analyses were performed from data so obtained. Numbers of binding sites, determined by Scatchard analyses, were expressed as fmol/mg membrane protein. Protein was assayed as described by Lowry <u>et al</u>. (1951) using bovine serum albumin as standard.

Infundibular stalks were also dissected, weighed and stored at -70°C until quantification of GnRH content. We have previously demonstrated that 96% of GnRH in the hypothalamus resides in the pituitary stalk in cattle (Estes et al., 1977).

Radioimmunoassays

Blood was stored for 4 h at room temperature, then at 4°C for an additional 12 h before centrifugation to obtain Concentrations of LH and FSH in serum samples from serum. each experiment were each determined in a single assay using double antibody procedures previously validated in this laboratory (Convey et al., 1976; Carruthers et al., 1980). Standard preparations of LH and FSH were NIH-LH-BS and NIH-FSH-Bl, respectively. Within assay coefficients of variation averaged 7.7 + 0.6% for LH and 16.2 + 0.6% for FSH and sensitivities of LH and FSH assays were 0.125 and 2.5 ng/tube, respectively. Serum estradiol was quantified by radioimmunoassay as previously reported (Carruthers and Hafs, 1980). Inter- and intra-assay coefficients of variation determined from six assays were 6.8% and 11.1% for a pool of serum from ovariectomized cows to which 5 pg/ml estradiol was added. The sensitivity of the estradiol assay was 0.8 pg/tube.

GnRH in infundibular stalks was extracted with 2 N acetic acid and quantified in a single assay using an antibody validated by Nett et al. (1973) and procedures described by Estes <u>et al</u>. (1977). Within assay coefficient of variation was 10.0% and the sensitivity of the GnRH assay was 3.0 pg/tube.

Statistical analyses

All data reported were tested for heterogeneity of variances amongst groups using Bartlett's test (Gill, 1978). Heterogeneity was found in variances of serum concentrations of LH before GnRH injections, and changes in concentrations of serum LH after GnRH (Δ LH). Therefore, data of these variables were subjected to logarithmic transformation before statistical analysis. Variances of changes in concentrations of serum FSH after GnRH (\triangle FSH) were also found heterogeneous and data were subjected to square-root transformation before statistical analysis. Data of these three variables are presented in Results as: means of raw data (means + SEM of data after transformation). All data were analyzed by one-way analysis of variances. Specific comparisons of means were conducted using Bonferroni's t test (Experiment A), or Dunnett's t test (Experiment B) (Gill, 1978).

RESULTS

A. Pituitary sensitivity to GnRH during proestrus

Mean serum concentrations of LH and FSH in animals given multiple injections of GnRH beginning -24, 0, 18 or 36 h after implant removal is shown in fig. 5. Changes in

fig. 5. Changes in serum LH and FSH concentrations in cows (six/group) after nine injections (arrows) of GnRH (500 ng/injection) beginning -24, 0, 18 or 36 h after removal of a progestagen implant. For serum LH, standard error of difference between two treatments means at the same time was <u>+</u> 15.1 ng/ml and standard error of difference between two means at different times for the same treatment was <u>+</u> 2.0 ng/ml. Corresponding values for serum FSH were <u>+</u> 71.4 and <u>+</u> 6.3 ng/ml, respectively.



Time From First Injection of GnRH (h)

concentrations of LH and FSH in serum in response to GnRH expressed as the difference (Δ) between mean pretreatment values and the single maximal gonadotrophin concentrations obtained during all the GnRH injections were used in the statistical analysis. Before the start of GnRH treatment, LH and FSH concentrations in serum were not different (P>.20) among groups, and were 1.8 (0.5 + 0.07) and 56.3 + 3.23 ng/ml, respectively. Changes in concentrations of LH and FSH (Ang/ml serum) of those beef cows given GnRH beginning -24, 0 and 18 h after implant removal were small and relatively stable, averaging 2.9 (0.8 ± 0.35) , 6.2 (2.0 + 0.35) and 6.4 (1.7 + 0.24) for LH, and 25.7 (5.7 + 0.79), 35.8 (7.3 + 0.66) and 35.8 (6.7 + 0.40) for FSH, respectively. However, animals given GnRH beginning at 36 h after progestagen implant removal had a greater change (P<.005) in serum LH and FSH than the other three groups $(\Delta LH = 33.4 (3.4 + 0.21) \text{ ng/ml} \text{ and } \Delta FSH = 97.3 (10.2 + 10.21)$ 0.75) ng/ml).

Although there was no change in the concentrations of estradiol in serum collected during each GnRH injection period, a linear increase (P<.05) in mean treatment concentrations of serum estradiol from 5.5 to 8.9 pg/ml was detected between -24 and 36 h.

B. <u>Pituitary binding of GnRH during proestrus/estrus</u> Concentrations of LH and FSH in serum of all three

treatment groups are presented in fig. 6. Data from the group slaughtered after onset of the preovulatory gonadotrophin surges were normalized to time of peak gonadotrophin surges. In all cases, maximal FSH concentration in serum coincided with that of LH. Preovulatory LH and FSH surges in those cattle resemble those of normally cycling cows in terms of magnitude, duration and general shape (Chenault <u>et al</u>., 1975; Dobson, 1978 and Rahe <u>et al</u>., 1980). The time of onset of estrus occurred at an average of -3.5 ± 0.7 h before peak gonadotrophin surges.

Concentration of estradiol was measured in the last sample obtained from each animal slaughtered at 0 or 24 hr, and also in samples obtained after first detection of estrus. During the period of estrus, mean estradiol concentrations in serum were higher (P<.05) than that of cows killed at 0 or 24 h (8.6 + 0.6 vs. 4.7 + 0.7 pg/ml).

Neither GnRH content in the infundibular stalk nor the affinity constant of GnRH binding sites were different (P>.25) amongst groups, averaging 1.4 ± 0.10 ng GnRH/mg stalk and $1.2 \pm 0.04 \times 10^{10}$ M⁻¹, respectively (data not shown). In addition, numbers of GnRH binding sites and pituitary contents of LH and FSH remained constant 0 and 24 h after implant removal (fig. 7). However, a significant decrease was detected in the number of GnRH binding sites during the 12 h after onset of estrus. This decline was

fig. 6. LH and FSH in serum collected during

proestrus/estrus after estrous synchronization. Values from animals killed at 0 and 24 h are means \pm SEM of samples collected at -4, -2 and 0 h before slaughter. Data obtained during the preovulatory gonadotropin surges were normalized to time of peak of LH and FSH values (from -4 to +6 h). Values are mean \pm SEM (when larger than the datum symbol) for the number of cows indicated in parentheses.



fig. 7. Number of GnRH binding sites and concentratons of gonadotrophins in bovine anterior pituitary glands during proestrus/estrus. Values from animals killed at 0 and 24 h after implant removal are means \pm SEM. Data from animals killed during the estrous period are plotted individually. Onset of estrus occurred -3.5 \pm 0.7 h before the peak gonadotrophin surges.



concurrent with the decreasing pituitary contents of LH and FSH.

DISCUSSION

The objective of this research was to determine whether the proestrous increase in the ability of the pituitary gland to release gonadotrophins in response to GnRH in cattle was associated with changes in number and/or affinity of GnRH binding sites of the pituitary gland. To be able to predict time of estrus with reasonable accuracy, I chose to use a scheme for synchronization of estrus. Because I did not know if pituitary responsiveness to GnRH would increase prior to estrus in cattle so treated, I deemed it necessary to measure change in responsiveness during the proestrous period following this synchronization treatment. In the present study, the quantity of LH and FSH released in response to GnRH increased several fold by 36 h after completion of the synchronization scheme. Thus, I demonstrated with this model that the responsiveness of the the pituitary gland is increased prior to the time of expected preovulatory surges of gonadotrophins. This result is in agreement with similar reports by Convey et al. (1976) in which estrus was synchronized in dairy heifers using $PGF_{2\alpha}$ alone.

If the increase in the ability of the pituitary gland to release gonadotrophins was directly associated with GnRH binding sites, one would expect to detect changes in

the number and/or affinity of GnRH binding sites prior to or concurrent with the increase in pituitary responsiveness to GnRH. No changes in either number or affinity of GnRH binding sites were detected up to the time of estrus. Although it is possible that I did not quantify GnRH binding sites at the appropriate time, I feel that this is unlikely. Several recent studies provide evidence that a similar conclusion could be gleaned from data generated with rats, hamsters and ewes. For example, increased responsiveness of the pituitary gland to GnRH occurs during proestrus in rats (Aijer et al., 1974) and hamsters (Arimura et al., 1972) and at estrus in ewes (Reeves et al., 1971). Yet, no change in number and affinity of GnRH binding sites in the pituitary glands was observed in the morning of proestrus in rats (Savoy-Moore et al., 1980; Clayton et al., 1980), and hamsters (Adams and Spies, 1981), or in ewes prior to, during and following estradiol-induced surges of gonadotrophins (Wagner et al., 1979). The only time during the estrous cycle when the number of GnRH binding sites increases in rats is between the days of metestrus and diestrus, a time when responsiveness of the pituitary gland to GnRH is quite low relative to that at proestrus (Aiyer et al., 1974).

During the period of estrus and preovulatory gonadotrophin surges, we observed a gradual decline in the number of GnRH binding sites that occurred in synchrony with depletions of pituitary contents of LH and FSH. Again this

is in agreement with data obtained from rats and hamsters. Marked decreases in numbers of GnRH binding sites were observed either in the late afternoon of proestrus coincident with the LH surge (rats: Savoy-Moore et al., 1980; Clayton et al., 1980) or on the day of estrus (hamster: Adams and Spies, 1981; rats: Reeves et al., 1982). The detection of decreased GnRH binding sites during estrus in the present study in conjunction with the observation of higher numbers of GnRH binding sites in ovariectomized cows when compared with intact cows (see Materials and Methods) provided evidence that this assay procedure was capable of detecting differences in the numbers of GnRH binding sites in the anterior pituitary gland. Therefore, the failure to detect changes in either the number or affinity of the binding sites concurrent with increased responsiveness of the pituitary glands to GnRH in the present study provides evidence that the change in the responsiveness of the pituitary gland during proestrus may not be directly related to a concomitant change in number or affinity of the GnRH binding sites in the gland.

Experiment II: Relationships amongst <u>in vitro</u> and <u>in vivo</u> gonadotrophin secretion and number of pituitary GnRH binding sites in postpartum beef cows

INTRODUCTION

After parturition, cattle go through a period of anestrus before resumption of estrous cyclicity. Insufficient secretion of LH from the anterior pituitary gland has been implicated as one reason for anestrus. At the onset of the anestrous period, pituitary LH content (Labhsetwar et al., 1964; Saiduddin and Foote, 1964; Saiduddin et al., 1968), GnRH-induced release of LH (Webb et al., 1977; Fernandes et al., 1977; Kelser et al., 1977; Schallenberger et al., 1978) and mean concentrations of serum LH (Erb et al., 1971; Short et al., 1972, Fernandes et al., 1977; Kesler et al., 1977; Schallenberger et al., 1978; Goodale et al., 1978) were lower than that observed just before the first postpartum estrus. Similar changes in these indices of LH secretion have been reported in sheep (Jenkin et al., 1977). Gradual restoration of GnRH-induced release of LH in human females during puerperium has also been observed (Jeppsson et al., 1974; LeMaire et al., 1974). Restoration of LH secretion to levels comparable to that observed prior to first estrus may be required for resumption of estrous cyclicity.

Interaction of GnRH with its binding sites in the plasma membrane of gonadotrophs is the first step in stimulation of LH secretion. Any changes in either the

number or affinity constant of the GnRH binding sites may alter the sensitivity of gonadotrophs to GnRH. Clayton et al. (1980) and Clayton and Catt (1981) reported that lactating rats have about one half of the number of GnRH binding sites in their pituitary glands as compared with that of rats in metestrus. On the other hand, data presented by Parfet et al. (1983) did not support the idea that GnRH binding sites were reduced during postpartum period in beef COWS. They found that the number of GnRH binding sites in the anterior pituitary gland decreased between days 10 and 20 after parturition and then remained constant until the first postpartum estrus. To date, no change in the affinity constant of GnRH binding sites in the pituitary gland has been reported. In the experiment described herein, I evaluated the relationship between the ability of the pituitary gland to release gonadotrophins in response to GnRH and the number and affinity constant of GnRH binding sites in pituitary glands of postpartum beef cows.

MATERIALS AND METHODS

Animals

Thirty two crossbred (primarily Hereford X Angus) beef heifers were obtained from the beef herd of the Upjohn Company, Kalamazoo, MI. They were approximately 2 years old when they were bred by artificial insemination between September 22 and October 16, 1981 after synchronization of

estrus with prostglandin $F_2\alpha$. These cattle averaged 479 <u>+</u> 13 kg and had an above average score on body condition just before parturition. They were fed to meet the nutritional requirement established by National Research Council (1976). Feeds consisted of corn silage supplemented with vitamins and minerals. All cattle were "halter-broken" and accustomed to handling prior to parturition.

These cattle were assigned randomly to be slaughtered on day 7, 14, 28, 42 or 56 after parturition and were housed in groups of five to six cows per pen in a cold confinement barn. Parturitions occurred between July 1 and July 24, 1982. Each cow was suckled by a single calf until the time of slaughter.

Blood samples

A pen was specially modified for collection of blood samples via a remote cannula fitted into a jugular vein of a cow and for observation of suckling events through a two-way mirror from an adjacent room. Cows and calves were acclimated to this pen 4 days prior to blood sampling. One day before the designated day of slaughter, cows were bled at 10-min intervals between 0800 and 1400 h. During the sampling period, cows were loosely restrained with halters and calves had free access to suckle. Suckling events and their durations during this period were recorded.

Blood was stored for 2 h at 37°C room temperature, then

at 4°C for an additional 24 h before centrifugation to obtain serum. Concentrations of LH, FSH and prolactin in serum were measured. Also, selected samples were analyzed for estradiol, progesterone and cortisol.

Beginning on day 21 after parturition, blood samples were also collected via venipuncture every 3 days until time of slaughter. These samples were assayed for progesterone. Based on serum concentrations of progesterone and morphology of ovaries, we determined when these cows resumed estrous cyclicity before the time of slaughter.

Anterior pituitary glands

Pituitary glands were collected and packed in ice within 15 min after the cows were killed. Anterior pituitary glands were isolated and bisected. Pituitary halves from each animal were weighed and then randomly assigned either to be stored in liquid nitrogen until quantification of GnRH binding sites or to be incubated <u>in</u> <u>vitro</u> to determine the ability of the gland to release gonadotrophins.

A. <u>Quantification of GnRH binding sites in anterior</u> pituitary glands.

The hemi-pituitary gland was homogenized in 15 ml of Tris-HCl buffer (pH 7.7, 10 mM Tris with 1 mM dithiotheritol) using an Omni mixer (50 ml container, Sorvall, Norwalk, CT) at maximum speed for 2 min. The

resulting slurry was further homogenized with a hand driven tissue grinder (glass to glass, Pyrex $\ddagger7726$, 0.15 mm clearance, 10 strokes). A portion of this homogenate was stored at -70°C until assayed for LH and FSH. A crude membrane fraction was prepared from the remaining homogenate as described in Experiment I. Binding assays were carried out for individual pituitaries and Scatchard analyses were performed from data so obtained. Numbers of binding sites are expressed as fmol/mg membrane protein. Protein was assayed as described by Lowry <u>et al</u>. (1951) using bovine serum albumin as standard.

GnRH analog, D-Ala⁶-des-Gly¹⁰-GnRH-ethylamide (GnRH-A; Peninsula Lab, San Carlos, CA) was iodinated by a lactoperoxidase-glucose oxidase method (Clayton <u>et al</u>., 1979). Specific activity calculated as described previously, ranged from 1152 to 2017 μ Ci/ μ g (Experiment I). The maximal percent of specific binding of $125_{I-GnRH-A}$ to an excess of membrane protein varied with iodinations and ranged 28 to 43%. Total radioactivity added to each assay tube was corrected for variation in maximal specific binding prior to Scatchard analysis. The assay was carried out as previously reported (Experiment I) and radioactivity was measured in a gamma spectrometer with a counting efficiency of 85%.

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B. Fresh vs frozen anterior pituitary gland

Bovine anterior pituitary glands were obtained from a local abattoir. After a pituitary gland was bisected, the halves were randomly assigned to be used fresh or after freezing in liquid nitrogen for 2 h followed by thawing over a 15-min period. Binding assays were run on each pituitary half as described previously. We found that neither the affinity contants nor numbers of GnRH binding sites estimated using crude membrane preparation from frozen pituitary halves was different from those prepared from fresh halves (Table 2). For convenience, we stored pituitary halves in liquid nitrogen until time of assay.

C. Ability of the anterior pituitary gland to release gonadotrophins

To determine changes in ability of anterior pituitary glands to release LH and FSH, one half of the fresh gland from each animal was sliced and diced into $1-2 \text{ mm}^3$ explants. Twenty to 25 mg of pituitary explants were incubated in 4 ml of culture medium under an atmosphere of 95% O₂ and 5% CO₂ at 37°C. Culture medium consisted of Hank's minimum essential medium and Medium 199 in 1:1 ratio supplemented with 5 mM L-glutamine (GIBCO, Grand Island, NY). Explants were first washed with culture medium at 15 min intervals for 1 h and then incubated in the absence of any treatment for 2 h to establish basal release of gonadotrophins into medium. At the end of 2 h, medium was

TABLE 2. Comparisons of affinity constants and numbers of GnRH binding sites between fresh and frozen halves of anterior pituitary glands

Pituitary No.	Fresh Half	Frozen Half
1	1.19	0.63
2	0.87	0.93
3	0.54	0.93
4	0.63	0.88
5	0.86	0.85
6	1.29	1.67
7	1.35	1.01
	0.96 <u>+</u> 0.12	0.98 + 0.12

Affinity Constants (X $10^{10} M^{-1}$)

paired-t test P>0.25

Numbers of binding Sites (fmol/mg protein)

Pituitary No.	Fresh Half	Frozen Half
1	69.7	75.1
2	65.0	44.5
3	22.3	28.4
4	52.7	86.1
5	115.8	151.1
6	133.8	111.7
7	201.2	196.9
	94.4 + 22.8	99.1 <u>+</u> 22.5

paired-t test P>0.25

changed and explants were challenged with 4 ng GnRH per flask for an addition 2 h. Again, medium was collected after the challenge. All samples of medium were assayed for LH and FSH.

Ovaries

Ovaries were collected from each cow after slaughter. Presence of newly formed corpora lutea was noted and used as one criterion to determine resumption of estrous cyclicity in these cattle.

Radioimmunoassays

Concentrations of LH in all serum and medium samples were determined in four assays using a double antibody procedure previously validated in this laboratory (Convey <u>et</u> <u>al</u>., 1976). Standard preparations of LH was NIH-LH-B8. Inter and intra-assay coefficients of variation were 12.1% and 11.6%, respectively. Serum and medium FSH was quantified using USDA-FSH-B1 as standard (Validations in Appendix III). Rabbit antibovine FSH (B-5) supplied by Dr. K.W. Cheng was used at 1:75,000 dilution. Inter and intra-assay coefficients of variation determined from three assays were 10.8% and 8.1%, respectively. Using NIH-prolactin-B4, concentrations of prolactin in serum were measured (Koprowski and Tucker, 1971) in a single assay with a intra-assay coefficient of variation of 9.1%.
Selected serum samples were assayed for estradiol and progesterone as previously described (Carruthers and Hafs, 1930; Louis <u>et al.</u>, 1973). Inter and intra-assay coefficients of variation were 15.0% and 7.9% for estradiol and 9.5% and 9.5% for progesterone. Serum cortisol was measured using a newly validated assay procedure developed in this laboratory (Purchas <u>et al.</u>, 1984). Within assay coefficient of variation determined from a single assay was 3.1%.

Statistical Analysis

A. Acyclic vs. cyclic cows

At slaughter, 11 of the 32 beef cows had corpora lutea on the ovaries and elevated concentrations of serum progesterone (> 1 ng/ml). This indicated that these 11 cows had resumed estrous cyclicity before slaughter (Table 3).

Table 3.	Distributi	on of	acyclic	and	cyclic	COWS	with	time
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from partu	irition			
Postpartum Days	N	Acyclic	Cyclic	
7	6	6	0	
14	6	6	0	
28	6	5	1	
42	6	3	5	
56	6	1	5	

With the variable of cyclicity in mind, animals were rearranged into the following six groups: those that were acyclic on postpartum days 7 (n=6), 14 (6), 28 (5) or 42 (3) and those that were cyclic on postpartum day 42 (5) or 56 (5) (total N=30). Due to lack of replication, the cow that was cyclic on day 28 and the other that was acyclic on day 56 were deleted from statistical anlaysis. Since beef cows resumed estrous cyclicity on different postpartum days and were slaughtered on fixed postpartum days, means of variables obtained on days 42 and 56 from cyclic cows were means of data from different days of the estrous cycle.

B. Evaluation of pulsatile pattern of serum hormones

To evaluate pulsatile pattern of serum LH, FSH and prolactin the following criteria were used:

- Pulse: a sample value that exceeded a preceeding value by 3 standard deviations (S.D.) established from control serum values run in the same assay (3 S.D. = 0.3 ng/ml for LH, 7.5 ng/ml for FSH and 8.8 ng/ml for prolactin).
- 2. Nadir: lowest point(s) between the defined peaks.
- 3. Sensitivity: the least amount of hormone that could be significantly detected by assay; i.e. significantly different from buffer control (0.125 ng for LH; 2.5 ng for FSH and 0.5 ng for prolactin).
- 4. Baseline for animals with pulsatile release: Mean baseline concentrations of hormone included all samples that were equal to nadir and within the range of nadir + assay sensitivity.

- 5. Baseline for animals with no pulsatile release: Mean baseline concentrations were equal to mean of all serum samples.
- 6. Amplitude: The difference between maximal value reached during a pulsatile release and the nadir preceding the release.

C. Analysis of Data

All data reported were tested for heterogeneity of variance among groups using Bartlett's test (Gill, 1978). Heterogeneity was found in variances of baseline concentrations and overall mean concentrations of serum LH, serum concentrations of cortisol, progesterone and estradiol. Therefore, data of these five variables were subjected to logarithmic transformation before statistical analysis. Data of these variables are presented in Results as: means of raw data (means <u>+</u> SEM of data after transformation). All data were analyzed by one-way analysis of variance. Specific comparisons of means were conducted using Bonferroni's t test (Gill, 1978).

RESULTS

Cows and calves

Thirteen bull calves and 17 heifer calves were born. Neither the sexes, birth weights of the calves nor gestation lengths were different among groups (P>0.25). The calves had a mean birth weight of 26.4 ± 0.6 kg, and gestation lengths averaged 277.8 ± 0.6 days. No major calving difficulty was encountered.

Suckling

There was no change (P>.20) in either the frequency or duration of suckling events observed at the times studied (Table 4). Mean frequency (number/6 h) and duration (min) were 2.3 \pm 0.2 and 8.6 \pm 0.3, respectively. Furthermore, these variables were not different between acyclic and cyclic cows.

In vitro release of gonadotrophins

A. Basal Release of LH and FSH

Among acyclic cows between days 7 and 42 after parturition basal release of LH into medium increased linearly (P<0.005) from 9.1 ± 1.5 to 20.2 ± 0.4 ng/ml medium/mg pituitary gland and basal release of FSH increased linearly (P<0.025) from 136 \pm 5.5 to 164 \pm 8.9 ng/ml medium/mg pituitary gland (fig. 8 and 9). By day 42 after parturition, basal release of LH and FSH from acyclic cows was comparable (P>0.10) to that of cyclic cows on day 42 and 56 after parturition.

B. GnRH-induced release of LH and FSH

The profile of GnRH-induced release of LH from

Frequencies and durations of suckling events in postpartum beef cows. Table 4.

		Suck1	ing Events
Postpartum Days	N	Frequency (no./6 h)	Duration (min)
7 Acyclic	ъ	2.8 ± 0.49ª	8.6 ± 0.59
14 Acyclic	9	2.7 ± 0.49	7.9 ± 0.75
28 Acyclic	Ŋ	1.2 ± 0.20	8.5 ± 0.29
42 Acyclic	ſ	1.7 <u>+</u> 0.88	10.8 ± 0.56
42 Cyclic	ß	3.0 ± 0.77	8.9 ± 0.96
56 Cyclic	2	2.4 ± 0.51	8.7 + 0.79
X	29	2.3 ± 0.24	8.6 ± 0.34

^aMean <u>+</u> SEM.

fig. 8. Concentration of basal and GnRH-induced release of LH from pituitary explants of postpartum beef cows. Values are means <u>+</u> SEM.



fig. 9. Concentrations of basal and GnRH-induced release of
FSH from pituitary explants of postpartum beef
cows. Values are means + SEM.



pituitary explants was parallel to basal release of LH (fig. 8). There was a linear increase (P<0.05) in GnRH-induced release of LH into medium from 17.1 ± 3.2 to 34.8 ± 6.3 ng/ml medium/mg pituitary gland between days 7 and 42 after parturition. GnRH-induced release of LH in acyclic cows on day 42 after parturition was similar (P>0.25) to that of cyclic cows on day 42 and 56 after parturition. GnRH-induced release of FSH into medium, however, did not vary (P>0.25) between days 7 and 56 after parturition among acyclic cows or between acyclic and cyclic cows (fig. 9).

Pituitary concentrations of LH, FSH and GnRH binding sites

Among acyclic cows, concentrations of LH and FSH expressed as μ g/mg pituitary tissue increased linearly (P<0.05) with time postpartum (fig. 10). Pituitary LH concentrations increased from 0.43 ± 0.04 to 0.76 ± 0.05 and pituitary FSH concentrations from 0.85 ± 0.02 to 0.99 ± 0.08 between days 7 and 42 after parturition. By day 42 after parturition, pituitary concentrations of LH and FSH of acyclic cows were similar (P<0.05) to those of acyclic cows on day 42 and 56 after parturition.

There were positive correlations (P<0.001) between LH concentration in pituitary glands and basal (r=0.76) and GnRH-induced (r=0.75) releases of LH from pituitary explants in culture. A positive correlation was also detected (r=0.53; P<0.005) between FSH concentration in the pituitary

fig. 10. Concentrations of LH and FSH in anterior pituitary
 glands of postpartum beef cows. Values are means
 + SEM.



gland and basal release of FSH from the explants.

Affinity constant and number of GnRH binding sites did not change (P>0.25) with time postpartum. They averaged 0.8 \pm 0.02 X 10¹⁰ M⁻¹ and 110.7 \pm 7.4 fmol/mg protein, respectively (Table 5).

Profiles of serum gonadotrophins

A. LH

Profiles of serum LH are presented in fig. 11. Frequency of episodes and mean baseline concentrations of LH did not change with time from parturition, or when cows resumed estrous cyclicity. There was an average of 1.5 + 0.3 pulses/6 h and mean baseline concentration was 0.9 (-0.2 + 0.05) ng/ml. In contrast, we detected a linear increase (P<0.05) in the overall mean concentrations of serum LH between days 7 and 42 after parturition in the acyclic cows. Furthermore, among acyclic cows, the amplitudes of LH pulses observed on days 14, 28 and 42 after parturition were significantly greater (P<0.05) than that on day 7 (2.4 \pm 0.2 vs. 1.3 + 0.4 ng/ml, respectively). The amplitude of pulsatile LH release between days 7 and 42 after parturition among acyclic cows was significantly higher (P<0.05) than that of cyclic cows (2.1 + 0.2 vs. 1.3 + 0.3 ng/ml,respectively).

B. FSH

Profiles of serum FSH were uneventful (fig. 12).

			Numbers of binding sites
Postpartum Days	N	Affinity Constants (X 10 ¹⁰ M-1)	(fmol/mg protein)
7 Acyclic	Q	0.79 ± 0.09	98.3 ± 13.8
14 Acyclic	Q	0.73 ± 0.05	119.9 ± 15.7
28 Acyclic	S	0.67 ± 0.06	129.0 ± 18.6
42 Acyclic	m	0.81 ± 0.04	81.8 ± 12.3
42 Cyclic	ß	0.71 ± 0.03	136.6 ± 25.8
56 Cyclic	Ŋ	0.31 ± 0.07	94.8 ± 11.4
×		0.75 ± 0.02	110.7 ± 7.40

pituitary glands of postpartum beef cows

Affinity constants and numbers of GnRH binding sites in anterior

Table 5.

fig. 11. Profiles of serum LH in postpartum beef cows. Values of frequencies and amplitudes of LH pulses are means <u>+</u> SEM. Data of baseline concentrations and overall mean concentrations of serum LH are means of raw data. Baseline concentrations and overall mean concentrations of serum LH were subjected to logarithmic transformation before statistical analysis (see Materials and Methods).



fig. 12. Profiles of serum FSH in postpartum beef cows. Values are means <u>+</u> SEM.



Although I observed episodic release of FSH, frequencies and amplitudes of the pulses, mean baseline concentrations and overall mean concentrations of FSH did not change between days 7 and 56 after parturition. They averaged 1.4 ± 0.2 pulses/6 h, 11.8 ± 3.2 ng/ml, 34.7 ± 0.8 ng/ml and $36.1 \pm$ 0.9 ng/ml, respectively. In addition, none of these variables were different (P>0.10) between acyclic and cyclic cows.

C. Resumption of episodic release of gonadotrophins

The number of cows showing episodic release of LH and FSH increased with time from parturition (Table 6). On days 7 and 14 after parturition, 58.4% of the acyclic cows showed episodic release of gonadotrophins and this increased to 83.4% on days 28 and 42. Among cyclic cows, 80% of them showed episodic release of FSH whereas 100% of them showed episodic release of LH. Every episodic release of FSH occurred in synchrony with an episodic release of LH; however, only 89.1% of episodic releases of LH were accompanied by release of FSH.

Profile of serum prolactin

Concentrations of serum prolactin at the onset of the blood sampling period were high and gradually returned to steady state within 1.5 to 2 h. Although this is typical of serum prolactin (Tucker, 1971), it made the process of

Table 6. Distribution of resumption of episodic release of gonadotrophins in postpartum beef cows

		-	t of cows shows	ing episodic release
Postpar	tum days	N	LH	FSH
7 Асус	elic	6	66.7 ₁	66.7 ₎
14 Acyc	clic	6	$50.0^{\text{J}}\overline{\text{X}} = 5$	58.4 $50.0^{3} = 58.4$
28 Acyc	elic	5	100.0	100.0
42 Acyc	clic	3	66.7 $\overline{X} = 8$	$66.7^{\int \overline{X}} = 83.4$
42 Cycl	lic	5	100.0	80.0
56 Cycl	lic	5	100.0	80.0

analyzing profiles of serum prolactin during the entire period difficult, if not impossible. Consequently, only data of concentrations of serum prolactin obtained during the last 4 h of the 6 h period were used to study episodic release of prolactin.

Not all suckling events were followed by release of prolactin (Table 7). The degree of association of suckling events and episodic release of prolactin increased from 13 to 75% between days 7 and 42 after parturition. The degree of association then declined to 40 and 38% on days 42 and 56 after parturition respectively in cyclic cows. When an episodic release of prolactin was associated with a suckling event, the amplitude of that release was significantly higher (P<0.001) than that not associated with a suckling event (33.5 + 5.1 vs 13.8 + 2.3 ng/ml, respectively).

Steroid hormone concentrations in serum

Concentrations of progesterone in serum were measured in samples collected at 0800 and 1400 h, whereas concentrations of cortisol in serum were assayed in samples obtained at 0800, 1000, 1200 and 1400 h during the blood sampling period. Serum concentrations of estradiol were determined in a composite of seven hourly samples obtained from each cow during the blood sampling period.

Among acyclic cows, serum concentrations of progesterone, estradiol and cortisol averaged 0.4 (-1.0 +

				Amplitudes of	Amplitudes of
		·	<pre>% suckling events</pre>	prolactin release	prolactín release NOT
		Baseline	followed by	associated with suckling	associated with suckling
Postpartum days	Z	Concentration (ng/ml)	prolactin releases ^a	(ng/m1)	(lm/gn)
7 Acyclic	'n	14.0 ± 1.22	13	8.9 (1)b	19.4 ± 4.3 (5)
14 Acyclic	Q	17.0 ± 2.88	31	31.6 ± 6.8 (6)	17.2 ± 5.9 (5)
28 Acyclic	S	17.8 ± 2.17	75	43.9 ± 12.5 (3)	12.4 ± 1.6 (5)
42 Acyclic	m	14.6 <u>+</u> 2.05	75	17.5 (1)	0
42 Cyclic	Ŋ	13.2 ± 1.54	40	33.5 ± 13.7 (4)	9.7 (1)
56 Cyclic	ŝ	13.2 ± 1.71	38	23.6 ± 0.6 (2)	10.5 ± 0.4 (3)

Table 7. Profiles of concentrations of serum prolactin in postpartum beef cows

^aNo. of suckling events during which prolactin increased [±] total no. of suckling events X 100.

^bNumber of observations.

^CNone observed.

0.04) ng/ml, 1.8 (0.51 \pm 0.10) pg/ml and 7.5 (1.8 \pm 0.15) ng/ml, respectively, and did not change between days 7 and 42 after parturition (Table 8). In cows that had resumed estrous cyclicity before slaughter, estradiol and cortisol concentrations in serum were not different (P>0.20) from those of acyclic cows. In contrast, concentrations of progesterone in serum of cyclic cows were significantly higher (P<0.001) than that of acyclic cows [3.3 (0.84 \pm 0.31) vs. 0.4 (-1.0 \pm 0.04) ng/ml, respectively].

Changes in concentrations of serum progesterone, estradiol and Table 8.

cortisol with time in postpartum beef cows

Postpartum		Progesterone	Estradiol	Cortisol
Days	z	(ng/ml)	(pg/m1)	(ng/m1)
7 Acyclic	9	0.4a (-0.97 + 0.08)b	1.7 (0.53 + 0.08)	5.3 (1.5 + 0.30)
14 Acyclic	9	0.3 (-1.1 ± 0.04)	1.5 (0.34 ± 0.12)	8.5 (2.0 ± 0.31)
28 Acyclic	S	0.4 (-1.0 ± 0.10)	2.0 (0.58 ± 0.25)	7.5 (2.0 ± 0.18)
42 Acyclic	m	0.4 (-0.87 ± 0.14)	2.5 (0.70 ± 0.49)	10.2 (2.2 ± 0.33)
		0.4 (-1.0 ± 0.04)	1.8 (0.51 ± 0.10)	7.5 (1.8 ± 0.15)
42 Cyclic	ŝ	1.2 (0.11 ± 0.13)	1.9 (0.53 ± 0.19)	11.2 (2.0 ± 0.42)
56 Cyclic	ъ	6.0 (1.8 ± 0.18)	1.5 (0.31 ± 0.26)	13.7 (2.6 ± 0.13)
		3.3 (0.84 ± 0.31)	1.7 (0.44 ± 0.16)	12.5 (2.3 ± 0.23)

^aMeans of raw data.

bMeans + SEM of data after logarithmic transformation.

The objective of this experiment was to determine the relationship between the number of GnRH binding sites in the anterior pituitary gland and restoration of the ability of the gland to release gonadotrophins during postpartum anestrus. I chose to study basal and GnRH-induced release of gonadotrophins from explants of a hemipituitary gland in vitro for comparison with changes in number of GnRH binding sites in the other half of the same pituitary gland. In previous in vivo studies, 4 to 6-fold increases in GnRH-induced release of LH into blood were observed during the first 20 days after parturition (Kesler et al., 1977; Fernandes et al., 1978). During the same period, basal concentrations of serum LH before GnRH increased only 2 to 3-fold. These results support the concept that responsiveness of pituitary glands to GnRH increased after parturition. In the present experiment using an in vitro system, both basal and GnRH-induced release of LH from pituitary explants into medium doubled between days 7 and 42 after parturition. There was no change in concentrations of LH in medium in response to GnRH when expressed as difference between basal and GnRH-induced release of LH from pituitary explants. Thus, under in vitro conditions, I detected an increase in the ability of the pituitary gland to release LH but no increase in responsiveness of the gland

to GnRH during postpartum anestrus. The reasons for the difference between results from <u>in vivo</u> and <u>in vitro</u> studies is not clear at this time.

Neither the affinity constant nor number of GnRH binding sites in the pituitary gland changed during the postpartum interval with the increased ability of pituitary explants to release LH. Parfet <u>et al</u>. (1983) reported in an abstract that there was an initial decrease in the number of GnRH binding sites in pituitary glands of beef cows between postpartum days 10 and 20, and then remained constant. Nevertheless, both their and my results provide evidence that there is no direct relationship between changes in the ability of the pituitary gland to release LH and the number of GnRH binding sites in the gland. This conclusion is similar to that deduced by Ferland <u>et al</u>. (1981) in rats and Experiment I in this dissertation.

In the present study, there is a possibility that the number of GnRH binding sites reported may not represent the actual quantity of GnRH binding sites present in the pituitary explants. Firstly, GnRH binding sites were quantified at time 0 relative to onset of explant culture, whereas concentrations of gonadotrophins were determined in media collected at 2 and 4 h after onset of explant culture to evaluate basal and GnRH-induced release of gonadotrophins. Numbers of GnRH binding sites may change during incubation periods.

Secondly, in this and other studies, a crude membrane fraction of anterior pituitary gland was used in the quantification of GnRH binding sites. This crude membrane preparation consists of plasma membrane as well as other intracellular membranes. Therefore, the total number of GnRH binding sites were actually measured. It is possible that the presence of GnRH binding sites not associated with plasma membrane makes the detection of any subtle change in the number of GnRH binding sites in the plasma membrane of gonadotrophs difficult. In view of this possibility and the fact that GnRH binding to secretory granules (Sternberger and Petrali, 1973; Morel et al., 1980) and nuclei (Millar et al., 1983) have been reported, the procedure for obtaining crude membrane of pituitary glands was modified so that I could remove most of the secretory granules and nuclei from the final preparation of membrane. Even with removal of secretory granules and nuclei, no change in either the number or affinity of GnRH binding sites was detected; yet, the ability of pituitary glands to release LH increased.

Saiduddin <u>et al</u>. (1968) observed that concentrations of pituitary FSH was reduced to one half during the first 30 days after parturition in beef cows. In contrast, I observed a 15% increase in concentration of pituitary FSH during postpartum anestrus. The difference between their study and my findings might be explained by the difference in quantification of FSH. In their study, bioassay was used, whereas in mine, radioimmunoassay.

Changes in concentrations of LH in the pituitary gland, on the other hand, were in agreement with that in previous studies (Labhsetwar <u>et al</u>., 1964; Saiduddin and Foote, 1964; Saiduddin <u>et al</u>., 1963) in which a 2-fold increase in concentration of pituitary LH was observed during the first 4 weeks after parturition. Hence, there are iAncreases not only in the ability of the pituitary gland to release LH, but also in the storage of LH in the gland with time during postpartum anestrus.

Moss et al. (1980) observed that a constant percentage of total LH was released from dispersed pituitary cells of postpartum ewes after stimulation with a maximal dose of GnRH. Thus, these authors hypothesized that only a certain percentage of LH in the anterior pituitary gland was readily releasable. If this hypothesis is correct, as the concentrations of pituitary LH increases during the postpartum anestrus as observed in this experiment, I expected increased amounts of LH to be available for release. Indeed, this phenomenon may explain the positive correlations between concentration of LH in the pituitary gland and either basal or GnRH-induced release of LH from pituitary explants in the present study. And these increases in storage and release of LH may be essential for the re-establishment of the first postpartum preovulatory surge of gonadotrophins.

Previous studies reported a gradual increase in mean

concentration of serum LH during postpartum anestrus (Erb <u>et</u> <u>al</u>., 1971; Short <u>et al</u>., 1972; Kesler <u>et al</u>., 1977; Fernandes <u>et al</u>., 1978; Goodale <u>et al</u>., 1978; Walters <u>et</u> <u>al</u>., 1982). My studies confirmed these observations. In addition, I observed that the overall mean concentration of LH increased as the ability of the pituitary gland to release LH <u>in vitro</u> increased. This increase in overall mean concentration of LH in serum was probably due in part to an increase in the amplitude, but not frequency of LH pulses on days 14, 28 and 42 during postpartum anestrus. This is in agreement with the observations of Walters <u>et al</u>. (1982) and Humphrey <u>et al</u>. (1983). In addition to an increase in amplitude of pulsatile release of LH, Rawlings <u>et al</u>. (1980) and Goodale <u>et al</u>. (1978) detected an increase in frequency of the LH pulses.

Regarding the ability of the pituitary gland to release FSH, only basal release of FSH from pituitary explants increased 20% between days 7 and 42 after parturition. Corresponding concentrations of pituitary FSH increased 15% during the same period. Although these increases in basal release of FSH from explants and concentrations of FSH in the pituitary gland were significant statistically, the physiological importance of these quantitatively small increases remains to be determined. In the present study, these increases were not reflected in the concentrations of serum FSH. No change in profile of serum FSH was observed during the period studied.

The discrepency between release of LH and FSH may be explained by a feedback mechanism selective for FSH. Bovine follicular fluids contain an inhibin-like substance which selectively inhibits GnRH-induced release of FSH, but not basal release of FSH from bovine pituitary cell cultures (V. Padmanabhan, personal communication). Bovine follicular fluid also had no effect on either basal or GnRH-induced release of LH from anterior pituitary cell cultures. Exposure of the pituitary gland to an endogenous inhibin-like substance might account for similar magnitudes of GnRH-induced release of FSH during postpartum anestrus. However, presence of inhibin in serum during postpartum anestrus remains to be determined. GnRH from the hypothalamus stimulates synthesis and release of gonadotrophins. GnRH exerts its trophic effect by first interacting with its binding sites in the plasma membrane of gonadotrophs. Therefore, any changes in affinity constants and numbers of GnRH binding sites in the plasma membrane of gonadotrophs may affect the equilibrium of the interaction between GnRH and its binding sites. To date, no change in affinity constant of GnRH has been observed. On the contrary, numbers of GnRH binding sites vary depending on the stages of estrous cycle and lactation in laboratory and domestic animals (see Introductions for experiment I and II for review).

Giguere <u>et al</u>. (1981) reported that treatment of pituitary cells of intact female rats with dihydrotestosterone led to 40% decreases in numbers of GnRH binding sites and similar decreases in the ability of the cells to release LH in response to GnRH. In a recent study, Wise <u>et</u> <u>al</u>. (1983) used a GnRH antagonist to occupy a portion of GnRH binding sites in pituitary glands to reduce number of free binding sites for GnRH. As the number of free binding sites for GnRH decreases, there is a corresponding decline in GnRH-induced release of LH from the pituitary gland. Although these decreases in numbers of GnRH binding sites were induced experimentally, results from these two studies

provide evidence that there is a positive correlation between number of GnRH binding sites in the pituitary gland and the ability of the gland to release gonadotrophins.

Concentrations of serum estradiol and progesterone vary at different stages of the estrous cycle in cattle. These ovarian steroids can modify the release of gonadotrophins from the pituitary gland by direct action on gonadotrophs (Padmanabhan and Convey, 1981). Furthermore, these ovarian steriods can also modify numbers of GnRH binding sites in the pituitary gland (see Review of Literature for review). It is possible that one of the sites of action of ovarian steroids is at the GnRH binding sites in the plasma membrane of gonadotrophs. This interaction may modulate the number of GnRH binding sites and hence the release of gonadotrophins. Increases in the ability of the anterior pituitary gland to release gonadotrophins is believed to be a prerequisite to development of the preovulatory surges of gonadotrophins and subsequent ovulation. Studying the relationship between number of GnRH binding sites in the pituitary gland and ability of the gland to release gonadotrophins would provide insight into hormone action so that one can eventually manipulate hormone secretion and regulate fertility in cattle, other domestic species and man. Assuming there was a significant correlation between number of GnRH binding sites in the pituitary gland and ability of the gland to release gonadotrophins, a treatment

regimen that will increase number of GnRH binding sites in the pituitary gland may potentially hasten the first estrous cycle in pubertal heifers and postpartum cows and also may synchronize the time of preovulatory surges and subsequent ovulations.

In this dissertation, I quantified numbers of GnRH binding sites in the anterior pituitary gland in two different physiological states. One physiological state is when the ability of the pituitary gland to release gonadotrophins increases just prior to preovulatory surges of gonadotrophins. The other is during the postpartum period when the ability of the pituitary gland to release gonadotrophins recovers. In both instances, there is no direct relationship between the number of GnRH binding sites in the pituitary gland and the ability of the pituitary gland to release LH. Ferland et al. (1981) also reported the absence of parallel changes in these two variables during the estrous cycle of the rat. In addition, these authors found that a single injection of estradiol suppressed both basal and GnRH-induced release of LH and FSH in female rats with no obvious change in the affinity constant or number of GnRH binding sites. Furthermore, after multiple injections of GnRH in rats in the afternoon of proestrus, these authors observed enhanced release of LH into blood but a loss of about 40% of GnRH binding sites in the pituitary gland. Thus, numbers of GnRH binding sites in

the pituitary gland may not be a lmiting factor in the ability of the gland to release gonadotrophins.

In the Review of Literature, I have proposed a model for GnRH action on gonadotrophins in the anterior pituitary gland. In that model, I have suggested a sequence of intracellular changes that may occur between the interaction of GnRH and its binding sites in the plasma membrane of gonadotrophs and the eventual release of gonadotrophins from gonadotrophs. Changes in one or more of these cellular events could increase the ability of the pituitary gland to release gonadotrophins without any change in number or affinity constant of GnRH binding sites. For example, maturation of a second messenger system or a calcium-dependent process after the formation of GnRH-binding site complexes may govern the eventual increase in the ability of the pituitary gland to release gonadotrophins. Studies have shown that either cAMP (Borgeat et al., 1972) or cGMP (Naor et al., 1978) might be the second messenger of GnRH action in stimulating gonadotrophin release. Interpretation of results from a study by Conn et al. (1979a), however, suggests that the influx of calcium into gonadotrophs following GnRH administration may be important for the release of LH. Any increases in concentration of cAMP, cGMP or calcium intracellularly could eventually lead to increased release of gonadotrophins from the pituitary glands.

The ability of the pituitary gland to release gonadotrophins may also be enhanced by previous exposure of the gland to GnRH (Aiyer et al., 1974; Foster, 1978). This phenomenon, known as self-priming, is exerted via a direct effect on the bovine pituitary gland (Padmanabhan et al., 1981). Development of the priming effect of GnRH takes about 1 h (Aiyer et al., 1974; Padmanabhan et al., 1981) whereas it takes at least 3 days to induce a 2-fold increase in number of GnRH binding sites (see Review of Literature for review). Increasing frequency of pulsatile release of LH prior to the preovulatory surge of LH (Rahe et al., 1980) and with time from parturition (Rawlings et al., 1980; Goodale et al., 1973) were observed. It is reasonable to suggest that the frequency of pulsatile release of GnRH from the hypothalamus also increased at these times. The selfpriming effect of GnRH may play an important role in preparing the pituitary gland to release gonadotrophins in response to GnRH released at estrus without a corresponding change in numbers of GnRH binding sites.

Ovarian steroids can also enhance synthesis and subsequent release of LH. Chronic treatments with estradiol and progesterone suppress synthesis of the alpha subunit of gonadotrophins (Landefeld <u>et al</u>., 1982). Acute administration of estradiol enhances whereas chronic exposure to estradiol suppresses alpha subunit synthesis (Landefeld <u>et al</u>., 1984). Regarding LH release, Padmanabhan <u>et al</u>. (1978) observed that estradiol enhanced basal and

GnRH-induced release of LH from bovine pituitary cells. Progesterone, in contrast, completely inhibits the ability of estradiol to increase basal and GnRH-induced release of LH (Padmanabhan and Convey, 1981). During proestrus, I observed a gradual increase in the concentration of estradiol in serum. In contrast, at parturition, the concentrations of progesterone and estradiol in serum decrease precipitously and remain low during postpartum anestrus (Smith et al., 1973; Edqvist et al., 1973). Thus, the pituitary gland is under positive feedback of estradiol during proestrus, whereas during postpartum anestrus it recovers gradually from an extended negative feedback of estradiol and progesterone. Both of these mechanisms could lead to increasing ability of the pituitary gland to release LH.

The discordant relationship between the number of GnRH binding sites in the pituitary gland and ability of the gland to release LH brings forth many questions. Some questions are:

- a) What is the mechanism by which the release of gonadotrophins is regulated?
- b) Does the same mechanism also regulate synthesis of gonadotrophins?
- c) Is the same mechanism involved in all physiological states?
- d) Do GnRH and/or ovarian steroids modify the mechanism? If so, how?
The relationship between the number of GnRH binding sites in the pituitary gland and change in the ability of the gland to release LH and FSH during proestrus and postpartum anestrus in beef cows was studied.

Estrus in beef cows was synchronized using a progestagen implant and a prostaglandin $F_{2}\alpha$ injection. Cows were given GnRH at 40-min intervals for 6 h beginning at -24, 0, 18 or 36 h after removal of the progestagen implant. Changes in concentration $(\Delta ng/ml)$ of serum LH after GnRH averaged 2.9, 6.2, 6.4, 33.4; whereas serum FSH averaged 25.7, 35.8, 35.8 and 97.3. Therefore, the responsiveness of the pituitary gland to GnRH increased by 36 h after implant removal. Other groups of cows subjected to the same synchronization regimen were slaughtered at 0 h, 24 h or at various times after onset of estrus. GnRH binding to crude pituitary membrane preparations was assessed. There was no apparent change in the affinity constant of GnRH binding sites with time after implant removal. The number of GnRH binding sites remained unchanged until the period of estrus during which a 50% decline was detected. This decline was coincident with decreasing pituitary concentrations of LH and FSH.

Thirty two primiparous suckled beef cows were slaughtered on either day 7, 14, 28, 42 or 56 from

parturition. One of 6, 5 of 8 and 5 of 6 cows resumed estrous cyclicity at time of slaughter on days 28, 42 and 56, respectively. Among acyclic cows between days 7 and 42, basal release of LH from pituitary explants doubled whereas basal release of FSH increased only by 20%. GnRH-induced release of LH, but not FSH, increased 2-fold during the same On day 42, basal and GnRH-induced release of LH and period. FSH from pituitary explants of acyclic cows were similar to that of cyclic cows on days 42 and 56. Concentrations of pituitary LH of acyclic cows doubled, whereas concentrations of pituitary FSH increased only 15% between days 7 and 42. Again by day 42, concentrations of pituitary LH and FSH of acyclic cows were similar to those of cyclic cows on day 42 and 56. During postpartum anestrus, although overall mean concentrations of serum FSH did not change, overall mean concentrations of serum LH increased. An increase in amplitude of pulsatile release of LH may explain this increase in overall mean concentration of serum LH. Numbers and affinity constants of GnRH binding sites remained constant during the period studied.

Based on the results obtained from beef cows, I conclude that during proestrus and postpartum anestrus there is no direct relationship between the ability of the anterior pituitary gland to release gonadotrophins and numbers or affinity constants of GnRH binding sites in a crude membrane preparation of the anterior pituitary gland.

Therefore, under these experimental conditions, number or affinity constant of GnRH binding sites in the pituitary gland of a beef cow may not be a limiting factor in the ability of the gland to release gonadotrophins. APPENDICES

APPENDIX I

Primary culture of bovine anterior pituitary cells

- I. Check list for chemicals
 - 1. 70% ethanol
 - Dulbecco's modified Eagle medium (Grand Island Biological Co. Cat. #430-1660)
 - 3. L-Glutamine (GIBCO; Cat. #320-5039)
 - 4. Penicillin-Streptomycin (Penicillin-G 10,000 U/ml and Streptomycin sulfate 10 mg/ml; GIBCO; Cat. #600-5145)
 - 5. Fungisone (Amphotericin B; 250 µg/ml; GIBCO; Cat. #600-5295)
 - 6. HEPES (N-2-Hydroxyethyl-piperazine-N'-2ethanesulfonic acid; Cat. #H3375)
 - 7. Sodium bicarbonate (Sigma; Cat. #S8875)
 - 8. Collagenese (type I: 150 U/mg; Sigma; Cat. #C-0130)
 - 9. Pancreatin (viokase; GIBCO; Cat. #610-5725)
 - 10. MEM esential amino acid without L-glutamine (GIBCO; Cat. #320-1130)
 - 11. MEM nonessential amino acid (GIBCO; Cat.
 #320-1140)
 - 12. Postpartum cow serum (low steroid concentration)
 - 13. Lauryl sulfate (Sodium salt; Sigma; Cat. #L-5750)

II. Pre-sterilized materials

Nalge filter units (0.22 µm; cat #245-0045) 50 ml disposable centrifuge tube (Corning; Cat. #25330) disposable petri dishes (Thomas; Cat. #3488-328) disposable syringes disposable hypodermic needles multiwell culture plates (Costar, Cambridge, MA; Cat. #3524)

III. Materials needed to be autoclaved before each culture

four 125 ml Erlenmeyer flasks
three 125 ml Erlenmeyer flasks with screw caps
 (Corning; Cat. #4985)
three stirring magnets
two 250 ml graduated cylinders
one 250 ml beaker
four 50 ml beakers
four medium bottles (Kimble; Cat. #14250)
five gauze pads (individually wrapped)
six Pasteur pipettes
6 X 400 ml H₂0 in 500 ml bottles

IV. Check list for equipment

1 centrifuge (set at room temperature)
1 Stadie-Riggs microtome and disposable blades
1 scapel and disposable blades
forceps
1 cutting board
1 hemocytometer
1 mixer
1 Selectapette (1 ml maximum volume; Clay Adams; Cat.
#4690) and pipette tips (Cat #4696)

V. Components of media

- 1. maintenance medium
 Dulbecco's modified Eagle medium (pH 7.4)
 supplemented with:
 - MEM essential amino acids without L-glutamine (10 ml/l medium)
 - 2. MEM nonessential amino acids (10 ml/l medium)
 - 3. L-glutamine (10 ml/l medium)
 - 4. Penicillin-Streptomycin (1 ml/l medium)
 - 5. Fungisone (1 ml/l medium)
 - 6. Sodium bicarbonate (3.7 g/l medium)
 - 7. HEPES (N-2-hydroxyethyl-piperazine-N'-2ethanesulfonic acid; 6.0 g/l medium; 25 mM)
- 2. Growth medium

Maintenance medium (pH 7.4) supplemented with:

- 1. L-glutamine (100 ml/l medium)
- 2. 10% postpartum cow serum

- VI. Dispersion and incubation of bovine anterior pituitary cells
 - anterior lobes of four to six bovine pituitary glands are isolated
 - 2. the anterior lobes are sliced into ~1 mm slices and a Stadie-Riggs microtome and diced into 1 mm³ pieces with scalpel
 - 3. wash pieces with maintenance medium (see previous section) until medium remains clear after wash
 - 4. dissolve 300 mg of collagenase in 100 ml medium and then filter mixture through a Nalge filter unit (.22 μ m)
 - 5. distribute enzyme solution to Erlenmeyer flasks (50 ml/flask)
 - 6. transfer washed pituitary pieces (~ 2 to 3 pituitary glands/flask) and stirring magnets to enzyme solution.
 - cap and set flask in water bath (37°C) and stir for 45-60 min.
 - 8. filter pituitary pieces (if any left) and tissue-enzyme mixture through layers of gauze pads into a 50 ml disposable centrifuge tubes
 - 9. centrifuge tubes at 250 X g for 5 min. at room temperature, and decant supernatant fluid.
 - 10. add 50 ml of medium to centrifuge tubes and vortex tubes to resuspend cell pellet.
 - 11. Centrifuge tubes at 230 X g for 4 min at room temperature, and then decant supernatant fluid
 - 12. repeat steps 9 and 10 once
 - 13. Add 4 ml pancreatin to 30 ml medium and then filter mixture through a Nalge filter unit (.22 μ m)

- 14. add filtered enzyme mixture to pellet, vortex tube to resuspend pellet and then transfer to an Erlenmeyer flask containing a stirring magnet
- 15. cap and set flask in water bath (37°C) and stir mixture for 15-30 min.
- 16. repeat steps 8-10
- 17. repeat step 11 twice
- 18. add approximately 100 ml of growth medium (see previous section) to cell pellet and vortex to resuspend cells.
- 19. count number of cells in a hemocytometer
- 20. dilute cell suspension to obtain ≈ 5 X 10⁵ cells/ml medium and then add randomly 1 ml of cell suspension to multiwell culture plates
- 21. grow cells under an atmosphere of 95% $O_2:5$ % CO_2 (0 h)
- 22. change growth medium at 48 and 96 h
- 23. treatments commence at 120 h
- 24. cells are washed with growth medium (without serum) five times at 10-min intervals
- 25. following repeat washes, cells are incubated with appropriate treatments in growth media without serum
- 26. Upon completion of incubation, media are collected. Cells are lysed with 0.5% lauryl sulfate (2 ml/well) for determination of hormone content of cells.

APPENDIX II

Dose response of bovine anterior pituitary cells to GnRH and D-Ala⁶-des-Gly¹⁰-GnRH-ethylamide (GnRH-A)

Bovine pituitary cells cultures was prepared as described in Appendix I. GnRH or GnRH-A was dissolved in 0.1% Knox gelatin in 0.05 M phosphate buffered saline and was administered on day 5 of culture. Media were collected 6 h after GnRH or GnRH-A treatment for measurements of LH and FSH.

The effect of increasing concentrations of GnRH $(10^{-12} \text{ to } 10^{-7} \text{ M})$ and GnRH-A $(10^{-13} \text{ to } 10^{-7} \text{M})$ on LH and FSH concentrations in media are shown in fig. 13 and 14, respectively. Cubic regression curves of concentration of medium LH or FSH versus log concentration of GnRH or GnRH-A are also presented.

fig. 13 LH concentration in media following 6 h incubation of bovine pituitary cells with increasing concentration of GnRH (10-12 to 10-7 M) or GnRH-A (10-13 to 10-7 M). Values are means \pm SEM.



fig. 14. FSH concentration in media following 6 h incubation of bovine pituitary cells with increasing concentration of GnRH (10^{-12} to 10^{-7} M) or GnRH-A (10^{-13} to 10^{-7} M). Values are means <u>+</u> SEM.



APPENDIX III

Radioimmunoassay for FSH

FSH in sera and media was assayed by a double antibody radioimmunoassay.

I. First Antibody

Rabbit anti-bovine FSH serum (B-5) was supplied by Dr. K.W. Cheng, University of Manitoba, Winnipeg, Manitoba, Canada. It was diluted 1:400 with 0.05 M EDTA in pH 7.0 phosphate buffered saline (0.05 M EDTA-PBS) and kept frozen at -60°C. Before use, the antiserum was further diluted to 1:75,000 with 1:400 normal rabbit serum in 0.05 M EDTA-PBS.

II. Iodinated Hormone

5 µg of highly purified (Dr. K.W. Cheng) bovine FSH (bFSH) dissolved in 20 µl of 0.5 M phosphate buffer (PBS) was pre-dispensed into conical vials (500 µl volume). Vials were keep frozen at -60°C until use. Chloramine T (5 µg/10 µl PBS) and 1 mCi of carrier free Na¹²⁵I (IMS-300, Amersham, Arlington Heights, IL) were mixed vigorously with bFSH for 0.5 min. The oxidation was terminated with sodium metabisulfite (10 µg/10 µl in PBS). A transfer solution (100 µl) containing 16% sucrose and 1 mg potassium iodide were added to the reaction vial. The mixture was then placed on a Biogel P-60 column (0.8 X 20 cm) which was

pre-coated with 2-3 ml of 2% bovine serum albumin in PBS (2% BSA-PBS). One ml fractions were collected in disposable test tubes containing 1 ml of 2% BSA-PBS. The most radioactive fraction corresponding to the 125_{I} -bFSH was used in the radioimmunoassay. The 125_{I} -bFSH was diluted to approximately 20,000 cpm/100 μ 1 with 1% BSA-PBS on the day of use.

III. Second Antibody

Anti-rabbit gamma globulin serum (anti-RGG) was obtained from sheep injected with rabbit gamma globulin (Fraction II, Miles Labs, Elkhart, IN) emulsified in Freund's adjuvant. Anti-RGG was diluted with 0.05 M EDTA-PBS to an appropriate concentration on the day of use.

IV. Assay Procedure

USDA-FSH-Bl was used as assay reference standard. Varying volumes of serum samples or standard were pipetted into disposable culture tubes (12 X 75 mm) and 1% BSA-PBS was added to bring the volume to 500 µl. Two hundred µl of diluted anti-bovine FSH serum, 100 µl of 125_{I} -labelled bFSH and 200 µl of diluted anti-RGG were added sequentially to the assay tubes at 24-h intervals. The contents of each test tube were mixed after addition of each reagent. The assay was incubated at 4°C. Seventy-two h after addition of anti-RGG, 3 ml of PBS was added to each test tube. The assay was then centrifuged at 2000 X g for 30 min. The supernatant fluid was discarded and pellets were counted in a gamma spectrometer.

V. Cross reactivity

Hormone specificity of binding of ¹²⁵I-labelled bFSH to antiserum was determined by incubating various amounts of highly purified bovine thyroid stimulating hormone (Dr. J.G. Pierce, University of California at Los Angeles) highly purified bovine LH (Dr. L.E. Reichert, Albany Medical College, NY) and highly purified bFSH (Dr. K.W. Cheng), NIH-growth hormone-B18, NIH-prolactin-B5 and USDA-FSH-B1 (Fig. 15). Cross reaction was calculated as follows:

amount of USDA-FSH-Bl required to reduce binding of 125I-FSH to 50% X 100% amount of other pituitary hormone required to reduce binding of 125I-FSH to 50%

Cross reaction of the anti-bovine FSH serum with bovine prolactin and growth hormone was <0.5% while cross reaction with highly purified bovine thyroid stimulating hormone and LH were approximately 2.6%. Highly purified bFSH was about 29 times more potent than USDA-FSH-B1 in displacing bound $125_{I-labelled}$ FSH from antibovine FSH serum. fig. 15. Cross reaction of anti-bovine FSH serum with various pituitary hormone preparations.

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Displacement of ¹²⁵I-labelled FSH by increasing volumes of cow serum (day 3 of estrous cycle), ovariectomized heifer serum and media from cultures of pituitary explants (2 pools) was parallel to the standard curves (fig. 16).

IV. Recovery of FSH

Known quantities of FSH (NIH-FSH-B1) added to cow serum, ovariectomized heifer serum and culture medium were recovered (124.8%, 132.3% and 93.0%, respectively).

VII. Sensitivity of Variation of FSH assay

The least amount of FSH that can be significantly detected by the assay, i.e. significantly different from buffer control was 2.5 ng. Inter- and intra-assay coefficients of variation from three assays were 10.8% and 8.1%, respectively.

VIII. Quality Control

FSH concentrations in various volumes of bovine sera or culture medium were measured in each assay to ensure parallelism between inhibition curves by sera or media and USDA-FSH-B1. fig. 16. FSH standard curve and inhibition curves by bovine sera and media from bovine pituitary explant cultures.

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