The effects of season and in vivo treatment with follicle-stimulating hormone (FSH) on testicular weight, serum testosterone and luteinizing hormone (LH) concentrations, testicular response to in vitro LH and prolactin (PRL) stimulation and $^{125}$I-hCG testicular binding capacity were examined in prepubertal bulls.

Twenty prepubertal, Holstein bulls (4 months of age) were assigned at random to receive subcutaneous injections of saline (control) or 5 mg FSH twice daily for 10 days. Ten bulls were utilized in each of two seasons, summer (August, 1978) and winter (March, 1979). Jugular venous blood was sampled at 12 hr intervals throughout the 10-day treatment period. On day 11, the bulls were castrated and the testes and epididymides were weighed and the testes subjected to further analysis.

The paired testes weight from bulls treated with FSH in the summer was 38% greater ($P < .01$) than that of control bulls ($39.3 \pm 1.6$ and $28.5 \pm 1.5$g, respectively) but did not differ between treatments in the winter. Neither season or FSH treatment had an effect on epididymal
weight (5.5 ± .5g).

Serum LH concentrations were not significantly different between control bulls in summer and winter (1.87 ± .22 and 1.73 ± .13 ng/ml, respectively). Bulls treated in vivo for 10 days with FSH displayed 33% greater (P = .06) serum LH concentrations than control bulls (2.39 ± .13 vs 1.80 ± .13 ng/ml, respectively). Serum LH concentrations remained constant during the 10-day treatment period. A diurnal variation in serum LH concentrations was not detected (2.04 ± .12 and 2.16 ± .13 ng/ml, AM and PM samples, respectively).

Serum testosterone concentrations in bulls receiving saline were not different between summer and winter (.29 ± .02 and .38 ± .03 ng/ml, respectively). In the winter, treatment of bulls with FSH stimulated a 24% increase (P < .05) in serum testosterone over control bulls (.47 ± .02 vs .38 ± .03 ng/ml, respectively).

Treatment of bulls with FSH stimulated a 2.4-fold increase (P < .05) in testicular testosterone content over controls (300.5 ± 67.3 vs 123.6 ± 47.1 ng/mg testicular DNA, respectively). To examine in vitro testosterone production in response to in vitro LH and PRL stimulation, testicular tissue from each bull was incubated in medium TC 199 for 3 hr at 34 C. The addition of 5 and 50 ng LH/ml incubation medium stimulated testosterone synthesis in the winter but not the summer. In the winter, testicular tissue from saline-treated bulls responded to 5 and 50 ng LH/ml media with a similar magnitude of testosterone synthesis as FSH-treated bulls. The addition of PRL (1000 ng/ml) to incubations of testicular tissue had no effect on testosterone production and did not have an additive effect in concert with LH.
Testicular binding capacity of $^{125}$I-hCG was not different between seasons (7.4 ± .9 and 6.9 ± .9 cpm x $10^4$ $^{125}$I-hCG/mg DNA, summer and winter, respectively) or between treatment of bulls with FSH or saline (9.7 ± 1.1 and 6.6 ± .6 cpm x $10^4$ $^{125}$I-hCG/mg DNA, respectively).

The results of this study demonstrate that in vivo treatment of immature bulls with 5 mg FSH twice daily for 10 days stimulates testicular function. The slight increase in serum LH cannot account for the magnitude of the observed increases in paired testes weight, serum testosterone and testicular content of testosterone. Thus, it appears that exogenous FSH is capable of stimulating the bovine testis and consequently may play a role in controlling the onset of puberty and spermatogenesis in the male bovine.
The Effects of Exogenous Follicle Stimulating Hormone on Serum Hormone Concentrations and In Vitro Testicular Responsiveness to Gonadotropins in Prepubertal Bulls

by

Stuart Alan Meyers

A THESIS
submitted to
Oregon State University

in partial fulfillment of the requirements for the degree of

Master of Science
Completed August 27, 1979
Commencement June 1980
APPROVED:

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Date thesis is presented  August 27, 1979

Typed by Melanie Turner for Stuart A. Meyers
Acknowledgement

I would like to dedicate my efforts involved in this thesis to the loving memories of my two grandmothers, Sarah Atkins and Hannah Meyers, who passed away during the period that I was at Oregon State. I would like to gratefully acknowledge the Oregon Agricultural Experiment Station and the Department of Animal Science for providing the funding that allowed me the freedom to pursue the area of study that most intrigued me. Especially, I would like to thank Drs. James E. Oldfield and Lloyd V. Swanson who were instrumental in obtaining these funds. I would also like to thank my parents for their continued support and love that helped make life less complex than it oftentimes appears. My heartfelt appreciation and gratitude go sincerely to Dr. Fredrick Stormshak, who, although unable to understand my penchant for the fly, showed me where the elusive Oregon trout lie. His unending support for me, appreciation of science, and great sense of humor served as an inspiration to me and helped make this experience worthwhile. Similarly, my fellow physiology graduate students and good friends at Oregon State served as an inspiration and I will always fondly remember the frequent times we shared. My dog, Tyrone, deserves special mention for his support for me and for his unceasing loyalty to my cause.

Finally, I wish to express my deepest appreciation to Kathryn Ellen Dusenbery, who typed the rough draft of this thesis, and whose sincere love and support for me was and continues to be my greatest inspiration.
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THE EFFECTS OF EXOGENOUS FOLLICLE STIMULATING HORMONE ON SERUM HORMONE CONCENTRATIONS AND IN VITRO TESTICULAR RESPONSIVENESS TO GONADOTROPINS IN PREPUBERTAL BULLS

INTRODUCTION

An understanding of the events surrounding the onset of puberty is essential to animal agriculture and to medicine, but is of particular importance to the artificial insemination (AI) and livestock industries. Currently, it requires approximately five years to evaluate the genetic qualities of young AI bulls. It would be economically beneficial to the AI industry and consequently to the dairy and beef industries to prove sires at an earlier age. This eventually may be accomplished by manipulation of the hypothalamo-hypophysial-gonadal axis to permit the induction of spermatogenesis. Perhaps semen could then be sampled earlier in the life of the potential sire. In addition, together with the induction of female reproductive cycles, the induction of spermatogenesis in rams during the non-breeding season could conceivably lead to an increase in the reproductive potential of sheep.

Unfortunately, the mechanisms leading to normal sexual development have not been elucidated and endeavors by researchers to influence early spermatogenesis have not succeeded. One goal of basic research in this area is to provide an understanding of sexual development so that future manipulations of these processes may be possible. In addition, understanding the normal processes of sexual maturation may
assist in the treatment of abnormal sexual maturation.

The objective of this research is to provide a further understanding of the endocrine regulation of the prepubertal testis-pituitary axis. This has been approached by studying the effects of pituitary hormones on \textit{in vivo} and \textit{in vitro} testicular steroidogenic responses and the effect of pituitary hormone treatment on testicular gonadotropin receptor activity.
REVIEW OF LITERATURE

Definition of Male Puberty

In its most general sense, puberty has been described as the period during which the two sexes become distinguished from one another (Marshall, 1922). Crew (1931) extended this description to include gonadal function, stating that puberty is characterized by the ability to elaborate functional gametes and by the ability and the desire to play the appropriate role in sexual union. The development of gametes, secondary sexual characters and behavioral sexuality is influenced by hormones produced by the developing gonad and brain. The development of the gonads, thus, is a primary limiting factor in sexual maturation. Donovan and van der Werff ten Bosch (1965) have included the endocrine functions of the testis in their working definition of puberty. These authors state that puberty is the "phase of bodily development during which the gonads secrete hormones in sufficient amounts to cause accelerated growth of the genital organs and the appearance of secondary sexual characters." These processes culminate, for the male, in the maturation of spermatogenesis, as well as the ability and desire to mate. The presence of concentrations of 50 to 100 million spermatozoa/gram of testis is indicative of the completion, or near completion, of puberty (MacMillan and Hafs, 1969; Swanson et al, 1979; Schanbacher, 1979).

The primary objective of this review will be to discuss the cur-
rent understanding of the development of bovine testicular endocrine function and the various influences on this process by other hormones and stimuli. The endocrine events surrounding the onset of puberty in the bull will be emphasized, with minor emphasis on endocrinological and histological events leading to this period. Where experimental evidence is lacking for the bovine, evidence from other species will be cited.

The Occurrence of Puberty in Cattle

Puberty has been estimated to be complete by 9 to 10 months of age in Holstein bulls (MacMillan and Hafs, 1969) and 11 to 13 months in Angus, Hereford and Limousin beef bulls (Swanson et al., 1971; Almquist and Cunningham, 1967; Schanbacher, 1979). At this time, the concentration of testicular spermatozoa has reached 50 million/gram testis, having first appeared as early as 5 to 7 months of age in Holstein bulls (MacMillan and Hafs, 1969) and 6 to 9 months in beef breeds (Abdel-Raouf, 1960; Schanbacher, 1979).

MacMillan and Hafs (1969) have described the development of the reproductive tract of the bull as being comprised of two general phases of accelerated growth. From 2 to 4 months of age, testicular RNA concentration and seminal vesicular DNA and RNA content increased, followed by a plateau. The second phase occurred from 7 to 9 months with respect to increased seminal vesicular RNA, fructose and citric acid content, reflecting increased androgen production. Body weight increased linearly from birth to 12 months of age (MacMillan and Hafs, 1968b). Testicular weight increased from birth to 12 months of age in a curvilinear manner (MacMillan and Hafs, 1969), apparently independent
of the observed biphasic development of other constituents of the reproductive tract.

**Histological Observations in Relation to Puberty**

Three phases of testicular development were described in beef bulls by Abdel-Raouf (1960). For purposes of this review three histological criteria will be considered: 1) the diameter of the seminiferous tubules and the formation of lumina, 2) the presence of Sertoli and germ cells within the tubules and 3) Leydig cell development.

**Tubular Diameter and Lumen Formation**

Abdel-Raouf (1960) observed that tubular development may be divided into three periods, which have been confirmed by the observations of MacMillan and Hafs (1969). In the first period, from birth to 20 weeks of age, the diameter of the tubules (actually the solid sex cords) increased two-fold from 45.78 μm to 94.15 μm. Lumina were totally absent prior to 20 weeks. From 20 to 36 weeks of age (period II) the relative increase in diameter was less than two-fold, reaching 172.04 μm. Lumina were first observed by Abdel-Raouf (1960) between the ages of 28 to 32 weeks whereas MacMillan and Hafs (1969) observed lumen formation as early as 16 to 60 weeks. In the third period, 36 to 60 weeks of age, the tubules approached mature diameters, around 200 μm.

Beef breeds are known to attain puberty at a slower rate and later age than dairy breeds (Swanson et al.; MacMillan and Hafs, 1968a) and this may explain the differences between the observations of
MacMillan and Hafs (1969) and Abdel-Raouf (1960).

**Presence of Germ Cells**

At birth only gonocytes and indifferent supporting cells are present within the sex cords. Some of the gonocytes undergo a progressive degeneration while others proliferate; however, none remain after 16 weeks of age. It is from these proliferating gonocytes that the spermatogonia arise and are apparent as early as 8 weeks of life. The spermatogonia give rise to primary spermatocytes which were observed at about 20 weeks of age. The indifferent supporting cells are of two sequentially distinct, but slightly overlapping populations: the cis- and the bis-type cells. Both of these cell types undergo maturational divisions and give rise to the Sertoli (sustentacular) cells between the 28th and 40th weeks of life. During this time, the secondary spermatocytes, having arisen from the primary spermatocytes prior to 28 weeks, are giving rise to spermatids and the first spermatozoa. The appearance of the first spermatozoa around this time has been observed recently in Limousin beef cattle (Schanbacher, 1979): however, MacMillan and Hafs (1969) observed the first spermatozoa in Holstein bulls as early as 20 weeks of age.

Around the time of puberty, tight junctions form between adjacent Sertoli cells, forming the blood-testis barrier which selectively prevents certain substances from entering the seminiferous tubules (Vitale-Calpe *et al.*, 1973). The blood-testis barrier insures that the intratubular fluid maintains a favorable environment for spermatogenesis and sperm metabolism. Protein and amino acid content of rete testis fluid is much less than that of blood plasma. Rete testis fluid
contains little glucose but contains a high concentration of inositol relative to blood plasma (White, 1974).

Leydig Cell Development

The development of Leydig cell function has been studied extensively (Bascom, 1923; Ficher and Steinberger, 1971; Lording and de Kretser, 1972; Feldman and Bloch, 1978). Most of these studies have centered on functional development of the Leydig cells, while relatively little attention has been given to the histological aspects of Leydig cells. The Leydig cells are situated in the intertubular (interstitial) spaces of the testis and are the primary biosynthetic source of testicular androgens.

Hooker (1944) observed that bovine Leydig cells differentiate very early postnatally from the mesenchymal cells of the interstitial spaces. However, the presence of Leydig cells has been observed as early as the 30 mm (30 to 45 days gestation) bovine embryo (Bascom, 1923).

The Role of Hormones in Testicular Function

The testis consists essentially of two functional tissue types: 1) the germinal epithelium of the seminiferous tubules, from which spermatozoa arise, and 2) the interstitial tissue in the intertubular space which synthesize androgens. The gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) regulate testicular function. In response to LH, the Leydig cells are stimulated to synthesize androgens, while the Sertoli cells secrete androgen-binding
protein (ABP) and estrogen in response to FSH stimulation in immature rats (Dorrington et al., 1976, 1978). Another product of the seminiferous epithelium is inhibin, a water-soluble protein suggested to be capable of selectively suppressing FSH levels in serum of castrate male rats, rabbits and rams (Franchimont et al., 1977). Lee et al., (1974) have shown that testosterone-free extracts of bovine testes exhibit inhibin activity. It is believed that Sertoli cells are the source of inhibin since media from Sertoli cell cultures contain an inhibin-like factor which is capable of suppressing FSH levels in pituitary cell cultures (Steinberger and Steinberger, 1977). However, despite the evidence for the existence of inhibin, conclusive proof of its existence awaits its isolation.

Hormone Mechanism of Action in the Testis

Luteinizing Hormone

In the testis the primary site of action for LH is the Leydig cell. Luteinizing hormone stimulates androgen secretion from Leydig cells in vivo (Lindner and Mann, 1960; Smith et al., 1973) and in vitro (Dufau et al., 1971, 1974; Catt et al., 1974; Mendelson et al., 1975), as does human chorionic gonadotropin (hCG). As the intertubular spaces are well vascularized, LH and hCG can readily gain access to the Leydig cells. Both hormones can stimulate 3',5'-cyclic adenosine monophosphate (cAMP) synthesis in isolated interstitial tissue preparations (Rommerts et al., 1974; Catt et al., 1974).

The first stage in the action of protein hormones, including LH and hCG, is the binding of the hormone to high affinity, specific
receptors located on the plasma membrane of the target cell. High
affinity binding of LH and hCG to Leydig cells has been demonstrated
in vitro (Catt et al., 1974; Mendelson et al., 1975), and is confined
only to the testis (Catt et al., 1974). Leydig cell binding of LH and
hCG increases with age in rats (Sharpe et al., 1973; Ketelslegers
et al., 1978) and bulls (McCarthy, 1978; Schanbacher, 1979) and is a
temperature-dependent saturable process with a relatively high equili-
bration association constant ($K_a = 2 \times 10^{10}$ M$^{-1}$). Radiolabelled LH
or hCG can be readily dissociated from Leydig cells by incubation with
excess unlabelled LH or hCG but not by other hormones such as prolactin
(PRL), FSH, or human growth hormone - indicative of highly specific
binding (Catt et al., 1972, 1974).

Once the hormone is bound to the membrane receptor, a stimulation
of cAMP levels is observed. This is believed to be accomplished by the
coupling of the receptor with membrane-localized adenyl cyclase which
converts ATP to $3', 5'$-cAMP, the second messenger. There is a complex
series of metabolic events involved between the stimulation of cAMP
levels and the release of testosterone. Either actinomycin D, an
inhibitor of gene transcription, or cycloheximide, which inhibits
translation, can block the effect of hCG on testosterone synthesis,
indicating that protein synthesis is required for testosterone synthesis
(Jungmann and Schweppe, 1972; Catt et al., 1974). Cyclic AMP increases
the activity of protein kinase in fractions of interstitial tissue
(Cooke et al., 1976). Protein kinase in the unstimulated cell exists
as an inactive holoenzyme. When stimulated by gonadotropins the holo-
enzyme dissociates into a regulatory subunit, which complexes with
cAMP, and the active catalytic subunit. In this manner protein kinase
is believed to phosphorylate cytoplasmic proteins which catalyze the conversion of cholesterol to pregnenolone (Armstrong et al., 1970), perhaps by influencing the availability of cofactors, availability of cholesterol, or cholesterol transport into mitochondria. In the ovaries of immature rats, it has been shown that hCG rapidly stimulates transcription by accelerating the incorporation of $^3$H-uridine into rapidly labelled nuclear RNA (Jungmann and Schweppe, 1972). Administration of 200 IU hCG to rats is followed by a 400 to 500% increase over control values of the Mn$^{++}$-dependent RNA polymerase (polymerase II) within 2 hours of injection. Similarly, the stimulation of Mg$^{++}$-dependent RNA polymerase (polymerase I and III) activity occurs within 10 minutes of hCG administration and appears to be required for the early action of hCG (Jungmann and Schweppe, 1972). It is believed that similar actions occur in the Leydig cells (Setchell, 1978).

**Follicle-Stimulating Hormone**

The primary target tissues of FSH in the testis are the seminiferous tubules, particularly the Sertoli cells. In contrast to LH, FSH stimulates cAMP formation and other processes in the tubules (Dorrington and Fritz, 1974), and, indeed, will not bind to interstitial cell preparations. Follicle-stimulating hormone stimulates the synthesis of ABP and estradiol in Sertoli cell preparations of the immature rat (Dorrington et al., 1978). Specific receptors for FSH have been demonstrated in the rat and bull testis (Desjardins et al., 1974; Steinberger et al., 1974; McCarthy, 1978; Cheng, 1975), and have been shown to be saturable, temperature-dependent, membrane-bound proteins of high affinity and low capacity. The concentration of FSH receptors in the
rat testis exhibits a sharp rise from 2 to 10 days of age, and declines from days 15 to 25, remaining constant thereafter (Ketelslegers et al., 1978).

Once FSH is bound to the membrane receptors of the Sertoli cell, the subsequent events are similar to the events in the mechanism of LH action. The stimulation of cAMP and protein kinase are observed rapidly after FSH is bound (Means et al., 1976). The stimulation of $^3$H-uridine incorporation into rapidly labelled nuclear RNA is seen concurrently with stimulation of RNA polymerase II activity which indicates transcription of messenger RNA. These events are coincident with synthesis of ABP (Means and Tindall, 1975). The function of ABP is unknown, but it is located within the seminiferous tubules, efferent ducts and epididymis, where it is believed to localize androgens. It is not found in serum or lymph of the rat (French and Ritzen, 1973). Hypophysectomy blocks ABP synthesis, but treatment with FSH or testosterone can restore ABP activity (Tindall and Means, 1976). To my knowledge ABP has not been demonstrated in the testes of bulls.

Prolactin

Very little is known about the mechanism of action of PRL in the testis. It has been demonstrated by autoradiography (Vanha-Perthula et al., 1973) and by saturation analysis (Aragona et al., 1977), that PRL binds to specific receptors on Leydig cells. Treatment of rats with PRL potentiates the effects of LH on restoration of spermatogenesis after hypophysectomy (Bartke et al., 1978) and increases the concentration of cholesterol esters in the testis of the mouse (Bartke, 1971). In bulls, PRL plus LH treatment had no significant effect over
LH alone on serum androgen concentrations (Smith et al., 1973). It is presumed that PRL acts similarly to LH and FSH in the stimulation of cAMP and protein kinase.

**Androgens**

Androgens are required for normal differentiation of the fetal reproductive tract, accessory organ development, male behavior and spermatogenesis (Setchell, 1978). In hypophysectomized male rats or monkeys, testosterone administration can prevent the subsequent regression of the seminiferous tubules (Walsh et al., 1933, 1934; Smith, 1944).

Most of the aspects concerning the mechanism of action of androgens have been elucidated in the ventral prostate of the rat (Mainwaring, 1975). The action of androgen in the testis is assumed to be analogous to its action in the prostate. High affinity androgen receptors have been demonstrated in germ cells, interstitial cells, and Sertoli cells of the testis. The first step in steroid hormone action is entry of the hormone into the cell, where the hormone forms a complex with high affinity, low capacity, cytoplasmic receptor proteins. The cytoplasmic receptor protein for androgens displays a much greater affinity for 5α-dihydrotestosterone than for testosterone, and it appears that the testosterone entering the cell must be converted to dihydrotestosterone prior to receptor association. The activity of the 5α-reductase enzyme required for this conversion has been demonstrated in androgen target tissues such as ventral prostate, testis and epididymis (Liao et al., 1973a; Galena et al., 1974; Tindall et al., 1972). Once formed, the dihydrotestosterone-receptor complex then undergoes a
temperature-dependent configurational change, or activation, and is
translocated to the nucleus in a temperature-dependent process. The
activated complex is believed to interact specifically with acceptor
sites on the chromatin (Mainwaring, 1975). The nature of these acceptor
sites is virtually unknown, but Mainwaring and Peterken (1971) have
speculated that the receptor complex is bound to DNA, and that specific-
ity of binding is determined by acidic, or non-histone, proteins
associated with the chromatin. In contrast, Liao et al.,(1973b)
believe that specificity is imparted by nuclear-associated RNA parti-
cles. Prior to release of the receptor complex from the nucleus, sever-
al nuclear events are stimulated. Stimulation of Mg\(^{++}\) and Mn\(^{++}\)-
dependent RNA polymerase is observed. Synthesis of ribosomal and mes-
senger RNA's, protein synthesis and phosphorylation occur, followed by
later events of DNA synthesis and mitosis (Setchell, 1978; Mainwaring,
1975).

**Estrogens**

The role of testicular estrogens is not fully understood. How-
ever, specific receptors for estrogens (E) have been demonstrated in
cytoplasmic and nuclear fractions of the rat testis interstitial
tissue (Brinkmann et al., 1972). The mechanism of action of estrogens
has been developed primarily from studies utilizing uterine and mammary
tissues, but is assumed to act similarly in the testis. Upon entering
the target cell, the estrogen molecule (E) associates with a high
affinity \( (K_a = 10^{10}) \) cytoplasmic receptor \( (R_c) \). The ER\(_c\) complex, which
sediments in a sucrose gradient of low ionic strength at around 8s, and
at high ionic strength at around 4s, then undergoes a temperature-
dependent transformation to a 5s, or active, complex. The 5s complex can readily enter the nucleus where it elicits a tissue-specific stimulation of RNA polymerase activity and protein synthesis following association with chromatin acceptor sites (Gorski and Gannon, 1976).

**Androgen Biosynthesis in the Testis**

The interstitial tissue of the testis is the primary source of steroid hormones, which originate from the Leydig cells. Hall et al., (1969) demonstrated that isolated rat testicular interstitial tissue was capable of converting \(^3\)H-cholesterol to \(^3\)H-androstenedione and \(^3\)H-testosterone during in vitro incubation. Isolated seminiferous tubules were not capable of this conversion. In addition, Cooke et al., (1972) reported that testosterone content of interstitial tissue increased with in vitro incubation time, but isolated tubular testosterone content decreased or remained unchanged at the termination of incubation.

It is of particular importance to this thesis to review the biosynthetic pathways involved in testicular steroid secretion by the testis (Figure 1). There are two possible sources of steroids within the testis: 1) cholesterol formed within the testis from acetate, or derived elsewhere in the body, and 2) acetate derived from the blood or as acetyl coenzyme A formed from testicular glucose metabolism. Once formed, cholesterol, a C30 compound, enters the mitochondria of the Leydig cell and, following a series of two hydroxylations and one side chain cleavage requiring nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen, is converted to pregnenolone, a key steroid in all subsequent conversions. This reaction is considered to be the
rate limiting step in steroid biosynthesis. Luteinizing hormone is believed to stimulate the formation of pregnenolone from cholesterol (Hall, 1966; Dufau et al., 1976).

Pregnenolone leaves the mitochondria and enters the cytoplasm where it may undergo two possible conversions, both of which lead to 17α-hydroxysteroids. Pregnenolone, a Δ<sup>5</sup> compound (C5-C6 double bond), with the enzyme 17α-hydroxylase, is converted to 17α-hydroxypregnenolone. The majority of the pregnenolone, however, is converted to progesterone, a C3-ketosteroid, by the enzyme 3β-hydroxysteroid dehydrogenase. This enzyme requires NAD<sup>+</sup> as a cofactor, and histochemical localization of it has provided further evidence that androgens are synthesized in the interstitial tissue (Bubenik, et al., 1975). Recently, the gonadotropins LH and FSH have been reported to stimulate the activity of the 3β-hydroxysteroid dehydrogenase-ketosteroid isomerase complex (Shaw et al., 1979). Progesterone is converted to 17α-hydroxyprogesterone by a 17α-hydroxylase enzyme that requires molecular oxygen and NADPH.

Two biosynthetic pathways, Δ<sup>4</sup> and Δ<sup>5</sup>, lead to testosterone. The "Δ<sup>4</sup>-pathway" leading to testosterone originates at 17α-hydroxyprogesterone, which is converted to Δ<sup>4</sup>-androstenedione by the enzyme 17,20-lyase. The Δ<sup>4</sup>-androstenedione is subsequently converted to testosterone by the reduction of the C16-keto group in the presence of NADPH and 17β-hydroxysteroid dehydrogenase. The "Δ<sup>5</sup>-pathway" utilizes the same two enzymes, however, the substrate entering the sequence is 17α-hydroxyprogrenolone rather than 17α-hydroxyprogesterone. The 17α-hydroxyprogrenolone is converted by 17,20-lyase to dehydroepiandrosterone which differs from Δ<sup>4</sup>-androstenedione by a C3-hydroxyl group
Figure 1. Biosynthetic conversions related to Leydig cell androgen production from cholesterol. The left-hand column illustrates the "Δ⁵-pathway" and the right-hand column illustrates the "Δ⁴-pathway".
CHOLESTEROL

$\Delta^5$-PREGNENOLONE $\rightarrow$ 3$\beta$-HYDROXYSTEROID DEHYDROGENASE $\rightarrow$ PROGESTERONE

$17\alpha$-HYDROXYLASE

$17\alpha$-HYDROXYPREGNENOLONE $\rightarrow$ 3$\beta$-HYDROXYSTEROID DEHYDROGENASE $\rightarrow$ 17$\alpha$-HYDROXYPROGESTERONE

17-20 LYASE

DEHYDROEPIANDROSTERONE $\rightarrow$ 3$\beta$-HYDROXYSTEROID DEHYDROGENASE $\rightarrow$ $\Delta^4$-ANDROSTENEDIONE

17$\beta$-HYDROXYSTEROID DEHYDROGENASE

$\Delta^5$-ANDROSTENEDIOL $\rightarrow$ 3$\beta$-HYDROXYSTEROID DEHYDROGENASE $\rightarrow$ TESTOSTERONE
and position of the double bond. This compound can be converted to either \( \Delta^4 \)-androstenedione by a C3 oxidation using 3\( \beta \)-hydroxysteroid dehydrogenase, or to \( \Delta^5 \)-androstenediol by a C17-reduction. Both \( \Delta^5 \)-androstenediol and \( \Delta^4 \)-androstenedione can be converted to testosterone by 17\( \beta \)-hydroxysteroid dehydrogenase. It is believed that the "\( \Delta^4 \)-pathway" is the predominant pathway in bulls (Lindner, 1969). However, Aman and Ganjam (1976) reported the presence of both androstenedione and androstenediol, indicating that both pathways may be functional in mature bulls.

In the immature rat, androstenedione and testosterone can be converted to estrone and 17\( \beta \)-estradiol, respectively, by aromatizing enzymes requiring oxygen and NADPH. Following entry of androstenedione or testosterone into the seminiferous tubules, aromatization occurs, which can be stimulated by FSH or cAMP (Dorrington et al., 1976; Dorrington and Armstrong, 1975). Estrone can be converted to estradiol in the presence of 17\( \beta \)-hydroxysteroid dehydrogenase, which has been observed in both interstitial and tubular compartments (Richards and Neville, 1973). The functions of testicular estrogens are not well understood but have been shown to have roles in gonadotropin feedback and control of testosterone biosynthesis, Leydig cell maturation, and spermatogenesis (Dorrington et al., 1978).

**Steroid Hormone Concentrations in Relation to Puberty**

**Prenatal Androgen Production**

Testicular androgens have been detected in the ram fetus as early as 30 days of gestation (Attal, 1969), indicating the presence of a functional Leydig cell population. Similarly, Mongkonpunya et al.,
(1974) reported that the male bovine fetus is capable of androgen production at least as early as 90 days of gestation. Testicular androstenedione was observed to increase with fetal age whereas testicular content of testosterone decreased with fetal age. It was suggested that either testosterone synthesis is actually reduced with advancing fetal age, or more testosterone is converted to androstenedione. Serum concentrations of androstenedione and testosterone were shown to be approximately ten-fold greater in serum from male fetuses than from female fetuses, were highest at 90 days of gestation and decreased at 180 and 260 days of gestation. In addition, serum concentrations of androstenedione and testosterone were comparable to each other at 90 days of gestation, but the ratio of testosterone to androstenedione decreased with advancing age, similar to testicular content of the two steroids.

In other species, the fetal testes are also capable of steroidogenesis. The fetal rat testis is capable of converting progesterone to testosterone in vitro (Ficher and Steinberger, 1971) and has been observed to do so as early as 15 to 20 days of gestation (Feldman and Bloch, 1978). The fetal rabbit testis has been reported to begin testosterone synthesis around 17.5 days of gestation, reaching a maximum at about 20 days, and decreasing thereafter (George et al., 1978). It has been suggested, for the rat, that a fetal population of Leydig cells undergoes degeneration prior to birth, and that a histochemically distinct second generation arises prior to puberty (Lording and deKretser, 1972). This has not been demonstrated to occur in the bull; however, the observations of Mongkonpunya et al. (1974) and Bascom (1923) make it tempting to hypothesize a similar process. Fetal androgens
appear to be necessary for normal development of the male reproductive tract by the stimulation of Wolffian duct development, which leads to the development of the epididymis and efferent tubules (Setchell, 1977). In addition, fetal androgens are necessary for masculinization of the brain which leads to male patterns of behavior and gonadotropin release (Naftolin and Ryan, 1975).

Postnatal Androgen Production

From birth to about 4 months of age, plasma testosterone in Holstein bulls is very low; ranging from undetectable levels at birth to about .59 ng/ml at 4 months of age (Rawlings et al., 1972). Secchiari et al., (1976) and Karg et al., (1976) reported similar findings of less than 1 ng/ml of serum until about 4 months of age in Fresian and Brown Swiss bulls, respectively. During the same period of development, androstenedione concentration was significantly greater than testosterone concentration in serum and testicular tissue, but was more variable and declined with advancing age (Rawlings et al., 1972; McCarthy et al., 1979a). In contrast, testosterone in plasma of Holstein bulls increases dramatically from 5 months of age to around 12 months of age (Rawlings et al., 1972; Karg et al., 1976; Secchiari et al., 1976; Schanbacher, 1979; McCarthy et al., 1979a). Lindner and Mann (1960) reported that the ratio of androstenedione to testosterone in the testis was greater than 1 prior to 4 months of age, less than 1 from 4 to 6 months, and less than .1 thereafter. A similar trend was reported by Rawlings et al., (1972). In addition, Kiser et al., (1974) observed that testes slices from 1- and 3-month-old bulls, incubated in vitro, synthesized predominantly androstenedione when
subjected to an LH stimulation, while testicular tissue from 5-month-old bulls synthesized predominantly testosterone.

The reasons for the decreased ratio of androstenedione to testosterone observed during sexual maturation are not fully understood. Possibly, maturation of steroidogenic enzyme systems are responsible for the shift from androstenedione to testosterone. Increased activity of 3β-hydroxysteroid dehydrogenase from 10 to 30 days of age was observed in male rat testes (Payne et al., 1977). In addition, Inano et al., (1967) and Payne et al., (1977) found increasing 17β-hydroxy-steroid dehydrogenase activity in male rats from about 20 to 60 days of age. It is also possible that availability of nicotinamide adenine dinucleotide cofactors in the Leydig cells serves to limit testosterone synthesis.

Pituitary Hormones in Serum During Puberty

Luteinizing Hormone

The concentrations of gonadotropins in sera of bulls around the time of puberty is not well established and varies with investigators. Lacroix et al., (1977a) reported that plasma LH increased slightly from birth to 5 weeks of age in Charolais bulls, varied widely from 5 to 20 weeks of age, then remained steady up to one year of age. McCarthy et al., (1979a) observed similar LH activity in sera of Holstein bulls from birth to one year of age, including a period of high variability and increased frequency of episodic LH releases per day occurring at 5 to 20 weeks of age. MacMillan and Hafs (1968b) reported an increase in serum LH from 6 to 10 months of age in Holstein bulls. In contrast,
serum LH in the rat is elevated very early postnatally, but declines from 10 to 20 days of age and increases very slowly, or not at all, as sexual maturation proceeds (Swerdloff et al., 1971; Odell and Swerdloff, 1974). In cattle, a similar high neonatal level of serum LH has not been reported. However, Rawlings et al., (1972) reported that plasma LH concentrations increased from 1 to 2 months of age, remained stable to 10 months, and again increased at 11 to 12 months of age. Karg et al., (1976) observed no change in plasma LH in relation to age in Brown Swiss bulls. In addition, Schanbacher (1979) observed no significant elevation in serum LH during the period of increased testosterone secretion or initiation of spermatogenesis in 3/4 Limousin beef bulls.

Follicle-Stimulating Hormone

MacMillan and Hafs (1968b) observed that pituitary content of FSH was lowest from 6 to 10 months of age, suggesting greater FSH release into blood around the time of puberty. Recently, several laboratories have reported the development of successful radioimmunoassays for serum FSH in bulls. Thus far, no detectable changes in serum concentrations of FSH have been reported during sexual maturation of Holstein (McCarthy et al., 1979), Brown Swiss (Karg et al., 1976) or 3/4 Limousin (Schanbacher, 1979) bulls. In contrast, increased serum concentration of FSH before puberty relative to after puberty has been reported in rats (Odell and Swerdloff, 1974) and rams (Lee et al., 1976). Consequently, the role of FSH in puberty is still poorly understood.
Prolactin and Growth hormone

Prolactin and growth hormone (GH) concentrations have not been well characterized in relation to age in bulls. Charolais bulls from birth to 1 year of age do not seem to have varying plasma PRL concentrations in relation to age (Lacroix et al., 1977b). Serum PRL concentrations have been reported to vary with photoperiod. Bourne and Tucker (1975), Lacroix et al., (1977b) and Sanford et al., (1978) have reported that serum PRL is greatest during periods of increasing photoperiod and lowest during periods of decreasing photoperiod. In addition, McCarthy et al., (1979a) found that PRL tended to be higher and more variable in pubertal bulls than in prepuberal bulls. However, average prolactin concentrations were not affected significantly by age (McCarthy et al., 1979a).

Serum GH concentrations were higher in prepubertal bulls than in pubertal bulls (McCarthy, 1978). The role of GH in sexual maturity and testicular functions has not been elucidated; however, it has been implicated in testicular function (Swerdloff and Odell, 1977).

Causative Factors of Male Puberty

Many factors influence the age at which puberty occurs. Inheritance and breed (Lunstra et al., 1978) as well as nutrition and body size (Johnson et al., 1971) are related to the time of onset of puberty in bulls. This thesis, however, will be concerned primarily with the physiological mechanisms leading to the occurrence of sexual maturation in the male.

Puberty is characterized in the male by an elevation in testos-
terone secretion which may be the result of increased gonadotropic stimulation of the Leydig cells. This may occur in several ways: 1) elevated serum levels of gonadotropins at the time of puberty, resulting in increased availability of gonadotropins at the testis, 2) decreased negative feedback sensitivity of the hypothalamo-pituitary unit to gonadal steroids, and/or 3) increased testicular response to gonadotropic stimulation. Each of these potential possibilities will be discussed with regard to their possible role in the induction of puberty.

**Increased Serum Gonadotropins**

In contrast to data pertaining to the rat which indicates that serum FSH is elevated prior to puberty (Ketelslegers *et al.*, 1978), serum FSH appears not to vary with age in bulls (Karg *et al.*, 1976; McCarthy *et al.*, 1979a; Schanbacher, 1979). There is as much evidence for unchanging serum LH concentrations in cattle (Rawlings *et al.*, 1972; Karg *et al.*, 1976; Schanbacher, 1979) as there is evidence for varying serum concentrations of LH (MacMillan and Hafs, 1968b; Lacroix *et al.*, 1977a; McCarthy *et al.*, 1979a). If indeed serum LH does change relative to puberty, how might this occur? It has been suggested (Grumbach *et al.*, 1974; Odell and Swerdloff, 1975) that increased neural stimulation of the hypothalamus, or decreased neural inhibition, by higher centers in the central nervous system results in elevated gonadotropin-releasing hormone (GnRH) stimulation of the pituitary at the time of puberty. MacMillan and Hafs (1968b) reported increased hypothalamic content of GnRH at 5 months of age in Holstein bulls. The authors also reported a decline in GnRH content at 6 months, followed by an increase until 8
to 10 months.

**Decreased Feedback Sensitivity**

The hypothalamus-pituitary unit has been demonstrated to be under the negative influence of gonadal steroids (Mongkonpunya et al., 1975a, b; Mccarthy and Swanson, 1976; Tannen and Convey, 1977) which are capable of suppressing hypothalamic secretion or pituitary response to synthetic GnRH. Castration of prepubertal or mature rams, rats, bulls, or monkeys (Pelletier, 1968; Swerdloff et al., 1971; McCarthy and Swanson, 1976; Resko et al., 1977) results in elevated serum LH and FSH levels as the hypothalamus-pituitary unit is released from feedback supression. The most commonly held theory of sexual maturity has been that of a progressive decrease in feedback sensitivity of the hypothalamus to gonadal steroids. Consequently, with approaching puberty it is hypothesized that the hypothalamus-pituitary unit requires progressively increasing concentrations of androgens to maintain gonadotropin secretion at basal levels. Thus, puberty would occur concurrently with increasing serum titers of androgens in concert with relatively stable gonadotropin levels. It has been demonstrated that injections of testosterone, as well as estradiol, reduce serum LH concentrations in intact and castrate rams and bulls, and that castrate animals require more testosterone to suppress LH levels post-pubertally than prepubertally (Bolt, 1971; Pelletier, 1970, 1973a; McCarthy and Swanson, 1976; Tannen and Convey, 1977).

Testicular secretions may have a direct site of action at the pituitary. Tannen and Convey (1977) reported that castrate bulls respond to GnRH with greater LH release than intact control bulls.
Testosterone replacement to castrates failed to return the castration-induced increase in serum LH to intact control levels unless replacement was initiated immediately following castration (Tannen and Convey, 1977). These results indicate that some secretory product of the testis, perhaps other than testosterone, may have a regulatory role at the level of the pituitary. Estradiol has been shown to suppress the castration-induced increase in serum LH at lower doses than required for testosterone. It would appear that any decreased feedback sensitivity is mediated at the level of the hypothalamus, since bulls of varying ages do not appear to differ in response of the pituitary to GnRH. Mongkonpunya et al. (1975 a,b) have demonstrated that intact Holstein bulls of 2, 4, and 6 months of age can respond to short-term or chronic GnRH stimulation equally well with elevated serum LH. However, only the 6-month-old bulls were capable of further responding to elevated LH levels with an elevation in testosterone secretion. The younger bulls can, however, respond to elevated LH with an increase in serum androstenedione (Kiser et al., 1974; Mongkonpunya et al., 1975 a,b).

Increased Testicular Responsiveness

It has been suggested that a change in hypothalamic sensitivity to inhibition by gonadal steroids is not the sole explanation for sexual maturation (Odell et al., 1973). Rather, in addition to decreased feedback sensitivity of the hypothalamus, an increase in gonadal sensitivity to gonadotropin stimulation could help explain the onset of puberty.

The ability of LH and hCG to stimulate increased prostate weight and testosterone biosynthesis (testicular responsiveness) in vivo and in vitro has been shown to increase with advancing age in male rats.
(Odell et al., 1973; 1974; Payne et al., 1977; Chen et al., 1977),
cattle (Kiser et al., 1974; Mongkonpunya et al., 1975 a, b; McCarthy
et al., 1979a) and sheep (Foster et al., 1978). Sexually immature male
rats showed no response to subcutaneous injections of LH 5 days after
hypophysectomy while sexually mature rats responded with increased
testis and prostate weights (Odell et al., 1973). However, when
immature hypophysectomized rats were given FSH injections, a signifi-
cant increase in testicular weight was observed following LH stimula-
tion. It was subsequently suggested that FSH induces testicular
sensitivity to LH in the male rat. Therefore, they suggested that
immature rats did not respond to LH stimulation because they had not
previously been exposed to FSH. Follicle-stimulating hormone treat-
ment of hypophysectomized immature rats caused an increase in the num-
ber of LH receptors per testis, increased in vitro testosterone prod-
uction in response to LH, and increased testis weight (Chen et al.,
1976). The nature of FSH-induction of testicular sensitivity has
been extensively studied. The LH-sensitive testicular response is
enhanced to a greater extent than would be expected from the increase
in testicular LH receptors following FSH treatment. The percentage
increase in testosterone response following FSH treatment was
observed to be much greater than the percentage increase in LH recep-
tors (Chen et al., 1976). Catt and Dufau (1973) concluded that maximal
testosterone production in response to hCG occurred when fewer than 1%
of the LH receptors were occupied. Thus Leydig cells contain a large
excess of LH receptors which are not necessary for maximal testosterone
synthesis, but are nonetheless regulated by a complex hormonal inter-
action. The role of large excesses of LH receptors is unknown but
probably acts as a backup system to ensure testicular response to existing LH levels.

Follicle-stimulating hormone may play a role in inducing the synthesis of steroidogenic enzymes, thus forcing the flux of androgen synthesis toward testosterone. Shaw et al. (1979) found that FSH can stimulate 3β-hydroxysteroid dehydrogenase activity which catalyses this conversion.

The maintenance and regulation of LH receptor concentrations and testicular response to LH has been observed to be under a complex control system by pituitary hormones and gonadal steroids. In addition to FSH, PRL and GH have been implicated in the control of testicular function (Bartke et al., 1978; Zipf et al., 1978). Prolactin has been observed to enhance the effect of exogenous LH on spermatogenesis, plasma testosterone, and the in vitro incorporation of $^{3}$H-acetate into testosterone (Bartke, 1971; Bartke and Dalterio, 1976). Prolactin treatment alone, or with GH, prevented the loss in LH receptors observed following hypophysectomy (Zipf et al., 1978). The actions of PRL and GH were found to be additive in preventing receptor loss. Zipf et al. (1979) also found that, although PRL was able to increase testicular LH receptors, it was unable to increase testicular responsiveness to LH. Growth hormone alone similarly increased LH receptor content in rat testes, but in contrast to PRL, was able to enhance testicular responsiveness to LH. Swerdloff and Odell (1977) reported similar effects of GH and PRL on testosterone secretion. Chen et al. (1977) demonstrated that treatment of hypophysectomized immature male rats with testosterone propionylate for 4 days, followed by FSH treatment, enhanced the effect of FSH on number of LH receptors, but did not affect the FSH-induced
testosterone responsiveness to LH. In contrast, treatment of rats with estradiol for 4 days following hypophysectomy had no effect on FSH-induced LH receptors but completely inhibited the FSH-induced testicular responsiveness to LH.

Summary: A Possible Mechanism for the Onset of Puberty

These results indicate that a complex interaction of gonadal steroids and pituitary hormones are, at least in part, responsible for the control of testicular metabolism and probably regulate the onset of puberty. Constant or elevated serum FSH acts to stimulate Leydig cell steroidogenic enzymes by way of some Sertoli cell product. In addition, it is conceivable that FSH acts on the Sertoli cells to stimulate estradiol secretion prior to puberty, which in turn inhibits testosterone production in the Leydig cells. As FSH stimulates the germinal epithelium, ABP and inhibin are also produced. With continued stimulation, inhibin levels possibly increase with advancing age and selectively decrease pituitary FSH release. When serum FSH concentrations are reduced, the Leydig cells are released from estradiol suppression and testosterone production increases. Testosterone then can maintain spermatogenesis while ABP concentrates the androgen within the seminiferous tubules.

In bulls, it is not known whether FSH, PRL, or GH play important roles in the control of the onset of puberty or induce the increased testicular responsiveness to LH that has been observed during sexual maturation (Kiser et al., 1974; Mongkonpunya et al., 1975a, b; Schanbacher, 1979; McCarthy et al., 1979a). In an effort to induce spermatogenesis in 4-month-old bulls, Haynes et al. (1977) chronically infused
thyrotropin-releasing hormone, which increases PRL, GH, and thyrotropin release from the pituitary, together with GnRH. The authors reported increased LH, PRL, GH, and thyrotropin in serum of bulls, but observed no affects on the advancement of spermatogenesis.

The purpose of the present research was to determine if in vivo FSH administration to 4-month-old Holstein bulls could 1) induce an increase in in vitro testicular responsiveness to LH and 2) induce increases in testicular LH receptors, testicular weight, and serum concentrations of LH and testosterone. In addition, it was also of interest to determine whether these effects differed with respect to season. I was also interested whether short term in vitro PRL treatment could alter in vitro testicular response to LH stimulation, and if an in vitro synergism between LH and PRL occurs in the bovine testis.
MATERIALS AND METHODS

I. Animals

Twenty Holstein bulls, 16 to 17 weeks of age, were utilized in an experiment designed to examine the effects of season and in vivo FSH treatment on in vitro testicular responsiveness to various doses of LH and PRL. Effects of treatment with FSH on testicular weight, serum concentrations of testosterone and LH, and LH binding capacity of testicular tissue were also examined. Part I (summer) was conducted in August, 1978 and part II (winter) was conducted in March, 1979. All animals were housed at the Oregon State University Dairy Center and were weaned at 6 weeks of age. Thereafter, the bulls received alfalfa hay ad libitum and a 16% crude protein concentrate ration.

II. Hormones and In Vitro Incubation Media

The Armour FSH-P (Lot #519E78) used for in vivo FSH injections was kindly donated by Dr. Lionel L. Reilly, Burns-Biotec, Inc., Laboratories Division, Omaha, NE. This preparation has been estimated by bioassay to contain 0 to 3% contamination with LH (Steelman and Pohley, 1953); however, Dr. Reilly indicated that this batch contained no detectable LH activity. Injections were given in .15M NaCl (saline) at a concentration of 1 mg/ml.

Bovine LH (NIH-LH-B8) and prolactin (NIH-P-B3) used in the in vitro incubations were obtained from the Pituitary Hormone Distribution Center, National Institute of Arthritis, Metabolism, and Digestive Diseases. Stock solutions of prolactin consisted of 1 mg PRL/ml of saline and stock solutions of LH used in vitro consisted of 1.5 µg
LH/ml in Gelatin-.01M phosphate-buffered saline (GPBS; Appendix IA). Stock solutions were stored at -20 C until used for incubations of testicular tissue. Prior to use, PRL (1 mg/ml) was diluted to 1 µg/ml in saline and used in subsequent in vitro incubations. Varying doses of these hormones were added to flasks with GPBS so that a constant volume of 130 µl was added to each flask.

For radioiodination, highly purified bovine LH (BLH, LER 1072-2), graciously supplied by Dr. L.R. Reichert, Jr., Department of Biochemistry, Emory University, Atlanta, Georgia, was dissolved in .01M NH₄HCO₃ buffer, pH 8.3 (Appendix II) at a concentration of 1 µg/µl. Highly purified human Chorionic Gonadotropin (hCG, Batch No. CR-121) was supplied by the Center for Population Research of the National Institute of Child Health and Human Development, National Institutes of Health. This preparation had an estimated biological activity of 11000-13000 IU/mg, and was dissolved in distilled water (DH₂O) at a concentration of 2 µg/µl for radioiodination. Unlabelled hCG used in receptor studies (section VIII B) was dissolved in .01M phosphate-buffered saline, pH 7.0, (PBS; Appendix Ia) at concentrations 1 to 100.0 ng hCG/40 µl. For receptor saturation studies, bovine LH (NIH-LH-B9) was used instead of hCG as it was more available than hCG. The bovine LH was dissolved in PBS at concentrations of 5 to 50 µg LH/30 µl to provide 1000-fold excesses of competitor for the radio-labelled hCG (¹²⁵I-hCG) levels.

Medium TC 199 10x solution (Difco Laboratories, Detroit, MI 48201) was used for incubations of testicular tissue. Immediately prior to use, this preparation was diluted 10-fold with sterile double distilled water and supplemented with 25 mM HEPES buffer (GIBCO, Grand
Island, NY 14072), Penicillin-G (68 mg/l, 1600 U/mg, Sigma Chemical Company, St. Louis, MO 63178), and streptomycin sulfate (100.00 mg/l, GIBCO, Grand Island, NY, 14072). In the winter portion of the experiment, 44 mM NaHCO₃ was added to increase buffering capacity (See Appendix III).

III. Blood Collection and Hormone Treatment

Blood was collected by jugular venipuncture at 12 hr intervals immediately prior to twice-daily hormone treatment for 10 days. At the time of blood collection, 8:00 a.m. and 8:00 p.m., bulls were haltered and secured in stanchions. Immediately following blood collection, bulls were injected with 5 ml Armour FSH-P (1 mg/ml) or saline. Four milligrams were injected subcutaneously in the scapular area and 1 mg was injected into the center of the scrotum approximately 4 to 5 cm from the ventral abdominal wall. Blood was allowed to clot at ambient temperature for 8 to 12 hr, then refrigerated at 4°C for 12 to 24 hr. Following refrigeration, blood samples were centrifuged at 2000 x g for 10 min and serum was aspirated and frozen at -20°C until assayed for testosterone and LH.

On the morning of day 11, blood samples were obtained and bulls were weighed. Within 30 min of blood collection, bulls were castrated using an emasculator following surgical exposure of the testes after removal of the bottom tip of the scrotum. Right and left testes were separated and transported to the laboratory at ambient temperature.

IV. Preparation of Testicular Tissue and Incubation Procedures

The tunica vaginalis was dissected away from each testis, and the
epididymides and testes were weighed separately. Following removal of
the tunica albuginea, a portion of the parenchymal tissue of the right
testes was cut into two 1-to 2-gram pieces. These were flash-frozen
either in PBS in liquid nitrogen (summer), or in 20% glycerol-PBS (GL-
PBS) in dry ice/methanol (winter), for subsequent LH/hCG receptor
analysis (section VIII) and DNA determination (section IX). The
remaining parenchymal tissue of the right testes was minced into
approximately 1 mm\(^3\) pieces at ambient temperature on a sheet of plex-
iglass using the opposing motion of 2 scalpel blades. The testicular
mince was rinsed 3 times with fresh TC 199, then aliquoted into 25 ml
Erlenmeyer flasks (5-40 mg/flask). Each flask was in a 34 C water
bath and contained 3 ml TC 199 plus one of the following in vitro
treatments, in triplicate: 0, 5, or 50 ng LH/ml media; 1000 ng PRL/ml
media; or 1000 ng PRL plus 50 ng LH/ml media. Triplicate unincubated
control flasks containing 3 ml TC 199 plus 130 ul GPBS also received
testicular mince. However, this tissue was removed 1 to 2 min later,
and the tissue and media were frozen separately.

Flasks were exposed for 3 to 5 seconds with 95% \(O_2\) and incubated
for 3 hr under a constant atmosphere (95% \(O_2\): 5% \(CO_2\)) at 34 C in a
Dubnoff Metabolic Shaking Incubator (30-40 cycles/min) at pH 6.8-7.2.
Adjustments of pH were made as necessary by addition of .1N NaOH or
.1N HCL.

Summer. Following incubations, media and tissue were separated.
Tissue was weighed after blotting excess media; 500 \(\mu\)l PBS was added to
each tissue sample and media and tissue samples were stored at -40 C
until assayed for testosterone.
Winter. The procedure was the same as for summer except that the tissue samples were placed back into their respective ice cold media and stored at -40 C.

V. Radioimmunoassay for Media, Tissue, and Serum Testosterone

A. Extraction Procedures

Summer. Media and serum samples were thawed and added to duplicate 15 x 85 mm assay tubes in the following quantities: FSH-treated bulls, 10 μl media; control bulls, 100 μl media; all serum; 500 μl. Every third sample was accompanied by a "tracer" tube to monitor for losses during extraction (extraction efficiency). Prior to addition of media or serum, "tracer" culture tubes received 10 μl (7000 cpm) [1,2,6,7-3H]testosterone (New England Nuclear, 98.8 Ci/mmol) in absolute ethanol, and evaporated under dry air. Two ml benzene: redistilled hexane (B:H, 1:2), was added to all tubes. Tubes were vortexed vigorously for exactly 30 sec and frozen at -20 C for 1 to 3 hr. After freezing of the aqueous phase, the solvent phase was decanted into 12 x 75 mm glass culture tubes, except for tracer tubes which were decanted into liquid scintillation counting vials. The solvent phase was evaporated under dry air. To the counting vials, 10 ml toluene-based scintillation fluid (Appendix IB) was added and the vials were counted in a liquid scintillation spectrometer (Packard Model 2425). The dried 12 x 75 mm culture tubes were assayed for testosterone (Part B, below).

Tissue samples were prepared for extraction by adding 500 μl PBS to the tissue samples, which had been frozen in 500 μl PBS, giving a
final volume of 1 ml. Seven thousand cpm \([1,2,6,7-^3\text{H}]\text{testosterone}\) (98.8 Ci/mmol) in 10 µl absolute ethanol was placed in a motor driven ground glass homogenizer for determination of extraction efficiency. Following addition of tissue and buffer, the samples were homogenized 30 to 60 seconds and then rinsed with 1 ml PBS. The rinse was added to the homogenate, giving a total volume of 2 ml. This homogenate was transferred to 25-ml tubes with Teflon-coated screw caps and extracted by vigorous hand shaking for 2 min in 10 ml B:H, then frozen (-20°C). After freezing the aqueous phase, the solvent phase was decanted into 15 x 85 mm culture tubes and evaporated under dry air. The inner walls of the tubes were rinsed with additional B:H solvent and were then dried. When dry, tubes were brought to a final volume of 2 ml with 95% ethanol. Five hundred microliter aliquots were transferred to liquid scintillation vials, and dried under air. Ten ml toluene-based scintillation fluid was added to each vial, and they were counted in a liquid scintillation spectrometer to determine the extraction efficiency, which averaged 91.0%.

Duplicate 100 µl aliquots of extracted steroid were transferred to 12 x 75 mm culture tubes and were air-dried for quantification of testosterone.

Winter. For the winter portion of the experiment, the media and tissue were extracted and assayed together for testosterone. After thawing, testicular tissue was separated from incubation medium and placed into a motor-driven ground glass homogenizer containing 3500 cpm \([1,2,6,7-^3\text{H}]\text{testosterone}\) (98.8 Ci/mmol) to monitor for losses during extraction. One ml incubation medium was added and tissue was thoroughly homogen-
(ca. 30 sec). The homogenate was transferred to 25-ml tubes with Teflon coated screw-caps; the homogenizer was rinsed with the remaining medium which was also transferred to the screw-cap tubes, resulting in a suspension of 3 ml. This was extracted as described for summer samples.

B. Radioimmunoassay Procedure for Testosterone

A single-antibody dextran-coated charcoal radioimmunoassay (RIA) was used for quantification of media, tissue, and serum testosterone concentrations. All assays were performed in 12 x 75 mm glass culture tubes. In each assay, two tubes for non-specific binding (NSB) and two tubes for total counts each received 200 μl GPBS. All other tubes, containing either standards (2 sets/assay) at 0, 25, 50, 100, 250, 500, 1000, or 2000 pg testosterone (Sigma Chemical Co., St. Louis, MO, Lot #61G-2340, Δ4-Androsten-17β-ol-3-one) in 100 μl absolute ethanol, or extracted media, tissue, or serum samples which had been previously air dried, received 200 μl testosterone antibody (Antiserum #666, testosterone-3-BSA, G.D. Niswender, Colorado State University). The antibody was diluted with GPBS to a concentration sufficient to bind 20 to 30% of the labelled testosterone. The tubes were vortexed vigorously for 5 sec and incubated at room temperature for 20 min after which 12000 cpm 3H-testosterone [1,2,6,7-3H]testosterone, (98.8 Ci/mmol in 100 μl) GPBS was added to all tubes. Tubes were vortexed gently for 5 sec and incubated overnight (16 to 20 hr) at 4 C.

After placing the tubes in an ice bath, 1 ml dextran-coated charcoal (Appendix IB) in PBS was added to all tubes except the total count tubes which received 1 ml PBS. Samples were vortexed gently for 5 sec, incubated 10 min at 4 C, and centrifuged at 2500 x g at 4 C for 10 min.
The supernatant (.6 ml) was transferred to scintillation vials together with 7 ml Triton-x-100-toluene scintillation fluid (Appendix IB). The contents were mixed and then counted in a liquid scintillation spectrometer.

Within-assay variability was determined from replicates (n = 10) of incubation media from a summer bull that were quantified for testosterone in one assay. The resulting coefficient of variation was 16.0%. The inter-assay variability was similarly determined from replicates of a serum sample from a mature bull that was quantified in 10 assays. The inter-assay coefficient of variation was 25.9%.

VI. Radioiodination of Bovine Luteinizing Hormone and Human Chorionic Gonadotropin

Human chorionic gonadotropin and BLH were iodinated by adaptations of the chloramine-T method of Greenwood et al. (1963). Five micrograms BLH (LER 1972-2) in 5 μl 0.01M NH₄HCO₃ buffer, pH 8.3, was added to a 1 ml vial, followed by the addition of .5 mCi ¹²⁵I (Amersham Searle, Arlington Heights, IL) in 25 μl 0.5M phosphate buffer (Appendix II). Forty microliters freshly prepared chloramine-T (1 μg/μl) in .05M phosphate buffer (column buffer, pH 7.0, Appendix II) was added and the oxidation reaction allowed to proceed for 2 min with gentle agitation. Fifty microliters freshly prepared sodium metabisulfite, Na₂S₂O₅ (2.5 μg/μl column buffer) was added to stop the reaction. Twenty-five microliters 2.5% bovine serum albumin-PBS (BSA-PBS) was added to reduce nonspecific binding of BLH to glass during gel chromatography, followed by 100 μl sucrose transfer solution (Appendix II). The entire mixture was layered on top of a 15 cm Bio-Gel P-60 column. The reaction vial
was rinsed with 70 μl potassium iodide-sucrose rinse solution (Appendix II) which was added to the top of the column. Buffer was added to the column and 15 1-ml fractions were collected in 12 x 75 mm glass culture tubes containing 1 ml GPBS. Free $^{125}$I was separated from hormone-bound $^{125}$I by elution with column buffer. A 10 μl sample of each fraction was counted for 0.1 min in a Packard Auto-Gamma Spectrometer (Model 5025) to characterize the elution pattern (Figure 2).

Human chorionic gonadotropin was iodinated and separated according to the procedures used for BLH, but with the following differences: To minimize oxidative damage to hCG, the quantity of chloramine-T was reduced to 5 μl (1 μg/μl in DH$_2$O) and allowed to react with 1.0 mCi $^{125}$I in 50 μl 0.5M phosphate buffer and 10 μg hCG/5 μl DH$_2$O. Less sodium metabisulfite (15 μg/6 μl DH$_2$O) was added to stop the reaction. In place of 2.5% BSA-PBS, GPBS was added to the reaction vial prior to addition of the sucrose transfer solution.

The peak activity of BLH- or hCG-bound $^{125}$I usually was eluted between fractions 4 to 7, while free (unbound) $^{125}$I was eluted between fractions 10 to 14 (Figure 2). For BLH, only those fractions in which at least 25% of $^{125}$I-B LH could be bound by the BLH-antibody (section VII) at a minimum dilution of 1:64000 were used in subsequent RIA's. For hCG, fractions (active fractions) with the greatest capability of binding to a receptor preparation (section VII. A) in the presence of 1000-fold excess unlabelled hCG were used in receptor studies. Tubes containing active fractions of hormone-bound $^{125}$I were sealed with parafilm and stored at -20 C.
Figure 2. Elution profiles of radioiodination reaction mixtures of LH (top) and hCG (bottom) on 15 cm Bio-Gel P-60 columns. For each hormone, the left peak represents $^{125}$I bound to hormone and the right peak represents free $^{125}$I. Each point represents the radioactivity in a 10 μl aliquot of each of 15 eluted 1-ml fractions.
VII. Radioimmunoassay for Serum Luteinizing Hormone

Serum LH was analyzed using the double antibody RIA of McCarthy and Swanson (1976). Serum samples were thawed and aliquots of 200 or 300 µl were diluted to a final volume of 500 µl with GPBS in 12 x 75 mm glass culture tubes. Dilutions were performed using a Micromedic Automatic Dispensing Pipette (Model 25004, Micromedic Systems, Inc., Philadelphia, PA). Three sets of standards (NIH-LH-B7) were interspersed within each assay and each set contained concentrations of 0, .08, .16, .32, .64, 1.28, 2.56, 5.12, 10.24, or 20.48 ng LH in 500 µl GPBS. The BLH antibody (guinea pig anti bovine LH-GPABLH, Lot GPI-3rd) was developed in this laboratory and diluted in 1:400 normal guinea pig serum (NGPS, Appendix IC) to the final concentration. Two hundred microliters of GPABLH, at a concentration predetermined to give about 25% binding (1:64000 to 1:96000), was added to standards and serum samples. Tubes for NSB received 200 µl 1:400 NGPS. Tubes were vortexed, incubated for 24 hr at 4 C and 100 µl $^{125}$I-BLH in GPBS (200000 cpm/ml GPBS was added to all assay tubes, including two total count tubes, which received only $^{125}$I-BLH. The tubes were vortexed. Following an incubation at 4 C for 24 hr, 200 µl gamma globulin antibody (Sheep anti-guinea pig gamma globulin-SAGPGG, developed in this laboratory, Lot 73-126, 3rd bleeding) diluted 1:25 in EDTA-PBS buffer (Appendix IC) was added to all but total count tubes. The tubes were vortexed, then incubated 72 hr at 4 C. After incubation, 3 ml cold PBS was added to each tube, except total count tubes, and the tubes were subjected to centrifugation at 2000 x g (4 C) for 30 min. The supernatant was decanted, tubes were inverted to drain for 15 to 30 min and the tops
were wiped.

The quantity of radioactivity contained in the precipitate of each tube (bound $^{125}\text{I-BLH}$) was determined using a Packard Auto Gamma Spectrometer (Model 5025) set to count for 10 min or 10,000 cpm.

VIII. Binding of $^{125}\text{I-hCG}$ to Testicular Homogenates

A. Preparation of Crude Membrane Fractions

Receptor studies were conducted utilizing a crude membrane fraction prepared from samples of flash-frozen testicular tissue from each bull (section IV). Testicular tissue samples were thawed in ice water, blotted dry, and placed into polyethylene centrifuge tubes containing cold 20% GL-PBS at a concentration of 1000 mg/2 ml GL-PBS. Samples were homogenized on ice using a Tekmar SDT Tissumizer (Tekmar Co., Cincinnati, OH) for 15 sec at setting 50 (medium speed), followed by dilution to 4 ml GL-PBS. Homogenates were filtered through 2 layers of cheesecloth and centrifuged at 1500 x g (4 C) for 20 min; the precipitate was placed on ice. The 1500 x g supernatant was aspirated and centrifuged at 30000 x g for 40 min. Following centrifugation, the supernatant was discarded and the precipitate combined with the 1500 x g precipitate, brought to original volume (2 ml) with cold GL-PBS, resuspended by vortexing vigorously. These precipitates (homogenates) were flash-frozen in dry ice-methanol and stored at -40 C until binding studies and DNA quantification were performed.

B. Determination of $^{125}\text{I-hCG}$ Binding to Crude Membrane Fractions

Binding studies were performed in 12 x 75 mm polyethylene culture
tubes coated with 5% BSA-PBS. Homogenates (section VII. A) from each bull were thawed in ice water. Duplicate total binding tubes for each sample received, in order; 90 μl PBS, 40 μl \( ^{125} \text{I-hCG} \) (100,000 cpm), and 100 μl testicular homogenate (150 to 270 μg DNA). To determine NSB, another set of duplicate tubes received 60 μl PBS, 40 μl \( ^{125} \text{I-hCG} \) (100,000 cpm), 30 μl (50 μg) unlabelled LH or hCG (1000-fold excess of the \( ^{125} \text{I-hCG} \) added) and 100 μl testicular homogenate. In addition, two total count tubes received only 40 μl \( ^{125} \text{I-hCG} \) (100,000 cpm) to determine the amount of labelled hormone that was added. All binding studies were performed in duplicate. These samples were gently vortexed and incubated for 24 hr at 25 C in a Dubnoff Metabolic Shaking Incubator (30 cycles/min), conditions determined to be optimal by McCarthy (1978) and Catt et al. (1972). Following the incubation, 1 ml ice-cold PBS was added to all tubes (except total count tubes) and the tubes were subjected to centrifugation at 6000 x g (4 C) for 30 min. Supernatants were discarded and the precipitates were washed by resuspending in 1 ml ice-cold PBS using vigorous vortexing, followed by centrifugation at 5000 x g (4 C) for 30 min. The resulting supernatants were discarded, and tubes were inverted for 30 min, then the tops were wiped dry. The quantity of \( ^{125} \text{I-hCG} \) bound to precipitate (receptor) was determined in all tubes using a Packard Auto-Gamma Spectrometer (Model 5025) set to count for 10 min or 10,000 cpm.

Specific binding of \( ^{125} \text{I-hCG} \) was determined in testicular homogenates as the difference between total \( ^{125} \text{I-hCG} \) bound to homogenates in the presence of 1000-fold excess of unlabelled hormone (NSB), [i.e. cpm totally bound - cpm NSB = cpm specifically bound]. The 1000-fold excess LH (section II) used in receptor saturation and binding capacity
studies was determined in a preliminary binding displacement experiment: A constant quantity of receptor (100 µl homogenate = 150 µg DNA) was incubated with a constant quantity of $^{125}$I-hCG (100,000 cpm) and increasing concentrations (0 to 100 ng hCG or LH) of unlabelled hormone (Figure 3). The 1000-fold excess hCG or LH was defined as 1000 times the quantity of unlabelled hormone capable of the greatest displacement of $^{125}$I-hCG. It was calculated that 50 ng unlabelled LH, or hCG, was capable of 90% displacement of 100,000 cpm $^{125}$I-hCG from the receptor. Thus, it was assumed that 50 µg of LH would be a 1000-fold excess of 100,000 cpm $^{125}$I-hCG, and this quantity was subsequently used in saturation and binding capacity studies.

Saturation studies were performed on crude membrane fractions prepared from testicular tissue of 3 bulls from each treatment (winter only) to determine receptor binding capacity (saturation). These studies (Figure 4) required a constant receptor concentration and increasing concentrations of $^{125}$I-hCG. It was determined that 100,000 cpm $^{125}$I-hCG was an appropriate quantity to achieve receptor saturation. Thus, this concentration, together with 50 µg unlabelled LH, was subsequently used to determine $^{125}$I-hCG binding capacity in homogenates prepared from all other bulls.

IX. Determination of Total DNA in Testicular Tissue

Total DNA in testicular tissue was determined by the diphenylamine method of Burton (1956). Parenchymal tissue of the right testes which was stored (-20 C) at the time of incubations was thawed and blotted dry. Tissue samples were homogenized in a motor-driven ground glass homogenizer in .3M perchloric acid (PCA) for 60 seconds at a concentra-
Figure 3. Binding displacement curve of $^{125}$I-hCG by increasing concentrations of unlabelled hCG (0—0) and LH (0—0). Each point represents cpm specifically bound performed in duplicate with a constant volume of .23 ml/tube and incubated at 25 C for 24 hr. Each tube received testicular homogenate containing about 150 μg DNA and 100,000 cpm $^{125}$I-hCG.
UNLABELLED hCG (O-O) OR LH (●) (ng/tube)
Figure 4. Saturation curve for $^{125}$I-hCG from 4-month-old bulls treated *in vivo* with FSH ( — ), $n = 2$ bulls, or saline ( — ), $n = 3$ bulls. A constant amount of testicular homogenate (150 µg DNA) was incubated in duplicate with increasing quantities of $^{125}$I-hCG with or without a 1000-fold excess unlabelled LH. Each point represents cpm specifically bound. Saturation of the binding sites in 150 µg testicular DNA was achieved by the addition of 100,000 cpm $^{125}$I-hCG. Addition of labelled hormone greater than this amount failed to increase specific binding of $^{125}$I-hCG in this concentration of tissue. Data from one of the winter FSH-treated bulls, bull #32, was removed from saturation analysis, as the receptor homogenate for saturation studies from this bull had accidentally thawed prior to use.
SPECIFICALLY BOUND $^{125}$I-hCG (cpm x 10$^{-3}$)

CPM ADDED $^{125}$I-hCG (x 10$^{-3}$)

- ○ FSH (n=2)
- ● SALINE (n=3)
tion of 250 mg/ml. Following precipitation of the total DNA in .5M PCA, 200 μl of the above homogenates or 100 μl of receptor homogenates (section VIII. A) were utilized in the colorimetric DNA determination.

X. Statistical Analysis

Testes and epididymal weights as corrected for body weight by analysis of covariance were analyzed with a 2 x 2 factorial arrangement of treatments. Data from in vitro testicular incubations and serum LH and testosterone determinations were analyzed by split plot analysis of variance and linear regression. Comparisons between specific in vitro treatment means were performed using Scheffe's Interval (Gill, 1973). Data from LH/hCG binding capacity studies were analyzed by analysis of variance with a 2 x 2 factorial arrangement of treatments or a one-way analysis of variance. Bartlett's test for homogeneity of variance (Snedecor, 1956) was performed when heterogeneity of variance was suspected.
RESULTS AND DISCUSSION

Combined Testes and Epididymal Weight

The bulls utilized in this study (n = 20) averaged 118.2 ± .5 [mean ± SE] days of age (range: 114 to 121 days) and 103.7 ± 5.7 kg bodyweight. These bulls are 28 to 34 kg lighter than the Holstein bulls of a similar age reported by MacMillan and Hafs (1968b) and McCarthy et al. (1979a; 138.3 ± 2.7 and 131.5 ± 5.6 kg, respectively). Bodyweight of the bulls in this study did not differ significantly between seasons or from twice-daily subcutaneous treatment with 5 mg FSH or saline for 10 days.

Bodyweight was positively correlated (P < .05) with paired testes (r = +.77) and epididymal weight (r = +.55). Consequently, analyses of covariance were performed on these data and values reported herein are adjusted using bodyweight to correct for testis and epididymal weights.

Testis weight following FSH treatment (Table 1) differed with respect to season (season x treatment interaction, P < .05). In the summer, testis weight was 38% greater (P < .01) in bulls treated with FSH (39.3 ± 1.6g) in comparison with control bulls (28.5 ± 1.5g). In the winter, testis weight did not differ between FSH-treated and control bulls (32.2 ± 1.5 vs 34.4 ± 1.6 g, respectively). These testis weights are in agreement with those reported by MacMillan and Hafs (1968a) and McCarthy et al. (1979a); however, in accordance with bodyweight, our bulls had slightly lower testis weights.
### TABLE 1. PAIRED TESTIS AND EPIDIDYMAL WEIGHT<sup>a</sup>

IN 4-MONTH-OLD BULLS TREATED 10 DAYS

IN VIVO WITH FSH OR SALINE

IN SUMMER AND WINTER

<table>
<thead>
<tr>
<th>Season and Treatment</th>
<th>Testis Weight&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Epididymal Weight&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Summer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>28.5 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.6 ± .5</td>
</tr>
<tr>
<td>FSH</td>
<td>39.3 ± 1.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.8 ± .6</td>
</tr>
<tr>
<td><strong>Winter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>32.2 ± 1.5</td>
<td>5.6 ± .5</td>
</tr>
<tr>
<td>FSH</td>
<td>34.4 ± 1.6</td>
<td>5.8 ± .5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SE adjusted for bodyweight.

<sup>b</sup>n = 5 observations/group.

<sup>c,d</sup>Differing superscripts within a column indicate P < .05.
Epididymal weight did not vary between season or in vivo treatment with FSH. Averaged across season and treatment, epididymal weight was 5.5 ± .5 g (Table 1), similar to the epididymal weights reported for bulls of this age by MacMillan and Hafs (1969).

In vivo treatment with FSH is capable of increasing testicular weight in immature male rodents (Davies, 1971; Davies et al., 1975; Means and Tindall, 1975; Chen et al., 1976, 1977). Increased testis weight has been attributed to an elevation in protein synthesis within the tubules, particularly by Sertoli cells, and is reflected by increased incorporation of $^3$H-lysine into testicular proteins (Davies et al., 1975). In addition, in vivo treatment with FSH may have induced Sertoli cell hyperplasia or hypertrophy, resulting in increased Sertoli cell cytoplasmic mass as reported for prepubertal mice (Davies, 1971). Epididymal weight was not affected by in vivo treatment with FSH for 10 days. Possibly this was not a sufficient length of time for seminiferous tubular secretions to reach the epididymis in quantities great enough to be detected as increased epididymal weight.

**Serum Luteinizing Hormone**

Serum LH concentrations (Table 2) in control bulls were not significantly different between bulls in the summer or those in the winter (1.87 ± .22 and 1.73 ± .13 ng/ml, respectively). This is not surprising in view of the findings of Bourne and Tucker (1975) who reported no changes in serum LH concentrations in response to altered photoperiod in immature Holstein and Guernsey bulls.

When data were pooled across seasons (Figure 5), bulls treated
TABLE 2. AVERAGE SERUM LH CONCENTRATIONS (ng/ml)\(^{a,b}\) DURING A 10-DAY \textit{IN VIVO} TREATMENT OF 4-MONTH-OLD BULLS WITH FSH OR SALINE IN SUMMER OR WINTER

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Summer</th>
<th>Winter</th>
<th>Overall treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.87 ± .22 (^{c})</td>
<td>1.73 ± .13</td>
<td>1.80 ± .13 (^{c})</td>
</tr>
<tr>
<td>FSH</td>
<td>2.81 ± .22 (^{d,e})</td>
<td>1.97 ± .13 (^{f})</td>
<td>2.39 ± .13 (^{d})</td>
</tr>
<tr>
<td>Overall season</td>
<td>2.34 ± .16</td>
<td>1.85 ± .09</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)Mean ± SE, based on \(n = 21\) serum samples taken every 12 hr during 10 day treatment.

\(^{b}\)\(n = 10\) bulls/season-treatment group.

\(^{c,d}\)Different superscripts within a column indicate \(P = .06\).

\(^{e,f}\)Different superscripts within a row indicate \(P = .06\).
Figure 5. Average LH concentrations in blood serum of 4-month-old bulls treated in vivo with 5 mg FSH (0---0) or saline (0---0) twice daily for 10 days. Samples were taken prior to twice-daily injections of FSH or saline at 12 hr intervals. Each point represents the mean serum concentration from 10 bulls. Since there were no seasonal differences, data were combined across seasons. Pooled SE was .29 ng/ml.
NUMBER OF DAYS ON TREATMENT

SERUM LH (ng/ml)

O --- O FSH

• • • SALINE
with 5 mg FSH twice daily for 10 days had 33% greater (P = .06) serum LH concentrations than saline-injected bulls (2.39 ± .13 vs 1.80 ± .13, respectively). Although the interaction of season x treatment was not significant, bulls treated with FSH in the summer exhibited a 50% increase (P < .05) in serum LH concentrations (Table 2) over control bulls (2.81 ± .22 vs 1.87 ± .22 ng/ml). In the winter, however, FSH treatment only resulted in a 14% increase (P > .10) in serum LH concentrations (1.97 ± .13 vs 1.73 ± .13 ng/ml).

It is difficult to envision how FSH administration could elevate serum LH concentrations. To my knowledge, there is no evidence to suggest the possibility that FSH is capable of altering LH release at the level of the hypothalamus or pituitary. Although FSH has not been demonstrated to do so in cattle, it is possible that treatment with FSH increased testicular testosterone and estrogen release into blood serum. Some combination of testosterone and estrogen may have increased pituitary release of LH, or altered the clearance rate of LH. However, both of these steroids are well known to reduce serum concentrations of LH in rams and bulls (Bolt, 1971; McCarthy and Swanson, 1976). Alternatively, some other FSH-induced testicular product may alter pituitary LH release. Haynes et al. (1978) reported that prostaglandin F$_2$α(PGF$_2$α) was capable of elevating serum LH concentrations in bulls within 40 min of injection. Although there is no evidence in the literature, it is possible that treatment with FSH stimulated increases in PGF$_2$α which in turn stimulated pituitary LH release. The possibility cannot be excluded that the elevation in serum LH concentration from treatment with FSH was due to LH-contamination of the FSH preparation. This is unlikely, however, since the LH-activity of the FSH-P preparation has been esti-
mated by rat testis bioassay to be less than 3% (Steelman and Pohley, 1953). A rat testis bioassay conducted in our laboratory (Appendix IV) also revealed negligible LH contamination in this preparation. In that study we failed to detect a release of testosterone into media following incubation of rat testes with 0.1 or 1.0 μg FSH-P/ml media. Furthermore, the half-life of exogenous LH has been estimated at 20 to 30 min (Yen et al., 1968; Smith et al., 1973) in humans and cattle and would certainly be unavailable for detection by 12 hr post-injection when blood was sampled from our bulls.

The effect of in vivo FSH treatment on serum LH concentration was not additive; serum LH concentrations remained stable during FSH- or saline-treatment. The regression of serum LH concentrations on days of treatment was not significant for bulls treated with FSH ($\hat{Y} = 2.71 - .0027X$) or saline ($\hat{Y} = 1.7 + .0007X$). However, occasional high concentrations of serum LH were detected in some bulls, indicating a probable episodic secretion pattern of LH. This was expected as Lacroix et al. (1977a) and McCarthy et al. (1979a) reported that bulls exhibit a period of increased variability and frequency of episodic LH release at 4 months of age. Our blood sampling regimen (twice-daily) did not lend itself to confirming these observations, but the data suggest that episodic secretion patterns occurred in our bulls and were unaffected by in vivo treatment with FSH.

In addition, differences in serum LH concentrations were not detected between morning and evening samples (2.04 ± .12 and 2.16 ± .13 ng/ml, respectively), indicating a lack of diurnal variation in serum LH concentration. However, these results may have been confounded by
the fact that our sampling times were not constant in relation to photoperiod between the two seasons. For example, evening blood samples (8:00 p.m.) were obtained in relative darkness in comparison to the evening samples in the summer. The lack of any diurnal variation in serum LH concentrations is in agreement with Smith et al. (1973), McCarthy and Swanson (1976) and McCarthy et al. (1979b) who did not observe a diurnal secretion pattern of LH, using more frequent sampling intervals than those of the present study.

Serum Testosterone

Similar to serum LH, serum testosterone concentrations (Table 3, Figure 6) in control bulls were not significantly different between seasons (.29 ± .13 and .38 ± .13 ng/ml, respectively, summer and winter) and are in agreement with serum testosterone concentrations of < 1 ng/ml reported for 4-month-old bulls (Rawlings et al., 1972; Karg et al., 1976; Secchiari et al., 1976; Lacroix et al., 1977a; McCarthy et al., 1979a). Subcutaneous injection of our bulls with 5 mg FSH twice daily for 10 days stimulated a 2-fold increase (P < .05) in serum testosterone concentrations over control bulls (.69 ± .04 vs .34 ± .02 ng/ml, respectively). However, the response of the bulls to this treatment differed with respect to season and number of days treated (treatment x season interaction, P = .06). In the summer, treatment with FSH resulted in a 210% increase (P < .05) in serum testosterone concentrations over control bulls (.90 ± .06 vs .29 ± .02 ng/ml, respectively). Similar treatment with FSH in the winter, however, only increased (P > .10) serum testosterone concentrations 24% over levels in control bulls
TABLE 3. AVERAGE SERUM TESTOSTERONE CONCENTRATION (ng/ml)\textsuperscript{a,b} DURING A 10-DAY IN VIVO TREATMENT OF 4-MONTH-OLD BULLS WITH FSH OR SALINE IN SUMMER OR WINTER

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Season</th>
<th>Summer</th>
<th>Winter</th>
<th>Overall Treatment</th>
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</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>.29 ± .02\textsuperscript{c}</td>
<td>.38 ± .03</td>
<td>.34 ± .02\textsuperscript{c}</td>
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<tr>
<td>FSH</td>
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<td>.90 ± .06\textsuperscript{d}</td>
<td>.47 ± .02</td>
<td>.69 ± .04\textsuperscript{d}</td>
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<tr>
<td>Overall Season</td>
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<td>.60 ± .04</td>
<td>.42 ± .02</td>
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</table>

\textsuperscript{a}Mean ± SE, based on n = 21 serum samples taken every 12 hr during 10 day treatment period.

\textsuperscript{b}n = 10 bulls/season-treatment group.

\textsuperscript{c,d}Different superscripts within a column indicate P < .05.
Figure 6. Average serum testosterone concentrations in 4-month-old bulls treated \textit{in vivo} in summer (top) or winter (bottom) with 5 mg FSH (0---0) or saline (0---0), twice daily for 10 days. Samples were taken at 12 hr intervals prior to twice-daily injections. Each point represents the mean serum concentration from 5 bulls. Pooled SE was .13 ng/ml.
(.47 ± .02 vs .38 ± .03 ng/ml, respectively).

In addition, in both seasons, serum testosterone concentrations were elevated in an additive manner during treatment with FSH (time x treatment x season, P = .06). In the summer, the regression coefficient \( \hat{Y} = .65 + .002X \) of serum testosterone concentrations on days treated was 6-fold greater (P < .01) than the similar regression coefficient for control bulls \( \hat{Y} = .26 + .0003X \). A similar relationship, although only of 2.3-fold difference existed between the regression coefficients for FSH and control bulls in the winter \( \hat{Y} = .39 + .0007X \) vs \( \hat{Y} = .37 + .003X \), respectively). In both seasons, the regression coefficient for control bulls was not significantly different from zero.

It is interesting that, in the winter, a 14% increase in serum LH concentrations during FSH treatment was accompanied in our bulls by a 24% increase in serum testosterone concentrations, while in the summer a 50% increase in serum LH was accompanied by more than a 200% increase in serum testosterone. If testosterone response to LH were linear, as suggested by the data of McCarthy et al. (1979a), a 50% increase in serum LH concentration would be expected to elicit an increase in serum testosterone concentrations of only 85%. Recently, Haynes et al. (1978) demonstrated that a single injection of 30 mg PGF\(_2\alpha\) to 1-year-old bulls was capable of directly elevating (P < .05) serum testosterone concentrations within 1 hr post-injection; too early for the concomitantly-induced increase in serum LH to elicit a release of testosterone. Possibly, FSH treatment of our bulls may have stimulated PGF\(_2\alpha\) release which could have enhanced testicular content of testosterone, as well as pituitary LH release, thus facilitating the LH-induced release of
testosterone. It is likely, however, that in addition to any indirect effects on pituitary LH release, the effects of FSH on elevation of serum testosterone were mediated by the stimulation of the testicular steroidogenic enzymes 3β- and 17β-hydroxysteroid dehydrogenases as reported by Shaw et al. (1979) and Payne et al. (1977) in rats. In this manner, testicular response to elevated serum LH may have been enhanced by treatment of bulls with 10 mg FSH daily for 10 days. The ability of FSH to synergize with LH on testosterone secretion has been reported for the rabbit testis (Johnson and Ewing, 1971); however, the exact nature of this relationship has not been fully determined.

**In Vitro Testosterone Biosynthesis**

As a means to further examine the endocrine response of the immature bovine testis to *in vivo* treatment with 5 mg FSH twice daily for 10 days, the direct effects of FSH treatment on LH-induced testicular sensitivity were studied in *in vitro* incubations of testicular tissue (Table 4, Figure 7). When both seasons are considered, treatment *in vivo* with FSH stimulated a 2.4-fold increase (P < .05) in testosterone content of unincubated tissue over control bulls (300.5 ± 67.3 vs 123.6 ± 47.1 ng/mg testicular DNA). This agrees with the increase in serum testosterone concentrations observed in our FSH-treated bulls, and suggests that serum testosterone concentration is a reflection of testicular content of this steroid.

Incubation of testicular tissue for 3 hr without in vitro hormone stimulation (0 ng LH) did not significantly increase testosterone synthesis (total media plus tissue testosterone content) for either FSH
<table>
<thead>
<tr>
<th>Season and In Vivo Treatment</th>
<th>In Vitro Treatment</th>
<th>LH (ng/ml media)</th>
<th>PRL (ng/ml media)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unincubated</td>
<td>0</td>
</tr>
<tr>
<td>Summer</td>
<td></td>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>Saline (5)</td>
<td></td>
<td>48.9±19.5</td>
<td>110.6±33.8</td>
</tr>
<tr>
<td>FSH (5)</td>
<td></td>
<td>202.0±69.7</td>
<td>610.9±165.3</td>
</tr>
<tr>
<td>Winter</td>
<td></td>
<td>198.3±82.6</td>
<td>227.4±109.6</td>
</tr>
<tr>
<td>Saline (5)</td>
<td></td>
<td>399.0±103.4</td>
<td>515.2±129.4</td>
</tr>
<tr>
<td>FSH (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aMean ±SE of 3 replicate tissue samples/in vitro treatment. Numbers in parentheses indicate number of animals.

bIncubation of testicular tissue for 3 hr at 34°C in an atmosphere of 95% O₂: 5% CO₂, pH ≈ 7.0

cBovine pituitary hormones, LH = NIH-LH-B8, PRL = NIH-P-B3
d,e,f,g Different superscripts within a column indicate P < .05.
h,i,j Different superscripts within a row indicate P < .05.
Figure 7. **In vitro** testosterone production (total ng media plus tissue/mg testicular DNA) following incubation of testicular tissue from each of 20 bulls. Bulls were treated *in vivo* in summer (top) or winter (bottom) with 5 mg FSH (clear bars), or saline (hatched bars), twice daily for 10 days. Testicular tissue from all bulls was unincubated or incubated for 3 hr with 0, 5, or 50 ng LH, 1000 ng PRL or 50 ng LH + 1000 ng PRL/ml of media; five bulls in each season-treatment group.
or saline-treated bulls in the winter. Likewise, testosterone synthesis was not induced during incubation of testicular tissue of control bulls in the summer. However, incubation of testicular tissue from bulls treated with FSH in the summer resulted in a 3-fold increase (P < .05) in testosterone synthesis (610.9 ± 165.3 vs 202.0 ± 69.7 ng/mg DNA) over unincubated control tissue.

When LH was present at 5 and 50 ng/ml media during incubations of testicular tissue, testosterone was synthesized in a dose-response relationship (P < .05; Table 4, Figure 7) in FSH and control bulls in the winter but not the summer. In the summer, addition of LH did not affect in vitro testosterone production. McCarthy et al. (1979a) demonstrated that testicular tissue from 4-month-old bulls responded to incubation with 50 ng LH with a 1.5-fold increase in testosterone synthesis, similar to the FSH and control bulls in the winter portion of this experiment. While it is possible that treatment of bulls in the summer with FSH simultaneously induced insensitivity to LH, the data of McCarthy et al. (1979a) suggest that our summer control bulls should have responded to LH stimulation. However, these authors incubated tissue from immature bulls in the fall (September) but not in the summer. Perhaps testicular tissue from immature bulls are incapable of responding to LH in the summer.

Alternatively, it is possible that our control bulls may have responded to LH stimulation predominately by the synthesis of androstenedione rather than testosterone. Since androstenedione was not quantified in this experiment, this possibility cannot be excluded. McCarthy et al. (1979a) reported that tissue content, as well as serum
levels, of androstenedione are about 5-fold greater than that of testosterone in 4-month old bulls. Kiser et al. (1974) demonstrated that incubated testicular tissue from bulls of this age group respond to LH stimulation primarily by synthesizing androstenedione rather than testosterone. Similarly, Lindner, (1969) earlier reported that infusion of hCG to immature bulls resulted in a 5-fold increase in serum androstenedione while testosterone remained relatively unchanged. It is not known whether there are seasonal differences in basal or LH-induced levels of androgens in sera or tissue of immature bulls. However, the data in Table 3 suggest that there are no such seasonal differences. Possibly, treatment of bulls with FSH stimulated testicular steroidogenic enzymes, as reported by Shaw et al (1979) and Payne et al. (1977), causing greater availability of androstenedione as substrate for conversion to testosterone.

Hence, our bulls may have been incapable of responding to LH in the summer; however, FSH treatment was still able to increase testosterone synthesis (Figure 7), although the mechanism of LH responsiveness may have been inadequate or blocked by FSH at that time.

Although 1.6-fold more testosterone was synthesized in vitro in the winter than in the summer in response to stimulation by 50 ng LH (752.8 ± 202.8 vs 460.7 ± 144.2 ng/mg DNA, respectively), treatment of bulls in vivo with FSH was effective only in the summer in enhancing (P < .05) in vitro testosterone production over control bulls. In the winter, both FSH and control bulls responded equally to in vitro LH stimulation with elevated testosterone production. The data for serum testosterone and in vitro testosterone production compliment each other in that
serum testosterone of FSH-treated bulls was elevated 3-fold (P < 0.05) in the summer over control bulls. Likewise, in the summer, testosterone content of unincubated tissue from FSH-treated bulls was 4-fold greater (P < 0.05) than testosterone content of unincubated tissue from control bulls (202.0 ± 67.7 vs. 48.9 ± 19.5 ng/mg DNA). In the winter, testosterone content of unincubated tissue from FSH-treated bulls was elevated 2-fold, while serum testosterone concentrations in the winter FSH-treated bulls were elevated only 1.2-fold. Thus, FSH treatment of immature bulls in the summer is capable of increasing testicular synthesis of testosterone and subsequently increasing serum concentrations of testosterone. However, seasonal factors prevented FSH from eliciting the same effects in both seasons. This effect of FSH on bovine androgen production, and induction of sensitivity of the testis to LH is in agreement with reports of a similar phenomenon in the rat (Odell et al., 1973; Chen et al., 1976, 1977). These authors demonstrated that pretreatment of hypophysectomized immature male rats with 40 to 80 g FSH/day stimulated increases in prostate and testis weights. In addition, Chen et al. (1976, 1977) reported that pretreatment of hypophysectomized immature rats induced increases in in vitro LH-induced testosterone production, and that this affect was beyond the effects of LH alone.

Testicular tissue from FSH- and saline-treated bulls was also incubated with PRL or PRL plus LH to determine whether PRL is capable of influencing bovine testicular function (Table 4, Figure 7). Although testicular tissue from bulls treated in vivo with FSH displayed greater (P < 0.05) in vitro testosterone production than control bulls, the
addition of 1000 ng PRL/ml to incubation medium did not stimulate increased testosterone synthesis. Similarly, across all seasons and in vivo treatment groups, 1000 ng PRL plus 50 ng LH/ml did not result in any increases in in vitro testosterone production over incubation with 50 ng LH/ml alone. Thus, PRL failed to synergize with LH to stimulate testosterone production in our short-term incubation system. These observations are in agreement with the in vivo findings of Smith et al. (1973). These authors reported that single injections of 4 mg PRL to mature bulls did not alter testosterone release into blood serum. In addition, injections of 4 mg PRL plus 2 or 4 mg LH did not alter serum testosterone concentrations from bulls receiving injections of 2 or 4 mg LH alone. However, that study utilized a single injection of PRL and did not elevate PRL exposure to the testes over an extended period of time, days or weeks. The present study similarly did not expose testicular tissue to PRL for an extended period of time. However, comparisons between the data of Smith et al. (1973) and our own data must be drawn with caution, since the former study was conducted in vivo while the latter was conducted in vitro. In contrast, Hafiez et al. (1972) demonstrated that plasma testosterone was greater following prolonged in vivo treatment with 5 μg LH plus 200 μg (twice daily for 3.5 days) PRL than treatment with LH alone (5 μg twice daily) in hypophysectomized rats. The lack of agreement between these studies could be due to species differences, or differences in duration of exposure of the testis to PRL.

In hypophysectomized rats, treatment with PRL was able to increase testicular responsiveness to LH (Bartke and Dalterio, 1976) and growth
of the testes in PRL-deficient hereditary dwarf mice (Bartke and Lloyd, 1970). Moreover, PRL administration to Golden hamsters with short-photoperiod-induced regression of the testes produced testicular growth and elevated testicular LH-receptor and plasma testosterone concentrations; LH and FSH had no affects alone (Bartke et al., 1975; Bex et al., 1978). Thus, although dairy cattle are not seasonal breeders and consequently display little reproductive seasonality, it is possible that endocrine effects mediated by seasonal PRL secretion could have influenced testicular responses to gonadotropin stimulation in the present study.

**Testicular Binding Capacity of $^{125}$I-hCG**

In order to determine if the observed elevation in serum testosterone concentrations and in vitro testosterone production following in vivo treatment with FSH was due to increased capacity of the testes to bind LH, studies were performed to examine the testicular binding capacity of LH. Saturation studies utilizing testicular receptor homogenates from 3 control bulls and 2 FSH-treated bulls indicated that about 100,000 cpm $^{125}$I-hCG was required to saturate the binding sites (Figure 4). Consequently, 100,000 cpm $^{125}$I-hCG was used in binding capacity studies with testicular homogenates from each of the 20 bulls.

When data were considered across in vivo treatments, testicular $^{125}$I-hCG binding capacity was not significantly different between summer and winter bulls ($7.4 \pm .9 \text{ vs } 6.9 \pm .9 \text{ cpm} \times 10^4 \text{ }^{125}\text{I-hCG/mg DNA}$, respectively, Figure 8). Thus, season did not influence the testicular binding of LH in our bulls and is supported by the observation that control bulls in both seasons were capable of binding equal quantities
Figure 8. Binding capacity of $^{125}\text{I}-\text{hCG}$ in 4-month-old bulls (n = 5/treatment group) following 10-day \textit{in vivo} treatment with 5 mg FSH (clear bars), or saline (hatched bars), twice daily in summer and winter. Binding capacity was defined as the quantity of $^{125}\text{I}-\text{hCG}$ specifically bound at equilibrium following 24 hr incubation of testicular tissue (150 \(\mu\)g DNA), from each of 20 bulls with 100,000 cpm $^{125}\text{I}-\text{hCG}$ alone or with 1000-fold excess LH (50 \(\mu\)g).
BOUND $^{125I}$-hCG (cpm x $10^{-4}$/mg TESTICULAR DNA)

- SALINE
- FSH

SUMMER

WINTER
of $^{125}$I-hCG (6.2 ± .4 and 7.0 ± 1.1 cpm x $10^4$ $^{125}$I-hCG/mg DNA, summer and winter, respectively).

Testicular binding capacity of $^{125}$I-hCG was not significantly different between FSH and control bulls in either season (8.6 ± 1.6 vs 6.2 ± .4 cpm x $10^4$ $^{125}$I-hCG/mg DNA, respectively for summer; 6.9 ± 1.6 vs 7.0 ± 1.1 cpm x $10^4$ $^{125}$I-hCG/mg DNA, respectively for winter) although, in the summer, testicular homogenates from FSH bulls bound 37% greater (P < .10) quantities of $^{125}$I-hCG than control bulls (Figure 8). Although this relationship was not significant, it may be meaningful in that it indicates that testicular tissue from the bovine may be capable of responding to FSH-induction of testicular LH receptors as reported for other species (Odell et al., 1973; Chen et al., 1976, 1977; Payne et al., 1977; Shaw et al., 1979). A possible increase in LH-binding capacity could aid in explaining how an increase of 50% in serum LH concentrations could stimulate a much greater increase of 210% in serum testosterone concentrations. There is disagreement in the literature as to whether the induction of LH receptors by FSH treatment serves to actually increase the LH response potential of the target tissue. Catt and Dufau (1973) reported that occupation of 1% of the LH-receptors resulted in maximal testosterone production in the rat testis. Occupation of more than 1% of the receptors failed to increase testosterone production over maximal levels. It seems therefore, that 99% of testicular LH-receptors are extra, or "spare" receptors. The function of spare receptors is unknown, but may serve to ensure that a population of hormone receptors is always available for response in target tissues. In addition, Catt et al. (1974) demonstrated that hCG or LH can stim-
ulate testosterone production by the rat testis, but that this occurs before increased cAMP levels can be detected. This raises doubt as to whether gonadotropins act only by the adenyl cyclase-cAMP system, and suggests that membrane receptors coupled to the adenyl cyclase system for LH or hCG are not the only route for cellular stimulation by these hormones.
GENERAL DISCUSSION

These data suggest that administration of 5 mg FSH twice daily for 10 days to prepubertal bulls is capable of altering testicular function, but that seasonal influences are presumably capable of modifying the effects of FSH treatment. Indeed, the present results demonstrate that in vivo treatment with FSH can induce increases in testicular weight, testosterone content of the testes, and serum concentrations of testosterone in prepubertal bulls. In addition, FSH treatment was capable of increasing serum LH concentrations either by stimulating pituitary release of LH directly, or through some intermediate FSH-induced product. However, in each of these cases, except for serum LH concentrations, the effects of FSH treatment were greater in magnitude in the summer than in the winter. The in vitro data demonstrates that, in the winter, regardless of FSH treatment, the testis was capable of responding to 50 ng LH, while in the summer, the bovine testis was incapable of responding to in vitro LH stimulation. In addition, testes from these immature bulls in the summer possessed slightly, but not significantly, greater capacity to bind LH in response to FSH treatment.

It appears that, although dairy cattle are non-seasonal breeders, a seasonal influence of endocrine nature may be involved in the development of testicular endocrine function and puberty. In addition, it appears that FSH may play a role in the sexual maturation of male cattle as reported for other species.
The nature of any seasonal influence is difficult to elucidate since many factors, including temperature and photoperiod, are associated with season. During the present investigation average photoperiod for August, 1978 and March, 1979 were 13.98 hr and 11.43 hr, respectively. Average temperature during the study period was 18.8 °C for August, 1978 and 7.5 °C for March, 1979. The most interesting difference, endocrinologically, between the two seasons was the influence of photoperiod. The two portions of the experiment were conducted six months apart; approximately midway between periods of decreasing photoperiod (August, 1978) and increasing photoperiod (March, 1979). Lacroix et al. (1977b) have reported that seasonal changes in photoperiod are accompanied by parallel changes in serum PRL concentrations in young bulls. These investigators reported that plasma PRL levels in immature beef bulls were lowest in November (3.3 ± 0.5 ng/ml) and increased until July, when the highest PRL levels were attained (15.5 ± 0.8 ng/ml). Plasma PRL concentrations declined thereafter. Similarly, by altering the duration of artificial light exposure to prepubertal bulls, Bourne and Tucker (1975) and Leining et al. (1979) have confirmed this observation. In both of these studies, bulls of 6-8 weeks of age were acclimatized to 8 hr light and 16 hr darkness (8L:16D). Serum PRL concentrations averaged 8 ng/ml. When the photoperiod was increased to 16L:8D for a 5 to 8 week period, serum PRL concentrations increased to 25-100 ng/ml (Bourne and Tucker, 1975) and 67 ng/ml (Leining et al., 1979).

In addition, Wetteman and Tucker (1974) have shown that serum PRL concentrations decreased from 10.8 ng/ml to 6.7 ng/ml when heifers were
exposed to a 10 C artificial environment following acclimatization at 21 C. It is probable, therefore, that serum PRL concentrations were higher in August than March in our bulls, but that this elevation was ineffective alone in altering testicular function. Thus, there were no differences in serum hormone concentrations or testicular weights across seasons in control bulls in the present study. However, when the bulls were exposed to 5 mg FSH twice daily for 10 days, the combination of endogenous PRL plus exogenous FSH may have been sufficient to stimulate increases in testicular weight and testosterone content, serum testosterone concentrations, and in vitro testosterone production.

Although serum FSH concentrations in cattle do not appear to change relative to puberty (Karg et al., 1976; McCarthy et al., 1979a; Schanbacher, 1979), this hormone cannot readily be discounted with regard to its role in sexual maturation. It is conceivable that constant serum FSH concentrations, coupled with seasonal changes in PRL, act in concert to induce testicular sensitivity to serum concentrations of LH. At the time of puberty, serum LH displays a pattern of increased frequency of stimulatory episodes, and the testis responds accordingly with increased testosterone secretion (Lacroix et al., 1977a; McCarthy et al., 1979a). Frequency of episodic LH peaks increased from around 4 peaks/24 hr. at 1 month of age to 12 peaks/24 hr at 4 months of age (McCarthy et al., 1979a). The authors report that peak height was relatively stable during this period; however, both frequency and height of episodic peaks declined thereafter.

The increase in testicular sensitivity is manifest as an increased activity of steroidogenic enzymes and concentration of LH receptors.
Consequently, the pubertal testis can respond to increased LH stimulation with increased testosterone production, which, in turn, is the major stimulatory hormone on sexual behavior and secondary sexual characteristics.

It is likely that the onset of puberty in the bull is regulated by a slightly different set of hormonal events than in the rat or the ram. In both of these latter species, and perhaps in other seasonally breeding species, FSH may play a more dominant role in the initiation of puberty and spermatogenesis, as this hormone is observed to be elevated in serum, prior to puberty in males. In the ram, PRL plays a major role in seasonality of sexual functions (Pelletier, 1973b; Lincoln and Davidson, 1977; Sanford et al., 1978). Possibly FSH induces puberty in concert with long-day increased serum PRL concentrations in rams, and increased serum PRL during each subsequent breeding season re-induces increased testicular sensitivity to LH, serum testosterone, libido, and spermatogenesis.

Studies similar to those reported in this thesis should be conducted utilizing a highly purified FSH preparation to further evaluate the roles of in vivo FSH treatment on bovine testicular function. In addition the effects of FSH on cellular and subcellular functions in the testis should be examined with regard to cellular replication, protein synthesis, and spermatogenesis. In this way, an understanding of the control of normal and abnormal sexual maturation in cattle and other domestic and wild species can perhaps be achieved.
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APPENDICES
Appendix I. Composition of Reagents Used in General Procedures and Radioimmunoassays

A. Reagents for General Procedures

0.01M Phosphate Buffered Saline, pH 7.0 (PBS)

- NaCl: 8.183 g/l
- Na$_2$PO$_4$·H$_2$O: 1.38 g/l
- Sodium Azide: 1.00 g/l

Bring to partial volume with distilled water.
Adjust pH to 7.0 with NaOH (Approximately 1.43 ml 5N NaOH/l).
Store at 4°C.

0.1% gelatin-PBS (GPBS)

Add 0.5 g Knox gelatin to approximately 400 ml PBS.
Heat and stir gently to dissolve.
Dilute to 500 ml with PBS.
Store at 4°C.

B. Reagents for Testosterone Radioimmunoassay

Toluene-Based Scintillation Fluid
Dissolve 5.0 g 2,5-diphenyloxazone (PPO) in one liter technical grade toluene

Triton-x-100-Toluene Scintillation Fluid
Dissolve 7.0 g PPO in one liter technical grade toluene and Triton-x-100 (2:1).
Dextran-Coated Charcoal
Dextran T-70 (Pharmacia) 0.25 g/l
Neutralized Norit (Sigma) 2.50 g/l
Bring to volume with cold PBS and mix well for approximately 2 hr prior to use, if used same day. Mix well at least 20 min before use otherwise. Keep on ice while in use. Store at 4 C.

C. Reagents for Luteinizing Hormone Radioimmunoassay

0.05M EDTA-PBS, pH 7.0 (EDTA-PBS)
Disodium EDTA 18.61 g
Dissolve in approximately 950 ml PBS. Adjust to pH 7.0 with 5N NaOH while stirring. Dilute to 1 liter. Store at 4 C.

1:400 Normal Guinea Pig Serum (NGPS)
Dilute 2.5 ml non-immune guinea pig serum with 1 liter EDTA-PBS. Divide into 100 ml portions. Store at -20 C.
Appendix II. Composition of Reagents Used in Radioiodination

0.01M Ammonium Bicarbonate Buffer, pH 8.3 (NH₄HCO₃ buffer)

\[ \text{NH}_4\text{HCO}_3 \] 0.79 g

Dilute to 1 liter with distilled water.
Adjust pH to 8.3 with 5N NaOH.
Store at 4 C.

0.5M Phosphate Buffer, pH 7.5

Monobasic (0.5M) \( \text{NaH}_2\text{PO}_4\cdot \text{H}_2\text{O} \) 69.01 g
Dissolve, dilute to 1 liter in distilled water.

Dibasic (0.5M) \( \text{Na}_2\text{HPO}_4 \) 70.98 g
Dissolve by heating, dilute to 1 liter in distilled water.

Mix monobasic and dibasic together to give pH 7.5.
Dispense in 1 ml portions and store at -20 C.
Store monobasic and dibasic buffers at 4 C.

0.05M Sodium Phosphate Buffer, pH 7.5 (column buffer)

Solution A
\[ \text{NaH}_2\text{PO}_4\cdot \text{H}_2\text{O} \] 2.78 g
Merthiolate 0.01 g
Dilute to 100 ml with distilled water.
Store at 4 C.

Solution B
\[ \text{Na}_2\text{HPO}_4 \] 14.21 g
Merthiolate 0.05 g
Dilute to 500 ml with distilled water.
Store at 4 C.

Use 16 ml Solution A, 84 ml Solution B, dilute to 400 ml
with distilled water.
Adjust to pH 7.5 with 5N NaOH.
Store at 4 C.
**Sucrose Transfer Solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Sucrose</td>
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<tr>
<td>KI</td>
<td>0.1 g</td>
</tr>
</tbody>
</table>

Dilute to 10 ml in distilled water

Store at -20 C in 1 ml portions

---

**Potassium Iodide-Sucrose Rinse Solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
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<tr>
<td>KI</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.001 g</td>
</tr>
</tbody>
</table>

Dilute to 10 ml in distilled water

Store at -20 C in 1 ml portions
Appendix III. Rat Testis Bioassay, Test of Buffering Capacity of Various Incubation Media

The objective of this experiment was to determine if the addition of 44 mM NaHCO₃ to medium TC 199 would 1) improve the buffering capacity and 2) improve or alter the steroidogenic potential of that medium.

Three mature OSU rats were sacrificed by decapitation and the testes removed. The testes were decapsulated, weighed, and each whole testis was placed into a flask containing one of the incubation media solutions indicated below. Control medium consisted of TC 199 as described in Materials and Methods (section II). All flasks were incubated for 3 hr as described in Materials and Methods (section IV). Testosterone was quantified in media only.

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Testis Wt. (g)</th>
<th>Medium (3 ml/flask)</th>
<th>Media Testosterone (ng/g testis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.36ᵃ</td>
<td>Control</td>
<td>13.68</td>
</tr>
<tr>
<td>2</td>
<td>1.29ᵇ</td>
<td>Control + 44mM NaHCO₃</td>
<td>19.92</td>
</tr>
<tr>
<td>3</td>
<td>1.35ᵇ</td>
<td>Control</td>
<td>10.59</td>
</tr>
<tr>
<td>4</td>
<td>1.35ᵇ</td>
<td>Control + 44mM NaHCO₃ + 30 μg LH</td>
<td>23.04</td>
</tr>
<tr>
<td>5</td>
<td>1.31ᶜ</td>
<td>Control</td>
<td>18.85</td>
</tr>
<tr>
<td>6</td>
<td>1.40ᶜ</td>
<td>Krebs-Ringer Bicarbonate + glucose</td>
<td>14.57</td>
</tr>
</tbody>
</table>

ᵃ,ᵇ,ᶜ' Similar superscripts indicate testes from same rat.

Results and Discussion

Average testosterone produced in incubations of rat testes in control media was 14.4 ± 2.4 ng/g testis. Addition of 44 mM NaHCO₃ caused a 38% increase in testosterone release into media (19.9 vs 14.4 ± 2.4 ng/g testis). As these data could not be analyzed statistically, this may or may not have been a significant increase. Therefore, it is
concluded that addition of this buffer does not alter the steroidogenic potential of TC 199.

The addition of 30 μg LH/ml media resulted in an increase in media testosterone content (23.0 vs 15.5 ng/g testis). In addition, incubation of a single rat testis in Krebs-Ringer Bicarbonate + glucose medium did not result in increased testosterone content of media in comparison with incubation of the other testis from the same rat in control media (14.6 vs 18.9 ng/g testis). Thus it is concluded that Krebs-Ringer Bicarbonate does not offer an improved incubation environment over medium TC 199.

The ability of TC 199 to resist changes in pH, as detected by visual observation of color stability of the phenol red indicator, was improved by the addition of 44 mM NaHCO₃. It is concluded that addition of 44 mM NaHCO₃ to TC 199 does not alter the steroidogenic potential of that medium, and, in addition, is effective in increasing buffering capacity of TC 199. Consequently, this buffer was utilized only in testicular incubations in the winter portion of the experiment described in this thesis.
Appendix IV. Rat Testis Bioassay for Luteinizing Hormone

The objectives of this experiment were to determine 1) the extent of contamination with LH within the Armour FSH-P that was used in in vivo treatment of immature bulls, and 2) whether two bottles of LH (NIH-LH-B8) differed in their ability to stimulate testosterone production in vitro by rat testes.

Eleven mature OSU rats were sacrificed by decapitation and the testes removed. Testes were decapsulated and the whole testes were assigned at random to flasks containing 3 ml medium TC 199 without 44 mM NaHCO₃. Flasks were then incubated (as described in Materials and Methods, section IV) with various doses of LH in duplicate. Two bottles of LH were tested for relative potency: (a) LH that had been used in the summer, 1978 in vitro incubations and (b) LH from an unopened vial of LH. In addition, Armour FSH-P used in in vivo injections was tested at two dosages in duplicate for relative LH-contamination. Media and incubation procedures used were as described in Materials and Methods, section IV, except that incubations were of 4 hr duration. Testosterone content of media only was quantified as described in Materials and Methods, section V.

Results and Discussion

Incubation of rat testes without hormone stimulation resulted in 5.04 ± .75 ng testosterone/ml media. Vial (a) LH appeared to stimulate testosterone release at doses of 1.0 and 10.0 ng LH/ml media whereas vial (b) LH did not stimulate testosterone release at 1.0 ng LH/ml media.
Vial b LH, however, at 10.0 and 100.0 ng/ml did appear to stimulate

### Experimental Design and Results of Incubation

<table>
<thead>
<tr>
<th>Vial of NIH-LH-B8</th>
<th>LH (ng/ml media)</th>
<th>FSH-P (µg/ml media)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ng testosterone/g testis-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>4.62</td>
<td>6.79</td>
</tr>
<tr>
<td></td>
<td>7.03</td>
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<tr>
<td>Mean</td>
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<td>5.23</td>
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<tr>
<td>b</td>
<td>5.07</td>
<td>3.31</td>
</tr>
<tr>
<td></td>
<td>3.40</td>
<td>2.59</td>
</tr>
<tr>
<td>Mean</td>
<td>4.24</td>
<td>2.95</td>
</tr>
</tbody>
</table>

testosterone by 30% over control flasks (6.72 and 6.69 vs 5.03 ng/ml).

In this bioassay system, stimulation of testosterone secretion by LH at 10.0 or 100 ng/ml appeared to be maximal, as these two LH doses were indistinguishable from one another.

Incubation of rat testes with .1 or 1.0 µg FSH-P/ml media did not stimulate testosterone secretion greater than the LH doses except for one testis at 1.0 µg FSH-P/ml, which secreted 16.4 ng testosterone/g testis. Testosterone production by this testis may have been stimulated by the LH-contamination of the FSH-P. However, this could be within the observed variation between all pairs of duplicate flasks. For example, testosterone release by the other testis at this dose of FSH-P was not different from testes incubated without hormone stimulation. If the FSH-P was to have as much as 10% LH contamination, testosterone release at the 1.0 µg FSH-P dose should equal that for testes incubated with 100.0 ng LH/ml. Although one testis did exhibit this effect, it cannot
be concluded that FSH-P contains 10% LH contamination since other testes incubated with FSH-P did not exhibit testosterone release over control testes. It would appear that the FSH-P contains closer to 0, .1 or 1.0% LH contamination since testes incubated with FSH-P displayed similar testosterone release to either incubations without LH or with .1 and 1.0 ng LH/ml.

It is concluded that, although much variation between incubated testes existed, vial (a) LH was slightly more potent in LH activity than vial (b) LH. Consequently, vial (a) LH was utilized in all in vitro testicular incubations. In addition, it is concluded that FSH-P probably does not contain more than 1% LH contamination. However, a more rigorous study needs to be performed with more replicate testes incubated with FSH-P before a definite conclusion can be made as to the LH contamination of this preparation.