PITUITARY AND GONADAL RESPONSES TO GONADOTROPIN RELEASING HORMONE IN BULLS

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
KRIT MONGKONPUNYA
1974

This is to certify that the

thesis entitled

Pituitary and Gonadal Responses to

Gonadotropin Releasing Hormone in Bulls

presented by

Krit Mongkonpunya

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology & Dairy Science

H. Allen Tucker
Major professor

May 17, 1974
Date

ABSTRACT

PITUITARY AND GONADAL RESPONSES TO
GONADOTROPIN RELEASING HORMONE IN BULLS

By

Krit Mongkonpunya

Two experiments were designed: 1) to determine the luteinizing hormone (LH) response to gonadotropin releasing hormone (GnRH) in bulls at 2, 4 or 6 months of age and the consequent testosterone response to increased endogenous LH; and 2) to determine blood levels of LH and testosterone and gonadal and epididymal sperm in pubertal bulls during and after chronic GnRH treatment.

Nine bulls, three each at 2, 4 and 6 months of age received three doses (200, 400 or 800 μg; intramuscular injection (im) of GnRH, one on each of 3 consecutive days. Jugular blood was sampled at frequent intervals and serum LH, testosterone and androstenedione were determined by radioimmunoassay.

Serum LH significantly increased ($P<0.01$) from 1 ng/ml before GnRH to a peak of about 24 ng/ml at 45 minutes, then it declined gradually to pretreatment concentrations within 6 hours. This pattern of LH response was similar in bulls at 2, 4 or 6 months of age. Peak responses after 200, 400 or 800 μg GnRH were not different. However, the duration of the LH response after 800 μg GnRH was longer than that after 200 μg GnRH. LH responses also differed significantly by days of treatment.

The time from GnRH injection to LH peak, magnitude of the peak and duration of serum LH response were significantly greater on day 1 than on days 2 or 3. These data indicated that the LH response of prepubertal bulls to 24-hour repeated GnRH administrations decreases.

All doses of GnRH stimulated serum testosterone but older bulls responded more than younger bulls. In 2-month-old bulls, testosterone averaged 0.4 ng/ml before GnRH, increased slightly to 0.6 ng/ml at 2-3 hours and declined to 0.3 ng/ml at 6 hours after GnRH. In 4-month-old bulls comparable values were 0.6, 1.2 and 0.5 ng/ml. In contrast, testosterone in 6-month-old bulls averaged 1.7 ng/ml before GnRH, increased significantly ($P<0.05$) within 1 hour (4.6 ng/ml) and peaked (5.3 ng/ml) at about 2 hours after GnRH. Since age was without effect on LH response to GnRH, this increased capability of the testis to release testosterone between 2 and 6 months of age suggested that the testis is more directly related to sexual maturation than the anterior pituitary.

Androstenedione concentrations of blood either before GnRH or after GnRH were only about 13% of those of testosterone. However, all doses of GnRH increased androstenedione significantly ($P<0.05$) at 2, 4 or 6 months of age, and this response was unaffected by age of the bull. On the average androstenedione increased about 3-fold from 120 pg/ml before GnRH to 300 pg/ml at 1-2 hours, and then declined to 60 pg/ml at 8 hours after GnRH. Similar to the LH response, androstenedione responses differed from the first to the third day. On day 1, androstenedione increased gradually until 4 hours after GnRH. On day 2 androstenedione peaked at 1 hour, and on day 3 it peaked at 2 hours. The general patterns of LH and androstenedione responses

to GnRH on each day were similar although androstenedione lagged behind LH. Since androstenedione significantly ($P<0.05$) increased after GnRH at all ages and the testosterone response was significant only in 6-month-old bulls, probably indicates that 2- or 4-month-old bulls possess a different mechanism for converting androstenedione to testosterone, or the processes of the conversion may be blocked or insufficiently stimulated in younger bulls.

Experiment II was designed as a triple split-plot with nesting, and involved 18 bulls, six each at 2, 4 and 6 months of age. Three bulls at each age either received saline or 40 μg GnRH (im) twice daily for 28 days. Two months after the last injection sperm numbers were measured in the epididymis and testis.

GnRH injections had no effect on growth rate or testicular dimensions measured throughout the experimental period (3 months). Testicular and body growth were significantly correlated ($r = 0.82$, $P<0.01$). Regardless of treatments, the youngest age at which I found sperm was 5 months, whereas appreciable numbers of testicular spermatozoa were found in all bulls at 9 months. The GnRH treatment failed to increase testicular or epididymal sperm counts.

Serum LH in saline-treated bulls was essentially constant, averaging 1.3, 1.5 and 1.7 ng/ml for 2, 4 and 6-month-old bulls, respectively; and it did not differ from the average pretreatment concentration in bulls subsequently treated with GnRH. Serum LH significantly increased ($P<0.01$) 30 minutes after GnRH. The greatest response was after the first injection (AM) on day 1; the values were 32.7, 27.4 and 14.7 ng/ml for 2, 4 and 6-month-old bulls, respectively. The comparable data after the second injection (PM) on day 1 were 23.6, 12.9 and 8.4 ng/ml.

Although the LH response on the average was greater in younger bulls this was not significant ($P>0.05$). The average (AM+PM) serum LH at 30 minutes after GnRH on day 1 from all bulls (20.0±4.6 ng/ml) was significantly ($P<0.05$) higher than those on day 7, 14, 24 and 28 which averaged 5.5±0.8, 6.5±0.9, 5.0±0.9 and 6.2±0.2 ng/ml respectively. Serum LH at 30 or 60 days after the end of treatment period (28 days) did not differ significantly between saline or GnRH-treated bulls; the values in the control bulls were 1.2, 0.5 and 0.6 ng/ml for 2-, 4- and 6-month-old bulls, respectively. The respective values in GnRH-treated bulls were 2.1, 0.3 and 0.4 ng/ml.

In 6-month-old bulls treated with GnRH, average testosterone concentrations (5.8±0.3 ng/ml) were significantly higher ($P<0.05$) than those at 2 or 4 months of age, which averaged 0.7±0.1 and 2.0±1 ng/ml respectively. Comparable data for the control group were 3.8±.4, 2.4±.2 and 0.5±.1 ng/ml for 6, 4 and 2 months, respectively. Serum testosterone in 6-month-old bulls treated with GnRH was also greater ($P<0.05$) than that in saline-treated bulls (5.8 vs 3.8 ng/ml), but this difference could not be detected in younger animals.

To summarize, the results indicated that LH released in response to GnRH was similar in bulls aged 2 to 6 months, but the testosterone responses increased during this interval. Unlike testosterone, androstenedione responses were similar in the bulls at 2, 4 or 6 months of age, but the magnitude of the responses was lower than that of testosterone. LH responses decreased with repeated injections of GnRH. Furthermore, repeated GnRH injections did not hasten puberty (sperm production) in the bull.

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By

KRIT MONGKONPUNYA

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Departments of Physiology and Dairy Science

1974

TO MY PARENTS

BIOGRAPHICAL SKETCH

The author was born in the District of Wungparng, Pacharng, Lumphoon, Thailand on August 1, 1938. He received his elementary education in Wungparng public school system and his secondary education in Prince Royal's College, a Presbyterian Missionary School in Chiengmai, Thailand. He entered the Agriculture College at Kasetsart University, Bangkok, Thailand and received a Bachelor degree of science in June 1961. His thesis was entitled "Nutritive value of paragrass".

Upon his graduation, he joined the Department of Biology, Kasetsart University as an instructor.

In 1965 he was awarded a scholarship from South East Asia Treaty Organization (SEATO) for graduate study at West Pakistan Agriculture University, Lyallpur, West Pakistan. He received his Masters of Science degree in physiology in July 1967. His thesis was entitled "Effects of day light and ambient temperature on reproductive performance in mice". Again, he rejoined the Department of Biology, Kasetsart University.

In September 1971 he was granted a scholarship from the Rockefeller Foundation to continue his graduate study in physiology and dairy science at Michigan State University. He is completing the requirements for the Ph.D. degree in May 1974.

ACKNOWLEDGMENTS

The author thanks Dr. Harold D. Hafs, Professor of Physiology and Dairy Science, for the advice, guidance and assistance he has extended throughout his study program.

Appreciation is also expressed to Dr. H. A. Tucker for his advice and help in preparing this manuscript.

The author is also grateful for the advice and consent of other guidance committee members, Drs. E. P. Reineke, J. L. Gill and J. E. Wilson.

The author also thanks Dr. R. Neitzel for statistical advice and computer programming. For their assistance in the laboratory and in collecting blood samples the author thanks his colleagues Dr. R. A. Bourne, Dr. L. A. Edgerton, Dr. J. A. Koprowski, Dr. Y. C. Lin, O. W. Smith, T. M. Louis, D. T. Vines, J. N. Stellflug, R. J. Kittok, T. W. Beck, B. E. Seguin, P. A. Noden and V. G. Smith.

A debt of gratitude is extended to Drs. E. M. Convey, W. D. Oxender, J. H. Britt and P. K. Kindel for their interest and advice during this study.

The financial support rendered by the Kasetsart University and The Rockefeller Foundation for a scholarship is appreciated.

Finally, the author thanks his wife, Aporn, whose encouragement and sacrifices proved invaluable.

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INTRODUCTION

The practical goals of research in physiology of reproduction in bulls are increased fertility and sperm production to cope with the increasing demand for semen from proven sires. Low libido, low sperm production and delayed puberty are problems which delay genetic progress in the artificial insemination industry.

Development of the reproductive system is under the influence of the hypothalamo-hypophyseal system. General relationships between the pituitary, hypothalamus and testis have been established in the adult. However, precisely when and how the hypothalamo-hypophyseal system participates in testicular maturation during puberty is unknown.

Following the determination of the structure of hypothalamic luteinizing hormone (LH) releasing hormone (Matsuo et al., 1971), evidence indicated that this decapeptide caused release of follicle stimulating hormone (FSH) as well as LH in many species, including cattle. This decapeptide has been called LH-RH or LH-RH/FSH-RH, but because of its ability to release both LH and FSH the term gonadotropin releasing hormone (GnRH) will be used in this thesis.

The purpose of this thesis was to study the effects of administration of GnRH on pituitary function and secondarily on gonadal function. Two experiments were designed to: 1) determine the LH and androgen responses to GnRH in bulls at 2, 4 or 6 months of age;

and 2) determine blood levels of LH and testosterone, and gonadal and epididymal sperm in pubertal bulls during and after chronic GnRH treatment.

REVIEW OF LITERATURE

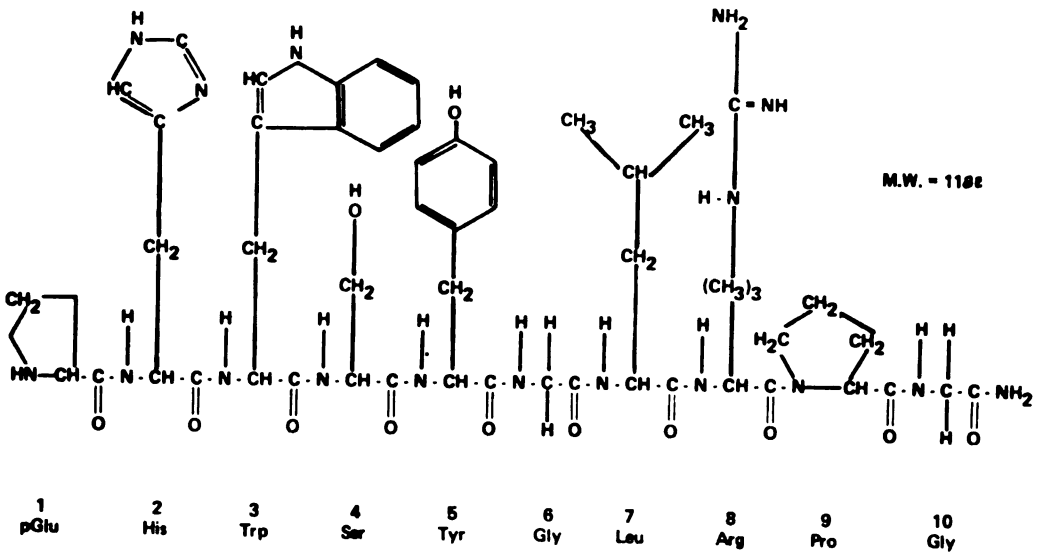
1. The Hormones of the Hypothalamus

In 1947, it was postulated that the anterior pituitary gland, which controls the peripheral endocrine glands, is regulated by factors or hormones originating in the hypothalamus (Green and Harris, 1947). Evidence accumulated that at least nine hormones from the hypothalamus control the biosynthesis and release of pituitary hormones. Among these hormones, two of them (thyrotropin releasing hormone (TRH) and gonadotropin releasing hormone (GnRH), Figure 1) have been isolated and synthesized and are available for investigation. The record of investigational development concerning the chemistry, physiological function and search for their chemical structure has been reviewed (McCann and Porter, 1969; Meites, 1970; Martini and Ganong, 1971; Schally et al., 1972; Guillemin and Burgus, 1972; Bogdanove, 1972; Schally et al., 1973; and Convey, 1973).

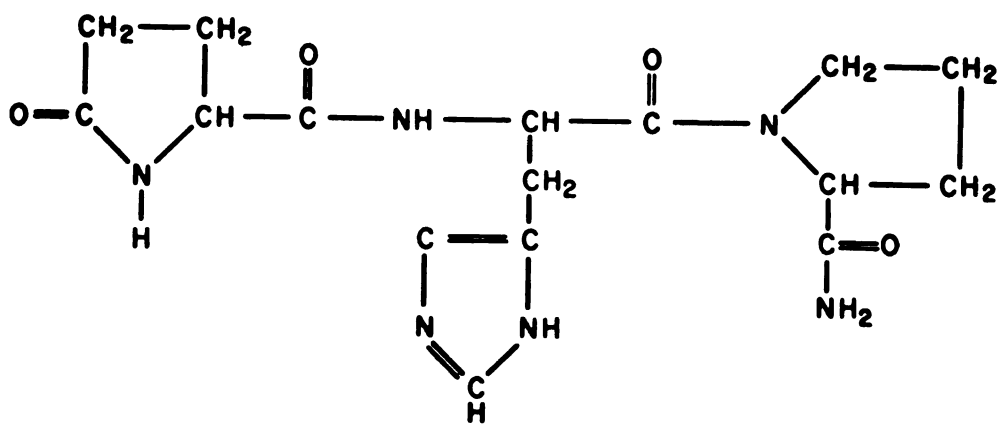
Following determination of the structure and synthesis of GnRH by Matsuo et al. (1971), it was demonstrated that GnRH preparations with the structure shown in Figure 1 have the same activity as the native substance purified from the hypothalamus. The view that one hypothalamic hormone (GnRH) could be responsible for stimulating the release of both FSH and LH is supported by considerable physiological and biochemical evidence. For example this decapeptide caused release of FSH and LH in rats (Schally et al., 1971c), sheep (Reeves et al., 1972), humans (Schally et al., 1971b; Rebar et al., 1973) and cattle

Figure 1.--Gonadotropin releasing hormone (GnRH) and thyrotropin releasing hormone (TRH). Amino acid sequences.

Gn-RH



TRH



(Pyro) Glu ——— His ——— Pro-(Amide)

(Zolman and Convey, 1973; Kaltenbach et al., 1973). Small doses of GnRH stimulated the release of FSH and LH in rat pituitaries cultured in vitro (Schally et al., 1972). Furthermore, chemical and enzymatic inactivation of GnRH (Schally et al., 1971a) was accompanied by loss of both FSH and LH releasing activities, and chromatographic and electrophoretic separational techniques failed to separate the two activities (Schally et al., 1971b).

Evidence against the concept of one hypothalamic hormone controlling the discharge of both LH and FSH was demonstrated by Johansson et al. (1972) and Currie et al. (1971). They reported that GnRH caused release only of LH. Moreover, another hypothalamic preparation essentially free of the GnRH moiety caused release of LH, and even greater amounts of FSH (Bowers, 1973). At low doses, this preparation stimulated only FSH release. Thus, Bowers (1973) proposed that the decapeptide (Figure 1) is LH-RH; and that FSH-RH is a separate hypothalamic releasing hormone of unknown structure.

In contrast to the effects of GnRH on LH and FSH, 25 μg of GnRH given intravenously to men had no significant effect on thyroid stimulating hormone (TSH), growth hormone (GH), adrenocorticotropin hormone (ACTH), insulin or blood glucose (Wagner et al., 1972).

Recently, using a radioimmunoassay (RIA) Nett et al. (1973) determined GnRH in serum of normal rams (71±1.4 pg/ml) and in serum of castrate ewes (128±6.1 pg/ml). The time required for one-half of GnRH activity to disappear from blood was about 6 minutes in sheep (Nett et al., 1973), and rats (Redding and Schally, 1973). Thus, it appears that GnRH normally reaches the peripheral circulation, but it is rapidly inactivated.

2. Effect of GnRH on Gonadotropin Release

The magnitude and time course of increased blood LH after GnRH administration vary among species, doses, routes of administration and physiological states of the experimental animals.

In cattle, the LH response is proportional to the dose of GnRH (Zolman et al., 1973), especially when given on the basis of body weight (Golter et al., 1973). There is also interaction between doses and routes of administration. Peck et al. (1973) found that intravenous and intrauterine administrations were equally effective; while Tarnavsky and Reeves (1973) found that intrapulmonary administration (intratracheal) was less effective than intramuscular. Golter et al. (1973) found that 1.3 μg GnRH given in acidified saline or in 2% carboxymethyl cellulose per kilogram of body weight caused similar LH responses, but a subcutaneous silastic implant of GnRH was less effective. They concluded that the rate and duration of release of GnRH are more important than the dose of GnRH in regulating LH release. Convey (1973) concluded that "GnRH causes release of LH in vivo and in vitro and pituitary responsiveness to exogenous GnRH varies with reproductive state probably as a result of variation in the steroid environment".

Chakraborty et al. (1973a) reported that the serum LH response in prepubertal female pigs subjected to repetitive treatment with GnRH (16 injections of 25 μg over 96 hours) was gradually decreased between the first and third injections. Thereafter, the LH response was consistent but at a lower level. Chakraborty et al. (1973b) also reported that infusion of GnRH at a rate of 2.3 μg per hour into anestrous ewes failed to maintain elevated serum LH concentrations after 3 hours of infusion.

3. Luteinizing Hormone Releasing Factor (LRF) in Cattle

To my knowledge, there are only two reports of hypothalamic content of LRF activity in cattle. Both used a partial purified extract of the hypothalamus in a rather insensitive and indirect bioassay. The LRF potency in the hypothalami of Holstein bulls from birth to 12 months of age was determined by the ovarian ascorbic acid depletion assay in rats (Macmillan and Hafs, 1968b). LRF activity was not detectable in the hypothalami from bulls less than 5 months of age; it increased between 6 and 10 months and the increase was associated with increased LH in the plasma. In heifers during the estrous cycle Hackett and Hafs (1969), using the same assay technique found that hypothalamic LRF was increased from day 20 to day 7 and decreased on days 11 and 18. The elevated LRF was associated with release of LH, ovulation, and luteal growth; the reduced LRF occurred during the period of maximal progestin secretion.

4. Gonadal Steroid Response to Increased LH After GnRH Administration

McCormack and Spies (1972) administered an extract from ovine stalk-median eminence into mature rhesus males and measured serum LH and testosterone. Testosterone began to rise later than LH, and increased 2- to 12-fold above baseline within 15 to 90 minutes after the beginning of treatment. In women experiencing abnormally infrequent or absence of menses, Newton and Collins (1972) found that GnRH increased plasma LH, FSH and estradiol 17-β. Kastin et al. (1972) found that men and prepubertal boys showed significant increases in estradiol after injection of GnRH. Similarly, blood LH and testosterone increased in response to GnRH in children at puberty (Roth et al., 1972 and 1973).

5. Functional Relationship Among Hypothalamus, Pituitary and Gonads

The integration of physiological functions of the hypothalamus, pituitary and gonads are not yet known with certainty. Keeping in mind that there might be species differences, few studies have been done on farm animals. Most of the evidence given in this section is from laboratory animals.

The general relationship between the hypothalamus and pituitary-gonadal axis has been actively investigated. Results may be summarized as follows. As described in the previous section, a hypothalamic hormone (GnRH) regulates the synthesis and release of pituitary gonadotropins (LH and FSH). Gonadal functions are stimulated due to the increase of gonadotropin concentrations in the blood. The positive effects of gonadotropins on the male reproductive tract are mostly a consequence of androgens produced by the testes (Donovan, 1970). Sampling every hour Katongole et al. (1971) and Smith et al. (1973) demonstrated episodic increases in serum LH. In individual bulls, about four LH peaks occurred during every 24-hour period. Most of the LH spikes were accompanied or followed within 1 hour by increases in serum testosterone. Gonadotropins can also act directly on the seminiferous tubules, since LH administration to hypophysectomized lambs caused proliferation of sertoli cells in the seminiferous tubules, while large doses of testosterone failed to show this effect (Courot, 1970). However, FSH is believed to stimulate the germinal epithelium of the testes, while the principal action of LH is to activate the Leydig cells for the production of androgens (Li, 1972). Possibly the actions of these two hormones are synergistic (Courot,

1970), and altering the FSH-LH ratio also may be involved in their physiological function (Charro et al., 1973).

At the molecular level, gonadotropin stimulation of androgen secretion evidently involves activation of Leydig cell adenyl cyclase activity (Dufau et al., 1973). However, there is evidence that the initial stimulation of steroidogenesis by gonadotropins may operate by pathways other than that directly involving adenyl cyclase and cyclic AMP formation (Catt and Dufau, 1973) because low gonadotropin levels (0.1 ng/ml), which stimulate testosterone release in isolated rat testis, failed to enhance the formation of cyclic AMP.

Gonadal steroids influence the release and synthesis of hormones from the hypothalamus and pituitary. Administration of estrogen or testosterone decreased serum LH concentration in rats (Bogdanove, 1963; Jackson, 1972; Ramirez and McCann, 1963; and Smith and Davidson, 1968), rams (Pelletier, 1970) and cattle (Odell and Kiddy, 1969). This is the so-called negative feedback control of steroids on gonadotropin secretion. The anatomical site of action of the negative feedback control is the anterior hypothalamus and pituitary. Thus, after injection of radioactive sex steroids into rats (Touhimaa and Niemi, 1972; Stern and Eisenfeld, 1971; Sar and Stumpf, 1973a,b), there was more radioactivity in the anterior hypothalamus and anterior pituitary than other parts of the brain. Sar and Stumpf (1973b) found that about 10-15% of cells in the anterior pituitary of rats retained radioactivity in their nuclei within 1 hour after injection of 1,2-^{3}H testosterone, while no accumulation of radioactivity was found in the other lobes of the pituitary. The cells retaining radioactivity were identified as gonadotrophs. The major component of the

radioactivity retained in the pituitary after the injection of ^{3}H-testosterone was identified as dihydrotestosterone (Stern and Eisenfeld, 1971).

Karsch et al. (1973) concluded that the negative feedback regulation of LH release is a finely tuned system with a set-point which appears to change as a function of time after castration in monkeys. Furthermore, progesterone can synergize with estradiol in suppressing LH release. Possibly, LH secretion is reduced by the combined inhibitory effect of progesterone and estradiol since castrated rats were less sensitive to progesterone than intact rats (Ramirez and McCann, 1963). Increased blood LH after castration differs with age and sex (Yamatomo et al., 1970). For example, in adult male rats serum LH increased sharply by 8 hours after castration and reached a plateau by 18 hours. In adult females the increment in LH per unit time was less than in males, finally reached concentrations comparable to those of the males 10 days after castration. Similar patterns of the increment in serum LH were also observed in immature males and females, but the concentrations were less than in the adults.

Though it has been demonstrated that the negative feedback of steroids can have effects at the pituitary or hypothalamus, there is no evidence that similar direct effects occur normally. Besides, we do not know the mechanism of action of the steroid feedback. It has been reported that aromatization of the steroids in the hypothalamus may be one mechanism essential for the negative feedback (Beyer et al., 1970; Reddy et al., 1973). Reddy et al. (1973) observed that the intact male hypothalamus was more active than that of intact female in the aromatization of androstenedione to estrone; castration increased this activity in both sexes. However, in rats, Swerdloff et al. (1972)

administered testosterone and dihydrotestosterone (an A-ring reduced steroid which is not convertible to estrogen) over a broad dose range and found that dihydrotestosterone was more potent than testosterone in suppressing serum LH, but they were equally potent in suppressing FSH. They concluded that conversion of androgens to estrogens was not required for inhibition of the hypothalamo-pituitary axis. Consequently, the mechanism of the negative feedback control system is still open.

6. Reproductive and Endocrine Changes in Fetal Through Pubertal Stage

A functional relationship may exist between the pituitary and gonads in the bovine fetus as early as 90 days of gestation (Oxender et al., 1972a; Kim et al., 1972; Mongkonpunya et al., 1974). In male fetuses, serum LH and testosterone were highest at 90 days of gestation and decreased as fetal age increased (Mongkonpunya et al., 1974). Nakai et al. (1972) demonstrated that castration of male fetal rats resulted in an increased percentage of basophilic cells in the pituitary, suggesting early existence of a feedback mechanism between the testes and the hypothalamic-pituitary axis. The gonads of the fetal horse at about 4 months were composed mainly of interstitial cells (Raeside et al., 1973). During fetal development Reyes et al. (1973) found the highest testosterone concentration in human testes at 8-12 weeks, but negligible quantities in ovaries or adrenals. They concluded that there was high androgen production in the testis at the time of male genital differentiation.

In bovine neonates during the first 7 days of life, the concentration of serum LH was relatively lower than in fetuses regardless of stage of gestation or sex (Oxender et al., 1972b). However,

testosterone secretion in bovine neonates has not been studied. In humans at birth, Forest et al. (1973) demonstrated that testosterone in umbilical cord blood was significantly higher in males than in females. In male infants, testosterone concentration increased from birth to 2-3 months, then gradually declined until 7 months when its concentrations stabilized until puberty.

Reproductive and endocrine development in Holstein and Hereford bulls from birth through 1 year of age were investigated in our laboratory (Macmillan and Hafs, 1968a,b; Macmillan and Hafs, 1969; Purchas, Macmillan and Hafs, 1970; Rawlings, Hafs and Swanson, 1972; Swanson et al., 1971a). The results from these studies can be summarized as follows:

a. Sperm Number.--Testicular sperm were found in some bulls at 5 to 7 months and in all bulls at 8 months. Average testicular sperm concentration increased about 14-fold between 7 to 12 months, and sperm concentration at 12 months was similar to that for mature bulls. In contrast, the sperm content of caudae epididymides at 1 year is only about 20% of that in mature bulls. It was concluded that Holstein bulls attain their mature rate of spermatogenesis by 1 year of age. The subsequent increases in sperm production with advancing age were primarily due to increases in testicular weight.

b. Pituitary and hypothalamic endocrine changes.--Pituitary weight increased linearly from birth to 1 year of age. While the concentration of pituitary LH was highest at 1 month, the total gland content was greatest at 6 months of age. Estimated total serum LH did not significantly change from birth to 2 months, increased to 4 months and increased again between 6 and 10 months of age; the two increases

in LH were associated with increases in hypothalamic GnRH. In contrast to these results, Odell and Kiddy (1969) and Odell et al. (1970) reported that there were no consistent changes in blood LH concentration during sexual maturation in heifers or bulls.

Concentration of pituitary FSH was greatest at 2 months, but pituitary content was highest at 5 and 6 months and both declined after 6 months of age. The data suggested that puberty in bulls commences at 2 months and is qualitatively completed by 10 months of age. The greatest changes occur between 6 and 10 months.

c. Development of reproductive tract.--Thirteen criteria related to androgen secretion were studied. The results indicated that the period of accelerated reproductive development associated with puberty was terminated by 9 to 10 months of age. It was also revealed that pubertal development may be biphasic; one phase occurred between 2 to 4 months and the other from 6 to 9 months of age - the periods of rapid increases in plasma LH. The second period of development was typified by appearance of more obvious external manifestations of puberty and by rapid onset of spermatogenesis.

d. Testicular and blood plasma androgens.--Using a gas-liquid chromatographic assay, Rawlings et al. (1972) found that testicular concentration of testosterone increased from birth to 5 months, decreased at 6 months and then generally increased to 11 months. Testicular androstenedione fell from 4 months to 7 months and remained stable at a low level thereafter. After 4 months of age, testosterone predominated. Plasma testosterone generally increased from undetectable concentrations at birth to about 4 ng/ml at 11 months of age. After

8 months of age, testicular and serum testosterone increased with the rising titers of plasma LH.

Mann et al. (1949 and 1960) reported a high correlation between androgen and secretory function of accessory organs which could be observed long before any sign of active spermatogenesis in bulls. Lindner (1959) and Lindner and Mann (1960) chemically identified endocrine constituents of bull testes from 28 days to 17 1/2 years of age and found testosterone and androstenedione could be detected as early as 39 days. The combined content of the two steroids per unit weight of tissue was not very different in the testes of very young calves or of mature bulls. However, androstenedione predominated in testes of young bulls whereas in mature bulls testosterone was predominent. Between the 2nd and 6th month of life the rate of development of seminal vesicles increased at an exponential rate and this was closely associated with testosterone content of the testes.

e. Histological development of bovine testes.--Hooker (1944 and 1970) reported that Leydig cells differentiated from interstitial mesenchymal cells in bulls by 4 months of age, increased in number until 2 years of age. Thereafter there was a gradual increase in size of the Leydig cells mainly due to vacuolation.

More recently Galina (1972) studied micromorphological changes in the immature equine, bovine, porcine and ovine testes from birth through 365 days of age. It was found that: 1) In immature testes, the seminiferous tubules were mostly irregular with two types of intratubular cells: supporting cells and gonocytes. The interstitial cell population showed important differences among these four species.

The interstitium of horse and pig neonates were populated by cells with large round nuclei, similar to mature Leydig cells, whereas in the immature bull and ram testes, only mesenchymal cells and fibroblasts were observed. 2) In the prepuberal phase, tubules with lumina were present with primary spermatocytes in their four meiotic stages. The number of primary spermatocytes increased throughout this stage, indicating a constant increase in the activity of the germ cells. However, the interstitium during this prepubertal phase did not show any noticeable difference from the early neonatal stage. 3) Characteristic of the pubertal phase was the presence of sperm in the seminiferous tubules, with enormous variation among individuals. The interstitium, which were differentiated from the mesenchymal cells, contained a juvenile form of Leydig cell.

Collectively, this evidence strongly indicates that steroidogenic activity in bovine testes is high in the first trimester of fetal life and gradually declines as gestation advances. Thereafter, synthesis is considerably lower until onset of puberty, at approximately 5-6 months of age. These changes are closely associated with secretion of LH and FSH from the anterior pituitary.

7. Current Hypotheses Concerning Mechanisms of Initiation of Puberty

"Puberty is that stage in development during which maturation of the sexual apparatus occurs and reproductive capability is attained" (Root, 1973). Puberty is a gradual sequential phenomenon influenced by many factors such as genetics, nutrition, environment, and hormonal conditions (Cole and Cupps, 1969). Studies of the testicular development in cattle have been limited. Based mostly on work in laboratory animals, three hypotheses of sexual maturation have been proposed.

These three theories, described in greater detail below, are open to question, and none has been proven. Most likely, sexual maturation is multifaceted, involving several mechanisms which are turned on sequentially.

a. A decrease in sensitivity of the hypothalamus and pituitary to sex steroid feedback (Smith and Davidson, 1968). The hypothalamic-pituitary system is more sensitive to the inhibitory effects of gonadal steroids in immature than in mature animals (Byrnes and Myer, 1951; Ramirez and McCann, 1963, 1965). Thus, several authors have suggested that the onset of puberty, at least in rats, might be associated with a decrease in the sensitivity of the higher nervous centers to sex steroid feedback resulting in increased gonadotropin release which precipitates puberty.

Moreover, the gonadotropin response of the pituitary to the stimulation of hypothalamic hormone also changes during the course of sexual maturation. Spona and Luger (1973) demonstrated that the male rat pituitary in vitro released greater amounts of LH in response to GnRH during prepubertal ages (10-40 days) than after puberty. In contrast Roth et al. (1972 and 1973) found that pituitaries of boys at prepubertal ages released amounts of LH which were significantly less than those at puberty. Recently August et al. (1972) demonstrated that early enlargement of human testes was associated with gradual increases in blood LH, testosterone and FSH, suggesting an upward shift in the threshold of the feedback mechanism (i.e., decreased sensitivity of the pituitary to testosterone feedback). Touhimaa and Niemi (1973) injected labelled ^{3}H-testosterone or ^{3}H-estradiol into fetal, neonatal or adult rats and found that the retention of radioactivity in the hypothalamus was higher in the neonate than in the adult. This

suggesting that a more avid steroid receptor mechanism existed in the neonate, and may contribute to the increased inhibitory effects of steroids on gonadotropin release before puberty.

As previously mentioned castration stimulates LH release. If steroids lose their ability to suppress gonadotropin release as animals age, one might expect castration to increase LH more at an early age. However, Crim and Geshwind (1972) found that the LH response after castration of rams was equivalent at 30 or 150 days of age. Furthermore, Foster et al. (1972) reported that pituitary LH did not differ in intact and castrated male or female lambs within 14 days after castration.

b. FSH induction of sensitivity to LH in Leydig cells. Based upon results from a series of experiments, Odell et al. (1973) postulated that a change in sensitivity of the higher nervous centers to inhibition by gonadal steroids could not be the sole explanation for the process of sexual maturation. For example, serum LH in male rats between birth and sexual maturation increased only slightly while FSH increased markedly between 12 and 21 days of age, then declined as sexual maturation proceeded. Moreover, hypophysectomized sexually immature males showed no increase in testicular weight after treatment with LH for 5 days, but testicular weight increased after FSH. Treatment of immature hypophysectomized rats with FSH for varying periods followed by FSH or FSH plus LH produced a significant increase in testicular weight while LH alone produced no effect. However, FSH plus LH produced greater effect than FSH alone and the difference became progressively greater with the time of FSH pretreatment. Thus, they postulated that FSH induces sensitivity of the testes to LH in male rats, and that sexual maturation may occur without alteration of steroid feedback sensitivity.

c. A decrease of 5α-reduction of androgens in Leydig cells. Folman et al. (1972) demonstrated that testes of immature rats formed larger quantities of dihydrotestosterone (35%) and other 5α-reduced metabolites of testosterone than the testes of mature rats (2-3%). It has been shown (Swerdloff et al., 1972) that dihydrotestosterone was more potent than testosterone in suppressing gonadotropin release. Besides, Baulieu et al. (1968) found that dihydrotestosterone was more effective in stimulation of cell division in prostate gland epithelium while testosterone was more effective in stimulation of prostatic secretions. Thus, possibly the mechanism of sexual maturation is associated with metabolic changes in the testes itself. In other words, during sexual maturation there is diminishing 5α-reduction or a decrease in the amount of the more effective inhibitors from the testes.

Testicular enzyme systems besides hydroxysteroid dehydrogenase complex and 5α-reductase, may be involved in sexual maturation. Monn et al. (1972) reported that two separate isoenzymes of cyclic nucleotide phosphodiesterase named "c" and "f" were found in adult rat testes while only "c" component was present in 20-day old rats. The increase in phosphodiesterase activity was paralleled by an identical increase in testicular adenyl cyclase activity between 20 and 50 days of age. Macindoe and Turkington (1973) reported, in rats, a close association between sexual maturation and ornithine decarboxylase and S-adenoxyl-L-methonine decarboxylase.

In my judgement, all of the factors and mechanisms mentioned above probably are involved one way or another in sexual maturation.

MATERIALS AND METHODS

1. Radioimmunoassay of Testosterone

Radioimmunoassay (RIA) of testosterone recently has been developed and validated by Smith (1972) in our laboratory. The procedures were described in detail elsewhere (Smith and Hafs, 1973). As compared with gas-liquid chromatography (GLC) or competitive protein binding assays, RIA has relatively higher sensitivity; testosterone can be measured in as little as 0.1 ml of bull's blood serum. However, the RIA procedure described by Smith was still laborious; only about 40 unknowns could be completed daily. A more efficient procedure was desirable because of the large numbers of samples in this research. Thus, the following procedure for RIA of testosterone was developed whereby more than 100 unknowns could be assayed within 1 day.

a. Testosterone Extraction.--Aliquants (100 µl) of each unknown were placed in three 15X85 mm disposable culture tubes and about 3000 dpm ^{3}H,1-2-testosterone (New England Nuclear, Boston, Mass.) was added to one of the triplicate tubes to estimate procedural losses. Each tube was vortexed with 2 ml nanograde benzene:hexane (1:2) for 30 sec, then stored at -20^{o}C for at least 1 hour to freeze the aqueous phase. The organic solvent from the tube with ^{3}H-testosterone was decanted into a scintillation vial, and the solvent in each of the other two extraction tubes was decanted into two 10X75 mm disposable culture tubes for RIA.

b. Radioimmunoassay (RIA).--The organic solvent was evaporated and 200 µl antibody[1] (diluted 1:3000 in 0.1% Knox gelatin in 0.1 M phosphate-buffered saline (PBS), pH 7.1; Appendix I.B.4) was added. Two sets of standard tubes containing 0, 20, 50, 100, 200, 500, 1000, 1500 and 2000 pg testosterone were included in each assay and treated similarly to the unknowns. After addition of antibody, each tube was vortexed 10 sec and incubated 30 min at room temperature. 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, and the tubes were vortexed 5 sec and incubated at 4^{o} for 12-24 hours. To separate free from antibody-bound testosterone, 1.0 ml of dextran-coated charcoal (0.25 g carbon decolorizing neutral Norit and 0.025 g Dextran 150 in 100 ml distilled water) was added at 4^{o}, and each tube was vortexed for 5 sec, equilibrated at 4^{o} for 10 min and centrifuged (2500 g) for 10 min at 5^{o}. Antibody-bound ^{3}H-testosterone in 0.5 ml of the supernatant fluid was measured in a liquid scintillation spectrometer. For comparison among assays, standard sera with high and low testosterone, and extracts from blank extraction tubes were assayed with each set of unknown serum samples.

c. Assay Validation.--Similar to progesterone RIA (Louis et al., 1973), preliminary studies revealed extraction efficiency of ^{3}H-1,2-testosterone was relatively constant (83±0.2%, n=240). The largest source of variance was among assays; thus the mass of testosterone determined in each set of unknowns was corrected for the average loss

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

of tracer determined within that set. The sensitivity of this assay was less than 20 pg testosterone, an amount which displaced about 8% ($P<0.01$) of the ^{3}H-testosterone bound to antibody.

I determined testosterone in aliquants of sera from 28 bulls by the modified assay and by the RIA described by Smith and Hafs (1973); results (Table 1) from the two methods were highly correlated ($r=0.81$) with the new method resulting in slightly (≈12%) higher testosterone values than the old method ($P<0.05$). The within-sample coefficient of variation for the 28 testosterone determinations by my method was 10%, less than the 31% for the other assay. The specificity of the antibody was described by Ismail, Niswender and Midgley (1972) and by Smith and Hafs (1973).

As another means of validating the testosterone RIA's, I purified eight extracts of unknown fetal serum samples and one steer serum sample on LH-20 Sephadex columns with two solvent systems. Firstly, the benzene:hexane extracts of steroids were eluted with chloroform: ethanol (98:2) which brought testosterone and androstenedione off the column together between 15 and 22.5 ml of effluent. Secondly, the elution from the first system was evaporated, dissolved in choloroform: heptane:ethanol (200:200:1, saturated with water), layered on an LH-20 Column and again eluted with chloroform:heptane:ethanol, to separate androstenedione from testosterone. By the second solvent system, androstenedione eluted between 20-25 ml and testosterone between 40-45 ml of effluent. The tubes containing the highest radioactivity representing androstenedione and testosterone were quantified by the present RIA to compare (Table 2) with the results of RIA directly applied to benzene:hexane extracts of sera without chromatography.

TABLE 1.--Comparison of two methods for assay of testosterone in bull sera.

Number of sample	New method		Old method[a]	
	Determination		Determination	
	1	2	1	2
	(ng/ml)			
1	5.0	6.1	6.0	6.7
2	3.5	3.6	4.6	4.2
3	3.1	3.3	2.4	2.7
4	4.2	4.3	3.2	3.6
5	4.2	3.7	3.6	2.8
6	4.5	4.2	5.1	4.4
7	3.4	3.5	2.8	3.4
8	4.8	4.0	4.4	7.2
9	4.5	5.4	3.5	5.0
10	2.2	1.7	1.2	1.2
11	4.4	5.8	4.1	2.9
12	3.0	3.5	3.2	3.4
13	3.9	3.9	2.6	2.2
14	1.7	1.8	1.4	1.8
15	5.6	5.9	4.4	6.6
16	2.2	2.2	1.2	0.6
17	6.5	6.6	5.0	4.1
18	3.5	3.3	2.0	3.2
19	4.2	4.7	3.2	3.0
20	3.1	3.2	3.1	3.0
21	1.7	1.1	1.5	1.0
22	3.0	2.8	2.3	2.5
23	3.4	2.8	2.6	3.6
24	4.2	4.3	3.4	3.4
25	4.4	5.5	6.0	0.6
26	2.8	2.6	1.7	3.7
27	3.7	3.7	5.4	4.4
28	1.7	2.2	1.4	1.1
Avg ± SE	3.66±.22	3.77±.27	3.26±.27	3.30±.32

[a]Smith and Hafs (1973).

TABLE 1.--Comparison of two methods for assay of testosterone in bull sera.

Number of sample	New method		Old method[a]	
	Determination		Determination	
	1	2	1	2
	(ng/ml)			
1	5.0	6.1	6.0	6.7
2	3.5	3.6	4.6	4.2
3	3.1	3.3	2.4	2.7
4	4.2	4.3	3.2	3.6
5	4.2	3.7	3.6	2.8
6	4.5	4.2	5.1	4.4
7	3.4	3.5	2.8	3.4
8	4.8	4.0	4.4	7.2
9	4.5	5.4	3.5	5.0
10	2.2	1.7	1.2	1.2
11	4.4	5.8	4.1	2.9
12	3.0	3.5	3.2	3.4
13	3.9	3.9	2.6	2.2
14	1.7	1.8	1.4	1.8
15	5.6	5.9	4.4	6.6
16	2.2	2.2	1.2	0.6
17	6.5	6.6	5.0	4.1
18	3.5	3.3	2.0	3.2
19	4.2	4.7	3.2	3.0
20	3.1	3.2	3.1	3.0
21	1.7	1.1	1.5	1.0
22	3.0	2.8	2.3	2.5
23	3.4	2.8	2.6	3.6
24	4.2	4.3	3.4	3.4
25	4.4	5.5	6.0	0.6
26	2.8	2.6	1.7	3.7
27	3.7	3.7	5.4	4.4
28	1.7	2.2	1.4	1.1
Avg ± SE	3.66±.22	3.77±.27	3.26±.27	3.30±.32

[a]Smith and Hafs (1973).

TABLE 2.--Comparison of RIA values with and without steroid purification on LH-20 Sephadex.

Fetal serum sample	Testosterone		Androstenedione	
	Solvent extract	Sephadex LH-20	Solvent extract	Sephadex LH-20
	(ng/ml)			
1	0.20	0.50	0.21	0.14
2	0.14	0.34	0.03	0.00
3	4.11	2.82	0.12	0.03
4	3.92	2.33	0.13	0.03
5	0.45	0.46	0.04	0.13
6	0.69	0.68	0.02	0.03
7	0.23	0.37	0.07	0.00
8	0.60	0.32	0.07	0.04
9 (steer serum)	0.02	0.26	0.00	0.04
Avg ± S.E.	1.15±0.5	0.90±0.3	0.08±0.02	0.05±0.01

The correlation coefficient for paired estimates of testosterone in the nine samples was 0.99. Average testosterone determined by RIA on solvent extracts of serum without chromatography did not differ significantly from those obtained by RIA after LH-20 chromatography, indicating that substances separated from testosterone by the chromatography did not compete appreciably with testosterone.

2. Radioimmunoassay (RIA) of Androstenedione

Regarding RIA of androstenedione, the procedure was similar to that for testosterone. However, there are some minor differences which are described below.

a. Androstenedione Extraction.--Aliquants of 200 µl of each serum sample were extracted with 2 ml of nanograde benzene:hexane (1:2). This volume of the serum samples was twice as much as used in testosterone extraction, because serum androstenedione level was lower than that of testosterone (Lindner, 1959; Lindner and Mann, 1960) and results from preliminary studies indicated that the androstenedione assay was not as sensitive as the testosterone assay (50 vs 20 pg). However, the ratio between serum and the organic solvent volumes should not be narrower than 1:5 because the serum often became emulsified during extraction, and this lowered extraction efficiency. The other steps for extraction were the same as for testosterone.

b. Radioimmunoassay (RIA).--Androstenedione antiserum[2] was diluted to 1:1000 in 0.1% Knox gelatin in PBS, and 200 µl of this dilution was used per assay tube. The specificity of the antibody was described by Mongkonpunya et al. (1974).

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[2]Dr. G. D. Niswender furnished androstenedione antiserum code number 866.

c. Assay Validation.--Similar to testosterone RIA, the extraction efficiency of ^{3}H-1,2-androstenedione was relatively constant (84.2±4.4%, n=28). The sensitivity of this assay was less than 50 pg and results obtained from the eight fetal serum samples and one steer serum (Table 2) indicated that the within-sample coefficient of variation was greater than for testosterone assay (52 vs 15%).

As a means of validating the androstenedione assay, similar to that described for the testosterone RIA, the results from RIA directly applied to benzene:hexane extracts without chromatography were compared with the results from RIA after androstenedione purification on LH-20 Sephadex (Table 2). The correlation coefficient for estimates with and without chromatography of the nine samples was 0.44. Average androstenedione determined by RIA on solvent extracts of serum without chromatography did not differ significantly from those obtained by RIA after LH-20 chromatography. Thus, substances separated from androstenedione by the chromatography did not compete appreciably with androstenedione.

3. Radioimmunoassay (RIA) of Luteinizing Hormone (LH)

The bovine LH assay has been described elsewhere (Oxender, 1971; Oxender et al., 1972b). The details of the procedure, including some minor changes, are as follows:

LH antiserum was developed by repeated injections of NIH-LH-B5 into guinea pigs (Appendix I.C.1). Purified bovine LH (LER-1072-2) used for iodination was supplied by Dr. L. E. Reichert Jr. (Emory University, Atlanta, Georgia). This preparation had an LH potency of 1.66 NIH-LH-Si units/mg and showed no FSH activity when tested at 3600 µg in the

Steelman-Pohley assay. It had a thyroid stimulating hormone (TSH) contamination estimated at 0.021 USP units/mg.

a. Radioiodination.--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°C. These vials were thawed immediately before iodination and 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 Corporation, 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 added to the vial, the vial was stoppered, and the contents were gently mixed by finger tapping. The reaction was stopped at exactly 2 minutes 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 (PBS-2.5% BSA) was added to diminish the loss of hormone adhering to the glass vial.

A 1X12 cm glass column packed with Bio Gel P-60 (Bio Rad Labs., Richmond, California) was equilibrated previously by passing 0.05 M sodium phosphate buffer pH 7.5 (Appendix I.A.2) through, and then 2 ml PBS-2.5% BSA was added and eluted with buffer to reduce non-specific binding of the protein hormone to glass. 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 hormone vial, recovered, and also layered beneath the buffer on

the column. The iodinated LH was eluted from the column under gravity with 0.05 M sodium phosphate buffer and 15 ml were collected in 1-ml aliquots from the column in 12X75 mm disposable glass tubes containing 1 ml of 2% Knox gelatin in PBS. The elution profile was determined by quantifying the radioactivity of 10 µl from each of the 15 tubes 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. The peak ^{125}I-LH tube was used in the LH RIA. The iodinated LH was quite stable; stored at 4°C, it could be used up to 2 weeks.

b. Radioimmunoassay.--Each unknown was assayed in dilution duplicate. Two selected dilutions made in PBS-.1% Knox gelatin (Appendix I.B.4) of each unknown were added to disposable glass culture tubes (12X75 mm) with a Hamilton microliter syringe (Hamilton Co., Whittier, California). PBS with 0.1% Knox gelatin was then added to give a total of 500 µl. As discussed by Hunter (1967), use of two dilutions provided evidence of the specificity of the assay which is not provided with duplicate determinations of the same dilution. Each lot of 100 tubes included 10 tubes containing 0, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24, and 20.48 ng of standard LH (NIH-LH-B8, National Institutes of Health, Endocrinology Study Section, Bethesda, Maryland; Appendix I.B.5).

Two hundred µl of LH antibody (Appendix I.B.7), hereafter referred to as first antibody, was added at a dilution of 1:200,000 to each of the culture tubes and the tubes were incubated at 4°C for 24 hours. Solutions of ^{125}I-LH for RIA were prepared by diluting the stock ^{125}I-LH with PBS containing 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°C for 24 hours.

Sheep anti-guinea pig gamma globulin (SAGPGG, Appendix I.C.2), later referred to as the second antibody, 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 hours. After each addition, the tubes were vortexed gently and covered during the incubation to retard evaporation.

Following the final incubation, 3 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 g for 30 minutes 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 minutes 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 minutes or for a total of 4000 or 10,000 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.

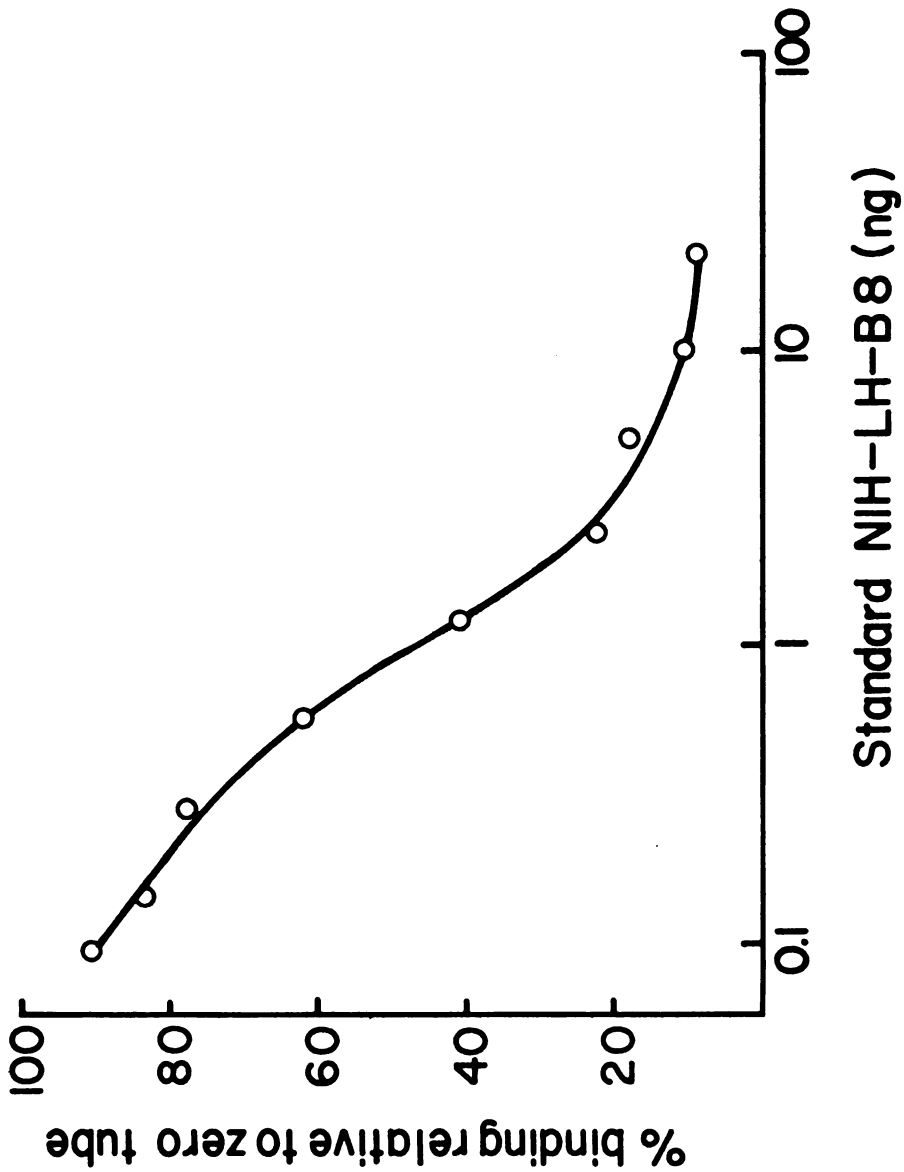
Control tubes were included in each assay to determine background radioactivity (tube containing 1:400 control guinea pig serum in place of the first antibody), total counts added (tube containing only ^{125}I-LH) and counts in the precipitate (tube containing no unknown or standard). Values for the duplicate standards were averaged and plotted as the percent of ^{125}I-LH precipitated at each dose of LH standard (Figure 2).

Oxender et al. (1972b) reported that the assay detected as little as 0.1 ng LH (NIH-LH-B5). Mean recoveries of 0.1, 0.5, 2.0 and 8.2 ng of bovine LH added to 100 µl of serum were 0.1, 0.4, 1.9 and 8.4 ng, although precision was reduced at the high level.

4. Blood Sample Collection and Intravenous Administration

One day before each experiment, jugular cannulation (Vikim Vinyl Tubing, Size V10, Bo Lab, Derry, N.H.) was performed in each animal. Approximately 20 cm of the 60 cm tubing was inserted into a jugular vein and affixed to the neck with tag cement (Nasco, Fort Atkinson, Wisconsin) on 7.6X12.7 cm adhesive tape. Each cannula was flushed with 50% glucose in 3.5% sodium citrate and sealed until used for blood collection. The blood sampling procedure included the following steps; 1) 5 ml of blood and citrate were withdrawn and discarded, 2) 10 ml of blood sample was taken and transferred into a 18X85 mm test tube, and 3) the cannula was flushed with 3.5% sodium citrate. When the cannula needed to be sealed for long periods or between long sampling intervals, 50% glucose in 3.5% sodium citrate was used to

Figure 2.--Dose response curve for NIH-LH-B8 standard.


% binding relative to zero tube
100
80
60
40
20
0.1
1
10
100
Standard NIH-LH-B8 (ng)

flush and fill the cannula. The frequency of blood sampling in each experiment are shown in the particular experiment and also with results of that experiment.

Regarding intravenous administration of GnRH through the cannula, the appropriate volume of normal saline containing GnRH was injected into the cannula and then the cannula was flushed with 5 to 10 ml 3.5% sodium citrate.

5. Design and Procedure of Experiments

a. Experiment I.

This experiment was designed to study the responses of serum LH, testosterone and androstenedione in pubertal bulls after treatment with GnRH. Three 3X3 Latin squares were used (Table 3). Nine bulls, three each at 2, 4 and 6 months of age (average 75, 93 and 137 kilograms body weight, respectively), and three doses of GnRH (200, 400 or 800 μg) were used. Each bull received the three doses of GnRH by intramuscular injection, one on each of 3 consecutive days. Jugular blood was sampled by indwelling cannula at intervals of 0.5, 0.25 and 0 hour before GnRH, and then at intervals of 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 12 hours after GnRH. Blood samples were left at room temperature for 2 to 4 hours, and then kept over night at 4°C before sera were separated by centrifugation at 2500 g for 15 min. The sera were kept at -20°C until assayed for LH, testosterone and androstenedione.

Since animal number 9 in Table 3, a 6-month-old bull, was found to be a cryptorchid, the results obtained from this animal were excluded from the analysis. Exclusion of the ninth bull could cause minor bias in estimates of differences by dose or by day. However, the sample average differences by dose or day were rather small. The significant interactions of day or dose with sampling interval for LH and androstenedione could be slightly biased.

TABLE 3.--Schedule for GnRH treatments to bulls in experiment I.

Age	Bull	Day 1	Day 2	Day 3
(mo)		μg		
2	1	200[a]	400	800
	2	800	200	400
	3	400	800	200
4	4	200	400	800
	5	400	800	200
	6	800	200	400
6	7	800	200	400
	8	200	400	800
	9	400	800	200

[a]Values are μg GnRH given intramuscularly.

b. Experiment II.

This experiment was designed as a triple split-plot with nesting, to study the chronic effect of GnRH on serum LH, testosterone and testicular development in pubertal bulls.

Nine bulls, three each at 2, 4 and 6 months of age, were each given intramuscularly 40 μg of GnRH twice daily (once between 0800 to 0900 hours and the other between 1600 to 1700 hours) for 28 days. Another three bulls at each age were injected with saline as controls. Blood samples were collected by vena puncture immediately before injections and then at 0.5 and 3 hours after injections on day 1, 7, 14, 24 and 28 of treatment. It should be mentioned that originally we planned to collect blood samples at 7-day intervals i.e. day 1, 7, 14, 21 and 28. Due to a severe snow storm on day 21, we sampled blood on day 24 instead. Blood and serum samples were handled as described before until LH and testosterone were quantified. At 2 months after conclusion of the treatment, the approximate period from beginning of spermatogenesis until sperm are available for ejaculation (Almquist and Amann, 1961), the bulls were castrated. Gonadal and extragonadal sperm were determined by the methods described by Macmillan and Hafs (1968a). Body weight and testicular dimensions (Foote et al., 1970; Hahn et al., 1969) were also recorded during the experimental period.

RESULTS AND DISCUSSION

Experiment I: Serum LH, Testosterone and Androstenedione After 200, 400 or 800 µg GnRH (im) in 2-, 4- or 6-Month-Old Bulls

a. LH Response:

Serum LH responses to GnRH are listed by age of bulls and by doses of GnRH in Tables 4 and 5, respectively. The analysis of variance of these data, but excluding those obtained before GnRH injections, is in Table 6.

Serum LH responses to GnRH did not differ significantly among ages (2, 4 or 6 months, Table 4) or among doses (200, 400 or 800 µg, Table 5) of GnRH. On the average serum LH significantly increased ($P<0.01$) from 1 ng/ml before GnRH to 19 ng/ml within 15 minutes after GnRH. The peak of 24 ng/ml was at 45 minutes, then LH declined progressively to 1 ng/ml within 6 hours after GnRH. This result resembles that reported by Miyachi et al. (1973). They found that LH and FSH increased to about the same extent at all ages after injection of GnRH in rats. In humans, Rebar et al. (1973) found that LH responses to 50, 150 and 450 µg GnRH iv were statistically indistinguishable. Our data suggest that all three of the GnRH doses used in bulls were above that which gives a maximal LH response.

Although serum LH after 200, 400 or 800 µg GnRH (Table 5) did not differ significantly, the duration of the LH response to intramuscular injection of 800 µg GnRH was longer than that after 200 µg GnRH (4 vs

TABLE 4.--Serum LH after injection (im) of GnRH into bulls at 2, 4 or 6 months of age.

Sampling interval after GnRH	Age (months) of bull		
	2	4	6
(hr)	(ng/ml)		
Before	1±1	1±1	1±1
0.25	19±3[a]	21±2	15±1
0.50	20±2	24±2	19±3
0.75	23±2	25±2	22±4
1.0	20±2	22±2	20±5
1.5	17±3	19±2	19±7
2.0	18±5	20±2	18±7
3.0	10±3	13±2	14±7
4.0	4±1	5±1	5±3
6.0	1±1	1±1	1±1
8.0	1±1	1±1	1±1
12.0	1±1	2±1	1±1

[a]Each value is the mean ± standard error of LH responses to 200, 400 and 800 µg GnRH.

TABLE 5.--Serum LH after injection (im) of 200, 400 or 800 µg GnRH into bulls.

Sampling interval after GnRH	Dose of GnRH (µg)		
	200	400	800
(hr)	(ng/ml)		
Before	1±1	1±1	1±1
0.25	20±4[a]	17±2	19±2
0.50	20±2	21±3	23±2
0.75	22±2	22±3	27±3
1.0	18±2	19±3	25±3
1.5	13±1	16±2	26±5
2.0	13±2	18±4	26±6
3.0	8±1	9±2	19±5
4.0	2±1	3±1	8±2
6.0	1±1	1±1	1±1
8.0	1±1	1±1	1±1
12.0	2±1	1±1	1±1

[a]Each value is the mean ± standard error of observations on three bulls at 2 and 4 months, and on two bulls at 6 months.

TABLE 6.--Summary of ANOVA for LH data after GnRH.

Source	df	SS	MS	F	P_I
Age	2	153.40	76.70	.73	N.S.
Day	2	424.18	212.09	2.03	N.S.
Animal/Age	5	1,572.97	314.59	3.01	<.10
Dose-Direct	2	565.44	282.72	2.70	N.S.
Error "a"	12	1,253.18	104.43		
Sampling Interval	10	19,572.49	1,957.24	77.54	<.01
Age X Interval	20	271.78	13.58	.54	N.S.
Day X Interval	20	1,709.69	85.48	3.38	<.01
Dose X Interval	20	1,238.48	61.92	2.43	<.01
Error "b"	170	4,291.14	25.24		

3 hours, respectively). This explains the significant interaction in LH response between the dose of GnRH and the sampling interval after GnRH injection (Table 6). This result is in agreement with the observation (Golter et al., 1973) that the durations of LH responses to 0.03, 0.3 and 3.0 μg GnRH per kilogram of body weight of Hereford bulls were 55, 220 and 366 minutes, respectively. Zolman et al. (1973) reported that peak and duration of serum LH responses in mature Holstein bulls varied with the dose of GnRH up to 160 μg when given intravenously. In my study the differences in LH responses among animals within age only approached significance (Table 6; $P<0.10$). Thus, the marked increase in serum LH which occurred after each dose of GnRH was rather consistent among animals.

The magnitude of LH peaks after GnRH in my prepubertal bulls was only about 60% of those reported by Golter et al. (1973) and Zolman et al. (1973), suggesting that adults may respond to GnRH with greater release of LH. Route of administration of the GnRH also could have affected this result. Examples of the influences of how steroid environment can affect the LH response to GnRH have been published. For example, Krey et al. (1972) demonstrated that in female monkeys, the LH responses to 10 μg GnRH at mid-luteal phase (day 19-20) was greater ($P<0.05$) than at other phases of the menstrual cycle. Similarly in women (Yen et al., 1972) the response was greatest during day 21-23 of the cycle. Convey (1973) concluded in his review that "the pituitary responsiveness to exogenous GnRH varies with reproductive state, probably as a result of variation in the steroid environment". Moreover, it has been hypothesized that the pituitary

of immature animals is more sensitive to negative steroid feedback as compared with that of mature animals (Smith and Davidson, 1968).

Mode of administration also can affect the LH response to GnRH. Peck et al. (1973) studied the release of LH in heifers during the late luteal phase of the estrous cycle after intravenous or intrauterine administration of GnRH. After 1 mg of GnRH, serum LH at 2 hours was 38.3 and 28.7 ng/ml for intravenous and intrauterine administration, respectively. Golter et al. (1973) studied different vehicles for GnRH in bulls, and found that 1.3 μg GnRH (sc) per kilogram of body weight in acidified saline or in 2% carboxymethyl cellulose showed comparable peaks of LH responses (41±10 vs 55±10 ng/ml), but the later vehicle prolonged ($P<0.05$) the duration of LH response.

Though there was no significant ($P>0.10$) difference between LH responses among days, a significant ($P<0.05$) interaction between day and interval indicated that peak and duration of LH responses differed by day of treatment (Figure 3 and Table 7). That is, serum LH responses on days 1, 2 and 3 rose sharply within 15 minutes after GnRH, but the magnitude of the peak on Day 1 was significantly greater ($P<0.05$) than those on days 2 and 3. While serum LH on days 2 and 3 declined gradually within 45 minutes after GnRH, serum LH on Day 1 plateaued until 2 hours after GnRH. Our data suggesting a decreasing responsiveness in LH release to repeated GnRH administration agrees with those reported by Chakraborty et al. (1973a,b). They found that the LH response in immature female pigs gradually decreased between the first and third injections of GnRH given at 6-hour intervals. In another experiment with anestrous ewes, infusion of GnRH failed to

Figure 3.--Serum LH after injection (im) of GnRH into bulls on three consecutive days.

Each point represents the mean ± standard error of eight observations, one in each bull.

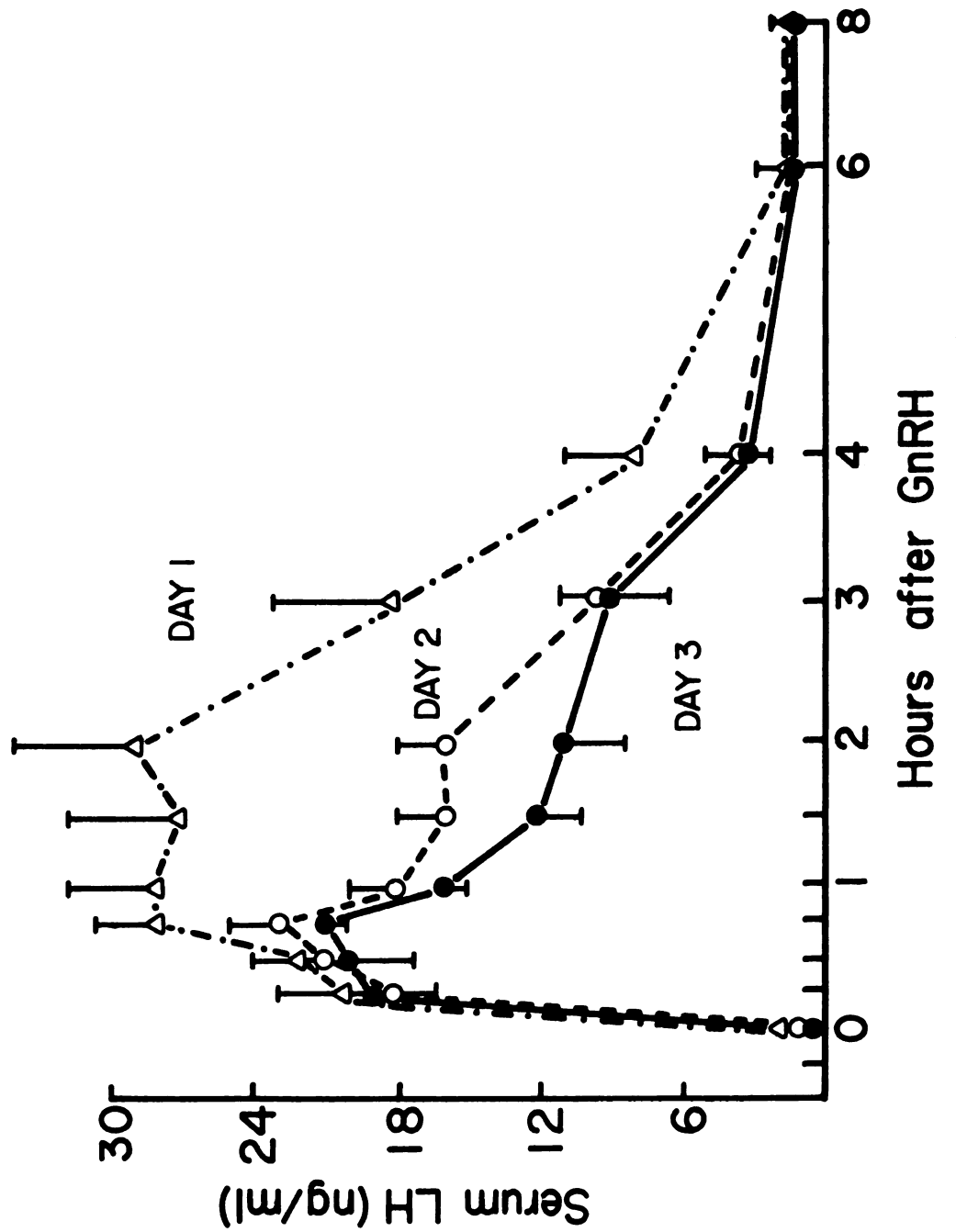

Serum LH (ng/ml)
30
24
18
12
6
0
1
2
3
4
6
8
Hours after GnRH
DAY 1
DAY 2
DAY 3

TABLE 7.--Serum LH after injection (im) of GnRH into bulls on three consecutive days.

Sampling interval after GnRH	Day of administration		
	1	2	3
(hr)	(ng/ml)		
Before	2±1[a]	1±1	1±1
0.25	20±3	18±2	19±3
0.50	22±2	21±3	20±2
0.75	28±3	23±2	21±1*
1.0	28±4	18±2*	16±1*
1.5	27±5	16±2*	12±2*
2.0	29±5	16±2*	11±3*
3.0	18±5	9±2	9±3
4.0	8±3	3±2	3±1
6.0	1±1	1±2	1±1
8.0	1±1	1±1	1±1
12.0	2±1	1±1	1±1

[a]Mean ± standard errors for eight bulls each given 200, 400 or 800 µg GnRH im.

*Significantly less than value on day 1.

maintain the LH response after 3 hours, because GnRH decreased the LH concentration and content in the pituitary.

b. Testosterone Response:

Since the serum LH responses to GnRH were similar at 2, 4 and 6 months of age regardless of the dose of GnRH, I decided to determine the testosterone response to increased endogenous LH.

Serum testosterone, determined in the same samples in which I determined LH, are shown in Table 8 and Figure 4. The summary of the analysis of variance of the data, omitting samples taken before GnRH, is listed in Table 9.

In 2-month-old bulls, testosterone averaged 0.4 ng/ml before GnRH, increased slightly to 0.6 ng/ml at 2-3 hours and declined to 0.3 ng/ml at 6 hours after GnRH ($P>0.05$). The comparable values were 0.6, 1.2 and 0.5 ng/ml in 4-month-old bulls ($P>0.05$). In contrast, in 6-month-old bulls, testosterone averaged 1.7 ng/ml before GnRH, increased significantly ($P<0.05$) within 1 hour (4.6 ng/ml) and peaked (5.3 ng/ml) at about 2 hours after GnRH injection, and then returned to 1 ng/ml 6 to 8 hours after GnRH. The lack of a significant increase in serum testosterone in 2- or 4-month-old bulls was not caused by a lack of LH in the blood as shown in Table 4. Hooker (1970) reported that differentiation of mesenchymal cells or fibroblastic cells of the testicular interstitium in bull calves began about 3 1/2 months of age and cytochemically they became fully developed as secretory cells during the next 6 1/2 months. These results clearly demonstrate a difference in sensitivity of the Leydig cells to increased endogenous LH in bulls at different ages, and suggests that this testicular change from 2 to 6 months of age is related with sexual maturation.

TABLE 8.--Serum testosterone after injection (im) of GnRH into bulls at 2, 4 or 6 months of age.

Sampling interval after GnRH	Age (months) of bull		
	2	4	6
(hr)	(ng/ml)		
Before	0.4±.1	0.6±.1	1.7±.5
0.5	0.4±.1[a]	1.0±.1	2.1±.4
1.0	0.5±.1	0.9±.2	4.6±.4
1.5	0.5±.1	1.0±.2	4.8±.2
2.0	0.6±.1	1.2±.1	5.3±.3
3.0	0.7±.1	1.0±.2	5.1±.2
4.0	0.6±.1	0.9±.2	4.2±.5
6.0	0.3±.1	0.6±.1	1.1±.4
8.0	0.2±.1	0.4±.1	0.6±.2

[a]Each value is the mean ± standard error of testosterone responses to 200, 400 and 800 µg GnRH.

Figure 4.--Serum testosterone after injection (im) of GnRH into bulls at 2, 4 or 6 months of age.

Each point represents the mean ± standard error of nine or six observations, three in each bull.

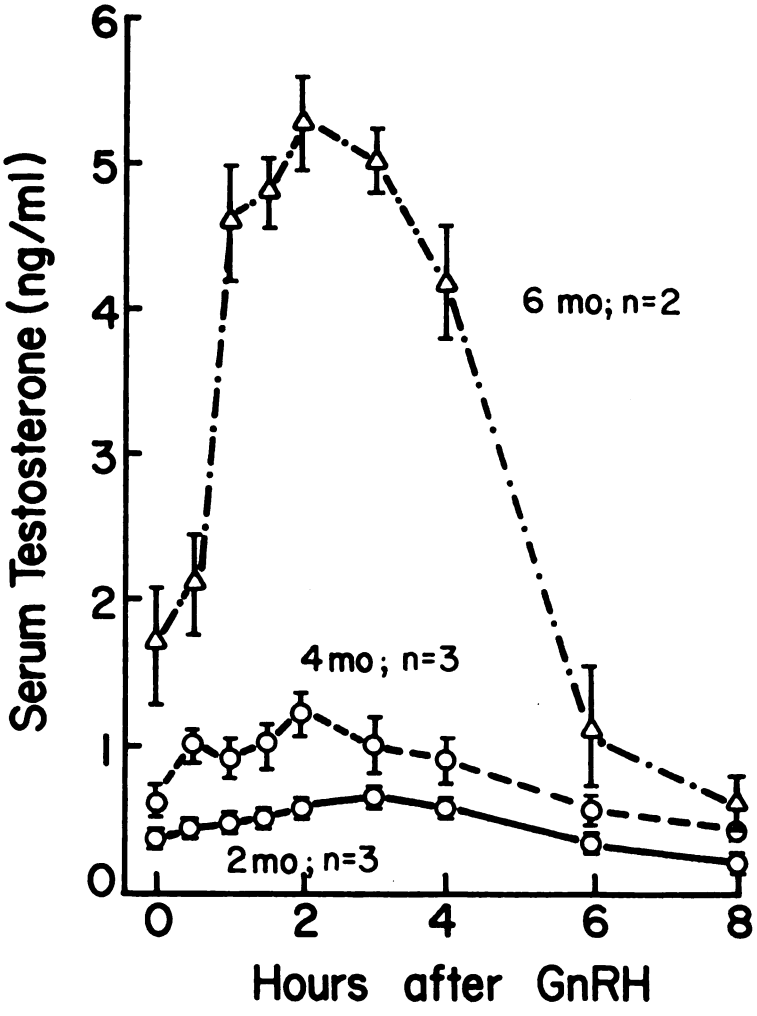

Serum Testosterone (ng/ml)
0
1
2
3
4
5
6
0
2
4
6
8
Hours after GnRH
6 mo; n=2
4mo; n=3
2mo; n=3

TABLE 9.--Summary of ANOVA for testosterone data after GnRH.

Source	df	SS	MS	F	P_I
Age	2	287.48	143.74	2,356.39	<.01
Day	2	.06	.03	.49	N.S.
Animal/Age	5	9.48	1.89	30.98	<.01
Dose-Direct	2	.21	.10	1.64	N.S.
Error "a"	12	.73	.061		
Sampling Interval	7	89.88	12.84	61.14	<.01
Age X Interval	14	94.10	6.72	32.00	<.01
Day X Interval	14	3.27	.23	1.09	N.S.
Dose X Interval	14	2.40	.17	.81	N.S.
Error "b"	119	25.32	.21		

Our results showing increased release of testosterone in 6-month-old bulls is in agreement with the results of Zolman and Convey (1973) who reported that 10, 40 and 160 μg of GnRH given to mature bulls caused significant increases in serum testosterone and androstenedione (184 and 75%, respectively).

In contrast to the LH response to GnRH, the significant day x interval interaction did not occur for testosterone (Table 9). Evidently, the reduced LH response on day 2 and 3 (Table 7) was still high enough to give a maximal testosterone surge. Smith et al. (1973) reported that, on the average, an increase in LH of only 2.4 ng/ml resulted in increased testosterone. Moreover, unlike LH, there was a significant difference in testosterone among animals within age. The coefficients of variation were 100, 80 and 73% in 2-, 4- and 6-month-old bulls, respectively; indicating that there was more variation in serum testosterone in younger than in older bulls.

c. Androstenedione Response:

Blood androstenedione at various intervals after GnRH is listed by ages of bulls in Table 10 and by days of treatments in Table 11. A graphic illustration of an interaction between day and interval is in Figure 5.

The concentrations of blood androstenedione were about 68, 83 and 93% lower than testosterone in 2-, 4- and 6-month-old bulls, respectively. In contrast to testosterone, blood androstenedione, on the average, after GnRH (200, 400 or 800 μg) increased significantly ($P<0.05$) at 2, 4 or 6 months of age, and on each day of treatment (Table 10 and 11). On the average androstenedione increased about three-fold from 119 pg/ml before GnRH to an average of 297 pg/ml at

Figure 5.--Serum androstenedione after injection (im) of GnRH into bulls on three consecutive days.

Each point represents the mean of eight observations, one in each bull.

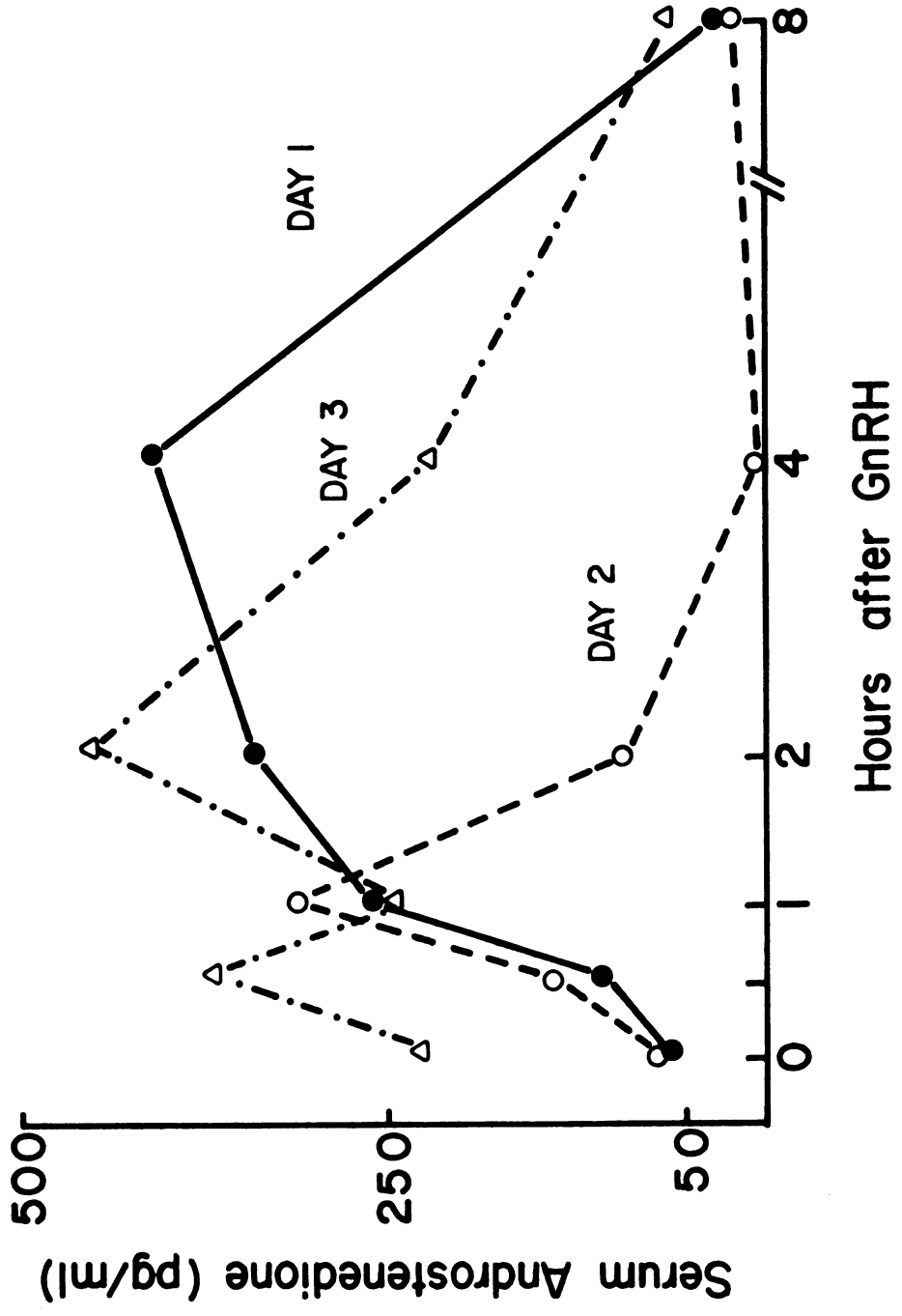

Serum Androstenedione (pg/ml)
500
250
50
0
1
2
4
8
Hours after GnRH
DAY 1
DAY 2
DAY 3

TABLE 10.--Serum androstenedione after injection (im) of GnRH into bulls at 2, 4 or 6 months of age.

Sampling interval after GnRH	Age (months) of bull		
	2	4	6
(hr)	(pg/ml)		
Before	130±50[a]	100±50	120±80
0.5	110±40	310±80	190±120
1.0	260±50	330±80	220±50
2.0	380±90	260±70	310±110
4.0	200±70	270±70	160±90
8.0	50±20	100±50	10±7

[a]Each value is the mean ± standard error of androstenedione response to 200, 400 and 800 µg GnRH.

TABLE 11.--Serum androstenedione after injection (im) of GnRH into bulls on three consecutive days.

Sampling interval after GnRH	Day 1	Day 2	Day 3
(hr)	(pg/ml)		
Before	63±54[a]	64±30	231±61
0.5	105±60	139±65	370±88
1.0	261±92	316±36	248±55
2.0	394±96	94±30	456±65
4.0	418±50	0±0	227±63
8.0	34±23	64±60	76±31

[a]Each value is the mean ± standard error of eight bulls each given 200, 400 or 800 µg GnRH im.

1 to 2 hours, and then declined to 58 pg/ml at 8 hours after GnRH. Neither the differences among ages nor those among days approached statistical significance. Doses of GnRH had essentially equal effects on stimulating serum androstenedione concentrations, although androstenedione differed significantly ($P<0.05$) among animals within age (Table 12).

A significant ($P<0.01$) interaction in the androstenedione responses associated with day and interval (Table 12) is illustrated in Figure 5. On day 1, androstenedione increased gradually until 4 hours after GnRH. On day 2, androstenedione peaked at 1 hour, and on day 3 it peaked at 2 hours. Serum LH also exhibited a significant day x interval interaction. Thus, the pattern of androstenedione response to GnRH was generally similar to that for LH although it lagged behind the LH response by 15 min to 2 hours. These data may suggest that LH affects serum androstenedione.

Thus, the testes of 2- or 4-month-old bulls are as capable of androstenedione synthesis as the testes of 6-month-old bulls, while testosterone synthesis was not evident in large quantity until 6 months. In the testes of 6-month-old bulls the peak of testosterone response after GnRH was about 20-fold greater than that for androstenedione (5.3 vs 0.3 ng/ml). Others (Lindner, 1959; Lindner and Mann, 1960) have reported that androstenedione is the predominant androgen before puberty in bulls. But this is the first report that androstenedione is capable of responding to LH stimulation before puberty. My data suggest that LH stimulation of testicular testosterone synthesis is probably blocked before 6 months of age possibly in part at the conversion of androstenedione to testosterone, since serum

TABLE 12.--Summary of ANOVA for androstenedione data after GnRH.

Source	df	SS	MS	F	P_I
Age	2	86,022	43,011	1.69	N.S.
Day	2	19,813	9,906	0.39	N.S.
Animal/Age	5	531,674	106,334	4.17	<.05
Dose-Direct	2	93,259	46,629	1.83	N.S.
Error "a"	12	305,820	25,485		
Sampling Interval	5	1,058,110	211,622	6.31	<.01
Age X Interval	10	291,543	29,154	.87	N.S.
Day X Interval	10	1,190,282	119,028	3.55	<.01
Dose X Interval	10	141,697	14,169	.42	N.S.
Error "b"	85	2,849,286	33,521		

androstenedione in young bulls (2 and 4 months of age) was increased significantly whereas testosterone was not increased. It would be interesting to test whether blockade of the activity of 17-β-hydroxysteroid dehydrogenase would cause the accumulation of androstenedione rather than testosterone after LH stimulation in postpubertal bulls. In other words, deficiency of 17-β-hydroxysteroid dehydrogenase may account for low testosterone in 2- and 4-month-old bulls relative to 6-month-old bulls, and activation of this enzyme may be associated with onset of testosterone synthesis at puberty.

In summary, results from this experiment indicated that serum LH increased about 24-fold within 45 minutes after GnRH injection, and declined to pretreatment concentrations within 6 hours after GnRH. This pattern of LH response to GnRH was similar at all ages of bulls and at all doses of GnRH, except that the higher dose (800 μg) of GnRH showed a longer duration of LH response. In addition, there was a decreased responsiveness in LH release to repeated administrations of GnRH. In response to the increased LH release, serum testosterone in 6-month-old bulls increased about 3-fold within 2 hours after GnRH, but in 2- or 4-month-old bulls serum testosterone was not significantly altered. Unlike testosterone, androstenedione significantly increased at all ages and the pattern of the androstenedione response to GnRH was generally similar to that for LH.

Experiment II: Effect of GnRH Injections Twice Daily for 28 Days on Serum LH and Testosterone, and on Reproductive Development in Pubertal Bulls

a. Body Weight:

Within the same age, the average body weights of bulls at the beginning of the experiment were not statistically different. The averages were 73, 117 and 173 kilograms for bulls at 2, 4 and 6 months of age, respectively. Average daily gain, measured during the experimental period, between control and GnRH-treated groups did not differ significantly. However, daily rate of gain during the experimental period increased with advancing age (0.50, 0.88 and 1.27 kilograms for 2, 4 and 6 months of age, respectively). Macmillan and Hafs (1968b) reported that the body weight of bulls at 2, 4 and 6 months were 73, 138 and 179 kilograms, respectively, and the increase in body weight with increased age was linear. They concluded that the period of puberty was not associated with marked changes in rate of growth.

b. Testicular Dimension:

The average testicular circumference, width and length are shown in Table 13. Statistical comparisons of these parameters between bulls given saline and bulls given GnRH were not significantly different ($P>0.05$). Chakraborty *et al.* (1973b) infused GnRH at 2.3 μg/hour into anestrous ewes for 20 hours and found no effect on ovarian follicular growth. Though their investigation cannot be directly compared with the present study in prepubertal bulls, experimental animals in both studies were sexually inactive. Macmillan and Hafs (1968a,b and 1969) demonstrated that testicular growth (weight, width and length) was

TABLE 13.--Average testicular dimensions of bulls.

Age at beginning	GnRH (mg/day)	Testicular dimension[a]		
		Circumference	Width	Length
(mo)		(cm)		
2	0	15.0	3.9	5.7
	40X2	14.5	3.8	5.8
4	0	19.5	4.4	7.4
	40X2	19.1	4.3	7.2
6	0	25.5	6.2	10.2
	40X2	26.5	6.1	9.8

[a]The averages of 4 measurements during the experimental period.

associated with increasing concentrations of hypothalamic LH releasing factor, plasma LH and body weight. Thus, failure of GnRH to stimulate testis growth could be attributed to the gradual decrease of the LH response to the repeated treatment of GnRH as demonstrated in Experiment I and in this experiment (Figures 6 and 7).

c. Testicular and Extratesticular Sperm Counts:

Testicular spermatozoa were found at 60 days after the last GnRH injection in all bulls except two which received GnRH treatment starting at 2 months of age (Table 14). At 9 months I found appreciable numbers of testicular spermatozoa, and this is in agreement with the report by Macmillan and Hafs (1968a); the youngest age at which they found sperm was 5 months. However, they reported nearly 3-fold more total testicular sperm than I found (average 0.16 vs 0.5 and 6 vs 15 billion at 7 and 9 months, respectively). These differences in sperm numbers are principally due to reduced (34 and 45% at 7 and 9 months, respectively) testicular weight and also sperm concentrations in the present study (63 and 52% for 7 and 9 months, respectively).

In my bulls, total epididymal sperm numbers, at 9 months of age were $3.28X10^9$, similar to the $4.00X10^9$ reported by Macmillan and Hafs (1968a). In general, epididymidal sperm can be enumerated more precisely than testicular sperm because more debris is present in testicular homogenates. Thus, although the rate of spermatogenesis in my bulls may have been somewhat less than those reported by Macmillan and Hafs (1968a), the general changes from 5 to 9 months of age were similar.

TABLE 14.--Gonadal size and sperm, and epididymidal sperm at 60 days after 28 days of GnRH treatment.

Bull age	GnRH	Total testes weight	Circumference of scrotum	Testicular sperm[a]		Total epididymal sperm[a]	
				Concentration	Total	Tail	Head-body
(mo)	(μg/day)	(g)	(cm)	($X10^6$/g)	($X10^9$/bull)	($X10^6$)	($X10^6$)
5	0	39.8±8.1	15.4±1.5	0.3±0.1	0.01±0.0	0	2.5 (2)
	40X2	45.9±12.9	17.5±2.3	0.5 (1)[b]	0.01 (1)	0	3.0 (1)
7	0	100.1±8.8	20.8±1.4	2.6±2.2	0.3±0.2	1.00 (1)	4.0 (1)
	40X2	74.5±6.1	20.2±0.9	0.5±0.0	0.03±0.0	4.00 (1)	3.7±1.2
9	0	245.3±24.5	29.2±1.3	25.8±3.0	6.2±0.3	1821.3±440.4	1465.3±536.5
	40X2	246.0±27.6	30.4±0.5	22.9±9.1	6.0±2.6	1750.7±962.3	1403.3±897.9

[a]Includes spermatids (stages VI, VII and VIII) and spermatozoa.

[b]Number of bulls, if less than three.

At 9 months of age all bulls had sperm in their testes and epididymides, but sperm numbers did not differ ($P>0.1$) between control and treated animals. At this age, the total sperm counts were greater in the tail (1,786 million) than in the head-body (1,434 million) of the epididymis. This observation is in agreement with reports by Almquist and Amann (1961) and Macmillan and Hafs (1968a); the tail of epididymis is the major storage organ for sperm awaiting ejaculation. However, at 5 or 7 months of age, sperm numbers were higher in the head-body epididymis. In the younger bulls, spermatogenesis had begun only recently and the sperm probably had not yet accumulated in the tail of epididymis.

d. Serum LH:

From Experiment I, intramuscular administration of GnRH into 6-month-old bulls increased serum LH and serum testosterone within 45 minutes. After injection of LH into mature bulls (Smith et al., 1973), testosterone doubled in blood within 1 hour, peaked at 1.5 to 2 hours and remained high for at least 4 hours. Thus, in the present experiment, blood was sampled at 0, 0.5 and 3 hours after injections of GnRH beginning between 0800-0900 hours and again between 1600-1700 hours on days 1, 7, 14, 24 and 28. Serum LH values in these samples are shown in Table 15. The summary of analyses of variance of the same data are in Table 16.

Administration of GnRH (40 μg im twice daily for 28 days) significantly ($P<0.01$) increased serum LH (Table 15) in all bulls. This effect was similar in bulls at all ages and between AM or PM injections. However, serum LH responses were significantly affected by day (1, 7, 14, 24 and 28) of the experimental period ($P<0.05$), and by the sampling

TABLE 15.--Serum LH response to saline or GnRH (40 μg, im) injected twice daily for 28 days in bulls

Day	AM-PM	Interval relative to injection	Age of bull: 2 months Saline	2 months GnRH	4 months Saline	4 months GnRH	6 months Saline	6 months GnRH
			(ng/ml)					
D_1	AM	0	0.7±0.3[a]	0.8±0.2	0.9±0.3	2.3±0.9	1.2±0.3	2.4±0.5
		0.5	0.3±0.2	32.7±20.2	1.1±0.5	27.4±6.8	1.2±0.5	14.7±1.9
		3.0	0.5±0.2	6.8±2.2	1.3±0.7	3.1±1.5	2.7±1.5	2.8±1.2
	PM	0	0.8±0.2	0.5±0.0	1.5±0.6	0.8±0.0	1.0±0.4	2.3±1.1
		0.5	0.6±0.2	23.6±19.3	0.7±0.1	12.9±4.7	0.9±0.4	8.4±2.5
		3.0	1.1±0.6	1.8±1.1	1.7±0.8	2.6±0.9	2.7±1.5	1.0±0.0
D_7	AM	0	0.8±0.3	1.0±0.2	1.8±0.5	1.7±1.0	0.8±0.2	2.9±0.4
		0.5	0.7±0.2	6.0±1.3	1.0±0.2	8.1±2.6	0.8±0.4	5.5±1.5
		3.0	4.8±1.0	4.2±2.2	3.1±1.5	2.9±1.0	4.8±2.3	3.4±1.5
	PM	0	3.0±1.9	1.4±1.0	0.6±0.0	0.6±0.2	1.9±0.5	1.1±0.3
		0.5	1.2±0.6	2.7±0.8	0.6±0.1	6.8±2.7	1.1±0.3	3.9±1.9
		3.0	1.5±1.2	2.1±0.5	2.1±0.3	1.7±0.4	1.1±0.5	1.4±0.4
D_{14}	AM	0	1.7±1.4	4.7±3.6	1.1±0.0	1.8±0.4	0.9±0.2	2.6±1.4
		0.5	0.8±0.4	6.7±3.1	0.8±0.2	5.7±1.4	1.4±0.3	2.9±0.7
		3.0	1.0±0.4	4.6±3.8	1.6±0.6	1.5±0.4	3.7±1.7	2.2±1.6
	PM	0	1.1±0.7	0.9±0.2	1.2±0.4	1.6±0.9	1.8±0.6	1.9±0.7
		0.5	0.7±0.4	8.0±3.9	0.7±0.2	8.5±0.6	1.2±0.1	7.2±2.5
		3.0	0.7±0.2	5.1±2.9	3.2±1.2	12.5±10.3	2.7±1.2	1.1±0.5

Table 15.--(Cont'd).

D_{24}	AM	0	1.6±0.7	1.7±1.2	1.0±0.4	1.0±0.3	1.4±0.2	1.0±0.4
		0.5	1.2±0.1	4.1±2.1	1.9±0.6	8.5±2.2	1.0±0.3	4.0±2.1
		3.0	1.1±0.4	2.0±0.8	1.6±0.9	1.2±0.4	0.6±0.1	1.8±0.8
	PM	0	0.7±0.2	1.6±0.7	1.6±0.9	6.8±4.0	1.2±0.4	1.5±0.4
		0.5	0.7±0.2	4.7±1.4	1.1±0.3	10.2±3.0	2.6±1.5	2.4±1.0
		3.0	1.4±1.0	2.1±1.1	1.4±0.2	2.5±0.5	0.9±0.0	1.0±0.3
D_{28}	AM	0	1.2±0.4	1.1±0.1	1.1±0.2	1.8±0.3	1.5±0.6	2.0±0.6
		0.5	2.7±1.7	12.7±8.9	1.0±0.3	10.6±1.6	0.9±0.1	2.9±0.9
		3.0	2.3±0.9	2.0±0.6	1.5±0.5	6.1±2.1	0.8±0.1	1.0±0.3
	PM	0	0.8±0.2	0.8±0.0	2.3±1.0	1.9±0.9	0.9±0.3	1.6±0.7
		0.5	0.7±0.2	1.4±0.1	1.2±0.3	7.5±1.2	5.0±3.9	1.8±0.5
		3.0	2.1±0.9	1.6±1.0	5.1±1.8	2.2±0.5	2.5±1.5	1.5±0.2
Mean			1.3±0.2	---	1.5±0.2	---	1.7±0.2	---

[a]Each value is the mean (± SE) of three bulls.

TABLE 16.--Summary of ANOVA for LH data during 2X40 μg GnRH daily for 28 days.

Source	df	SS	MS	F	P_I
Treatment (T)	1	1151.36	1151.36	14.369	0.003
Age (A)	2	108.74	54.37	0.679	N.S.
TA	2	178.14	89.07	1.112	N.S.
Bulls/TA (B)	12	961.56	80.13		
Days (D)	4	422.70	105.67	3.146	0.02
TD	4	604.59	151.15	4.500	0.004
AD	8	97.40	12.17	0.362	N.S.
TAD	8	146.69	18.34	0.546	N.S.
BD	48	1612.20	33.59		
AM-PM (H)	1	42.39	42.39	3.794	N.S.
TH	1	58.87	58.87	5.269	0.04
AH	2	30.69	15.34	1.373	N.S.
TAH	2	6.60	3.30	0.295	N.S.
BH	12	134.07	11.17		
DH	4	140.63	35.16	8.789	0.0005
TDH	4	163.67	40.92	10.229	0.0005
ADH	8	71.03	8.88	2.219	0.04
TADH	8	44.32	5.54	1.385	N.S.
BDH	48	192.01	4.00		

Table 16.--(Cont'd).

Sampling Interval (I)	2	1093.21	546.60	9.799	0.001
TI	2	1373.56	686.78	12.312	0.0005
AI	4	103.71	25.93	0.465	N.S.
TAI	4	165.86	41.46	0.743	N.S.
BI	24	1338.73	55.78		
DI	8	1018.85	127.36	4.487	0.0005
TDI	8	1015.57	126.95	4.473	0.0005
ADI	16	268.18	16.76	0.591	N.S.
TADI	16	250.02	15.63	0.551	N.S.
BDI	96	2724.83	28.38		
HI	2	55.39	27.69	5.942	0.008
THI	2	63.38	31.69	6.799	0.005
AHI	4	58.72	14.68	3.149	0.032
TAHI	4	5.29	1.32	0.284	N.S.
BHI	24	111.86	4.66		
DHI	8	156.48	19.56	2.614	0.012
TDHI	8	134.96	16.87	2.254	0.030
ADHI	16	135.48	8.47	1.131	N.S.
TADHI	16	94.72	5.92	0.791	N.S.
BDHI	96	718.42	7.48		
Total	539	17054.85			

intervals relative to time of GnRH injection ($P<0.01$). Several two- and three-way interactions were statistically significant (Table 16), but their physiological significance is obscure.

Considering only the saline control group (Table 15), average serum LH during the treatment period did not differ significantly among age groups (1.3, 1.5 and 1.7 ng/ml for 2, 4 and 6 months, respectively). Serum LH levels in control bulls between 2 and 9 months of age were essentially within the same range (Table 17). The small increase in LH concentration between 4 and 5 months of age was not significant ($P>0.05$). This finding is in agreement with that reported by Odell et al. (1970), who found no marked differences in mean LH concentrations in bulls and heifers between 1 and 5 months of age. However, Macmillan and Hafs (1968b) found LH levels increased between 2 and 4 months and again between 6 and 10 months of age. In contrast to serum LH, serum testosterone increased dramatically between 2 and 9 months of age. These data suggest two possibilities: 1) only minute amounts of LH are required for stimulation of testosterone synthesis in Leydig cells; and 2) other hormonal factors may be involved in the stimulation of testosterone synthesis.

Before GnRH was given to these animals serum LH averaged 1.8±0.2 ng/ml; this was comparable to that in the control group (1.3±0.1 ng/ml). Serum LH markedly increased at 30 minutes after GnRH injection (Table 15 and Figure 6) and declined to or approached pretreatment values within 3 hours after GnRH. It was observed that serum LH concentrations at 30 minutes and 3 hours after GnRH were correlated ($r = 0.57$). This relationship is probably only a reflection of the time required for degradation of LH in blood. In contrast, serum LH levels before GnRH

TABLE 17.--Serum LH and testosterone in control bulls treated with saline twice daily for 28 days.

Age at the beginning	Age at sampling	Day of expt.	LH	Testosterone
(mo)			(ng/ml)	
2	2	1	0.7±.1[a]	0.7±.2
	3	28	1.6±.3	0.6±.1
	4	58	0.7±.2	0.1±.0
	5	88	1.8±.3	1.7±.6
4	4	1	1.2±.2	1.7±.7
	5	28	2.0±.6	2.6±.5
	6	58	0.9±.4	2.2±.4
	7	88	0.1±.1	2.1±.7
6	6	1	1.6±.4	3.2±.8
	7	28	1.9±.7	3.8±.4
	8	58	0.6±.4	3.6±1.1
	9	88	0.6±.2	2.8±1.1

[a]Each value is mean ± standard error of six observations from each of three bulls. Blood samples were taken at 0, 0.5 and 3 hours after saline injection at 8 AM and 5 PM on each day; except days 58 and 88.

Figure 6.--Serum LH at 0, 0.5 or 3 hours after injection (im) of GnRH into bulls on days 1, 7, 14, 24 or 28.

Each bar represents the mean ± standard error of eighteen observations, two in each bull. Control values were averages of 0, 0.5 and 3 hours because they did not differ significantly.

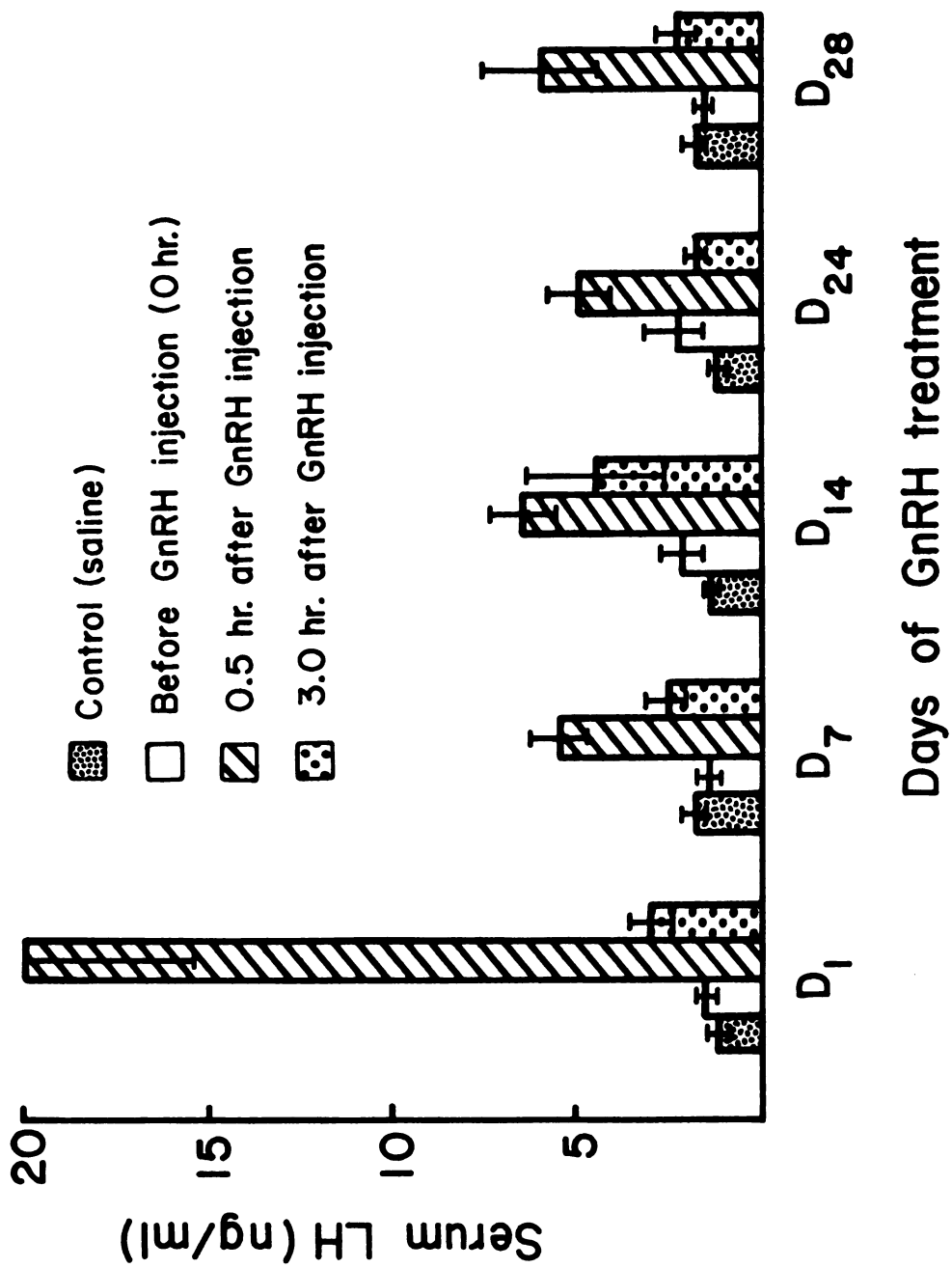

Control (saline)
Before GnRH injection (0 hr.)
0.5 hr. after GnRH injection
3.0 hr. after GnRH injection
Serum LH (ng/ml)
20
15
10
5
D1
D7
D14
D24
D28
Days of GnRH treatment

TABLE 18.--Serum LH and testosterone in bulls treated with GnRH twice daily for 28 days.

Age at the beginning	Age at sampling	Day of expt.	LH	Testosterone
(mo)			(ng/ml)	
2	2	1	11.0±5.0[a]	1.1±0.2
	3	28	3.3±1.6	0.4±0.1
	4	58	2.1±0.8	0.3±0.1
	5	88	---	---
4	4	1	8.2±2.6	1.8±0.3
	5	28	5.0±0.9	2.5±0.4
	6	58	0.1±0.1	2.8±0.8
	7	88	0.5±0.2	4.7±1.4
6	6	1	5.3±1.3	5.0±0.9
	7	28	1.8±0.2	6.2±0.8
	8	58	0.1±0.1	0.8±0.3
	9	88	0.7±0.2	7.3±2.2

[a]Each value is mean ± standard error of six observations from each of three bulls. Blood samples were taken at 0, 0.5 and 3 hours after GnRH at about 8 AM and 5 PM on each day; except days 58 and 88.

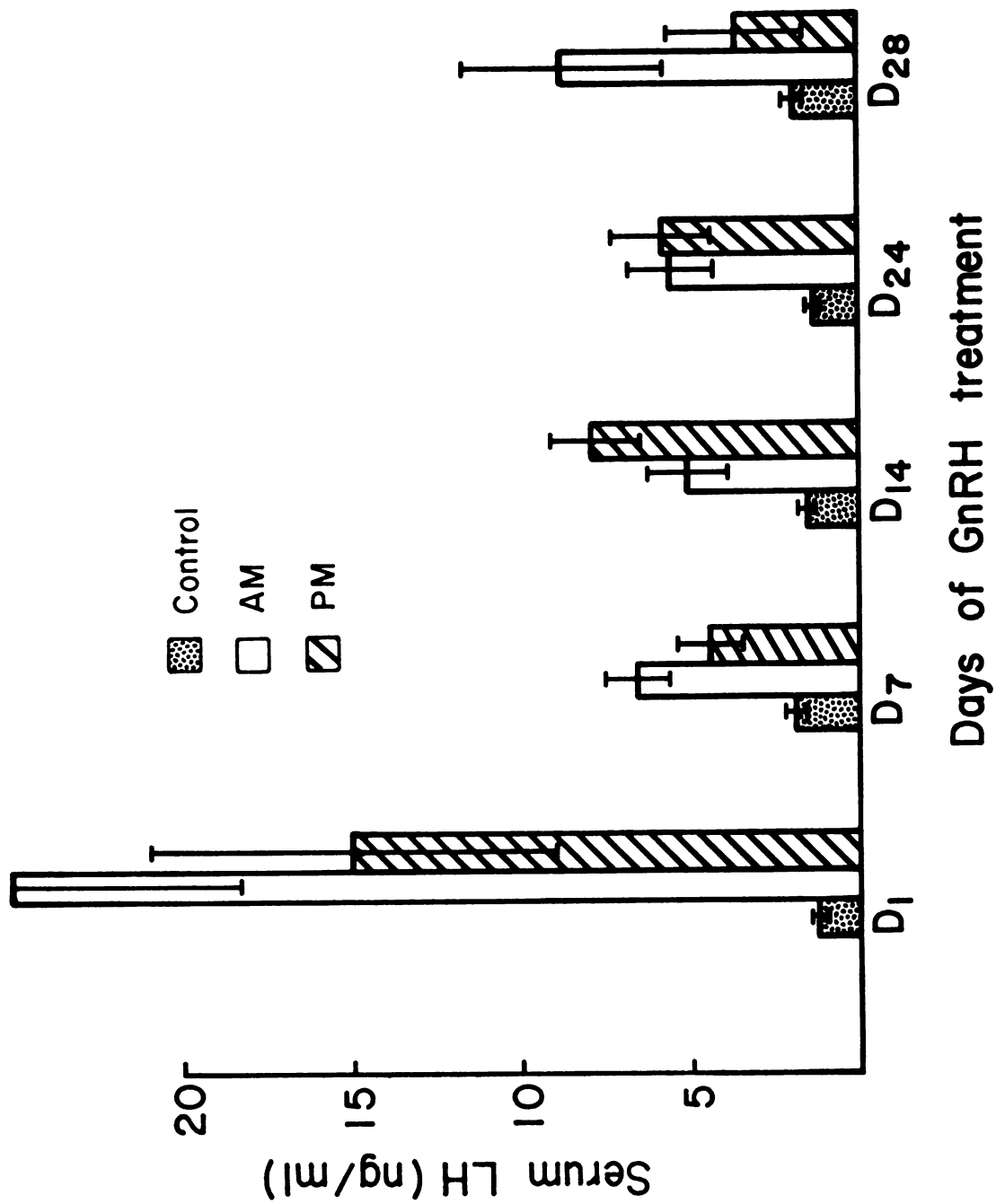
was not associated with LH concentrations at 30 minutes (r = 0.02). The serum LH response to GnRH at 30 minutes after GnRH was greatest following the first injection (AM) on day 1 (Figure 7); the values were 32.7, 27.4 and 14.7 ng/ml for 2-, 4- and 6-month-old bulls, respectively (Table 15). Comparable data after the second injection (PM) on day 1 were 23.6, 12.9 and 8.4 ng/ml, respectively (Table 15). The average (AM+PM) serum LH concentrations at 30 minutes after GnRH on day 1 from all bulls (20.0±4.6 ng/ml) were significantly ($P<0.05$) higher than those on days 7, 14, 24 and 28 which averaged 5.5±.8, 6.5±.9, 5.0±.9 and 6.2±.2 ng/ml, respectively (Figure 6). Notice that the values from day 7 to 28 are consistent; the principle decrease in the LH response to GnRH occurred before day 7.

Although the younger bulls apparently had a greater LH response, this was statistically insignificant ($P>0.05$). Moreover, two-way interactions between age and other factors (treatment x age, day x age, AM-PM x age and interval x age; Table 16) were not significant ($P>0.05$) suggesting that the pituitaries of animals at different ages responded equally to the GnRH treatment. This finding together with that of Macmillan and Hafs (1968b) who found that GnRH was undetectable in the hypothalami from bulls before 5 months of age suggests that probably GnRH in the hypothalamus is a limiting factor for LH release before puberty. This result is in accord with those of Experiment I and Miyachi et al. (1973) who found that, in rats, LH and FSH increased to about the same extent at all ages after injection of GnRH.

Although there was no significant difference between the LH after AM or PM injections of GnRH, significant interactions between day and AM-PM, and between sampling interval and AM-PM (Table 16) were due to

Figure 7.--Serum LH at 0.5 hour after injection (im) of GnRH into bulls in the morning (AM) or evening (PM).

Each bar represents the mean ± standard error of nine observations, one in each bull. Control values were averages of AM and PM because they did not differ significantly.


Serum LH (ng/ml)
20
15
10
5
Control
AM
PM
D1
D7
D14
D24
D28
Days of GnRH treatment

the fact that the AM injections on day 1, 7 and 28 caused greater LH release than PM injections. In contrast, AM injections on days 14 and 24 were less effective than PM injections (Figure 7). I have no explanation for this change in sensitivity of pituitary to GnRH.

Serum LH concentrations (Table 18) on days 58 and 88 (30 and 60 days after the last GnRH injection) were similar to those of the control animals (Table 17). These values were significantly ($P<0.05$) less than those during GnRH treatment, between days 1 and 28, indicating that after withdrawal of GnRH administration serum LH concentration declined to a normal range.

In Experiment I, intramuscular injection of GnRH (200, 400 and 800 μg) resulted in a greater LH peak and longer duration of LH response on day 1 than on days 2 or 3. Similarly, repeated injections of GnRH into immature female pigs (Chakraborty et al., 1973a) and infusion of GnRH into anestrus ewes (Chakraborty et al., 1973b) resulted in decreasing LH responses. In the pig, the LH response declined from the first to the third injection and thereafter remained constant throughout a 6-hour interval of 16 injections. In the anestrous ewe, the LH response to GnRH infusion declined progressively until LH returned to preinfusion values at 20 hours. Results from these three investigations are in general agreement with the finding in Experiment II, that the LH response diminishes upon repeated injection of GnRH.

e. Serum Testosterone:

Serum testosterone determined at 1 month intervals during the experimental period (3 months) in control and GnRH-treated groups are shown in Table 17 and 18, respectively. Serum testosterone generally increased between 2 and 9 months. In bulls starting on the experiment

at 6 months of age, GnRH injections increased the average testosterone concentrations 31% ($P<0.05$) as compared with saline injections.

Serum testosterone values and the summary of analysis of variance are listed in Tables 19 and 20. The overall analysis of variance showed no significant difference in serum testosterone responses between saline and GnRH, nor among days that blood samples were collected (days 1, 7, 14, 24 and 28). Significant differences ($P<0.05$) were obtained among ages (2, 4 or 6 months), between AM and PM treatments and among the sampling intervals relative to the time of the GnRH injection.

The testosterone data (Table 19) in both control and treated bulls markedly fluctuated. Overall average serum testosterone in the two treatment groups (three bulls at each age) did not differ significantly (2.2±.2 and 2.8±.2 ng/ml for saline and GnRH, respectively). The comparable data for LH were 1.5±0.1 and 4.4±0.5 ng/ml. This increase in LH after repeated GnRH should have been sufficient to cause normal elevation in testosterone because Smith et al. (1973) reported that during a normal 24-hour period episodic increases in serum LH which averaged only 2.4 ng/ml, caused significant increase in testosterone (>2 ng/ml).

This seeming failure of endogenous LH to stimulate testosterone is most likely associated with non-responsiveness of the Leydig cells of bulls at 2 and 4 months. For example the analysis of variance (Table 20) showed that testosterone was significantly affected by age. In the 6-month-old bulls treated with GnRH, average testosterone (5.8±0.3 ng/ml) was significantly ($P<0.05$) higher than those in 2- or 4-month-old bulls treated with GnRH which averaged 0.7±0.1 and 2.0±0.1 ng/ml (Table 19) respectively. The comparable data for control

TABLE 19.--Serum testosterone response to saline or GnRH (40 μg, im) injected twice daily for 28 days in bulls at 2, 4 or 6 months of age.

Day	AM-PM	Interval relative to injection	Age of bull: 2 months Saline	2 months GnRH	4 months Saline	4 months GnRH	6 months Saline	6 months GnRH
			(ng/ml)					
D_1	AM	0	0.4±0.1[a]	0.5±0.4	0.4±0.1	1.6±1.1	3.4±0.8	4.5±3.4
		0.5	0.4±0.1	0.5±0.4	0.1±0.1	1.9±1.3	2.1±0.5	5.5±3.0
		3.0	0.3±0.1	1.5±0.6	0.5±0.3	1.6±0.8	1.3±0.4	5.1±1.8
	PM	0	1.2±0.0	1.2±0.9	4.0±1.0	1.5±0.3	5.4±3.9	3.6±2.6
		0.5	1.0±0.2	1.1±0.9	3.6±1.8	1.8±0.6	5.6±3.5	7.6±2.8
		3.0	0.7±0.1	1.6±0.4	1.6±0.9	2.6±1.0	1.4±0.4	3.8±1.5
D_7	AM	0	0.3±0.2	0.5±0.2	1.7±1.0	1.3±0.9	1.4±0.1	7.8±1.8
		0.5	0.3±0.2	0.2±0.0	1.6±1.1	1.1±0.7	0.9±0.3	7.4±1.6
		3.0	0.3±0.3	0.6±0.1	1.2±0.7	1.3±0.7	6.8±3.0	2.2±0.7
	PM	0	0.8±0.3	1.1±0.3	3.1±0.7	1.7±0.6	7.3±1.7	7.3±1.3
		0.5	0.6±0.2	0.9±0.2	2.3±0.3	1.6±0.6	8.5±3.2	5.4±1.4
		3.0	0.4±0.2	1.2±0.5	4.4±0.5	2.4±1.4	2.5±1.5	4.1±1.2
D_{14}	AM	0	0.2±0.1	0.1±0.1	1.9±0.9	2.1±1.2	6.4±2.0	7.1±1.7
		0.5	0.3±0.2	0.1±0.1	1.4±0.6	2.1±1.2	4.6±1.1	5.4±1.5
		3.0	0.3±0.3	0.2±0.1	2.6±1.2	0.9±0.3	2.6±0.8	5.0±2.2
	PM	0	0.4±0.3	0.8±0.2	1.8±0.9	1.7±0.5	6.7±2.6	6.5±3.4
		0.5	0.5±0.3	1.1±0.4	2.0±1.5	1.6±0.5	5.8±3.1	7.9±3.3
		3.0	0.5±0.3	1.3±0.8	4.3±0.5	2.2±0.5	5.5±2.3	3.7±1.5

Table 19.--(Cont'd).

D_{24}	AM	0	0.4±0.2	0.3±0.2	2.6±1.4	1.3±0.7	1.9±1.1	8.7±2.1
		0.5	0.4±0.2	0.4±0.1	1.2±0.7	1.7±1.1	1.2±0.5	6.6±2.8
		3.0	0.4±0.3	0.1±0.1	1.5±0.5	0.9±0.3	4.3±1.5	7.4±1.3
	PM	0	1.0±0.5	1.4±0.8	2.8±0.8	3.6±1.3	2.6±1.3	6.8±2.5
		0.5	0.7±0.2	0.8±0.2	2.7±1.1	3.9±1.7	2.1±1.0	4.8±1.5
		3.0	0.5±0.3	0.5±0.1	4.6±0.5	2.2±0.8	1.3±0.5	3.3±1.3
D_{28}	AM	0	0.3±0.2	0.1±0.1	3.2±1.6	2.2±1.1	5.0±0.6	6.8±3.3
		0.5	0.3±0.2	0.0±0.0	1.5±0.7	2.6±0.8	4.6±0.6	6.5±1.8
		3.0	0.4±0.3	0.1±0.1	3.0±1.6	2.9±0.7	2.6±1.5	3.8±2.4
	PM	0	0.6±0.2	0.6±0.2	1.0±0.3	2.7±1.0	4.2±1.5	7.0±1.0
		0.5	0.8±0.4	1.0±0.3	3.9±1.1	1.5±0.7	3.1±1.4	7.2±1.5
		3.0	0.9±0.3	0.7±0.2	3.1±1.3	3.4±2.1	3.1±1.1	6.1±2.7
Mean			0.5±0.1	0.7±0.1	2.4±0.2	2.0±0.1	3.8±0.4	5.8±0.3

[a]Each value is the mean (± SE) of three bulls.

TABLE 20.--Summary of ANOVA for testosterone data during 2X40 μg GnRH daily for 28 days.

Source	df	SS	MS	F	P_I
Treatment (T)	1	53.33	53.33	1.626	N.S.
Age (A)	2	1630.62	818.31	24.946	0.0005
TA	2	137.99	68.99	2.103	N.S.
Bulls/TA (B)	12	393.64	32.80		
Days (D)	4	17.19	4.30	1.056	N.S.
TD	4	23.79	5.95	1.462	N.S.
AD	8	58.29	7.29	1.791	N.S. (0.1)
TAD	8	45.95	5.74	1.411	N.S.
BD	48	195.31	4.07		
AM-PM (H)	1	54.47	54.47	5.844	0.05
TH	1	12.48	12.48	1.339	N.S.
AH	2	9.79	4.89	0.525	N.S.
TAH	2	16.18	8.09	0.868	N.S.
BH	12	111.84	9.32		
DH	4	15.86	3.96	0.673	N.S.
TDH	4	18.49	4.62	0.785	N.S.
ADH	8	50.33	6.29	1.068	N.S.
TADH	8	23.21	2.90	0.492	N.S.
BDH	48	282.79	5.89		

Table 20.--(Cont'd).

Sampling Interval (I)	2	21.03	10.51	7.788	0.002
TI	2	6.38	3.19	2.362	N.S.
AI	4	81.89	20.47	15.163	0.0005
TAI	4	9.10	2.27	1.685	N.S.
BI	24	32.40	1.35		
DI	8	9.28	1.16	0.408	N.S.
TDI	8	38.61	4.83	1.696	N.S.
ADI	16	35.39	2.21	0.777	N.S.
TADI	16	44.11	2.76	0.969	N.S.
BDI	96	273.15	2.85		
HI	2	6.97	3.48	1.237	N.S.
THI	2	6.12	3.06	1.086	N.S.
AHI	4	28.54	7.14	2.534	N.S. (.06)
TAHI	4	13.86	3.47	1.231	N.S.
BHI	24	67.58	2.82		
DHI	8	20.89	2.61	0.574	N.S.
TDHI	8	34.68	4.33	0.953	N.S.
ADHI	16	41.85	2.62	0.575	N.S.
TADHI	16	93.56	5.85	1.286	N.S.
BDHI	96	436.52	4.55		
Total	539	4459.47			

animals were 3.8±0.4, 2.4±0.2 and 0.5±0.1 ng/ml for 6-, 4- and 2-month-old bulls, respectively. Testosterone in 6-month-old bulls treated with GnRH was significantly greater ($P<0.05$) than that of saline-treated bulls of the same age (Figure 8). This significant difference was not observed in 2- or 4-month-old bulls (Figure 8).

I expected to find an acute testosterone release into the serum after GnRH treatment of 6-month-old bulls. However, this pattern of response was not observed (Figure 8). Considering only the first two injections of GnRH on day 1 in 6-month-old bulls, I observed a trend toward this pattern (Figure 9). However, the difference between GnRH and saline was not significant ($P>0.05$). Infrequent sampling between 0.5 and 3 hours may account for our failure to observe a sharp release of testosterone.

The data in Table 19 showed marked variation in serum testosterone in AM and PM samples. Average serum testosterone of all samples taken in the AM (2.2±0.2 ng/ml) was significantly ($P<0.05$) lower than that in PM samples (2.8±0.2 ng/ml). However this AM vs PM difference ($P<0.05$) in average testosterone occurred in the saline-treated group (1.7±0.2 and 2.7±0.2, respectively), and not in the GnRH-treated bulls (2.7±0.3 vs 3.0±0.3 ng/ml). Lack of a significant difference between AM and PM serum testosterone, in GnRH-treated animals was probably due to the fact that the LH and testosterone release patterns were regularly controlled with exogenous GnRH.

Testosterone levels in GnRH-treated animals slightly increased ($P>0.05$) during the treatment (2.6, 2.7, 2.8, 3.0 and 3.1 ng/ml for day 1, 7, 14, 24 and 28, respectively), whereas saline-treated animals did not change (1.9, 2.5, 2.7, 1.8 and 2.3 ng/ml for day 1, 7, 14, 24 and 28, respectively).

Figure 8.--Serum testosterone at 0, 0.5 and 3 hours after injection (im) of saline or GnRH into bulls at 2, 4 or 6 months of age.

Each point represents the mean ± standard error of thirty observations, ten in each bull. Values at 0 hour were averages of serum testosterone in blood collected prior to each repeated injection of GnRH.

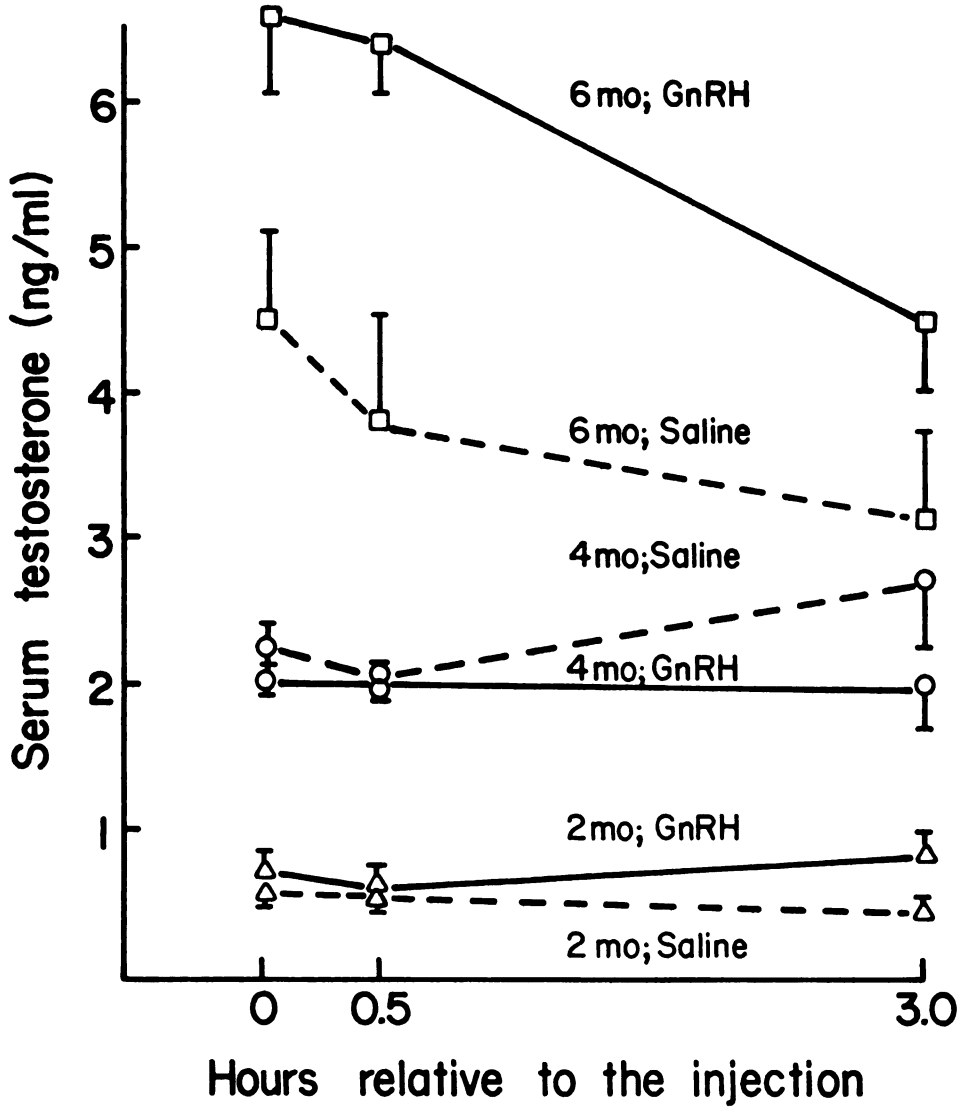
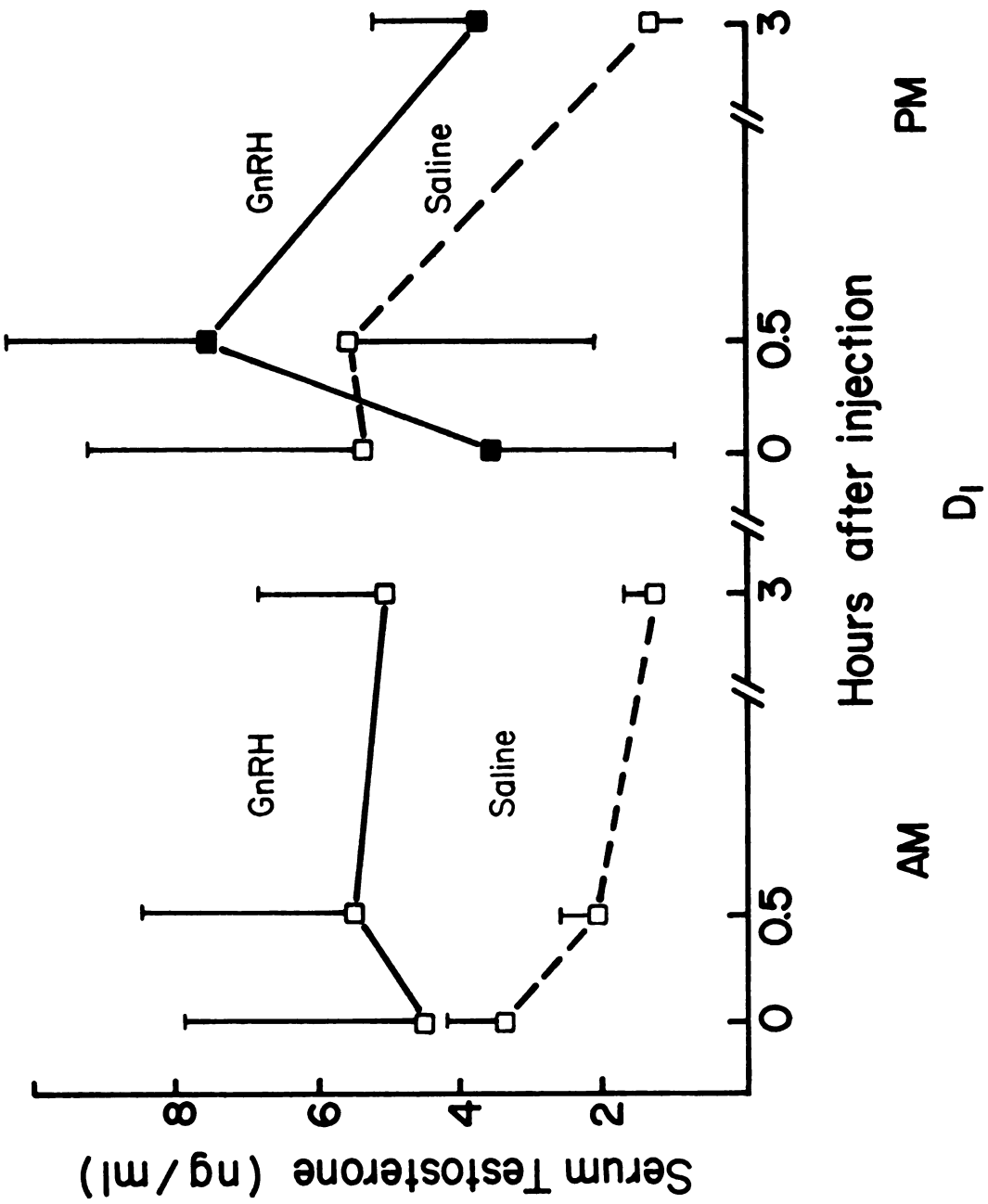

Serum testosterone (ng/ml)
6
5
4
3
2
1
6 mo; GnRH
6 mo; Saline
4 mo;Saline
4 mo; GnRH
2 mo; GnRH
2 mo; Saline
0
0.5
3.0
Hours relative to the injection

Figure 9.--Serum testosterone on day 1 (AM or PM) at 0, 0.5 or 3 hours after injections (im) of saline or GnRH into bulls at 6 months of age.

Each point represents mean ± standard error of three observations, one in each bull.


Serum Testosterone (ng/ml)
8
6
4
2
0
0.5
3
0
0.5
3
Hours after injection
AM
PM
GnRH
Saline
GnRH
Saline
D1

Experiment II confirms the results of Experiment I, that GnRH is equally effective in stimulating LH release at 2, 4 or 6 months of age. However, the LH response cannot be maintained at a high level by repeated injections of GnRH or by a constant rate of infusion of GnRH (Chakraborty et al., 1973a,b). The effect of released LH on steroidogenesis differed markedly among ages. The 6-month-old bulls responded with greater testosterone than 2- or 4-month-old bulls. However, these changes in LH and testosterone after GnRH treatment did not alter body growth, testicular development or testicular and epididymal sperm numbers. From these results, it is possible that the critical time for turning on puberty in bulls is between 5 and 6 months of age. This speculation is also supported by the fact that testicular sperm (Macmillan and Hafs, 1968a) and GnRH activity in the hypothalami (Macmillan and Hafs, 1968b) were first detectable in bulls at this age.

GENERAL DISCUSSION

At least three hypotheses have been proposed to explain the onset of puberty. Smith and Davidson (1968) reviewed the evidence for a decrease in the responsiveness of the hypothalamus and pituitary to negative steroid feedback from birth to puberty. This, according to the theory, results in increased release of gonadotropins. However, Odell and Kiddy (1969) demonstrated that there was no significant change in serum LH during prepuberty in cattle. Administration of FSH during the critical pubertal period can hasten the puberty in rats (Odell et al., 1973) and they proposed that FSH induces sensitivity of the gonad to respond to LH. Finally, it was speculated (Folman et al., 1972) that a decrease in 5α-reduction of androgens within the testes is responsible for a decrease in the negative feedback of steroids on the hypothalamo-pituitary axis and hence stimulates sexual maturation.

Leydig cells become functional during fetal life. In male bovine fetuses, serum testosterone was highest at about 90 days of gestation (Kim et al., 1972; Mongkonpunya et al., 1974). In addition, when testicular tissue slices of 90-day bovine fetuses were incubated for 2 hours in medium containing 0.5 ng of LH per ml, testosterone in the homogenate was significantly greater than in those without LH or in fresh tissue (Kiser, Lin and Oxender, unpublished data). The source of gonadotropins which naturally influence the function of these Leydig cells is not known with certainty; it could be fetal, placental or maternal.

Soon after birth, the Leydig cells go into a period of regression (dedifferentiation?). Galina (1972) demonstrated that the immature bull and ram interstitium were populated with mesenchymal cells and fibroblasts. However, he found that the interstitium of testes of immature horses and pigs were similar to mature Leydig cells, suggesting a different pattern of sexual development among species. Another experiment in our laboratory (T. E. Kiser, Y. C. Lin and K. Mongkonpunya, unpublished data) demonstrated testosterone synthesis in response to LH *in vitro* was about three-fold greater in testicular slices from 5-month-old bulls than in testicular slices from 3-month-old bulls. Thus, the evidence indicates that the Leydig cells in bull testes synthesize testosterone in response to LH early during the fetal period, regress before birth, then develop again during puberty. The factor(s) which are responsible for regulating these changes in the Leydig cells are unknown.

If one accepts that Leydig cells are under the control of gonadotrophins, how can LH be selectively effective by age? The first hypothesis to explain trophic hormone stimulation of steroidogenesis was proposed by Haynes and Berthet (1957), Haynes (1958) and Haynes *et al*. (1959) who demonstrated that ACTH increased adrenal adenosine 3',5'-monophosphate (3',5' CAMP, or cyclic AMP) and this increased phosphorylase activity stimulated corticosteroid production. Haynes and his colleagues demonstrated that cyclic AMP was as effective as ACTH in stimulation of corticosteroid production. Thus, it was concluded that "the activation of adrenal cortical phosphorylase by ACTH is mediated through adenosine 3',5'-monophosphate".

Following the proposal of Haynes many investigators demonstrated that stimulation of testicular functions by LH and FSH also are mediated through cyclic AMP (Dorrington et al., 1972; Rommerts et al., 1973; Dufau et al., 1973). However, recently Rubin et al. (1973) demonstrated that increased cyclic AMP levels are not necessarily associated with the physiological response to tropic hormone stimulation, and maximum steroid release may occur long after cyclic AMP has fallen to control levels. They suggested that probably there are other unidentified cellular messengers which directly involve the mediation of the tropic hormone stimulation. In the discussion, Rubin et al. (1973) pointed out that many chemicals (such as prostaglandins) may have fundamental roles as messengers.

Unfortunately, none of this evidence regarding the mechanisms of action of tropic hormone stimulation explain the selective effect of LH on the androgen synthesis at different ages. Since Catt and Dufau (1973) proposed that the action of LH was related to the number of receptor sites in the target tissues, possibly immature animals have fewer or inactive LH receptor sites. But whether the number of receptors could fall from early fetal life to birth, then increase during puberty is unknown.

Results from the present experiment indicated that GnRH administration (40 μg, im, twice daily for 28 days) did not hasten puberty (sperm production and testosterone secretion). Therefore, it is possible that sexual maturation may involve other hormonal changes besides LH and FSH. For example, prolactin has been shown to involve sexual maturation in rats (Moger and Geschwind, 1972), and mice (Musto et al., 1972). In addition, Swanson et al. (1971a) reported

a significant inverse correlation between pituitary prolactin and seminal vesicular fructose and positive correlation (.31) between serum prolactin and testosterone concentrations. Thyrotropin releasing hormone (TRH) causes release of thyrotropin (Koch et al., 1972), prolactin and growth hormone (Vines et al., 1973; Convey et al., 1973). Therefore, chronic administration of GnRH plus TRH might be a possible means to hasten puberty in bulls.

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APPENDICES

APPENDIX

I. Composition of reagents used in radioimmunoassay.

A. Reagents for radioiodination

1. 0.5 M sodium phosphate buffer, pH 7.5
   Monobasic (0.5 M)
   Add 69.005 g $NaH_2PO_4 \cdot H_2O$ to distilled water.
   Dissolve, dilute to 1 liter.
   Dibasic (0.5 M)
   Add 70.98 g Na_2HPO_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 portions, 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 g
   Merthiolate ---------- 0.01 g
   Dilute to 100 ml with distilled water.
   Solution B
   $NaHPO_4/7H_2O$ ----------26.825 g
   Merthiolate ---------- 0.05 g
   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. Discard chloramine-T remaining in vial.
   *30 mg for GH

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 g
   KI ---------- 0.1 g
   Dilute to 10 ml with distilled water.
   Dispense in 1 ml portions, store at -20°C.

6. Rinse solution
   Sucrose ---------- 0.8 g
   KI ---------- 0.1 g
   Bromphenol blue ---------- 0.001 g
   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 g
   Monobasic phosphate ---------- 100 ml
   (See Appendix A.1)
   Dibasic phosphate ---------- 260 ml
   (See Appendix A.1)
   Merthiolate ---------- 1.75 g
   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 g
   Add approximately 950 ml PBS.
   Adjust pH to 7.0 with 5 NaOH while stirring.
   Dilute to 1 liter, store at 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 g EWA (Sigma Chemical Corp.) or 10 g 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 of Knox gelatin.
   Using graduate 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 Electrobalance.
   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 vial, 4 ml each.

6. 1:400 normal guinea pig serum (NGPS).
   Obtain blood from guinea pig that has 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 1:400 with 0.05 M PBS-EDTA, pH 7.0.
   Dispense in small quantities, store at -20°C.
   On day of use, dilute the 1:400 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 required concentration with 0.05 M PBS-EDTA, pH 7.0.
   Store at 4°C or at -20°C.

C. Antibody and anti-gamma globulin production

1. Guinea pig anti-LH
   0.5 or 1.0 mg NIH-LH-B5 was dissolved in water and Freund's complete adjuvant added (1:1 ratio).
   1.1 or 0.6 ml of the emulsion per guinea pig was injected subcutaneously in 4 scapular region sites.
   The above procedure was repeated 15 and 30 days later substituting Freund's incomplete adjuvant for adjuvant.
   Antisera was collected by cardic puncture 46 and 78 days after the initial injection.

2. Sheep anti-guinea pig gamma globulin
   Guinea pig gamma globulin (Fraction 11, Pentex, Inc., Kankakee, Illinois) (40 mg), streptomycin (100 mg) and penicillin (1000 I.U.) was emulsified in 5 ml of water plus 5 ml Freund's complete adjuvant.
   10 ml was subcutaneously injected in 8 scapular sites of a 75 kg goat.
   The above procedure repeated 15 days later substituting Freund's incomplete adjuvant for adjuvant.
   Antisera was collected 30 days after the second antigen injection by jugular vein puncture.

II. Preparation of liquid scintillation fluids.

A. Steroid scintillation fluid

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

Mix until dissolved.

B. Bray's solution

Naphthalene	240 g
PPO	16 g
Dimethyl POPOP	0.8 g
Ethylene Glycol	80 ml
Methanol	400 ml
p-dioxane	3264 ml

Mix until dissolved.

III. Composition of a reagent used in determination of sperm number.

A. STM solution

Sodium Chloride	68 g
Triton X-100	8 ml
Merthiolate	0.8 g

Bring volume to 8000 ml with water.