TESTICULAR AND BLOOD PLASMA ANDROGEN LEVELS IN THE MALE BOVINE FROM BIRTH THROUGH PUBERTY

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ABSTRACT

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By

Norman C. Rawlings

A technique was developed to quantify the low levels of testosterone and androstenedione found in the blood and testes of bulls from birth through puberty. The technique involved the conversion of the steroids to heptafluorobutyrate derivatives and quantification by gas liquid chromatography with electron capture detection. A total of sixty-five Holstein bulls were killed in groups of five at monthly intervals from birth to 12 months of age. Twenty-nine Hereford bulls were bled at the eleventh, twelfth, and thirteenth month and slaughtered two weeks after the last bleeding.

In the Holstein bulls plasma concentration of testosterone increased from birth to 5 months of age, fell to 6 months of age, rose until 11 months and finally declined at 12 months of age. Testicular testosterone concentration showed a similar trend from 4 months of age. Androstenedione concentration in the testicle fell from 4 months of age to a very low level at 6 and 7 months, then

rose slowly until 11 months and finally declined to a low level at 12 months of age. In the plasma, androstenedione was very variable for the first 5 months and then almost zero until a spurious rise in concentration at 12 months of age. The plasma concentration of androstenedione did not reflect or parallel testicular concentration. The ratio of testosterone to androstenedione in the testis varied from 0.65:1 at 4 months of age to 48:1 at 8 months, and 8:1 at 12 months of age.

Testosterone and androstenedione concentrations were higher in the Hereford bull serum than in the Holstein bull plasma, but tended to decrease with age. Values for the testes and serum at slaughter were also higher, but this elevation may have been due to stress of slaughter. In the serum the difference could have been a breed difference or perhaps that these beef bulls were less mature physiologically.

The changes in concentrations of testosterone and androstenedione in both tissues with age were compared to changes for various other endocrinological and reproductive criteria by correlation analysis. These analyses suggested the following conclusions. Testosterone synthesis and secretion increased with age, stimulated by increased circulating levels of LH. Testosterone in turn stimulated the seminal vesicular content of fructose and citric acid. The biphasic pattern of increased circulating LH and increased seminal vesicular secretions (rising from 2 to

4 months of age, declining to 6 months, and increasing to 12 months of age) was paralleled somewhat by testosterone synthesis and secretion. Androstenedione did not appear to be involved in these relationships and probably lacked androgenic properties. It may have been more involved with the growth of the testis, and its synthesis and secretion by the testis did appear to be stimulated by LH in bulls older than 6 months of age. The role of both androgens in the initiation of spermatogenesis was not clear, but testosterone did appear to have some relationship to gonadal sperm numbers, especially at initiation of spermatogenesis.

FSH was probably not related to the stimulation of steriodogenesis during puberty; its role may have been more involved with growth of the testis and reproductive tract and the initiation of spermatogenesis. Testosterone and androstenedione levels declined to stable adult levels no sooner than 11 months of age, but growth and activity of other reproductive criteria of these same animals indicated that puberty ended at about 9 months of age.

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BIOGRAPHICAL SKETCH

Norman C. Rawlings was born in Bristol, England, on July 10, 1946. Lived in the small village of Dundry, Somerset, where he attended a nursery and junior school run by the Church of England. At age 11, he moved to a new comprehensive school at Chew-Stoke, Somerset, but due to a promotion his father received he moved to Leicester where he initially attended Lancaster boys school. He later passed the 13 plus examination and was again moved to Moat Road Intermediate school, Leicester, where he obtained passes in eight subjects at the General Certificate of Education ordinary level. This allowed him to transfer to Gateway boys school, Leicester (a grammar school) to study for the advanced level GCE in Chemistry, Botany and Zoology obtaining passes in Botany and Zoology and also a special subject, Use of English.

He was accepted by the University of London, Wye College, in 1964 to study for a degree in Agriculture. He graduated in June 1968 with a BSc pass degree (second division) after three years at College and one year mandatory practical work on a farm in South Wales.

In September 1968 he was awarded a graduate research assistantship by the Department of Dairy, Michigan State University. This position allowed him to study for a Masters degree, with a major in Reproductive Physiology and Endocrinology, which he completed in the fall of 1970.

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I am very grateful to the Dairy Department at Michigan State University for the opportunity to study for a Masters degree, and also for financial support (NIH Research Grant HD-03039) without which it would not have been possible for me to attend graduate school in America.

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TABLE OF CONTENTS

THESU

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

INTRODUCTION

The demands on modern commercial animal production necessitate the utmost efficiency of the producer if he is to profit or even survive. Even small improvements in any aspect of animal production may bring large rewards. Genetic improvement of stock whether for beef or dairy purposes is permanent, but slow. Sons of superior sires must be kept unproductive for several years while they mature and are progeny tested, and even then they may be discarded as inferior. Studies have been performed in our laboratory to describe pituitary hormonal and reproductive changes during sexual maturation in bulls. Knowledge likely to come from studies such as these may allow us to hasten the process of sexual maturation, allowing commercial AI centers to obtain "proofs" on bulls at an earlier age and significantly reduce the unproductive portion of the life of the bull. By the same token, sperm production might be increased and fertility enhanced by treatment with exogenous hormones.

An important approach to these ends would be to identify causal relationships between levels of various

hormones and sperm production or fertility. A more complete description of the reproductive and endocrine changes from birth through puberty in the male bovine is prerequisite to these goals. The present study on androgen levels complements reproductive and endocrine data obtained earlier on the same bulls. To the author's knowledge no one has attempted previously to measure androgen levels from birth through puberty in the bull.

As recently as ten years ago, analysis of steroidal compounds by gas liquid chromatography was realized. In the last three or four years, methods for the quantification of steroid hormones involving gas liquid chromatography have appeared. These techniques were insensitive or not amenable to the routine analysis of large numbers of biological samples. The initial part of this study was development of a sensitive and accurate assay for androgens that could be performed routinely and with some speed.

REVIEW OF LITERATURE

Between birth and one year of age, the bull goes through many endocrine and physiological changes associated with sexual development. At birth the testis consists of solid sex chords but by one year of age the seminiferous tubules have attained mature form, spermatogenesis occurs and the young bull is capable of paternity (Abdel-Raouf, 1960; Baker et al., 1955).

Among many definitions, puberty has often been defined as the age at which sexual maturity is attained. But Donovan and van der Werrf ten Bosch (1965) defined it as including the entire period when the gonads secrete hormones in amounts sufficient to cause accelerated growth of the genital organs and the appearance of secondary sexual characters.

Many endocrine, physiological and behavioural criteria may be used to define the termination of puberty. In Holstein bulls ejaculation can occur at 38 weeks of age (Bratton et al., 1959), but many bulls lack libido at this age. Bratton et al. also showed that at 43 weeks semen production had started as determined by ejaculation into an artificial vagina. Sperm in the epididimis and proximal ductus differentia were obtainable by

electroejaculation at this age. (Wolf et al., 1965.) Some consider puberty complete when sperm are found in these regions. All of these criteria vary with genetic and environmental factors. This subject is reviewed by Macmillan (1967) and will not be developed further as the purpose of this review is to consider the role of androgens and their interactions with other endocrine and physiological factors during puberty, principally in the male bovine. Since data on the bovine are limited, other species will be included where necessary to develop hypotheses.

1. Gonadotropins

Clark (1935); McQueen--Williams (1935) and Lauson et al. (1939), were among the first to measure total gonadotropins in the pituitary. Pituitary total gonadotropin potency in male rats increased gradually during the first two weeks of life, faster in the third and fourth weeks and then declined.

Burr et al. (1970) reported that luteinizing hormone (LH) levels in the plasma of boys rose at ten years of age and reached a plateau at 13 years of age (see Table 1). Similarly, Yen (1969) detected LH in the plasma of boys at eight years of age and LH levels rose until 14 years of age with a very rapid increase between 10 and 12 years. He estimated a threefold increase in plasma LH between 8 and 14 years of age and concluded that sexual maturity was attained at 14 years of age. Skinner et al. (1968),

TABLE 1.--Gonadotropin Levels in Plasma of Humans at Various Ages.

Age	Plasma LH		Plasma FSH
(years)	--------(miu/ml)--------		(µg FSH/ml)
5	4.03[a]	--	1.18[a]
6	4.02	--	1.40
7	3.97	--	1.27
8	4.27	1.1 ±0.29[b]	1.24
9	4.08	0.63±0.04	1.30
10	4.42	0.86±0.31	1.67
11	4.44	1.2 ±0.11	1.77
12	4.80	2.1 ±0.27	2.09
13	4.55	4.2 ±0.83	3.11
14	4.99	4.1 ±0.30	2.86
15	5.46	4.0 ±0.45	2.25

[a]Burr et al. (1970)

[b]Johannan et al. (1969)

in an extensive survey of sexual maturation in the ram, noted that LH levels in the blood fell for the first 42 days of life and then rose markedly. However, levels of plasma LH were only weakly correlated with other parameters.

Burr et al. (1970) reported rising levels of plasma follicle stimulating hormone (FSH) in boys to 12 years of age, after which FSH levels declined (see Table 1). This fits well with data of Yen and Vicia (1970) who found that plasma FSH rose to 14 years of age and then declined. Kragt et al.(1968) determined that levels of FSH in the pituitary of rats increased fifteenfold from 10 days to

35 days of age and then stabilized at levels typical of adults. Gonadotropins remain fairly constant in man from 20 years through 90 years (Kent and Acone, 1965). The variation of plasma FSH levels with age for the rat are shown in Table 2.

TABLE 2.--Gonadotropin Levels in Anterior Pituitary of Male Rats at Various Ages.

Age (days)	Pituitary FSH (µgFSH/gland)	Age (days)	Pituitary FSH (µgFSH/gland)
11	18[a]	45	221
16	43	50	206
21	30	60	374
22-40	79-166	70	744
40	195		

[a]Pearce and Brown (1970)

Reece and Turner (1937) reported that pituitary prolactin potency appeared to increase slowly from birth to one year of age in bulls and then reached a plateau. In male guinea pigs, pituitary prolactin potency increased parallel to body weight from 170 to 880 g, but prolactin potency in male rats did not change between 80 and 340 g body weight. Reece and Turner (1937) also made observations on the male rabbit, here pituitary prolactin potency in immature animals was three times greater than in adults.

2. Development of the Testis

a) Growth and Spermatogenesis

A log log plot of testis weight as a function of body weight in rats and man revealed two distinct slopes with a change in slope at puberty (Spencer, 1968). In rats, the second part of the slope was less steep than the first. That is, testis growth showed positive allometric growth in the first phase and negative allometric growth in the second phase, but the picture was reversed in man. This result is substantiated by Burr et al. (1970) who observed that testicular growth in boys was slow up to 12 years of age and then proceeded more rapidly. Skinner (1968) showed a sharp rise in the testicular weight of ram testes at 42 days of age. Thus testicular development in rams resembles that in man.

The changing allometric growth for rat testes agrees well with nucleic acid data. The DNA concentration of rat testes declined rather rapidly from 2 to 4 weeks of age and then more gradually to 10 weeks (Fujii and Koyama, 1962). Desjardins et al. (1968) noted a similar trend, but a marked increase from birth to 15 days. RNA varied similarly. Fujii and Koyama (1962) concluded that sexual function of the testis commenced at 3 weeks of age based on the fact that the RNA:DNA ratio increased after 3 weeks of age.

Abdel-Raouf (1960) delineated five stages of reproductive development of bulls based on the morphology of the seminiferous tubules. The infantile stage consisted of the first two postnatal months and was marked by solid sex chords and foetal type cells. The proliferative stage lasted from 2 to 4 or 5 months. Spermatogonia appeared during this period. Lumen formation occurred and primary spermatocytes appeared in the third or prepubertal stage. Spermatids and later sperm appeared during the pubertal stage which lasted from 32 to 44 weeks. During the final post-pubertal or adult stage, mainly quantitative development took place and the testis increased in size.

Martig (1969) observed that the fertility of beef bulls and percentage of normal sperm increased from 1 to 2 years. Most bulls were relatively fertile at one year near completion of puberty. In man spermatogenesis is initiated between 12 and 14 years of age.

b) Steroidal Secretroy Activity

Experiments have been conducted to determine the type and site of steroid hormone secretion. Knapstein (1968) injected radioactive acetate or cholesterol into the spermatic artery of an adult male human. Blood collected from the spermatic vein revealed incorporation of radioactivity into testosterone and androstenedione. Incubation experiments by Bell (1968) and Hall (1969) revealed conversion of progesterone or pregnenalone to

androgens by the seminiferous tubules. As cholesterol is the major substrate for steroidogenesis it was concluded that the Leydig cells produced most of the androgens secreted by the testis.

In bulls, Leydig cells differentiate from intertubular mesenchymal cells in some cases by four months of age (Hooker, 1944), increase in number until two years of age, and increase in size in the mature bull mainly due to vacuolation.

The androgen secretory activity of the Leydig cells also increases during the period of sexual maturation. Lindner (1959) and Lindner and Mann (1960) observed that the ratio of androstenedione to testosterone changed from 1:1 in testes of calves at four months of age to 1:10 at nine months of age. Lindner (1961b) could detect only testosterone in the spermatic vein blood taken from a ram and two boars all between 3 and 4 months of age. Skinner (1968) detected androgens in the ram testis at birth; the ratio of androstenedione to testosterone at this time was 1:1. The testicular content of testosterone increased with testicular size but was highly variable. Androstenedione content fell until 56 days of age and then rose slightly to mature levels.

In vitro incubation studies provided evidence to support the findings of Lindner and Skinner. Becker and Snipes (1968) incubated radioactive androstenedione and testosterone with guinea pig testicular tissue. Testis

from a 10-week old animal showed a steady state equilibrium of androgens favoring androstenedione. However testosterone predominated in testis from 7-month old animals. Studies on the adult Rhesus monkey (Resko, 1967) showed a 10.1 ratio of testosterone to androstenedione. Pre-pubertal monkeys had small amounts of both steroids in their testicles, but testosterone was undetectable in the blood. By three years of age, androstenedione was detected in the blood and testosterone approached adult levels. The immature rat testis contained much androstenedione and little testosterone (Strickland, 1970). Androstenedione did not accumulate in the immature testis. Thus there was no block to its conversion to testosterone. It was concluded that testosterone reductase may play a role in puberty. In the mature testis, testosterone accumulated and androstenedione formation decreased.

Tables 3, 4 and 5 summarize testicular and plasma androstenedione and testosterone levels at different ages for three species. Little comment is needed, but it is interesting to note the large variability of androgen levels during sexual maturation. The increase in plasma testosterone in guinea pigs (Resko, 1970) between days 15 to 30 coincides with the first behavioural signs of sexual maturity. Plasma testosterone levels in man from 20 through 90 years are fairly constant but the utilization and metabolic clearance rate may drop with age (Kent and

Acone, 1965). Some normal adult levels of testosterone and androstenedione from the plasma and testis of several species are given in Table 6.

Probably the most interesting hypothesis of this section is that the testis gains the ability to convert androstenedione to testosterone in large amounts beginning at puberty. This agrees with the hypothesis of Strickland (1970) that testosterone reductase may be an important enzyme of puberty.

TABLE 3.--Variation of Plasma and Testicular Testosterone and Androstenedione with Age in the Rat.

Age	Testosterone		Androstenedione	
	Plasma	Testicle	Plasma	Testicle
(days)	(ng/ml)	(µg/g)	(ng/ml)	(µg/g)
1	0.27[a]	0.194	ND[d]	0.096
5	0.21	0.134	ND	0.043
10	0.09	0.122	ND	ND
15	0.10	Trace[b]	ND	ND
30	0.15	Trace	0.13	0.008
40	0.64	0.006	0.18	0.004
60	1.10	0.043	0.15	0.003
90	2.04	0.111	0.12	0.015
120	1.14	--	0.57	--[d]

[a]Resko et al. (1968)

[b]Response not great enough to quantify

[c]None detected

[d]Sample lost

TABLE 4.--Variation of Plasma Testosterone with Age in the Human.

Age	Plasma Testosterone	Age	Plasma Testosterone
(years)	(ng/ml)	(years)	(ng/ml)
<10	<0.2-0.5[a]	30-39	5.6
10-16	<0.2-5.4	40-49	4.7
22-59	3.4-14.9	50-59	5.9
20-40	5.0[b]	60-69	5.9
31	0.08±0.10[c]	70-79	5.8
20-29	6.6[d]	80-93	2.8

[a]Van der Molen (1965)

[b]Panicucci (1965)

[c]Saez and Bertrand (1968)

[d]Kent and Acone (1965)

TABLE 5.--Variation of Plasma and Testicular Testosterone and Androstenedione with Age in the Guinea Pig.

Age	Testosterone		Androstenedione	
	Plasma	Testicle	Plasma	Testicle
(days)	(ng/ml)	(µg/g)	(ng/ml)	(µg/g)
1	0.09[a]	0.092	0.49	0.043
5	ND[b]	--[d]	0.42	--
15	Trace[c]	0.082	0.18	0.118
30	0.42	0.049	0.45	0.030
40	0.25	0.014	--	0.016
60	0.70	0.066	0.25	0.016
90	1.69	0.121	--	0.022
120	1.13	0.194	0.34	0.018
250	1.98	--	--	--

[a]Resko et al. (1970)

[b]None detectable

[c]Response not great enough to quantify

[d]Sample lost

TABLE 6.--Normal Adult Levels of Testosterone and Androstenedione from Plasma and Testicles of Different Species as Estimated by Gas Liquid Chromatography.

Androgen	Species	Plasma	Testis	Reference
		(ng/ml)	(µg/g)	
Testosterone	Rat	41.00	19.50	Bardin (1969)
	Rat	50.40	--	Bardin (1969)
	Rat	1.14	0.11	Resko et al. (1968)
	Rat	5.04	--	Frick et al. (1969)
	Rabbit	3.76	--	Haltmeyer et al. (1969)
	Rabbit	2.10±0.40	--	Seiki et al. (1968)
	Ram	--	17.24±7.57	Skinner et al. (1968)
Androstenedione	Rat	9.20	0.02	Resko et al. (1968)
	Ram	--	1.24±0.62	Skinner et al. (1968)

3. The Hypothalamic-Pituitary-Testis Axis

a) Maturation of the Axis

In immature animals, the pituitary gland is a relatively labile structure and its future pattern of gonadotropin secretion is determined by the effect of steroidal hormones working through and with the modifying influence of the hypothalamus. Harriss and Jacobson (1952) transplanted pituitaries from immature to mature animals and vice versa. They found that the activity of the transplanted glands was dependent on the age of the host, not the gland. This suggested a modifying influence of the hypothalamus.

Androgen sterilization of female rats within the first few days of life is a fairly well known phenomenon (Gorski, 1966) and leads to constant estrus. This treatment is not effective after ten days of post natal life. These observations suggest the possibility of modification of the hypothalamic-pituitary axis during early life. From work undertaken by Harris and Levine (1965) with the male rat, it appeared that the normal mechanism underlying the future patterns of sexual behaviour and gonadotropin secretion are organized in the first few days of life by testicular secretion in male rats. If no testicular secretion is present then this pattern was fixed in a female form.

The gonads exert a continuous effect on the pituitary in the rat, probably principally via the hypothalamus. Libman et al. (1953) castrated male rats at birth and

found pituitary basophils enlarged by seven days and even more by 15 days. However pituitary castration cells, similar to those seen in the castrated adult, did not appear until 45 days of age. This suggested a build-up of gonadotropins in the pituitary in the absence of modifying influence of the gonads. Immature males had lighter pituitaries than immature females (Van Rees and Paesi, 1955). Castration caused male pituitary weight to be increased by 32% but that of the female by only 7%. This suggested that androgens may retard pituitary growth. Some more recent evidence agrees with the hypothesis that the hypothalamus is involved with steroidal modification of pituitary activity. Domer and Staudt (1969) histologically investigated the effect of castration in various areas of the hypothalamus and found that only the ventro medial nucleus was affected. Immediately after birth, this nucleus was large in females and smaller in males, and castration of the male allowed it to enlarge towards a female status.

Recent evidence utilizing new techniques to measure plasma LH substantiates pituitary and histological evidence of a build-up of pituitary gonadotropins after castration. Ramirez and McCann (1963) observed that gonadectomy of male rats increased the levels of plasma LH. This effect was twice as large in the immature as compared to the mature male rat. They also showed that the increase in LH was suppressed by administration of

testosterone propionate, and the suppression was greater in the immature rat. The differences between mature and immature rats suggest a change in the sensitivity of the hypothalamus to androgens during puberty. Kurcz (1969) produced some important evidence that supports this hypothesis. Male rats injected with large doses of testosterone immediately after birth were incapable of an increase in gonadotropin output. If the treatment was delayed until five days after birth, however, gonadotropin secretion developed normally. Kurcz (1969) concluded that the early injection desensitized the hypothalamus and damaged gonadotropin secretory cells. The very drastic long term effects of steroids on the sensitive early postnatal hypothalamus were demonstrated in work by Ladosky and Kerikowski (1969). One-day old rats injected with either estradiol benzoate or testosterone propionate showed delayed spermiogenesis at 35 to 60 days. This had recovered by 90 days but the accessory reproductive glands were still very small. Ulrich (1968) suggested that the rate of utilization of sex steroids did not alter at puberty, probably because the hypothalamus became less sensitive to the minute amounts of these steroids circulating during infancy and higher levels were needed to inhibit gonadotropin output. Hence higher levels of steroids were found in the blood. The increased levels of protein and steroidal hormones together stimulated sexual maturation.

The ability of the liver to metabolize steroids increases at puberty. Thus the high sensitivity of the immature hypothalamus to steroids may be due in part to the slower rate of inactivation of steroids by the liver. Denef (1968) showed that testosterone given immediately after birth in female and castrated male rats prevented the development of a female type steroid metabolism. Treatment later in life had only a temporary effect. This suggests that the liver has the potential to develop a female pattern of steroid metabolism unless modified by androgens, and also that the liver may play some role in the maturation of the hypothalamus.

Changes in levels of steroid secretion and parallel changes in the hypothalamus do not seem to be associated with increases of secretion of luteinizing hormone releasing factor (LHRF) by the hypothalamus. Ramirex and Mcann (1963) showed no difference in LHRF activity in the median eminence in mature or immature rats. However Macmillan (1967) could not detect LHRF in bulls up to four months of age but found a sudden increase at five months, a decline at six months, an increase at eight months and little change to welve months of age. When viewed with the changing levels of plasma LH a possible role of LHRF in sexual maturation is suggested.

b) Correlations and Interactions between the Pituitary, Testis, and Hypothalamus

Tables 1, 2, 3, 4 and 5 provide a comparison of gonadotropin levels and androgen levels in various species

during sexual maturation. Burr (1970) found that LH levels in the plasma of boys rose at ten years and reached a plateau at 13 years, this was associated with a small increase in testicular growth. FSH rose in the plasma at 12 months of age. Testicular size rose slowly at first, but much more rapidly during the period of rapidly increasing FSH levels. Later FSH levels fell while LH levels continued to rise. This suggests that maintenance of the testis requires less FSH than the active growth phase. At the peak of LH secretion, testosterone levels rose in the blood, plasma levels of FSH increased and spermatogenesis was initiated.

Testicular weight was associated with plasma levels of FSH in the rat (Kragt and Ganong, 1968) but Skinner (1968) reported that FSH in the ram was not highly correlated to testosterone plasma levels after 40 months of age.

The relationships described have more meaning in terms of the roles of hormones in sexual maturation. Lostroh (1969) investigated some of these roles in an experiment where male rats were hypophysectomized and treated with FSH and LH two months later. These treatments initiated spermatogenesis and androgen secretion as noted by accessory organ growth. Separately, neither hormone exerted this effect. High sperm output was correlated with high androgen titers, but testosterone did not initiate spermatogenesis.

FSH has been shown to stimulate testicular RNA and protein synthesis and this property seemed fairly specific for FSH and gonadotropins with FSH activity (Means, 1969). FSH also has been shown to stimulate phospholipid synthesis in the testis even if the LH activity was destroyed with urea (Yokoe and Hall, 1970).

The mechanism of stimulation of the testis by FSH and LH at the molecular level, albeit in different structures, are probably similar. Connell and Eiknes (1968) and Murod et al. (1969) performed in vitro incubation studies with rat and canine testis, respectively, and demonstrated that FSH and LH stimulated the production of cyclic AMP from ATP, and also that FSH, LH and cyclic AMP stimulated testosterone synthesis. It appears as if the major role of FSH is in stimulation of testicular growth via protein synthesis.

Kar (1966) injected ovine FSH and LH into Rhesus monkeys. There was a small increase in the size of the seminiferous tubules and Leydig cells with concomitant stimulated testosterone secretion. No greater effect was noted with combined FSH and LH, and he concluded that LH had its effect on the interstitial cells and FSH on the seminiferous tubules. Similar experiments in the rat yielded less conclusive results, and he concluded that the timing of gonadotropin injections with the phase of development of the testis was very important. These

experiments associated LH with the Leydig cells, the major site of androgen production.

Several investigators have reported that gonadotropins stimulate testicular steroidogenesis by enhancing the 20α-hydroxylation of cholesterol to pregnenolone (e.g. Dorfman, 1969; Hall and Young, 1968). Oshima (1967) injected radioactive progesterone into male rats followed by an injection of testosterone propionate. The androgen markedly reduced the utilization of the progesterone substrate by the testis. Upon withdrawal of testosterone propionate treatment progesterone utilization increased, but the testis only converted it as far as androstenedione and 17α-hydroxy-progesterone. After a longer period of testosterone withdrawal, the 17α-hydroxylase and C_{17}-C_{20} lyase enzyme system recovered by 17β-hydroxy steroid dehydrogenase was much slower to recover. Oshima suggested that LH stimulates these enzyme systems.

Literature reviewed in this section suggests that FSH stimulates protein synthesis and general growth of the testis, laying the foundation for steroid secretion while LH acts directly on the enzyme systems involved in androgen secretion. Both gonadotropins seem necessary to initiate spermatogenesis.

4. Attempts to Accelerate the Process of Sexual Maturation

Most approaches to this problem have involved administration of hormones implicated in initiation and

maturation of spermatogenesis, or maturation of the reproductive tract, or in stimulation of endogenous levels of these hormones. It has been known for a long time that mating increases the circulatory and testicular levels of testosterone. This was shown recently by Herz et al. (1969). This response probably reflects a neuroendocrine reflex mediated by the hypothalamus causing release of LH which stimulates synthesis of testosterone.

Lindner (1961a) increased spermatic vein levels of androstenedione eight fold in a 90-day old calf, but could not stimulate testosterone output with HCG administered for 50 days prior to androgen determination. Saez and Bertrand (1968) attained a twentyfold increase in plasma testosterone levels in prepubertal boys with injections of HCG, but in adults only a two or threefold increase was noticed. He hypothesized that the lower levels of LH in the prepubertal boys allowed much more capacity for stimulation. Fariss (1969) reported that injections of 5 or 10 iu HCG gave the maximum testosterone response in hypophysectomized male rats, testosterone levels in the plasma peaked 45 minutes after injection and then declined rapidly. However, when male rats were given HCG immediately after birth during the period of hypothalamic differentiation (Dorner, 1969) testicular size and androgen secretory activity increased only to result in hypogonadism and small accessory sex organs in the adult. Data from adult male rabbits (Haltmeyer, 1969; Saginar and

Horton, 1968) showed that either HCG or copulation increased plasma levels of androstenedione and testosterone very rapidly. Morkow et al. (1968) examined the effect of HCG on the testis in more detail. He injected 100 iu HCG for 15 days into adult male guinea pigs and noted an increase in size and number of Leydig cells. Within these cells, the smooth and rough endoplasmic reticulum and the Golgi apparatus were increased thus indicating a potential for a vast increase in metabolic activity.

Wakeline (1959) injected pregnant mare serum gonadotropin (PMS), HCG, testosterone propionate and human post menopausal gonadotropin (HMG) for seven days into intact male rats from 25 or 28 days of age. All of the hormones increased accessory organ weight, presumably by stimulating testosterone synthesis. Only PMS accelerated appearance of spermatids in the testis but none of the treatments accelerated initiation of spermatocytogenesis. Woods and Simpson (1961) reported that ovine FSH and LH did not hasten sexual maturity in intact young male rats, but a combination of the two gonadotropins hastened maturity in hypophysectomized animals. They maintained that the immature pituitary produces an "anti-gonadotropin." The rat testis was responsive to gonadotropins between birth and six days of age (Price and Ortiz, 1944), but the maximum response to exogenous gonadotropins as measured by seminal vesiculat weight was between 20 and 26 days. More recently Sandler and Hall (1968) administered LH to male

rats at 20, 70, 240 and 360 days of age. There was no difference in response between the latter three ages, but injection of LH at day 20 resulted in greater conversion of sholesterol to testosterone and androstenedione with testosterone predominating. Sandler (1968) hypothesized that the greater response in the young rats was due to lower levels of endogenous LH. Mann et al. (1960) could detect no change in testis weight, seminal vesicular weight, or fructose and citric acid in calves given HCG before 12 weeks of age. However these responses were increased by testosterone propionate in calves of the same age. The lack of response to HCG was probably due to the lack of ability of the young testis to secrete testosterone before 12 weeks of age.

Clomiphene acetate stimulates testosterone secretion from the testis by blocking the feedback control mechanism at the hypothalamus. Bardin et al. (1967) injected 100-200 mg of clomiphene acetate into adult male humans for 6 to 9 days and caused a small increase in plasma LH and testosterone levels within 2 to 6 days. But after six days there was a large increase in both criteria. In adult human males, clomiphene citrate stimulates plasma levels of FSH and testosterone (Kulin et al. 1969). But in the prepubertal boy, FSH and testosterone levels are suppressed. The young hypothalamus is very sensitive to low levels of steroids which reduce gonadotropin output. Treatment with clomiphene citrate probably does not compete

completely for binding sites in the hypothalamus thus allowing some steroid feedback to occur.

Kalra and Prasad (1967) demonstrated that clomiphene also inhibits spermatogenesis at the primary spermatogenesis at the primary spermatocyte stage in the adult male rat. Injections of 75 µg of FSH per day could not counter this. However 200 µg per day of TP advanced spermatogenesis to the spermatid stage and when the two treatments were combined spermatogenesis was complete. The level of testosterone propionate administered was sufficient to inhibit gonadotropin secretion, but for testosterone propionate alone to maintain spermatogenesis in the face of clomiphene citrate it required injections of 1 mg per day. Both FSH and testosterone propionate were required to restore testicular weight, but only 15 µg per day was necessary to maintain the accessory organs. Work by Heller (1970) seems to qualify the previous comments. He found that in normal men clomiphene citrate had two primary actions in sperm production. At low dose levels there was a stimulation of cell numbers but at high dose levels he observed many abnormal spermatids and a fall in the number of ejaculated spermatozoa.

Methalibure has properties similar to clomiphene. Skinner (1969) found that it increased testicular and accessory organ growth rate in pubescent male rabbits. Petry et al. (1968) experimented with mesteralone (1-methyl-androstane-17β-ol-3-one) a strong androgen which

exhibits no inhibition of gonadotropin secretion nor any deleterious effect on sperm numbers. This unusual combination of properties may be because the A ring of this steroid is saturated and thus cannot be converted to estrogens which have strong inhibitory effects at the hypothalamus.

This survey is by no means complete as there are a whole spectrum of drugs that have been investigated for properties that would hasten sexual maturity or at least early production of fertile sperm. An attempt has been made to outline some of the techniques, and physiological and endocrinological interactions that offer some promise. It is clear that the dose levels of drugs and hormones and the period of sexual maturity at which they are administered are of prime importance.

5. Development of the Accessroy Sex Glands

The accessory reproductive glands develop under the influence of testosterone. Before assays for blood and testicular levels of testosterone were available, seminal vesicular fructose, citric acid and nucleic acid were taken as indicators of the androgenic status of animals.

Androstenedione administered to a castrated male calf from 2.5 to 6 months of age delayed seminal vesicular growth and secretion (Skinner et al. 1968). He concluded that androstenedione was devoid of any androgenic properties, being mainly an anabolic steroid. This view is corroborated by Baranas (1969) who castrated 6-month old

calves and injected testosterone or androstenedione. Both promoted growth and nitrogen retention, but only testosterone showed any androgenic properties.

Desjardin et al. (1968) observed that rat seminal vesicular RNA and DNA increased beginning around day 20 after birth. This was before the animals were sexually mature and before the onset of fructose secretion. Rabinovitch and Lutwak-Mann (1951) injected testosterone into castrated rats, the earliest response of the seminal vesicles was an increase in RNA levels. Ritter (1969) performed a similar experiment, he noticed a rise in RNA one or two days after the start of testosterone treatment with a return to control levels by two to six days. Seminal vesicular DNA started to rise one or two days after the start of testosterone treatment and was complete by six days. Protein concentration remained constant in the glands. Ritter noted that similar responses occurred in the prostrate gland, but they were somewhat more delayed.

Measurements of the growth of the seminal vesicles (Pearce and Brown, 1970) and prostate gland (Kragt and Ganong, 1968) of the rat revealed a rapid postnatal growth phase for the seminal vesicle for 21 days. From then on the growth curve was fairly flat until 40 days. The prostrate gland had two periods of grwoth, 10 to 20 days, and 30 to 40 days; it is interesting to note that pituitary LH declined rapidly during the last period.

Secretory activity of the seminal vesicles does not seem to be correlated highly with any changes in DNA or RNA. Porter and Melampy (1952) and Levey and Szego (1955) could not detect a significant amount of fructose until 30 to 37 days in the rat. Seminal vesicles in guinea pigs commenced secretory activity at 20 days, long before spermatozoa appeared in the testis. In the bull, Abdel-Raouf (1960) observed a marked rise in citric acid and fructose secretion of the seminal residue at 24 weeks of age. Fructose levels rose rapidly to 42 weeks, whereas the rate of increase in citric acid was negligible after 36 weeks. Lindner and Mann (1960) showed an increase of seminal vesicular secretory activity due to increasing blood testosterone levels. Asdell (1955) noticed that bull seminal vesicles grew slower than the body until such a time when higher testosterone levels were expected, but Abdel-Raouf (1960) believes the seminal vesicles maintain a steady rate of growth until 68 months. In the rams, Skinner (1968) detected fructose in the seminal vesicles at birth, however citric acid was not detected until 14 days of age and even then there existed a 10:1 ratio between fructose and citric acid. During sexual maturation seminal vesicular fructose and citric acid content and concentration were both correlated to plasma testosterone levels.

Recently more evidence has been gathered to support the hypothesis of androgenic stimulation of the growth

and secretion of accessory reproductive glands. Treter (1969) demonstrated binding of testosterone by the seminal vesicle of the rat. Unhjem and Treter (1968) injected radioactively labelled testosterone into male rats and found it became associated with macromolecules of the prostrate gland, but not of the kidney or muscle. Belham (1969) demonstrated that testosterone was taken up by the prostate gland, but not bound specifically before it was converted to dihydrotestosterone. In an experiment Unhjem and Treter (1968) and Kouarski et al. (1969) found that the highest concentration of testosterone was in the prostrate gland and that dihydrotestosterone was found nowhere else except in reproductive accessory glands.

The literature reviewed in this section indicates that the growth and secretory activity of accessory reproductive organs are very much dependent on circulating androgen levels.

6. Testosterone and Androstenedione in the Testes and Plasma as Measures of Androgenic Status

Until recently, the androgenic status of an animal was estimated by the growth and secretory activity of the accessory reproductive organs because they were found to respond uniformly to exogenous testosterone and to treatments thought to stimulate endogenous testosterone secretion. In intact animals, the response of the accessory glands to androgenic stimulation is to the total androgenic

stimulation and may not represent one or two steroid hormones.

In the present study testosterone and androstenedione were measured in the blood and testes as a reflection of the androgenic status of the bull. Several factors could confuse the inferences which may be made from this study, and these will be briefly discussed in light of the current literature. Of all the androgenic compounds produced in the body testosterone is the most potent. As discussed earlier, androstenedione has little or no androgenic properties and most other metabolites are less potent than testosterone. Van der Molen et al. (1965) considered free testosterone in plasma reflected the androgenic status of the male. Horton and Tait (1965) estimated that the bulk of testosterone was secreted by the testis and not produced in the blood. Only 7% of the blood androstenedione was converted to testosterone, however 36% of blood androstenedione was formed in the blood from testosterone. The rest was secreted by the testis. The pulmonary site is an important site for the conversion of testosterone to androstenedione. Kirschner et al. (1965) estimated that 20% of the blood testosterone was produced by the adrenals, either by direct synthesis and secretion or by conversion of peripheral metabolites. Van de Wiele and Mcdonald (1963) agreed with this. This value seems high and disagrees with Horton's estimates. Rosenfeld et al. (1969) found

no relationship between secretory function of the adrenal gland and growth and development of the testis.

All of the testosterone in the blood may not be in an active form; some may be bound to proteins. The testosterone binding affinity in the blood varies with age in man (August, 1969; Pohlman et al., 1969). It is low in newborn infants, increases in prepubertal boys, but falls in adults. There does not seem to be any relationship between testosterone levels in the blood and the binding affinity of blood proteins for testosterone.

Several conjugated forms of the androgens are present in the blood. These are generally regarded as excretory or inactive forms of the steroids, but recently the steroid conjugates and their hepatic mesenteric circulation have been suggested as an important mechanism for control of blood levels of these steroids. Among the conjugates, only the sulfates of androstenedione and testosterone are secreted by the testis (Laatikainen et al., 1969). The glucuronides probably form the main excretory form (Ismail to Loraine, 1969; Robel et al., 1965). Little testosterone glucuronide is excreted. Most testosterone is reduced at the A ring to a 5β androstane 3α-17β-doil before conjugation. Table 7 shows the levels of androgen conjugates in the blood. It is interesting to note that the quantities are almost as large as the free androgens.

TABLE 7.--Normal Adult Levels of Testosterone and Androstenedione in Free and Conjugated Form from Plasma of Man as Estimated by Various Techniques.

Hormone	Technique[a]	Plasma	Reference
		(µg/100ml)	
Testosterone	GLC-HF	0.34 to 1.49	Van der Molen (1965)
Testosterone	GLC-HF	0.50	Panicucci (1965)
Testosterone	PB	0.85	Frick (1969)
Testosterone	GLC-HF	0.58±0.29	Surace et al. (1965)
Testosterone	GLC-EC	0.42±0.60	Brownie et al. (1964)
Testosterone	Enzymatic	0.10±0.40	Finkelstein et al. (1961)
Testosterone	Enzymatic	0.56	Forchielli et al. (1963)
Testosterone	DID	0.74	Hudson et al. (1963)
Testosterone	DID	0.80±0.25	Riondel et al. (1963)
Testosterone	DIDer	0.72 (0.32-1.07)	Kirschner et al. (1965)
Testosterone	DIDer	0.65	Segree et al. (1964)
Testosterone	PB	1.14±0.42	Rosenfield (1969)
Androstenedione	GLC-HF	0.10 to 0.25	Van der Molen (1965)
Androstenedione	GLC-HF	0.00 to 0.09	Van der Molen (1965)
Androstenedione	GLC-HF	0.23 to 0.51	Saginar and Horton (1968)
Testosterone glucuronide	GLC-HF	0.68 (0.99 to 0.59)	Van der Molen (1965)
Testosterone glucuronide	GLC-HF	0.30 (0.09 to 0.59)	Van der Molen (1965)
Androstenedione sulfate	GLC-HF	0.02 to 0.10	Brooksbank (1968)

[a]GLC-HF = gas liquid chromatography with hydrogen flame detector,
GLC-EC = gas liquid chromatography with electron capture detection.

[b]DID = double isotope dilution.
PB = protein binding.
DIDer = double isotope derivative.

There are several techniques available to quantificate androgen levels in biological fluids and probably two major fluids from which meaningful estimates could be made. Table 7 lists normal adult levels of testosterone and androstenedione from the plasma of man as estimated by various techniques. The techniques used are gas liquid chromatography with hydrogen flame or electron capture detection, enzymatic techniques, double isotope dilution and derivative methods and the protein binding assay. All of these techniques yield surprisingly similar results considering the large variation that exists between human subjects. Blood and urine are the two major biological fluids in which androgen levels have been studied. Measuring levels of androgens in the urine are not reflective of levels available to the tissues.

Sampling of the blood pool may affect the androgen picture. Ismail and Loraine (1969) demonstrated a circadian rhythm of testosterone secretion in the blood of man. Seiki et al. (1968) showed that testosterone levels are higher in the inferior vena cava as compared to the carotid artery in rabbits. These factors and stress factors could all add some variability to the levels of androgens estimated from the blood sample.

Taking everything into consideration, levels of testosterone in the plasma and testes are perhaps the most reliable estimates of the androgenic status of an animal. Androstenedione is probably eliminated in this

respect and blood levels of this steroid are confusing because of its adrenal secretion and the conversion of testosterone to androstenedione for excretory purposes.

MATERIALS AND METHODS

1. Development of Techniques

a) Objective

Information on methodology for the assay of plasma and testicular levels of testosterone and androstenedione was scarce and incomplete. No routine methods were available for fast accurate assay of these two androgens in tissues for any species. The reported methods are tedious, time consuming, and untested with sufficient numbers to render them generally acceptable. Most of the analytical problems were related to the extreme purification of the androgens required by the high sensitivity required to measure the minute quantities of androgens in bulls. My aim was to develop an accurate technique that would be relatively simple in operation and sufficiently rapid that it could be operated routinely for the assay of large numbers of biological samples.

b) Introduction

The quantification of testicular and plasma androgens demands a very sensitive technique as the levels of these hormones found in bull tissues are in the range of

nanograms per milliliter of blood. Previous to 1960, no such technique existed. µg and mg levels of androgens had been assayed in bioassays such as the Cockerel comb assay and secretory responses of various accessory reproductive organs, these bioassays are not sufficiently sensitive for measurement of androgens in bulls. Spectrophotometric techniques seemed feasible, but they also lacked the sensitivity required by the low levels in most biological samples.

Chromatography in general works on the basic principle that the material to be purified or the mixture to be resolved is repeatably distributed between two phases, one is stationary, while the other flows past it. In gas chromatography, the mobile phase is gaseous.

In 1952 James and Martin showed that it was possible to separate compounds using gas liquid partition chromatography. Various compounds were investigated following this, such as fatty acids and organic amines. Several gas phase detectors were developed including the hydrogen flame ionization detector between 1957 and 1958.

During 1959-1960 the possibility of achieving a separation of steroids by gas liquid chromatography was under study in several laboratories. The main problem was that the solid phases then available would bind steroids tenaciously except at very high temperatures when the steroids would break down.

Vandenheuvel, Sweeley and Horning, 1960, demonstrated that thin films (1 to 3% of liquid phase) could be used for separation of many steroids without loss of functional groups at temperatures only a little higher than 200C and with retention times of as little as 15 minutes. This paved the way for the use of thin film columns, prepared with deactivated supports such as silanized acid washed diatamaceous earth.

The use of gas liquid chromatography for separation and quantification of steroids quickly evolved. A simplified scheme showing the adaptation of gas liquid chromatography for isolation and purification of steroids follows. A gas column 3 to 6 feet long, 0.4 mm i.d. is filled with finely divided inert solid, such as diatamaceous earth or ground fire brick impregnated with a nonvolatile liquid phase. The mixture of compounds to be analyzed is introduced to the column, and flash evaporated at one end of the column which is held at constant temperature throughout its length. The volatilized compounds are swept through the column by a constantly flowing stream of an inert gas such as argon, helium or nitrogen. Each component of a mixture of compounds moves on the column at a rate determined by its ratio of partition between the gas phase and the nonvolatile liquid (stationary) phase. Individual compounds in the gas emerging from the column are usually detected by physical or chemical means. Data are automatically recorded on a chart as a series of

peaks. The area under each peak is proportional to the quantity of that component in the mixture, which is identified by standards.

The hydrogen flame detector was used in our early studies and a brief introduction to the theory of its operation is warranted at this point. The sample is swept by the carrier gas through the column and into the hydrogen flame. As it enters the flame, molecules are ionized, forming positive and negative ions. The extend of this ionization depends on the nature of the compound (molecular structure, degree of unsaturation, etc.), and the temperature of the flame. An electric potential is applied between the hydrogen jet and a collector ring above it so that the jet is negatively charged and the collector positively charged. When a compound is ionized the electrons are attracted to the collector and flow via a biasing circuit and create a voltage drop across the input load resistor. This is amplified by the electrometer and the output is presented on a potentiometric recorder in the form of a chromatographic peak. The response of the ionization detector depends on the number of molecules per unit time entering the detector. Thus, increasing the flow rate or increasing the flame temperature would increase sensitivity. The degree of ionization is roughly proportional to the number of carbon atoms per molecule in any given organic compound. Inorganic compounds such as hydrogen, nitrogen, carbon dioxide and

and water are not ionized and hence not detected. A general rule is that a compound must have either a carbon-carbon linkage or a carbon-hydrogen linkage to be detectable with hydrogen flame detectors.

According to Guerra-Garcia et al. (1963) sensitivity of the hydrogen flame detector for adequate testosterone detection is 0.05 mg. This was obviously not sensitive enough to accurately quantify plasma levels of androgens, but seemed sufficiently sensitive to quantify testicular levels. Based on estimates by Lindner and Mann (1960) I expected to find approximately 6 μg of testosterone in 4 to 5 g of testicular paranchyma. I thought this may be enough, with careful calculation to produce a quantifiable response using the hydrogen flame detector.

c) Use of the Hydrogen Flame Detector

We homogenized 4 to 5 g of testicular tissue and extracted it with diethyl ether following the purification scheme of Armstrong et al. (1964). The extract was dried and transferred to a silica gel thin layer plate which was developed in two dimensions; first in hexane:ethyl acetate (5:2 vv) and second in methylene dichloride: diethyl ether (5:2 vv). The areas corresponding to standard androstenedione and testosterone on the thin layer plates were scraped from the plate. Steroids in the scraped silica gel were eluted and the androgens were taken up in methanol for gas liquid chromatographic analysis.

Standard curves for testosterone and androstenedione gave a linear response from 50 to 1,000 ng. I did not test less than 50 ng. My extracts of testes contained severe organic contamination. The most sensitive setting on the gas chromatograph (without completely masking the androgen peaks with contaminant peaks) revealed approximately 4 ng of testosterone. At a 1 ml dilution, this was equivalent to 4 μg of testosterone in the 4 to 5 g of testis extracted. Under these conditions of purification, 16 ng injected into the gas liquid chromatograph (peak of 2 cm^2) was the lower limit for accurate quantification. Further work revealed that the bulk of contaminants were from the testis extract, and the extraction efficiency was less than 10%. Thus, it appeared that a better method of purification and a more sensitive detection system were required to quantify testicular and especially plasma levels of androgens.

This preliminary work did however reveal retention data. The data for testosterone and androstenedione on various columns and at various conditions are summarized in Table 8. Column packing, carrier gas flow rate and oven temperature all influence retention time. Liquid phases can be divided into nonpolar or nonselective phases, and polar or selective phases. OV-1 and SE-30 are nonselective phases separating steroids mainly on a basis of molecular size and shape. Thus retention times for any percentage liquid phase are shorter with less difference

TABLE 8.--Retention Data for Testosterone (Test.) and Androstenedione (Androst.) on Various Gas Liquid Chromatographic Columns and at Various Conditions.

Steroid	Column	Temperature			Gas flow rates			Retention Time
		Oven	Flash heater	Detector heater	Carrier gas	Oxygen	Hydrogen	
		----------(C)---------			---------(ml/min)--------			(mins)
Test.	OV-1 3%	215	248	248	70	300	40	11.36[a]
		218	257	249	70	300	40	11.12
		222	263	262	50	275	40	8.96
	SE-30 1%	220	---	---	50	---	--	4.40
	QF-1 2%	222	252	268	75	300	40	20.00
Androst.	OV-1 3%	183	292	308	50	300	40	Unrecognized
		203	292	308	50	300	40	Unrecognized
		213	292	308	50	300	40	14.24
		223	292	308	50	300	40	10.00
		233	292	308	50	300	40	6.00
		234	292	308	50	300	40	4.24
	SE-30 1%	183	292	308	50	300	40	8.72
		203	292	308	50	300	40	4.00
		213	292	308	50	300	40	2.48
		223	292	308	50	300	40	0.96
		233	292	308	50	300	40	0.76
		243	292	308	50	300	40	With solvent front
	QF-1 2%	222	252	268	75	300	40	32.72
	XE-60 2%	222	252	268	75	300	40	32.00

[a]Retention times are averages of from 2 to 6 observations.

between steroids than for selective phases such as QF-1 or XE-60. The latter phases separate steroids more on the basis of charge properties of the molecules. Both increasing the carrier gas flow rate and increasing oven temperature decrease the retention time.

d) Electron Capture Detector

Browrie et al. (1964) suggested that the electron capture detector was 1,000 times more sensitive than the hydrogen flame detector when haloacetates of steroids were quantified.

The functional mechanism of electron capture detection is based on reduction of an electric current flow due to the removal of free electrons from the system by sample components eluted from a gas chromatographic column. The current is produced by a radioactive tritium foil emitting electrons (beta radiation) which flow between an anode and a cathode. The drop in current due to capture of electrons by materials eluted from the column is recorded, amplified and presented as a peak on the recorder. The potentialacross the cell is applied as a pulse. This increases the dynamic range of linear response as it prevents large ion migration and plating out of large ions on the cell foil which could reduce sensitivity. An inert nonelectron capturing carrier gas must be used such as 90% argon, 10% methane. No purge gas is necessary unless quantities of sample greater than 1 µg are introduced onto the column. The response of

substances in this detection system depends on their ability to capture electrons. Cargon and hydrogen do not capture electrons readily. Thus many organic solvents can be used because they give no response. Oxygen captures electrons readily, and alcohols to a lesser extent; thus most steroids should produce a reasonable response. Water and many inorganic and organic contaminants will also produce a response; thus the carrier gas must be very dry and the samples extremely pure. Halogens capture electrons readily and consequently linking of halogens to the steroid molecule increases their electrol capture response, as will be developed later. One disadvantage of the tritium foil detector is its breakdown and release of radioactivity at high temperatures. The temperature of the detector should be kept below 225C. Unfortunately this causes long retention times and necessitates columns with a low percentage of nonpolar liquid phases.

Free androstenedione and testosterone standards quantified using the electron capture detector showed that the detector was approximately 25 times more sensitive to androstenedione and 15 times more sensitive to testosterone than hydrogen flame detection. The difference between the two androgens is probably due to the extra oxygen group on androstenedione.

With the increased sensitivity of the electron capture detector I decided to try to quantify free testosterone and androstenedione. The main problem was

the large solvent and contaminant front produced by the biological samples. I planned to separate the two androgens from the contaminants and from each other at relatively low temperatures on nonselective columns by selective derivitization of steroids only. I decided to investigate the properties of trimethylsilyl and O-methyloxime trimethyl silyl derivatives. The derivatives were prepared by the method of Luukainen et al. (1961).

e) Trimethylsilyl Ether of Testosterone

Pyridine (0.2 ml) and 0.15 ml of hexamethyl disilazine were added to microgram amounts of testosterone, 0.05 ml of trimethyl chlorosilane also was added as a catalyst. The reaction continued over night in a dessicator.

The mixture was extracted with 0.5 to 0.3 ml of hexane and centrifuged, and the hexane was transferred to another tube. The mechanism of reaction with the hydroxyl group is unknown. According to Chambaz and Horning (1969), the hydroxyl on the seventeenth carbon of testosterone should be completely derivatized with the 20% trimethyl chlorosilane catalyst mixture that I employed. Gas liquid chromatographic analysis on a 1% SE-30 column, however, did not show one clear peak. Whether this was due to incomplete reaction or separation of the isomers etc. was not determined as I was not so much interested in the mechanics as using the derivatives as a tool in quantitative analysis.

f) O-Methoxime-Trimethylsilyl Derivatives of Testosterone and Androstenedione

0.2 ml of a solution of 14 mg of methoxyamine hydrochloride in 1 ml of pyridine were added to the sample. The reaction mixture was left at room temperature in a dessicator overnight. Hexamethyl disilazine (0.5 ml) was added the next morning. After a further six hours, the reaction mixture was dried under nitrogen and extracted with hexane. In the second part of the reaction the methoxyamine and hydrochloride acts as a catalyst. Ketones at the three and seventeen positions should be readily converted to the O-methyloxime-trimethylsilyl derivatives.

A second technique of adding the hexamethyl disilazane with the pyridine and methoxyamine hydrochloride overnight gave a better conversion of the steroids to the O-methoxime-trimethylsilyl derivative.

Both androstenedione and testosterone-O-methoxime-trimethyl silyl derivatives gave a single peak with electron capture on a 1% SE-30 column. The reaction mechanisms are not clear. Testosterone forms an O-methoxime-trimethylsilyl derivative at carbon 3. I did not investigate the derivative to ascertain whether a trimethylsilyl derivative also had formed at carbon 17. The position of derivative groups on the androstenedione molecule also were unknown; both carbon 3 and 17 are easily derivatized in this reaction.

All derivatives gave a lower response with the electron capture detector than the free steroids. The lowered response seemed to be proportional to the removal of the oxygen and hydroxylelectron capturing groups. Greatest response was seen at a pulse interval of 50 and a carrier gas flow rate of 40 ml per minute. Retention data for these derivatives are listed in Table 9. It will be noted that the trimethylsilyl group increases the retention time markedly but the combined O-methoxime-trimethylsilyl group does not increase the retention time much more.

On a nonselective column, retention times depended on molecular shape and size, and the increased molecular size of the derivatives would enhance retention times and aid separation. This property would be more applicable to group separations and qualitative analysis. As the derivatives did not enhance sensitivity nor the problem of contaminants, I used them no more. An important use of trimethylsilyl derivatives is to prevent thermal deomposition. An example of this is the loss of the side chain for steroids of the andrenocorticoid group containing the cortisone or cortisol side chain. Direct gas liquid chromatographic analysis of these results in loss of the side chain, but the O-methoxime-trimethylsilyl derivatives of these steroids are stable under most gas liquic chromatographic conditions.

TABLE 9.--Some Retention Time Data[a] for Trimethylsilyl and O-methoxime-Trimethylsilyl Derivatives of Testosterone and Androstenedione.

Carrier gas flow rate	Compound Analyzed	Retention times
(ml/min)		(mins)
35	Androstenedione	12.5[b]
40	Androstenedione	12.0
60	Androstenedione	10.1
40	Testosterone	14.0
30	Testosterone trimethylsilyl ether	22.0
40	Testosterone trimethylsilyl ether	16.0
60	Testosterone trimethylsilyl ether	14.0
40	Testosterone methoxamine	15.0
40	Testosterone methoxamine trimethylsilyl ether	17.0
40	Androstenedione methoxamine trimethylsilyl ether	18.0
30	Androstenedione methoxamine	15.0
40	Androstenedione methoxamine	14.8

[a]Column temperature, 180C; electron capture detector, 212C; flash heater, 207C; and 1% SE-30 columns.

[b]Retention times are averages of several observations.

At this point it seemed necessary to try to reduce the levels of organic and inorganic contamination of the extracts analyzed by electron capture gas liquid chromatography, and to try to find some means of increasing the response of testosterone and androstenedione in the electron capture detector. The latter problem was tackled first.

g) Halogen Derivatives of Androgens

As stated before halogens are strongly electron capturing. If a large molecule containing many halogen units could be attached to the steroid nucleus extremely high sensitivity could be attained.

Brownie et al. (1964) described a technique for the determination of plasma testosterone utilizing gas liquid chromatography with electron capture detection. This technique involved forming a chloracetate derivative of testosterone. The lowest level that could be accurately quantified was stated to be 1 ng.

For nanogram quantities of testosterone the technique for monochloracetylation was as follows. One-half ml of monochloroacetic anhydride in tetrahydrofuran (100 mg/10 ml) and 0.1 ml pyridine were added to the purified androgen extract. The reaction continued over night at room temperature in a dessicator. One ml of water was added to stop the reaction. The reaction solution was extracted with 1 ml ethyl acetate three times. The pooled extracts were washed in 1 ml of 6 N

hydrochloric acid twice, and twice in 1 ml of distilled water. Finally it was dried and taken up in hexane. This was a reaction with the hydroxyl group at carbon 17. The yield of derivative was high but to remove any underivatized steroid Brownie purified the derivatives on thin layer chromatography. However my experience showed the chloracetate derivative to be unstable on thin layer chromatography. The monochloracetate of testosterone had an extremely long retention time on most gas liquid chromatographic column phases. For these reasons and the fact that new halogen derivatives existed with shorter retention times, greater molar response, greater ease of preparation and greater stability, I decided to experiment with heptafluorobutyric anhydrides. This steroid derivative depended for its sensitivity on attaching a 7 fluorine chain at one or more positions on the steroid nucleus. It was claimed that derivatives of this type could lower the limit for accurate quantification to 0.1 ng, a level that should make the assay of blood androgen levels feasible.

Clark and Wotiz (1963) first used heptafluorobutyrate esters. Several methods of preparation have been suggested. Clark and Wotiz (1963) dried the extract of 1 ml of plasma under nitrogen and reacted this with 2 µl of heptafluorobutyric anhydride and 1 ml of tetrahydrofuran in 1 ml of hexane for 30 minutes at 60C. The reagents were then driven off under nitrogen at 60C and the derivatized

samples were dissolved in 50 µl of hexane. This method gave the 3, 17-diheptafluorobutyrate of testosterone and 3-monoheptafluorobutyrate of androstenedione. Table 10 summarizes several techniques and their sources.

When preparing the monoheptafluorobutyrates the level of pyridine was critical. If it was too low, the diheptafluorobutyrate formed. If it was too high, recoveries were very low. In my initial attempt to prepare heptafluorobutyrate derivatives of testosterone and androstenedione, varying amounts of steroid were used, from 0.5 to 500 µg. A 1:1 ratio of benzene to heptafluorobutyric anhydride was added to the steroids which were then heated to 70C for 40 minutes. The yield of this reaction was believed to be greater than 75% but tritiated androgens were included with each reaction to check yields. The reagents were evaporated under nitrogen at 60C and the residue was transferred with 0.5 ml of acetone three times to a cellulose thin layer plate (Whatman CC .41). The thin layer plates were developed in acetone:water (3:1 v/v) and required 2.25 hours to develop. Under ultra violet light, many ultra violet absorbing areas were apparent, and the radioactivity was spread from the origin continuously to the solvent front. These observations suggested that either the derivatization had failed or that the derivatives were unstable on cellulose thin layer plates.

TABLE 10.--Reaction Conditions and Products for the Preparation[a] of Testosterone and Androstenedione heptafluorobutyrates (HFB).

Quantity of testosterone	Benzene	Pyridine	HFB	Derivative	Source
2 to 6 µg	50 µl	---	100 µl	Di HFB	Exley (1967)
2 mg	0.1 ml	---	0.1 ml	Di HFB	Exley (1967)
0.1 to 5.0 ng	2.5 µl	---	2.5 µl	Di HFB	Exley (1967)
5 to 10 ml female plasma	500 µl	5	10 µl	Mono HFB	Clark (1963)
100 mg	100 ml	1 ml	1 ml	Mono HFB	Petruzzi (1967)
10 ml female, 5 ml male plasma	1 ml	0.1 ml	20 µl	Mono HFB	Petruzzi (1967)

[a]All reacted for between 30 and 60 minutes, between 60C and 70C.

Next, I attempted to use a derivatization procedure using 1 ml of benzene, 100 µl of pyridine and 20 µl of heptafluorobutyric anhydride. Again the reactants were heated at 70C for 1 hour. The reaction was stopped by addition of N hydrochloric acid, and the mixture was washed three times with distilled water. The derivatives were finally extracted with chloroform and transferred to a silica gel thin layer plate which was developed in benzene:ethyl acetate (4:1 v/v).

Inspection of the plates under ultra violet light revealed no ultra violet light absorbing areas with an RF similar to that of underivatized testosterone, but two areas were found with RF greater than underivatized testosterone. These were probably the mono- and diheptafluorobutyrate esters, as the replacement of hydroxyl or ketone groups by less polar groups should decrease the polarity of the steroid, hence increasing its RF on silica gel. Androstenedione gave an ultra violet light absorbing area with an RF greater than underivatized androstenedione. This was probably the monoheptafluorobutyrate ester. However the yield was very low and most of the ultra violet light absorbing material migrated with the RF of underivatized androstenedione. Derivatives prepared by the former method (involving only benzene and heptafluorobutyric anhydride) also gave the same ultra violet absorbing areas on silica gel thin layer plates developed as above. The plates still showed some

evidence of breakdown products, various spots of ultra violet light absorbing areas appearing between the major areas mentioned above for the derivatives prepared by the technique involving pyridine.

Gas liquid chromatographic analysis of these samples showed major peaks with retention times similar to those later proven to be the mono- and diheptafluorbutyrate esters of testosterone. Many contaminant peaks were present, their nature was unknown but they were probably breakdown products and various contaminants from glassware, solvents, etc. The best results were obtained for testosterone by increasing the amount of pyridine from 100 µl to 300 µl in the second technique outlined at the beginning of page 52. A very dense monoheptafluorobutyrate area was noted on the thin layer plate, while no free steroid and very little diheptafluorobutyrate were found. Much of the interfering ultra violet material was eliminated by prerinsing all glassware with acetone. The efficiency of the reaction was improved by using only freshly glass distilled benzene, (used for 1 week and then redistilled). Pyridine was also glass distilled and stored over potassium hydroxide. Only fresh heptafluorobutyric anhydride was used (used with 2 to 3 months of preparation). Thus the following technique was evolved for testerone.

h) Monoheptafluorobutyration of Testosterone

To approximately 10 μg of testosterone, 2 or 3 drops absolute ethanol was added and dried to remove water and 1 ml of a solution of heptafluorobutyric anhydride in benzene (0.2 ml of heptafluorobutyric anhydride in 10 ml of benzene) was added to the evaporated eluate. Pyridine (0.1 ml) was originally added but 0.2 or 0.3 ml or more seemed necessary to prevent the formation of the diheptafluorobutyrate. The reaction was performed in glass stoppered tubes for 1 hour at 70-80C. The mixture was then washed with 1 ml of 1 N hydrochloric acid and then twice with 1 ml of distilled water, vortexing each time for at least 15 seconds. The lower acid and water layers were removed with a Pasteur pipette and the tubes were centrifuged in a milk testers centrifuge for five minutes after removal of the bulk of the last wash to get the tubes as dry as possible. The remaining sample was then dried under nitrogen at 60C and taken up in acetone for thin layer chromatography on silica gel benzene:ethyl acetate (4:2 v/v).

The monoheptafluorobutyrate of testosterone appeared as a single peak with a retention time of 13 minutes on a 1% SE-30 column, an oven temperature of 203C, and a carrier gas flow rate of 40 ml/minutes. The introduction of the heptafluorobutyrate group to the testosterone molecule increased its response with the electron capture detector 1,000 fold. Having improved my techniques it was then

possible to prepare the androstenedione monoheptafluorobutyrate by the method of Exley et al. (1967) using equal amounts of benzene and heptafluorobutyric anhydride. The derivative was purified on cellulose thin layer plates or silica gel plates in the systems previously outlined (see page 52) with only minor breakdown. It gave a single peak on a 1% SE-30 column with a retention time of 6.5 minutes, under the same conditions outlined (see page 54) for testosterone. Blanks run through the derivatization techniques gave no response on analysis by gas liquid chromatography. Greatest response to the androgen derivatives was obtained with a pulse interval of 50 microseconds and a gas flow rate of 40 ml/minute or less. Both techniques of thin layer chromatography still caused some breakdown of the derivatives with a resultant loss of steroid.

Challis and Heap (1969) used gel filtration on sephadex LH-20 to purify the heptafluorobutyrate of estrone, 17-β-estradiol and 20-β hydroprogesterone. They showed that the heptafluorobutyrates were stable under purification, probably because they were never exposed to the atmosphere. Sephadex LH-20 was not practical for our use as the androgens were similar in molecular size, shape, and polarity and I could not separate them from their heptafluorobutyrates on a molecular sieve basis in a range of solvent systems. Assuming that exposure on a thin surface was responsible for the breakdown of the

derivatives, various solid phases were experimented to develop a useful separational procedure. Columns (5 x 50 mm) were packed with silica gel GF-254-E (Merk A.G. Darmstadt). A mixture of benzene:ethyl acetate (95:5 v/v) was chosen after many attempts with other solvents, as it seemed to separate the heptafluorobutyrates from the underivatized steroids. The column had a void volume of 0.5 ml and ran at a rate of 0.1 ml per minute. Table 11 summarizes the fractions in which various compounds were eluted from the column.

TABLE 11.--Solvent Fractions Containing Various Steroidal Hormones and their Heptafluorobutyrate Derivatives as Eluted from Silica Gel Microcolumns.[a]

Steroid	Fraction	Steroid	Fraction
Testosterone	2.5 to 3 ml testosterone	MHFB[b]	1 to 2 ml
Androstenedione	3.5 to 4.5 ml androstenedione	MHFB	1.5 to 2.5 ml
Progesterone	2.7 to 3.2 ml progesterone	MHFB	1 to 2 ml

[a]Silica gel column (5 x 50 mm) eluted with benzene:ethyl acetate (95:5 v/v).

[b]Monoheptafluorobutyrate.

Fractions (0.5 ml) were collected and the elution patterns were ascertained by incorporating tritium labelled steroids and by gas liquid chromatography of the fractions. Although there was no breakdown of the derivatives during purification with silica gel and no contaminants were found, the chromatographic separation of the derivatives from free steroids was not good and not always repeatable.

Consequently columns were packed with 60 to 80 mesh florosil (Fisher Scientific Company, New Jersey, USA) to the same dimensions as the silica gel columns. Steroids on these columns were eluted with various solvent systems and Table 12 shows the solvent systems and separations achieved.

The underivatized steroids came off very slowly and had to be flushed off with a more polar solvent such as methanol. Gas liquid chromatographic analysis (electron capture detection) showed that there had been no breakdown of the derivatives during purification on florosil microcolumns. The samples were however heavily contaminated and this was remedied by washing the florosil in methanol and distilling all solvents in glass before use. It was also noted that even tygon tubing was susceptible to extraction by organic solvents giving intolerable contamination.

This system of purification was used only in the proof of our assay because so little free steroid was

TABLE 12.--Fractions Containing Various Steroids and Their Heptafluorobutyrate Derivatives as Eluted from Florosil Microcolumns.[a]

Solvent System	Steroid	Fraction	Steroid	Fraction
Benzene:ethyl acetate (95:5 v/v)	Progesterone	>19 ml	Progesterone MHFB[b]	1 to 8 ml
Benzene:ethyl acetate:water (91:9:0.6 v/v)	Testosterone	>13 ml	Testosterone MHFB	1 to 7 ml
Benzene:ethyl acetate:water (91:9:0.6 v/v)	Androstenedione	>12 ml	Androstenedione MHFB	1 to 6 ml

[a]Florosil microcolumn (5 x 50 mm).

[b]Monoheptafluorobutyrate.

ever detected when the heptafluorobutyrate was purified. The yield of this reaction was probably better than 95%. Mass spectral analysis and the repeatability of estimates as shown in the Results and Discussion section made it unnecessary to purify the heptafluorobutyrate derivatives from unreacted free steroids as long as extreme care was taken in their preparation (see page 74).

The preparation procedures were modified before the assay was used routinely. All extracted and isolated steroids were dried under nitrogen at 60C and vacuum dessicated for a minimum of 12 hours before derivatization to remove all moisture. Androstenedione monoheptafluorobutyrate was prepared as previously outlined and progesterone monoheptafluorobutyrate was prepared in the same way. The original method for preparing testosterone monoheptafluorobutyrate was discarded as pyridine was very difficult and noxious to work with, also traces of diheptafluorobutyrate nearly always formed. I decided to use the same technique as for androstenedione to prepare the diheptafluorobutyrate of testosterone. The final scheme of preparation is shown in the section below on the final methods used.

i) Development of an Extraction and Purification Scheme for Plasma and Testicular Testosterone and Androstenedione

From my experiences with preparing and analyzing standard androgen heptafluorobutyrate ethers by gas liquid

chromatography and electron capture detection, reagents and glassware used for routine laboratory work required scrupulous purification and cleaning to keep contamination at an acceptable level. Reagent requirements and techniques for preparation of glassware are outlined in the final materials and methods for routine assay section below.

My original scheme for the purification of androstenedione and testosterone (see page 39) was obviously unacceptable for gas liquid chromatography with hydrogen flame or electron capture detection. The main problems were poor extraction efficiency and poor removal of organic contaminants.

Surace et al. (1965) presented a scheme for purification of plasma testosterone. Briefly it was as follows. Blood plasma was extracted three times with three volumes of diethyl ether. The pooled extracts were then washed with 10 ml normal sodium hydroxide three times, once with 10 ml of 3% sodium bicarbonate and three times with 10 ml of water. The extract was then dried and redissolved in 10 ml of 70% methanol. This was stored for 12 hours at -18C and centrifuged at the same temperature, and the supernatant fluid was removed. The precipitate of neutral lipids was washed with 5 ml ice cold 70% methanol. The supernatant fluid was washed twice with 5 ml of petroleum ether. The petroleum ether was re-extracted with 10 ml of 70% methanol. The pooled methanolic

extracts were diluted with an equal volume of water and back extracted three times with 20 ml of benzene. The benzene extract was dried and chromatographed by ascending thin layer chromatography using the solvent system benzene:ethyl acetate (60:40 v/v).

The main advantage of this scheme seemed to be the early removal of neutral lipids. It was modified initially as follows. Four to five g of testicular parenchyma were homogenized in saline and extracted with a minimum of two volumes of ether three times. A ratio of ether to homogenate less than 2:1 resulted in emulsions. The ether extracts were dried and taken up in 25 ml of 70% methanol and stored at -15C overnight. The next day they were centrifuged at 1,200 g and -20C for 20 minutes. At first, an attempt was made to dry the 70% methanol supernatant, but as this took so long it was diluted with an equal volume of water and back extracted with 30 ml of benzene three times. This partition often resulted in emulsion, but 30 ml of benzene minimized this problem while volumes less than this nearly always resulted in an emulsion. The benzene was evaporated and the extract applied, relatively free of neutral lipids, to silica gel thin layer plates. The plates were developed using ascending thin layer chromatography. The first solvent system was hexane: ethyl acetate (5:2 v/v) to remove any remaining traces of neutral lipids from the steroids, and the second at an angle of 90° to the first was benzene:ethyl acetate

(60:40 v/v), to separate the androgens from each other and other steroid hormones. Testosterone and androstenedione were eluted, derivatized and analyzed by gas liquid chromatography with electron capture detection. These samples still gave a large solvent front on the gas chromatograph when analyzed. Inorganic contaminants from the silica gel were suspected of contributing to this. Before further use all silica gel was washed three times with methanol. Analysis of extracts of silica gel before and after washing showed that a large contaimination was contributed by silica gel and that this could be removed by methanolic washing.

Several thin layer plates were sprayed with concentrated sulphuric acid to visualize steroids and possible contaminants (see Figure 1). The neutral lipids had migrated further than androstenedione or testosterone but the latter two were not separated well and were contaminated by lipoproteins.

Nienstadt (1967) proposed a technique for defatting steroids. This technique was tested in an attempt to remove every nonsteroidal lipid component of the ether extracts. The dried ether extract was dissolved in 5 ml of 5% octanol in hexane and applied to a 5 x 50 mm florosil microcolumn. Fatty impurities were eluted from the column with 20 ml of 5% octanol, and the octanol was displaced from the active sites of the florosil with 5 ml of 1% ethanol in hexane. The steroids were then eluted with

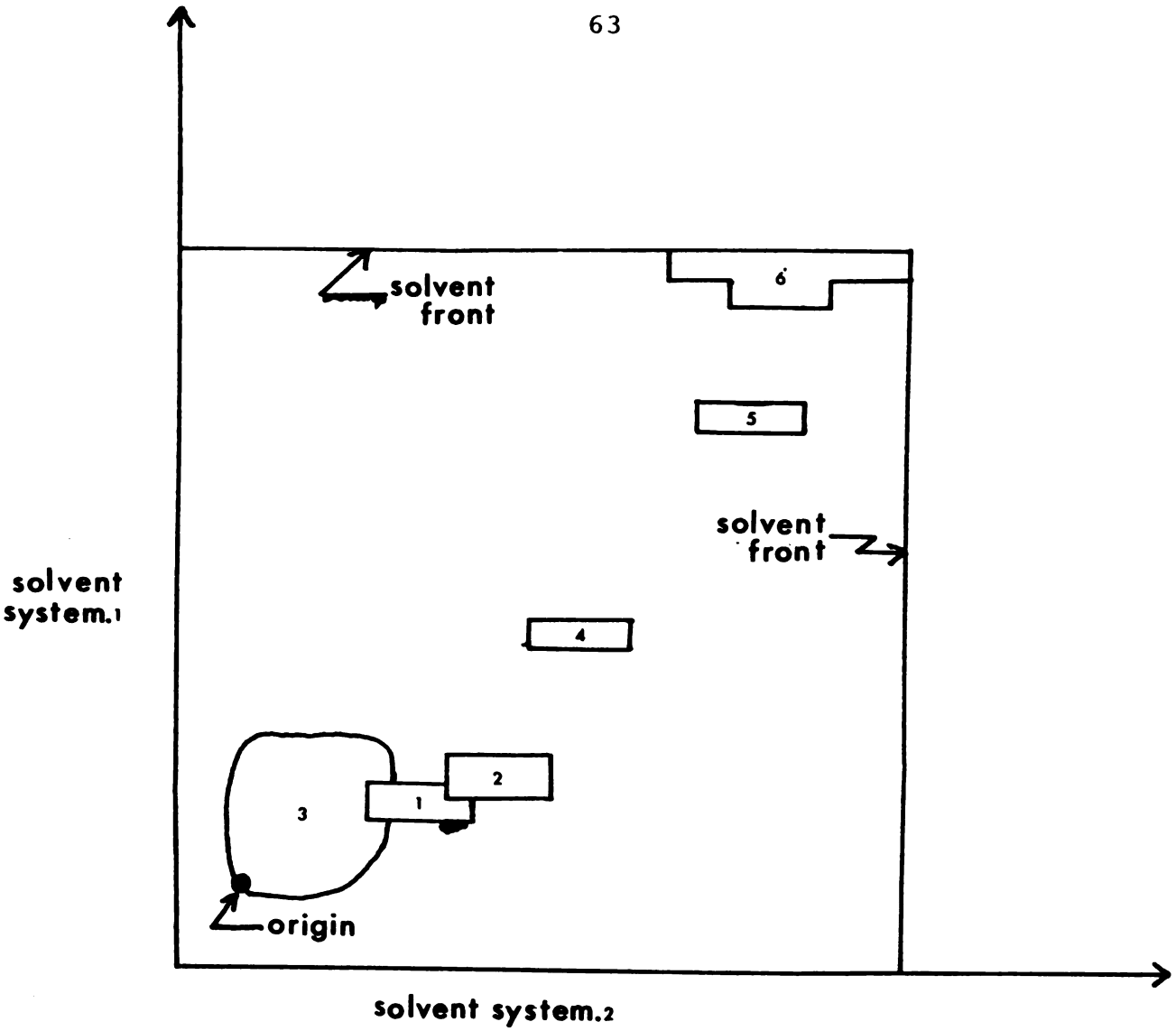

Figure 1.--Identification of Steroidal Constituents[a] of a Bovine Testicular Extract on Thin Layer[b] Chromatography Plates.

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[a]Areas showing colour reaction with concentrated sulphuric acid are (1) testosterone, (2) androstenedione, (3) probably conjugated lipo proteins, (4) dehydroepiandrosterone, (5) cholesterol and (6) esterified cholesterol.

[b]Silica gel GF-254.

[c]Solvent systems are (1) hexane:ethyl acetate (5:2 v/v) and (2) benzene:ethyl acetate (6:4 v/v).

10 ml of ethyl acetate. Based upon addition of isotopic testosterone and androstenedione to the ether extracts and liquid scintillation counting of the various extracts, I estimated that 90% of the androgens were eluted in the ethyl acetate fraction. This fraction was developed on thin layer chromatography (as outlined above), and visualization of the various components was achieved with concentrated sulphuric acid. The quantity of cholesterol was much reduced as was the component that previously overlapped androstenedione and testosterone. However gas liquid chromatographic analysis of the two androgens still revealed some interferring peaks with retneion times similar to the two androgens and a smaller but still unacceptable front.

As this technique was tedious and the resultant androgenic fractions required further purification, I decided to try to improve the methanolic precipitation of lipids and design a new thin layer chromatographic system to purify and separate steroidal hormones. The results of this work comprise the present method used for routine assay of testosterone and androstenedione outlined on page 66 to page 78.

Recovery of androstenedione and testosterone as measured by the recovery of labelled steroids ranged from 35% to 65% (with a few extreme values), with a mean of 50% over twenty samples. Table 13 shows the counts per minute collected from the various fractions and containers

TABLE 13.--Average Distribution of Radioactivity in Various Discarded Fractions and Containers for a Total of Twenty Samples.

Fraction/Container		Counts per minute
Cylinders in which testicular extraction performed		29[a]
Heptane (pooled)		880
Diluted methyl alcohol		960
Fatty Precipitates		2,000
Tubes from which residue transferred to TLC plates		36[a]
Amount of radioactive steroid added to testicular tissue,		
	3H-1,2-testosterone	8,700[a]
	3H-7-androstenedione	6,050
	Total	14,750
	Total losses	3,840 (26%)

[a]Considered to be background.

after they had been used in the extraction and purification system. The fatty precipitate accounted for most of the androgen lost and the heptane and methanol partitions accounted for additional losses. The remaining loss was in the thin layer chromatography step. Not all of the androgens were scraped from the plates, but the loss was not estimated.

Several water blanks were extracted and run through the assay. None produced any response upon analysis by gas liquid chromatography with electron capture detection at a retention time similar to that of either androgen assayed. Steps taken to check the authenticity, accuracy and precision of the assay are discussed in the Results section.

2. Final Materials and Methods for the Routine Quantification of Testicular and Plasma Testosterone and Androstenedione using Gas Liquid Chromatography with Electron Capture Detection

a) Preparation, Storage and Treatment of Materials and Samples

Reagents.--All solvents were nanograde (Mallinckrodt), with the exception of heptane which was spectrophotometric grade (Mallinckrodt), and all were used without further distillation. Acetic acid was analytical grade (Mallinckrodt), and water used was double glass distilled.

Heptafluorobutyric anhydride was obtained from K and K Laboratories (Plainview, New York) and Peninsula

Biochemicals (Florida) in 10-g lots, and was stored in brown glass bottles in a dessicator. A portion of the heptafluorobutyric anhydride was prepared in our laboratory by the method of Clark and Wotiz (1963). Freshly distilled heptafluorobutyric acid (5 ml) was refluxed with a large excess of phosphorus pentoxide (60 g) for 1 to 2 hours. Heptafluorobutyric anhydride was distilled over phosphorus pentoxide and the fraction which boiled between 108 to 110C was collected.

When stored unopened the heptafluorobutyric anhydride was used up to nine months after purchase with no detectable deterioriation, however when the bottle was opened, signs of breakdown of the reagent appeared over a period of five months. Benzene to be used in the reaction to derivitize standards also was stored in the dessicator.

Glassware.--All glassware was washed four times with Alconox detergent, (Alconos Incorporated, New York) and rinsed in tap water five times, in distilled water five times, and in double distilled water five times, and then air dried. Finally, before use, all glassware was rinsed three times in nanograde acetone and air dried.

Steroids.--Standard testosterone (Δ^4-androsten-17 β ol-3one), androstenedione (Δ^4-androsten-3, 17-dione) and progesterone (Δ^4-pregnen-3, 20-dione) were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio) and used without further purification. 3^H-7-progesterone and

3^{H}-7-Δ^{4} androstenedione were purchased from Nuclear Chicago (Des Plaines, Illinois), 3H-1,2-progesterone from New England Nuclear (Boston, Massachusetts), and 3H-1,2-testosterone from Amersham Searle (Des Plaines, Illinois). All isotopes were repurified by thin layer chromatography in the same systems as used in the assay system.

Measurement of Radioactivity.--Radioactivity was measured in a Mark 1 liquid scintillation spectrometer, manufactured by Nuclear Chicago (model number 6860). This was equipped with a teletypewriter print out system (Teletype Corporation, Stokie, Illinois) with facilities to punch counts per minute onto a computer tape. This sytem was used throughout the study and two scintillation fluids were employed. Brays solution, consisting of 4 g PPO, 0.2 g dimethyl POPOP, 60 g napthalene, 20 ml ethylene glycol, 100 ml methanol and 816 ml of paradioxane, in approximately 1 litre of solution, was used when the sample to be counted contained water or could not be dried. It was found to be excellent in minimizing quenching and gave most efficient radioactivity counting under these conditions. The second scintillation fluid was designed especially for efficient counting of steroid isotopes and consisted of 7.5 g PPO, 75 g POPOP, 120 g napthalene, 500 ml xylene and 500 ml paradioxane. All samples were counted in glass vials (various sources), generally 10 ml of Brays solution or 5 ml of steroid scintillation fluid were used.

Gas Liquid Chromatography.--A Hewlett Packard Model 402 gas chromatograph was used throughout this study. The instrument was a dual column type, equipped with two hydrogen flame detectors. A tritium foil electron capture detector was mounted on one column. This column had two exit ports allowing the use of either the hydrogen flame or the electron capture detector.

Experimental Animals, Collection and Storage of Samples.--a)Holstein Bulls. Sixty-five Holstein Friesian bulls were used in this study which was part of a larger study conducted by K. L. Macmillan to investigate endocrine and reproductive development of the Holstein bull from birth through puberty. Five bulls were slaughtered at birth followed by five per month until 12 months of age. Blood was collected at slaughter in heparinized tubes and centrifuged at 1800 g. The plasma was frozen and stored at -15C until assayed. Testes were removed at slaughter, frozen whole and stored at -15C until assayed.

b) Hereford Bulls. Thirty-four Hereford bulls, part of a long-term genetic study, were available to be bled at 11, 12 and 13 months of age. Twenty-nine of the animals were slaughtered at 13 months of age, one week after the last bleeding. All Hereford blood samples were allowed to form a clot and then were centrifuged. The serum was frozen and stored at -15C until assayed. Testes were removed at slaughter, frozen whole and stored at -15C

until assayed. Several other endocrine and reproductive criteria were measured.

b) Methodology.

Serum.--To a 100 ml glass stoppered cylinder, approximately 4,000 cpms each of tritium labelled testosterone and androstenedione were added (to account for extraction losses) with a Lambda pipette. Serum was then added followed by ether at a ratio of two volumes of ether to one of serum. The contents of the cylinder then were shaken for two minutes and the ether extract removed with a 10 ml pipette into a 40 ml polypropylene centrifuge tube. The extraction was repeated twice more and the extract then dried thoroughly under nitrogen. The tube walls were washed down three times with ether (to concentrate the extract to the base of the tube), and again thoroughly dried.

Five ml of 70% methanol was added to the extract and gently vortexed, a further 20 ml of 70% methanol were then added by running the methanol down the walls while rotating the tube. The tubes were capped with parafilm and stored overnight at -15C. This step served to precipitate most of the neutral lipids in the extract. The tubes had to remain in the freezer for at least 12 hours and no longer than 24 hours to achieve an effective lipid precipitation. After this period the tubes were centrifuged at approximately 6,000 g for 0.5 hour at -15C. Care was taken when transferring the tubes from the freezer to

the centrifuge to ensure that they did not warm. Following centrifugation, the 70% methanol was immediately decanted into a 100 ml glass stoppered cylinder.

Ten ml of heptane was added to the 70% methanol and the cylinders were gently everted 5 to 10 times. The heptane (upper layer) was removed with a 10 ml pipette and discarded. The operation was repeated. This partition removed most of the remaining neutral lipid material.

Following the heptane partition the 70% methanol was further diluted with 40 ml of water, to render any steroids present fairly insoluble. This solution was then back extracted with methylene dichloride to remove steroidal material as follows. Twenty ml of methylene dichloride were added and the cylinders were everted five to ten times as before. The methylene dichloride (lower layer) was removed with a 10 ml pipette and transferred to a 40 ml conical tube. This extract was then dried under nitrogen. Care was taken not to remove any of the diluted methanol as this would greatly hinder drying at this stage. The tube walls were washed down three times with methylene dichloride:benzene (1:1 v/v) to concentrate the residue to the tip of the tube, and the dried extract was taken up in 0.5 ml of the same solvent mixture.

The extract was transferred to a silica gel thin layer plate with a Pasteur pipette, and the tube was rinsed twice in the same solvent. Washings also were transferred to the thin layer plate. The sample was

spotted in the lower left hand corner of the plate, and standard androstenedione and testosterone were spotted in the lower right hand corner. The plate was developed by ascending chromatography in the solvent system ether: dimethyl formamide:acetic acid (99:1:1 v/v). This system was designed to drive all remaining neutral lipids above and away from the androgens I was concerned with.

The plates were dried and turned 90° so that the androgen sample spots were again on its lower edge. Once again standard steroids were applied away from the sample androgen areas and the plate was developed in chloroform: methanol (99:1 v/v). This system served to separate the two androgens concerned, and to separate them from other major neutral steroids (see Figure 2).

The acetic acid in the first solvent system tended to alter the developmental pattern of the lower two edges of the plate. Thus a 2-inch corridor to the right of the plate in the second system was isolated by scoring a line down the plate with a Pasteur pipette. This gave a flat solvent front in the second system as compared to one sloping up to the right on plates not scored as above. The sloping solvent front was due to faster migration of the solvents on the right hand side of the plate.

The standard androgens were visualized on the plates under ultra violet light. A line was scored from the front edge of each standard spot across the plate, parallel with the appropriate edge. An area about 3 cm^2 was scraped below

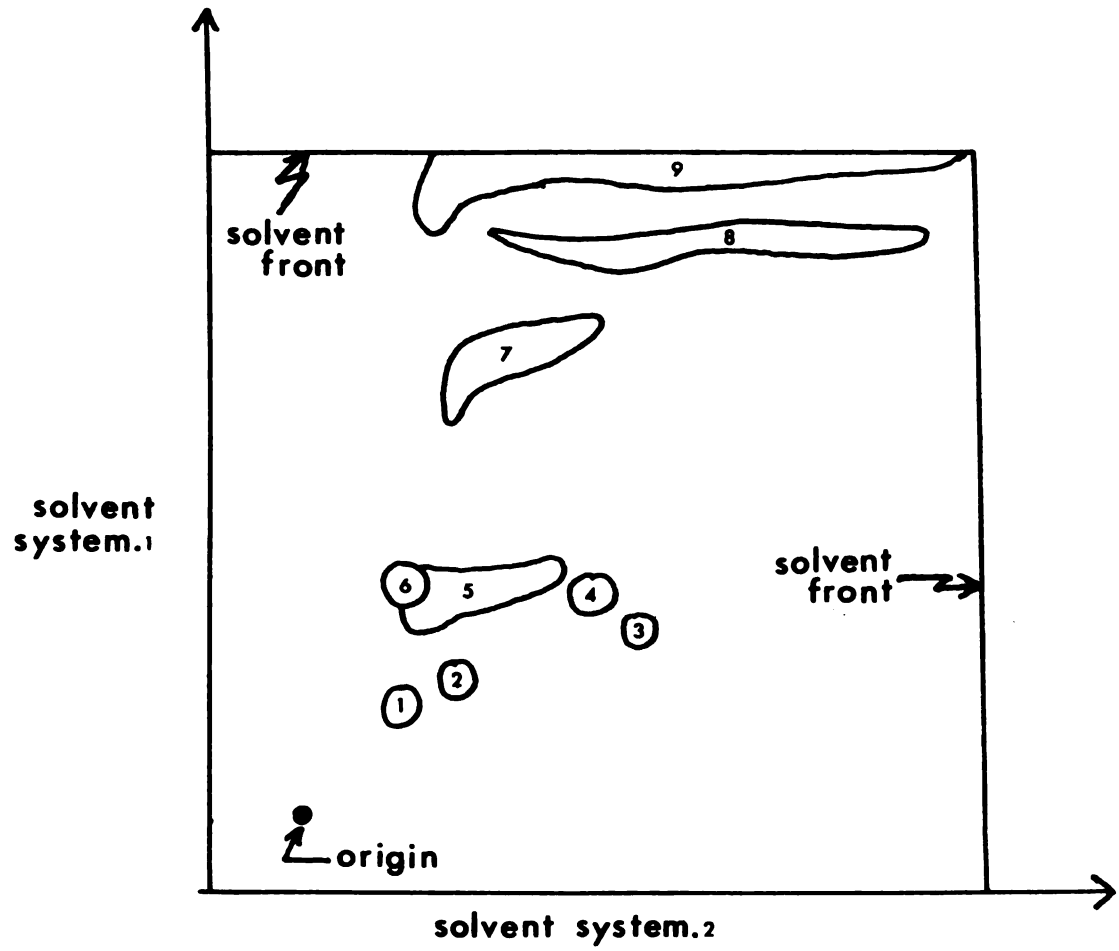

Figure 2.--Positions of Steroids and Various Contaminants on Silica Gel Thin Layer Plates after Development on Ether:dimethyl formamide:acetic acid (99:1:1 v/v) (1) and Chloroform:methanol (99:1 v/v) (2), as Revealed by Ultra Violet Light and Concentrated Sulphuric Acid.

Areas showing colour reaction with concentrated sulphuric acid are (1) 20 hydroxyl progesterone, (2) testosterone, (3) androstenedione, (4) progesterone, (5) lipoprotein or phospholipid, (6) dehydroepiandrosterone, (7) cholesterol, (8) free fatty acids, (9) cholesterol palmitate.

each line at the point of intersection. It was necessary to scrape well behind the standard areas because the sample androgens nearly always run slower than the standard androgens. Care was taken to leave at least 0.25 inch of silica gel between the two areas scraped to ensure no overlap.

The silica gel bearing the sample androgens was placed in a 12 ml conical centrifuge tube and eluted with 2 ml of ethyl acetate three times. Each tube was vortexed for 15 seconds and centrifuged at 298 g for 1 to 2 minutes. The extract was poured off into either a 12 or 15 ml conical centrifuge tube with a ground glass stopper.

The pooled extracts were dried and the androgens were carefully concentrated to the tip of the tube with two to four washes of acetone. The extracts at this stage had to be thoroughly dried under nitrogen. If storage of the samples was necessary it was done by simply storing the silica gel scrapings in ethyl acetate in a sealed tube.

The dried extracts were vacuum dessicated overnight. When the vacuum was broken, nitrogen rather than damp air was allowed to fill the dessicator. To each tube of either androstenedione, testosterone or progesterone, 50 µl of benzene was added, followed by 50 µl of heptafluorobutyric anhydride. The tubes were then stoppered and heated in a water bath at 75C for 30 minutes. The reaction coupled a 7-fluorine chain onto the steroid nucleus.

The steroids were concentrated to the tip of the tube and taken up in hexane. The quantity of hexane depended on the suspected concentration of steroid; 500 µl was usually used for most serum and testicular extracts. Between 1 and 4 µl of this solution was injected into the gas liquid chromatograph column, again the amount depended on the estimated concentration of steroid.

A 3-foot, 1 to 5% OV-1 column was used with an oven temperature between 190 and 198C. The flash heater and the electron capture detector were set between 200 and 210C. A pulsed DC current was applied to the detector with a pulse interval of 50 microseconds. The electrometer was operated at range 10, and attenuations between 4 and 128; again this depended on the concentration of steroid suspected in the sample. Argon-methane carrier gas was used with no purge gas. The carrier gas was supplied at 40 p/si and between 55 and 65 ml per minute flow rate. Care was taken not to apply to the column amounts of steroid exceeding 1 µg. If this amount was to be exceeded, a purge gas or hydrogen flame detector had to be used. With such large quantities of steroid, if no purge gas was used with the electron capture detector, severe contamination of the cell foil could result. Chart recorder speed was set at 0.25 inches per minute.

With each batch of samples derivatized duplicate 10 µg amounts of standard testosterone, androstenedione and progesterone were derivatized. Before each set of

of samples were chromatographed, 10 ng of each duplicate of testosterone and androstenedione were chromatographed with 10 ng of progesterone as an internal standard. This established the ratios between the internal standard and the androgens which were used in the quantification formula described later. It also established retention times for the steroids in question. The latter was important as retention times vary from day to day. It was also necessary to have the data to separate the androgen and internal standard peaks from interfering peaks of contaminants.

The carrier gas flow rate and oven temperature controlled the retention times on a particular column, the amount of steroid injected however may have given minor fluctuations. At a carrier gas flow rate of 60 ml/min (rotameter reading of 2.85 at 38.75 p/si), an oven temperature of 193C and flash heater 199C, the retention time of androstenedione and testosterone was 6.1 minutes; and of progesterone was 10.2 minutes. These settings gave a reasonable compromise between sensitivity and an acceptable retention time. Maximum response at pulse 50 was gained at a rotameter reading of 2.3 and an oven temperature of 194C, but the reduced carrier gas flow rate gave an intolerably long retention time for routine use.

Each sample was injected with a known quantity of internal standard. The area under each peak was measured by the method of height times width at half height; the technique although not perfect gave the most accurate

assessment of the area as compared to such methods as triangulation or planimeter measurements.

An aliquot was removed for liquid scintillation counting to calculate extraction losses, usually before gas liquid chromatographic analysis. But if only a small quantity was removed for the latter analysis, the remaining sample was eluted into a liquid scintillation vial with three washings of acetone.

All peak areas were standardized to a 1 µl injection at a sensitivity of 10 x 64 and quantification calculated from the formula on page 78.

Testicular Homogenates.--A section of testicular parenchyma was weighed, diced, and placed in a 7 ml glass homogenizer. Approximately 4,000 cpms each of tritium labelled testosterone and androstenedione were added to this with a Lambda pipette to account for extraction losses. Five ml of saline also was added, and the material was homogenized. Up to 3 g of tissue could be homogenized in this way. The homogenate was poured into a 100 ml glass stoppered cylinder and the homogenizer was thoroughly rinsed three times with ether. The rinsings were poured into the cylinder. Ether was added to the homogenate in the cylinder at a rate of three volumes of ether to one of tissue, and sometimes at a ratio of 4:1 for very fresh tissue samples. The contents of the cylinder were shaken and extractions performed as for the serum samples. The testicular homogenate extract was treated exactly as the serum extract from this point on, however the techniques

outlined to remove neutral lipids had to be rigidly followed as testicular parenchyma, especially fresh samples, apparently contained more lipid.

Quantification Formula.--Quantity (in μg) of testosterone injected into the gas liquid chromatograph column was calculated from the following formula:

$$\text{Testosterone} = \left(\frac{Xs}{Xp}\right)\left(\frac{CS}{Cx}\right)\left[\frac{(Tx)(288.4)}{(Ts)(484)}\right] \quad (0.01), \text{ where}$$

Xs = cpm of testosterone (^3H) initially added to plasma,

Xp = cpm of ^3H) in the aliquot removed prior to GLC,

Cs = area (cm^2) of 20 ng of internal standard,

Cx = area (cm^2) of internal standard from sample,

Ts = area (cm^2) of 0.01 μg testosterone monoheptafluorobutyrate,

Tx = area (cm^2) of testosterone monoheptafluorobutyrate from the sample,

288.4 = MW of testosterone, and

484 = MW of testosterone monoheptafluorobutyrate.

The value obtained from the formula above has to be corrected for dilution and for the amount of tissue used to express the result on a per g or per ml basis.

RESULTS

1. Assay for Testicular and Plasma Levels of Testosterone and Androstenedione

a) Introduction

Success of the quantitative determination of steroids in biological samples by gas liquid chromatography with electron capture detection depends on scrupulous attention to detail. Although the technician or researcher with average skill can successfully use this technique in routine analysis, if he is not willing to adhere rigidly to the necessary standards of cleanliness and accuracy then it is futile to even commence work. Purification of the desired steroids from other steroids as well as organic, and inorganic contaminants is important. The success of the assay also depends on the precision at each stage of the procedure, the stability of the derivative, and the linear response of the detector.

b) Linearity of the Detector Response

The response of the electron capture detector to testosterone diheptafluorobutyrate and androstenedione monoheptafluorobutyrate was found to be linear in the

range of quantities that were measured. For testosterone it was 0.001 to 10 ng, and for androstenedione it was 0.003 to 10 ng.

c) Sensitivity

The quantities of steroid that gave a peak of sufficient area to accurately quantify were 0.001 ng for testosterone diheptafluorobutyrate and 0.003 ng for androstenedione monoheptafluorobutyrate. Levels less than this could be detected but not quantified with acceptable accuracy. Biological samples giving a response in this category were recorded as having "trace" levels of the particular hormone.

Most biological samples were diluted in 500 µl of solvent. If 1 µl of this solution was introduced onto the gas liquid chromatographic columns, then the quantification limits were 0.1 ng testosterone and 0.3 ng androstenedione per ml of blood plasma. Larger quantities could be injected and the samples could feasibly be concentrated to a smaller volume but the solvent front and background noise became severe problems.

Figures 3 and 4 show representative traces of testosterone diheptafluorobutyrate and androstenedione monoheptafluorobutyrate from plasma and testicular extracts with progesterone monoheptafluorobutyrate as an internal standard.

Figure 3.--Gas Liquid Chromatographic Tracing of Testosterone[a] from 10 ml of Plasma.

Testosterone peak equivalent to approximately 16 pg. Conditions were oven temperature 195C, flash evaporator and electron capture detector 200C, 3-foot, 1% OV-1 column, argon/methane 95:5 carrier gas, supplied at 40 p/si and 60 ml/minute, sensitivity attenuation 10 x 4 and a pulse interval of 50 microseconds.

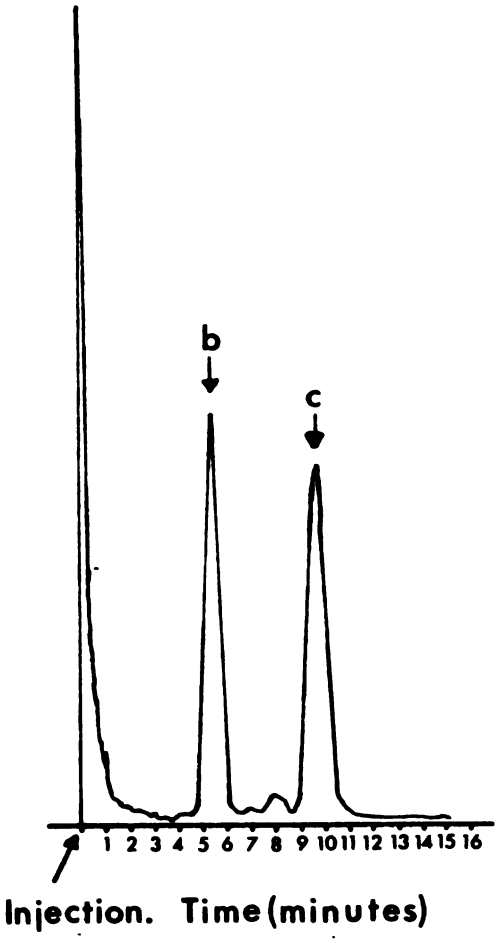

Figure 4.--Gas Liquid Chromatographic Tracing of Testosterone.[b] From 4 g of Testis with 10 ng of a Progesterone Internal Standard.[c]

Testosterone peak equivalent to approximately 470 pg. Conditions were as for Figure 3 except oven temperature, flash evaporator, and electron capture detector temperature were elevated 5C, also attenuation was 10 x 64.

d) Stability of Heptafluorobutyrate Derivative

Purification of testosterone diheptafluorobutyrate, androstenedione monoheptafluorobutyrate and progesterone monoheptafluorobutyrate on florosil microcolumns, as discussed in the Materials and Methods section (page 35) revealed no significant breakdown of the derivative and better than 95% derivative formation. These observations were based on the fact that isotopes added to the three steroids yielded virtually no activity in the fractions from the microcolumns that should contain the free steroids if they were present. The derivatives were often stored in hexane at 5C for several weeks without any evidence of degeneration as shown by the size and appearance of peak traces.

e) Recoveries

These are dealt with in the Materials and Methods section (page 35 and Table 13).

f) Specificity

Specificity of determination of androgens by gas liquid chromatography depends on purifying the steroids of interest and thus removing all organic and inorganic contaminants plus all other steroids that could interfere with the response of the desired steroids.

During the development of the procedure, all glass-ware and reagents used were rinsed or extracted with organic solvents and the residues were analyzed by gas

liquid chromatography with electron capture detection after reaction with derivatized reagents. These extraction procedures were repeated after attempts had been made to clean and purify glassware and reagents by the techniques described in the methodology section, and none of the rinsings or extractions gave a response when analyzed except silica gel, which gave a large front but no peaks and with retention times similar to either androgens or the progesterone derivative.

During the routine analysis of biological samples no interfering peaks were observed and it was assumed that all other steroids that could interfere had been eliminated by the purification procedure. The peaks from the biological samples believed to be androstenedione monoheptafluorobutyrate or testosterone diheptafluorobutyrate gave identical retention times as standard preparations of the steroids on several columns with different liquid phases (see Materials and Methods section). Analysis of these standard steroids by gas liquid chromatography mass spectrometry revealed typical scans for androstenedione monoheptafluorobutyrate, testosterone diheptafluorobutyrate and progesterone monoheptafluorobutyrate. Finally water blanks were analyzed by the routine procedure, none of these gave any response to electron capture detection.

g) Accuracy

The precision of the complete extraction and analysis method was estimated by replicate analysis of known amounts of testosterone or androstenedione (10 ng) added to 5 ml of saline. The range of values obtained is shown in Table 14. The average estimate and standard error of this mean for testosterone was 10.66±0.34 ng and for androstenedione was 11.48±0.76 ng.

h) Precision

Replicate analyses were performed on testis samples, these values are shown in Tables 15 and 16. The standard deviation for duplicates was 0.17. Thus, the precision of measurement of testosterone and androstenedione from biological samples was reasonable. One source of error in this estimate may have been the varying mass of hormone present as the radioactive tracer. Radioactively labelled steroids of high specific activity were used; 1.050 Curies/mM for ^{3}H-7-androstenedione and 44.1 curies/mM for ^{3}H-1,2-testosterone. After purification few biological samples exceeded a combined amount of 6,000 cpms of ^{3}H-1,3-testosterone and ^{3}H-7-androstenedione. Isotopes (5,000 cpm) of each hormone (10,000 cpm combined) derivatized, diluted in 500 µl of hexane, and 1 µl injected onto the gas liquid chromatographic column produced no response with electron capture detection. Most samples contained less than 2,000 cpm ^{3}H-7-androstenedione and 2,000 cpm ^{3}H-1,2-testosterone. This was equivalent to

TABLE 14.--Replicate Analysis of Standard Testosterone and Androstenedione added to Saline.

Replicate Determinations	Testosterone	Androstenedione
	----ngs-----	------ngs------
Amount added	10	10
Replicate Determinations, 1	12.67	14.68
2	10.10	13.32
3	9.19	10.97
4	10.84	14.14
5	11.52	10.94
6	8.01	8.71
7	9.49	12.39
8	11.43	9.43
9	13.24	8.74
10	10.83	---
11	10.57	---
12	10.74	---
13	9.62	---
14	10.80	---
15	10.91	---
Mean plus standard error	$\bar{X} \pm e_{\bar{X}}$	

TABLE 15.--Duplicate Analysis of Bull Testes for Testerone and Androstenedione.

	Testosterone		Androstenedione	
Bull	1	2	1	2
	(µg/gm)			
127	2.072	1.462	0.108	0.133
132	0.205	1.231	0.010	0.009
134	0.255	0.197	0.015	0.028
121	1.343	1.316	0.035	0.058
124	1.854	1.829	5.172	4.539
109	0.212	0.176	0.030	0.025

TABLE 16.--Analysis of Variance of the Duplicate Androgen Analysis in Table 15.

Source	df	ss	ms
Testosterone			
Among bulls	5	6.44	--
Within bulls	6	0.19	0.03[a]
Total	11	6.63	--
Androstenedione			
Among bulls	5	38.56	--
Within bulls	6	0.20	0.03[a]
Total	11	38.76	--

[a]Standard deviation between duplicates 0.17 for both steroids.

0.0008 ng of androstenedione introduced to the gas liquid chromatograph system and 0.000023 ng of testosterone, assuming dilution in 500 µl and a 1 µl injection. The limit for reasonably accurate quantification was 0.003 ng for androstenedione and 0.001 ng for testosterone. It was obvious that the mass of testosterone would give no interference in the quantification of ^{3}H-1,2-testosterone. Some question existed for ^{3}H-7-androstenedione. Calculations of mass made from the specific activity given by the supplier suggested it should give a GLC response at the levels that were used. However repeated analysis of up to 5,000 cpm of the tracer gave no response. Levels much higher (20,000 cpm) gave a small response equivalent to those classified as "trace" among biological samples. Consequently, no correction was made on biological samples for the mass of radioactive tracers. From the specificity and accuracy of the assay for androstenedione, and the fact that ^{3}H-7-androstenedione gave no response upon analysis at the levels used, it was assumed that the specific activity of the Tracer was greater than indicated by the supplier. The latter could be explained by the fact that the radioactively labelled steroids were constantly repurified by thin layer chromatography, before and during routine use.

2. Holstein Bulls

a) Testicular Testosterone and Androstenedione

Testosterone and androstenedione concentrations in the testes of the Holstein bulls are presented as monthly averages with standard errors in Table 17. These data are presented only from 4 to 12 months of age because the testes of the younger bulls were lost previously.

The values among bulls of similar age were very variable (Table 17, Appendix Table 1). Analysis of variance for testicular testosterone over the period of 4 to 12 months of age revealed significant differences at the 10% level of probability. Analysis of the testicular testosterone data by orthogonal contrasts showed (1) an increase from 4 to 5 months ($P<0.05$), (2) a depression from 4 and 5 months to 6 and 7 months ($P<0.01$), (3) a general increase from 6 to 11 months and (4) a decrease at 12 months ($P<0.010$). In other words, testicular concentration of testosterone was high at 4 months of age, plunged to a low level at 6 months and increased to 11 months of age. After 11 months, it fell to the lowest level at 12 months of age.

An analysis of variance for testicular androstenedione concentrations over the period 4 to 12 months of age gave an F value only approaching significance ($P<0.10$). Orthogonal contrasts revealed a significant reduction in testicular concentrations of androstenedione from months

TABLE 17.--Average Testicular and Plasma Concentration of Testosterone and Androstenedione in Holstein Bulls from Birth to 1 Year of Age.

Age	Testis		Plasma	
	Testosterone	Androstenedione	Testosterone	Androstenedione
(Months)	----------(ng/g)----------		----------(ng/ml)----------	
Birth			0.00	0.00
1			0.72±0.64	10.00±9.53
2			0.39±0.18	0.31±0.19
3			0.59±0.19	1.85±1.72
4	1,083±341[a]	1,666±1,173	0.76±0.28	0.00
5	3,157±1,860	732±274	2.98±1.74	7.12±7.12
6	321±48	175±131	0.33±0.11	0.00
7	473±205	31±21	2.48±1.34	0.00
8	1,597±473	33±5	4.82±2.02	0.01±0.00
9	729±341	45±14	2.40±1.50	0.01±0.00
10	1,087±493	81±36	2.44±1.94	0.00
11	1,856±490	222±81	3.73±1.80	0.00
12	288±38	32±3	0.50±0.16	1.83±1.63

[a]Mean ± standard error.

4 to 7 to the last 5 months ($P<0.05$). Concentrations at months 6 and 7 were lower than at months 4 and 5 ($P<0.025$), and the difference in testicular androstenedione between months 4 and 5 approached significance ($P<0.10$). The concentration of testicular androstenedione fell sharply and continuously to 7 months of age, remained stable at a low level until 9 months of age and then appeared to rise until 11 months, only to fall back to a low level at 12 months of age.

It should be pointed out that the orthogonal contrasts for analyses of testicular androgens were set up after the data were collected because the original contrasts were designed to include observations at birth, 1, 2 and 3 months of age. This restriction does not apply to any of the other data in this thesis.

Table 18 shows the change of the ratio of testicular testosterone to androstenedione with age from 4 to 12 months of age. At 4 months of age there appeared to be more androstenedione than testosterone, but the ratio increased until 8 months of age when the ratio of testosterone to androstenedione was 48:1. The data suggested a decrease in the ratio of 12 months of age (9:1), due to a fall in testosterone concentration between 8 and 9 and between 11 and 12 months, and the gradual increase of androstenedione concentration to 11 months of age.

TABLE 18,--Testosterone Androstenedione Ratio from Birth to 1 Year of Age in the Testes and Plasma of Holstein Bulls.

Age	Testosterone/Androstenedione	
	Testis	Plasma
Birth	Lost	ND[a]
1	Lost	0.072
2	Lost	1.26
3	Lost	0.32
4	0.65	ND[b]
5	4.31	0.41
6	1.83	ND[b]
7	14.95	ND[b]
8	48.29	482.00
9	15.87	240.00
10	13.34	ND[b]
11	8.34	ND[b]
12	8.76	0.27

[a]Testosterone and androstenedione were not detected.

[b]Androstenedione was not detected.

b) Plasma Testosterone and Androstenedione

Table 17 lists the monthly means and standard errors for testosterone and androstenedione concentrations in the plasma. The plasma concentrations of testosterone and androstenedione were highly variable among bulls within age.

Analysis of variance of testosterone concentrations in the plasma revealed that variations from birth to 12 months of age were significant at the 20% level of probability. Orthogonal contrasts revealed one significant contrast ($P<0.01$); plasma concentrations of testosterone were significantly higher in the last 6 months than during the first 7 months. Despite the large fluctuations, there seemed to be a gradual increase of testosterone concentration in the plasma from birth to 11 months of age followed by a precipitous drop at 12 months of age.

Plasma concentrations of androstenedione were extremely variable and there were no significant differences between months as revealed by analysis of variance or orthogonal contrasts. Despite enormous fluctuations during the first 6 months, no androstenedione was detected in the blood again until 12 months of age. The testosterone to androstenedione plasma concentration was extremely variable (Table 18).

3. Hereford Bulls

Table 19 shows the serum concentrations of testosterone and androstenedione at 11, 12, and 13 months of age, with values from slaughter blood (13 months of age plus 2 weeks). Also presented are the testicular concentrations of testosterone and androstenedione from testes removed at slaughter. Serum concentrations of testosterone appeared to decline from 11 months to 12 and 13 months, but this difference was not significant. Concentrations of plasma androstenedione appeared to drop over the 2-month period, but again an analysis of variance revealed no significant difference among these values. The slaughter values for both parameters were higher than the value for the 13 month bleeding, but analysis of variance showed that only the difference between testosterone concentration at slaughter and the value obtained from the 13 month bleeding approached significance ($P<0.10$). This suggested a general release of steroids from the testis caused by the stresses associated with confinement and slaughter.

TABLE 19.--Average Plasma Concentrations of Testosterone and Androstenedione in Hereford Bulls of 11, 12, and 13 Months of Age, with Serum and Testicular Levels of Testosterone and Androstenedione at Slaughter.[a]

Age	Testis		Serum	
	Testosterone	Androstenedione	Testosterone	Androstenedione
(months)	----------(µg/gm)----------		----------(ng/ml)----------	
11			5.54 ± 0.78	4.27 ± 1.69
12			4.26 ± 0.74	3.69 ± 1.46
13			4.34 ± 0.61	1.85 ± 0.69
13[a]	0.887 ± 0.113	0.036 ± 0.005	6.70 ± 1.10	3.54 ± 1.71

[a]Values recorded on slaughter samples taken 2 weeks after the last bleeding.

GENERAL DISCUSSION

The changing ratio of testosterone to androstenedione in the testes with age of the Holstein bulls was in good agreement with other investigators and in several species. Lindner (1959) and Lindner and Mann (1960) observed that the ratio of testosterone to androstenedione in the testis changed from 1:1 in a male calf at 4 months of age to 10:1 at 9 months of age. Skinner (1968) showed that this ratio was 1:1 in the ram testis at birth. He observed an increase of testicular testosterone with age, but found that levels were highly variable as was apparent in the present study. He found that androstenedione content fell until 56 days of age and then rose slightly to mature levels. This seemed to generally parallel the findings of the present study in the testes of Holstein bulls.

Studies in the guinea pig (Becker and Snipes, 1968), in the Rhesus monkey (Resko, 1967), and in the rat (Strickland, 1970) also substantiated the findings of this study. The Hereford bulls slaughtered at 13 months of age had a ratio of testicular testosterone:androstenedione

of 12:1 but slightly higher levels than found in the Holstein bulls at 12 months of age.

This is the first report of androgen levels in bull blood, but results were not clear. The Holstein bulls generally showed an increasing plasma concentration of testosterone to 11 months, and then a decline to 12 months, but the rise to 11 months was by no means smooth. There was no smooth decline of plasma androstenedione concentration from the early months to the later ones. Rather, two or three large peaks of androstenedione occurred until 5 months of age with values much higher than any testosterone values, and in most cases due to a large androstenedione value calculated for one bull. After 5 months however no androstenedione was detected in the blood until 12 months of age when a large but non-significant rise occurred.

Resko et al. (1968) demonstrated a general rise in androstenedione plasma concentrations with age until a mature level was reached in the male rat. However, Resko (1970) in the guinea pig seemed to show a picture somewhat similar to the present study with rather eratic androstenedione plasma concentrations declining to 60 days and then increasing slightly to a mature level at 120 days.

The plasma androstenedione and testosterone concentrations for the 3-monthly bleedings of the Hereford bulls (11, 12 and 13 months) seemed to be higher than equivalent

Holstein values, as were the testicular values. This was probably a breed difference. The Hereford bull plasma androstenedione did not show a rise between 11 and 12 months, but rather a steady decline to 13 months establishing approximately a 2:1 ratio of testosterone to androstenedione, in agreement with other investigators. The Hereford bull androgen levels could be higher because they were less mature than the Holstein bulls; it is known that beef bulls mature slower than Holstein bulls. Aside from the elevation at slaughter, the plasma testosterone concentrations suggested a leveling off at what could be a mature adult concentration.

Correlations were analyzed between various androgen values and between androgen levels and other endocrinological and physiological criteria concerned with reproductive development in the bull (see Tables 20, 21, 22 and 23). The Holstein bull androgen data collected in this study complemented the larger study conducted by Macmillan (1967). This study was entitled "Endocrine and Reproductive Development of the Holstein Bull from Birth through Puberty." Criteria measured by Macmillan (1967) that are pertinent to the present study are as follows: (1) gonadal and extra gonadal sperm numbers (Macmillan and Hafs, 1968a), (2) testicular DNA, seminal vesicular fructose and citric acid (Macmillan and Hafs, 1969); and (3) pituitary FSH and plasma LH (Macmillan and Hafs, 1968b). Plasma LH was estimated by the method of acetone precipitation and

TABLE 20.--Various Overall Correlation Coefficients (r) for Holstein Reproductive and Endocrinological Data from Birth to 12 Months of Age.

	r values			
	Plasma Testosterone	Testis Testosterone	Plasma Androstenedione	Testis Androstenedione
Plasma testosterone				
Testis testosterone	0.47[b]			
Plasma Androstenedione	0.24[e]	-0.11		
Testis androstenedione	-0.13	0.16	0.07	
Plasma LH concentration (OAD)	0.17	0.18	-0.01	-0.29
Plasma LH content (OAD)	0.63[c]	0.38[b]	-0.03	-0.17
Plasma LH content (RIA)	0.33	0.54	-0.07	0.30[e]
Plasma LH concentration (RIA)	0.06			
Pituitary FSH concentration	0.14			
Pituitary FSH content	0.00	0.15	-0.16	0.11
Seminal vesicular fructose total	0.44[a]			
Seminal vesicular fructose concentration	0.46[a]	0.01	-0.13	-0.35[d]
Testicular DNA concentration			0.24[e]	0.39[c]
Testicular citric acid concentration	0.28[d]	-0.14	-0.15	-0.32[d]
Gonadal sperm total	0.13			
Gonadal sperm concentration	0.13	0.18	0.39[d]	0.10
Epididymal sperm total	-0.18	0.01	0.38[d]	0.21

[a] iP<0.001
[b] jP<0.01
[c] kP<0.02
[d] lP<0.05
[e] mP<0.10

RIA = quantified by Radioimmunoassay.
OAD = quantified by ovarian ascorbic acid depletion.

TABLE 21.--Various Within Month Correlation Coefficients (r) for Holstein Reproductive and Endocrinological Data from Birth to 12 Months of Age.

[a]Plasma LH Content

[b]Plasma LH Content

[c]Plasma testosterone concentration

[d]Testis testosterone concentration

[e]Plasma androstenedione concentration

[f]Testis androstenedione concentration

[g]Plasma testosterone content

[h]Testis testosterone content

[i]Plasma androstenedione content

[j]Testis androstenedione content

[k]Seminal vesicular fructose concentration

[l]Testis sperm concentration

[m]Seminal vesicular fructose concentration

[n]Testis sperm concentration[f]

[o]Testis DNA concentration

[p]Testis sperm concentration

[q]Testis DNA concentration

[r]Testis sperm concentration

	Correlations												
Age	r_{ab}	r_{ac}	r_{bc}	r_{bd}	r_{cd}	r_{ce}	r_{cf}	r_{de}	r_{df}	r_{ef}	r_{eg}	r_{fg}	r_{fh}
0													
1	0.33		0.33			0.65				0.40			
2	0.92[t]		-0.16			0.90[t]				-0.67[w]			
3	0.58		-0.03			0.99[t]				-0.30			
4	-0.53	0.63		0.87[v]	-0.30	0.79[v]		-0.41		-0.15		0.67	
5	-0.06	-0.45	-0.11	-0.67	-0.46	0.29		-0.15		0.85[u]		0.58	
6	-0.15	0.52		-0.70[w]	-0.69	0.31		0.37				0.26	
7	0.43	0.58	0.91[u]		0.96[s]	0.14	0.91[v]	0.17	-0.19	-0.89[t]	0.84[w]	-0.71	
8	-0.18	0.16	0.08	0.17	0.97[t]	0.61	0.70	0.59	0.56	-0.17	0.00	0.67[w]	0.51
9	0.63	0.86[u]		0.49	0.87[u]	0.92	0.75[w]	0.79[v]	0.63	0.71[w]	0.11	0.58	0.96
10	0.69[w]	0.78[w]		0.80[w]	0.62	0.17	0.51	0.86[v]	0.10	-0.30	-0.68[w]	0.18	0.31
11	0.45	0.85[k]		0.71	0.33	0.72	0.36	-0.24	0.40			0.62	-0.23
12	0.52	0.35	0.82[v]	-0.05	0.53	0.15	0.26	0.71[w]	-0.35	0.75[w]	0.81[w]	0.37	-0.08
Overall	0.33[u]	0.54[s]	-0.07	-0.30[w]	0.47[t]	0.46[s]	0.13	0.01	0.18	0.24[w]	0.39[v]	0.39[u]	0.10

[s]$p<0.001$

[t]$p<0.01$

[u]$p<0.02$

[v]$p<0.05$

[w]$p<0.10$

TABLE 22.--Various Correlation Coefficients (r) for Tissues made on Material Collected at Slaughter[f] from Hereford Bulls.

	r values			
	Plasma Testosterone	Testis Testosterone	Plasma Androstenedione	Testis Androstenedione
Plasma testosterone concentration		0.63[a]		
Plasma androstenedione concentration				0.24
Plasma LH concentration	0.38[d]	0.56[a]	0.28	0.55[b]
Plasma prolactin concentration	0.31	0.05	-0.22	0.00
Pituitary FSH concentration	0.14	0.06	0.38[d]	0.04
Seminal vesicular fructose concentration	0.19	0.23	0.03	0.22
Seminal vesicular citric acid concentration	0.06	-0.10	-0.22	0.01
Gonadal sperm concentration	0.05		-0.40[d]	
Epididymal sperm total	0.05		-0.37[e]	

[a] $P<0.001$

[b] $P<0.01$

[c] $P<0.02$

[d] $P<0.05$

[e] $P<0.10$

[f] 13 months of age plus 2 weeks.

TABLE 23.--Correlation Coefficients (r) for Serum Androgens and Serum Gonadotropins from Herefords at 11, 12 and 13 Months of Age.

Age		Plasma LH concentration	Plasma Prolactin concentration
(Months)			
11	Plasma androstenedione concentration	0.10	-0.15
	Plasma testosterone concentration	0.18	-0.29
12	Plasma androstenedione concentration	0.01	-0.27
	Plasma testosterone concentration	-0.04	-0.16
13	Plasma androstenedione concentration	0.17	-0.16
	Plasma testosterone concentration	-0.04	0.06

[a]P<0.001

[b]P<0.01

[c]P<0.02

[d]P<0.05

[e]P<0.10

ovarian ascorbic acid depletion, but the recovery of LH from the plasma of the bulls was only 20%. More recently (Swanson, 1969) re-quantified plasma LH by radioimmunoassay. There is some variance between the two estimates and these will be discussed (see Table 20).

Data presented in this study for androgen levels in Hereford bulls were also analyzed parallel to other reproductive and endocrimological criteria (see Tables 22 and 23). These criteria were estimated by Swanson et al. (1970) and included: (1) gonadal and extra gonadal sperm numbers; (2) seminal vesicular fructose and citric acid; and (3) pituitary FSH and plasma LH.

The correlation between plasma testosterone concentration and testicular testosterone concentration for Holstein bulls was significant. This suggested that as testosterone rose, plasma testosterone also did and not vice versa as is often the case for the secretions of other endocrine glands. The within month correlations between plasma and testicular testosterone revealed a non significant negative correlation at months 4, 5 and 6, however the correlations were positive from months 7 to 12, thus months 7, 8 and 9 were significant (see Table 21). This qualifies the overall correlation and suggests that during months 4, 5 and 6 testosterone secretion has not reached a maximum and that release into the blood depletes the testicular stores of testosterone. The overall correlation between testicular androstenedione and plasma androstenedione

concentration was not significant (see Table 20). This indicated that increased levels of androstenedione in the testis after 4 months of age were only partially released into the blood, the larger portion being rapidly converted to other steroids such as testosterone. Also, that high levels of androstenedione in the blood after 4 months of age did not reflect secretion by the testis, but probably metabolism of plasma testosterone or perhaps secretion from the adrenal gland. Horton (1965) estimated that 36% of blood androstenedione was produced by the metabolism of blood testosterone. Evidence by Kirschner (1965) also suggested the adrenals as being an important source of androstenedione.

The relationship between testicular and plasma concentrations of androgens for Hereford bulls at slaughter (13.5 months of age) showed similar relationships. Plasma testosterone concentration was significantly correlated with testicular testosterone concentration (see Table 22), and plasma concentration of androstenedione showed a small non-significant correlation with testicular androstenedione concentration (see Table 22). For the Holstein bulls the correlation between plasma testosterone concentration and plasma androstenedione concentration approached significance (see Table 20), and the correlation of testicular testosterone concentration and androstenedione concentration, although not significant was positive (see Table 20). After 4 months of age, increased synthesis

of testosterone in the Holstein bull testis seemed to be accompanied by small increases in androstenedione synthesis. This seemed feasible as androstenedione is synthesized as a precursor for testosterone and some of the intermediate metabolite was likely to be detected.

As for androgens in the blood, as a major portion of the testosterone was apparently converted to androstenedione possibly in preparation for excretion, increases in androstenedione concentration could reflect increases in testosterone concentration. The correlation of plasma testosterone and androstenedione (see Table 20), although not highly significant, was larger than the similar testicular correlation (see Table 20), probably because it included the earlier months when androstenedione was the predominant steroid. Increased concentrations of blood androstenedione at this early date were probably accompanied by smaller increases in testosterone, as little testosterone was produced from the androstenedione pool in the testis at this time.

Testicular concentration of androstenedione and plasma concentration of testosterone were negatively correlated, but the correlation was small and non-significant (see Table 20). A similar non-significant correlation was noted for testicular concentration of testosterone and plasma concentration of androstenedione (see Table 20). This seemed to confuse the issue, but may be explained by the fact that the correlations were only

made on values from 4 to 12 months of age and during this period the two hormones were only vaguely correlated in the testis or within the blood. However, in the blood, the testosterone and androstenedione concentrations seemed to follow each other more closely and this is acceptable if the bulk of blood androstenedione is regarded as a metabolite of blood testosterone, when the steroidal levels were cross correlated the correlation was negative. The lack of significant correlations suggest no important relationship between testicular production and plasma concentration of androstenedione.

Plasma LH (Macmillan and Hafs, 1968c, Appendix Table 3) in the Holstein bulls, as measured by the ovarian ascorbic acid depletion method, rose from 2 months of age to 4 months, declined to 6 months and then increased to 12 months of age. This biphasic pattern of secretion was not clearly reflected in plasma testosterone concentration but appeared to be reflected in the testicular values. When measured by radioimmunnoassay plasma LH appeared to rise from 1 month of age to 2 months, declined to 4 months, rose again to 5 months, fell to 6 months and then rose slowly to 9 months of age. A sharp rise was noted between 10 and 11 months but the levels of LH in the plasma fell again at 12 months of age (Swanson, 1970; Appendix Table 3). It appears that the levels of LH in the plasma for the first 6 months were higher on average than for months 6 to 10. This is interesting as the most rapid reproductive

development was made in the latter period. The values as measured by radioimmunoassay are probably more realistic as the assay procedure is more sensitive and accurate than the ovarian ascorbic acid depletion bioassay. Also, the LH precipitated from the blood of the bulls to be used in the latter assay was accomplished at a very low recovery rate.

A correlation over all months showed that testicular and plasma content of testosterone were both significantly correlated to plasma content of luteinizing hormone in the Holstein bulls, no matter which set of LH figures were used (see Table 20). Within month correlations between plasma LH (as measured by radioimmunoassay) and testicular or plasma testosterone levels show a more or less continuous stimulation of testosterone synthesis in the testis of Holstein bulls within each age group from 5 to 12 months of age (see Table 21). Release of testosterone into the blood appears to be related, within monthly age groups, to plasma LH levels during two periods, 1 to 3 months and 9 to 12 months (see Table 21). Testosterone levels in the plasma generally rose between these two periods, thus it seems feasible to conclude that release of testosterone from the testis from 4 to 8 months occurs without LH control.

A correlation over all months revealed that androstenedione content of the testis and plasma were negatively correlated to plasma content of LH, as measured by ovarian

ascorbic acid depletion, but the correlations were far from significant (see Table 20). Using LH figures from radioimmunoassay a similar correlation resulted with plasma androstenedione, but with testis androstenedione a positive correlation approaching significance was revealed (see Table 20).

Within month correlations between plasma LH and testicular or plasma androstenedione levels were also analyzed (see Table 21). Plasma levels of androstenedione in the early monthly age groups (1 to 6 months) seemed to show little correlation with LH levels in the plasma. In the testis it appeared that within the early months (4 to 7 months) androstenedione levels decreased with increasing levels of LH in the plasma, but in older bulls they increased parallel with plasma LH.

These correlations between androgen levels in the testis and plasma of Holstein bulls suggest that LH stimulates testosterone synthesis mainly by stimulating the testicular enzymes concerned with the conversion of androstenedione to testosterone. Also, that considerable stimulation is seen, especially in older animals, at an earlier stage in steroidogenesis.

Oshima (1967) and Strickland (1970) both suggested a stimulatory role for LH on the enzyme system responsible for hydroxylation at the 17 carbon of androstenedione. High levels of androstenedione in the testes of young animals suggested that ample availability of cholesterol

and acetate substrate, but it was not until later stages of sexual maturity that the chain of enzyme reactions were carried as far as testosterone in large quantities, probably due to the stimulation of increasing LH titers in the blood.

Data from the Hereford bulls bled at 11, 12 and 13 months of age revealed no significant correlations between plasma concentrations of LH (see Table 23). If anything these correlations suggested a decreasing dependence of testosterone secretion on circulating LH with age. As shown by the Holstein data, the dependency was probably greater in developing animals when increasing circulating levels of LH were stimulating androgen synthesis and secretion. However, within month correlations between testis or plasma androgens and circulating LH levels suggested a lower correlation between the criteria in the more mature bull, especially at month 12 (see Table 21).

Androstenedione concentrations in the blood of the Hereford bulls at 11, 12 and 13 months of age showed no significant correlations with LH concentrations in the blood (see Table 23). At these ages LH probably only maintained testicular androgen secretion, unlike pubertal animals where large increases of both criteria were seen. If this is true, then in mature animals, a more critical assessment of androgen and LH are necessary to detect relationships between the two, above the large between animal variations that exist. It is interesting to note

the apparent lack of stimulation of plasma testosterone levels by plasma LH levels between 4 and 8 months of age in the Holstein bulls (see Table 21). It may be that testis release of androgens at this period and in mature bulls is not under any LH control. In older animals, androstenedione seemed to be more prominent in the blood, and the ratio of testosterone to androstenedione was only 2:1. Less androstenedione may have been converted to testosterone in the testis and/or more was released into the blood. This may reveal, as was suggested for the Holstein bulls, a stimulatory effect of LH earlier in the steroidogenic pathway than conversion of androstenedione to testosterone. Alternately, that enzyme systems converting androstenedione to testosterone may reach a steady state in the mature animal. Production of androstenedione would not be rate limiting to testosterone synthesis and a steady state secretion of androstenedione could be established.

Data from Hereford bulls at slaughter revealed a significant correlation between testicular concentration of testosterone and serum LH concentration (see Table 22), as well as a significant correlation between testicular androstenedione concentration and serum LH (see Table 22). Similar correlations for serum concentrations of testosterone and androstenedione with serum LH concentration approached significance (see Table 22). These data probably reflected a general stimulation of hormone

synthesis due to stress. Several investigators suggested that gonadotropins stimulated steroidogenesis by enhancing the 20αhydroxylation of cholesterol to pregnenalone (e.g. Dorfman, 1969; Hall and Young, 1968).

Pituitary FSH potency showed no relationship to androgen synthesis or secretion in bulls. Correlations of pituitary FSH potency in the Holstein (Macmillan and Hafs, 1968) and Hereford (Swanson et al., 1970) bulls with testicular and plasma concentrations of testosterone and androstenedione were all non-significant except for the correlation with plasma androstenedione concentration at slaughter for the Hereford bulls (see Tables 20 and 22).

Means (1969) demonstrated the ability of FSH to stimulate testicular protein biosynthesis and concluded that the activity was specific for FSH and gonadotropins with FSH activity. After experiments involving administration of exogenous FSH and LH into Rhesus monkeys, Kar (1966) concluded that the major role of FSH in the testis was to stimulate growth via protein synthesis. Thus, although FSH may be important in preparing the machinery for androgen secretion the role of supervising secretion is probably more in the hands of LH.

Plasma prolactin concentration was determined for the Hereford bulls (Swanson et al., 1970) but it was not significantly correlated to any of the androgen criteria (see Table 23). Correlations of plasma prolactin with

androgen data at slaughter were highest and androstenedione criteria gave consistently negative correlations with plasma prolactin concentration. The role of prolactin in the bull is not clear.

Sperm were not detected in the testis or epididymis until 6 or 7 months of age for the Holstein bulls studied, all bulls showed sperm in these areas by 8 months of age (Macmillan and Hafs, 1968; Appendix Table 4). For these bulls, no significant overall correlations were found between testicular or epididymal sperm content and testicular testosterone and androstenedione or plasma testosterone concentration (see Table 20). However, plasma androstenedione concentration was significantly correlated to gonadal sperm concentration and total spididymal sperm (see Table 20).

The correlations between androgen levels and testicular sperm numbers were examined on a within month basis (see Table 21). The correlation between testis or plasma testosterone and testicular sperm numbers was greatest at 8 and 9 months of age, decreasing from then on. This suggests that testosterone may play a role in the initiation of spermatogenesis and to a lesser extent with the following increase in sperm numbers. Androstenedione did not appear to be consistently related in any way to the initiation and maturation of spermatogenesis in Holstein bulls.

The concentration of testosterone in the slaughter serum of the Hereford bulls was not significantly correlated with gonadal sperm concentration or total epididymidal sperm (see Table 22). However, androstenedione was significantly correlated to gonadal sperm concentration and the correlation with total epididymidal sperm numbers approached significance.

A combination of FSH and LH was necessary to stimulate and maintain spermatogenesis in hypophysectomized rats (Lostrah, 1969). It is possible that a combination of gonadotropins and androgens were necessary for the initiation of spermatogenesis in bulls. The DNA concentration of Holstein bull testes declined to 10 months of age (Macmillan and Hafs, 1968; Appendix Table 5) due to increases in the size of the seminiferous tubules. This trend was reversed at 10 months of age when large numbers of sperm appeared in the testis. Changes in testosterone levels did not appear to parallel these changes, but testicular androstenedione concentration was significantly correlated to the concentration of testicular DNA over the whole period (see Table 20), and the correlation of plasma androstenedione and testicular DNA concentration approached significance (see Table 20). Analysis of within month correlations between plasma or testis androstenedione concentrations and testis DNA concentration was made (see Table 22). It appears from this that testicular androstenedione may be involved with DNA

synthesis in the testis and that this could be a local effect not mediated via the systemic circulation. Androstenedione may be involved with protein synthesis and testicular growth. No clear relationship between testosterone and androstenedione and spermatogenesis was revealed by this study, but testosterone appeared to be involved in at least the initiation of spermatogenesis, if not maturation and maintenance.

Accessory gland growth and secretory criteria are acknowledged classically to reflect the androgenic status of an animal. In this study, seminal vesicular secretory activity was shown to reflect the changing levels of plasma testosterone but androstenedione did not seem to be related to accessory sex organ activity. The fructose and citric acid contents of the seminal vesicles of the Holstein bulls increased from 1 month of age to 1 year with a more rapid increase during the last 6 months (Macmillan and Hafs, 1969; Appendix Table 6). Over the whole period testicular testosterone concentration was not significantly correlated with either seminal vesicular citric acid concentration (see Table 20), or fructose concentration. But plasma testosterone was significantly correlated to seminal vesicular fructose concentration and citric acid concentration. Plasma testosterone content was also significantly correlated to seminal vesicular fructose content. Plasma androstenedione concentration was not significantly correlated to seminal vesicular fructose

content. Plasma androstenedione concentration was not significantly correlated to seminal vesicular fructose content or citric acid concentration (see Table 20). Testicular androstenedione concentration however was significantly negatively correlated to seminal vesicular fructose concentration, and to citric acid concentration (see Table 20). Plasma testosterone concentration was positively correlated to seminal vesicular fructose concentration within each month, with large and even some significant correlations (see Table 21) occurring biphasically at 2, 3 and 4 months and 8 and 9 months of age. Within month correlations between testis testosterone concentration and seminal vesicular fructose concentration were basically positive with significant correlations at 9 and 10 months of age (see Table 21). The correlation was negative at 4 and 5 months of age, this may reflect the negative correlation between testis and plasma testosterone concentrations that existed at this age.

The correlations between testicular androstenedione and seminal vesicular secretions were not surprising as testicular androstenedione decreased with age in Holstein bulls and was very low after 6 months of age, or the time when the most rapid increases in seminal vesicular activity occurred. The picture for blood levels of androstenedione was less dramatic, probably because levels of androstenedione to 6 months of age were highly variable

and then almost zero until 12 months of age. Neither were correlations between testosterone and seminal vesicular secretions surprising; plasma testosterone concentration did not seem to increase as biphasically as testicular testosterone concentration, but there was a low point at 6 months which coincided with the end of the first phase of increasing seminal vesicular secretory activity and the beginning of the second phase. The overall relationship of testicular testosterone to seminal vesicular secretion is difficult to explain, however the within month correlations show the expected relationships. It would be expected that blood levels would be more correlated to target organ activity but the blood levels of testosterone were strongly correlated to testicular values. One can only surmise that had all of the testicular values from birth to 1 year been available then the correlation would have been greater.

However testosterone in the blood apparently stimulated seminal vesicular secretory activity, and androstenedione played no such role. This view is corroborated by Baranas (1969) who castrated 6-month old bovine males and injected testosterone or androstenedione. Both promoted growth and nitrogen retention but only testosterone showed any androgenic properties. Skinner (1968) found that androstenedione depressed seminal vesicular growth and secretion.

The relationships between androstenedione or testosterone and seminal vesicular secretory activity for the Hereford bulls was not clear at all (see Table 22). All of the correlations were made on slaughter material which appeared to be confused by an elevation of hormonal levels due to stress. None of the correlations made were significant, testicular testosterone concentration showed, as for Holstein bulls, a small positive correlation with seminal vesicular fructose concentration, and a small negative correlation with citric acid concentration. Testicular androstenedione gave small positive correlations with seminal vesicular concentration of fructose and citric acid. Serum testosterone concentration was poorly correlated with either secretory product (see Table 22). Androstenedione concentration in the plasma gave a negative correlation with citric acid concentration in the seminal vesicle, and a small positive correlation with fructose concentration in the same accessory gland (see Table 22).

SUMMARY AND CONCLUSIONS

A technique for routine assay of androstenedione and testosterone from biological samples has been evolved. The salient features of this assay were extraction of tissue with diethyl ether, purification by methanolic precipitation of lipids and thin layer chromatography to isolate the androgens. Quantification was performed on the diheptafluorogutyrate derivative of testosterone and the monoheptafluorobutyrate derivative of androstenedione utilizing gas liquid chromatography with electron capture detection. The sensitivity of the assay was good enough to allow quantification of plasma levels of testosterone and androstenedione to as little as 0.001 ng. Specificity, accuracy and precision of the technique also were acceptable. This assay was employed in a study of testosterone and androstenedione in the blood and testis of bulls from birth through puberty.

A total of sixty-five Holstein bulls were killed in groups of five at monthly intervals from birth to 12 months of age. The testicular parenchyma and plasma from these animals were assayed for testosterone and androstenedione. A group of twenty-nine Hereford bulls were bled at 11, 12

and 13 months of age and slaughtered two weeks after the last bleeding. Androgens in tissues from these animals were assayed as for the Holstein bulls.

Testicular concentration of testosterone from Holstein bulls increased during months 4 (1.08 µg/g) and 5 (3.15 µg/g), and during months 7 (0.17 µg.g) and 11 (1.86 µg/g). A decline was noted at 6 (0.32 µg/g) and 12 (0.29 µg/g) months. The concentration of testicular androstenedione fell sharply from months 4 (1.66 µg/g) to 7 (0.03 µg/g), remained stable at a low level until 9 months of age and then appeared to rise until 11 months (0.22 µg/g), only to fall to a low level at 12 months of age. These fluctuations in concentration produced a ratio of testosterone to androstenedione of 0.65:1 at 4 months of age, 48:1 at 8 months and 8:1 at 12 months of age. Despite large fluctuations there seemed to be a gradual increase of testosterone concentration in the plasma of Holstein bulls from 0.00ng/ml at birth to 3.73ng/ml at 11 months of age followed by a precipitous drop to 0.50 ng/ml at 12 months of age. Despite great fluctuations during the first 6 months, no androstenedione was detected in the blood again until 12 months of age. Testerone and androstenedione levels in the Hereford bulls were generally higher than in Holsteins even though the concentrations in the blood of Herefords appeared to fall from 11 (testosterone 5.5 ng/ml, androstenedione 4.25 ng/ml) to 13 (testosterone 4.32 ng/ml, androstenedione 1.83 ng/ml)

months. It was not clear if this was a difference due to breed or physiological maturity. The plasma androgen concentrations recorded at slaughter may have been elevated due to stress. However a 2:1 ratio of testosterone to androstenedione was established in the blood.

The relationships between the plasma and testicular steroids quantified were examined over the first year of life in bulls. Correlation analysis was also performed between testicular and plasma androstenedione or testosterone and several endocrinological and physiological criteria associated with sexual maturation. Among these criteria were plasma LH concentration, pituitary FSH potency, gonadal and extra gonadal sperm numbers and seminal vesicular secretory activity.

In the Holstein bulls, plasma testosterone concentration reflected testicular testosterone concentration from 4 to 12 months of age. No such relationship existed for androstenedione. After the first 4 months of age, little androstenedione was released from the testis. Most was probably converted to testosterone. In the blood, the major part of androstenedione probably originated from metabolism of testosterone or from adrenal secretion.

Testosterone synthesis and secretion increased with the rising titers of plasma LH during the first year of life in Holstein bulls, but there was an indication that testosterone secretion from the testis may be independent

of LH between months 4 and 8 and in mature bulls. Androstenedione and LH appeared to be negatively correlated in young bulls but after 6 or 7 months of age this relationship was positive. Few of the within month correlations were significant. It was suggested that LH stimulated steroidogenesis mainly by stimulating the conversion of androstenedione to testosterone, but some stimulation at an earlier stage of steroidogenesis was also apparent especially in the older bulls.

After one year of age, data from the Hereford bulls suggested little correlation of LH to testosterone or androstenedione. Thus LH may only maintain androgen secretion in the mature animal and not directly stimulate it. The biphasic pattern of increasing LH secretion (rising from 2 to 4 months of age, declining to 6 months, and then increasing to 12 months of age) was reflected more noticeably in plasma than in testicular testosterone concentrations. Large between animal and between month variations tended to obscure this result.

Pituitary FSH potency was not significantly correlated with either androstenedione or testosterone in either breed. FSH may be involved with general stimulation of growth and protein synthesis in the testis of young bulls during sexual maturation.

No significant relationships were found between serum prolactin and plasma or testicular androstenedione or

testosterone in the Herefords. The role of prolactin in the male was not clear.

Gonadal and extra gonadal sperm numbers during maturation of the Holstein bulls were shown to be significantly correlated only to plasma androstenedione concentration on the basis of overall correlations. The Hereford slaughter data suggested a negative relationship between blood levels of androstenedione and gonadal and extra gonadal sperm numbers. Testosterone concentration in the plasma seemed to have little relationship with sperm numbers in this breed. Correlations made within each age group between testosterone or androstenedione and gonadal sperm numbers revealed a positive relationship between testis and plasma testosterone and gonadal sperm numbers. Although few of the correlations were significant, a role was suggested for testosterone in the initiation of spermatogenesis. Androstenedione did not exhibit any clear relationship at all with spermatogenesis. It is probable that a combination of gonadotropins and androgens is necessary for the initiation of spermatogenesis in bulls.

The fructose and citric acid content of the seminal vesicles of the Holstein bulls increased from 1 month of age to 1 year, with a more rapid increase during the last 6 months. The changing levels of plasma testosterone were found to reflect this pattern. Androstenedione levels in the maturing bull did not appear to be related

to increased secretory activity of the accessory glands. The correlations made between Hereford bull seminal vesicular activity and serum or testicular testosterone and androstenedione levels showed no significant relationships. This was confusing, it could be that the stress of slaughter had altered hormonal levels or that when maintenance rather than active growth in the mature animal was the case then no strong relationship between gonadal and accessory glands could be seen.

Puberty based on testicular and circulating levels of testosterone and androstenedione would appear to continue until at least 11 months of age in bulls. After this age, the levels of testosterone and androstenedione decline to adult levels. Testosterone appears to be the major androgenic steroid in bulls responding to increasing circulating levels of LH and stimulating accessory gland secretion in a biphasic pattern over the first year of life. Androstenedione lacks androgenic properties, and any specific function during puberty is unclear although it may promote testicular growth.

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APPENDICES

APPENDIX TABLE 1

PLASMA AND TESTICULAR TESTOSTERONE AND ANDROSTENEDIONE VALUES FOR INDIVIDUAL HOLSTEIN BULLS BY MONTH

APPENDIX TABLE 1.--Plasma and Testicular Testosterone and Androstenedione Values for Individual Holstein Bulls by Month.

Age	Bull No.	Plasma Testosterone Conc. (ng/ml)	Plasma Testosterone Total (µg/animal)	Plasma Androstenedione Conc. (ng/ml)	Plasma Androstenedione Total (µg/animal)	Testis Testosterone Conc. (µg/g)	Testis Testosterone Total (µg/testis)	Testis Androstenedione Conc. (µg/g)	Testis Androstenedione Total (µg/testis)
Birth	1	ND[a]	ND	ND	ND				
	2	ND	ND	ND	ND				
	3	ND	ND	ND	ND				
	4	ND	ND	ND	ND				
	5	ND	ND	ND	ND				
	Mean	--	--	--	--				
1 Month	179	Trace[b]	Trace	Trace	Trace				
	180	Trace	Trace	Trace	Trace				
	182	3.29	4.81	48.10	70.37				
	183	Trace	Trace	ND	ND				
	184	0.30	0.45	1.87	2.80				
	Mean±SE	0.72±0.64[c]	1.10±0.94	10.00±9.53	18.30±17.37				
2 Months	175	0.30	0.77	0.64	1.65				
	176	1.10	2.94	Trace	Trace				
	177	0.28	0.67	ND	ND				
	178	0.29	0.70	0.91	2.20				
	185	Trace	Trace	Trace	Trace				
	Mean SE	0.40±0.18	1.02±0.50	2.34±0.19	0.97±0.56				
3 Months	164	Lost	Lost	Lost	Lost				
	165	Lost	Lost	0.39	1.33				
	166	0.86	3.08	Trace	Trace	1.91	31.76	6.47	107.42
	167	0.70	2.23	Trace	Trace				
	168	0.22	0.81	7.00	25.63				
	Mean±SE	0.59±0.19	2.04±0.66	1.85±1.72	6.75±6.30	1.91	31.76	6.47	107.42
4 Months	163	0.81	3.87	Trace	Trace	Lost	Lost	Lost	Lost
	170	0.56	2.76	ND	ND	1.45	33.67	0.70	16.23
	171	Trace	Trace	ND	ND	0.62	16.73	0.61	16.49
	172	1.74	8.26	ND	ND	0.41	7.57	0.18	3.42
	174	0.68	3.09	ND	ND	1.85	44.03	5.17	122.76
	Mean±SE	0.76±0.28	3.60±1.34	--	--	1.08±0.34	25.50±8.21	1.67±1.17	39.73±27.85
5 Months	156	0.51	2.68	ND[a]	ND	Lost	Lost	Lost	Lost
	158	Lost	Lost	ND	ND	8.09	288.56	0.29	10.38
	160	1.80	9.17	ND	ND	3.96	122.46	1.23	38.13
	161	1.49	7.81	ND	ND	0.27	9.55	0.23	7.97
	162	8.12	43.45	35.6	190.51	0.30	7.96	1.18	31.74
	Mean±SE	2.98±1.74	15.78±9.33	7.12±7.12	38.10±38.10	3.16±1.87	107.79±66.15	0.73±0.27	22.06±7.57

6 Months	150	0.41	2.54	ND	ND	0.39	21.46	0.03	1.88
	153	Trace[b]	Trace	ND	ND	0.41	11.85	0.04	1.00
	154	0.37	2.45	ND	ND	0.28	13.99	ND	ND
	155	0.52	3.62	ND	ND	ND	ND	0.70	29.90
	157	Lost	Lost	Lost	Lost	0.21	12.11	0.12	6.82
	Mean±SE	0.33±0.11[c]	2.16±0.76	--	--	0.32±0.05	11.18±3.45	0.18±0.13	7.92±5.62
7 Months	143	4.46	34.89	ND	ND	0.62	39.05	ND	ND
	144	1.19	8.61	ND	ND	0.16	9.35	0.02	1.25
	145	Trace	Trace	Trace	Trace	0.12	9.79	Lost	Lost
	146	ND	ND	ND	ND	0.26	18.97	Lost	Lost
	147	6.75	41.58	Trace	Trace	1.22	50.18	0.07	3.03
	Mean±SE	2.48±1.34	17.02±8.87	--	--	0.47±0.21	25.47±8.19	0.03±0.02	1.43±0.88
8 Months	136	Lost	Lost	Lost	Lost	1.48	201.85	0.02	2.55
	137	4.01	31.76	ND	ND	1.00	148.75	0.03	4.18
	138	10.40	87.72	Trace	Trace	3.30	282.51	0.05	4.48
	142	4.10	32.47	Trace	Trace	1.70	148.58	0.04	3.27
	148	0.77	5.88	ND	ND	0.51	28.54	0.03	1.63
	Mean±SE	4.82±2.02	39.46±17.24	--	--	1.60±0.47	162.05±41.40	0.03±0.01	3.22±0.52
9 Months	120	0.52	4.61	Trace	Trace	0.17	26.11	0.02	3.46
	121	2.11	18.94	ND	ND	1.34	221.19	0.04	5.76
	122	0.35	3.24	Trace	Trace	0.16	23.62	0.04	5.73
	125	0.76	7.02	Trace	Trace	0.22	30.04	Lost	Lost
	126	8.25	80.99	Trace	Trace	1.76	389.83	0.09	19.65
	Mean±SE	2.40±1.50	22.96±14.77	--	--	0.73±0.34	138.16±73.35	0.05±0.01	8.65±3.71
10 Months	123	0.38	3.72	Trace	Trace	Lost	Lost	Lost	Lost
	124	10.10	97.62	ND[a]	ND	1.80	209.05	0.16	18.16
	127	1.68	17.59	ND	ND	2.07	406.41	0.13	26.67
	132	Trace[b]	Trace	ND	ND	0.21	32.82	0.01	1.37
	134	Trace	Trace	ND	ND	0.28	47.38	0.03	4.89
	Mean±SE	2.44±1.94[c]	23.40±18.35	--	--	1.09±0.49	173.92±87.18	0.08±0.04	12.79±5.88
11 Months	108	0.01	0.12	ND	ND	1.17	209.51	Lost	Lost
	113	5.60	67.33	ND	ND	3.70	816.29	0.34	78.09
	117	1.49	16.53	Lost	Lost	1.97	289.20	0.39	57.22
	118	7.80	83.67	ND	ND	1.46	229.26	0.08	12.83
	119	Lost	Lost	Lost	Lost	0.97	157.04	0.08	13.38
	Mean±SE	3.73±1.80	41.91±19.96	--	--	1.86±0.49	340.26±120.87	0.22±0.08	40.38±16.31
12 Months	101	0.61	7.38	Trace	Trace	0.41	49.41	0.03	3.04
	102	Lost	Lost	Lost	Lost	0.33	62.19	0.04	8.21
	104	0.89	11.15	6.70	83.95	0.28	57.40	0.03	6.62
	107	0.38	5.09	ND	ND	0.21	48.34	Lost	Lost
	109	0.13	1.32	0.61	6.19	0.21	56.94	0.03	8.08
	Mean±SE	0.50±0.16	6.24±2.06	1.83±1.63	2.54±20.52	0.29±0.04	54.86±2.62	0.03±0.00	6.49±1.20

APPENDIX TABLE 2

PLASMA TESTOSTERONE AND ANDROSTENEDIONE VALUES FOR INDIVIDUAL HEREFORD BULLS BY MONTH AND AT SLAUGHTER, AND TESTIS TESTOSTERONE AND ANDROSTENEDIONE VALUES

APPENDIX TABLE 2.--Plasma Testosterone and Androstenedione Values for Individual Hereford Bulls by Month and at Slaughter, and Testis Testosterone and Androstenedione Values.

	Plasma								Testis	
	11		12		13		Slaughter[a]		Slaughter[a]	
Bull	Test.[b]	Androst.[c]	Test.	Androst.	Test.	Androst.	Test.	Androst.	Test.	Androst.
	(ng/ml)								(µg/g)	
836	11.2	0.01	119.0	0.01	4.73	ND[d]	5.81	1.11	0.972	0.015
845	2.78	0.97	12.9	ND	4.55	ND	2.98	ND	0.425	LR[e]
837	5.34	ND	LR	17.30	7.48	ND	3.73	T[e]	0.772	0.020
864	LR	LR	ND	25.10	1.38	4.82	3.75	T	0.530	0.009
853	12.30	8.66	2.94	T	3.89	1.62	LR	LR	0.392	0.009
852	3.29	0.69	Lost	1.87	5.86	ND	11.30	1.57	2.327	0.067
855	LR	LR	Lost	Lost	LR	LR	3.07	ND	1.275	0.040
880	7.73	T	10.10	2.34	3.50	4.45	LR	LR	0.759	0.064
840	T	T	6.00	1.06	6.48	LR	17.70	T	1.533	0.119
823	3.15	ND	2.91	3.43	2.26	ND	4.35	T	0.304	0.011
829	1.33	3.46	ND	4.96	T	14.70	3.78	ND	0.660	0.031
854	13.60	14.70	0.68	ND	9.38	ND	15.20	T	1.835	0.075
881	ND	Lost	Lost	Lost	8.20	T	12.70	1.41	0.983	0.044
811	10.20	13.90	0.91	18.10	2.16	ND	17.50	Lost	1.820	0.064
832	11.60	Lost	LR	LR	10.40	ND	9.76	15.00	2.118	0.046
847	11.80	1.66	1.89	ND	4.09	T	1.26	3.33	0.358	0.019
862	12.20	T	0.91	ND	1.13	1.90	2.04	ND	0.491	0.014
801	2.30	4.97	T	ND	2.26	T	2.09	1.03	0.256	0.016
872	1.10	ND	2.29	ND	1.60	LR	5.48	1.52	1.227	0.033
882	10.50	1.49	LR	LR	8.70	9.00	8.69	0.94	1.436	0.039
817	1.36	Lost	6.59	LR	11.00	ND	3.06	ND	0.404	0.045
849	4.17	1.53	5.16	LR	T	LR	T	35.10	1.120	0.052
821	1.30	ND	4.72	LR	2.16	LR	8.80	20.80	1.377	0.065
816	2.80	T	8.65	ND	3.20	ND	Lost	Lost	0.527	0.043
879	6.46	LR	4.90	T	LR	5.37	2.17	T	0.166	0.019
858	4.94	T	6.68	9.53	3.77	ND	5.10	1.84	0.693	0.023
877	2.44	T	3.33	ND	6.00	T	LR	1.30	0.235	0.009
813	9.43	23.60	9.26	T	LR	LR	13.80	Lost	0.240	0.007
824	6.57	37.50	3.89	ND	Lost	1.36	3.28	T	0.477	0.009
818	2.17	T	3.01	LR	LR	4.72				
810	3.55	0.94	1.14	ND	T	T				
863	6.00	T	ND	LR	2.98	ND				
802	T	1.08								
Mean	5.54	4.27	4.26	3.69	4.34	1.85	6.70	3.54	0.887	0.036
± SE	±0.78	±1.69	±0.74	±1.46	±0.61	±0.69	±1.10	±1.71	±0.113	±0.005

[a]13 months plus 2 weeks

[b]Testosterone

[c]Androstenedione

[d]None detected

[e]Low recovery

APPENDIX TABLE 3

AVERAGE PLASMA CONCENTRATIONS AND TOTAL PLASMA CONTENT OF LUTEINIZING HORMONE AND THE AVERAGE RATIO BETWEEN TOTAL PLASMA CONTENT AND TOTAL PITUITARY CONTENT OF LUTEINIZING HORMONE IN HOLSTEIN BULLS FROM BIRTH TO 12 MONTHS OF AGE

APPENDIX TABLE 3.--Average Plasma Concentrations and Total Plasma Content of Luteinizing Hormone and the Average Ratio Between Total Plasma Content and Total Pituitary Content of Luteinizing Hormone in Holstein Bulls from Birth to 12 Months of Age.

Age	LH		
	Plasma Concentration	Plasma Content	Plasma[c] Pit. Ratio
(months)	(ug/l)[a]	(ug/animal)[b]	
Birth	0.48[d]	0.59[d]	1.97
1	0.41[d]	0.63[d]	0.33
2	0.17 ± 0.03	0.43 ± 0.08	0.34
3	0.34 ± 0.09	1.17 ± 0.36	0.49
4	0.35 ± 0.09	1.72 ± 0.45	0.88
5	0.29 ± 0.08	1.51 ± 0.41	0.53
6	0.24 ± 0.06	1.50 ± 0.43	0.61
7	0.30 ± 0.09	2.14 ± 0.59	1.50
8	0.42 ± 0.10	3.32 ± 0.77	2.38
9	0.42 ± 0.12	3.90 ± 1.22	1.51
10	0.50 ± 0.14	5.08 ± 1.48	2.33
11	0.38 ± 0.10	4.31 ± 1.07	1.87
12	0.47 ± 0.11	5.47 ± 1.18	2.22

[a]ug NIH-LH-B3 equivalent per liter.

[b]ug LH per liter x body wt. (kg) x 0.035.

[c]ug plasma LH per animal ÷ mg pituitary LH per animal.

[d]Estimates derived from pooled samples.

APPENDIX TABLE 4

GONADAL SPERM CONCENTRATION AND SPERM NUMBERS IN HOLSTEIN BULLS FROM 5 to 12 MONTHS OF AGE

APPENDIX TABLE 4.--Gonadal Sperm Concentration and Sperm Numbers in Holstein Bulls from 5 to 12 Months of Age.

Age	Number of Bulls	Sperm Concentration	Total Sperm
(months)		(10^6 sperm/g parenchyma)[a]	(10^9 sperm/ testis)[a]
5	1	4.24	0.15
6	2	6.80	0.34
7	3	4.26	0.27
8	5	27.87 ± 4.52[b]	2.62 ± 0.62
9	5	50.37 ± 3.80	7.80 ± 1.39
10	5	38.99 ± 1.77	5.41 ± 0.62
11	5	52.83 ± 4.19	8.59 ± 1.38
12	5	57.33 ± 5.97	10.69 ± 1.97

[a]Include spermatids (stages VI to VIII) and spermatozoa.

[b]± SE: only computed when average included 5 bulls.

APPENDIX TABLE 5

CHANGES IN TESTICULAR NUCLEIC ACID CONCENTRATION AND CONTENT AND THE RNA:DNA RATIO FOR HOLSTEIN BULLS FROM BIRTH TO 12 MONTHS OF AGE

APPENDIX TABLE 5.--Changes in Testicular Nucleic Acid Concentration and Content and the RNA:DNA Ratio for Holstein Bulls from Birth to 12 Months of Age.

Age	DNA Conc.[a]	RNA Conc.[a]	Total DNA	Total RNA	RNA/DNA
(months)	(mg/g)	(mg/g)	(mg/testis)	(mg/testis)	
Birth	7.18	5.05	13.64	9.71	0.70
1	6.26	5.32	17.28	15.39	0.90
2	5.43	5.94	34.93	37.80	1.11
3	4.64	5.19	43.40	49.26	1.13
4	4.09	6.13	79.72	120.35	1.54
5	3.95	5.56	111.23	157.19	1.43
6	4.01	5.55	168.12	235.30	1.39
7	3.35	4.94	194.59	290.15	1.47
8	3.34	4.56	302.30	414.56	1.38
9	3.28	4.59	510.86	722.27	1.40
10	3.27	4.34	448.40	599.16	1.33
11	3.95	4.67	607.15	732.70	1.27
12	3.60	4.72	666.83	873.30	1.31

[a]Concentration

APPENDIX TABLE 6

CHANGES IN THE WEIGHT OF THE PAIRED SEMINAL VESICLES AND THEIR DNA, RNA, CITRIC ACID AND FRUCTOSE CONTENTS FOR HOLSTEIN BULLS FROM BIRTH TO 12 MONTHS OF AGE

APPENDIX TABLE 6.--Changes in the Weight of the Paired Seminal Vesicles and Their DNA, RNA, Citric Acid and Fructose Contents for Holstein Bulls from Birth to 12 Months of Age.

	Weight	Total DNA	Total RNA	Total Citric Acid	Total Fructose
(months)	(g)	----------	(mg)	----------	
Birth	3.29	11.93	12.37	2.17	0.26
1	1.95	5.45	4.38	1.43	0.12
2	3.08	10.77	11.43	2.86	0.51
3	5.95	21.29	27.85	5.09	1.61
4	12.56	42.75	60.09	11.11	4.82
5	16.24	45.79	62.74	16.56	8.89
6	15.61	40.58	58.68	17.07	12.80
7	21.12	55.68	69.91	46.26	20.74
8	22.71	50.79	67.25	54.79	31.82
9	40.18	87.49	132.37	76.11	101.90
10	36.20	86.65	114.19	81.81	59.52
11	44.15	86.08	135.27	108.83	98.52
12	44.61	103.90	148.90	130.88	81.50

APPENDIX TABLE 7

PLASMA CONCENTRATION AND CONTENT OF LUTEINIZING HORMONE IN HOLSTEIN BULLS FROM BIRTH TO 12 MONTHS OF AGE AS MEASURED BY RADIOIMMUNOASSAY

APPENDIX TABLE 7.--Plasma Concentration and Content of Luteinizing Hormone in Holstein Bulls from Birth to 12 Months of Age as Measured by Radioimmunoassay.

Age	Bull No.	Plasma LH	
		Conc.	Content
(months)		(µg/l)	(µg/bull)
0	1	0.38	0.39
	2	1.00	1.22
	3	0.40	0.63
	4	1.74	2.05
	5	0.00	0.00
	Mean±SE	0.71±0.21	0.86±0.36
1	180	0.66	0.98
	183	0.52	0.80
	179	0.13	0.19
	182	0.65	0.92
	184	0.55	0.81
	Mean±SE	0.50±0.06	0.74±0.14
2	175	1.51	3.83
	176	2.66	7.10
	177	1.85	4.42
	178	1.40	3.38
	185	0.40	1.05
	Mean±SE	1.63±0.23	3.96±0.97
3	164	--	--
	165	0.73	2.49
	166	1.73	6.19
	167	0.89	2.83
	168	1.04	3.81
	Mean±SE	1.10±0.15	3.83±0.83
4	163	0.21	1.01
	170	0.19	0.94
	171	0.97	4.97
	172	0.00	0.00
	174	1.89	8.60
	Mean±SE	0.65±0.24	3.10±1.62
5	156	0.58	3.04
	158	0.88	4.61
	160	0.82	4.18
	161	1.54	8.07
	162	0.84	4.49
	Mean±SE	0.93±0.11	4.88±0.84

APPENDIX TABLE 7.--con't.

Age	Bull No.	Plasma LH	
		Conc.	Content
(months)		(µg/l)	(µg/bull)
6	150	0.43	2.66
	153	0.41	2.37
	154	0.81	5.36
	155	0.12	0.84
	157	0.36	2.07
	Mean±SE	0.42±0.08	2.66±0.74
7	143	--	--
	144	0.06	0.43
	145	0.55	3.70
	146	0.30	2.04
	147	0.65	4.00
	Mean±SE	0.39±0.09	2.54±0.82
8	137	0.57	4.70
	138	0.08	0.63
	136	0.68	5.74
	142	0.60	4.75
	148	0.03	0.23
	Mean SE	0.40±0.10	3.21±1.15
9	120	0.00	0.00
	121	1.07	9.61
	122	0.00	0.00
	125	0.02	0.18
	126	0.76	7.46
	Mean SE	0.37±0.15	3.45±2.10
10	124	0.70	6.63
	127	0.61	6.39
	123	0.00	0.00
	132	0.30	3.15
	134	0.00	0.00
	Mean SE	0.32±0.10	3.23±1.46
11	108	1.66	19.32
	113	2.13	25.61
	118	1.68	18.64
	117	1.48	15.86
	119	1.13	12.42
	Mean±SE	1.61±0.12	18.37±2.18

APPENDIX TABLE 7.--con't.

Age	Bull No.	Plasma LH	
		Conc.	Content
(months)		(µg/l)	(µg/bull)
12	101	1.15	13.91
	102	0.92	10.59
	104	1.82	22.80
	107	0.46	6.17
	109	1.54	15.63
	Mean±SE	6.17±0.17	13.82±2.76