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Title: INFLUENCE OF STEROIDS AND GONADOTROPIN RELEASING HORMONE ON SERUM LUTEINIZING HORMONE LEVELS IN PREPUBERTAL CASTRATE HOLSTEIN BULLS

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Factors involved in regulating the release of luteinizing hormone (LH) from the anterior pituitary were investigated using prepubertal Holstein bulls. These factors included the effects of castration, steroid replacement therapy and synthetic gonadotropin releasing hormone (GnRH) on serum LH concentrations.

In three castration studies involving eight bulls, 4 to 6 months of age, serum LH levels increased from less than 1 ng/ml before castration to 2.6 $\pm$ 0.82 ng/ml during a period 6 to 12 hr after castration and exhibited random fluctuations thereafter. It is concluded that serum LH levels increase when the pituitary-hypothalamic system is released from inhibition by testicular products (steroid or non-steroid).

Blood sampling at 5 (trial 1) and 10 (trial 2) minute intervals in two groups of steers demonstrated that serum LH fluctuations

were non-rhythmical, and the magnitude of fluctuations varied between studies (trial 1 - range 1.3 to 3.3 ng/ml; trial 2 - range 2.1 to 11.8 ng/ml).

Intravenous (iv) injection of androgens [androstenedione, testosterone and testosterone propionate (TP)] at dosages of 0.5 to 1024 μg/kg BW and intramuscular (im) injection of androgens (TP and dihydrotestosterone benzoate) at dosages of 0.5 to 16.0 μg of free steroid per kg BW failed to reduce serum LH levels or alter fluctuations. An im TP dosage of 100 mg (2 x daily for 3 days) to two steers reduced serum LH from 5.9 ± 0.55 ng/ml before treatment to 1.6 ± 0.13 ng/ml after treatment and reduced fluctuations. Intravenous injection of 17β-estradiol (0.5 and 2.0 μg/kg BW) suppressed serum LH levels from 2.7 ± 0.10 ng/ml before injection to 1.3 ± 0.13 ng/ml for a period 0.5 to 4.0 hr after injection and im injection of estradiol benzoate (dosages equivalent to 0.5 and 2.0 μg $E_2$/kg BW) decreased serum LH concentrations from 5.2 ± 0.42 ng/ml pre-treatment to 3.2 ± 0.18 for the 12 hr period after treatment. Intravenous injection of these estrogens reduced LH levels faster than im injection, but iv injection maintained suppression for a shorter time period.

Ten, 40, 80 and 160 μg of GnRH iv caused an increase in serum LH concentrations to greater than 30 ng/ml. The LH peak heights and LH peak areas (total LH discharge) were not related to dosage administered. A second study using 2.5, 5.0, 7.5 and 10 μg GnRH

produced LH peaks of 15.1, 25.2, 120.5 and 36.1 ng/ml, respectively, at 20 to 30 min after injection. Correlations between dosage and serum LH peak height or peak area were non-significant, but a significant dose-response was obtained by deleting the steer receiving 7.5 μg GnRH (peak height 120.5 ng/ml) from the analysis.

A final experiment was conducted to determine if the suppressive action of testosterone operates at a hypothalamic or pituitary site. Testosterone propionate (100 mg actual T) was injected twice daily for 4 days im into 4 Holstein steers and 5 μg GnRH (the minimum effective dosage) was administered before (day 1), during (day 5) and after (day 6) TP administration. TP reduced serum LH levels from 5.8 ± 0.90 ng/ml on day 1 to 2.4 ± 0.21 ng/ml on day 5 and 2.1 ± 0.06 ng/ml on day 6. Analysis by paired t-test showed no significant difference between GnRH-induced LH peak heights or LH peak areas between days 1 and 5 and days 1 and 6. Testosterone propionate did not markedly alter the sensitivity of the pituitary to GnRH. Thus it appears that testosterone acts at the hypothalamus to inhibit the discharge of luteinizing hormone-releasing hormone.

# Influence of Steroids and Gonadotropin Releasing Hormone on Serum Luteinizing Hormone Levels in Prepubertal Castrate Holstein Bulls

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## INTRODUCTION

Continual progress in increasing the efficiency of animal agriculture is essential if animal products are to remain a competitive protein source for the world's growing population. Improving efficiency by increasing the number of offspring born per female has been recognized and is currently under intensive investigation. However, the importance of the male in increasing production is more obscure. Selection programs have produced high quality sires and artificial insemination (AI) has facilitated matings of superior sires to many females. In this way, genes for potentially valuable production traits from select, proven sires can be transmitted to a greater number of offspring to increase the rate of genetic progress. Proving sires from any species of farm animal is a slow and costly process, especially in the dairy business where lactation information from offspring of each sire must be evaluated. Any decrease in the time involved in proving sires could benefit the industry. If semen could be collected from bull calves soon after birth, matings required for proving a bull could be made by AI before bulls normally attained

puberty. Advancing puberty will require a more thorough understanding of pituitary-gonadal physiology of the male than is currently known.

Furthermore, knowledge gained from basic research on anterior pituitary-gonadal interrelationships in the male would be useful in correcting certain pathological conditions causing infertility in valuable boars, bulls, rams and stallions, or extending their effective breeding life.

Research in this thesis deals with the pituitary-gonadal system and attempts to define regulation of the anterior pituitary by steroid hormones in cattle. This is accomplished by monitoring pituitary function (by measuring serum LH levels by radioimmunoassay) after castration and after administration of steroids and gonadotropin releasing hormone.

# REVIEW OF LITERATURE

This review will discuss pituitary-gonadal relationships in the male with special emphasis given to farm animals. Research on this subject in males has been limited, especially in farm animals, due primarily to the high costs of large animal research. For this reason it is necessary to refer to investigations in humans and laboratory animals for a more complete picture of these relationships.

## Spermatogenesis

Pituitary participation in rat gonadal function was demonstrated as early as 1927 (Smith, 1927) and the components, follicle stimulating hormone (FSH) and luteinizing hormone (LH), soon discovered (Zondek, 1930), were proposed to control spermatogenesis and Leydig cell function, respectively (Greep, Fevold and Hisaw, 1936; Greep and Fevold, 1937).

Removal of the pituitary results in a reduction in spermatogenesis in rats and a complete loss of spermatogenesis in primates. Gonadotropin replacement therapy in hypophysectomized animals reveals that both FSH and LH, acting in synergism, are required for maintenance of testicular structure and function. Prolactin (PRL) and growth hormone (GH) have also been suggested to participate in spermatogenesis, and androgens, produced by the Leydig cells which

are stimulated by gonadotropins, are a major factor in sperm production. Although androgens have been shown to maintain gametogenesis, they cannot restore sperm output in hypophysectomized rats after sperm production has ceased (Steinburger, 1971).

Investigations of the components responsible for sperm production in bulls have revealed that the onset of spermatogensis is accompanied by pronounced alterations in the pituitary gonadotropins, FSH and LH (MacMillan and Hafs, 1968). Sperm are present in the testes at six months of age and approach adult concentrations by one year (Hafs, 1974). This increase in sperm production is accompanied by a decrease in pituitary FSH (Swanson et al., 1971), an increase in plasma LH (MacMillan and Hafs, 1968) and an increase in plasma testosterone (Rawlings, Hafs and Swanson, 1972). Swanson et al. (1971) suggested that the release of FSH (decreased pituitary FSH content) participates in spermatogenesis while Rawlings et al. (1972) concluded that increased plasma LH is related to the onset of testosterone secretion and spermatogenesis.

## Androgen Secretion

In mature animals the relationship of LH release to testosterone secretion has been demonstrated in the ram (Sanford, Howland and Palmer, 1973a; Sanford, Palmer and Howland, 1973b) and the bull (Katongle, Naftolin and Short, 1971). Three to five LH peaks occurred

randomly in rams within each 24-hr period and were followed by increases in serum testosterone from baseline levels of 0.3-1.5 ng/ml to 8.5 $\pm$ 0.9 ng/ml within 1 hr following each LH peak (Sanford et al., 1973b). Katongle et al. (1971) reported four to ten LH peaks in 24 hr in bulls and stated that each LH peak was followed by increases in testosterone from 2-4 ng/ml before the LH peak to 16-20 ng/ml after the LH peak. Following LH peaks of 23.8 ng/ml, induced by administration of synthetic gonadotropin releasing hormone in bulls, testosterone increased from 1.7 ng/ml to 5.2 ng/ml 2 hr after injection (Mongkonpunya et al., 1973). Furthermore, sexual stimulation in rams (Sanford et al., 1973a) and bulls (Katongle et al., 1971) results in immediate elevations in serum LH followed within 40 min by serum testosterone peaks.

Exogenous hormone treatment can also affect testosterone production. Intravenous injection of 500 IU of human chorionic gonadotropin (HCG), a compound high in LH activity, effected increases in testosterone at 0.5 and 2 hr post-injection (Katongle et al., 1971). Of the pituitary hormones, only LH augmented serum testosterone while PRL, thyroid stimulating hormone (TSH), GH and FSH did not significantly increase androgen output (Sequin and Hafs, 1973). Serum testosterone levels following LH plus PRL treatment were not significantly different than levels obtained after LH alone (Smith et al., 1973).

## Regulation of Gonadotropins

Extremely important in the regulation of all pituitary hormones, releasing hormones have been accepted as the primary link between neural influences and the hypophysis (Green and Harris, 1947). These peptides, produced by the hypothalamus and released in the median eminence, travel through the portal vasculature of the stalk median eminence and pituitary to regulate adenohypophysial functions. Releasing hormones are suggested to exist for all of the pituitary hormones (Meites, 1970); several have been isolated, sequenced and synthesized (Blackwell and Guillemin, 1973). Schally et al. (1971) speculated that FSH and LH may be regulated by a single releasing hormone, and later a decapeptide was isolated possessing both FSH and LH releasing activity from porcine (Schally et al., 1971a) and ovine hypothalami (Amoss et al., 1971). Matsuo et al. (1971b) determined the sequence of porcine luteinizing hormone-releasing hormone-follicle stimulating hormone-releasing hormone (LHRH-FSHRH), and Burgus et al. (1972) found the sequence of ovine LHRH-FSHRH to be identical.

The development of synthetic releasing hormones [i. e., gonadotropin-releasing hormone (GnRH) (Matsuo et al., 1971a), thyrotropin-releasing hormone (TRH) (Boler et al., 1969) and growth hormone-releasing hormone (GH-RH) (Veber et al., 1971) ] has

permitted intensive investigation of clinical and basic mechanisms of pituitary control.

Synthetic GnRH, found to have releasing activity similar to purified LHRH-FSHRH in rats (Schally et al., 1972), effects the release of LH in domestic animals and its use has been suggested for controlling fertility and treating infertility in farm animals (Convey, 1973). The release of LH after GnRH injection has been characterized in the bovine (Zolman et al., 1973), ovine (Arimura and Debeljuk, 1972), and porcine (Chakraborty et al., 1973). Further work in boars (Pomerantz et al., 1974), rams (Galloway, 1974) and bulls (Golter et al., 1973) indicated that small doses of GnRH are capable of producing surges in LH greater than those normally occurring during random fluctuations in the male and a dose-response was shown in each species. Both Golter et al. (1973), using intramuscular injections of 0.03, 0.3 and 3.0 μg/kg body weight (BW), and Zolman et al. (1973), using intravenous injections of 10, 40 and 160 μg of GnRH, showed that the peak serum LH levels in bulls and time to peak serum LH levels increased with increasing dose.

The influence of age upon LH release was investigated in pubertal bulls at 2, 4 and 6 months of age and no difference in response to 200, 400 or 800 μg GnRH given intramuscularly was observed (Mongkonpunya et al., 1973). In addition Pomerantz et al. (1974) observed no difference in response between adult and pubertal male

Gottingen miniature pigs given 2.0 μg/kg GnRH through indwelling intravenous cannulae.

Reeves, Arimura and Schally (1970) observed that castrate animals are markedly more sensitive to purified LH-RH preparations. Wethers given 27 ng purified LH-RH (AVS-77-3 No. 320-339) responded with increases in serum LH of 160 ng/ml whereas intact ewes and rams given an equivalent dose had increases in serum LH of only 7 to 14 ng/ml. That the same holds true for pigs was shown in intact and castrate miniature male pigs (Pomerantz et al., 1974).

The suggestion was made that the pituitary responsiveness to purified LH-RH changes during the estrous cycle as a result of alterations in circulating steroids (Reeves, Arimura and Schally, 1971a; Arimura et al., 1972; Zolman et al., 1973). Experimental evidence has supported this hypothesis; ewes were indeed more sensitive to LH-RH at estrus than at any other time (Reeves et al., 1971a).

Exogenous steroids can also influence the action of GnRH on the hypophysis. Exogenous estrogens augmented the LH release after GnRH in female rats (Arimura and Schally, 1971), ewes (Reeves, Arimura and Schally, 1971b) and women (Kastin et al., 1970). Progesterone administration causes a reduction in pituitary sensitivity to LH-RH in the rat and ewe (Debeljuk, Arimura and Schally, 1972a).

Steroids also alter the sensitivity of the pituitary in the male. In intact male rats, pretreatment with estradiol benzoate (EB), testosterone propionate (TP) or TP plus EB suppressed LH release after GnRH administration (Debeljuk, Arimura and Schally, 1972b). The action of progestagens, similar to that in the female, decreased the pituitary response to GnRH treatment (Debeljuk, Vilchez-Martinez and Arimura, 1973).

Similar manipulation of the steroid environment in large animals has been limited. In castrate rams, 600 mg TP caused a reduction in GnRH-induced serum LH peak heights as compared to animals given only the vehicle (corn oil) and GnRH (Pelletier, 1973).

## Feedback Regulation

The gonadotropins of the male are regulated by negative feedback of steroidal and non-steroidal testicular products on the hypothalamus or pituitary (Greep and Jones, 1950; Davidson, 1966). Basic proof that this system exists lies in changes in pituitary and plasma gonadotropins after castration. This phenomena was first reported in 1929 when increased pituitary gonadotropins were observed in male and female rats following castration (Engle, 1929; Evans and Simpson, 1929).

Following gonadectomy, elevated plasma FSH and LH values have been recorded in rats (Gay and Midgley, 1969), humans (Walsh,

Swerdloff and Odell, 1973) and rams (Pelletier, 1968; Crim and Geschwind, 1972) by use of radioimmunoassay. In contrast, MacDonald and Clegg (1966) observed no changes in serum LH as measured by the Parlow bioassay technique in rams after castration.

Post-castration serum gonadotropin changes as yet have not been thoroughly documented in bovine males, however, changes in pituitary FSH and LH concentrations were recently studied (Parlow, Bailey and Foote, 1973). In steers, 1 year after gonadectomy, FSH doubled and LH was halved as compared to intact bulls of comparable age and breed.

Androgens, produced by the Leydig cells, are the principal testicular products involved in maintaining normal gonadotropin levels in intact males (Gay and Dever, 1971). But suggestions that testosterone is not the sole testicular product involved in regulation of gonadotropins (Greep and Jones, 1950; Ramirez and McCann, 1965; Gay and Dever, 1971) has encouraged consideration of two other factors. These are estrogens, secreted by the Leydig cells (McCullagh and Shaffenburg, 1952) or Sertoli cells (Lacy, 1967), and "inhibin," a hypothetical product of the seminiferous tubules (Vidgoff et al., 1939; Swerdloff et al., 1971). Two factors provide evidence for the involvement of estrogens in the gonadotropin inhibiting activity of the testes: (1) estrogens are present in the testes and (2) estrogens

are extremely potent in suppressing FSH and LH in man (Walsh et al., 1973).

Evidence for involvement of "inhibin" includes the selective increase in FSH after pathologically or clinically induced destruction of the germinal epithelium (Walsh et al., 1973).

The influence of steroid administration on pituitary secretion in the male has been investigated using intact and castrate rams. The intact male is more sensitive to testosterone negative feedback than the castrate animal. A single intramuscular injection of 5 mg TP suppressed LH release in normal rams (Bolt, 1971) whereas 10 mg TP/45.5 kg BW daily for 1 wk (Crim and Geschwind, 1972) and 400 (Pelletier, 1970) or 600 mg TP (Pelletier, 1972) at one injection were used in castrates. The single 5 mg dose reduced serum LH for the entire 53-hr sample period and suppressed all random fluctuations characteristic of normal rams. Crim and Geschwind (1972) sampled serum only once each week so a profile of the testosterone-initiated suppression could not be obtained. The 400 mg dose of TP decreased LH for 5 to 7 days (Pelletier, 1970) and the 600 mg dose of TP caused a two-part reduction in LH at 12 hr and at 72 hr with a return to normal 24 to 48 hr post-injection.

Crim and Geschwind (1972) castrated rams at 30, 60, 90, 120 and 150 days of age, treated the rams with TP (10 mg/45.5 kg from 2 to 4 weeks post-castration) and observed equivalent reductions in

gonadotropins in all age groups. They concluded that the feedback system was fully functional in rams as early as 30 days of age.

The action of other steroids in the ram was examined by Bolt (1971). Estradiol (5 mg) and progesterone (100 mg) given separately by intramuscular injection and had effects indistinguishable from testosterone (i. e., decreased serum LH levels and reduced fluctuations).

The suppressive action of testicular products, steroidal or non-steroidal, may occur at the pituitary and/or higher centers in the brain. Early investigators found what seemed to be evidence for feedback at the hypothalamus or median eminence. Testosterone implanted into the median eminence caused testicular and accessory gland atrophy in rats (Lisk, 1962) and dogs (Davidson and Sawyer, 1961), indicating reduced stimulation of the Leydig cells by gonadotropins. Androgen implanted directly into the pituitary had no regressive effects on the gonads or accessory sex glands. Mapping the hypothalamic areas indicated that feedback centers were located in the medial basal hypothalamus (Lisk, 1962). Also, Mittler and Meites (1966) found that large doses of testosterone decreased FSH-RH activity in the hypothalamus in castrate male rats and Schally et al. (1967) determined that testosterone had no effect on LHRH-FSHRH action at the pituitary. However, recent work is in opposition to the concept of the hypothalamus being the sole feedback site, as

intrapituitary implants of testosterone exerted direct inhibition of pituitary gonadotropin release in male rats (Kingsley and Bogdanove, 1973).

# MATERIALS AND METHODS

## Experimental Design

The studies contained within this thesis are divided into four major experiments, each of which consist of a number of trials. In most cases the trials within an experiment follow in logical succession with each subsequent experiment relying on information gained in a preceding experiment. Considerable variation exists in the design of each trial depending on the animals available, treatments desired and goals pursued.

The many separate trials can be categorized into four experiments: (1) castration, (2) steroid replacement therapy, 3) gonadotropin releasing hormone treatment (GnRH) or (4) steroid replacement therapy plus GnRH. Experiments 2, 3 and 4 were carried out in animals which had been previously castrated a variable length of time before the experiments were conducted. In general, each trial entailed treatment, blood sample collection and quantitation of serum LH in the collected samples.

## Animals

Holstein bull calves, provided by the OSU Dairy Center, were used for these investigations. During the course of these studies,

calves were housed at the OSU Dairy Center and maintained by the OSU Dairy Center staff.

## Blood Collection

Five or 10 ml blood samples were collected by jugular venipuncture or by indwelling jugular cannulae. Samples collected by venipuncture were drawn with 10 ml disposable syringes fixed with 1 inch 20 ga needles. The blood was placed in 10 ml tubes and allowed to clot for 4 to 6 hr at room temperature.

After freeing the blood clot from the sides of the tube with a spatula, the samples were refrigerated and later centrifuged at 1110 x g in a refrigerated centrifuge (Ian Sorvall Inc., Norwalk, Connecticut) for 10 min at 4C. The serum was decanted into labeled storage vials and frozen until assayed.

In trials requiring frequent sampling, animals were fitted with one of two types of indwelling cannulae: (1) intramedic PE 60 tubing, I.D. 0.030 inch, O.D. 0.048 inch (Clay Adams, Parsippany, New Jersey) or (2) medical grade vinyl tubing, I.D. 0.058 inch, O.D. 0.080 inch (Becton, Dickinson and Company, Rutherford, New Jersey).

When inserting the cannula, the animal was restrained and the head turned back to permit easy access to the jugular vein. Before insertion, the tubing was disinfected in tincture of Zephiran chloride and the point of entrance on the neck was cleaned with 70% ethanol.

The jugular vein was located by palpation and engorged by retarding the venous flow posterior to the intended point of cannulation. A 1.5 inch 15 ga stainless steel needle for PE 60 or a 1.5 inch 12 ga stainless steel needle for vinyl tubing was used to puncture the vein. About 35 cm of a 100 cm length of tubing was passed through the needle and into the vein, after which the needle was removed. The cannula was affixed to the neck at the point of exit by adhesive tape and branding cement. An 8 x 15 cm length of adhesive tape, containing a short slit through which the cannula was first passed, was attached with branding cement to the neck with the adhesive side facing outward. Additional tape of the same size was applied directly over the initial piece with adhesive surface facing inward, leaving the cannula secured between two adhesive surfaces. The remaining tubing was attached at the withers with tape as before and a three-way Teflon stopcock (Pharmaseal Inc., Toa Alta, Puerto Rico) was connected to the distal ends of the tubing via a blunted needle (21 ga for PE 60, 16 ga for vinyl).

The cannulae were flushed with 3.5% sodium citrate-2% Liquamycin (Pfizer Inc., New York, N.Y.) between sampling to maintain patency of the catheters. At sampling times, 3 ml of fluid were withdrawn and discarded, 5 or 10 ml of blood were obtained, and 5 ml citrate-Liquamycin were infused to flush and fill the tubing. The blood was handled as described earlier.

## Injections

Two routes of administration, intravenously (iv) in the jugular vein or intramuscularly (im) in the rump or thigh, were used in these experiments. Those hormones given iv were infused via indwelling venous cannulae and flushed with 3 ml citrate-Liquamycin or injected with a disposable syringe and 18 ga x 1.5 inch needle. Intramuscular injections were given with a 20 ga x 1 inch needle with an appropriately sized disposable syringe. Two million units (10 cc) Combiotic (Pfizer Inc., New York, N. Y.) were injected im into each animal after each trial to prevent infection.

The drugs used in these studies were obtained from three sources: (1) Sigma Chemical Co. (St. Louis, Missouri), $\Delta^4$-androsten-17$\beta$-ol-3-one (testosterone), $\Delta^4$-androsten-17$\beta$-ol-3-one-propionate (testosterone propionate), $\Delta^4$-androsten-3, 17-dione (androstenedione), 5$\alpha$-androstan-17$\beta$-ol-3-one benzoate (dihydrotestosterone benzoate), and $\beta$-estradiol-3-benzoate (estradiol benzoate); (2) Schwarz-Mann (Orangeburg, New York), 1, 3, 5(10)-estratrien-3, 17$\beta$-diol (estradiol) and (3) Abbott Laboratories (North Chicago, Illinois), synthetic gonadotropin releasing hormone.

## Experiment 1. Castration

Three trials were conducted in June 1972, December 1972, and

September 1973 involving three, one and four animals, respectively, to determine the acute effects of castration on serum LH levels. Also, two trials were conducted to investigate serum LH fluctuations in the chronic castrate animal by obtaining blood samples at frequent intervals for a short period of time.

In the animals to be castrated, the testes and their surrounding tunica albuginea were exposed by bilateral longitudinal incisions in the ventral portion of the scrotum. Number two chromic gut (Ethicon Inc., Somerville, New Jersey) was used to ligate the spermatic cord 5 to 10 cm proximal to the testes at which time an emasculator was used to mascerate and sever the spermatic cord just distal to the ligation.

All bulls used in the castration studies were 4 to 6 months of age and all blood sampling was by jugular venipuncture.

In trial one, bulls weighing approximately 140 kg were castrated and blood was sampled hourly beginning 5 hr before to 5 hr after castration, then every 6 hr for 47 hr, then daily at noon for 9 days and finally twice on the 12th day at 1430 and 2100 hr. In a second trial, one bull was monitored by hourly sampling for 11 hr succeeding castration. A final castration study utilized four animals, ranging in weight from 130 to 220 kg. Blood samples were obtained just prior to castration, then hourly for 12 hr.

Four chronically castrate steers, 4 months post-castration, and four chronically castrate steers, 2 months post-castration, were involved in two studies in October 1972 and December 1973, respectively, to investigate changes in serum LH occurring over short intervals. In both cases, 10 ml blood samples were taken by cannulae and handled as described previously. Commencing at 1640 hr, the animals in the first study were bled every 5 min until 1735 hr, while in the second study sampling was at 10 min intervals from 1000 to 1200 hr.

## Experiment 2. Steroid Replacement Therapy

Three groups of castrate males received steroid treatments in a series of studies designed to define the suppressive effects of steroids on LH release. Trial one was conducted 12 days post-castration in three steers (5 months old) fitted with PE 60 cannulae. Two dosages of testosterone (T) (0.5 and 2.0 μg/kg BW), androstenedione (A) (2.0 and 8.0 μg/kg BW) and 17β-estradiol ($E_2$) (0.5 and 2.0 μg/kg BW) were randomly assigned to each of the three animals on each of 4 consecutive days, allowing for two observations per dose level (Table 1). Hormones were injected iv in 10 ml of 10% ethanol-0.9% saline (1:9) at 0900 hr each day. Five ml samples were collected at 15 min intervals from 0800 to 0930 hr, 30 min intervals from 0930 to 1700 hr and a final sample at 2100 hr each day of the experiment.

Table 1. Assignment of Steroid Treatments and Dosages Administered Intravenously to 5-Month-Old Steers Castrated 12 Days Previously (Trial 1).

| Day | Animal | Body Weight (kg) | Hormone Treatment[a] | Dosage (μg/kg BW) | Quantity Injected (μg) |
|---|---|---|---|---|---|
| 1 | 12 | 139.2 | A | 8.0 | 1113.6 |
| | 13 | 141.5 | T | 0.5 | 70.8 |
| | 14 | 120.0 | A | 2.0 | 240.0 |
| 2 | 12 | as above | $E_2$ | 0.5 | 69.6 |
| | 13 | | $E_2$ | 2.0 | 283.0 |
| | 14 | | T | 2.0 | 240.0 |
| 3 | 12 | as above | $E_2$ | 0.5 | 69.6 |
| | 13 | | T | 0.5 | 70.8 |
| | 14 | | $E_2$ | 2.0 | 240.0 |
| 4 | 12 | as above | T | 2.0 | 278.4 |
| | 13 | | A | 8.0 | 1132.0 |
| | 14 | | A | 2.0 | 240.0 |

[a] A = Androstenedione; T = Testosterone; $E_2$ = 17β-Estradiol

Because testosterone and androstenedione failed to alter serum LH levels in trial 1, a second steroid experiment involved higher dosages of T and A administered to steers (8 months old) castrated 4 months previously. Testosterone (6.7 and 26.7 μg/kg BW) and A (26.7 and 106.6 μg/kg BW) were assigned as shown in Table 2 and given intravenously via the jugular vein in 10 ml of 30% ethanol-0.9% saline. From 0815 to 1000 hr and from 1000 to 1800 hr, 5 ml of blood were collected by cannulae at 15 and 30 min intervals, respectively. Steroids were injected immediately after the 0945 hr sample.

The third study differed from the previous experiments in that (1) hormones were administered im and (2) esters of steroids were used rather than non-esterified steroids to determine if the route of administration or the solubility of the steroid affected its ability to suppress LH. Four Holstein steers, 7 months old, castrated 2 months previously, were given 1 ml im injections of testosterone propionate (TP) (0.5, 4.0, or 16.0 μg/kg free steroid), estradiol benzoate ($E_2B$) (0.5 or 2.0 μg/kg), dihydrotestosterone benzoate (DHTB) (0.5, 4.0, or 16.0 μg/kg) or vehicle (corn oil). The animals were subjected randomly to a steroid or control treatment at 0930 hr every second day on each of 5 different days (Table 3) with the restriction that each animal received each steroid while those receiving the same steroid twice did not do so on consecutive treatment days or at the same dosage. Esterified steroid dosages are expressed in terms of

Table 2. Assignment of Steroid Treatments and Dosages Administered Intravenously to 8-Month-Old Steers Castrated 4 Months Previously (Trial 2).

| Day | Animal | Body Weight (kg) | Hormone Treatment[a] | Dosage (μg/kg BW) | Quantity Injected (mg) |
|---|---|---|---|---|---|
| 1 | 4 | 244.9 | A | 26.7 | 6.54 |
| | 10 | 279.0 | T | 6.7 | 1.87 |
| | 11 | 205.9 | T | 26.7 | 5.50 |
| | 18 | 204.1 | A | 106.6 | 21.76 |
| 2 | 4 | as above | T | 26.7 | 6.54 |
| | 10 | | A | 106.6 | 29.74 |
| | 11 | | A | 26.7 | 5.50 |
| | 18 | | T | 6.7 | 1.37 |

[a]A = Androstenedione; T = Testosterone

Table 3. Assignment of Steroid Treatments and Dosages Administered Intramuscularly to 7-Month-Old Steers Castrated 2 Months Previously (Trial 3).

| Day | Animal | Body Weight (kg) | Hormone Treatment[a] | Dosage of Esterified Steroid (μg/kg BW) | Total Quantity of Ester Injected (mg) | Equivalent Dosage of Nonesterified Steroid (μg/kg BW) |
|---|---|---|---|---|---|---|
| 1 | Y-4 | 193 | EB | 2.76 | 0.53 | 2.0 |
| | Y-1 | 151 | Control | - | - | - |
| | R-3 | 260 | TP | 0.60 | 0.16 | 0.5 |
| | B-2 | 193 | TP | 4.76 | 0.92 | 4.0 |
| 3 | Y-4 | 193 | Control | - | - | - |
| | Y-1 | 151 | EB | 0.67 | 0.10 | 0.5 |
| | R-3 | 260 | DHTB | 21.76 | 5.66 | 16.0 |
| | B-2 | 193 | DHTB | 5.44 | 1.05 | 4.0 |
| 5 | Y-4 | 193 | DHTB | 21.76 | 4.20 | 16.0 |
| | Y-1 | 151 | DHTB | 0.68 | 0.10 | 0.5 |
| | R-3 | 260 | Control | - | - | - |
| | B-2 | 193 | TP | 19.04 | 3.68 | 16.0 |
| 7 | Y-4 | 193 | TP | 19.04 | 3.68 | 16.0 |
| | Y-1 | 151 | TP | 0.60 | 0.09 | 0.5 |
| | R-3 | 260 | DHTB | 0.68 | 0.18 | 0.5 |
| | B-2 | 193 | EB | 2.76 | 0.53 | 2.0 |
| 9 | Y-4 | 193 | TP | 4.76 | 0.92 | 4.0 |
| | Y-1 | 151 | DHTB | 5.44 | 0.82 | 4.0 |
| | R-3 | 260 | EB | 0.69 | 0.18 | 0.5 |
| | B-2 | 193 | Control | - | - | - |

[a]TP = Testosterone propionate; DHTB = Dihydrotestosterone benzoate; $E_2B$ = Estrdiol benzoate

free steroid injected. Five ml samples of blood were taken by cannulae every 15 min from 0800 to 0930 hr and every 30 min from 0930 to 2130 hr.

Four levels of TP, each in a total volume of 50 ml of 67% ethanol-0.9% saline (2:1) were given to four steers in a fourth steroid study (Table 4) to determine if an effective dose could be attained. Levels of TP were randomly assigned. The animals were bled by jugular venipuncture every 30 min from 0800 to 1400 hr, once at 1600 hr and every 4 hr through 72 hr post-injection. The hormone was injected (iv) after collection of the 1000 hr sample. An effective, suppressing dosage of TP still had not been attained. Therefore, in a fifth trial, two steers, randomly selected from the steers used in Trial 4, were injected with 100 mg TP (84.0 mg actual T) twice daily at 0800 and 2000 hr for 3 consecutive days. The steroid was injected in 2 ml corn oil im into alternate sides of the rump region. The bleeding schedule was as follows: day 1, 0600, 0630, 0700, 0730, 0800, 1400, 2000 hr; day 2, 0200, 0800, 1400, 2000 hr; day 3, 0200, 0800, 1400, 2200, 2230, 2300, 2400 hr and day 4, 0600, 1200, 1800, 1830, 1900 hr. No steroid injections were given on day 4.

Table 4. Assignment of Steroid Treatments and Dosages Administered Intravenously to 8. 5-Month-Old Holstein Steers Castrated 3. 5 Months Previously (Trial 4).

| Animal | Body Weight (kg) | Hormone Treatment | Dosage of Esterified Steroid (μg/kg BW) | Total Quantity of Ester Injected (mg) | Equivalent Dosage of Nonesterified Steroid (μg/kg BW) |
|---|---|---|---|---|---|
| R-3 | 280 | testosterone propionate | 76. 2 | 21. 34 | 64 |
| Y-4 | 198 | " | 304. 6 | 60. 31 | 256 |
| Y-1 | 157 | " | 609. 3 | 95. 66 | 512 |
| B-2 | 211 | " | 1218. 6 | 257. 12 | 1024 |

## Experiment 3. Gonadotropin Releasing Hormone Treatment

An initial trial was conducted in four Holstein steers (7. 5 months of age, 2. 5 months post-castration) in a 2-day experiment to investigate the LH response after treatment with varying doses of GnRH. The animals received randomly assigned dosages of GnRH (Table 5) in 10 ml saline through an indwelling cannula followed by a flush of 5 ml 3. 5% sodium citrate-2% Liquamycin on each of 2 successive days. Blood samples were taken every 15 min for 90 min before GnRH injection and at 10 min intervals for 60 min succeeded by 30 min samples through 4 hr post-injection.

Reduced dosages of GnRH were used in a second study in an attempt to determine the minimum effective dosage of GnRH (dosage

Table 5. Assignment of Gonadotropin Releasing Hormone Dosages to 7.5-Month-Old Holstein Steers Castrated 2.5 Months Previously.

| Animal | Body Weight (kg) | Day 1 (μg) | Day 2 (μg) |
|---|---|---|---|
| Y-4 | 179 | 160 | 10 |
| Y-1 | 138 | 80 | 40 |
| B-2 | 186 | 40 | 80 |
| R-3 | 257 | 10 | 160 |

required to evoke an easily detectable increase in serum LH). Dosages of 2.5, 5.0, 7.5 and 10 μg GnRH in saline were administered to animals B-2, R-3, Y-1 and Y-4, respectively, at 8.5 months of age and 3.5 months post-castration. The steers were bled by venipuncture 30 min previous to injection, immediately prior to injection and then at 10, 20, 30, 40, 50 and 60 min and at 1.5, 2, 3 and 4 hr following injection.

## Experiment 4. GnRH and TP

Combining information gained from previous experiments with both TP and GnRH, a final experiment was designed to investigate the influence of TP on GnRH-induced LH release. The experiment was conducted from March 1 to 7, 1974 with March 1 being referred to as day 1. The four steers (10 months old and 5 months post-castration) received identical treatment throughout the experiment which consisted

of, in brief, GnRH on day 1, TP on days 1, 2, 3, 4, 5, and GnRH on days 5 and 6. Testosterone propionate, 119 mg in 2 ml corn oil (equivalent to 100 mg T), was injected im in alternate sides of the rump at 2000 hr on day 1, 0800 and 2000 hr on days 2, 3, 4, and 0800 hr on day 5. Two ml of corn oil was given on day 1 at 0800 hr, day 5 at 2000 hr, and day 6 at 0800 and 2000 hr to maintain the constant presence of corn oil throughout the experiment with or without the presence of TP. Five μg GnRH (the minimum effective dosage) in 5 ml of 0.9% saline was infused rapidly into the left jugular vein at 1200 hr on days 1, 5 and 6 via a short length of small-diameter Tygon tubing fitted with a 1.5 inch 18 ga needle at one end and a three-way valve at the other. Each dose of GnRH was followed by 3 ml saline to flush the catheter. Ten ml blood samples via the jugular vein (venipuncture) were taken at 0800 and 2000 hr on days 1 through 8. Also, on days 2, 3, 4 and 7 (days on which no GnRH was given), frequent blood samples were obtained every 30 min from 1200 to 1400 hr. On days 1, 5 and 6 (GnRH treatment days) blood was collected every 30 min from 1000 to 1200 hr, every 10 min from 1200 to 1300 hr, and every 30 min from 1300 to 1500 hr.

## Radioimmunoassay

### Production of Guinea Pig Anti-Bovine LH (GPABLH; first antibody)

Antibody for the radioimmunoassay was successfully produced

after several injections of 1.0, 0.5 or 0.1 mg of bovine LH (BLH) (NIH-LH-B7) into guinea pigs. BLH was diluted in 1.0 ml 0.9% saline and 1.2 ml adjuvant to yield a total volume in excess of 2 ml. The first injection contained Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, Michigan) whereas all subsequent injections included Freund's incomplete adjuvant (FICA). After homogenization for 2 min, the emulsion was injected intradermally at numerous sites in the back and in the footpads. A total of seven injections were given at 3 week intervals. Nine days following the third and all later injections 2 ml blood was taken be heart puncture with a 2.5 ml syringe and 22 ga x 1 inch needle.

After separation by centrifugation, 0.1 ml of the serum was diluted 1:400 with 1:400 normal guinea pig serum (Appendix 1.B.4) and frozen until use. All bleedings were subjected to a titration procedure using antiserum dilutions of 1:400-1:128,000 to determine binding ability. By this method, bleedings three and four from one guinea pig which received 0.1 mg BLH were found to bind approximately 30% of BLH-$I^{125}$ at a dilution of 1:64,000 which was considered adequate for use in the assay. Yalow and Berson (1968) state that maximum assay sensitivity occurs when, in the absence of unlabeled hormone, 33 1/3% of the labeled hormone (BLH-$I^{125}$) is antibody-bound or when the bound to free ratio equals 0.5. Consequently, the

antibody titer was adjusted so that approximately 33 1/3% of the BLH-$I^{125}$ was antibody bound and 67 2/3% remained free.

The antiserum was also tested for specificity with other bovine anterior pituitary hormones, for precision (repeatability), accuracy (recovery) and sensitivity (minimum amount detectable).

Production of Sheep Anti-Guinea Pig Gamma Globulin (SAGPGG) and Sheep Anti-Rabbit Gamma Globulin (SARGG) (second antibody)

Twenty-five mg (0.5 mg/kg body weight) guinea pig gamma globulin (Miles Laboratories, Kankakee, Illinois) and 25 mg rabbit gamma globulin (Nutritional Biochemicals Corporation, Cleveland, Ohio) were dissolved in 5 ml basic isotonic saline and homogenized with 5 ml of either FCA (initial injection) or FICA (all subsequent injections) and 0.75 ml Combiotic. The emulsion was injected subcutaneously at numerous sites in the scapular and back regions of a 50 kg ewe. The ewe received seven injections at 3 wk intervals and a "booster" injection 4.5 months after the last injection. Approximately 500 ml of blood were withdrawn from the jugular vein 9 days following the third and all later injections, and at monthly intervals for 4 months following the bleeding after the last injection. Evaluation of the 10 bleedings revealed that adequate precipitation of BLH-GPABLH complexes occurred at a 1:2 dilution of SAGPGG with

0.05 M EDTA-PBS (Appendix I. B. 2) and that precipitation of BLH-RABLH (Rabbit anti-bovine LH supplied by G. D. Niswender, Dept. of Physiology and Biophysics, Colorado State University, Fort Collins, Colorado) complexes occurred at a 1:12 dilution of SARGG with EDTA-PBS.

Radioiodination

The iodination procedure was adapted from the technique of Greenwood, Hunter and Glover (1963). Highly purified BLH (LER 1072-2), graciously supplied by Dr. L. R. Reichert, Jr. (Dept. of Biochemistry, Emory University, Atlanta, Georgia), was dissolved in 0.01 M $NH_4HCO_3$ pH 8.3 buffer (Appendix I. A. 1) at a concentration of 1 µg/µl. Sinha et al. (1972) reported that use of ammonium bicarbonate buffer rather than $H_2O$ for dissolving mouse growth hormone resulted in superior iodinations, higher binding and a more sensitive curve so this buffer was used in our iodination procedure. BLH aliquots of 2.5 and 5.0 µl were dispensed into 1 ml vials and frozen until needed.

For each iodination a vial of 2.5 or 5 µl BLH was removed from the freezer and 29 µl 0.5 M phosphate buffer (Appendix I. A. 3) containing 0.5 mc $I^{125}$ (Amersham Searle, Arlington Heights, Illinois) was added to the vial. Thirty µl freshly prepared chloramine-T (1 µg/µl) (Appendix I. A. 4) in 0.05 M phosphate pH 7.5 buffer (column

buffer) (Appendix I. A. 2) was added to the vial and the entire mixture was agitated by finger tapping for precisely 2 min, at which time 50 µl freshly prepared sodium metabisulfite (2. 5 µg/µl) (Appendix I. A. 5), diluted in column buffer, was added to quench the reaction. To reduce nonspecific binding of BLH during gel chromatography, 25 µl 2. 5% BSA (Appendix I. A. 6) was added to the vial. One hundred µl Transfer Solution (Appendix I. A. 7) was added to the vial, the entire contents mixed and then drawn into a Pasteur pipette and carefully layered in column buffer above the Bio-Gel P-60 bed of a column (0. 9 x 15 cm). Remaining BLH-$I^{125}$ in the vial was rinsed with 70 µl Rinse Solution (Appendix I. A. 8) and also layered on the column.

The components, free $I^{125}$ and $I^{125}$ bound to LH (BLH-$I^{125}$), were eluted with column buffer. Fifteen 1-ml fractions of column effluent were collected in culture tubes containing 1 ml 0. 1% gelatin-PBS (Appendix I. B. 3). After mixing, 5 µl aliquots of each fraction were placed in culture tubes and the radioactivity of each fraction was determined by counting for 1 min in an auto gamma counter (Packard Instrument Co. Inc., Downers Grove, Illinois). A sample elution curve is displayed in Figure 1. Tubes containing fractions from the first peak, the BLH-$I^{125}$ peak, were sealed with parafilm and frozen. Each fraction within the first peak was diluted to 200, 000 cpm/ml with 0. 1% gelatin-PBS buffer (Appendix I. B. 3) and incubated with antibovine LH serum (100 µl diluted 1:32, 000 to 1:128, 000 in

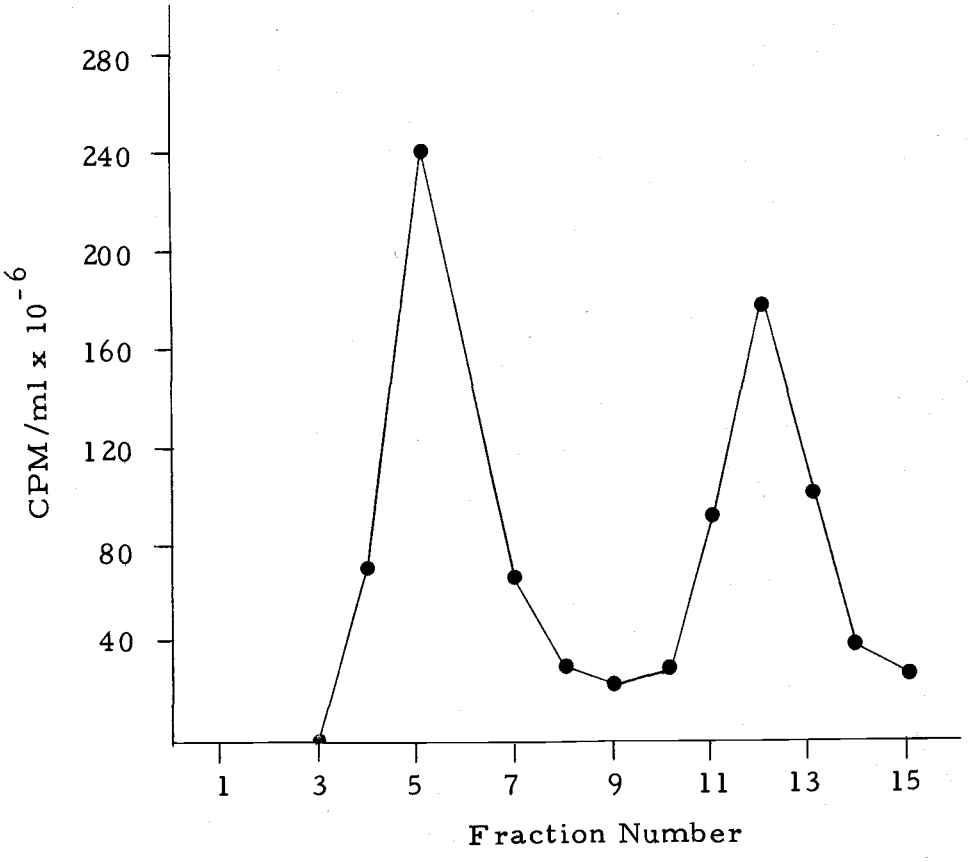


Figure 1. Sample elution curve after passage of radioiodinated bovine luteinizing hormone through a Bio-Gel P-60 column. The first peak contains BLH bound $I^{125}$ and the second peak contains free $I^{125}$.

1:400 normal rabbit or normal guinea pig serum, Appendix I. B. 4) and anti-gamma globulin serum (200 µl of SARGG or SAGPGG diluted 1:10 or 1:2, respectively, 0.05 M EDTA-PBS pH 7.0, Appendix I. B. 2) to determine if the iodinated LH was recognized by the antiserum. Only fractions in which at least 30% of the BLH-$I^{125}$ could be bound by the antibody at a minimum dilution of 1:64,000 were used in subsequent RIA's (Table 6).

Radioimmunoassay Procedure

The double antibody radioimmunoassay for bovine LH described by Niswender et al. (1969) was modified for use in our laboratory. Serum samples or NIH-LH-B7 standards (Appendix I. B. 5) were added to 12 x 75 mm culture tubes with 0.1% gelatin-PBS buffer (Appendix I. B. 3) sufficient to make 500 µl total volume. Two hundred µl of GPABLH or rabbit anti-bovine LH (RABLH) [nearly half of the samples assayed for this thesis were determined with RABLH (Lot B225), compliments of Dr. G. D. Niswender, and the remainder were determined with GPABLH (Lot GPI-3rd) prepared in this laboratory] diluted to a concentration of 1:32,000 to 1:128,000 with 1:400 normal rabbit or guinea pig serum (Appendix I. B. 4) was added to each tube, the contents gently mixed and the assay tubes incubated at 4 C for 24 hr. One hundred µl BLH-$I^{125}$, 200,000 cpm/ml, was added, the contents were mixed and incubated for 24 hr at 4 C. To

Table 6. Example of the Effect of First Antibody Dilution on BLH-$I^{125}$ Binding of Various Iodinated Fractions from the First Eluted Peak.

| Iodinated Fraction | Dilution of GPABLH | % BLH-$I^{125}$ Bound |
|---|---|---|
| F-4 | 1:32,000 | 16.9 |
| | 1:64,000 | 13.5 |
| | 1:128,000 | 11.5 |
| F-5 | 1:32,000 | 32.2 |
| | 1:64,000 | 25.2 |
| | 1:128,000 | 19.4 |
| F-6 | 1:32,000 | 55.5 |
| | 1:64,000 | 41.0 |
| | 1:128,000 | 27.4 |

enable precipitation of the BLH-$I^{125}$ first-antibody complexes (RABLH or GPABLH), 200 µl of second antibody (sheep anti-rabbit gamma globulin-SARGG or sheep anti-guinea pig gamma globulin-SAGPGG) were added 24 hr after BLH-$I^{125}$, and the tubes were vortexed and incubated for 72 hr at 4 C. All components were added with Hamilton repeating syringes (Hamilton Co., Reno, Nevada). At the completion of the 72 hr incubation period, 3 ml PBS (4 C) was added to each tube and the assay tubes were subjected to centrifugation (1732 x g) for 30 min at 4 C. After pouring off the supernatant, the radioactivity of the samples was determined by counting in an auto gamma counter programmed for 10 min or 10,000 counts, whichever occurred first.

# RESULTS AND DISCUSSION

## Validation of the Radioimmunoassay for Bovine Luteinizing Hormone

The LH RIA procedure using RABLH (supplied by Niswender) proved adequate in sensitivity, accuracy, specificity and precision for our work. The assay and antiserum were evaluated by Niswender in a manner similar to that used by Niswender et al. (1969) and bovine serum values obtained in our laboratory are comparable to values reported in the literature.

A thorough validation was conducted to determine accuracy, precision, sensitivity, and specificity of the GPABLH developed in our laboratory and previously shown to possess sufficient antibody titer. Accuracy was determined by measuring recovery of 0.61, 1.02, 2.05, 4.10, 8.19, 12.29 or 16.38 ng of BLH (NIH-LH-B7) added to 100 µl of bovine serum. Recovery of BLH was excellent up to and including 8.19 ng/tube (108.7% $\pm$ 2.65%) as shown in Figure 2. But recoveries were in excess of 200% in tubes to which 12.29 and 16.38 ng/BLH had been added.

To determine if a similar increase in LH recovery occurred in serum samples high in endogenous LH, various volumes of pooled serum containing ~26 ng/ml were assayed. Serum LH concentrations corrected to ng/ml for 400 µl (~10.4 ng/tube) and 500 µl

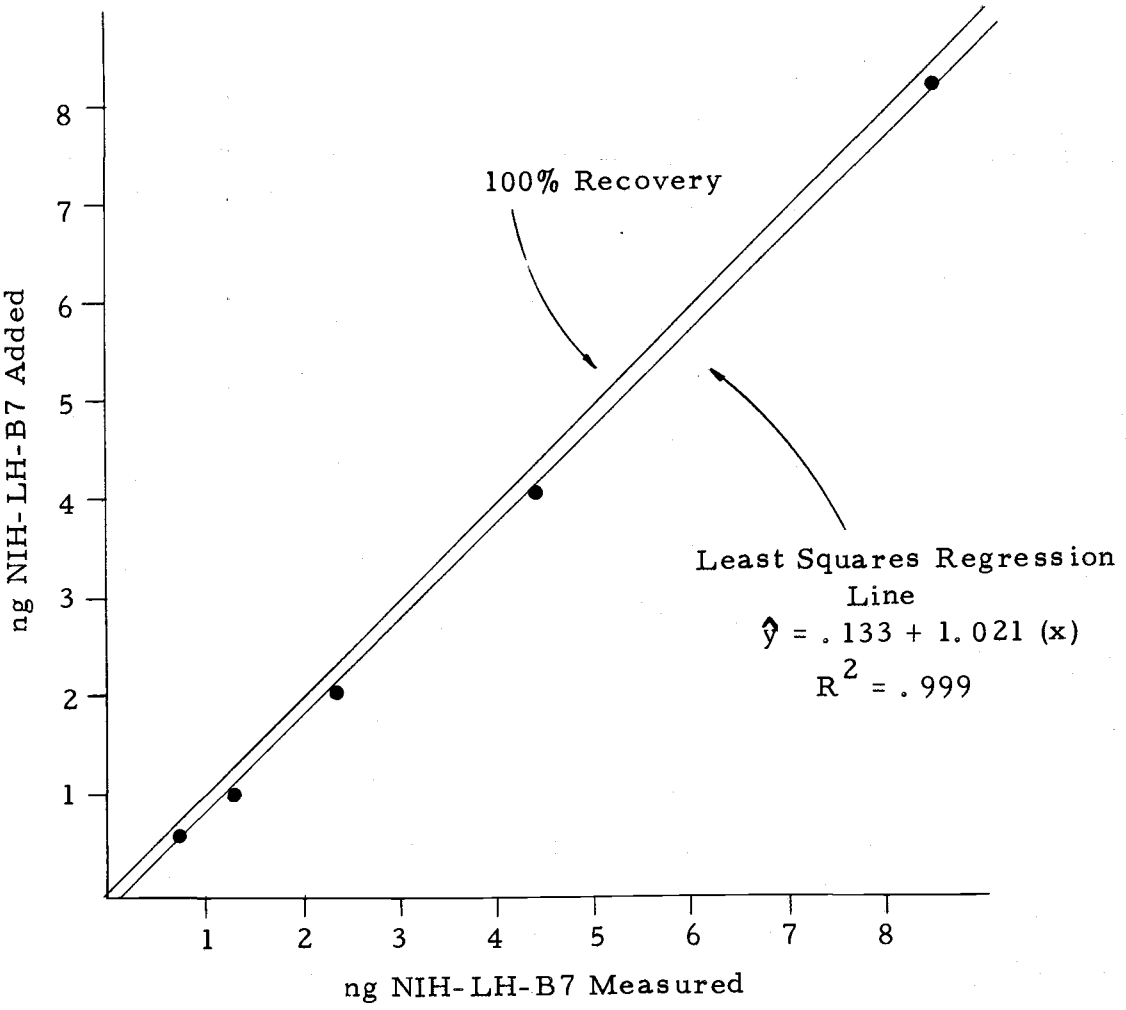


Figure 2. Recovery of exogenous bovine LH (NIH-LH-B7) added to 100 µl bovine serum.

(~13.0 ng/tube) were 127% and 153%, respectively, of the mean concentration determined from the other assayed volumes (50, 100, 200 and 300 µl). Inflated recoveries at high concentrations of LH per assay tube indicate that serum samples high in LH should be run at dilutions providing less than 10 ng/tube. All values obtained from serum dilutions having greater than 10 ng/tube with the antibody (GPABLH) prepared in this laboratory will be markedly inflated.

An interassay coefficient of variation of 12.8%, calculated from high and low pooled serum run as internal standards in each assay, indicates adequate precision and compares favorably with variation of 19.7% reported by Golter et al. (1973).

BLH concentrations of 0.2 ng/tube or 0.4 ng/ml are easily and repeatably detectable in bovine serum samples, demonstrating that this assay procedure using GPABLH (Lot No. GP1-3rd) contains excellent sensitivity for routine determination of serum BLH.

Antibody cross reactivity with PRL (NIH-P-B3), FSH (NIH-FSH-B1), and GH (NIH-GH-B16) was insignificant as shown by non-parallelism of these curves at less than 100 ng/tube with BLH standards (NIH-LH-B7) (Figure 3). Parallelism of TSH (NIH-TSH-B5) at less than 100 ng/tube with the BLH curve was as expected and is due, possibly, to alterations in immunoreactivity of TSH during purification. Swanson (1970) has shown that in a similar assay, serum pools containing high and low concentrations of TSH did not

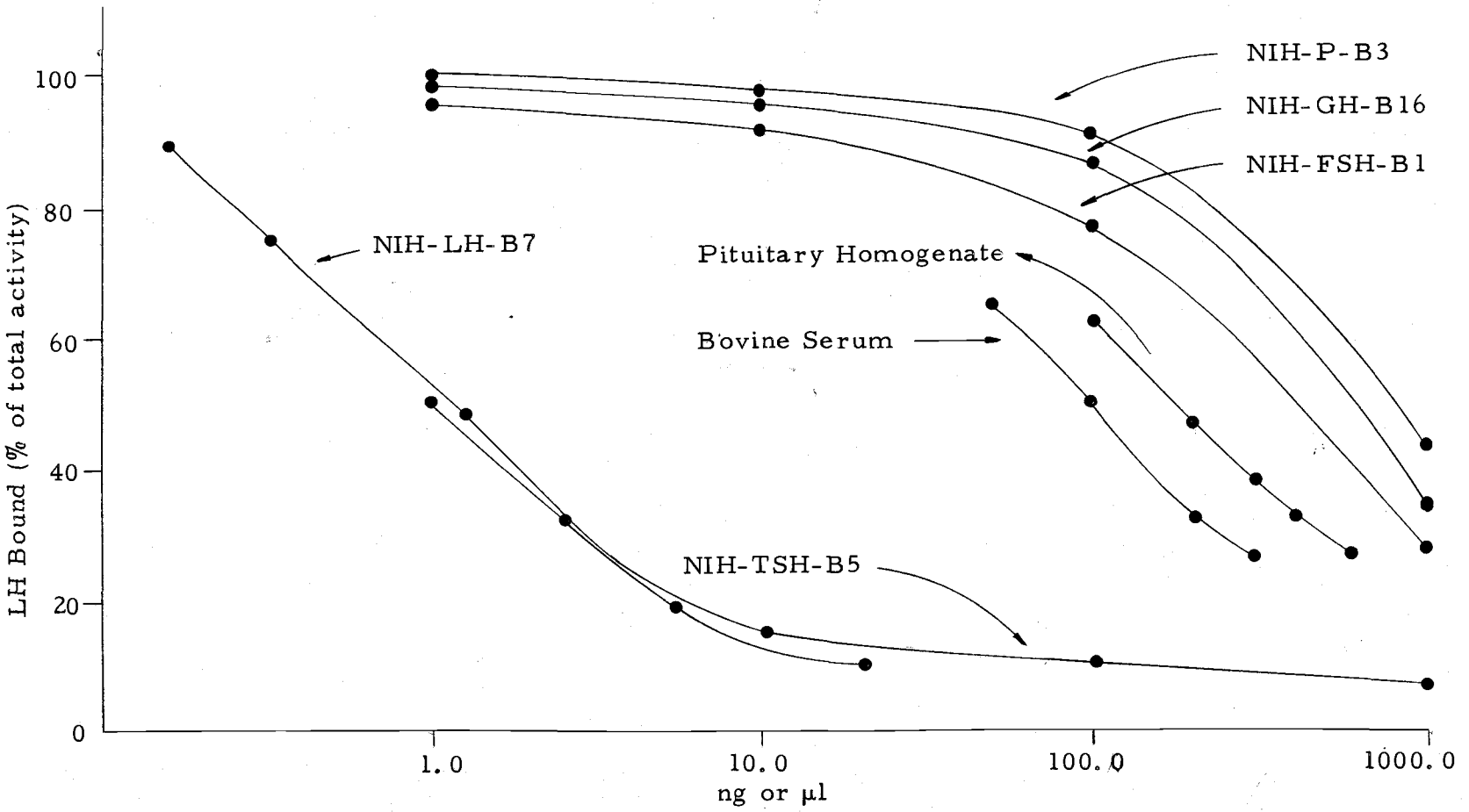


Figure 3. Dose-response curve for NIH-LH-B7 standard (ng), other NIH pituitary hormones (ng), bovine serum (µl) and a pituitary homogenate (µl).

react differently in the BLH assay, indicating no substantial cross-reactivity of endogenous TSH. Dilutions of serum and pituitary homogenates were parallel to the BLH curve, showing that the components estimated were immunoreactive BLH.

## Experiment 1. Castration

Serum LH increased dramatically after castration in all animals in trials 1, 2 and 3 (Figure 4). Of the three animals castrated in trial 1, one responded with a six-fold increase in serum LH 2 hr following castration. It is suspected that this increase in LH was not due to castration, but was one of the normal fluctuations which occurs in intact bulls (Katongle et al., 1971). Serum LH levels in all three animals were elevated six- to seven-fold at 11 hr post-castration. After reaching elevated levels serum LH became unstable and oscillated from 0.7 to 16.4 ng/ml. Estimates of fluctuations in serum LH during the sampling periods (Table 7) indicate that only a slight increase in serum LH occurred during the first 5 hr post-castration and that serum LH remained stable during this period. A substantial elevation in serum LH occurred in all animals by 11 hr post-castration and serum LH remained high throughout the sampled period. This indicated that the anterior pituitary was released from testicular inhibition sometime between 5 and 11 hr after castration.

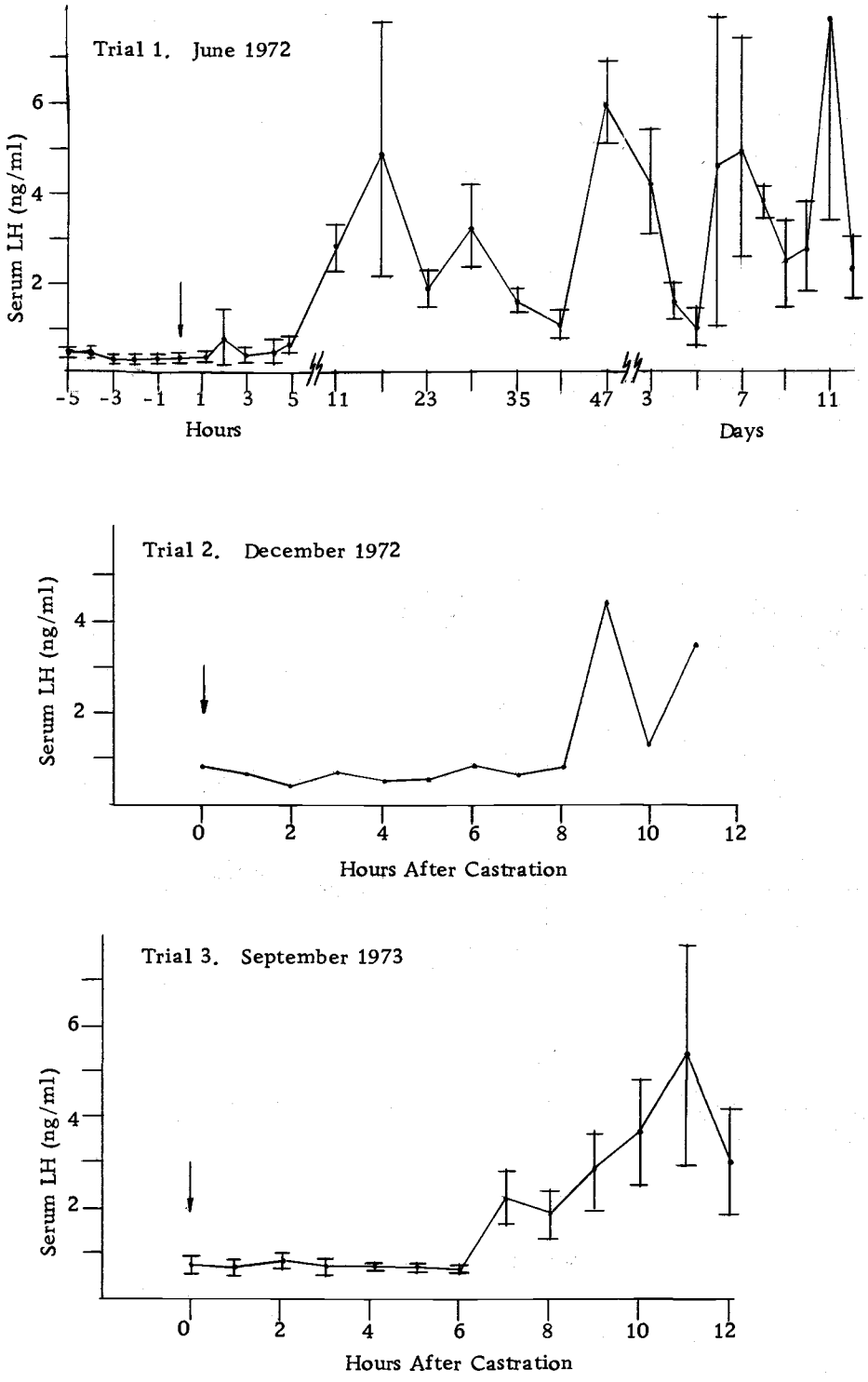


Figure 4. Serum LH concentrations of Holstein bull calves castrated between 4. 5 and 5. 5 months of age. 0 hr, indicated by the arrow, represents the time of castration. The 0 hr sample was collected just prior to castration in all cases. Each point represents the mean serum LH concentration (± SE) from 3 animals in trial 1 and 4 animals in trial 3.

In the single bull castrated in trial 2, serum LH remained below 1 ng/ml until 9 hr after castration when serum LH levels increased to 4.5 ng/ml, decreased to 1.1 ng/ml at 10 hr and increased again to 3.6 ng/ml at 11 hr post-castration (Figure 4, Table 7).

Serum LH increased from pre-castration levels in four bulls at 7 to 11 hr post-castration (trial 3). Serum LH increased during the 6 to 12 hr period after castration (Figure 4, Table 7), but fluctuated from 0.7 to 12.0 ng/ml as indicated by the larger standard error during this period.

Post-castration serum LH spikes in trials 1, 2 and 3 did not appear to occur at regular intervals although the frequency could not be determined accurately because of the 1 hr intervals between blood sampling.

Gonadectomy of these bull calves removed the pituitary-hypothalamic axis from suppression of negative feedback by non-steroid or steroidal testicular products. Similar increases in serum LH following castration have been documented in male and female rats (Gay and Midgley, 1969), man (Walsh, Swerdloff and Odell, 1973), ewes (Reeves, O'Donnell and Denorscia, 1972; Roche et al., 1970), rams (Pelletier, 1968), female rhesus monkeys (Atkinson et al., 1970), cows (Hobson and Hansel, 1972) and prepubertal heifers and bulls (Odell, Hescox and Kiddy, 1969).

Table 7. Serum LH after Castration of Holstein Bull Calves.

| Time from Castration | Collection Interval | n[a] | Serum LH (ng/ml) |
|---|---|---|---|
| | Trial 1 June 1972 | | |
| -5 to 0 hr | hourly | 6 | 0.4 ± 0.03[b] |
| +1 to 5 hr | hourly | 5 | 0.5 ± 0.11 |
| 11 to 47 hr | 6-hour | 7 | 3.1 ± 0.54 |
| 3 to 12 days | daily | 10 | 3.4 ± 0.59 |
| | Trial 2 December 1972 | | |
| 0 to +5 hr | hourly | 6 | 0.6 ± 0.07[c] |
| 6 to 11 hr | hourly | 6 | 1.9 ± 0.70 |
| | Trial 3 September 1973 | | |
| 0 to +5 hr | hourly | 6 | 0.7 ± 0.04[d] |
| 6 to 12 hr | hourly | 7 | 2.8 ± 0.49 |

[a]Number of blood samples from each animal during each time period.

[b]Mean ± SE of 3 bulls castrated at 4.5 months of age.

[c]Mean ± SE of 1 bull castrated at 6.0 months of age.

[d]Mean ± SE of 4 bulls castrated at 5.0 months of age.

Odell et al. (1969) collected blood samples daily and reported changes in serum LH in three prepubertal bulls from means of 4.6, 1.0 and 3.9 ng/ml during a 10-day pre-castration period to 10.7, 2.9 and 9.4 ng/ml, respectively, during a 10-day post-castration period. Although the pre-castration and post-castration levels in two of these animals were higher than reported in our study, the magnitude of the post-castration increases are comparable (from 0.3, 0.7 and 0.7 to 3.1, 1.9 and 2.8 in trials 1, 2 and 3, respectively). The study by Odell et al. (1969) did not define the acute pituitary response to castration as was done in our study, but the increase in serum LH between 7 and 11 hr post-castration in our studies corresponds to ovariectomy studies in the heifer in which serum LH levels were increased at 6 and 12 hr post-ovariectomy (Hobson and Hansel, 1972).

The time required for disappearance of testicular steroids from the systemic circulation after castration of bulls has not been documented, but work in male rhesus monkeys (Resko and Phoenix, 1972) and male rats (Coyotupa, Parlow and Kovacic, 1973) indicates that testosterone decreased to undetectable levels (less than 0.06 ng/ml) in the rat and to 25% of original levels (1.1 ng/ml) in rhesus monkeys by 2 hr post-castration. The reduction in circulating testosterone levels after castration as reported in the literature occurred before the initiation of increases in serum LH observed in

in these studies. However, the time course for the disappearance of other testicular products (i. e., estrogens and "inhibin") from the circulation after castration has not been investigated and their disappearance may also be involved in post-castration increases in serum LH.

Serum LH levels from two studies using 5 to 10 min sample collections in chronically castrate animals are presented in Figure 5. Steers in the first study at 4 months post-castration had only minor fluctuations in serum LH (range 1. 3 to 3. 3 ng/ml). Mean levels and deviations from the mean were similar between animals, as indicated in Table 8. In the second trial, using four steers 2 months post-castration, serum LH deviated from the mean with a range of 2. 1 to 11. 8 ng/ml. The standard errors (Table 8) are larger than those in trial 1, reflecting greater variation within animals, and the mean levels are also higher. Within animals the interval between peaks is not repeatable and all oscillations appear to be random. Because the fluctuations were greater at 2 months post-castration (trial 2) than at 4 months post-castration (trial 1), it appears that the number and magnitude of LH spikes decreases as time from castration increases, although this has not been documented by other workers. Butler et al. (1972) compared serum LH of ewes ovariectomized for less than 21 days with serum LH levels from ewes ovariectomized for longer periods and found no difference in frequency of LH spikes. The

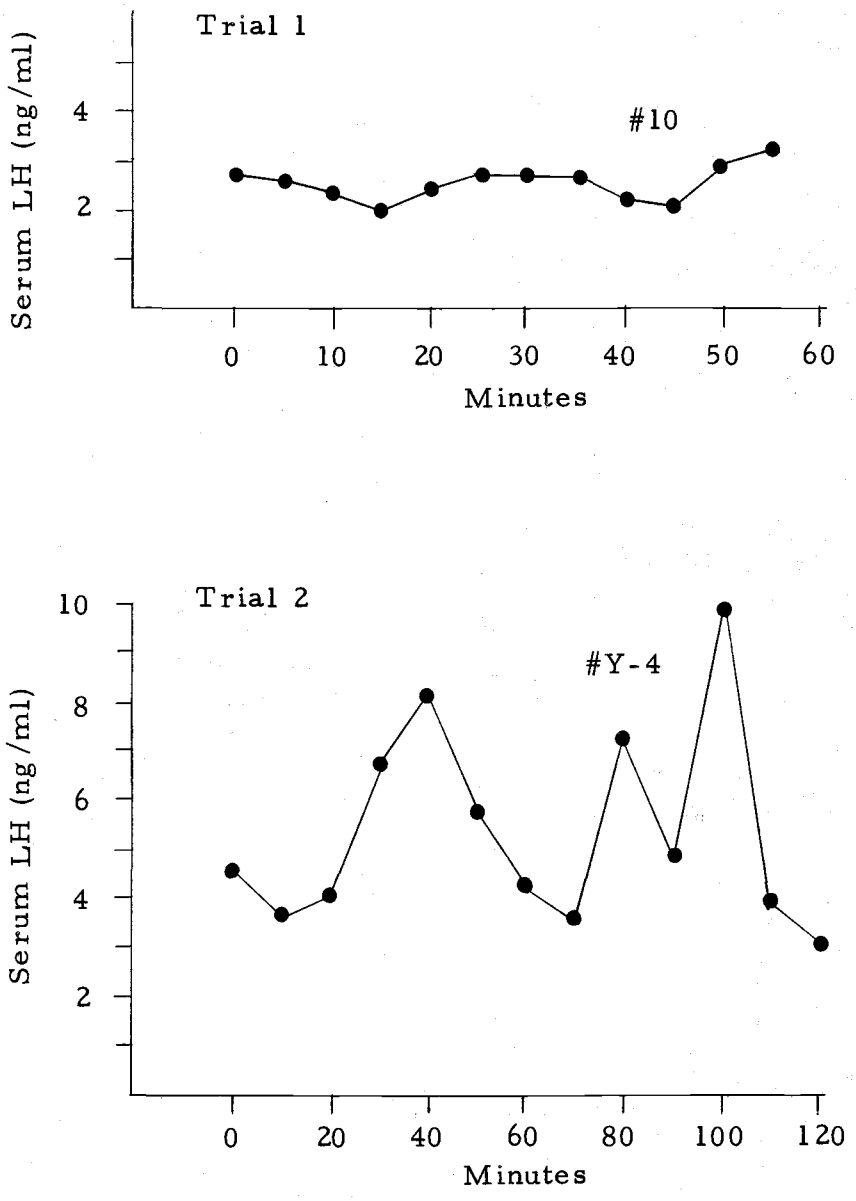


Figure 5. Serum LH concentrations from typical animals in two studies conducted in October, 1972 (trial 1) and December, 1973 (trial 2) at 4 months and 2 months post-castration, respectively. Steers in trial 1 were sampled at 5 min intervals and steers in trial 2 were sampled at 10 min intervals.

Table 8. Serum LH in Chronically Castrate Holstein Steers.

| Animal No. | n[a] | Serum LH (ng/ml) |
|---|---|---|
| | Trial 1[b] | |
| 4 | 5 | 2.1 ± 0.14 |
| 10 | 12 | 2.5 ± 0.11 |
| 11 | 12 | 2.5 ± 0.14 |
| 18 | 12 | 1.9 ± 0.10 |
| Mean | | 2.3 ± 0.07 |
| | Trial 2[c] | |
| B-2 | 13 | 6.2 ± 0.67 |
| R-3 | 13 | 3.7 ± 0.33 |
| Y-1 | 13 | 3.9 ± 0.71 |
| Y-4 | 13 | 5.3 ± 0.59 |
| Mean | | 4.8 ± 0.32 |

[a]Number of blood samples from each animal.

[b]Mean ± SE of serum LH concentrations from blood samples obtained at 5 min intervals for 60 min in steers castrated 4 months previously.

[c]Mean ± SE of serum LH concentrations from blood samples obtained at 10 min intervals for 120 min in steers castrated 2 months previously.

discrepancy (higher mean serum LH levels and greater fluctuations in trial 2) shown in Table 8 could be due to animal variation between trials. No rhythmic fluctuations existed in either group (2 or 4 months post-castration) which agrees with work by Roche et al. (1970) in which they stated that LH levels were completely uncontrolled after ovariectomy of ewes.

In contrast, Butler et al. (1972) demonstrated pulsatile discharges of LH at regular intervals of 45 to 75 min in ovariectomized ewes (sampled at 15 min intervals). Reeves, O'Donnell and Denorscia (1972) also observed rhythmic oscillations with a periodicity of 52 min in ewes ovariectomized 50 days previously. Similar rhythmic discharges were observed by Dierschke et al. (1970) in rhesus monkeys. Diekman and Malven (1973) investigated rhythmicity of LH release in ewes immediately after ovariectomy and found rhythmic peaks with intervals of 55 to 100 min.

The short overall length of the sampling periods in trials 1 and 2, 60 and 120 min respectively, may have been responsible for failure to detect rhythmic oscillations. The absence of rhythmic oscillations could also be characteristic of these steers or characteristic of all castrated male bovine. It is suspected that a longer sampling period would show a trend toward rhythmicity of serum LH spikes as seen in other species after castration.

## Experiment 2. Steroid Replacement Therapy

Intravenous administration of $E_2$ to chronically castrate Holstein bull calves suppressed serum LH levels within 2 hr (Figure 6a). Estradiol was effective in suppressing serum LH levels and abolished the LH spikes for a period of 0.5 to 4.0 hr after injection, indicated by the smaller standard errors during this period (Table 9). Serum LH levels remained suppressed to 8 hr post-injection. However, three of the steers produced spike releases of LH during this post-injection period and three of the four animals had serum LH peaks of 7.8, 6.8, and 9.6 ng/ml at 12 hr post $E_2$ treatment. No serum LH spikes were detected in one steer treated with $E_2$ between -1 hr and 12 hr post-injection as it had received $E_2$ on the previous day. This animal was not included in calculation of the means and standard errors of Figure 6a.

Intravenous injection of T or A had no effect on serum LH levels (Tables 9 and 10). An animal typical of those receiving androgen treatment is presented in Figure 6b (T 0.5 μg/kg BW). The steers in trial 1 not receiving $E_2$ showed rhythmic fluctuations in serum LH with 30 min sampling (Figure 6b), unlike the sporadic fluctuations seen with 5 and 10 min sampling in Experiment 1. The great differences in the regularity and amplitude of LH fluctuations between these two studies may be partially accounted for by variation

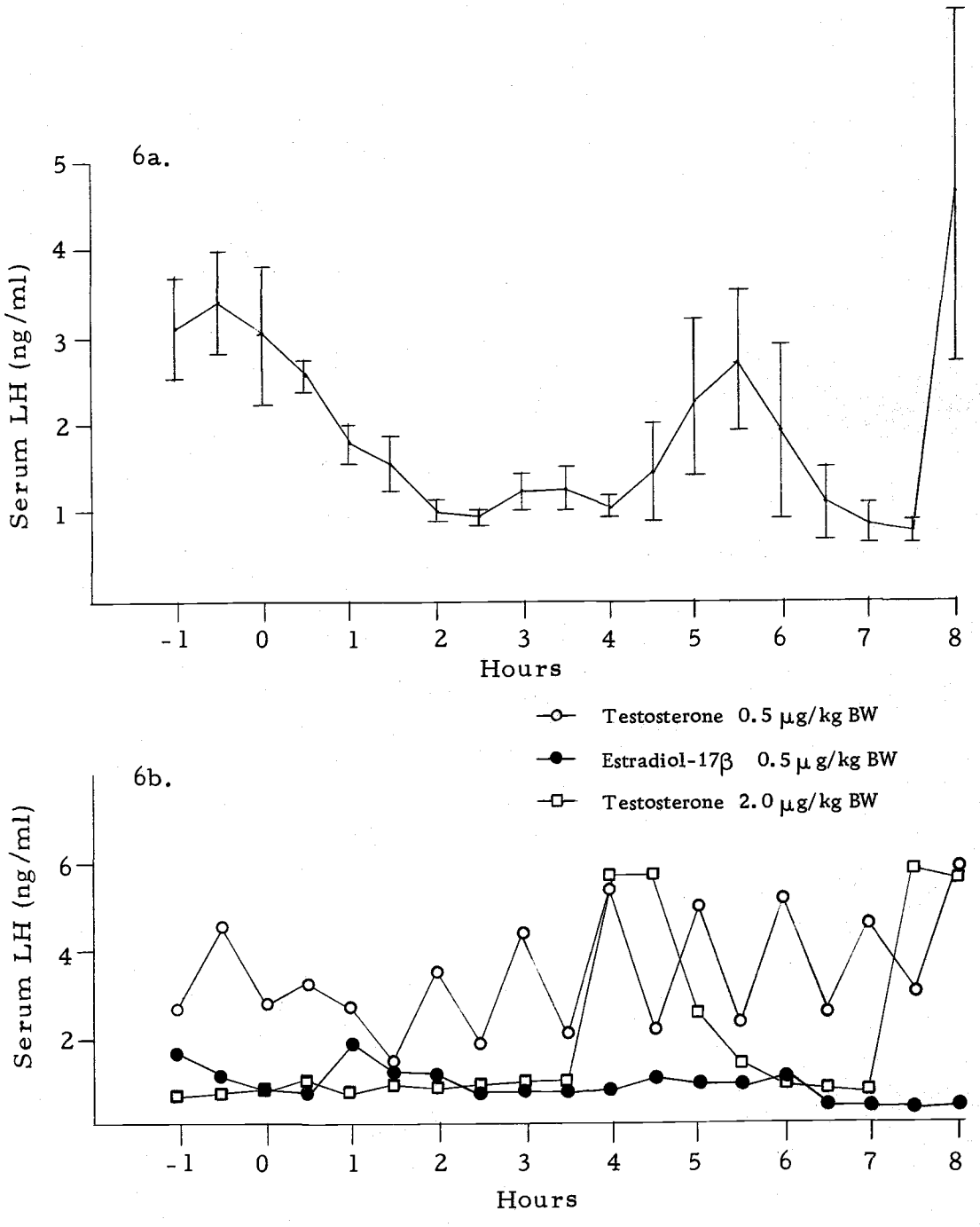


Figure 6. a. Each point represents the mean serum LH concentrations (± SE) of three animals administered estradiol-17β (0.5 or 2.0 μg/kg BW).

b. Serum LH concentrations for individual animals injected intravenously with 0.5 μg/kg BW testosterone, 2.0 μg/kg BW testosterone (estradiol-17β on 2 previous days) or 0.5 μg/kg BW estradiol-17β (0.5 μg/kg BW on the previous day). The time of injection (0 hr) is indicated by the arrow.

Table 9. Serum LH Concentrations (ng/ml) before and after Intravenous Injection of Two Dosages of Estradiol ($E_2$), Testosterone (T) or Androstenedione (A) to 5-Month-Old Holstein Steers Castrated 2 Weeks Previously.

| Treatment | Dosage[a] (μg/kg BW) | n[b] | Time Period | | | | |
|---|---|---|---|---|---|---|---|
| | | | -1.0 to 0.0 hr[c] | n | 0.5 to 4.0 hr | n | 4.5 to 8.0 hr |
| E | 0.5 | 3 | 2.1 ± 0.44[d] | 8 | 1.3 ± 0.16 | 8 | 1.3 ± 0.29 |
| | 2.0 | 3 | 3.3 ± 0.37 | 8 | 1.5 ± 0.14 | 8 | 2.0 ± 0.51 |
| T | 0.5 | 3 | 2.9 ± 0.36 | 8 | 3.0 ± 0.34 | 8 | 3.3 ± 0.37 |
| | 2.0 | 3 | 2.1 ± 0.53 | 8 | 1.9 ± 0.31 | 8 | 3.1 ± 0.48 |
| A | 2.0 | 3 | 3.9 ± 0.55 | 8 | 3.2 ± 0.42 | 8 | 3.4 ± 0.40 |
| | 8.0 | 3 | 2.8 ± 0.37 | 8 | 2.8 ± 0.31 | 8 | 3.2 ± 0.28 |

[a]Two steers were administered each dosage of each steroid.

[b]Number of blood samples taken from each animal in that time period.

[c]Steroids given at 0.0 hr.

[d]Mean ± SE.

Table 10. Serum LH Concentrations (ng/ml) for 8-Month-Old Holstein Steers Given Two Dosages of Testosterone (T) and Androstenedione (A) Intravenously.

| Treatment | Dosage[a] (μg/kg BW) | n[b] | Time Period | | | | |
|---|---|---|---|---|---|---|---|
| | | | -1.5 to 0.0 hr[c] | n | +0.5 to 4.0 hr | n | 4.5 to 8.0 hr |
| T | 6.7 | 4 | 2.5 ± 0.20[d] | 8 | 2.6 ± 0.24 | 8 | 2.9 ± 0.12 |
| | 26.7 | 4 | 3.3 ± 0.34 | 8 | 3.7 ± 0.20 | 8 | 3.7 ± 0.19 |
| A | 26.7 | 4 | 3.7 ± 0.26 | 8 | 3.8 ± 0.28 | 8 | 4.0 ± 0.21 |
| | 106.6 | 4 | 2.8 ± 0.15 | 8 | 3.3 ± 0.15 | 8 | 3.0 ± 0.17 |

[a]Two steers were administered each dosage of each steroid.

[b]Number of blood samples from each steer in each time period.

[c]Steroids given at 0.0 hr.

[d]Mean ± SE.

in the age at which the animals were castrated, variation in the time from castration to the time at which the experiments were conducted and inherent between animal variation.

Androgen treatments in trials 1 and 2 did not alter the frequency, number or peak heights of serum LH peaks, excluding one animal in trial 1 that received T (2.0 μg/kg BW) after receiving estradiol on two previous days. The serum LH levels in this steer are shown in Figure 6b. The LH levels were suppressed during the pre-treatment period, but two sporadic peaks occurred at 4 hr and 7 hr after androgen injection. Excluding the steers treated with $E_2$ and the steer treated with T after $E_2$ treatment on each of two previous days, an average of 5.9 LH peaks occurred in the remaining treated steers in the 8 hr post-treatment period with a mean periodicity within animals ranging from 64 to 98 min. Steers given androgens in trial 2 did not show regular oscillations in serum LH, similar to observations in these same four steers with 5 min sampling in Experiment 1 (Figure 5, trial 1).

In trial 3, intramuscular injection of $E_2B$ resulted in a slight reduction of serum LH during the period 0.5 to 6.0 hr post-injection, but a substantial decrease from pre-treatment levels occurred during the period 6.5 to 12 hr post-injection (Table 11).

Steers given $E_2B$ earlier were used as controls or given other steroids on later days at 2-day intervals as outlined in Table 3. On

Table 11. Serum LH Concentrations (ng/ml) before and after Intramuscular Injections of Estradiol Benzoate ($E_2B$), Testosterone Propionate (TP) and Dihydrotestosterone Benzoate (DHTB) to 7-Month-Old Holstein Steers at 2 Months Post-Castration.

| Treatment | Dosage[a] (μg/kg BW) | n[b] | Time Period | | | | |
|---|---|---|---|---|---|---|---|
| | | | -1.5 to 0.0 hr[c] | n | +0.5 to 6.0 hr | n | 6.5 to 12.0 hr |
| $E_2B$ | 0.5 | 4 | 3.9 ± 0.35[d] | 12 | 3.4 ± 0.23 | 12 | 2.3 ± 0.09 |
| | 2.0 | 4 | 6.4 ± 0.60 | 12 | 4.1 ± 0.29 | 12 | 2.8 ± 0.34 |
| TP | 0.5 | 4 | 3.0 ± 0.24 | 12 | 4.1 ± 0.54 | 12 | 2.8 ± 0.23 |
| | 4.0 | 4 | 5.1 ± 0.23 | 12 | 5.9 ± 0.47 | 12 | 4.8 ± 0.22 |
| | 16.0 | 4 | 3.8 ± 0.20 | 12 | 4.5 ± 0.31 | 12 | 5.0 ± 0.30 |
| DHTB | 0.5 | 4 | 3.8 ± 0.84 | 12 | 3.5 ± 0.68 | 12 | 5.5 ± 0.92 |
| | 4.0 | 4 | 3.7 ± 0.69 | 12 | 4.1 ± 0.29 | 12 | 3.9 ± 0.25 |
| | 16.0 | 4 | 2.0 ± 0.20 | 12 | 2.5 ± 0.28 | 12 | 2.9 ± 0.40 |
| Control | - | 4 | 4.1 ± 0.57 | 12 | 4.0 ± 0.42 | 12 | 4.0 ± 0.30 |
| Control[e] | - | 4 | 1.9 ± 0.14 | 12 | 2.1 ± 0.16 | 12 | 1.5 ± 0.08 |

[a]Dosages are expressed in terms of free steroid injected. Two steers were administered each dosage of each steroid.

[b]Number of blood samples from each animal in each time period.

[c]Steroids given at 0 hr.

[d]Mean ± SE.

[e]Controls receiving $E_2B$ 2 days earlier.

these subsequent days of treatment, they were bled at half-hour intervals for 13.5 hr. Carry-over of the $E_2B$-induced reduction of serum LH from the previous treatment was observed in three of four animals. One steer with a mean pre-treatment serum LH of 7.3 ng/ml received $E_2B$ and two days later LH levels were suppressed to less than 2 ng/ml throughout the 13.5 hr control bleeding period, indicating suppression of LH for at least 60 hr. Samples were not collected from this steer on subsequent days so the time of termination of LH inhibition is not known.

The serum LH in two other steers given $E_2B$ remained suppressed for 98 and 55 hr before peaks occurred. Only 144 hr after $E_2B$ injection did LH levels regain pre-treatment levels and fluctuations. No carryover of $E_2B$-induced suppression could be observed in the fourth steer because samples were not collected after the day of treatment.

TP and DHTB failed to reduce serum LH or interfere with inherent fluctuations (Table 11). Most fluctuations appeared random or uncontrolled although in some animals over short time periods LH spikes occurred at intervals of nearly 60 min.

Each of the four steers served as controls as a part of the treatment regimen and were bled for 13.5 hr every 0.5 hr without receiving steroid injections. Two, however, were treated with $E_2B$ 2 days previously so were not considered valid controls. The

remaining two steers that had not received previous $E_2B$ had serum LH patterns similar to those seen after androgen treatment (Table 11).

Higher levels of TP administered iv also failed to reduce serum LH levels in four steers sampled at 4-hr intervals for 72 hr (Table 12). Mean LH levels and the number of fluctuations within animals were not altered by the treatment.

Finally, 100 mg of TP given im twice daily for 3 days suppressed serum LH levels on the fourth day in both animals treated (Table 13). One steer had reduced levels by the second day of treatment but some minor oscillations persisted.

These studies with steroid replacement therapy indicate that in the castrate male bovine, estrogens are extremely potent in suppressing serum LH levels, whereas androgens (testosterone, testosterone propionate, androstenedione and dihydrotestosterone benzoate) are ineffective at comparable doses. Only extremely large doses of testosterone (non-physiological) given for several days were capable of suppressing LH levels. Previous work with androgens in farm animals involved high levels. The lowest dose reported was a single im injection of 50 μg T/kg BW given to intact rams which suppressed serum LH levels for a 53-hr sample period. In the castrate ram, 220 μg TP/kg BW/day (im) for 2 weeks (Crim and Geschwind, 1972) and single im injections of 4 mg TP/kg BW (Pelletier, 1970) and 6 mg

Table 12. Serum LH Concentrations (ng/ml) before and after Intravenous Injection of Four Dosages of Testosterone Propionate to Holstein Steers (8.5 Months of Age--3.5 Months after Castration).

| Animal No. | Dosage[a] (μg/kg BW) | Time Periods | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | -2.0 to 0.0 hr[c] | n | Day 1 | n | Day 2 | n | Day 3 | |
| R-3 | 64 | 5 | 3.4 ± 0.45[d] | 10 | 4.6 ± 0.70 | 10 | 3.6 ± 0.22 | 10 | 3.2 ± 0.37 |
| Y-4 | 256 | 5 | 4.3 ± 0.69 | 10 | 4.3 ± 0.52 | 10 | 5.2 ± 0.75 | 10 | 7.2 ± 1.56 |
| Y-1 | 512 | 5 | 3.4 ± 0.71 | 10 | 4.8 ± 0.70 | 10 | 10.3 ± 1.94 | 10 | 11.3 ± 1.88 |
| B-2 | 1024 | 5 | 4.5 ± 0.22 | 10 | 7.1 ± 1.21 | 10 | 7.1 ± 1.22 | 10 | 6.0 ± 0.62 |

[a]Dosages are expressed in terms of free steroid administered.

[b]Number of blood samples taken from each animal in each time period.

[c]Testosterone propionate given at 0.0 hr.

[d]Mean ± SE.

Table 13. Serum LH Concentrations (ng/ml) after 100 mg (im) Testosterone Propionate Twice Daily for 3 Days to 9.5-Month-Old Holstein Steers Castrated 4 Months Previously.

| Day[a] | n[b] | Mean ± SE[c] |
|---|---|---|
| 1 | 3 | 5.9 ± 0.55 |
| 2 | 4 | 3.9 ± 0.62 |
| 3 | 8 | 3.5 ± 0.71 |
| 4 | 5 | 1.6 ± 0.13 |

[a]Testosterone propionate was administered on days 1, 2 and 3.

[b]Number of blood samples from each steer each day.

[c]Mean ± SE are for two steers and for all observations each day.

TP/kg BW (Pelletier, 1972) were capable of suppressing serum LH concentrations.

TP dosages as high as 16 μg/kg im and 1024 μg/kg BW iv failed to suppress serum LH in these studies.

Plasma concentrations of testosterone subsequent to these treatments were not determined. A search of the literature failed to reveal any studies in which testosterone was measured after injection, but estrogens have been measured after iv and im administration of 17β-estradiol to rhesus monkeys. Following iv administration of 0.1, 0.2 and 0.5 μg estrogen/kg to female monkeys, serum estrogen levels rose to 180, 322 and 928 pg/ml, respectively

(Yamaji et al., 1972). Calculations using a serum volume of 3.5% of BW for a 5.0 kg monkey (175 ml serum volume) show that the maximum amount of estradiol present in the circulation at one time (175 ml x 180, 322, or 928 pg/ml) equals about 6% of the injected dosage. Intramuscular injection of 3 and 15 μg/kg BW to rhesus monkeys produced estradiol concentrations greater than 2000 pg/ml (Yamaji et al., 1971). The total circulating estrogens in this case were equal to 2% of the injected dose. Using values of 6% and 2% for iv and im injections, respectively, the highest single dosage of TP given in my experiments (1024 μg/kg BW iv and 16 μg/kg BW im) should theoretically produce a maximum circulating steroid concentration of 2100 ng/ml immediately after iv injection and 168 ng/ml after im injection. Both these levels are far in excess of normal peak T levels of 20 ng/ml in the male bovine (Katongle et al., 1971).

Inhibition of LH discharge must require not only certain minimal testosterone levels but also must require the presence of circulating testosterone for a finite time period. After iv treatment with 0.1, 0.2 and 0.5 μg/kg BW $E_2$, serum estradiol was detectable up to 2 hr post-injection (Yamaji et al., 1972), and after im treatment with 3 to 15 μg/kg $E_2$, serum $E_2$ was detectable for 8 to 12 hr post-injection (Yamaji et al., 1971). Experiments with radioactively-labeled T have shown that less than 0.25% of the original radioactivity is present in the serum 6 hr after injection in man (Wang et al.,

1967). In addition, heifers infused with $E_2$ at the rate of 2.6, 10.4 or 41.7 μg/hr for 24 hr had serum $E_2$ concentrations of 3.6, 9.2 and 90.5 ng/ml, respectively, at the end of the infusion period. Eight hr after termination of the infusion $E_2$ levels were 0.8, 4.0 and 6.5 ng/ml, respectively (Swanson, unpublished). Consequently, even with these large doses of TP serum T would be markedly reduced, if not non-detectable, by 12 hr post-injection.

In an attempt to overcome the problem of rapid disappearance of T from the circulation, steers in the final steroid replacement therapy experiment were treated with 100 mg TP twice daily for 3 days (Table 13). The continued presence of T induced suppression of LH levels by the fourth day.

Although it is generally agreed that T is the major regulator of LH secretion in the male (Gomes and VanDemark, 1974), evidence is accumulating for a role of estrogens. The extreme potency of estrogen in suppressing serum LH levels has been shown by our own data in castrate bulls as well as in other species. Gay and Dever (1971) observed $E_2B$ to be 100 times more effective than TP in preventing the post-castration rise in serum LH concentrations in male rats. Also, $E_2$ is more effective in suppressing FSH and LH in intact human males than T (Naftolin, Ryan and Petro, 1971).

In the male, three sources of estrogen capable of playing a role in regulating gonadotropins, have been documented. First, the

testes are a source of estrogens (Lacy, 1967) and produce both estrone ($E_1$) and $E_2$, at least in man (Longcope, Widrich and Sawin, 1972). Peripheral concentrations range up to 107 and 37 pg/ml for $E_1$ and $E_2$, respectively, in normal men.

In addition to testicular production of estrogen, peripheral conversion of androgens could contribute to circulating estrogen titers in the blood (Longcope, Kato and Horton, 1969; MacDonald, Rombaut and Siiteri, 1967). Thirdly, additional conversion of androgens to estrogens has been shown to occur in the brain in vivo and in vitro. Flores et al. (1973) demonstrated that A and T undergo aromatization to $E_1$ and $E_2$, respectively, in perfused homogenates of hypothalamic and limbic tissue from immature and adult rhesus monkey brains. Aromatizing enzymes have been identified in the anterior hypothalamus of rats capable of converting A to $E_1$ (Naftolin et al., 1972) and T and A were shown to be converted to $E_2$ and $E_1$, respectively, by rat brain tissue. The highest concentration of aromatizing enzymes was found in the limbic structures (Weisz and Gibbs, 1974).

It is possible then that testicular estrogens or testicular androgens converted to estrogens in the circulation or in the brain are involved in the regulation of gonadotropins. Naftolin, Ryan and Petro (1971) stated that conversion of T to $E_2$ provides a basis for steroid effects on the hypothalamus.

In contrast to studies suggesting the importance of estrogens, Beyer, Jaffe and Gay (1972) have suggested that the conversion of T to dihydrotestosterone (DHT) is required for the suppressive action of androgens at the brain and the enzyme involved in the conversion of T to DHT, 5$\alpha$-reductase, has been detected in the hypothalamus. DHT, a non-aromatizable androgen (cannot be converted to an estrogen), was significantly more effective in reducing serum LH concentrations in rats than T. High dosages of DHTB were not administered in my experiments, but low dosages of DHTB were as ineffective as low dosages of TP in suppressing serum LH. This indicates that DHT may not be a potent suppressor of LH in steers. That T was effective in reducing serum LH concentrations only at high dosages, that DHT was not effective at low dosages and that estrogen was effective at low dosages suggests that feedback of estrogens or androgens converted to estrogens in the blood or in the brain may actively participate in regulation of gonadotropins in bulls.

## Experiment 3. Gonadotropin Releasing Hormone Treatment

Following iv administration of GnRH to castrate Holstein bulls, serum LH levels increased to greater than 30 ng/ml in all animals (Table 14, trial 1). The maximum serum LH concentrations (ng/ml) were not related to the dosage administered ($r = 0.18$). The total LH discharge from each animal, as estimated by determining the

Table 14. Serum LH Characteristics after Administration of Gonadotropin Releasing Hormone to Holstein Steers.

| Animal | Dose (μg) | Peak Height (μg/ml) | Time to Peak (min) | Time to Return to Baseline[c] (hr) | Area of LH Peak (arbitrary units) |
|---|---|---|---|---|---|
| | | | Trial 1[a] | | |
| R-3 | 10 | 32.4 | 20 | 2.0 | 557 |
| Y-4 | 10 | 32.0 | 20 | 3.0 | 672 |
| Y-1 | 40 | 55.8 | 30 | 3.5 | 1832 |
| B-2 | 40 | 35.6 | 30 | 4.0 | 1056 |
| Y-1 | 80 | 77.8 | 120 | 4.0 | 4795 |
| B-2 | 80 | 33.4 | 30 | 3.0 | 970 |
| R-3 | 160 | 37.6 | 40 | 4.0 | 1209 |
| Y-4 | 160 | 40.8 | 40 | 4.0 | 1742 |
| | | | Trial 2[b] | | |
| B-2 | 2.5 | 15.1 | 20 | 4.0 | 104 |
| R-3 | 5.0 | 25.2 | 30 | 3.0 | 221 |
| Y-1 | 7.5 | 120.5 | 30 | 3.0 | 970 |
| Y-4 | 10.0 | 36.1 | 20 | 3.0 | 357 |

[a]Steers were approximately 7.5 months of age (2.5 months post-castration).

[b]Steers were approximately 8.5 months of age (3.5 months post-castration).

[c]Baseline was determined within each anomal from a mean of the pre-treatment samples.

area of the LH peaks with a planimeter (Table 14), also was not significantly correlated with dosage ($r = 0.36$). But the higher correlation coefficient indicated that evaluating the peak area is a more effective method of examining the response to GnRH than LH peak height. As shown in Table 14, between animal variation (especially the large release by animal Y-1) makes it difficult to detect a dose-response in these steers. In addition, it is possible that the absence of a dose-response is due to nearly maximal stimulation of LH release by these dosages of GnRH.

The second trial, involving lower dosages of GnRH, also produced nonsignificant correlations between dosage and peak heights ($r = 0.42$) and dosage and total area of the LH peaks ($r = 0.51$). Deletion of animal Y-1 from the analysis, leaving only the steers that received 2.5, 5.0 and 10 μg GnRH, gave significant correlations ($p < 0.05$) between dosage and peak height ($r = 0.99$) and dosage and LH peak area ($r = 0.99$)

An increase in peak serum LH concentrations with increasing dosage of GnRH is characteristic of males, as shown by Reeves et al. (1970) in wethers, Pomerantz et al. (1974) in pigs and Zolman et al. (1973) in bulls. Mature dairy bulls given 10, 40 and 160 μg GnRH responded with serum LH peaks of 7.2, 19.3 and 39.1 ng/ml at 20, 53, and 113 min after injection, respectively. The time from injection to the serum LH peak increased with larger dosages of

GnRH, but this did not occur in my trials. Greater sensitivity to GnRH has been reported in castrate animals than intact animals (Reeves et al., 1970) which may explain the higher peak LH concentrations in my study as compared to results reported by Zolman et al. (1973). A dose-response more closely parallel to those reported would probably have been achieved if additional animals could have been used at each dosage level.

## Experiment 4. GnRH and TP

As shown in Table 15, the 4-day TP treatment (119 mg/twice daily) reduced serum LH levels from 5.8 ± 0.90 ng/ml on the day before initation of TP treatment to 2.4 ± 0.21 ng/ml on day 5 (the last day of treatment) and to 2.1 ± 0.06 ng/ml on day 6. These data are based on seven observations across all four animals on each day. Two days following the last TP treatment (day 7) mean serum LH concentrations began to increase (2.8 ± 0.40 ng/ml) from the previous days due to elevated levels in steer R-3 on day 7 (6.4 ± 0.05 ng/ml). The mean serum LH concentration of the remaining three animals for day 7 was 1.6 ± 0.06 ng/ml indicating that steer R-3 had "escaped" from TP suppression earlier than the others.

The number of days required for suppression of serum LH concentrations varied between animals (Table 15). A marked reduction in LH concentrations occurred in steers Y-1 on day 2, B-2 on

Table 15. Serum LH Concentrations (ng/ml) before, during and after Testosterone Propionate Treatment. [a]

| Day[b] | Animal | | | | |
|---|---|---|---|---|---|
| | Y-1 | B-2 | Y-4 | R-3 | Mean ± SE |
| 1 | 3.9 ± 0.12 | 11.1 ± 2.68 | 5.1 ± 0.47 | 3.4 ± 0.30 | 5.8 ± 0.90 |
| 2 | 2.6 ± 0.30 | 5.7 ± 0.80 | 4.9 ± 0.82 | 3.7 ± 0.27 | 4.2 ± 0.37 |
| 3 | 1.9 ± 0.40 | 8.1 ± 1.55 | 1.9 ± 0.09 | 3.6 ± 0.35 | 3.9 ± 0.62 |
| 4 | 1.3 ± 0.05 | 2.3 ± 0.13 | 1.9 ± 0.05 | 5.6 ± 0.51 | 2.8 ± 0.34 |
| 5 | 1.8 ± 0.14 | 2.0 ± 0.08 | 2.3 ± 0.13 | 3.7 ± 0.60 | 2.4 ± 0.21 |
| 6 | 1.8 ± 0.09 | 2.2 ± 0.12 | 2.1 ± 0.04 | 2.3 ± 0.14 | 2.1 ± 0.06 |
| 7 | 1.2 ± 0.03 | 1.8 ± 0.02 | 1.9 ± 0.05 | 6.4 ± 0.05 | 2.8 ± 0.40 |

[a] Mean ± SE are presented for the 7 observations from each animal each day. Blood samples were collected at 0800, 1000, 1030, 1100, 1130, 1200 and 2000 hr on days 1, 5 and 6 and 0800, 1200, 1230, 1300, 1330, 1400 and 2000 hr on days 2, 3 and 4.

[b] TP treatment (119 mg twice daily, im) was given on days 2, 3, 4 and 5.

day 4, Y-4 on day 3 and R-3 on day 6. Serum LH values had decreased to less than 50% of day 1 concentrations in three of the four steers by days 5 and 6.

Administration of 5 μg GnRH iv on the day before TP treatment (day 1) at 4 (day 5) and 28 hr (day 6) after the last TP injection failed to show any marked differences in pituitary responsiveness to GnRH (Table 16).

LH peak heights compared between days 1 and 5 and days 1 and 6 by a paired t-test revealed no significant differences between days. A comparison of serum LH peak areas, as determined with a planimeter, between day 1 and day 5 and day 1 and day 6 by a paired t-test also failed to show differences between days. Most serum LH peaks were greater in this experiment using 5 μg GnRH than peaks in Experiment 3 (Table 14). Assay variability or error in the amount of GnRH weighed out for the two separate stock solutions could contribute to the greater values in Experiment 4.

Objective evaluation of the mean peak areas from each day (Table 16) suggests that more LH is released after TP treatment (days 5 and 6) than before treatment (day 1).

The high dosage of TP was effective in decreasing serum LH concentrations by 50% in all animals while GnRH treatment was equally effective in releasing LH on days 1, 5 and 6. No difference in LH released between days appeared to exist; however, more LH was

Table 16. Serum LH Characteristics after Treatment (iv) with 5 μg GnRH [GnRH was Administered before, during and after Testosterone Propionate (TP) Treatment (119 mg; 2x/day for 4 days)].

| Criteria | Animal | | | | |
|---|---|---|---|---|---|
| | Y-1 | B-2 | Y-4 | R-3 | Mean ± SE (N = 4) |
| | | Day 1[b] | | | |
| Time to peak (min) | 10 | 10 | 20 | 20 | - |
| Peak height (ng/ml) | 101.9 | 55.9 | 57.0 | 33.9 | 62.2 ± 14.27 |
| Time to return to baseline (hr) | 2.0 | 1.5 | 2.0 | 2.0 | - |
| Area[a] | 682 | 464 | 482 | 250 | 469.5 ± 88.28 |
| | | Day 5[c] | | | |
| Time to peak (min) | 30 | 10 | 20 | 20 | - |
| Peak height (ng/ml) | 80.0 | 106.1 | 75.9 | 14.9 | 69.2 ± 19.31 |
| Time to return to baseline (hr) | 2.0 | 3.0 | 2.5 | 2.5 | - |
| Area | 1072 | 1417 | 721 | 159 | 842.3 ± 268.43 |
| | | Day 6[d] | | | |
| Time to peak (min) | 10 | 20 | 20 | 20 | - |
| Peak height (ng/ml) | 63.8 | 104.9 | 68.0 | 56.9 | 73.4 ± 10.74 |
| Time to return to baseline (hr) | 2.5 | 3.0 | 2.5 | 2.5 | - |
| Area | 644 | 1172 | 668 | 556 | 760.0 ± 139.43 |

[a] Area of LH peak determined with a planimeter and expressed in arbitrary units.

[b] Day 1--GnRH given before TP treatment.

[c] Day 5--GnRH given 4 hr following last TP treatment.

[d] Day 6--GnRH given 28 hr following last TP treatment.

released in several animals on days 5 and 6 than day 1. An explanation for this could be that TP treatment inhibits LH release by decreasing the discharge of releasing hormone while not altering LH synthesis, causing increased storage of releasable LH available for discharge by GnRH.

These results indicate that TP causes no detectable reduction in pituitary sensitivity to GnRH. This is contrary to findings by Debeljuk et al. (1973) who observed that testosterone, dihydrotestosterone, 17$\alpha$-hydroxyprogesterone, 20$\alpha$-hydroxyprogesterone and 5$\alpha$-hydroxyprogesterone significantly decreased the release of LH and FSH after GnRH treatment in castrate male rats. Debeljuk et al. (1972) concluded that the suppressive activity of sex steroids is directed, at least in part, at the pituitary level. Earlier findings by Gersten and Baker (1970) and Kingsley and Bogdanove (1971) suggested that both estrogens and androgens can act directly at the pituitary. Although it is becoming apparent that sex steroids are involved at the pituitary in modulating gonadotropin release, this was not substantiated in this study.

It is well established that steroids also act on the hypothalamus where they influence the secretion of releasing hormones (Davidson, 1969), and it appears that this is the major mechanism involved in the decreased serum LH concentrations in these steers. To further substantiate this, quantitation of luteinizing hormone-releasing

hormone levels in serum before and after steroid treatment is needed. However, a more comprehensive study involving prolonged steroid treatment to allow steroid-brain interactions to reach equilibrium and a combination of steroids (estrogens and androgens) to subject steers to a steroid environment similar to that in bulls might show some alterations in pituitary sensitivity to exogenous GnRH.

# SUMMARY AND CONCLUSIONS

Serum LH concentrations in prepubertal Holstein bulls were quantified by radioimmunoassay before and after castration, steroid replacement therapy and GnRH treatment.

Serum LH concentrations were less than 1 ng/ml in intact Holstein bulls 4 to 6 months of age. Serum LH levels increased to $3.1 \pm 0.54$ ng/ml at 11.0 to 47.0 hr post-castration in trial 1, to $1.9 \pm 0.70$ ng/ml at 6.0 to 11.0 hr post-castration in trial 2 and to $2.8 \pm 0.49$ ng/ml at 6.0 to 12.0 hr post-castration in trial 3. The increase in serum LH occurred from 7 to 11 hr after castration, indicating release of the pituitary or hypothalamus from inhibition by testicular products (steroid or non-steroid). LH concentrations oscillated from 1.3 to 3.3 ng/ml when sampled every 5 min over a 55 min period in one group of steers (4 months post-castration) and from 2.1 to 11.8 ng/ml when sampled every 10 min over a 120 min period in a second group of steers (2 months post-castration). Serum LH peaks did not occur at regular intervals in these studies. The magnitude of LH fluctuations varied between animals and the number and magnitude of LH peaks may decrease with time from castration.

Administration of low dosages of androgens by two different routes to prepubertal castrate bulls was ineffective in reducing serum

LH to pre-castration levels or in altering the fluctuations. Intravenous administration of T (0.5 to 32.0 μg/kg BW) and A (2.0 to 128.0 μg/kg BW) via the jugular vein as well as intramuscular injection of androgen esters (TP and DHTB) at dosages equivalent to 0.5, 4.0 and 16.0 μg/kg BW of T and DHT failed to alter post-castration serum LH patterns.

High dosages of TP (equivalent to 64, 256, 512 and 1024 μg/kg BW) administered iv also were ineffective in reducing serum LH levels. However, 100 mg of TP given for 3 days (~884 μg T/kg BW each day) reduced serum LH levels from 5.9 ± 0.55 ng/ml pre-treatment to 1.6 ± 0.13 ng/ml on the day after the last treatment in two steers 9 months of age (BW~190 kg). Not only are high levels of T required to suppress serum LH concentrations, but T must remain in the circulation for a finite time period.

Dosages of $E_2$ (0.5 and 2.0 μg/kg BW), given intravenously, reduced serum LH levels from 2.7 ± 0.10 ng/ml before treatment to 1.3 ± 0.13 ng/ml for the 4 hr period after treatment. Administration of comparable dosages of $E_2$ by intramuscular injection of $E_2B$ also reduced serum LH levels. A decrease in serum LH levels after intramuscular injection of $E_2B$ occurred 4 hr after injection whereas a decrease in serum LH after intravenous injection of $E_2$ occurred about 2 hr after injection.

Of the steroids injected, estrogens were extremely potent in suppressing serum LH levels and androgens were affective only at high dosages (non-physiological) and after repeated injections. This information indicates that estrogen may be important in regulating gonadotropins in the male alone, or in combination with androgens.

GnRH treatment (10, 40, 80 and 160 μg) increased serum LH levels from $\leq$ 5 ng/ml to > 30 ng/ml within 40 min. A distinct dose-response was not evident due to a small sample size and extreme between animal variation. Lower GnRH dosages of 2.5, 5.0, 7.5 and 10 μg, used to determine the minimum effective dosage, caused a graded response with serum LH peaks of 15.1, 25.2, 120.5 and 36.1, respectively. By deleting the steer receiving 7.5 μg GnRH which was extremely sensitive to GnRH, a significant dose-response was obtained.

These steers were more sensitive to GnRH (released more LH) than intact bulls given comparable dosages of GnRH by other workers.

Treatment of four steers with 100 mg T (119 mg TP 2x/day for 4 days) decreased serum LH levels from 5.8 $\pm$ 0.90 on day 1 (before treatment) to 2.1 $\pm$ 0.06 2 days following the final injection. This TP treatment failed to alter pituitary sensitivity to GnRH. Serum LH peak heights and total LH released (peak area) after 5 μg GnRH were not different between day 1 (before TP treatment), day 5 (after 4 days of TP) and day 6 (28 hr after the last TP treatment).

It has been reported that androgens and other steroids alter pituitary sensitivity to GnRH in males and females of many species, but this was not substantiated in these studies in steers. To the contrary, it appears that in steers, the major affect on TP in decreasing LH release occurs by inhibiting LH-RH release at the hypothalamus.

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# APPENDIX

# APPENDIX

## Appendix I. Composition of reagents used in radioimmunoassay.

### A. Reagents for radioiodination

1. 0.01 M Ammonium Bicarbonate buffer pH 8.3
   $NH_4HCO_3$ . . . . . 0.79 g
   Dilute to 1 liter with distilled water.
   Adjust pH to 8.3 with 5N NaOH.
   Store at 4 C.

2. 0.05 M Sodium Phosphate buffer pH 7.5 (Column buffer)
   Solution A
   $NaH_2PO_4 \cdot H_2O$ . . . . . 2.78 g
   Merthiolate . . . . . 0.01 g
   Dilute to 100 ml with distilled water.

   Solution B
   $Na_2HPO_4$ . . . . . 14.21 g
   Merthiolate . . . . . 0.05 g
   Use 16 ml Solution A, 84 ml Solution B, dilute to 400 ml with distilled water.
   Adjust to pH 7.5 with NaOH.
   Store at 4 C.

3. 0.5 M Sodium Phosphate buffer pH 7.5
   Monobasic (0.5 M) $NaH_2PO_4 \cdot H_2O$ 69.01 g
   Dissolve, dilute to 1 liter in distilled water.
   Dibasic (0.5 M)
   $Na_2HPO_4$ . . . . . 70.98 g
   Dissolve by heating, dilute to 1 liter in distilled water.
   Mix 16 ml monobasic, 84 ml dibasic and 100 ml $H_2O$ to give pH 7.5.
   Dispense in 1 ml portions and store at -20 C.
   Store monobasic and dibasic buffers at 4 C.

4. Chloramine-T, 1 μg/μl
   Tightly pack chloramine-T into vials and cover with aluminum foil immediately upon receiving.
   Store at -20 C.

Within 30 min of each iodination, discard approximately 1/2 the contents of a vial, dispense 10 mg chloramine-T, discard the remainder.
Dissolve in 10 ml column buffer.

5. Sodium Metabisulfite, 2.5 μg/μl
Dilute 25 mg $Na_2S_2O_5$ to 10 ml with column buffer.
Use within 30 min of preparation.

6. PBS-2.5% Bovine Serum Albumin (BSA)
Dissolve 25 g BSA (Sigma Chemical Corp.) in PBS by stirring on magnetic mixer.
Dilute to a volume of 1 liter.
Filter through Whatman No. 1 filter paper.
Store at 4 C.

7. Transfer Solution

| | |
|---|---|
| Sucrose | 1.6 g |
| KI | 0.1 g |

Dilute to 10 ml in distilled water.
Store at -20 C in 1 ml portions.

8. Rinse Solution

| | |
|---|---|
| Sucrose | 0.8 g |
| KI | 0.1 g |
| Bromphenol blue | 0.001 g |

Dilute to 10 ml in distilled water.
Store at -20 C in 1 ml portions.

B. Reagents for radioimmunoassay

1. 0.01 M Phosphate Buffered Saline, pH 7.0 (PBS)

| | |
|---|---|
| NaCl | 143 g |
| $NaH_2PO_4$ monobasic phosphate (see Appendix I. A. 3) | 100 ml |
| $Na_2HPO_4$ dibasic phosphate (see Appendix I. A. 3) | 260 ml |
| Merthiolate | 1.75 g |

Dilute to 17.5 liter in distilled water.
Adjust pH to 7.0 if necessary.
Store at 4 C.

2. 0.05 M EDTA-PBS, pH 7.0
   disodium EDTA . . . . . 18.61 g
   Dissolve in approximately 900 ml PBS.
   Adjust pH to 7.0 with 5N NaOH while stirring.
   Adjust volume to 1 liter.
   Store at 4 C.

3. 0.1% Gelatin PBS
   Add 0.5 g Knox gelatin to approximately 400 ml PBS.
   Heat and stir to dissolve.
   Dilute to 500 ml with PBS.
   Store at 4 C.

4. 1:400 Normal Guinea Pig Serum or Normal Rabbit Serum
   Add 39.9 ml 0.05 M EDTA-PBS pH 7.0 to 0.1 ml non-immune guinea pig or rabbit serum.
   Store at -20 C.

5. Bovine LH standards (NIH-LH-B7)
   Prepare stock solution of 1 μg/ml (1 ng/μl) in 0.1% gelatin-PBS.
   Fill nine 50 ml volumetric flasks with approximately 45 ml 0.1% gel-PBS at room temperature.
   Carefully pipette 8, 16, 32, 64, 128, 256, 512, 1024 or 2048 μl of stock into volumetrics with appropriate Hamilton syringes.
   Fill volumetrics to 50 ml volume with 0.1% gel PBS.
   Stopper and invert 25 times.
   Transfer the contents of each volumetric to a beaker and while stirring, dispense 2 ml portions to 12 x 75 mm culture tubes.
   Seal tubes with parafilm.
   Quick freeze all standards in a dry ice-methanol bath.
   Store at -20 C.