The effect of a progesterone implant and GnRH infusion on blood LH and progesterone levels in postpartum cows

by Pi-Hsueh Shirley Li

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Animal Science
Montana State University

Abstract:
Six mature 15-16 days postpartum cows were selected for calving date, breed and weight and paired for experimental purposes. One individual of each pair served as a treated cow and the other as a control. All cows were nursing calves throughout the entire experiment. Three treated cows received a subcutaneous silastic progesterone implant at either 15 or 16 days postpartum. Six days later they were treated for 37 treatments with 50 ug synthetic gonadotrophinreleasing hormone (GnRH) in one ml of acidified saline at 2-hr intervals over a period of 72 hr via indwelling jugular catheters. Two hr after treatment 37, the progesterone implant was removed and thirty hr posttreatment 37 the treated cows received 500 ug of GnRH (treatment 38). The three control cows were infused with 1 ml of acidified saline at the same time interval. Rectal palpation of the ovaries was conducted periodically to assess changes in ovarian size and follicular growth. Blood samples were collected via jugular catheters, at times (30, 60, 90 and 120 min) following treatments 1, 2, 3, 4, 5, 13, 25, 37 and 38. Serum LH and progesterone levels were measured by radioimmunoassay.

Data were analyzed by the method of least squares. Serum LH concentrations were observed to peak between 30 and 60 min following treatments 1, 2, 3, 4, 13, 37 and 38 and dropped at 90 and 120 min posttreatment. Following treatments 5 and 25, serum LH concentration peaked at 90 min and dropped at 120 min. The time for maximum LH response during the experiment was found to be 150 min. The mean peak LH concentration of treated cows was 8.40±1.57 ng/ml compared with the mean basal level of 3.18+0.10 ng/ml for control cows ($P<0.05$).

The LH responses were significantly greater ($P<0.01$) following treatments 2 and 3 (18.88 and 12.70 ng/ml) than the mean serum LH concentration of the control cows. LH release appeared ($P<0.10$) to be cubical in response to GnRH infusions. Least squares progesterone mean in treated cows was 0.85±0.052 ng/ml compared with the mean of 0.25+0.017 ng/ml for control cows ($P>0.05$). LH and progesterone were not correlated significantly ($P<0.05$) during the 37 treatments ($r=0.064$), nor were they correlated significantly ($P>0.05$) during treatments I through 5 ($r=0.18$). Ovarian activity as determined by rectal palpation was increased in two of the three treated cows. The mean LH peak in response to the 500 ug GnRH infusion (treatment 38) was 7.72±0.60 ng/ml, however, none of the cows ovulated.

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Date Aug. 14, 1975

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by

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A thesis submitted in partial fulfillment
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ABSTRACT

Six mature 15-16 days postpartum cows were selected for calving date, breed and weight and paired for experimental purposes. One individual of each pair served as a treated cow and the other as a control. All cows were nursing calves throughout the entire experiment. Three treated cows received a subcutaneous silastic progesterone implant at either 15 or 16 days postpartum. Six days later they were treated for 37 treatments with 50 ug synthetic gonadotrophin-releasing hormone (GnRH) in one ml of acidified saline at 2-hr intervals over a period of 72 hr via indwelling jugular catheters. Two hr after treatment 37, the progesterone implant was removed and thirty hr post-treatment 37 the treated cows received 500 ug of GnRH (treatment 38). The three control cows were infused with 1 ml of acidified saline at the same time interval. Rectal palpation of the ovaries was conducted periodically to assess changes in ovarian size and follicular growth. Blood samples were collected via jugular catheters, at times (30, 60, 90 and 120 min) following treatments 1, 2, 3, 4, 5, 13, 25, 37 and 38. Serum LH and progesterone levels were measured by radioimmunoassay. Data were analyzed by the method of least squares. Serum LH concentrations were observed to peak between 30 and 60 min following treatments 1, 2, 3, 4, 13, 37 and 38 and dropped at 90 and 120 min post-treatment. Following treatments 5 and 25, serum LH concentration peaked at 90 min and dropped at 120 min. The time for maximum LH response during the experiment was found to be 150 min. The mean peak LH concentration of treated cows was 8.40 ± 1.57 ng/ml compared with the mean basal level of 3.18 ± 0.10 ng/ml for control cows ($P<0.05$). The LH responses were significantly greater ($P<0.01$) following treatments 2 and 3 (18.88 and 12.70 ng/ml) than the mean serum LH concentration of the control cows. LH release appeared ($P<0.10$) to be cubical in response to GnRH infusions. Least squares progesterone mean in treated cows was 0.85 ± 0.052 ng/ml compared with the mean of 0.25 ± 0.017 ng/ml for control cows ($P>0.05$). LH and progesterone were not correlated significantly ($P<0.05$) during the 37 treatments ($r=0.064$), nor were they correlated significantly ($P>0.05$) during treatments 1 through 5 ($r=0.18$). Ovarian activity as determined by rectal palpation was increased in two of the three treated cows. The mean LH peak in response to the 500 ug GnRH infusion (treatment 38) was 7.72 ± 0.60 ng/ml, however, none of the cows ovulated.

INTRODUCTION

Many factors influence the interval from parturition to first ovulation. For good reproductive performance the interval to a highly fertile ovulation must be 80 days or less, however, numerous studies with beef cattle have reported that about 25% of the cattle are not reproductively active by 80 days postpartum. The occurrence of ovulation without estrus is one form of early postpartum ovarian activity in some of the cows. The frequent absence of behavioral estrus in connection with early ovulations suggest that either the steroid hormone levels or the hypothalamic centers on which they act are abnormal, however, little is known about the endocrine physiology in postpartum cows. Treatments which would initiate estrous cycles early in the postpartum period would enhance reproductive performance. One of the hormones which may have some potential in stimulating postpartum ovarian activity is gonadotrophin-releasing hormone (GnRH).

The amino acid sequence of GnRH isolated from porcine hypothalamic tissue was determined in 1971 as (pyro) Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly (NH_2), and this decapeptide was then artificially synthesized. Treatment with synthetic GnRH has resulted in an increased release of FSH and LH in various species of animals. The half-life of GnRH has been found to be between 4 to 7 min. It seems reasonable to assume that if GnRH could be released to stimulate the release of gonadotrophins over a longer period of time more changes in ovarian morphology would result and ovulation would be induced.

The objectives of this study were to 1) evaluate the effect of repeated infusion of GnRH over an extended period of time on serum LH concentration and ovarian changes and 2) determine the effect of prolonged exposure to progesterone implant on pituitary responsiveness to GnRH.

LITERATURE REVIEW

Postpartum Endocrine Physiology in the Cow

Postpartum interval. Postpartum interval refers to the interval beginning with parturition and ending with a designated event, such as first estrus, first ovulation, first breeding, conception or complete involution of the uterus. First ovulation refers to the first ovulation postpartum, whether or not it is accompanied by estrus. First estrus refers to those cases which are also accompanied by first ovulations (Casida, 1968). There is a wide variation in the reported time (32 to 78 days) from parturition to first estrus (Casida and Venzke, 1936; Shannon et al., 1952; Olds and Seath, 1953; Menge et al., 1962). First ovulation after calving has been reported at 14 days (Wagner and Hansel, 1969), 15 days (Morrow et al., 1966), 19 days (Menge et al., 1962) and 35 days postpartum (Casida and Wisnicky, 1950). The first estrus did not coincide with first ovulation. This discrepancy was probably due to the occurrence of ovulations without estrus (Menge et al., 1962). On the basis of rectal palpations and clinical observations, the time period needed for uterine involution to occur has been reported to be from 25 (Morrow et al., 1966) to 50 days (Buch et al., 1955). Studies based on histologic criteria suggest a parturition to involution interval of 25 to 30 days for most animals (Gier and Marion, 1968; Wagner and Hansel, 1969). Wilbank and Cook (1958) reported that nursing calves delays the return to postpartum cyclic activity and depresses fertility. The onset of post-parturient ovarian activity and ovulation does not appear to be directly related to the rate of uterine involution

(Buch et al., 1955), although some conditions of the uterus can delay involution and the recommencement of estral cyclicity (Morrow et al., 1966). There are some factors, such as suckling (Wiltbank and Cook, 1958), nutrition (Wagner and Oxenreider, 1971) and season (Buch et al., 1955), reported to effect the interval from parturition to first estrus.

Exogenous hormone therapy. Numerous experiments have been performed to alter postpartum physiology with exogenous steroid hormones. A wide variety of postpartum hormone treatments, with only a few exceptions, have been ineffective in shortening postpartum intervals.

Casida and Wisnicky (1950) did not find significant effect on postpartum intervals by injecting Holstein cows with 20 mg of diethylstilbestrol dipropionate within 9 hours after calving. Work by Foote et al. (1960) indicated that a single injection of progesterone suspended in carboxymethyl cellulose 14 days postpartum tended to delay both estrus and ovulation in postpartum beef cows. However, Foote and Hunter (1964) treated postpartum beef cows with progesterone and estrogen or estrogen alone and found that the average intervals from calving to uterine involution, estrus and ovulation were significantly decreased.

Saiduddin et al. (1968) obtained shorter postpartum intervals to estrus, ovulation and conception in response to exogenous progesterone and estradiol at various stages postpartum in beef cows. Brown et al. (1972) found that progesterone feeding followed by estrogen administration had a greater effect in reducing the interval lengths to first

estrus, first ovulation and conception, especially during the early postpartum intervals. Maher et al. (1973) reported that human chorionic gonadotrophin treatment 27 to 34 hr after removal of progesterone implant in beef cows may decrease the time from calving to estrus and ovulation.

Pituitary levels of gonadotrophin in the postpartum cows. The data on pituitary gonadotrophin levels in the postpartum cows in many reports may be summarized by stating that luteinizing hormone (LH) levels generally rise after parturition while follicular stimulating hormone (FSH) is usually at a peak level at parturition and declines following parturition.

Labhsetwar et al. (1964) found that FSH was highest at calving and decreased after parturition. They also found that follicular development was greatest 21 days postpartum when pituitary FSH was lowest.

Saiduddin and Foote (1964) found an increase in pituitary LH from day 5 to day 30 postpartum in suckled cows by utilizing the ovarian ascorbic acid depletion (OAAD) assay. Saiduddin et al. (1966) studied the effects of suckling on pituitary LH activity during the first 30 days postpartum and found that pituitary LH content was increased at days 10, 20 and 30 postpartum when compared to levels found at parturition.

Quenedo et al. (1967) found that the pituitary LH concentration was 9.0 ug/mg of dry anterior pituitary in beef cattle at 20 days after

calving. Wagner, Saatman and Hansel (1969) reported that pituitary LH concentration at 7 and 30 days postpartum in milked cows was 2.07 ug/mg of fresh anterior pituitary and 2.62 ug/mg, respectively. No effect of suckling on pituitary LH content was found.

The results of Labhsetwar et al. (1964), Saiduddin and Foote (1964), Saiduddin et al. (1966), Quenedo et al. (1967) and Wagner et al. (1969) may be interpreted as a pituitary accumulation of LH preparatory for ovulation and a continual more gradual release of FSH to stimulate ovarian follicular development in preparation for ovulation.

Blood levels of LH and prolactin in the postpartum cows. Ingalls, Hafs and Oxender (1971) measured serum LH and prolactin in heifers from 30 days before parturition until first estrus postpartum. They found that serum prolactin concentration was from 50 to 100 ng/ml until 2 days before parturition, exceeded 200 ng/ml during the 2 days before parturition, began declining at parturition and reached a level of about 60 ng/ml at 60 hr postpartum, and ranged from 50 to 100 ng/ml thereafter. LH in blood serum did not change measurably.

Arije, Wiltbank and Hopwood (1971) observed LH and prolactin levels in cows from 3 to 4 weeks prepartum to the second postpartum estrus and reported that LH levels were less than 1 ng/ml before parturition and remained between 1 and 1.5 ng/ml during postpartum interval with periodic peaks of up to 3 ng/ml after 2 weeks postpartum. Prolactin levels were below 50 ng/ml during late gestation, and increased to above 300 ng/ml from 2 days before parturition to 20 days postpartum. In

the remainder of the postpartum period, prolactin levels remained between 100 to 200 ng/ml.

Hypothalamic Control of Gonadotrophins

Hypophysiotropic hormones. The hypothalamus is known to contain releasing hormones. It controls the secretion of LH and FSH from the anterior pituitary gland (Guillemin, 1964). This control is mediated by neurohumoral substance(s) designated LH-releasing hormone (LH-RH) and FSH-releasing hormone (FSH-RH) (Schally et al., 1968) instead of LH-releasing factor (LRF) and FSH-releasing factor (FRF). LH- and FSH-releasing activity can be extracted from the hypothalamus.

Campbell, Feuer and Harris (1964) reported that intrapituitary infusion of crude extracts of median eminence induced a high percentage of ovulatory response in rabbits. Nikitovitch-Winer (1962) demonstrated ovulation in the rat by intrapituitary injection of crude bovine median eminence extract. McCann (1962) observed an ovarian ascorbic acid depletion on intravenous injection of crude acidic extracts of rat stalk median eminence tissue into immature rats pretreated with gonadotrophins. Although these experiments indicated that the extracts of median eminence from various species contained a mediator for release of ovulation hormone, little was known about the chemical nature of this mediator.

Fawcett, Reed, Charlton and Harris (1967) reported that partially purified LRF obtained from beef hypothalami induced ovulation when injected into the pituitaries of estrus rabbits. Schally and Bowers

(1964) obtained highly purified LRF from bovine hypothalamus. These preparations were highly active in depleting ovarian ascorbic acid in immature female rats, in inducing a rise of plasma LH in ovariectomized estrogen-progesterone treated rats and in stimulating the release of LH into the incubation medium from isolated rat pituitary *in vitro*.

An FSH-releasing action of hypothalamic extracts were demonstrated shortly after the discovery of LRF. These extracts were found to elevate plasma FSH in spayed female rats in which the release of FSH had been inhibited either by the injection of estrogen and progesterone or by lesions in the median eminence, and a dose-response relationship was obtained (Dhariwal *et al.*, 1965; Igarashi and McCann, 1964, Igarashi, Nallar and McCann, 1964). Several laboratories reported a depletion of pituitary FSH after administration of hypothalamic extracts in castrated, steroids-blocked rats (Kuroshiman *et al.*, 1966; Saito *et al.*, 1967). FRF also increased FSH release into incubated medium from pituitary *in vitro* (Kuroshiman *et al.*, 1965; Mittler and Meites, 1964; Watanabe and McCann, 1968).

LH-RH has been isolated from porcine hypothalami and physiological studies indicated that it causes release of both FSH and LH (Schally *et al.*, 1971). The isolated porcine LH-RH showed FSH-RH activity in ng doses. In various chromatographic systems no FSH-RH activity could be detected in hypothalamic fractions except those corresponding to LH-RH (Schally *et al.*, 1971). Schally *et al.* (1971) then suggested that one single hypothalamic hormone, designated FSH-RH/LH-RH may control the

release of both FSH and LH. Gonadotrophin-releasing hormone (GnRH) will be used for convenience in later section of this review to describe the action of this hormone.

Recently, the structure of GnRH has been determined as a decapeptide (Matsuo et al., 1971a; Baba et al., 1971) and the decapeptide has been artificially synthesized (Matsuo et al., 1971b). The synthetic decapeptide was found to possess the same biological properties as the natural pure LH-RH.

Anatomical organization of the regulatory system of anterior pituitary secretion. The hypothalamic hormones are secreted by neurons in the basal hypothalamus and pass to the pituitary via the hypothalamic-hypophyseal vessels to exert their effects on specific cell types (Gay, 1972). Long and short portal vessels serve as the method of communication between hypothalamic neurons and hormone secreting cells of the anterior pituitary gland. Releasing factors may be secreted on or near the capillary network of the portal vessels and be rapidly transported to the cells of the anterior pituitary gland (Gay, 1972).

Neural mechanisms controlling gonadtrophin secretion. The pituitary gonadotrophin secretion may have dual neural control which appears to be different between the rhesus monkey and the rat. Little is known about the neural mechanisms controlling gonadotrophin secretion in most species. However, in the rhesus monkey, the control systems which govern basic and surge secretion of gonadotrophins are resident within

the medial basal hypothalamus (Knobil, 1974). While in the rat, the first level of control is the tonic secretion of gonadotrophin, situated in hypophysiotropic area of the hypothalamus (Halasz et al., 1962), which stimulates the production of FSH and LH is able to maintain ovarian follicular growth and estrogen secretion (as reviewed by Flerko, 1966). The hypophysiotrophic area includes the whole of the arcuate nucleus, the ventral part of the anterior periventricular nucleus and the parvecellular region in the retro-chiasmatic area (Halasz et al., 1962).

The other is the "release regulating mechanism", located in the preoptic-suprachiasmatic area of the hypothalamus. This neural mechanism triggers the burst of LH secretion responsible for ovulation. In the absence of this mechanism, the hypophysiotropic structures still function normally and gonadotrophins are secreted at a basal rate which evokes the constant vaginal estrus syndrome (as reviewed by Flerko, 1966).

This concept is further demonstrated by Halasz (1968) and by Schneider et al. (1969). Surgical transection of the anterior hypothalamus abolishes the cyclic release of LH in the female but not the basal secretion of LH in either sex (Halasz, 1968). It has been proposed that the preovulatory surge of LH is controlled by one group of neurons, while the basal secretion in both sexes is regulated by other neurons (Gay, 1972). Schneider, Crighton and McCann (1969) studied the effect of electrical lesions in the suprachiasmatic region on the

content of LRF stored in the stalk-median eminence and reported that the LRF concentration in the stalk-median eminence of rats with suprachiasmatic lesions induced constant vaginal estrus. Hypothalamic LRF concentrations were significantly lower than in spayed rats without lesions. They concluded that LRF-secreting neurons may be located in the suprachiasmatic area and be responsible for the discharge of ovulatory amounts of LH.

Pituitary secretions of gonadotrophins are controlled directly by hypothalamic secretion of GnRH. The hypothalamus is stimulated by estrogens to release GnRH (Gay, 1972). It has been known that increasing levels of serum estrogen are associated with the preovulatory release of LH in diestrous ewes (Goding et al., 1969) and cycling cows (Christensen et al., 1971). Kato and Villee (1967) studied the pattern of distribution of labeled estradiol in ovariectomized rats and found that radioactive estradiol was incorporated into the anterior hypothalamus. Their findings suggest that the anterior hypothalamus is a direct target tissue of estradiol. The autoradiographic results of Pfaff (1968) that the anterior portion of the hypothalamus concentrates estradiol support the conclusion of Kato and Villee.

Antagonism Between Prolactin and Gonadotrophin Production

The high levels of prolactin in early lactation were thought to be related to postpartum anestrus (Han, 1973). Release of FSH and LH is inhibited by the milking stimulus, and the ovaries are relatively inactive except in the rat and mouse (as reviewed by McCann and Porter,

1969). Considerable evidence exists for a reciprocal relationship of hypothalamic control of gonadotrophin and prolactin secretion. The evidence is, 1) when endogenous prolactin secretion is suppressed by median eminence implants of prolactin through autofeedback mechanism, the pituitary response by increasing gonadotrophin secretion, 2) the intensity of the suckling stimulus from the nursing young responsible for gonadotrophin suppression is also responsible for increased prolactin secretion, 3) when the pituitary responds to a prolactin stimulator (perphenazine) and gonadotrophin suppressor (methallibure), serum prolactin is increased, 4) histological changes exist for a reduction in size and number of secretory granules in pituitary secreting cells and an increase in size of gonadotrophs in response to prolactin producing pituitary tumors (MtT-WID pituitary tumors) and 5) various neurotransmitters when injected into the third ventricle of the brain produce inverse effects on the secretion of gonadotrophins and prolactin (as reviewed by Han, 1973).

Gonadotrophin-Releasing Hormone (GnRH)

Recently the amino acid sequence of GnRH has been established. This molecule has been synthesized and the synthetic hormone is available for investigation in farm animals. The following review will be confined to the chemistry and physiological function of synthetic GnRH.

Chemistry. Two laboratories independently reported isolation of porcine (Schally et al., 1971) and ovine (Amoss et al., 1971) GnRH.

Matsuo et al. (1971a) identified the peptide structure of porcine GnRH as (pyro) Glu-His-Trp-Ser-Try-Gly-Leu-Arg-Pro-Gly(NH_2), by the use of the combined Edman-dansyl procedure coupled with the selective tritiation method of C-terminal analysis. The N-terminal groups were identified by dansylation. The decapeptide was then synthesized by the solid phase method (Matsuo et al., 1971b) and shown to possess the same biological properties as the natural pure porcine (Schally et al., 1972) GnRH, when tested in laboratory animals.

Physiology. GnRH causes release of LH in sheep, cattle and pigs. Domanski and Kochman (1968) demonstrated that intra-anterior pituitary infusion of eight to ten hypothalamic equivalents were effective in inducing ovulation in ewes during late anestrus but not during mid anestrus.

Gay, Niswender and Midgley (1970) found an increase in serum LH in the anestrous ewes with repeated injections of two hypothalamic equivalents, but no ovulation occurred. When a total dose of 400 ug synthetic GnRH was administered intramuscularly in two equal injections to late anestrous ewes, serum LH levels were increased to 39.1$\pm$6.8 ng/ml (Reeves et al., 1972). This LH level could be comparable to the pre-ovulatory surge (25 to 30 ng/ml) (as reviewed by Convey, 1973). Apparently, the sensitivity of the pituitary to GnRH is increased in late anestrous ewes. In contrast, Reeves, Tarnavsky and Chakraborty (1974) treated ewes with synthetic GnRH at three periods of anestrus.

They found no difference of pituitary responsiveness to GnRH at the three stages of anestrus (early, mid and late).

Reeves, Arimura and Schally (1970) studied the effects of different doses (1, 3, 9 and 27 ug) of purified porcine GnRH administered intracarotidly to diestrous ewes, wethers and a ram. They found a significant increase in serum LH in each animal after administration of GnRH at all dosages. The maximum LH response in the ewes and ram was 3 ug of GnRH. Greater doses (9 and 27 ug) of GnRH could not induce higher levels of serum LH in the ewe or the ram. However, wethers responded to increasing doses of purified GnRH with a peak of 160 ng/ml after 27 ug administration with a linear increase. It appeared that the pituitary is more responsive to GnRH in the castrate than in the intact sheep.

Golter et al. (1973) studied the dose response relationship of changes in serum LH in response to synthetic GnRH in bulls and the effects of various carrying media on this response. The results were summarized in tables 1 and 2, respectively.

The results obtained seemed to show a dose related increase in serum LH and a significant influence on the LH response to the synthetic decapeptide depending on the carrying media used.

TABLE 1. SERUM LH RESPONSE TO SYNTHETIC LH-RH/FSH-RH IN BULLS ***

LH-RH/FSH-RH[a] ug/kg body weight	Peak serum LH (ng/ml)[b]	Time to peak serum LH (min)	Length of LH serum response (min)
0.0	1.9±0.4[c]	--	--
0.03	2.7±0.4	30±6	55±15
0.3	14.8±2.1*	40±0	220±12
3.0	46.2±7.0**	137±15	366±33

[a] Pure synthetic LH-RH/FSH-RH (Hoechst).
[b] NIH-LH-B7 standard.
[c] Mean ± SE.
* Different from control $P < .05$.
** Different from control $P < .01$.
*** From Golter et al. (1973).

TABLE 2. BOVINE SERUM LH RESPONSES TO SYNTHETIC LH-RH/FSH-RH[a] IN VARIOUS CARRYING MEDIA**

Injection media	Peak serum LH (ng/ml)[b]	Time to peak serum LH (min)	Length of LH serum response (min)
Acidified saline	41±10[c]	130±13	285±40
2% CMC	55±10	180±23	600±92*
Silastic 732 RTV	4±4*	150±121	--

[a] Containing 1.3 ug LH-RH/FSH-RH (Hoechst)/kg body weight.
[b] NIH-LH-B7 standard.
[c] Mean ± SE.
* Different from acidified saline $P < .05$.
** From Golter et al. (1973).

Zolman et al. (1973) demonstrated a linear increase in LH response with increasing levels of GnRH in bulls and a quadratic increase in luteal phase heifers. They reported that the nature of the bovine LH response to GnRH differs between sex. The male bovine pituitary is more responsive to GnRH than that of females. Zolman et al. (1973) also observed a dose related increase in LH release in vitro when bovine pituitary tissue was exposed to 1 or 4 ng purified porcine GnRH, suggesting that GnRH acts directly on the pituitary to cause LH release.

Classical physiochemical methods of LH-RH purification have failed to separate LH-RH and FSH-RH activities. Schally et al. (1972) thus suggested that the isolated FSH and LH-releasing hormone controls the secretion of both FSH and LH from pituitary. Redding et al. (1972) have reported that synthetic GnRH in the rat stimulates not only the release of LH but also FSH in vitro. Reeves et al. (1972) described changes of serum FSH after GnRH treatment in sheep. Results of Arimura et al. (1972) indicate that synthetic LH-RH can stimulate a considerable release of FSH as well as LH in vivo, during prolonged infusions. This report supports the concept that LH-RH decapeptide also has FSH-RH activity. Arimura et al. (1973) also observed an FSH response to GnRH in humans. Kaltenbach et al. (1974) showed the effect of synthetic GnRH on FSH release in heifers.

In contrast, several researchers suggest that separate hypothalamic hormones release FSH and LH (Johansson et al., 1972; Bowers et al., 1973). Johansson et al. (1972) incubated the homogenates of porcine hypothalamic tissue in the presence of ^{14}C-glutamic acid and ^{14}C-glutamine which allow the biosynthesis of hypothalamic hormones and fractionated this material from above biosynthesis. They obtained two different groups of fractions each of which showed activities for the release of both FSH and LH. On the basis of the ratio of the levels of FSH and LH which were released, one group indicated the presence of the known decapeptide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 and the other group revealed the exceptionally high release of FSH. If this latter fraction was further fractionated by partition chromatography using Sephadex G-25 and the resulting samples assayed in vitro by using female rat pituitary, the ratio for FSH/LH release was greater than that observed with synthetic LH-RH. This observation indicates that the decapeptide reported by Matsuo et al. (1971a) is only LH-RH. A partially purified fraction of FSH-RH having low LH-RH activity from porcine hypothalamus released a greater amount of FSH than the LH-RH decapeptide (Bowers et al., 1973). Because the relative FSH/LH releasing ratio is greater than that of the LH-RH decapeptide, Bowers et al. then concluded that the LH-RH decapeptide is only the physiological regulator of LH, and FSH-RH is a separate hypothalamic releasing hormone.

Apparently, more biological and chemical evidences are needed to determine whether one or two hypothalamic hormones are responsible for the release of FSH and LH.

Most of the experimental studies with GnRH performed *in vivo* are based only on acute experiments. An injection of GnRH induced a considerable rise of serum LH associated with a very slight increase in serum FSH (Arimura *et al.*, 1972; Rennels *et al.*, 1971). However, GnRH can "stimulate a considerable release of FSH as well as of LH *in vivo* if given by prolonged infusion" (Arimura *et al.*, 1972).

Evans and Nikitovitch-Winer (1969) demonstrated that chronic administration of crude sheep hypothalamic extracts could reactivate the pituitary graft. The trophic action of such extracts could be due to a complex of the treatment, since crude hypothalamic extracts seemed to contain various hypophysiotropic hormones and other substance.

Arimura *et al.* (1973) observed a significant stimulation of FSH cells of the pituitary grafts and a lesser change in LH cells in hypophysectomized female rats bearing pituitary grafts. They also found large Graafian follicles in GnRH treated animals and higher levels of circulating estrogens. This estrogen level was not great enough to induce vaginal cornification. Thus, prolonged exposure might be essential for full stimulation of FSH release by the GnRH decapeptide.

The pituitary gland in the immature pig also responds to repeated stimulation with exogenous synthetic GnRH by releasing LH into circulation (Chakraborty *et al.*, 1973).

During the normal estrous cycle, regression of the corpus luteum and fall in progesterone is followed by an increase in serum estrogen concentration in the cow (Henricks et al., 1972; Wettemann et al., 1972) and ewe (Moore et al., 1968; Obst et al., 1971). Soon after the peak or at the peak of serum estrogen, serum LH increased in the cow (Wettemann et al., 1972) and ewe (McCracken et al., 1971; Scaramuzzi et al., 1971). It has been known that 17_{β}-estradiol causes the preovulatory surge of LH secretion in the sheep (Goding et al., 1969; Beck and Reeves, 1973). Jonas et al. (1973) noted an increase in both FSH and LH after infusion of 17_{β}-estradiol into anestrous ewes. Ying, Fang andGreep (1971) also noted an increase in both serum FSH and LH levels in the intact immature female rats when a single injection of estradiol benzoate was given. The peak serum FSH and LH concentrations of individual estradiol treated ewes were similar to preovulatory surges of FSH and LH concentrations reported by Goding et al. (1969) and Reeves et al. (1971). The isolated GnRH was reported to induce an increase in both serum FSH and LH in the ewe (Reeves et al., 1972; Jonas et al., 1973). Since estradiol also induces an increase in both serum FSH and LH in the anestrous ewe (Reeves, Beck and Nett, 1974), this study supports the suggestion by Jonas et al. (1973) that estradiol acts via the hypothalamic decapeptide.

The estrogen-induced LH surge can be blocked by prior administration of progesterone (Cumming et al., 1971; Scaramuzzi et al., 1971) or by endogenous progesterone (Bolt et al., 1971; Cumming et al., 1971).

Hilliard et al. (1971) reported that intrapituitary infusion of LH-RH in rabbits could cause LH release from the pituitary and this release could be blocked by pretreatment with progesterone. However, this blocking effect of progesterone was not observed by Cumming et al. (1972), when the dose of progesterone was in the higher range of normal secretion for the luteal phase in the ewe, and the dose of GnRH was submaximal. It appeared that physiological amount of progesterone could be unable to prevent GnRH induced pituitary LH release. But the suppression of ovulation by progesterone could be overcome by raising the dose of GnRH (Hilliard et al., 1971). Debeljuk et al. (1972) found that 5 mg of progesterone was not able to suppress the effect of 1 ug purified GnRH on the rat pituitary gland, but it was effective in suppressing the response to lower doses of GnRH. Thus, the dose of GnRH is a very important factor in evaluating the suppressive activity of progesterone.

The first report to demonstrate that synthetic GnRH induces LH release in postpartum lactating cows was Britt, Kittock and Harrison (1974). They treated lactating Holstein cows with subcutaneous-implants containing 100 ug of GnRH on day 14 postpartum. Serum LH increased from 1.9 ± 0.4 ng/ml at GnRH treatment to 15.0 ± 1.9 ng/ml at 4 hr and declined to 4.7 ± 0.6 ng/ml at 6 hr. All treated cows ovulated on day 15 without estrous behavior, but progesterone response after GnRH seemed to be similar to that observed during normal estrous cycle. The interval to

first ovulation in GnRH treated cows was shorter than for saline treated control cows based on palpation per rectum, but the interval to first estrus was similar for both groups. This probably means that GnRH treatment can shorten the interval to first ovulation, but not the interval to first estrus. Therefore, GnRH may be a useful agent in stimulating ovarian cycles in the postpartum cows.

Radioimmunoassay of Protein Hormones

The principle of radioimmunoassay (RIA) is based on the immunological properties of foreign proteins. It is characteristic of protein hormones from a particular species to stimulate antibody formation when injected into a second species of animal. The antibody specifically binds to its hormone. RIA were first developed for insulin (Berson et al., 1956). This method depends upon highly specific reactions between a protein hormone and its antibody. The radiolabeled hormone molecules compete with the nonlabeled hormone molecules for the binding sites on the antibodies. Since only a small amount of antibody is present, its binding capacity for the hormone is limited and constant. Therefore, when increasing amounts of unlabeled hormone are added to the assay, the antibody binding sites are progressively saturated and the antibody can bind less of the radiolabeled hormone. In general, the more unlabeled hormone present in the serum sample, the less labeled hormone will be bound to the antibody and vice versa. The hormone bound to antibody is then separated from the unbound or free hormone by utilizing either electrophoresis, salt precipitation, gel

filtration, charcoal and silica, ion exchange resins, solid phase antibody methods and double antibody precipitation. After separating the bound hormone from the unbound hormone, the quantity of labeled hormone bound to the antibody can be measured. Quantitation of the unknowns is accomplished by comparing the radioactivity of the unknown samples with standard hormone preparations reacted under identical conditions (Wright and Taylor, 1967). The potential advantages of RIA over bioassay methods are their high sensitivity, specificity, simplicity and applicability to assay large numbers of samples at one time. However, it should be clear that what is being measured is immunological and not biological activity of the hormone, because immunological and biological specificities may reside at completely different sites on the hormone molecule. If the hormone remains intact, immunological and biological measurement may give identical results; if metabolic degradation of the hormone occurs, a given immunoassay may detect substances that are not biologically active (Berson and Yalow, 1964).

Midgley (1955) using human chorionic gonadotrophin (HCG) demonstrated a RIA for measuring human LH. Anti-HCG antiserum was obtained by repeated subcutaneous injection of rabbits with partially purified HCG emulsified in complete Freund's adjuvant. HCG was iodinated with ^{131}I by the method of Greenwood, Hunter and Glover (1963). This method utilized chloramine-T to oxidize iodide to iodine, which then reacts with tyrosine residues in HCG. Sodium metabisulfite was used to stop

the reaction by reducing the excess iodine to iodide. Anti-rabbit gamma globulin (anti-RGG) was used as a second antibody to bind anti-HCG-bound complex. The precipitate was then separated from unbound hormone by centrifugation and counted in a gamma counter.

Radioimmunoassay of Steroid Hormones

The principle and development of RIA was discussed in the above review section. Louis, Hafs and Seguin (1973) described a simple and rapid RIA for progesterone utilizing a ^{3}H-progesterone label. In this method, 100 ul of serum samples were extracted with benzene-hexane (1:2 V:V), then stored at -20C for one hour to freeze the aqueous portion. The organic solvent was poured off into scintillation vials and then assayed by RIA. The organic solvent was evaporated and 200 ul antiprogesterone were added. After 30 min of incubation at room temperature, 24,000 cpm ^{3}H-progesterone was added to each tube. The tubes were incubated at 4C for 4-20 hr. Five hundred ul of dextran-coated charcoal were used to separate free and antibody-bound progesterone. Antibody bound ^{3}H-progesterone in 0.5 ml of the supernatant were measured in a liquid scintillation spectrometer. The sensitivity of this assay was less than 25 pg progesterone.

Midgley, Niswender and Ram (1969) proposed a hapten-RIA to quantitate low molecular weight estrogenic steroids. In this method, any small molecular weight steroids can act as a hapten and become immunogenic when conjugated to a protein. When steroid hormone was

conjugated to bovine serum albumin (BSA), antibodies against the steroid hormone could be obtained by immunization with steroid-BSA conjugates. The BSA portion of the conjugate was radioiodinated and used in combination with antibodies specific for the hapten. Abraham (1969) developed the first hapten RIA for estradiol-17_{β}. The solid phase method (Catt, 1967) was used to separate free and bound steroid. The sensitivity of this assay was 30 pg/ml plasma and recovery ranged from 30 to 60 percent.

Midgley et al. (1971) demonstrated the preparation of radioiodinated steroids. Steroid derivatives were conjugated to the methyl ester of tyrosine. The phenolic tyrosine ring was available for iodination. Iodine -125 was used as a label rather than ^{3}H because of the greater specific activity of ^{125}I and easier and less expensive sample preparation.

MATERIALS AND METHODS

Hormones. The synthetic GnRH was donated by Abbott Laboratories, Agriculture and Veterinary Products Division (North Chicago, Illinois). This substance was dissolved in physiological saline containing 0.01M acetic acid and stored at 4C between treatment periods. Silastic rubber implants (1.27 cm diameter by 14 cm length) impregnated with progesterone (Sigma Chemical Company, St. Louis, Missouri) were made by mixing the progesterone, silastic rubber and hardening agent together and pumping this mixture into a plastic hose. On the day of insertion, a 14 cm length was removed from the hose. Each progesterone implant was weighed at insertion and at removal to obtain the rate of release of progesterone from the implant.

Experimental animals. Hereford, Angus and Hereford x Holstein crossbred mature cows were selected from the Montana State University beef herd and paired as shown in table 3 for experimental purposes.

TABLE 3. COMPOSITION OF EXPERIMENTAL TRIALS

Trial No.	1	2	3
Treated	Cow 33	Cow 125	Cow 623
Control	Cow 929	Cow 018	Cow 609
Breed	Angus	Hereford x Holstein	Hereford

All pairs were selected for calving date, breed and weight. One individual in each trial served as a treated cow and the other as a control. All cows were nursing calves throughout the entire experiment.

Treated cows were surgically implanted with a removable subcutaneous silastic progesterone implant at either 15 or 16 days postpartum. Six days after the insertion of progesterone implant, the three cows were treated with synthetic GnRH by 37 consecutive infusions of 50 ug at 2 hr intervals over a period of 72 hr through the indwelling jugular catheters. Each infusion was considered as one treatment. Two hours after treatment 37, progesterone implant was removed. The treated cows then received 500 ug of GnRH 30 hr post-treatment 37 (treatment 38). Three control cows were similarly infused 1 ml of acidified saline at the same time interval and received no progesterone implant.

Prior to infusion of GnRH or acidified saline all cows were cannulated. Cannulation of the jugular vein was carried out by using a 12-guage thin-walled, hypodermic needle to puncture the vein. Silastic tubing (O.D. 0.085 cm, I.D. 0.040 cm) was then inserted into the jugular vein via the 12-gauge hypodermic needle. Female adapters were inserted into the silastic tubing and taped to the skin. All cannulas were heparinized and capped for later infusion and blood collection. The cannulated cows were confined at the Montana State University Beef Center in small lots.

Collection of blood samples. Blood samples were collected via jugular catheters. Pre-treatment blood samples were collected at -30 and -1 min before the first 50 ug/ml of GnRH treatment. Post-treatment

blood samples were collected at 30, 60, 90 and 120 min after treatments 1, 2, 3, 4, 5, 13, 25, 37 and 38. Blood samples were kept at 4C overnight, and the serum was separated by centrifugation and stored frozen until assayed for LH and progesterone.

Ovarian examination. Rectal palpation of the ovaries was conducted periodically to assess changes in ovarian size and follicular growth. The ovarian diameter and its related size is listed in table 4. Cows

TABLE 4. OVARIAN DIAMETER AND RELATED SIZE.

Diameter (mm)	7	8-12	13-17	18-22	23-17	28-32	33
Size	Very small	Small	Medium small	Medium	Medium large	Large	Very large

in both treated and control groups underwent a surgical observation of the ovaries conducted between 35 and 40 days postpartum to determine if ovulation had occurred.

LH assay. All serum samples were assayed by a double antibody radioimmunoassay (RIA) as described by Niswender et al. (1969) with slight modifications. The theory behind the RIA is the immunological response of the animal to induce antibody (the primary antibody) formation against the injected antigen (LH). The labeled LH molecules compete with the unlabeled LH molecules (either standards or unknowns) for binding sites on the antibodies. When increasing amounts of unlabeled LH are added to the assay, the limited binding sites of the antibody are progressively saturated and the antibody can bind less

of the labeled LH. A second antibody is used to separate the bound LH from the free LH so that the radioactivity of bound LH can be measured.

The anti-bovien LH serum (primary antibody, Rabbit antiserum, Lot No. 225) was kindly donated by Dr. Gordon D. Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins, Colorado. Ten ml of double distilled water were added to the lypholized anti-bovine LH serum. This solution was distributed into 10 serum bottles to avoid repeated freezing-thawing. The 1 ml aliquots of anti-bovine LH serum were snap frozen in dry ice and pentane, sealed with parafilm and stored at -10C. This antiserum against LH was diluted 1:400 in 0.05M EDTA-phosphate buffered NaCl (PBS, 0.01M) at pH 7.0 containing 1:10,000 merthiolate and frozen at this concentration. Prior to assay the anti-bovine LH serum was diluted with non-immune normal rabbit serum (NRS, Colorado Serum Co., Denver, Colo.) previously diluted 1:400 in 0.05M EDTA-PBS (at pH 7.0 with 1:10,000 merthiolate) to give the final working solution. The anti-bovine LH serum was used at a concentration of 1:12,500. Small aliquots for running one assay were frozen and stored as described above. The anti-bovine LH serum was thawed rapidly one day before assay and stored overnight at 4C. The diluted antiserum was stored in an ice bath during addition to tubes. All reagents for LH assay were kept at 0-4C by placing in containers of ice.

Sheep anti-rabbit gamma globulin (anti-RGG) was used as second antibody and prepared by repeated subcutaneous injection of RGG (fraction II, Pentex, Inc., Kankakee, Illinois) emulsified in complete Freund's adjuvant. Anti-RGG was diluted with PBS to a working concentration of 1:5 and stored at -10C.

Purified bovine LH (NIH-LH-B7) was used for the preparation of assay standards. Standards were prepared in PBS-1% egg white (EW) with concentrations equal to 0, .1, .2, .4, .8, 1.6, 3.2, 6.4, 12.8 and 25.6 ng/ml PBS-1% EW. Standard solutions in 2 ml aliquots corresponding to each point were prepared, snap frozen and stored at -10C.

Highly purified bovine LH (LER-1072-2) was obtained from Dr. L. Reichert (Department of Biochemistry, Emory University, Atlanta, Georgia) and radioiodinated at room temperature by a modification of the method of Greenwood et al. (1963). Twenty-five ul of 0.5M sodium phosphate buffer (pH 7.5) was added to 1 mCi ^{125}I (Na ^{125}I in 0.1 N NaOH, New England Nuclear, Boston, Mass.) in the V vial and transferred to the iodination vial containing 2.5 ug of the purified LH in distilled water (1 ug/ul). Thirty ug of Chloramine-T (2 ug Ch-T/ul 0.05M sodium phosphate buffer, pH 7.5) were added and the mixture was agitated for 2 min by finger tapping the vial. The reaction was stopped by adding 60 ug of sodium metabisulfite (2 ug $Na_2S_2O_5$/ul 0.05M $NaPO_4$, pH 7.5). One hundred ul of transfer solution containing 16% sucrose, 1 mg KI and 10 ug bromophenol blue were added and the mixture was layered

beneath the buffer on the surface of the BIO-Gel P-60 column (50-100 mesh, Bio Rad Labs, Richmond, Calif), previously coated with 1.5 ml of PBS-5% EW to reduce non-specific binding of protein. Seventy ul rinse solution containing 8% sucrose and 1 mg KI were used twice to rinse the vial and transfer to the column to increase the recovery of the LH-^{125}I. Twenty 1 ml aliquots of the column eluate were collected in tubes containing 1 ml of PBS-5% EW. Each tube was mixed carefully. One tenth ml was taken from each collection tube and counted for 0.1 min on an automatic gamma counter (Automatic Gamma Count System, Model 4233, Manufactured by Nuclear Chicago Co.) to find the peak tube suitable for use in the assay (figure 1). The contents of the peak tube were diluted with PBS-0.1% EW so that 100 ul gave 40-50,000 cpm (IX working solution). The IX working solution was distributed into small plastic bottles with the amount for each assay and stored at -10C.

In the assay, disposable culture tubes (12 x 75 mm) were used. Standard curves were run in triplicate. Samples from any given cow were assayed within a single assay in duplicate to reduce between-assay variation. Prior to assay, the tubes were placed in stainless steel test tube racks and stored at 0-4C. Two-hundred ul of standards or unknowns were added to tubes containing 450 ul of PBS-1% EW. Control tubes containing 1:400 NRS instead of antibody and 100 ul of LH-^{125}I were used to determine non-specific binding in standards or unknowns. Fifty ul of diluted anti-bovine LH serum were added to each

tube, and the contents were mixed and incubated at 4C for 24 hr before addition of 100 ul of LH-^{125}I. After another 24 hr period at 4C, 200 ul of anti-RGG were added to each tube to precipitate the antibody complex. After 72 hr of incubation at 4C, all tubes received 3 ml of cold PBS, except the total count tubes, and were centrifuged at 2000 rpm for 30 min (Model PR-2 Centrifuge, International Equipment Co., Boston, Mass.). The radioactivity remaining in the precipitate after decanting was measured in an automatic gamma counter for 1 minute. The standard curve (figure 2) was plotted on a three-cycle semi-log paper. Percent bound $\left(\frac{\text{cpm standard point conc.}}{\text{cpm zero point conc.}}\right)$ was plotted in the linear Y-axis and nanograms of LH were plotted in the logarithemic X-axis. The sensitivity of the assay was 0.1 ng of LH or 0.5 ng/ml when 200 ul of serum was assayed. The amounts of the unknowns present in the assay tubes were calculated from the standard curve. These values were corrected by a factor of the dilution of serum (200 ul) in the tubes containing the unknowns. The correction factor was 5.

Progesterone assay. Serum progesterone was measured by RIA. The antisera to progesterone-11α-bovine serum albumin and progesterone 6β-bovine serum albumin were supplied by Dr. Gordon D. Niswender and used at the final dilution of 1:3,000. Antiserum was diluted to 1:50 with 1:400 stripped NRS and stored at -10C. Further dilution of anti-progesterone serum was made with stripped 1:400 NRS. Stripped 1:400 NRS was prepared by adding 1 ml of NRS to 399 ml of buffer A, a 0.1M

sodium phosphate buffer (pH 7.0) containing 5.38 gm NaH_2PO_4, 16.35 gm Na_2HPO_4, 9 gm NaCl, 1 gm Na Azide and 1 gm Knox gelatin in 1 liter double distilled water to make a 1:400 NRS. Eight gm of ethanol washed Florisil for every 100 ml of 1:400 NRS were added, and the resulting mixture was placed on a magnetic stirrer for about 2 hours. Following the stirring, the supernatant was placed in 50 ml centrifuge tubes and centrifuged at 2000 rpm for 30 min, after which the supernatant was carefully decanted into bottles and stored at 4C until used.

Progesterone-1, 2, 6, 7-^{3}H (SA 105 Ci/mM) was obtained from New England Nuclear, Boston, Mass. Fifty ul of this substance were purified on a 3-inch Sephadex LH-20 (Pharmacia Find Chemicals, Inc., Piscataway, N.J.) column to remove free tritium. The eluting solvent was benzene: methanol (90:10 V:V). Approximately 0.5 ml were collected in each of 80 tubes. Ten ul from each tube were transferred to a scintillation vial. Fifteen ml of counting fluid (Bray's solution) were added to each vial. The vials were counted and the results plotted with cpm versus tube number. The content in the peak tube and the first descending tube were transferred to a 125 ml Nalgene bottle and dried down under a stream of nitrogen. Fifty ml of buffer A were added to the bottle containing the ^{3}H-progesterone and 100 ul was counted. This was the stock solution. From the stock solution, dilutions were prepared for the assay (12,000 cpm/200 ul) and the recovery 2,400 cpm/100 ul) solutions. All ^{3}H-progesterone solutions were stored at 4C until used.

Progesterone obtained from Sigma Chemical Company, St. Louis, MO, was used as standards without further purification. A stock solution was prepared and concentrations of 2560, 1280, 640, 160, 80, 40, 20, 10 pg per 100 ul were prepared by serial dilution with double distilled ethanol, and stored at 4C until needed.

Buffer A, buffer B and buffer A+ charcoal were used as working buffers in this assay system. Buffer A was prepared as described earlier. Buffer B was the same as buffer A, except it contained 5% Knox gelatin. Buffer A+ charcoal (dextran-coated charcoal) was prepared with 500 ml buffer A, 2.5 gm Dextran T-80 (No. 24751, Sigma Chemical Co., St. Louis, MO) and 1.25 gm charcoal "Novit a" (Matheson Coleman and Bell, Los Angeles, CA) with fines removed. The fines were removed by washing several times with double distilled water and aspirating after the charcoal settled for about 2 hours. The washed charcoal was dried in an oven at 100C.

The scintillation fluid was Bray's solution and prepared with 21 gm of 2,5-diphenyloxazale, 0.9 gm phenyloxazolylphenyl and 300 gm napthalene, which was dissolved in 3 liters of p-dioxane.

The first step in performing the RIA was the pre-extraction. One-half ml samples of either serum, lab standard or water blank (double distilled water) were added to 16 x 125 mm disposable culture tubes. One-hundred ul of ^{3}H-progesterone (app. 2,400 cpm) for recovery were added to each tube. The tubes were mixed and placed at 4C overnight.

In order to determine a value for total count (recovery), 100 ul of ^{3}H-progesterone for recovery were added to each of 4 scintillation vials which were capped until the end of assay when Bray's solution was added. Extraction tubes containing either serum, lab standard or water blank were removed from the refrigerator and 3 ml of benzene: hexane (1:2 V:V) were added to each tube. The tubes were mixed for 30 sec at #5 speed on a vortex mixer, and placed in the freezer for 30-60 minutes. After this time, the tubes were removed from the freezer and the solvent layer was poured into the corresponding tube containing 1 ml of double distilled water. These tubes were then mixed at #4 speed for a few seconds and placed in the freezer until the water layer was frozen. The solvent layer was poured off into a corresponding scintillation vial and all the steps above were repeated for the second extraction.

After the solvent from the second extraction was in the scintillation vials, the vials were completely dried under a stream of nitrogen, and 1 ml of double distilled ethanol (room temperature) was added to each vial. The vials were quickly capped to prevent evaporation and the inner walls were carefully rinsed with the 1 ml of ethanol.

Two 300 ul aliquots were taken from each scintillation vial by means of an Eppendorf pipette (Brickman Instruments, Inc., Westbury, N.Y.) and added to duplicate disposable tubes (12 x 75 mm). The contents in the tubes were dried under a stream of nitrogen, and the scintillation vials were air dried for recovery. These recovery vials were used

to determine the procedural losses. The amount of ^{3}H-progesterone for recovery extracted from the serum samples was determined as follows:

$$\text{Recovery} = \frac{\text{cpm of recovery vials}}{(\text{cpm of total count vials}) \times 0.4}$$

This value was used to later correct the amount of progesterone obtained from the unknowns in each extraction.

Duplicate standard curves were prepared by using a Micromedic Automatic Pipetor (Model 25004, Micromedic Systems, Inc., Horsham, PA) to aliquot 100 ul of each standard concentration to corresponding tubes. Each 100 ul aliquot was flushed from the pipetor with 200 ul of double distilled ethanol. Each standard curve was arranged before and after sample assay tubes. The unknown samples, lab standards, water blank, and standard curves were evaporated under nitrogen in a 45C sand bath.

Two-hundred ul of anti-progesterone anti-serum were added to each tube and incubated at room temperature for 30 minutes. After incubation, 200 ul of ^{3}H-progesterone for assay were added to each tube. The tubes were gently mixed, and incubated at 4C for 14 to 16 hours. Following the incubation period 100 ul of cold buffer B, 1 ml of cold buffer A+ charcoal were added to all tubes to remove the unbound ^{3}H-progesterone. The samples were then quickly mixed by shaking the racks, and incubated at 4C for 10 min, then centrifuged at 2500 rpm for 10 min in a refrigerated centrifuge. Three-hundred ul of the supernatant were transferred into counting vials. Fifteen ml of Bray's

solution were added to all counting vials which included the four total count vials for recovery, the recovery vials, the standard curve vials, the serum sample vials and the four empty vials (background). Counts per minute were determined during a 5 min counting interval in a Beckman liquid scintillation LS-100 system (Beckman Instruments, Inc., Salt Lake City, Utah). Total count samples were included to determine the amount of ^{3}H-progesterone added to each tube. These samples contained ethanol, anti-progesterone, tritiated progesterone for assay, and buffer A which was used instead of the buffer A+ charcoal suspension.

The raw data (cpm) from the counter printout tape was first corrected for background by subtracting the average background cpm from each value. The average percent bound for each standard point was calculated and plotted against the corresponding picogram level. By comparing the average standard curve to the percent bound values obtained from each assay tube, a value was obtained which was expressed in picograms per 0.3 ml of unknown serum. This value was then corrected by dividing it into the percent recovery for that sample to adjust for procedural losses. This particular value, corrected for recovery, was then corrected again by multiplying a factor of 6.66, because only 0.5 ml of serum was extracted, and only 0.3 ml of supernatant from each tube was counted. The final correction was done to express the answer as picogram per ml of serum.

Analysis of data. Least squares analysis of variance (Harvey, 1968) were conducted at the MSU Computing Center to analyze all data. Comparison of the mean peak serum LH concentrations were made by Student's t-test. Correlation coefficient was found by using Corrxy program in Ministat modified by Dr. R. E. Lund, Department of Mathematics, Montana State University.