PITUITARY RESPONSIVENESS TO CONSECUTIVE GnRH CHALLENGES IN INTACT AND CASTRATE ONE MONTH OLD BULLS

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ABSTRACT

PITUITARY RESPONSIVENESS TO CONSECUTIVE GnRH CHALLENGES IN INTACT AND CASTRATE ONE MONTH OLD BULLS

By

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Holstein bulls were one-month old when assigned to one of three treatment groups to: 1) remain intact (N=5), 2) be castrated (N=6), or 3) be castrated then receive 10 mg testosterone thrice daily (N=4). On day ten of this experiment jugular blood was taken at 15 min intervals from 0900 to 1500 hr. Episodic peaks of LH were observed which ranged in magnitude from 1 to 12.9 ng/ml with a frequency of between 0 to 5 per 6 hr. Serum testosterone increased after each episodic LH peak in intact bulls.

All animals received 3 successive injections of GnRH (20 μg, i.m.) at 2 weeks, one every 12 hr for 36 hr. Average magnitude of LH response (area under LH response curve) to GnRH was greater ($P < 0.01$) in steers (91.4 ± 8.2) than in intact bulls (63.7 ± 8.3) or steers treated with testosterone (35.7 ± 8.4). Magnitude of LH release in response to the first GnRH challenge (82.7 ± 7.6) was greater ($P < 0.01$) than that of the second (58.9 ± 11.9) or third (60.4 ± 9.6).

A significant treatment by time interaction indicated that there was a change in magnitude of LH release in response to the first, second or third GnRH challenge and the degree of change was dependent upon treatment. Both intact bulls and steers given testosterone, but not steers receiving no testosterone, showed a decrease ($P < 0.001$) in response to the second and third GnRH challenge relative to the first. No further decrease was noted between the second and third challenges. In bulls, both androstenedione and testosterone increased after GnRH ($P < 0.05$, $P < 0.10$, respectively).

We conclude 1) that the mechanism responsible for episodic release of LH is present and operative in the one-month old bull and dependent upon testosterone treatment, 2) gonadal secretion and administration of testosterone affect the ability of the synthetic mechanism of the pituitary to restore releasable pools of LH as early as one month of age.

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A THESIS

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TO MY PARENTS

BIOGRAPHICAL SKETCH

Kenneth Joel Tannen was born on August 6, 1950 in New York City, New York. He attended public schools 122 and 143 in the Bronx and graduated from the Scholarship program of DeWitt Clinton High School in June, 1968. He entered the Polytechnic Institute of Brooklyn (now called the Polytechnic Institute of New York) and received a B.S. in the biological sciences in June, 1972.

In the fall of 1972, he began his post-graduate work at Michigan State University in the Department of Physiology. He received his Master's degree in January, 1975.

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INTRODUCTION

Synthesis and release of luteinizing hormone (LH) by the anterior pituitary is controlled in part by hormones secreted by the gonads and hypothalamus. Evidence for a negative feedback of testicular secretion on LH release is the increase in baseline LH concentration in blood that occurs after castration. Evidence for a positive hypothalamic control of LH release from the anterior pituitary came with isolation from hypothalamic tissue of a gonadotropin-releasing hormone (GnRH), which when administered exogenously, caused rapid increases in blood concentrations of LH, and to a lesser extent, follicle stimulating hormone (FSH) concentrations.

Purposes of these experiments were to: 1) evaluate the effect of the presence of the testis and its associated hormones on baseline LH concentration and episodic release of LH, and 2) evaluate the effect of castration and testosterone replacement on the magnitude of LH release induced with exogenous GnRH in immature bulls.

Development of the reproductive system is under direct control of the hypothalamo-hypophyseal system. In the adult, there are established relationships between the

members of the hypothalamo-hypophyseal-gonadal axis. By examining pituitary function of one month old bull calves, information may be gained as to how the pituitary and hypothalamus are effected by the developing reproductive organs of the immature animal.

REVIEW OF LITERATURE

I. Episodic Release of Luteinizing Hormone

Episodic release of a hormone is characterized by the appearance of fluctuating hormone concentrations in blood and the peak and nadir hormone concentrations are significantly different within each episode or event.

Episodic Luteinizing Hormone (LH) release has been reported in the rat (Gay & Midgely, 1969; Gay & Sheth, 1972), sheep (Bolt, 1971; Butler et al., 1972; Diekman & Malven, 1973; Riggs & Malven, 1974), cattle (Smith et al., 1973; Monkonpunya et al. (in press)), and monkeys (Atkinson et al., 1970; Dierschke et al., 1970). Rhythmic patterns of LH release were found in serum of rats 45 days post-gonadectomy; intervals between peaks varied from 20 to 60 min. Mean serum LH concentration was increased 20 fold relative to serum collected pre-castration. Patterns of release were characteristic for individual rats. Butler et al. (1972), reported that peaks in serum LH concentration occurred in intact ewes every 2 hrs. and ovariectomy reduced occurrence of these peaks to between 45 and 75 min. In addition, basal values of serum LH was increased 400% at 3 wks. after castration. Bolt (1971) observed fluctuations in serum levels of LH in intact rams when blood was sampled

at five hr intervals. Similarly, Riggs and Malven (1974) reported that 8-12 month old wethers castrated at one month, had rhythmic surges of serum LH which occurred approximately every 30 min with a range of 27-65 min. Here also, basal serum LH concentrations were significantly elevated in castrates relative to intact controls.

Circhoral oscillation of plasma LH concentrations were observed in ovariectomized adult rhesus monkeys (Dierschke et al., 1970) at two weeks to seven months post-ovariectomy; periodicity was 75 min ± 20 s.d. and magnitude was 40 to 92% of the calculated mean.

Mongkonpunya et al. (1975), reported an average of 3.7 episodic increases in serum LH concentration in intact bulls at 9 months when blood was sampled at 1 hr intervals during 24 hrs. The frequency of these pulsatile surges of LH was increased to 6.5 at 21 days post-castration.

Castration and the administration of exogenous gonadal steroids both exert significant effects upon the pattern of LH release. Gay and Sheth (1972), observed a post-castration increase in serum LH in rats with rhythmic patterns of LH release initiated by castration. No consistent difference was noted between patterns of LH release by gonadectomized male and female rats. Butler et al. (1972) and Diekman and Malven (1973) reported evidence for rhythmic patterns and increased concentrations of serum LH in mature ewes at 18 hrs

after ovariectomy. However, Diekman and Malven (1973) were unable to demonstrate increased serum LH concentration in ovariectomized ewes at 7 to 10 months although the appearance of rhythmic patterns of LH release were reported 42 to 137 hrs post-operatively. In addition, estradiol inhibited LH rhythmicity within 20 hrs and reduced basal serum levels of LH within 80-140 hrs.

The average time interval between episodes of LH release in wethers, was shown to be less than half that for ovariectomized ewes (Riggs and Malven, 1974). Estradiol decreased mean levels of serum LH as early as 3-7 days post-implanatation and only partially inhibited post-castration LH rhythmic pattern of release.

A 10-fold increase in plasma LH over basal concentrations was reported in gonadectomized monkeys at 20 days post-castration and episodic releases of LH were evident (Atkinson et al., 1970). Yamiji et al. (1972), inhibited pulsatile LH discharges and suppressed serum LH concentrations in rhesus monkeys by either a single injection or prolonged infusion of estradiol.

Monkonpunya et al. (in print) were unable to alter neither the number nor change the magnitude of episodic releases of LH by exogenous testosterone administered 3 X a day to castrate bulls.

Insight into the possible mechanism and origins of rhythmic patterns of LH release have been gained through experimentation using pharmacologic blockers and hypothalamic deafferentation. Chlorpromazine and haloperidol cause a brief interruption of the pulsatile rhythm of LH release in the rhesus monkeys which was associated with decline in plasma LH (Bhattacharya et al., 1972). The pulsatile pattern of LH release was resumed 3 to 10 hrs thereafter and was increased in magnitude but decreased in frequency; this pattern mimics the effect of exogenous estradiol. Phentolamine and phenoxybenzamine, α-adrenergic blocking agents, act similarly to chlorpromazine and haloperidol while the β-blocker, propanolol had no effect. This led these researchers to conclude that both norepinephrine receptors and dopaminergic receptors are involved in the episodic release of LH and that estradiol might act at these sites to decrease activity of these neurons and effect rhythmicity of LH release.

Blake and Sawyer (1974), using stereotaxic techniques in ovariectomized rats, were able to interrupt anterior, anterior lateral, and posterior lateral nerve projections to the medial basal hypothalamus without inhibiting the pulsatile circulating levels of LH after ovariectomy. Even with complete deafferentation of the medial basal hypothelamus, the constant estrus animals showed a regular fluctuating

pattern in plasma LH concentration which led the experimenters to speculate that the control of the fluctuating pattern may be inherent to the medial basal hypothalamic-hypophysial unit.

II. Factors Effecting Pituitary Release of LH and Responsiveness to GnRH

A. Endogenous steroids: effect on magnitude of LH release by GnRH

Evidence for a change in magnitude of LH release in response to a GnRH challenge during the estrous cycle or anestrous has been reported. Reeves et al. (1970), observed a decrease in LH response to GnRH in ewes, administered during diestrus relative to that observed at estrus. Later, Nett et al. (1974) and Hooley et al. (1974) found that LH release by GnRH was greatest during proestrus and minimal during diestrus or anestrus in ewes. This lead them to speculate that endogenous steroid ratios, particularly estrogen-progesterone, probably effect the sensitivity of the pituitary to GnRH.

Gordon and Reichlin (1974), administered stalk median eminence extracts to rats during early proestrus and estrous and found increased LH response to GnRH. These researchers also reported that pentobarbital blocked the increased pituitary responsiveness to GnRH and speculated that pituitary sensitization was caused by hypothalamic factors which were stimulated by rising estrogen concentrations.

Zolman et al. (1974), demonstrated in heifers a significant relationship between serum levels of estrone and estradiol and the LH response to GnRH challenges between days 15 of the estrous cycle and following luteal regression.

B. Basal release of LH

Spona (1973), working with rat anterior pituitaries in vitro, observed changes in LH release by pituitaries from rats killed at intervals from birth to 60 days. LH release was minimal in the neonate, and at 40 days. These results led to speculation that pituitary baseline output of LH may change with animal age.

In 1970, Wakabayashi and McCann examined basal LH release from rat anterior pituitary halves collected from intact and castrated males. They reported significantly higher levels of LH released from pituitaries of castrates as compared to controls. One year later, work from the same laboratory (Watson et al., 1971) revealed a ten-fold increase in serum LH of male rats after castration, and these results have been duplicated and confirmed by other laboratories (Root & Duckett, 1973; Debeljuk et al., 1973; Eldridge et al., 1974).

Age and sex of castrate animals have been shown as significant factors in modifying the basal release of LH. Negro-Vilar et al. (1973), reported finding minimal

increases in serum LH in male rats castrated at 15 days of age with a maximal LH response noted in animals castrated at 58 days of age, while Eldridge et al. (1974), found increased serum levels of LH by eight hours post-castration in male rats as compared to forty-eight hours post-castration in female rats. In the bovine, Mongkonpunya et al. (in press) demonstrated that maximum increase in serum LH concentration was achieved by 14 days post-castration in 9 month old bulls.

Gonadal steroids have been shown to influence control over baseline levels of LH release. To establish more precisely the method of gonadal control, the effect of administration of exogenous gonadal steroids was investigated: in rats by Debeljuk et al. (1972, 1974); Eldridge et al. (1974); Spona (1974), in pigs by Pomerantz et al. (in press), in cattle Hobson & Hansel (1974); Mongkonpunya et al. (in press), and in humans by Isurugi et al. (1973). In summary, these researchers demonstrated that they were able to suppress baseline LH below control levels in intact animals and reduce the post-castration rise of LH in castrated animals with various doses and treatments of gonadal steroids such as testosterone, dihydrotestosterone and estradiol or combinations of these steroids. These results indicate that gonadal steroids exert a negative feedback control on LH.

C. LH response to GnRH challenge

Isolation and synthesis of gonadotropin releasing hormone (GnRH) in 1971 provided researchers with the means to circumvent hypothalamic control when investigating release of luteinizing hormone from the anterior pituitary. Now, GnRH could be used to directly test pituitary LH reserves and release rates in animals in various physiological states.

Conflicting evidence has appeared concerning changes in pituitary sensitivity to GnRH with advancing age. In the rat, Spona and Luger (1973) reported decreased LH response to GnRH at 20 and 40 days of age. Pomerantz et al. (1974) found no difference in peak LH response to a GnRH challenge in prepubertal and adult pigs.

Castration, on the other hand, had a profound effect on anterior pituitary responsiveness to GnRH. Wakabayashi and McCann (1970) observed greater release of LH from pituitaries of castrates following a GnRH challenge in vitro as compared to intacts. They speculated the pituitary gland increases its ability to synthesize as well as release LH following castration. In vivo work performed by Watson et al. (1971); Root & Duckett (1973); and Debeljuk et al. (1974) in rats, demonstrated a significant increase in the LH response to GnRH after castration as compared to similarly challenged intact animals.

In sheep, Reeves et al. (1970) injected several different doses of purified porcine GnRH into ewes, wethers and a ram, and reported finding LH peaks in wethers which were 5 times higher than those observed in ewes and the ram. Similar differences between peak LH responses after GnRH in barrows and boars were recently reported by Pomerantz et al. (1974). They speculated that differences in LH serum levels might be partially due to decay rates of serum LH which they found to be 33% of normal in the castrates.

Steers showed greater than two fold increases in LH response to a 40 µg GnRH compared to their pre-castration response (Mongkonpunya et al., in press).

To summarize, the absence of gonadal feedback upon the hypothalamic hypophyseal axis, appears to increase the system's ability to release stored quantities and or increase synthesis of LH in response to an exogenous GnRH challenge. To determine whether gonadal steroids specifically, are involved in changes in pituitary responsiveness to GnRH, investigators administered exogenous steroids and examined their effect on GnRH induced LH release.

A series of experiments performed over a three year period by Debeljuk and co-workers (1972, 1973, 1974) elucidated the effects of specific steroids upon GnRH induced LH release in rats. In all cases, these researchers administered specific steroid treatment forty-eight hours prior to

a 0.1 µg injection of GnRH. Their findings revealed: 1) testosterone, estradiol benzoate, dihydrotestosterone and combinations of these steroids were successful in inhibiting GnRH induced LH release in both castrate and intact male rats; 2) estradiol can augment the LH response to GnRH in castrated male rats; and 3) estradiol can be stimulator or inhibitor depending upon time the steroid was administered relative to time of castration; estradiol given day 1 post-castration, decreased magnitude of LH release but increased the GnRH induced LH rise thereafter. In contrast, Foxcroft et al. (in press) reported that gilts receiving estradiol via silastic implants had significantly reduced baseline LH and the peak LH response to GnRH relative to untreated controls.

Hooley et al. (1974), found they could completely suppress GnRH induced LH release by continuous infusion of progesterone (500 µg/hr for 76 hrs) in ewes.

Hobson and Hansel (1974) examined the in vitro response of pituitary tissue from ovariectomized heifers to GnRH at three and eighteen hours after treatment with estradiol in vivo. A positive effect of estradiol on LH release was not observed until several hours after initial exposure to estradiol. On the basis of these results, the authors suggested that the initial suppressive effects of estradiol occur at the hypothalamus or higher centers and the increase

in LH release to GnRH which develops after several hours results from a direct effect of estradiol on the pituitary.

Administration of 20 mg testosterone thrice daily for seven days beginning at three weeks post castration did not effect magnitude of GnRH induced LH release in steers (Mongkonpunya et al., in press). The authors presently concede that the apparent ineffectualness of testosterone to lower GnRH induced LH release may have been due to the period of time which expired between the time of castration and the initiation of steroid therapy (personal communication). Administration of testosterone to men suffering hypogonadotropin eunichoidism in humans, decreased pituitary responsiveness to a GnRH challenge (Isurugi et al., 1973).

Evidence that gonadal steroids may effect higher centers which control LH release came from work performed on sheep. Nett et al. (1974), demonstrated an average three-fold increase in circulating levels of GnRH in castrate ewes suggesting that the gonads suppress the release of GnRH into the portal blood system.

In summary, the evidence to date indicates that gonadal steroids exert significant effect on the responsiveness of the anterior pituitary to release stores of LH.

The ability of the anterior pituitary to replenish releasable stores of LH was shown using the procedure of repeated administrations of GnRH. Thus, Chakroborty et al.

(1973) found that if they treated prepubertal female pigs with GnRH every six hours for 96 hours, LH response to GnRH by the third injection was significantly decreased as compared to initial values and remained so throughout the duration of the experiment. Similarly, Rippel et al. (1974) observed that a minimal period of 96 hours between consecutive injections of GnRH was required for complete recovery of original LH response in anestrous ewes. Evidence indicates that the pituitary requires a definite time interval between successive GnRH challenges in order to replenish releasable stores of LH.

D. Possible mechanisms and sites of action of GnRH

Insights into the mechanisms and sites of action of GnRH have recently been elucidated by employing binding site studies and experiments with protein inhibitors. Using rat pituitary cell cultures, Grant et al. (1973) were able to demonstrate two binding sites for GnRH on rat anterior pituitary cells. The first binding site showed high affinity, low capacity and high specificity for GnRH and is suspected of being the physiological GnRH receptor. The second site displayed low affinity, high capacity and partial specificity for GnRH. Spona (1974) also found two binding sites for GnRH in rat cell cultures but noted that one site disappeared if castrated rat pituitary cells were used.

It is generally acknowledged that low doses of estradiol will cause release of LH from rat anterior pituitary and that dopamine will release GnRH from stalk median eminence. Schneider and McCann (1970) reported inhibition of these responses *in vitro* if the protein synthesis inhibitors, puromycin and cycloheximide were added to the incubation media. They conclude that both the estradiol stimulation of LH release from the anterior pituitary and the dopamine induction of GnRH release from Stalk median eminence are being produced via intermediary peptides or proteins. One could speculate that gonadal steroid inhibition of GnRH release is activated through a protein intermediary.

III. Changes in Steroid Output by the Testes with Age

Since the time of Aristotle, the presence of functioning testes was known to be needed for the development of male secondary sex characteristics. This premise was clearly demonstrated by the classic experiment of Berthold in 1849 using domestic fowl. Since that time, research exploring factors responsible for the appearance of secondary sex characteristics and spermatogenesis has occupied the time of many investigators.

Recently, with the development of the competitive protein assays for androgens, changes of testicular and blood

plasma concentrations of androgens have been reported for several species. In the bovine fetus, Kim et al. (1972) measured serum levels of testosterone from male and female calves at various times during gestation. At all times examined, male fetuses had significantly higher testosterone levels than females and these elevated levels decreased approximately 2 months prior to parturition. Reyes et al. (1973) reported finding approximately 1.4 μg testosterone per mg testis tissue in human fetus but no appreciable amounts in the fetal adrenals. They also observed a decline in testicular testosterone concentrations occurring just after the decrease in maternal human chorionic gonadotropin.

Changes in testicular and blood plasma androgens in bulls from birth through maturity was the subject of several extensive studies (Lindner, 1959; Skinner et al., 1968; and Rawlings et al., 1972). In summary, these investigators reported finding androgens in the testes of bulls from birth through 17 years of age. The ratio of testosterone to androstenedione in testicular tissue and blood plasma varied significantly with age. During the first 4 months of age, androstenedione was the predominate testicular androgen which fell to low levels by 7 months of age. Testosterone, on the other hand, declined at 5 months only to again increase to near adult levels by 11 months. Lindner also noted that the androstenedione in the blood of calves was rapidly metabolized

by blood enzymes to epitesoterone, the biologically inactive 17 α epimer of testosterone.

Histological explanations for development of secondary sex characteristics and spermatogenesis of the bull had been extensively researched for the past 50 years (Bascom, 1923; Hooker, 1944; Santamarina and Reece, 1957; and Abdel-Raouf, 1960). Bascom (1923) observed interstitial cells appearing in the testes of bovine embryos at 30 mm crown-rump length (CR) and were present continuously from that stage to the adult animal. He noted a relative decrease in the interstitial cell number about the time of birth followed by a subsequent increase post partum. Hooker (1944) stated that in the young calf, the intertubular spaces were filled with mesenchymal cells which were later to differentiate into either fibroblasts or Leydig cells. Abdel-Raouf (1960) examined testicular tissue from bulls ranging in age from 2 days to 6 years. He divided testicular development into the following 5 major phases: 1) infantile phase-- birth to 2 months: sex cords are composed of fetal cells and indifferent basal supporting cells appear in the solid cords; 2) proliferation phase--2-5 months: spermatogonia appear and increase in number; increases are noted in the number of indifferent supporting cells; 3) prepubertal phase--5 to 8 months post-natally: tubule lumen forms; central and basal supporting cells decrease in number; primary spermatocytes

appear as well as spermatids, sperm and Sertoli cells appear toward the end of this phase; 4) pubertal phase--8 to 11 months: indifferent supporting cells all change to Sertoli cells; numbers of spermatogonia and Sertoli cells have reached adult limits; primary spermatocytes increase due to active divisions of spermatonia; 5) post pubertal phase--11 months to senility: there is an increase in sperm production.

Finally, with the development and availability of radioactively labelled precursors of testosterone and cholesterol, enzymatic changes in the biosynthetic pathway for androgens have been dilineated.

Linder (1969) reports the biosynthetic pathway for androgens in bulls is as follows: acetate $\longrightarrow$ cholesterol $\longrightarrow$ 20 α-hydroxycholesterol $\longrightarrow$ Δ^5-pregnenolone $\longrightarrow$ progesterone $\longrightarrow$ 17 α-hydroxyprogesterone $\longrightarrow$ androstenedione $\longrightarrow$ testosterone. Tamaoki et al. (1969) summarized factors which influence androgen production. Testicular tissue of immature rats demonstrated low activity of Δ^5-3 β-hydroxysteroid dehydrogenase and isomerase when compared to adult levels. These enzymes are related to testosterone formation from pregnenolone and their activity can be enhanced by administration of exogenous gonadotropins. Basically, in the immature animal the following was noted: 1) significant activity of catabolic enzymes which convert androgen intermediates to

non-androgen products; 2) there is active catabolism of androgens, particularly testosterone, into non-androgenic or less androgenic products; 3) net synthesis of cholesterol and pregnenolone from cholesterol was not yet active in testes but could be stimulated by exogenous gonadotropins *in vivo*; and 4) enzyme systems related to androgen production were not active enough.

Testicular enzyme systems have also been shown to be stimulated by gonadotropins (Hall & Young, 1968) by stimulating the conversion of cholesterol to 20 α-hydroxycholesterol. These enzyme systems also demonstrated autoinhibition; that is, inhibition by their own end products. Evidence for this effect was presented by Oshima (1967) who administered exogenous testosterone proprionate and noted a decreased incorporation of labelled precursors into androgens.

MATERIALS AND METHODS

I. Design and Procedure of Experiments

A. Episodic release experiment

This experiment was designed to show evidence of random, spontaneous increases in serum LH in one month old bulls which were intact, castrated and castrated with testosterone replacement therapy.

Seventeen 1 month old bulls (average b.w. 70.7 $\pm$ 3.8 kg) were assigned to one of three treatment groups and were: 1) left intact (N=5); 2) castrated (N=6), or 3) castrated and injected with testosterone (N=4). During the course of the experiment, one animal from group 3 died and another animal from the same treatment group was diagnosed cryptorchid. The results obtained from these animals were excluded from analysis.

On day 0 of the experiment, animals in groups 2 and 3 were castrated using established veterinary techniques. Animals in the castrate plus testosterone group were immediately started on a schedule of thrice daily injections of testosterone. Ten mg testosterone (Δ^4-Androstene-17 β-ol-3-one; Sigma Chemical Co.) dissolved in 1 ml safflower oil (Pacific Vegetable Oil Corporation; San Francisco, California)

was administered by intramuscular injection at 0700, 1500, and 2300 hours daily throughout the duration of the experiment.

On day 10, jugular cannulation (Vikim Vinyl Tubing, size V 10, Bo Lab, Derry, New Hampshire) was performed on each animal. Approximately 20 cm of the 50.8 cm length of tubing was inserted into a jugular vein and affixed to the neck with tag cement (Nasco, Fort Atkinson, Wisconsin) on 7.6 x 12.7 cm adhesive tape. Each cannula was flushed with 50% dextrose in 3.5% sodium citrate and sealed until used for blood collection. The blood sampling procedure included the following steps: 1) 3 ml of blood and citrate were withdrawn and discarded, 2) 5 ml of blood was taken and transferred into a 18 x 85 mm test tube, and 3) the cannula was flushed with 3.5% sodium citrate and sealed.

Starting at 0900 on day 11 post-castration, 5 ml of jugular blood were withdrawn from each animal every 15 min for 6 hr (25 samples). Blood samples were left at room temperature for 2-4 hr, and then at 4°C overnight before sera were separated by centrifugation at 2500 x G for 15 min. Sera were stored at -20° until assayed for LH, androstenedione and testosterone.

B. Consecutive GnRH challenges

This experiment was designed as a 3 x 3 split plot with repeat sampling, to study the anterior pituitary's ability

to replenish releasable stores of LH after consecutive challenges with GnRH.

The same bulls and treatment groups used in the previous experiment were used here. On day 14, 2 weeks post-castration, each bull received three injections of GnRH, one every 12 hours for 36 hours. The challenge consisted of 20 µg GnRH (lot 3549-215 b + c, Ref: 4545-187, Abbott Laboratories, North Chicago, Illinois) dissolved in 1 ml of 0.85% sodium chloride solution and was administered intramuscularly. Jugular blood was sampled by indwelling cannula at intervals of -20 and 0 min before GnRH and then at intervals of 10, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min after GnRH. Blood and serum samples were handled as described before, until LH, testosterone and androstenedione were quantified.

Numbers of episodic LH peaks were analyzed, after square root transformation of the data, by one way analysis of variance (Steel and Torrie, 1960). Magnitude of the episodic peaks of LH was analyzed as a nested design classification with unequal numbers (Sokal and Rohlf, 1969). Selected contrasts were compared using orthogonal contrasts (Sokal and Rohlf, 1969).

Variance of the LH response to GnRH was analyzed by the split plot method for repeat measurements as described by Gill and Hafs (1971). Selected contrasts were compared using orthogonal contrasts (Sokal and Rohlf, 1969).

Changes in serum androstenedione and testosterone in intact animals were analyzed by a split-split plot analysis of variance. Testosterone changes over time were also analyzed using linear regression analysis.

II. Radioimmunoassay of Testosterone

A rapid procedure for radioimmunoassay (RIA) of testosterone has recently been developed and validated by Mongkonpunya et al. (1975) in our laboratory. In comparison to assay techniques employing gas-liquid chromatography or competitive protein binding, RIA has greater sensitivity and testosterone can be accurately quantitated in as little as 100 µl of serum.

Assay procedures utilized duplicate aliquots of serum (either 100 or 200 µl) which were placed in 15 x 80 mm disposable culture tubes. To account for procedural losses, 3000 dpm ^{3}H-1,2-testosterone (New England Nuclear, Boston, Massachusetts) was added to a third aliquot of a representative number of unknowns randomly placed throughout each assay (10 to 20 per assay). Two ml of nanograde benzene: hexane (1:2) were added to each tube and they were allowed to set for 30 min at room temperature. The contents of each tube was vortexed for 30 sec and stored at -20° for at least 1 hr to freeze the aqueous phase. With precautions taken to prevent thawing the aqueous phase, the organic solvent

from tubes with ^{3}H-testosterone was decanted into scintillation vials and that destined for RIA was decanted into 12 x 75 mm disposable culture tubes.

Testosterone (Sigma Chemical Company) standards were pipetted from a stock solution having a concentration of 10 ng/ml and at least three sets of standards containing 0.0, 0.02, 0.05, 0.10, 0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 ng were included in each assay. Testosterone standards and serum extracts were dried by air and 200 µl of antibody[1] (diluted from 1:3000 in 0.1% Knox gelatin in 0.1 M phosphate-buffered saline (PBS), pH 7.1; Appendix I.B.1) was added. The content of each tube was mixed for 20 sec on a vortex and allowed to incubate at room temperature for 30 min. Then about 30,000 dpm ^{3}H-testosterone (^{3}H-1,2,6,7-testosterone; New England Nuclear; 91 c/mM diluted in 200 µl 0.1% Knox gelatin PBS, pH 7.1) was added to each tube. The contents of the tubes were vortexed for 5 sec and incubated at 4° for 12-24 hr. To separate free from bound testosterone, 0.5 ml of dextran-coated charcoal (0.025 gm Dextran 150 and 0.25 gm carbon decolorizing neutral Norit in 100 ml distilled water) was added at 0°, and the contents of each tube was vortexed for 5 sec, equilibrated in an ice bath for 10 min and centrifuged (2500 x G) for 10 min at 4°. A 0.5 ml

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[1]Dr. G. D. Niswender furnished rabbit antiserum to testosterone-3-oxime-bovine serum albumin; antiserum code number 667.

aliquot of the supernatant fluid was diluted with 5 ml of liquid scintillation fluid (Appendix II.A) for quantification of radioactivity in a liquid scintillation spectrometer (Nuclear Chicago Corporation, Isocap 300). For comparison among assays, sera with high and low testosterone concentrations, and extracts from blank extraction tubes are assayed with each set of unknown serum samples.

III. Radioimmunoassay of Androstenedione

The procedure for radioimmunoassay (RIA) of androstenedione was similar to that for testosterone and was developed and validated in our laboratory by Mongkonpunya et al. (1975). Minor differences between these assay systems are described below.

Aliquots of 100 µl of serum sample were extracted due to the expected higher concentration of androstenedione than testosterone in calf blood. Androstenedione antiserum[1] was diluted to 1:1000 in 0.1% Knox gelatin in PBS. To separate bound and free androstenedione, 0.5 ml of 1.0% carbon decolorizing neutral Norit and 0.5% Dextran T 70 in glass distilled water was added to each tube.

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[1]Dr. G. D. Niswender furnished androstenedione antiserum prepared against 6 α-succinyl androstenedione; antiserum code number 866

IV. Radioimmunoassay of Luteinizing Hormone (LH)

A. Preparation of antiserum

Fifteen mg NIH-LH-B8 were dissolved in 15 ml of 0.85% NaCl and Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) added in a 1:1 ratio, emulsified in a Servall Omni-mixer (Ivan Sorvall Inc., Norwich, Connecticut). Twelve guinea pigs (approximately 400-475 gm body weight (BW) were injected intradermally in the dorsal region at 10-20 sites per animal. The above procedure was repeated 17 and 31 days later except Freund's incomplete replaced complete adjuvant. Five days after the last immunization each guinea pig was anesthetized with ether and 10 ml blood collected by intracardiac puncture. The blood was allowed to clot at room temperature, then placed at 4° overnight. The following day, the blood was centrifuged at 2500 x G for 20 min, the sera decanted and stored at -20°.

B. Radioiodination of LH

Purified bovine LH (LER-1072-2) had been previously dispensed into 1 ml vials (2.5 µl of a 1 µg/µl solution in glass distilled water) and stored at -20°. The contents of these vials were thawed immediately before iodination. Iodination was performed at room temperature. Twenty-five µl of 0.5 M sodium phosphate buffer at pH 7.5 (Appendix I.A.1) was added to the hormone and mixed. One mCi of ^{125}I (80-140 mCi/ml., Amersham/Searle Corp., Arlington

Heights, Illinois 60005) was added, and the contents gently mixed.

Forty µg chloramine-T (Appendix I.A.3., Eastman Organic Chemicals, Rochester, New York) was next added to the vial, the vial stoppered, and the contents were mixed by rotating the vial. The reaction was stopped at exactly 2 min by adding 125 µg sodium metabisulfite (Appendix I.A.4.). After thorough mixing, 25 µl of 2.5% bovine serum albumin (BSA, Nutritional Biochemicals, Inc., Cleveland, Ohio) in 0.01 M phosphate buffered saline (PBS) pH 7.0 was added to diminish the loss of hormone adhering to the glass vial.

A 1 x 12 cm glass column packed with Bio Gel P-60 (BioRad Labs., Richmond, California) equilibrated with 0.05 M sodium phosphate buffer pH 7.5 (Appendix I.A.2.) then washed with 2 ml PBS-2.5% BSA to reduce non-specific binding of the hormone. One hundred µl of transfer solution (Appendix I.A.5.) was added to the vial with iodinated LH and the contents of the vial were layered beneath the buffer on the surface of the column. Seventy µl of rinse solution (Appendix I.A.6.) was added to the reaction vial, recovered, and also layered beneath the buffer on the column. Iodinated LH was eluted from the column with 0.05 M sodium phosphate buffer and 15 ml were collected in 1 ml aliquots from the column in 12 x 75 mm disposable glass tubes containing 1 ml of 2% Knox gelatin in PBS. The elution profile was determined by

quantifying radioactivity in 10 µl from each of the 15 fractions in an automatic gamma counter (Nuclear Chicago Corp., Des Plaines, Illinois). The first peak represented iodinated LH and the second peak represented free ^{125}I. ^{125}I-LH in the tube containing the greatest quantity of radioactivity was used in the LH RIA. The iodinated LH was quite stable; stored at 4°, it could be used up to 2 weeks.

C. Radioimmunoassay

Each unknown bovine sera sample was assayed in dilution duplicate. Two selected dilutions made in PBS-0.1% Knox gelatin (Appendix I.B.4.) of each unknown were added to disposable glass culture tubes (12 x 75 mm) using an automatic pipette (Micromedic Automatic Pipette, high speed model 25004, Huntsville, Alabama). To each lot of 100 tubes was included 12 tubes containing 0.088, 0.125, 0.176, 0.25, 0.352, 0.5, 0.704, 1.0, 1.408, 2.0, 4.0 and 8.0 ng LH (NIH-LH-B8, National Institutes of Health, Endocrinology Study Section, Bethesda, Maryland; Appendix I.B.5.).

Two hundred µl of LH antibody (first antibody; Appendix I.B.7.) was added at a dilution of 1:600,000 to each of the culture tubes and the tubes were incubated at 4° for 24 hr. Solutions of ^{125}I-LH for RIA were prepared by diluting the stock ^{125}I-LH with PBS-0.1% Knox gelatin so that 100 µl contained about 20,000 cpm. One hundred µl of ^{125}I-LH solution was then added to each tube. Incubation was continued at 4° for 24 hr.

Sheep anti-guinea pig gamma globulin (SAGPGG, second antibody, Appendix I.B.8.) was diluted to a titer which would optimally precipitate the guinea pig gamma globulin. The second antibody formed an antigen-antibody-antibody complex large enough to be precipitated by centrifugation. Two hundred µl of SAGPGG was added to each tube and incubation continued for 72 hr. After each addition, the tubes were vortexed gently and covered during the incubation to retard evaporation.

Following the final incubation, 2.5 ml of cold PBS (Appendix I.B.1.) was added to each tube to dilute the unbound ^{125}I-LH. The bound ^{125}I-LH was precipitated by centrifugation at 2500 X G for 30 min in a refrigerated centrifuge with a swinging bucket rotor (Sorvall Model RC-3, Ivan Sorvall, Inc., Norwalk, Connecticut). The supernatant fluid was decanted and the tubes allowed to drain for 30 min and remaining fluid adherent to the neck and lip of the tube was removed with absorbent tissue. The bound ^{125}I-LH of the precipitate was then quantified in an automatic gamma counter. Samples were usually counted for 10 min or for a total of 4000 counts, whichever accumulated first. This information was punched automatically on paper tape by a Teletypewriter (Teletype Corp., Skokie, Illinois). The standard curve was calculated by multiple regression analysis on a CDC 3600 computer. The values for standard LH assay fit

linear, quadratic and cubic components of the regression equation: correlation coefficients were consistently 0.99 to 1.00. These regression coefficients were entered manually into an Olivetti computer (Programma 101, Olivetti Underwood, New York, New York). The counting time for each unknown was entered into the computer from the punched tape through a Punched Tape Editor (Beckman Model 6912 Tape Editor, Beckman Instruments, Inc., Fullerton, California) and LH concentrations in the unknowns were computed automatically.

Control tubes were included in each assay to determine background radioactivity (tube containing 1:400 control guinea pig serum in place of the first antibody), total counts added (tube containing only ^{125}I-LH) and counts in the precipitate (tube containing no unknown or standard, zero tube). Values for the duplicate standards were averaged and plotted at the percent of ^{125}I-LH precipitated at each dose of LH.

D. Assay validation

Guinea pig anti-bovine LH sera were diluted to 1:1000, 1:10,000, 1:20,000, 1:40,000, 1:60,000, 1:80,000, 1:100,000, 1:200,000, 1:400,000, 1:600,000 in normal guinea pig control sera dissolved 1:400 in PBS-EDTA (Appendix I.B.2.). Each dilution was used as first antibody and added to 3 sets of 12 tubes containing LH as mentioned previously. Radioimmunoassay was then performed as if the sets of standards were

unknown samples. First antibody (guinea pig anti-LH sera) dilutions which demonstrated 20-30% binding when compared to the total precipitate tubes were selected for cross-reactivity, parallelism and total recovery studies.

The ability of first antibody to recognize LH from other bovine anterior pituitary hormones in solution was tested by radioimmunoassay of these hormones using the designated first antibody and ^{125}I-LH. To separate 12 x 75 mm culture tubes was added 0.0, 0.1, 1, 10, 25, 50, 100, 250, 500, and 1000 ng of bovine prolactin (NIH-P-B3), growth hormone (NIH-GH-B17), follicle stimulating hormone (NIH-FSH-B1), and thyroid stimulating hormone (NIH-TSH-B4 and Dr. J. G. Pierce-TSH). Two hundred µl of the selected guinea pig LH antisera dilution was added and radioimmunoassay carried out as before.

To demonstrate bovine sera parallelism within the same assay system, first antibody was added to culture tubes containing 10, 20, 25, 40, 50, 75, 100, 125, 150, 200, 250 and 300 µl of standard bovine sera containing high and low concentrations of LH. Radioimmunoassay of these samples was performed as before.

The ability of the antibody to differentiate quantities of LH in blood sera was demonstrated by the addition of 1.0 or 2.5 ng NIH-LH-B8 to 100, 200 and 300 µl of sera containing low and high concentrations of LH. The assay system

should then be able to differentiate and detect the differences in sera LH content due to the addition of the exogenous hormone.

All the validation procedures were undertaken within one radioimmunoassay.

RESULTS

I. Validation of Assay

Results of the bovine pituitary hormone cross-reactivity study are shown in Figure 1. A typical dose response curve with NIH-LH-B8 is shown on this figure. The useful range of assay sensitivity for measuring bovine luteinizing hormone was between 0.1 and 4 ng (86 to 18% binding). Increasing the concentration of pooled bovine serum gave a dose response curve which was parallel ($P < 0.01$) to the LH standard curve. When 1 ng of NIH-B8 luteinizing hormone was added to various dilutions of bovine serum, it was quantitatively recovered (approximately 92% efficient).

Specificity of the assay was excellent. NIH-GH-B17 cross-reacted with this antibody to the extent of about 1.4% but all cross-reactivity could be accounted for by the quantity of LH contamination in the NIH-GH-B17 preparation. NIH-TSH-B17 was as effective as LH in displacing I^{125} LH from the antibody, yet bovine TSH purified by Dr. J. G. Pierce displaced less than 0.2% of the label. This discrepancy between preparations is probably due to the fact that in the preparation of NIH-TSH, LH activity of the preparation is selectively destroyed by oxidation with hydrogen peroxide.

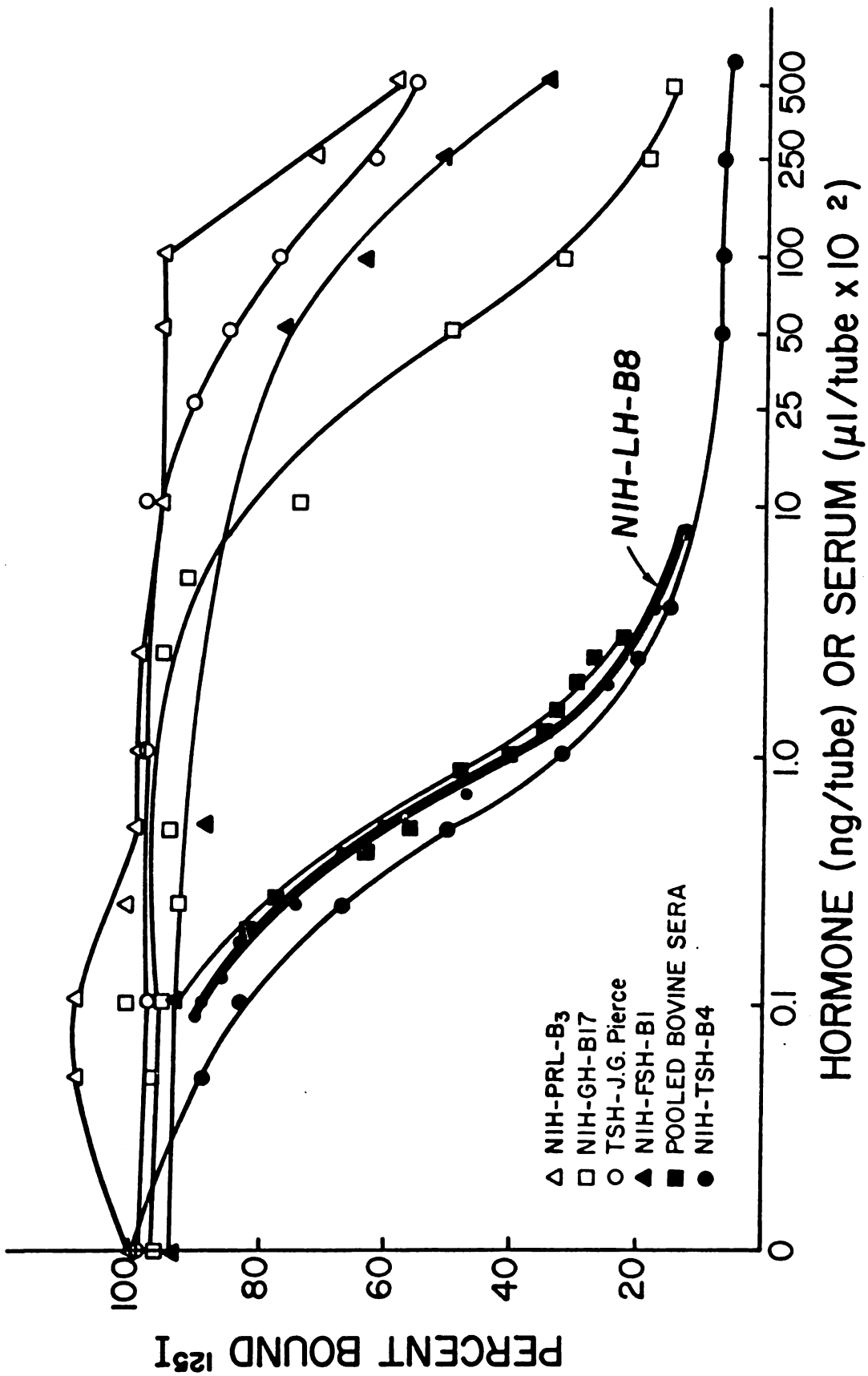


Figure 1. LH standard curve and antibody cross-reactivity curves.

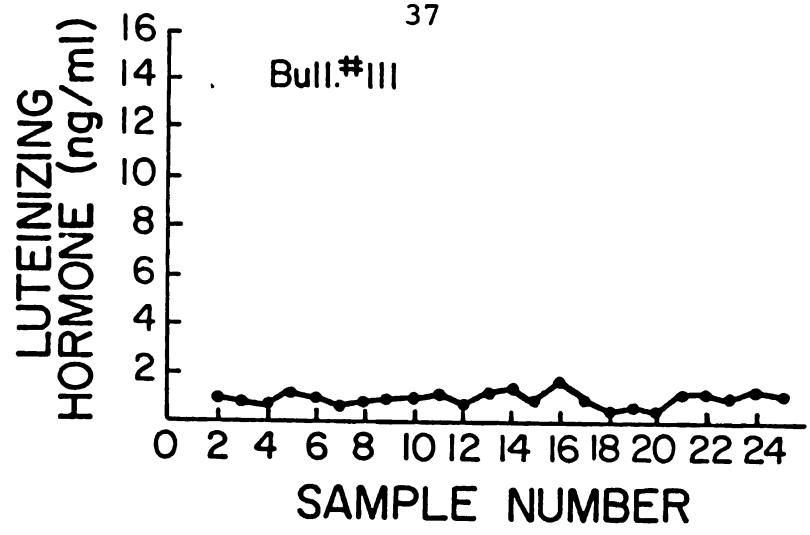
It would appear then that the antibody is cross-reacting with a subunit or fragment of the LH molecule which remained in the NIH-TSH preparation after the parent molecule was destroyed. The cross-reactivity with LH of NIH-FSH-B1 was less than 0.3% and NIH-PRL-B3 showed only 0.05% cross-reactivity. Thus, the radioimmunoassay is highly specific and sensitive for bovine luteinizing hormone.

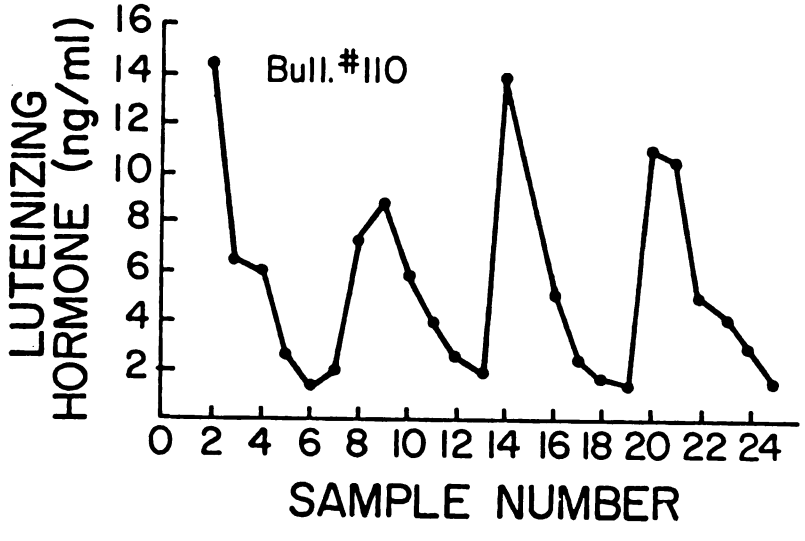
II. Episodic Release of LH

A. LH response

At least one episodic peak of LH was observed in serum collected during the 6 hr sampling period, in 2 of 4 steers given testosterone, 5 of 6 steers receiving no replacement testosterone and 3 of 4 intact bulls. For purposes of this study, an episodic peak of LH was defined as a difference in serum LH equal to or greater than one nanogram between nadir and peak. Patterns of episodic LH release are shown on Figure 2; the figure illustrates fluctuating LH serum levels in a representative steer or bull from each treatment group. The number of episodic peaks of LH ranged from 0 to 5 within the sampling period. Analysis of variance (Table 1) revealed a highly significant effect ($P < 0.001$) of treatment on the number of random episodic LH peaks with steers showing a greater number of peaks than either steers given testosterone or intact bulls. The number of episodic increases

Figure 2. Patterns of episodic LH release for a steer given testosterone (Bull #111), an untreated steer (Bull #110) and an intact bull (Bull #118).


Bull.#111
LUTEINIZING HORMONE (ng/ml)
16
14
12
10
8
6
4
2
0 2 4 6 8 10 12 14 16 18 20 22 24
SAMPLE NUMBER


Bull.#110
LUTEINIZING HORMONE (ng/ml)
16
14
12
10
8
6
4
2
0 2 4 6 8 10 12 14 16 18 20 22 24
SAMPLE NUMBER

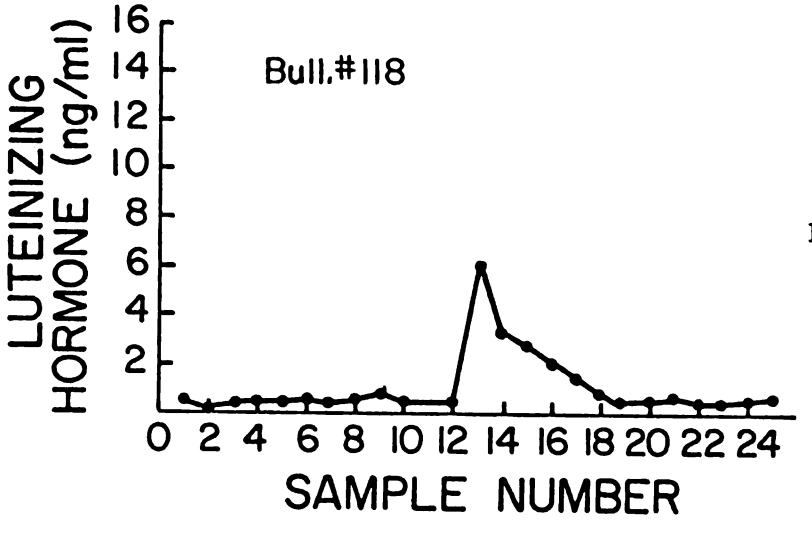

Bull.#118
LUTEINIZING HORMONE (ng/ml)
16
14
12
10
8
6
4
2
0 2 4 6 8 10 12 14 16 18 20 22 24
SAMPLE NUMBER

Figure 2

Table 1.--Summary of analysis of variance for number of episodic[a] peaks of luteinizing hormone.

Source	dF	SS	MS	F	P_1
Total	14	14.39			
Treatments	2	10.78	5.39	17.9	0.001
Animal	12	3.61	0.30		

[a]An increase greater than or equal to 1 ng LH/ml serum.

in serum LH of steers was shown by orthagonal contrasts of treatment means to be greater than the comparable values for steers given testosterone or intact bulls.

Episodic peaks of LH in serum ranged in magnitude from 1 to 12.9 nanograms between nadir and peak. Analysis of variance revealed no significant difference in magnitude of episodic LH peaks due to treatment (Table 2). But animal differences in magnitude of the episodic peaks was significant ($P < 0.005$).

Table 2.--Summary of analysis of variance for magnitude of episodic peaks[a] of luteinizing hormone (LH).

Source	df	SS	MS	F	P_1
Total	26	313.34			
Treatments	2	59.23	29.62	1.20	N.S.[b]
Animal/Treatment	7	173.31	24.76	5.20	< 0.005
Peaks/Animal	17	80.81	4.75		

[a]An increase greater than or equal to 1 ng LH/ml serum.
[b]Not significant ($P < 0.05$).

B. Profile of testosterone during sampling period

The profile of serum testosterone levels of each treatment group are shown in Figure 3. Serum testosterone in steers administered exogenous testosterone decreased from 10.3 ± 2.4 ng/ml at 0900, one hour after testosterone injection, to 1.7 ± 0.1 ng/ml at 1500, one hour before the next testosterone treatment. Comparison of mean serum testosterone concentration of steers (0.89 ± 0.04) and bulls (1.1 ± 0.06) reveals no significant differences. There were 2 episodic increases in serum LH observed in these bulls and each was followed by an increase in serum testosterone which averaged 0.5 ng/ml over baseline.

III. Luteinizing Hormone Release by Gonadotropin-Releasing Hormone

A. Magnitude of LH release

Pituitary responsiveness to each GnRH challenge was quantitated by measurement of the area under the LH response curve--Table 3. The characteristic LH response for each treatment group after each GnRH challenge is shown in Figures 4, 5 and 6.

Analysis of variance revealed a significant effect of treatment, time of treatment (first, second or third GnRH challenge), and a significant treatment by time interaction, Table 4. Comparison of the overall treatment means showed that the average magnitude of LH response to GnRH was greater

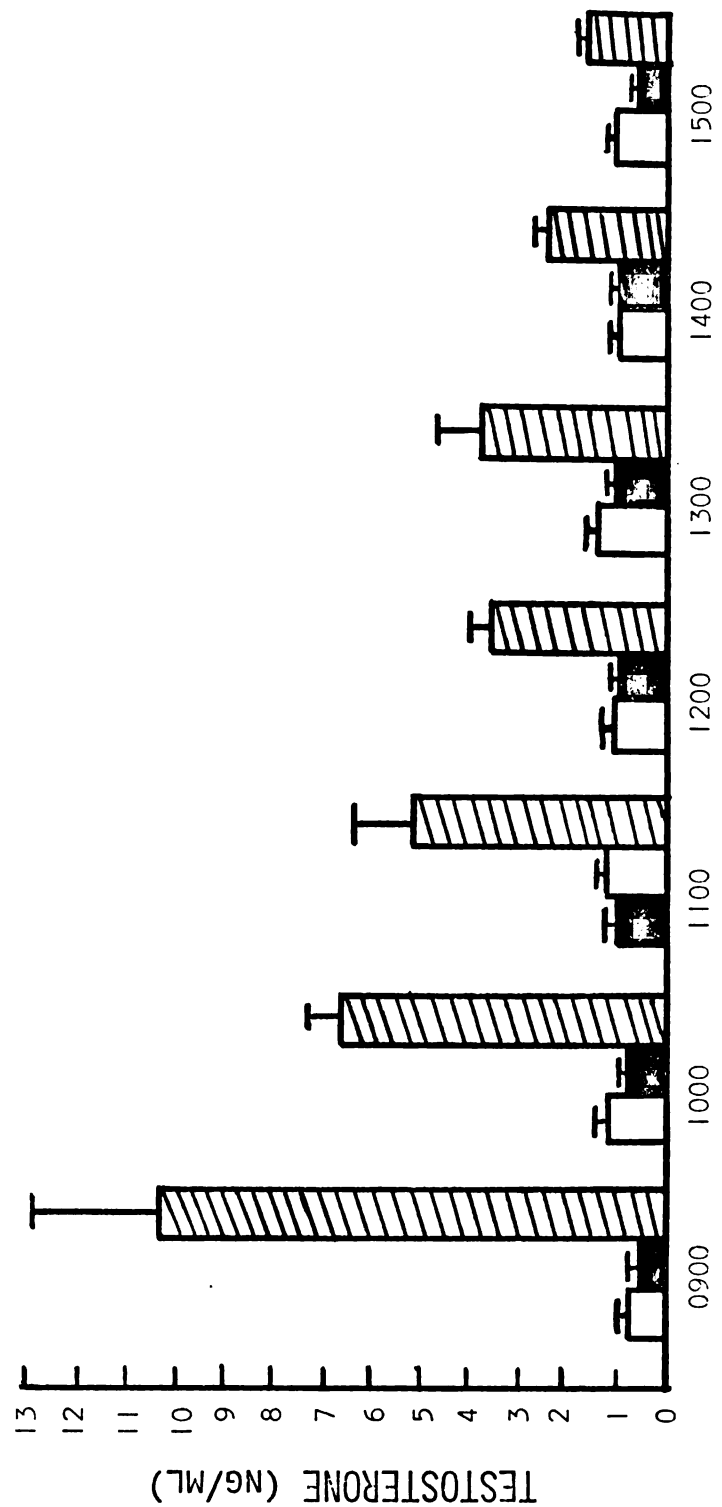


Figure 3. Serum testosterone levels of intact bulls (white bars), untreated steers (black bars) and steers given testosterone (shaded bars) at selected times during sampling period.

Table 3.--Luteinizing hormone (LH) response to Gonadotropin-releasing hormone as measured by average peak LH values and the area under the LH response curve.

Time[a]	Treatment	LH Response	
		Peak (ng/ml)	Area (cm^2)
1	Intact	12.96±3.91[b]	82.32±16.48[b]
	Castrate	15.15±1.56	92.23±10.27
	Castrate + Testosterone	10.75±2.59	68.75±13.91
2	Intact	10.48±2.37	48.10±12.49
	Castrate	16.02±4.08	96.88±18.15
	Castrate + Testosterone	2.33±0.55	15.52± 2.44
3	Intact	10.22±1.91	60.68±11.73
	Castrate	15.48±3.15	85.22±15.46
	Castrate + Testosterone	5.53±2.08	22.93± 4.37

[a]Numbers represent first, second and third GnRH challenges, each are twelve hours apart.

[b]Standard error of the mean.

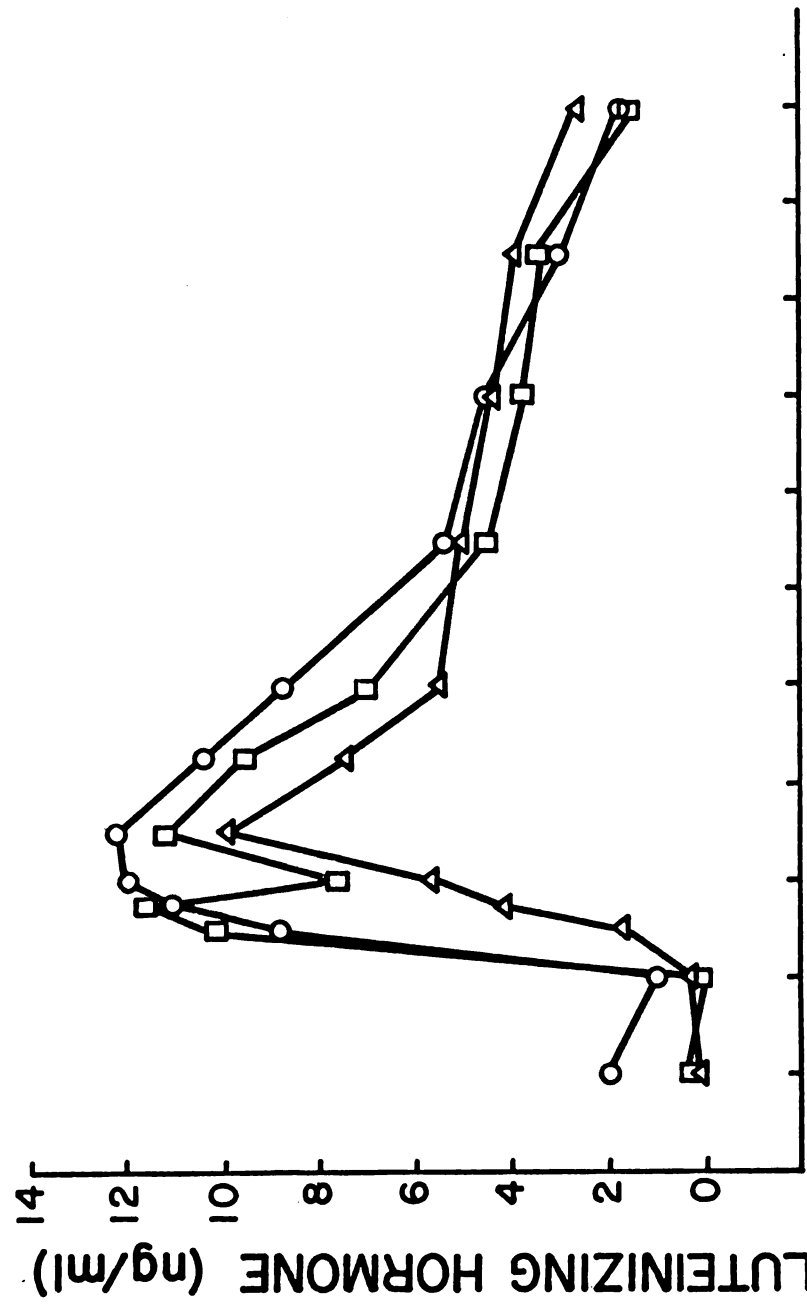


Figure 4. Serum LH in intact bulls (□—□), untreated steers (○—○), and steers given testosterone (△—△) after the first GnRH challenge.

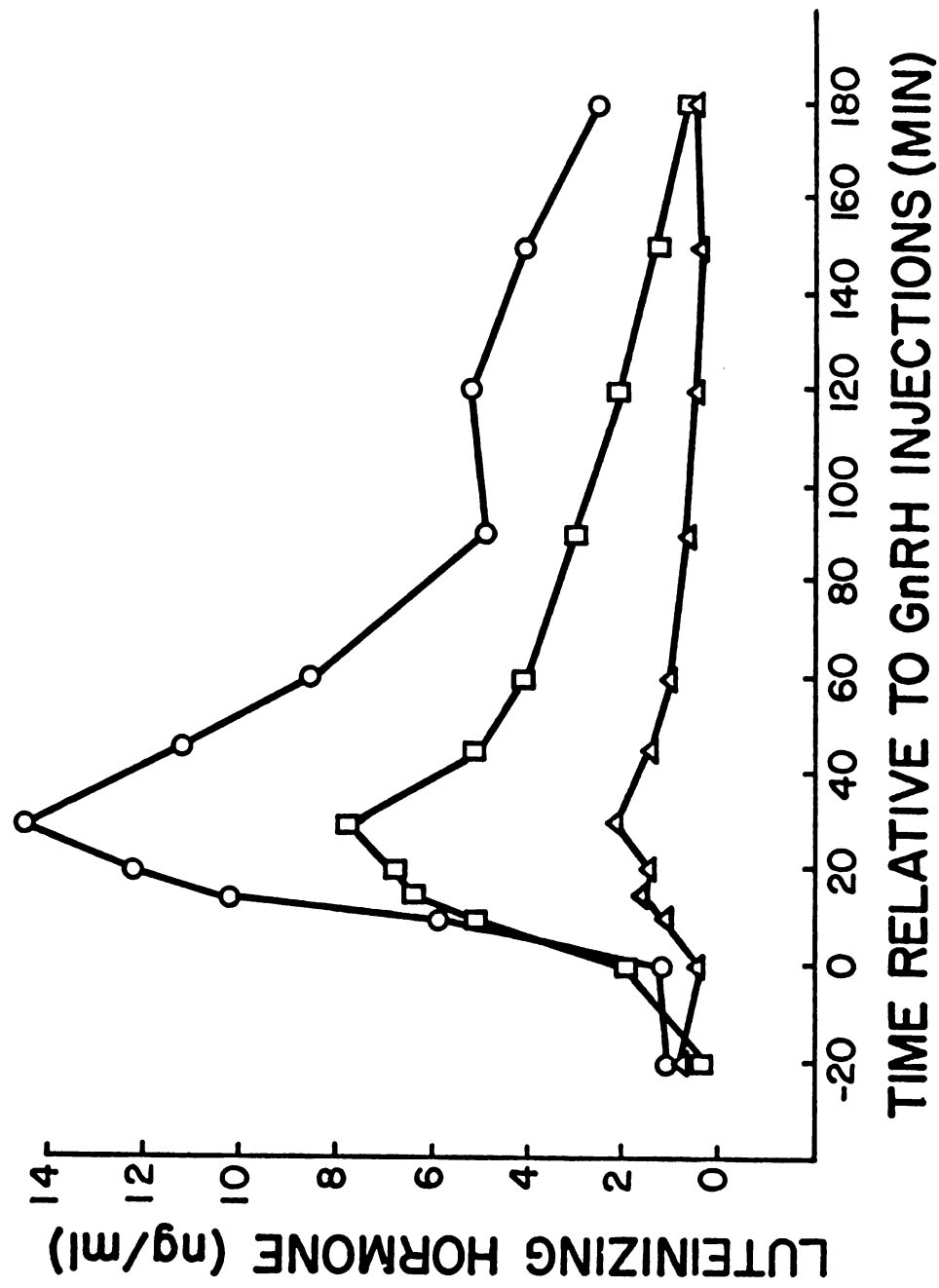


Figure 5. Serum LH in intact bulls (□—□), untreated steers (○—○), and steers given testosterone (△—△) after the second GnRH challenge.

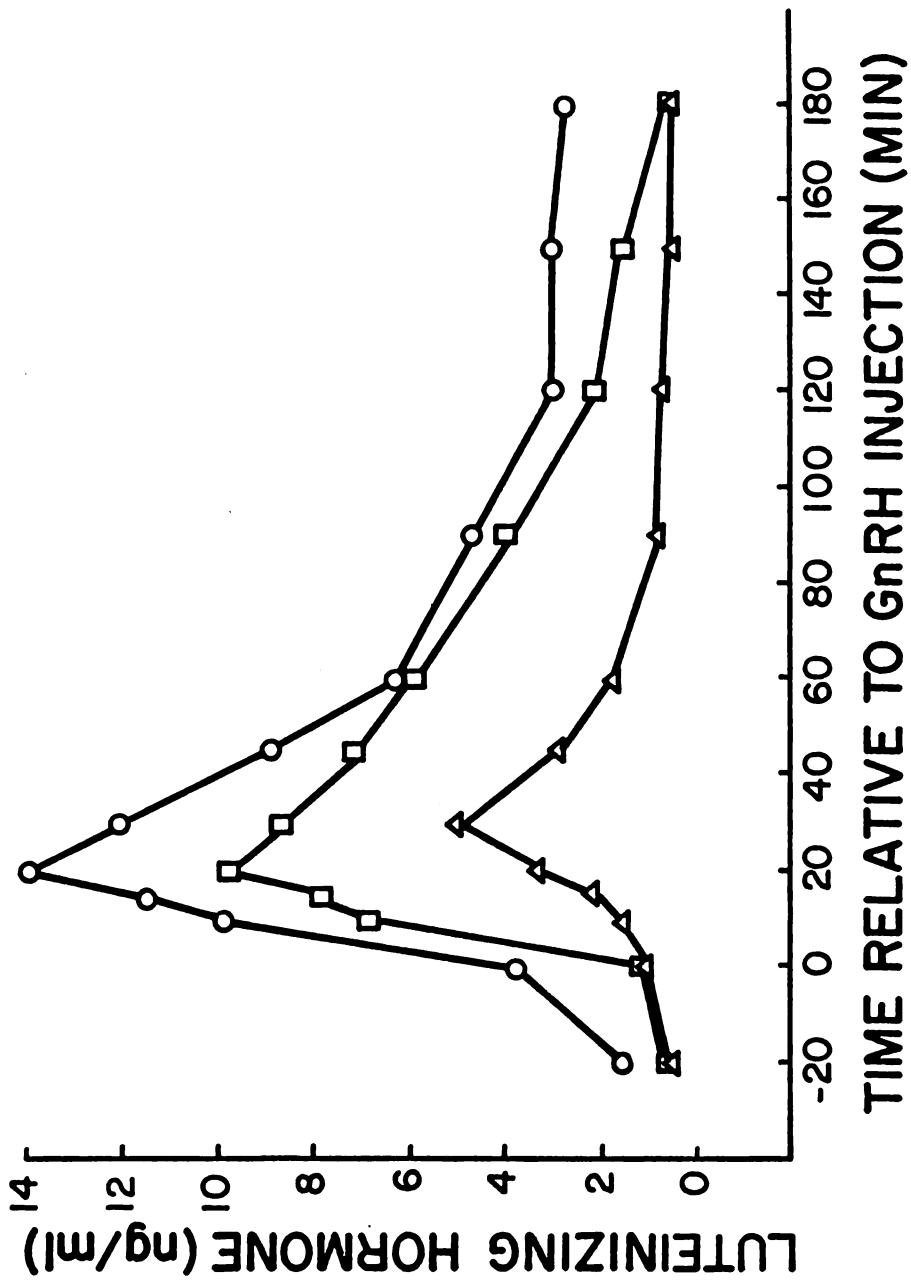


Figure 6. Serum LH in intact bulls (□—□), untreated steers (○—○), and steers given testosterone (△—△) after the third GnRH challenge.

Table 4.--Summary of analysis of variance for luteinizing hormone after gonadotropin-releasing hormone.

Source	df	SS	MS	F	P_1
Total	44	66882.2			
Treatment	2	22644.96	11322.5	5.6	< 0.025
Animal/Treatment	12	24067.6	2005.6		
Time	2	5302.3	2651.1	6.3	< 0.01
Time X Treatment	4	4757.7	1189.4	2.8	< 0.05
Error	24	10110.0	421.5		

($P < 0.01$) in steers (91.4 $\pm$ 8.2) than in intact bulls (63.7 $\pm$ 8.3) or steers treated with testosterone (35.7 $\pm$ 8.4). In addition, magnitude of LH release in response to the first GnRH challenge (82.7 $\pm$ 7.6) was greater ($P < 0.01$) than the second (58.9 $\pm$ 11.9) or third (60.4 $\pm$ 9.6). A significant treatment by time interaction indicated that there was a change in magnitude of LH release in response to the first, second or third GnRH challenge and that the degree of change was dependent upon treatment. Steers responded to GnRH with an increase in release which did not differ ($P < 0.01$) between each GnRH challenge. When compared to the first GnRH challenge, a decrease ($P < 0.001$) in LH response to the second and third GnRH challenge, in steers given testosterone and intact bulls, was observed.

No significant difference in the LH response among the three treatment groups to the first GnRH challenge was shown. By the second challenge, 12 hours later, we find significantly greater ($P < 0.001$) LH response to GnRH in steers compared to steers administered testosterone. This difference in LH response between these two treatment groups is also highly significant ($P < 0.001$) at the third GnRH challenge. LH response by steers given testosterone is significantly greater ($P < 0.001$) at the first GnRH challenge compared to the LH response in these animals 12 hours later, after the second GnRH challenge.

B. Androstenedione and testosterone response after gonadotropin-releasing hormone

Overall changes in serum levels of androstenedione and testosterone in intact bulls after GnRH were quantitated. Serum androstenedione increased ($P < 0.05$) from 0.25 ± 0.04 ng/ml at time 0, to a peak of 0.47 ± 0.1 ng/ml by two hours post-GnRH injection. Serum testosterone also increased, from a value of 0.47 ± 0.06 ng/ml to 0.79 ± 0.13 ng/ml by three hours post-injection. Although, testosterone peaked at three hours post-injection the significance level was borderline ($P < 0.10$). Both the androstenedione and testosterone levels at time 0 were high relative to 1/2 hour post-injection (Figure 7).

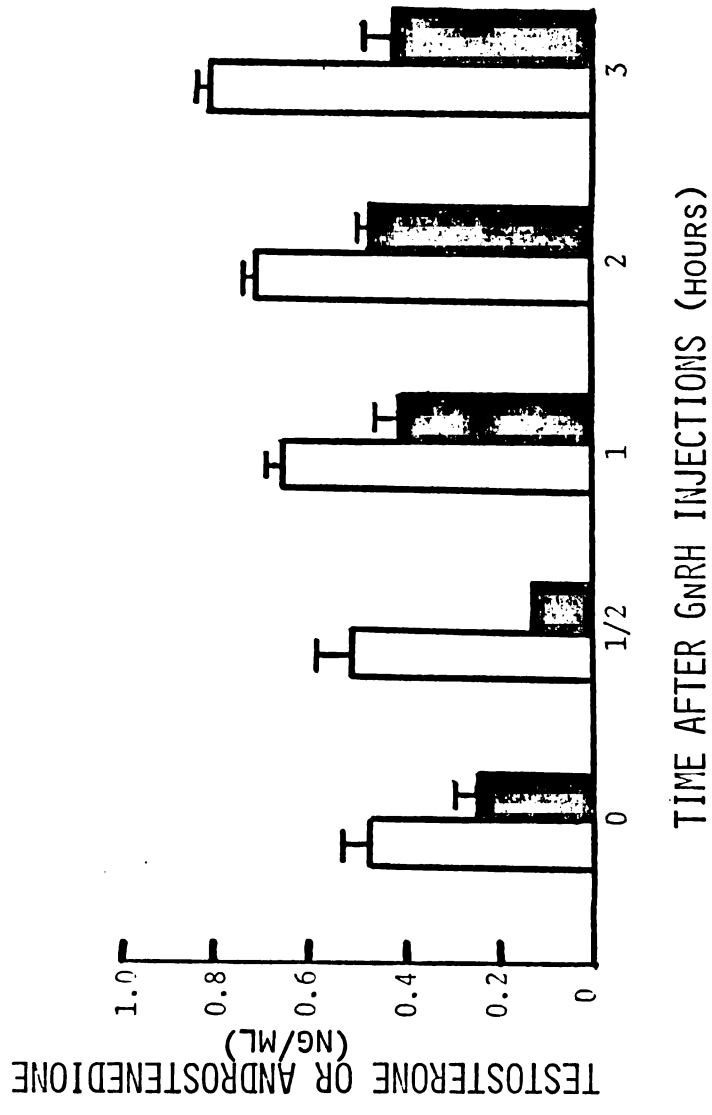


Figure 7. Serum testosterone (white bars) and androstenedione (black bars) in intact bulls after GnRH.

GENERAL DISCUSSION

Demonstration of episodic release of LH in these young bulls and steers indicates that a mechanism for control of this phase of LH release is present and functioning in bovine males as early as one month of age. Patterns of episodic release in one-month old bulls exhibited variations in magnitude and number within and between treatment groups. Castration increased number but not magnitude of episodic LH peaks appearing in steers, which is similar to the results reported for wethers (Riggs and Malven, 1974). That exogenous testosterone will counteract the effect of castration upon the episodic release of LH is supported by similar findings using other gonadal steroids in ewes (Riggs and Malven, 1974) and in monkeys (Yamiji et al., 1972).

Variations in patterns of episodic release observed herein did not differ from those patterns observed in prepubertal bulls and steers (Mongkonpunya et al., 1975). But, Mongkonpunya et al. (1975) were unable to revert number or magnitude of episodic releases of LH to that characteristic of bulls by administering exogenous testosterone to steers. This apparent discrepancy may result from the fact that testosterone replacement therapy in our experiment was

initiated at the time of castration instead of 21 days post-gonadectomy as in Mongkonpunya's study (1975). Diekman and Malven (1973) reported finding episodic LH release after ovariectomized ewes only after 42 to 137 hr post-surgery. Perhaps, by the time Mongkonpunya et al. (1975) administered testosterone, the hypothalamic and pituitary factors responsible for episodic release had become in part irreversibly changed to a higher set point of LH release and had become relatively insensitive to the inhibitory effect of the dose of testosterone administered. Alternatively, differences may be associated with age of the experimental animals and/or dose of testosterone. By administering 20 mg testosterone thrice daily, Mongkonpunya and co-workers were able to raise serum testosterone in 9 month old steers to a peak of approximately 12 ng/ml which was twice the average pre-castration level. We, on the other hand, were able to achieve a peak serum testosterone level in 1 month old steers of approximately 14 ng/ml which was about 14 times higher than levels found in intact controls. This proportionately higher effective level of serum testosterone may partially account for the differences in response seen in these two experiments.

The most generally accepted concept for the occurrence of puberty is that it represents a progressive loss of hypothalamic sensitivity to the inhibitory effects of feedback

of sex steroids (R. V. Short, 1972). Therefore, a proportionately higher dose of testosterone would be required in 9 month old prepubertal bulls to inhibit the increase occurrence of random LH release after castration.

It has been shown *in vitro* that testes taken from 1 month old bull calves are capable of responding to LH stimulation with the release of androgens into the incubation media (Kiser *et al.*, 1974). In support of those results, we have demonstrated an increase in serum testosterone *in vivo* which followed closely the appearance of an episodic LH peak in bull calves.

The decrease in magnitude of LH release by GnRH from first to second or third GnRH challenges in steers treated with testosterone and bulls but not in untreated steers suggests that there is a refractory period involved in the mechanism controlling GnRH induced LH release in 1 month old bovine males which is intensified or prolonged by testosterone. Evidence for this refractory period has been shown in other species by Rippel *et al.* (1974) in ewes and Chakraborty *et al.* (1973) in prepubertal gilts.

In bulls or steers given testosterone, the reduced LH response by the second GnRH challenge may be due to reduced releasable stores of pituitary LH. In this case, the presence of functioning gonads or the maintenance of relatively high serum levels of androgens inhibit the pituitary's

ability to restore released pools of LH. Evidence for a reduced rate of restoration of releasable LH stores can be demonstrated by stimulating pituitary LH release with GnRH at time intervals shorter than those needed for complete recovery of releasable LH stores. Rippel et al. (1974) reported that anestrous ewes required approximately 96 hours between GnRH challenges for complete pituitary recovery.

In one month old bull calves or steers given testosterone, it appears that a period of 12 hours between consecutive GnRH challenges is insufficient for total recovery of releasable pituitary stores of LH, yet this interval appears adequate for pituitary recovery in untreated steers. This again would indicate that either steers have an increased recovery rate or that the gonads and testosterone inhibit pituitary recovery of releasable LH stores.

Previously mentioned work by Rippel and Chakraborty also indicated that total pituitary content of LH in ewes and pigs which had received consecutive injections or chronic infusion of GnRH was unchanged when examined 6 to 24 hr after GnRH treatment. They also reported that the pituitary released only 20 to 30% of the total pituitary content of LH in response to a GnRH challenge. With this in mind, we can account for the reduced LH response seen at the second GnRH challenge, which seems to have been due to the release of newly synthesized hormone. Castration may cause a greater

rate of LH synthesis as evidenced by higher baseline LH values seen in steers. This would account for the unaltered LH response to GnRH seen in steers. Further experimentation is required where dosage of GnRH is increased and time interval between successive challenges decreased to better qualify the effect of gonadal secretions upon releasable pools of pituitary LH.

The mechanism controlling LH release by exogenous GnRH in 1 month old bulls and steers appears to be different from the control mechanism in prepubertal bulls. LH response to a GnRH challenge in castrated prepubertal bulls is significantly greater than the pre-castration LH response to a similar GnRH challenge (Mongkonpunya et al., 1975). A similar phenomenon was seen in 1 month old bulls and steers in this study but only after the second consecutive GnRH challenge. Pituitary sensitivity to androgen withdrawal or treatment in terms of LH response to GnRH may be different depending upon the age of the animal.

Over time, androstenedione increased in magnitude after each GnRH challenge. The increase in serum adrostenedione followed shortly after the serum increase in LH. Serum testosterone increased after GnRH and the difference between baseline and peak serum testosterone levels approached significance. This demonstrated that androgen production by immature bull testes can be stimulated by increased serum

levels of LH. After examination of these data, we believe that increased serum testosterone after LH stimulation in immature bulls could have been proven statistically if our experimental sample was larger. As it is, our results approach borderline significance ($P < 0.01$).

Although production of androstenedione by immature bull testes far exceeds testosterone production (Linder, 1959), low levels of androstenedione seen in bull serum may be due to rapid metabolism in the blood, primarily from testosterone and epi-testosterone.

SUMMARY AND CONCLUSION

One month old bull calves were assigned to one of three treatment groups: intact, castrated and castrated with testosterone replacement therapy. At ten days after initiation of the experiment, frequent blood sampling revealed random episodic increases in serum LH. Episodic peaks ranged in magnitude from 1 to 12.9 ng/ml between nadir and peak with frequency of occurrence ranging between 0 to 5 peaks within the 6 hr sampling period. Frequency of occurrence of LH peaks was greater in steers than in bulls or steers administered testosterone. Frequency of episodic peaks of bulls and steers given testosterone were not significantly different. Thus, we have established that one month old bull calves have episodic releases of LH which are increased in frequency following castration. We also find that replacement therapy with exogenous testosterone returned frequency to those of intact bull calves. We conclude that the mechanism responsible for episodic release is present and operative in the one month old bull and dependent upon testosterone treatment.

At 14 days after initiation of this experiment, all treatment groups were administered three successive GnRH injections intravenously; one every 12 hours for 36 hours.

Overall treatment means showed that the average magnitude of LH response to GnRH was highest in untreated steers (91.4 ± 8.2), followed next by intact bulls (63.7 ± 8.3) than steers given testosterone (35.7 ± 8.4). In addition, the magnitude of pituitary LH release in response to the first GnRH challenge (82.7 ± 7.6) was greater than the second (58.9 ± 11.9) and this reduced response was unchanged by the third challenge (60.4 ± 9.6). Serum androstenedione levels in intact bulls increased significantly over pre-injection concentrations after each GnRH challenge. Serum testosterone also increased after GnRH, but small sample size prohibited the establishment of definite statistical significance of this increase.

Our data indicate a role for gonadal steroids in the control of the release of LH from the pituitary as evidenced by the post-castration increase in serum LH concentration and the magnitude of LH response to GnRH. We conclude, therefore, that gonadal secretions affect the ability of the synthetic mechanisms of the pituitary to restore releasable pools of LH as early as one month of age.

Exogenous testosterone administration can reduce the GnRH induced LH response in the castrated month old bull. Testosterone, therefore, affects the pituitary's ability to recover from rapid and successive GnRH challenges as early as one month of age. A possible mechanism by which androgens

control anterior pituitary LH release is suggested.

To better illustrate the pituitary LH synthesis and release mechanism just described, visualize three equally sized hour glasses with hinged and latched bottom plates. The top chamber of each glass represents pituitary synthesis of LH, the bottom, pituitary LH stores. The rate at which sand flows from top to bottom represents the rate by which pituitary LH stores are replenished by the synthesis of new hormone. An LH response to GnRH is represented by the unlatching of the bottom hour glass plate and the subsequent loss of sand stored in the bottom chamber. To illustrate our experimental treatment groups, let an hour glass with a medium sized neck represent the intact bulls, one with a large neck as castrates and the last with a small neck as castrates administered testosterone. At the first unlatching (GnRH challenge) all the sand (LH) is emptied from the bottom chambers. If we shortly thereafter unlatch the bottom plates again, we find more sand would be emptied from the hour glass with the wide neck (castrates) when compared to the medium necked (intacts) which in turn would be greater than the amount emptied from the small necked glass (castrates given testosterone). Androgens, and the presence of the gonads govern the hour glass neck size and thus the rate of replenishment of the bottom chambers.

Lastly, endogenous increases in serum LH stimulate increases in circulating levels of androgens, thus demonstrating that the cause and effect relationship of the hypophyseal-gonadal axis is functioning in the one month old bovine male.

APPENDICES

APPENDICES

I. Composition of reagnets used in radioimmunoassay

A. Reagents for radioiodination

1. 0.5 M sodium phosphate buffer, pH 7.5

   Monobasic (0.5 M)

   Add 69.005 gm $NaH_2PO_4 \cdot H_2O$ to distilled water. Dissolve, dilute to 1 liter.

   Dibasic (0.5 M)

   Add 70.98 gm NaH_2PO_4 to distilled water. Heat to dissolve, then dilute to 1 liter.

   Mix monobasic and dibasic to give pH 7.5.

   Dispense in 1 ml portion, store at -20°C.

   Store the monobasic and dibasic buffers at 4°C.

2. 0.05 M sodium phosphate buffer, pH 7.5

   Solution A

   $NaH_2PO_4 \cdot H_2O$---------- 2.78 gm

   Merthiolate---------- 0.01 gm

   Dilute to 100 ml with distilled water.

   Solution B

   $NaHPO_4/7\ H_2O$----------26.825 gm

   Merthiolate---------- 0.05 gm

   Dilute to 500 ml with distilled water.

   Use 16 ml Solution A, 84 ml Solution B, dilute to 400 ml with distilled water.

   Adjust pH to 7.5 with NaOH if necessary.

   Store at 4°C.

3. Chloramine-T

   Upon receiving chloramine-T, dispense into small, tightly sealed vials, cover with foil, and store at -20°C.

   Dilute 10 mg chloramine-T to 10 ml with 0.05 M $NaPO_4$, pH 7.5 buffer. Use within 30 minutes of preparation.

4. Sodium metabisulfite, 2.5 µg/µl

   Dilute 25 mg $Na_2S_2O_5$ to 10 ml with 0.05 M $NaPO_4$, pH 7.5 buffer. Use within 30 minutes of preparation.

5. Transfer solution
   Sucrose ---------- 1.6 gm
   KI ---------- 0.1 gm
   Dilute to 10 ml with distilled water.
   Dispense in 1 ml portions, store at -20°C.

6. Rinse Solution
   Sucrose ---------- 0.8 gm
   KI ---------- 0.1 gm
   Brom phenol blue ---------- 0.001 gm
   Dilute to 10 ml with distilled water.
   Dispense in 1 ml portions, store at -20°C.

B. Reagents for Radioimmunoassay

1. 0.01 M phosphate buffered saline, pH 7.0 (PBS)
   NaCl ---------- 143 gm
   Monobasic phosphate ---------- 100 ml
   (See Appendix A.1)
   Dibasic phosphate ---------- 260 ml
   (see Appendix A.1)
   Merthiolate ---------- 1.75 gm
   Dissolve in distilled water and transfer to a large container.
   Dilute to 17.5 liters with distilled water.
   Adjust pH to 7.0, if necessary, store at 4°C.

2. 0.05 M EDTA-PBS, pH 7.0
   Disodium EDTA ---------- 18.612 gm
   Add approximately 950 ml PBS.
   Adjust pH to 7.0 with 5 N NaOH while stirring.
   Dilute to 1 liter, store at 0-4°C.

3. PBS--1% egg white albumin (PBS--1% EWA) or PBS--1% bovine Serum Albumin (PBS--1% BSA)
   Add 990 ml PBS to beaker.
   Add 10 gm EWA (Sigma Chemical Co.) or 10 gm BSA.
   Mix over magnetic mixer.
   Filter through Whatman No. 1 filter paper.
   Store at 4°C.

4. PBS--0.1% Knox gelatin (PBS--0.1% Knox)
   Weigh about 1 gm Knox gelatin.
   Using graduated cylinder, add appropriate volume of PBS to make 0.1%.
   Mix over magnetic mixer.
   Store at 4°C.

5. LH Standard
   Weigh 5-10 µg of NIH-LH-B8 on Cahn Electro-balance.
   Using 10 ml pipette, add PBS--0.1% Knox at appropriate volume to make a dilution of 1 µg/ml.
   Then, with volumetric flask, further dilute to 40 ng/ml.
   Store at -20°C in small semen vials, 4 ml each.

6. 1:400 normal guinea pig serum (NGPS)
   Obtain blood from guinea pigs that have not been used to develop antibodies.
   Allow blood to clot, recover serum and store the serum in convenient quantities at -20°C.
   Add 2.5 ml of appropriate serum to a 1 liter volumetric flask, dilute to 1 liter with 0.05 M PBS-EDTA, pH 7.0.
   Divide into 100 ml portions and store at -20°C.

7. Guinea pig anti bovine LH (GPABLH, identified in our laboratory as antibody I)
   Dilute the antisera to 1:600 with 0.05 M PBS-EDTA, pH 7.0.
   Dispense in 200 µl aliquots and store at -20°C.
   On day of use, dilute the 1:600 antisera to the required concentration using 1:400 NGPS as diluent.

8. Anti-gamma globulin
   Use sheep anti-guinea pig gamma globulin (SAGPGG) in LH assay.
   Dilute antisera to -equired concentration (presently being used at 1+30) with 0.05 M PBS-EDTA, pH 7.0.
   Store at 4°C or at -20°C.

C. Anti-gamma Globulin Production

1. Sheep anti-guinea pig gamma globulin
   Guinea pig gamma globulin (Fraction II, Pentex, Inc., Kankakee, Illinois) (40 mg), streptomycin (100 mg) and penicillin (100 I.U.) was emulsified in 5 ml Freund's complete adjuvant and 5 ml of water.
   10 ml was intradermally injected in 10-20 scapular sites of a 75 kg ram.

The above procedure repeated 15 days later substituting Freund's incomplete adjuvant for adjuvant.
Antisera was collected 30 days afrer the second antigen injection by jugular vein puncture.

II. Preparation of liquid scintillation fluid

A. Steroid scintillation fluid

Naphthalene	480 gm
PPO	30 gm
POPOP	0.3 gm
Xylene	2000 ml
p-dioxane	2000 ml

Mix until dissolved.

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