Effect of selection for female reproductive rate on testicular physiology and histomorphology in Rambouillet rams

by Horacio C?ardenas Seijas

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

Montana State University

Abstract:
Objectives of this study were to determine if scrotal circumference (SC), testicular weight (TW), seminal characteristics, histological components of the testicular parenchyma, capacity and affinity of the oLH/hCG receptor, ability and sensitivity of testicular parenchyma to secrete testosterone (T) in vitro after stimulation by oLH or hCG differed among Rambouillet rams from lines selected for low (LL) or high (HL) female reproductive rate and from a random-bred control line, (CL). Two trials were conducted, one during the winter of 1989 and the other during the winter of 1990. Lines had been selected for 19 and 20 years for each trial, respectively. Scrotal circumference (SC), testicular weight (TW) and in vitro T secretion after hCG were evaluated in Trial 1. These characteristics and in vitro secretion of T after oLH, receptor capacity and affinity, testicular histomorphology and seminal characteristics were evaluated in Trial 2. Rams among lines did not differ (P>.10) in SC and TW but LL rams had lower (P<. 05) percentage of progressively motile sperm than HL and CL rams. Volume percentage of Leydig cells were higher (P<.10) in HL rams than in LL and CL rams whereas volume percentage of vascular tissue was higher (P<.10) in LL rams than in HL and CL rams. Volume percentage of seminiferous tubules or seminiferous tubule diameter did not differ (P>.10) among rams of. these lines. Receptor capacity was higher (P<.10) but affinity was lower (P<.10) for oLH than for hCG in rams of these lines. Receptor capacity for hCG per g of testicular parenchyma did not differ (P>.10) among rams of these lines but capacity for oLH was higher (P<.10) in HL rams than in the other lines. Affinity for hCG was higher (P<.10) in HL and LL rams than in CL rams, however affinity for oLH did not differ (P<.10) among rams of these lines. There were line by time and dose by time interactions (P<.10) for in vitro T secretion by testicular parenchyma after oLH or hCG stimulation in rams of these lines. Selection applied upon female reproductive rate altered certain seminal, histomorphological and physiological male reproductive characteristics in Rambouillet sheep.

EFFECT OF SELECTION FOR FEMALE REPRODUCTIVE RATE ON TESTICULAR PHYSIOLOGY AND HISTOMORPHOLOGY IN RAMBOUILLET RAMS

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I would like to dedicate this work to my wife Luz and my parents Victor and Elena.

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ABSTRACT

Objectives of this study were to determine if scrotal circumference (SC), testicular weight (TW), seminal characteristics, histological components of the testicular parenchyma, capacity and affinity of the oLH/hCG receptor, ability and sensitivity of testicular parenchyma to secrete testosterone (T) in vitro after stimulation by oLH or hCG differed among Rambouillet rams from lines selected for low (LL) or high (HL) female reproductive rate and from a random-bred control line (CL). Two trials were conducted, one during the winter of 1989 and the other during the winter of 1990. Lines had been selected for 19 and 20 years for each trial, respectively. Scrotal circumference (SC), testicular weight (TW) and in vitro T secretion after hCG were evaluated in Trial 1. These characteristics and in vitro secretion of T after oLH, receptor capacity and affinity, testicular histomorphology and seminal characteristics were evaluated in Trial 2. Rams among lines did not differ ($P>.10$) in SC and TW but LL rams had lower ($P<.05$) percentage of progressively motile sperm than HL and CL rams. Volume percentage of Leydig cells were higher ($P<.10$) in HL rams than in LL and CL rams whereas volume percentage of vascular tissue was higher ($P<.10$) in LL rams than in HL and CL rams. Volume percentage of seminiferous tubules or seminiferous tubule diameter did not differ ($P>.10$) among rams of these lines. Receptor capacity was higher ($P<.10$) but affinity was lower ($P<.10$) for oLH than for hCG in rams of these lines. Receptor capacity for hCG per g of testicular parenchyma did not differ ($P>.10$) among rams of these lines but capacity for oLH was higher ($P<.10$) in HL rams than in the other lines. Affinity for hCG was higher ($P<.10$) in HL and LL rams than in CL rams, however affinity for oLH did not differ ($P<.10$) among rams of these lines. There were line by time and dose by time interactions ($P<.10$) for in vitro T secretion by testicular parenchyma after oLH or hCG stimulation in rams of these lines. Selection applied upon female reproductive rate altered certain seminal, histomorphological and physiological male reproductive characteristics in Rambouillet sheep.

INTRODUCTION

Reproductive rate is an important characteristic in sheep production. Improvement of reproductive rate by means of selection has been based primarily on characteristics that can be measured only in females such as ovulation rate or number of lambs born per lambing ewe. Because of the sex limited nature of these traits, selection based upon male characteristics has never been used to improve reproductive rate in domestic animals except for research purposes. A review of the literature of selection for reproductive rate in sheep, cattle and mice indicates that this type of selection induces direct responses in females under selection and, at the same time, indirect changes in reproductive characteristics of the male offspring. Recently, it was reported that changes in certain female characteristics related to reproductive rate occur when selection is applied to male reproductive traits.

It was hypothesized that genes that control physiological processes related to reproductive rate may be the same in males and females, therefore, selection to improve reproductive rate in domestic species could be based upon characteristics measured in males. This type of selection has the advantage of increased responses due to shorter generation intervals and higher selection intensities that can be applied in males relative to females.

The present review focuses on a) the physiology of reproduction of adult male sheep with emphasis on the physiology and histomorphology of the testis and the endocrinological control of testicular function, and b) the correlated responses in testicular characteristics of the male offspring when selection is applied to female reproductive rate.

REVIEW OF LITERATURE

Anatomy of the Adult Ram Testis

The testes are the primary organs of reproduction in male mammals. In sheep, they are paired ovoid organs located between the rear legs inside a sac-like structure called the scrotum.

Weight of the testis in sheep varies with age. Each testis weighs about 3 g by 1 month of age, 40 g by 4 months and 115 g by 6 months. The large change in testicular weight between 4 and 6 months of age, occurs soon after the interstitial cells (steroid producing cells) of the testis become fully functional (Gier and Marion, 1970). Further increase of testicular weight after 24 weeks of age, when rams reach full maturity (Ashdown, 1988), is considered insignificant and in general, testicular weight decreases as males get older. The size of each testis in relation to body weight of rams is about 0.5% (Setchell, 1984).

Blood concentrations of certain hormones and season of the year are known to influence testicular weight and size. A linear relationship was observed between testicular size and the concentrations of the pituitary hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) and the testicular steroid hormone, testosterone (Courot and Ortavat, 1981). Seasonal variations in testicular diameter and weight

appear to be related to photoperiod since these characteristics decrease coincident with increasing photoperiod and increase as photoperiod decreases (Lincoln, 1981; Thimonier, 1981).

Anatomically, the testis is covered by a capsule which is formed by three layers of tissue, an outer layer of visceral peritoneum, the tunica vaginalis, a middle layer referred to as the tunica albuginea that consists of fibroblasts, collagen and "myofibroblasts" and, on the inside, the tunica vasculosa which is an extension of the testicular interstitium (Setchell and Brooks, 1988). The tunica albuginea is the most prominent layer of the testicular capsule. The testicular artery penetrates the tunica albuginea and runs around the inferior pole and up the anterior border of the testis, at which point it breaks into branches which enter the testicular parenchyma (Davis et al., 1970).

The testicular capsule surrounds the parenchyma, which is composed of two elements; highly coiled tubes known as the seminiferous tubules and the interstitium. The diameter of the seminiferous tubules of the ram varies between 200 to 260μm (Hochereau-de Reviers et al., 1979; Lunstra and Shanbacher, 1988). Both ends of each seminiferous tubule open into the rete testis, which is a complicated network of intercommunicated channels located longitudinally in the central part of the testis (mediastinum). Spermatozoa that are produced in the seminiferous tubules are transported,

suspended in a fluid, to the rete testis and then leave the testis through the efferent ducts.

A basement membrane forms the outer wall of the seminiferous tubules. This membrane is composed of a number of layers: several inner non-cellular layers followed by layers of myoid cells and collagen fibers and then, on the outside, a layer of endothelial cells (Bustos-Obregon and Courot, 1974).

Inside the seminiferous tubules there are two types of cells; germ cells and somatic cells known as Sertoli cells. Just before puberty, germ cells begin their transformation into spermatozoa through the process of spermatogenesis. Sertoli cells are polymorphous cells, located at the periphery, against the internal side of the basement membrane. Adjacent Sertoli cells form junctional complexes that contribute to the formation of the blood-testis barrier (Fawcett, 1975; Waites and Gladwell, 1982). In the ram, proliferation of Sertoli cells occurs mainly in utero, but a relatively small increase in number of Sertoli cells occurs between birth and puberty. After puberty there is no further increase in number of Sertoli cells (Hochereau-de Reviers et al., 1987). Proliferation of Sertoli cells is markedly influenced by FSH (Orth, 1984). In sheep, the number of Sertoli cells per testis by 18 to 24 months of age varies with breed and ranges from 20×10^8 to 50×10^8 cells (Barenton et al., 1983; Hocherau-de Reviers et al., 1987).

The interstitium of the testicular parenchyma contains blood and lymphatic vessels, interstitial or Leydig cells, fibroblasts, nerves and collagen fibers. In the ram Leydig cells are found singly or in small clusters with no clear association with blood vessels. The primary function of these cells is steroid hormone synthesis and secretion. The connective tissue in the interstitium is abundant and includes many fibroblasts and much collagen. There are blood and lymphatic capillaries in almost all interstitial areas. This arrangement of elements in the interstitium of the ram is similar to that observed in cattle, elephants, monkeys and humans (Fawcett et al., 1973).

Other cell types are also present in the testicular interstitium. These include macrophages, lymphocytes, plasma cells and mast cells. The function of mast cells in the testicle is not well known (de Kretser and Kerr, 1988) and macrophages have been studied mainly in rat testis in relation to their localization and function. They appear to be endocytically active (Miller et al., 1983).

Testicular Physiology in Sheep

The testes produce male gametes, the spermatozoa, and perform critical endocrine functions, the synthesis and secretion of steroids (androgens) and inhibin, a protein hormone.

Endocrine Function of the Testis

Androgen Synthesis. Leydig cells synthesize and secrete androgens male mammals (Baillie et al., 1966; Christensen and Gillim, 1969; Hall et al., 1969; Christensen, 1975). Testosterone is the predominant androgen synthesized in the testis; other androgens are androstenedione and dehidroepiandrosterone which are less active than testosterone (Eik-Nes and Hall, 1965).

Development of Leydig cells in the testis exhibits a biphasic pattern in most mammals; the first phase occurs during gonadal differentiation (the fetal period of life) and the second around puberty (Gondos et al., 1976). In the pig an additional phase in Leydig cell development takes place in the perinatal period between 2.5 weeks before until 2.5 weeks after birth (Van Straaten and Wensing, 1978). A definitive population of Leydig cells is established in pigs (Van Straate and Wensing, 1978; Lunstra et al., 1986) and rats (Kaler and Neaves, 1980) just after puberty; however humans exhibit a decline in Leydig cell number (Kaler and Neaves, 1978) while horses experience an increase in Leydig cells with advancing age (Johnson and Neaves, 1981).

Ultrastructure of the ovine Leydig cell has been described only recently by Lunstra and Shanbacher (1988). A list of references on Leydig cells of other mammalian species can be found in the review by Christensen (1975). Leydig

cells of rams contain a large oval or elongated nucleus, numerous mitochondria, abundant densely packed smooth endoplasmic reticulum (SER), a characteristic of steroid secreting cells, and well developed Golgi apparati. The overall arrangement of organelles within Leydig cells of rams is similar to those found in other vertebrates including humans. Variation in some details, like number of lipid droplets or amount of SER among species has been reported (Christensen, 1975). Lunstra and Shanbacher (1988) reported that the percentage of testicular volume occupied by Leydig cells in rams was 2.7%, the volume of an individual Leydig cell was 416μm^3 and the total number of Leydig cells per paired testes was 26.4×10^9.

Synthesis of testosterone and other androgens by Leydig cells requires cholesterol as a precursor. Cholesterol can be either taken up from the circulation or synthesized inside Leydig cells from acetate. Most of the cholesterol (60 to 85%) required for steroidogenesis is synthesized in the SER of the Leydig cell and stored as lipid droplets (Morris and Chaicoff, 1959, Weibin and Chaikoff, 1961). Blood cholesterol to be used in steroid synthesis by Leydig cells is thought to be derived from low-density lipoproteins (Freeman and Ascoli, 1983).

The conversion of cholesterol to testosterone occurs by several enzymatic reactions that include three hydroxylations, two dehydrogenations, one isomerization and two C-C cleavages

(Hall, 1988). Cholesterol is transformed into pregnenolone in the mitochondria after two hydroxylations and one C-C cleavage between C 20-22. Hydroxylations are important reactions that take place in the mitochondrias of Leydig cells and are catalyzed by enzymes called monooxygenases. These reactions require NADPH and molecular O_2, the latter needs to be activated by cytochrome P_{450}. All reactions involved in the transformation of pregnenolone to testosterone take place in the SER. Pregnenolone is converted into progesterone by an oxidation and an isomerization. Pregnenolone and progesterone are C-21 steroids. The conversion of progesterone into androstenedione, a C-19 steroid, proceeds with a 17-α hydroxylation and a C 17-20 cleavage. Testosterone is formed by reduction of the 17-keto group of androstenedione in a reversible reaction catalyzed by the enzyme 17-ß hydroxysteroid dehydrogenase. The rate limiting step in the synthesis of testosterone is the formation of pregnenolone from cholesterol (Stryer, 1988).

Leydig cells of stallions and boars synthesize estrogens in addition to androgens (Bedrok and Samuels, 1969; Raeside and Renaud, 1982; Allrich et al., 1983). Furthermore, estrogens are probably synthesized in the seminiferous tubules because aromatization enzymes necessary to synthesize estrogens from testosterone are found in these structures in several species (Stainberger et al., 1979) including sheep (Setchell, 1978).

Secretion of Testosterone. Leydig cells, like other steroid secretory cells, store minimal amounts of their synthesized products (Eik-Nes, 1975). In the ram and in males of other species testosterone is secreted into the blood in a pulsatile manner (Sanford et al., 1974; Ortavat et al., 1982; Hall, 1988). The secretion of testosterone is regulated mainly by LH (Courot, 1967; Amman and Shanbacher, 1983). Testosterone and the other androgens diffuse directly into the blood, lymph and seminiferous tubules (Lindner, 1969).

Biological Functions of Androgens. Androgens exert important functions in male reproduction. These hormones control the development and maintenance of functionality of internal and external genitalia, appearance of secondary sexual characteristics, stimulate the process of spermatogenesis, interact with the hypothalamus-pituitary axis and stimulate the development of the musculo-skeletal system (Reeves, 1987; Hall, 1988).

Inhibin. The existence of the hormone called inhibin, a non-steroid substance that regulates pituitary function was first proposed by McCullag (1932), based on his studies on the endocrine activity of the testis. Currently, it is known that inhibin is a protein hormone, apparently produced in males only in the Sertoli cells of the seminiferous tubules, that exerts an inhibitory action on synthesis and secretion of FSH

from the hypophysis by mechanisms that remain to be elucidated (Steinberger and Ward, 1988).

Spermatogenesis

The process of formation of spermatozoa is called spermatogenesis. Spermatozoa have a precise function to carry genetic information of the male to the oocyte.

Spermatogenesis is usually divided into two continuous phases: spermatocytogenesis and spermiogenesis. The first phase includes the transformation by mitotic divisions of the stem cell A_0-spermatogonia (2n) into other types of spermatogonia (A_1, A_2, A_3, A_4, intermediate and B) which then divide to form primary spermatocytes (2n). In this series of divisions each new type of germ cell divides to produce the succeeding cell type. Primary spermatocytes undergo a reductional division (meiosis) to form secondary spermatocytes (result of first meiotic division) which then divide without further DNA synthesis to form the aploid cells known as spermatids (Garner and Hafez, 1987). In the ram, three type A, one intermediate and 2 type B spermatogonial generations have been identified (Hochereau-de Reviers, 1987).

During spermiogenesis spermatids undergo condensation of the nuclear chromatin, formation of the tail and development of the acrosome to become fully formed spermatozoa. These cells are then released into the lumen of the seminiferous

tubules by a process known as spermiation (Garner and Hafez, 1987). Additional morphological and physiological changes occur in spermatozoa during their passage through the epididymis. These changes are collectively called maturation (Voglmayr, 1975). The final result of spermatogenesis is a population of highly specialized cells with a very peculiar form consisting of a flattened head that contains the nucleus of the cell and a tail necessary for cell motility.

Within any cross section of a seminiferous tubule, different cell types of the spermatogenic process form defined associations that appear one after the other over a particular period of time. The appearance of series of associations repeated in a cyclic manner during the reproductive life of the male. The time elapsed between the disappearance of one association and the appearance of the same association is called the cycle of the seminiferous epithelium. In sheep, the duration of this cycle is 10.4 days (Hochereau-de Reviers et al., 1987) and the number of associations of germ cells, based on the morphology of the acrosome in the developing spermatid is 12 (Setchel, 1978). The duration of spermatogenesis in the ram is 47 days (Amman and Shanbacher, 1983).

Daily Sperm Production. Daily sperm production is the number of sperm produced per day by the testes (Amman, 1970) and represents one way by which efficiency of spermatogenesis

can be evaluated in a particular individual or species. Daily sperm production can be estimated by determining the cycle of the seminiferous epithelium by quantitative histological analysis of the testicular parenchyma, from counting number of spermatids in testicular homogenates and from daily sperm output measured in repeated ejaculates (Amman, 1970).

Estimation of daily sperm production was 4.81×10^9 in Ile-de-France and 3.54×10^9 in Romanov sheep during the breeding season (Hocherau-de Reviers et al., 1990). Based upon data from the literature, Amann et al. (1976) concluded that daily sperm production is highly variable between species, however when it was expressed as daily sperm production per g of testicular parenchyma it became relatively constant (approximately 21×10^6 to 25×10^6 cells per day) for most species, except dairy bulls and stallions (12×10^6 and 16×10^6 cells per day, respectively).

Epididymal Sperm Reserves. After being released into the lumen of the seminiferous tubules, sperm cells are transported to the epididymis where they undergo a process called maturation (Voglmayr, 1975; Holtz and Smidt, 1976) and are stored in the cauda epididymis and proximal deferent duct (Amman and Shanbacher, 1983). Exact changes that sperm undergo during maturation are not completely understood, however during this process sperm cells develop progressive motility, undergo morphological changes which include

reorganization of tail organelles, nuclear chromatin and plasma membrane, and changes in metabolism; all factors resulting in the acquisition of fertilizing capacity (Bedford, 1975; Orbegin-Crist and Olson, 1984). The average time required for sperm cells to transit through the epididymis of different mammals is approximately 10 days (Robaire and Hermo, 1988). Sperm transit through the epididymis and epididymal sperm reserves were estimated to be 16.4 days and >165 x 10^9 cells respectively for Ile de France rams and 10.4 days and >135 x 10^9 cells, respectively for Suffolk rams.

Spermatozoa are stored in the cauda epididymis until ejaculation or for more than 30 days if they are not ejaculated (Orbegin-Crist et al., 1975). Older sperm cells are selectively destroyed and reabsorbed after degradation (Bedford, 1975). In sexually "rested" rams a great number of sperm can be found in the urine (Lino et al., 1967).

Daily Sperm Output. The number of ejaculated sperm that can be obtained per day is called daily sperm output (Amann and Shanbacher, 1983). This is an important characteristic because fertility is correlated with the number of sperm deposited by the male or by artificial insemination in the female reproductive tract. If an adequate number of ejaculations are collected at short and regular intervals for several weeks, daily sperm output becomes highly correlated with and approaches daily sperm production (Amann, 1970).

Daily sperm production in mammals are markedly different and Chang (1945) reported that daily sperm output in rams was approximately 8.6×10^9 cells.

Sperm cells are ejaculated as part of the semen. The other component of the semen is the seminal plasma, a fluid formed by the secretions of the accessory organs of the male reproductive tract. Seminal characteristics differ among domestic animals. In rams, the volume of the ejaculate is 0.8 to 1.2 ml; the concentration of sperm cells is 2×10^9 to 3×10^9 per ml; the percentage of motile sperm is 60-80% and the percentage of morphologically normal sperm is 80 to 95% (Garner and Hafez, 1987).

Regulation of Testicular Function

Steroidogenesis

Luteinizing hormone, sometimes called Interstitial Cell Stimulating Hormone (ICSH), stimulates synthesis and secretion of testosterone by Leydig cells (Brady, 1951; Hall and Eik-Nes, 1962; Connell and Eik-Nes, 1968; Eik-Nes, 1975). Luteinizing hormone promotes de novo synthesis of testicular steroids and has almost no effect on secretion of preformed steroids from the Leydig cells (Eik-Nes, 1975). In rats, Hall et al. (1979) demonstrated that LH stimulated the transport of cholesterol to the inner mitochondrial membrane where side-

chain cleavage of this molecule takes place.

Luteinizing hormone is a 28.3 kD glycoprotein (Sairam, 1983) synthesized and secreted by cells (gonadotropes) of the hypophysis (Pearce and Parsons,1981; Pierce, 1988). This hormone consists of two polypeptide chains or subunits, α and ß, that are glycosylated at specific residues and are internally cross-linked by disulfide bonds. The hormone is biologically active only when both subunits are present and actively interacting (Pierce and Parsons, 1981).

In the male, LH is secreted in pulses which induce a pulsatile secretory response in testosterone (Sanford et al., 1974; Ellis and Desjarnis, 1982; Ortavat et al., 1986; Hall, 1988). The biological significance of pulsatile secretion of testosterone is not understood (Ellis and Desjarnis, 1982).

The action of LH in Leydig cell function, is mediated by a specific receptor located in the plasma membrane to which the LH molecule binds with high affinity (Catt and Dufau, 1977). The LH receptor is a dimeric transmembrane glycoprotein with a molecular weight of approximately 90 kD (Rajaniemi et al., 1989; McFarland et al., 1989; Loosfelt et al.,1989).

Using cDNA techniques for sequencing proteins, McFarland et al. (1989) predicted that the LH receptor should consist of a 341-residue hydrophilic extracellular domain and a 333-residue region containing seven hydrophobic transmembrane segments; a total of 674 amino acid residues with a molecular

weight of 75 kD. Because of glycosylation of the extracellular domain of the natural receptor, its molecular weight was calculated to be 93 kD. Similarly, Loosfelt et al.(1989), by means of cDNA clones encoding porcine LH receptor, predicted a 333-residue extracellular domain, which precedes a 266-residue domain that displays seven possible transmembrane segments. However, they found three other types of clones corresponding to small proteins in which the transmembrane domain was absent. Furthermore, and demonstrating that there is not complete agreement about the structure of the LH receptor, Rajaniemi et al. (1989) predicted only one transmembrane segment.

The LH molecule probably binds to the extracellular domain of its receptor (McFarland et al., 1989) by mechanisms that are not completely understood. Rajaniemi et al.(1989), hypothesized that the LH receptor binds one hormone molecule and that the most important interaction of the gonadotropin with the receptor occurs through its α-subunit, after the ß-subunit recognizes the receptor. Katikineni et al. (1980) found that binding of Human chorionic gonadotropin (hCG), a hormone with LH-like activity, to rat testicular membranes progresses from a loose to a tight binding; they hypothesized that this change may represent the beginning of the degradation of the hormone-receptor complex.

Recently, it was reported that the LH molecule exhibited disulfide isomerase activity (Boniface and Reichert, 1990).

This activity is similar to the function of thioredoxin an enzyme that catalyzes the formation of disulfide bonds. This common function is probably due to the amino acid sequence, Cys-Gly-Pro-Lys, found in the ß-subunit of LH and the active site of thioredoxin. It was suggested that this catalytic activity may be involved in the generation of hormone-receptor complexes, and may be an explanation of irreversible hormone binding to receptors found by Katikineni et al. (1980).

Binding of LH to its receptor stimulates binding of GTP to the membrane. This results in the activation of specific membrane proteins that participate in the activation of adenylate cyclase, which in turn stimulates cAMP synthesis from ATP. Cyclic AMP, acting as a second messenger, stimulates the phosphorylation of Leydig cell proteins through the action of cAMP-dependent protein kinases. The phosphorylated proteins presumably stimulate synthesis and secretion of steroids (Catt and Dufau, 1977; Pierce and Parsons, 1981; Hunzicker-Dunn and Birnbauner, 1985).

McFarland (1989) found structural similarities of the LH receptor with the G Protein-couplet receptor family. In this group of receptors, the formation of the hormone-receptor complex activates many molecules of G-protein located in the plasma membrane which then activate adenylate cyclase (Gilman, 1987; Stryer, 1988). Other authors suggest that Ca^{++}/phospholipid-dependent protein kinase may be involved in the process of LH stimulation in addition to cAMP for protein

phosphorylation (Tähkä, 1986; Hall, 1988).

Besides the endocrine regulation of Leydig cell function by LH, other studies have demonstrated that paracrine and autocrine mechanisms may be involved in the control of Leydig cell function. There are data to support interactions of Leydig cells with seminiferous tubules (Bergh, 1982; Wilton and Kretser, 1984), macrophages (Bergh,1985) and lymphocytes (Guo et al., 1990). Interleukin-1, a protein secreted by macrophages, decreased hCG binding to Leydig cells, blocked the hCG-stimulated secretion of testosterone and synthesis of cAMP in rat testicular tissue in vitro (Calkis et al., 1988). Similarly, Interleukin-2, a protein secreted by T lymphocytes, has a potent inhibitory effect on Leydig cell steroidogenesis through the inhibition of cAMP formation and the conversion of several androgen precursors to testosterone (Guo et al., 1990). It was also found that interferon gamma inhibits steroidogenesis in cultured porcine Leydig cells (Orava et al., 1989).

Regulation of the number of LH receptors is exerted mainly by LH itself and probably by other hormones (prolactin, FSH, estrogen) and other unknown factors of Sertoli cell origin (Tähkä, 1986). Increasing doses of LH, above biological concentrations, first induced an increase in number of receptors (up-regulation) and then a reduction (down-regulation) in LH receptors number. Down-regulation is usually related to an impairment of the Leydig cell to secrete

testosterone with additional hormone stimulation, a phenomenon also referred to as desentization (Dufau et al., 1979; Dufau et al., 1984). The block in steroidogenesis in desentisized Leydig cells is produced beyond adenylate cyclase activation and cAMP and pregnenolone formation. Presumably this block occurs in the conversion of 17α-hydroxyprogesterone to androgens precursors (Cigorraga et al., 1978; Dufau et al., 1979).

In human females, LH receptors also interact with hCG. It is now recognized that LH and hCG are hormones with similar chemical structure (Pierce and Parsons, 1981). They bind to the same receptor by similar mechanisms in both males and females and elicit qualitatively equivalent biological responses (Eik-Nes, 1975; Pierce and Parsons, 1981; Strickland et al., 1985; McFarland et al., 1988; Rajaniemi et al., 1988). However, studies performed in male rats and rams showed that even when the biological responses induced by LH and hCG are comparable the behavior of each hormone during its biochemical interaction with the receptor appears to differ. Hutaniemi and Catt (1981) found a higher association constant (K_a) of rat testicular receptors for hCG than for hLH or oLH, although the quantitative binding for the three hormones were similar. Contrary to these results, Sairam et al. (1988) reported similar affinities but markedly different capacities for oLH and hCG in testicular membrane preparations of rams.

Spermatogenesis and Sertoli Cell Function

Spermatogenesis is controlled primarily by the endocrine system. Two hormones are necessary for spermatogenesis; FSH stimulates and maintains the functions of Sertoli cells and testosterone, which is necessary for spermatid maturation and Sertoli cell function (Garner and Hafez, 1987; Bardin et al., 1988; Sun et al., 1990). Luteinizing hormone is required indirectly because it stimulates Leydig cells to synthesize and secrete testosterone that diffuses into the seminiferous tubules to exert its actions on spermatogenesis. Sun et al. (1990) found that the process of transformation of round to elongated spermatids was mainly testosterone dependent with limited effects of pituitary factors. The levels of testosterone necessary for this purpose in the rat was only 10-15% of those observed in normal rats.

A high correlation was found between total number of A1 spermatogonia and Sertoli cells, which was interpreted as the existence of important influences of Sertoli cell population on sperm production (Hochereau-de Reviers et al., 1987). Therefore, multiplication of Sertoli cells and establishment of an adequate population was considered important for sperm production.

The arrangement of Sertoli cells in the seminiferous tubules where they make contact to each other and to germ cells contributes to the organization and cohesion of the

seminiferous epithelium and the coordination of spermatogenesis. Some metabolites reach and leave the sperm cell by passing through the Sertoli cells which is facilitated by the closeness between both types of cells. Furthermore, Sertoli cells can reabsorb the residual bodies of spermatids and germ cells undergoing degeneration by phagocytosis (Courot et al., 1970).

The junctional contacts between Sertoli cells assist in the formation of the blood-testis barrier which makes the fluid of the seminiferous tubules and rete testis different from lymph and blood (Fawcett, 1975). The blood-testis barrier acts excluding molecules in such a manner so as to protect and isolate developing germ and sperm cells from mutagenic agents and the immune system (Bardin et al., 1988). Most proteins present in the fluid of the seminiferous tubules are secreted by Sertoli cells. These proteins and other compounds secreted by Sertoli cells provide the proper environment for the development of germ cells (Mather et al., 1983).

Sertoli cells are thought to secrete up to 100 proteins (Bardin et al., 1988) including testes specific proteins and some proteins that are normally found in serum (e.g., transferrin, Wright et al., 1981). Androgen binding protein (ABP) and inhibin are the best known proteins secreted by Sertoli cells. The function of inhibin was reviewed previously. Androgen Binding Protein has a molecular weight

of 85 kD and exhibits androgen-binding activity. It has been postulated to act in the following manner: a) as a carrier of testosterone within the Sertoli cell and from the testis into the epididymis, and b) to maintain high concentrations of androgens in the seminiferous tubules and epididymis necessary for sperm development (Bardin et al., 1988).

Other compounds secreted by Sertoli cells are growth factors (Feig et al, 1980), plasminogen activator (Lacroix et al., 1977), steroids (Welsh and Wiebe, 1978) and several others that may play a role in paracrine and autocrine regulation of testicular function (Tähkä, 1986).

Follicle stimulating hormone, like LH, is a glycoprotein consisting of an α and ß subunits with a molecular weight of 29 kD (Pierce, 1988). This hormone binds to its receptor located in the membrane of the Sertoli cell and stimulates the adenylate cyclase cascade which results in phosphorylation of proteins involved in the activation of a particular Sertoli cell functions (Bardin et al., 1988). The participation of Ca^{++} as a second messenger working through calmodulin for phosphorylation, has also been proposed as another mechanism of action of FSH on Sertoli cells (Means, 1981).

Factors that control Sertoli cell funtions are numerous and include FSH, insulin, insulin-like growth factors, glucagon, calcitonin, androgens, estrogens, progesterone, several vitamins and pro-opiomelanocortin-derived peptides (Bardin et al., 1988). These numerous factors, besides the

paracrine effects from other components of the testicle, make the mechanisms of control of Sertoli cell functions highly complex and not well understood. Recent findings on paracrine regulation of Sertoli cell function indicate that mRNA for the ß-subunit of inhibin changes according to the state of the cycle of the seminiferous epithelium, which suggested that certain germ cell factors may alter gene expression in Sertoli cells (Bhasin et al., 1989). Luteinizing hormone may also influence expression of the Inhibin ß-subunit gene (Krummen et al., 1990).

Hypothalamic-Hypophyseal-Testicular Axis

The two gonadotropins, LH and FSH, that play major roles in control of testicular function are synthesized and secreted by the adenohypophysis. The hypothesis that the pituitary controls testicular function was proposed about 50 years ago and it was confirmed in sheep by Courot (1967) who prevented testicular regression by injecting pituitary extracts that contain LH and FSH into hypophysectomized rams.

Gonadotropin secretion is controlled by the central nervous system through the hypothalamus and by feedback mechanisms from the testicles. Environmental factors also influence gonadotropin secretion by way of the central nervous system (Fink, 1988).

Neurons from different parts of the hypothalamus, secrete

a number of peptides that control pituitary function (Guillemin, 1978). Among them is a decapeptide called Gonadotropin-releasing hormone (GnRH, Matsuo et al., 1971). Gonadotropin-releasing hormone is released in a pulsatile manner into the hypophyseal portal vessels (Fink and Janieson, 1976; Levine et al., 1982; Carty and Locatelli, 1988) and is transported to the adenohypophysis where it stimulates the synthesis of LH and FSH and the release of pulses of LH and the slow and gradual release of FSH by the same pituitary cells (Fink, 1988). Gonadotropin releasing hormone appears to act on gonadotropes by altering intracellular Ca^{++} and the phosphatidylinositol cascade (Mason and Waring, 1985).

Androgens exert a negative feedback on secretion of LH and FSH (D'Occhio et al., 1982) and inhibin inhibits secretion of only FSH (Stainberger and Ward, 1988). The negative feedback of testosterone apparently works by inhibiting binding of GnRH to its receptors on the gonadotropes (Clayton and Catt, 1981; Kitahara et al., 1990) and by inhibiting release of GnRH from the hypothalamus (Levine and Duffy, 1988; Caraty and Locatelli, 1988). Endogenous opiods modulate the secretion of LH probably by acting at the level of the hypothalamus. It was found that naloxone, an opiod antagonist, produces an increase in serum LH in males and females (Cicero et al., 1979; Ebling and Lincoln, 1985). Other studies have demonstrated that alpha-adrenergic receptor stimulation can induce LH release and that there is a

synergistic interaction between the opiod and the adrenergic systems on modulating GnRH release (Barraclough and Wise, 1982).

Selection for Reproductive Rate and Testicular Function

Bradford (1972) considered the following characteristics as components of reproductive rate in female sheep: age at puberty, frequency of parturition, ovulation rate, litter size, survival of lambs and length of reproductive life. All of them have physiological components that work and are controlled by complex molecular mechanisms which are ultimately dictated by the genome.

When selection for or against female reproductive rate characteristics was performed a number of positively correlated changes were reported to occur in certain reproductive traits in males. Land (1973), found correlated responses in testis weight while selecting for ovulation rate in mice and positive correlations between testis diameter and prolificacy in sheep. He hypothesized that many aspects of male and female reproduction may be partially controlled by the same physiological factors (Land, 1974) and also postulated that it should be possible to select indirectly for female reproductive rate based upon male reproductive characteristics, particularly gonadotropin levels or those traits affected by these hormones. Bindon and Turner (1974)

come to similar conclusions after finding differences in LH concentrations in Merino ram lambs from lines of different prolificacy. Theoretically, increased reproductive rate in females could be obtained indirectly by selection applied to highly genetically correlated male reproductive characteristic (Walkey and Smith, 1980).

The hypothesis that common physiological mechanisms control reproductive rate characteristics in males and females has been partially confirmed by other studies. Higher LH concentrations were found in males from breeds of high prolificacy relative to males from other breeds of lower prolificacy (Thimonier and Pelletier, 1972; Land and Carr, 1975; Carr and Land, 1975).

Similar studies with lines of sheep in which females had been selected for different reproductive rates, did not reflect correlated changes in male reproductive characteristics. For instance, Booroola and control Merino rams did not differ in total daily sperm production and LH and FSH serum concentrations (Bindon et al., 1985) and secretion of testosterone after stimulation by pregnant mere serum gonadotropin (Hochereau-de Reviers et al., 1990). Furthermore, Rambouillet rams from lines in which females were selected for high or low reproductive rates did not differ in scrotal circumference, paired testicular weight, paired testicular volume, paired epididymal weight, epididymal sperm reserves or libido characteristics (Curry, 1990).

Correlated changes in female sex-limited traits after selection for or against male reproductive characteristics have also been studied. Haley et al. (1989) selected ram lambs for high and low LH and FSH response to GnRH injection. They found that females from the selection lines exhibited positive correlated responses in LH and FSH secretion after GnRH injection, ovulation rate and number of lambs born during the first breeding season of thier productive life. In a recent report by Haley et al. (1990), selection for high or low testicular size, produced only a small positive correlated indirect response to selection in litter size per ewe mated and genetic correlations close to zero in ovulation rate and litter size per ewe lambing.

Results presented in this review indicate that the hypothesis of the common mechanisms of control of reproductive processes in males and females has been confirmed by some studies particularly those related to LH concentrations. It appears that males and females of breeds or lines of sheep of different prolificacy, also differ in serum LH concentrations. However, when male reproductive characteristics on which LH can exert direct or indirect actions, were investigated, a decrease in relationship was observed between male reproductive characteristics and female reproductive rate.

STATEMENT OF THE PROBLEM

Lines of sheep of different reproductive rates have been produced by selection based upon female sex-limited characteristics over a certain number of generations. Indirect effects of this type of selection have been observed in males who have shown correlated changes in reproductive traits such as scrotal circumference, testes size and serum LH concentrations. However, there is little or no information on changes of LH receptors, testicular histomorphological characteristics and in vitro or in vivo testosterone secretion.

In vitro techniques to evaluate endocrine characteristics of testicular parenchyma have the advantage of the separation of the tissue from the confounding effects of extra-testicular factors that normally influence serum hormonal concentrations. In vitro techniques have not been used in rams to evaluate differences in capacity of testosterone secretion or sensitivity of the testicular parenchyma to secrete testosterone in response to a gonadotropin challenge.

A practical application of this knowledge may be the contribution to the finding of a genetically correlated male reproductive trait with a female characteristic of reproductive rate that can be used as a selection criterion for faster improvement of reproductive efficiency in sheep.

Objectives of this study were to determine if selection

for or against female reproductive rate in Rambouillet sheep altered the following reproductive characteristics of male offspring: 1) capacity and sensitivity of testicular parenchyma to secrete testosterone in vitro after stimulation by LH or hCG, 2) capacity and affinity of the LH/hCG receptor, 3) histological components of the testicular parenchyma, 4) scrotal circumference and testes weight, and 5) semen characteristics.

MATERIALS AND METHODS

Trial 1

Animals and Husbandry

Trial 1 of this study was performed during the winter of 1989. Twenty two-month-old Rambouillet rams from lines that have been selected for high (HL) and low (LL) reproductive rate and from a random-bred control line (CL) were housed at the Fort Ellis Experiment Station of Montana State University, Bozeman. During the experiment all animals were kept in a pen and had free access to a mix of alfalfa and grass hay, water and mineralized salt supplement.

By the time this trial was performed the lines had been selected for approximately 19 years. Details of the selection procedure and management of these lines have been described by Shoenian and Burfening (1990).

Orchiectomy

Seven HL, 7 LL and 9 CL rams were allocated randomly to be orchiectomized at 3 successive times over the experimental period, so that similar number of rams from each line were orchiectomized at the beginning, middle and end of the experiment.

Just before orchiectomy, the scrotum of each ram was shaved and the testis pushed toward the bottom of the scrotum to measure scrotal circumference at the widest point.

Rams were bilaterally orchiectomized under a surgical plane of anesthesia induced and maintained with thyamylyl sodium (4% in 0.9% sterile saline; Biotal^{R}, Bio-Ceutics Laboratories, Inc, St. Josephs, Mo). Immediately after orchiectomy, the testes were transported to the laboratory in a prewarmed styrofoam container. Weight of each testicle was recorded after removal of the epididymis and tunica albuginea.

In Vitro Stimulation of Testosterone Secretion

Tissue Processing. Testicular parenchyma was processed for in vitro stimulation of testosterone secretion using the procedure of Berardinelli et al. (1989) which was developed and validated in this laboratory. An additional component for this validation is presented in Appendix I.

Briefly, testicular parenchyma from the left testis was bisected longitudinally and thin strips (0.5cm wide and 0.5cm deep) were excised from the caput to the cauda. Strips were cut into small pieces and finely minced and macerated with scissors in a petri dish and then placed on a warm plate at 34°C. Aliquots of 250 mg of macerate were weighed and transferred to sterile-prewarmed 25 ml erlenmeyer flasks to which 10 ml of Tissue Culture Media 199 (ME 199, Cellgro^{TM},

Mediatech, Wash. DC) with sodium bicarbonate and buffered with HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid; Fisher Scientific, NJ) to pH 7.00 was added. Each flask was gently mixed and placed immediately into a Dubnoff metabolic shaker.

Incubations and Tissue Rinsing. All incubations were performed under a O_2:CO_2 (95% : 5%) atmosphere in a Dubnoff metabolic shaker set at 34°C and 90 cycles·min^{-1}.

Before the start of the four hours of incubation, when the parenchymal tissue was actually evaluated for testosterone secretion, ME 199 of each flask was changed three times (tissue rinsing) after periods of 15 min of incubation. Tissue rinsing was found to be necessary because it appeared to remove residual testosterone and gonadotropins present in the fluid accompanying the tissue mince that could interfere with the experimental stimulation of testosterone secretion and its evaluation (Berardinelli et al., 1989).

Doses of hCG and Sampling. Ten minutes after the third tissue rinse hCG (US Biochemical Corp., Cleveland, OH; cat. no. 13662, 5,000 IU per vial) was added to the flasks. Doses of hCG were: 0, 0.0025, 0.025 and 2.5 IU per ml of ME 199. Doses were prepared immediately before use, evaluated in quadruplicate and added to each flask in 100μl of ME 199.

Samples (300μl) of ME 199 were taken from each flask at

0 (just before addition of hCG), 1, 2, 3 and 4 h of incubation for assay of testosterone. Samples were kept frozen at -20°C until they were assayed for testosterone.

Testosterone Assay. Testosterone was assayed without extraction by a single antibody RIA validated in this laboratory (Berley et al., in press) and modified for testosterone determination in ME 199. Testosterone antiserum (rabbit) was purchased from Calbiochem (Lajolla, CA) and used at the dilution of 1:750. Tritiated testosterone was obtained from New England Nuclear. Antibody bound and free testosterone were separated by dextran-coated charcoal. Sensitivity of the assay was 9 pg and the inter- and intra-assay coefficients of variation for samples that inhibited binding at 29% and 75% were 4.5 and 9.2%, respectively.

Trial 2

Animals and Husbandry

Twenty five Rambouillet rams, 22 months of age, from the same selection lines as those described for Trial 1, were used for Trial 2 which was performed during the winter of 1990. Management of animals for this part of the study was the same as that described for Trial 1.

Semen Collection and Evaluation

Rams were trained for semen collection with an artificial vagina during the last week of November, 1989. Two ejaculates were collected at 3-week interval (first and fourth week of December, 1990) from rams that responded to training. Immediately after collection volume, color, contamination and odor of each ejaculate were recorded. Percentage of progressively motile sperm, percentages of live and dead sperm, concentration of sperm cells and percentages of morphologically normal sperm and sperm cells with head and tail abnormalities were estimated in each ejaculate using standard procedures outlined in Hafez (1987).

Orchiectomy, Tissue Processing and
In Vitro Testosterone Secretion

Scrotal circumference was measured as in Trial 1 and tone of each testis determined with a tonometer (Lane MGF. Inc. Denver, CO). Procedures for orchiectomy, determination of testis weight, processing of parenchymal tissue and in vitro stimulation of testosterone secretion were the same as those described in Trial 1 with the following modifications. After orchiectomy the right testis was fixed by perfusion for histomorphometric evaluations. One longitudinal half of the left testis was frozen at -20°C for determinations of LH/hCG

receptor characteristics and the other half was processed for in vitro stimulation of testosterone secretion. In Trial 2, only the weight of the left testis was obtained. Time from orchiectomy to the beginning of tissue rinsing was 1 h. This period was found to be very important to obtain a proper response of testosterone secretion to gonadotropin. Details of the effect of time after orchiectomy on testosterone secretion after stimulation of LH in vitro are given in Appendix I.

Both hCG and oLH (NIDDK-oLH-I-3, Bethesda, MD) were used to stimulate testosterone secretion by testicular parenchyma. Doses of hCG were the same as those used in Trial 1. Doses of LH were 0.13, 1.3 and 130 $ng \cdot ml^{-1}$ of ME 199. These doses of LH were equivalent to hCG doses on a molecular basis using the molecular weight of oLH as 28.3 kD and that of hCG as 37 kD (Sairam et al., 1988) and the equivalence of 15,000 IU of hCG per mg of the hormone. Thus, the concentrations of gonadotropin tested were 0, 4.5, 45.9 and 4,594.6 pM.

Because of the possibility of inhibition of testosterone secretion by high doses of LH, an additional dose of 13 $ng \cdot ml^{-1}$ of ME 199 was included to ensure maximum stimulation of testosterone secretion.

Testis Perfusion, Fixation and Embedding

The right testis of each ram was perfused immediately

after orchiectomy by a procedure modified from Glauert (1975). Perfusions were performed at room temperature through the testicular artery at a point close to the caput epididymis with a buffered perfusion fixative consisting of 3% glutaraldehyde:1% formaldehyde in 0.075 M cacodylate buffer.

The perfused testis was then cut into 3 parts; top, middle and bottom. Four 2 x 2 x 4 mm pieces were obtained from each part and fixed overnight at 4°C by immersion in buffered 2% glutaraldehyde solution. The pieces were then postfixed in buffered 1% OsO_4 fixative for at least 2 h, dehydrated in ethanol and embedded in araldite epoxy resin 502. Additional details of perfusion, fixation and embedding are presented in Appendix II.

Histomorphometry

Five thin sections (1 μm) were cut from each block of embedded tissue and stained with toluidine blue and basic fuchsin according to a modified procedure from Hoffmann et al. (1983).

Two sections from the five obtained from each block were chosen at random and photographed at 125X magnification using a light microscope. Details of two representative areas (one simple and one complex) of interstitium within each area included in the 125X pictures were then photographed at 1250X magnification.

Areas occupied by seminiferous tubules, interstitium and cross sectional areas (round sections) of seminiferous tubules were determined in the 125X photomicrographs. These measurements were used to estimate the percentages of the volume of the testicle (volume percentages) occupied by seminiferous tubules and interstitium (Lunstra and Shanbacher, 1988) and the seminiferous tubule diameters, respectively (Appendix III).

Areas occupied by Leydig cells with and without nuclei, vascular tissue and other structures of the interstitium were measured in the 1250x photomicrographs. These determinations were used to estimate the volume percentages of each of these type of structures in the testis (Lunstra and Shanbacher, 1988). Cross sections of Leydig cell nuclei were also determined in the 1250X photomicrographs.

Areas were measured directly from photomicrographs with a digitizer pad (Kurta^{R} IS/ONE, Phoenix, AZ) and calculations were performed by a computer using the program Sigma-Scan^{TM} (Jandel Scientific, Corte Madera, CA).

Leydig cells and Leydig cell nuclei were assumed to be round and their volumes were obtained by first calculating the diameter from the cross sectional area ($A=\pi r^2$), adjusting the diameter according to Abercrombie (1946) and then using this corrected value for the calculation of the volume ($V=4\pi r^3/3$).

Number of Leydig cell per g of parenchyma was calculated by dividing the total volume of Leydig cells in 1 g of

parenchyma by the Leydig cell volume (Allrich et al., 1983). A similar procedure was employed to calculate the number of Leydig cells per testis. Volumes of parenchyma were calculated from weights using a specific gravity of 1.04. Although specific gravity of the testicular parenchyma of each ram was determined an average value was used for all calculations of testicular volume because specific gravity did not differ among lines. Other details on histomorphometric techniques and calculations are presented in Appendix III.

Radioreceptor Assay

Preparations of Labeled Hormones. Iodination grade oLH (NIDDK-oLH-I-3, Bethesda, MD) and hCG (hCG #CR-125, NICHD, NIH, Bethesda, MD) were labeled with iodine-125 (^{125}I, New England Nuclear) by the lactoperoxidase method (See Appendix IV) and purified by gel chromatography in Sephadex G-75 columns (Pharmacia, Uppsala-Sweden). Hormones were eluted from the columns with 25 mM Tris-HCl (pH 7.5), containing 10 mM of $MgCl_2$ and 1 $mg \cdot ml^{-1}$ of bovine serum albumin (BSA). Elution fractions were tested for trichloroacetic acid precipitable protein and only those fractions that had at least 60% of the total radioactivity in the precipitate were used for the radioreceptor assays. The specific activity of each preparation was calculated based upon the percentage of incorporation determined by trichloroacetic acid protein

precipitation and were 27.4 $\mu Ci \cdot \mu g^{-1}$ for ^{125}I-oLH and 38.9 $\mu Ci \cdot \mu g^{-1}$ for ^{125}I-hCG.

Testicular Membrane Preparation. Testicular membranes were prepared by a modified procedure adapted from Sairam et al. (1988). Briefly, frozen testicular parenchyma was thawed overnight at 4°C. All further processing of membrane preparations and receptor assay were performed at 4°C. Approximately 25 g of testicular parenchyma was weighed, cut into small pieces with scissors, homogenized using a Polytron^R homogenizer (Brinkmann Instruments Co., Switzerland) and then filtered through four layers of gauze. Homogenizations were carried out in 50 ml of 25 mM Tris-HCl buffer (pH 7.5) containing 25 mM of sucrose.

The filtrate was centrifuged initially at 500 x g for 15 min, then the supernatant was recovered and centrifuged at 30,000 x g for 30 min. The pellet that was obtained in this centrifugation step was resuspended in 25 mM Tris-HCl buffer (pH 7.5) and centrifuged again at the same speed. Pellets were resuspended in the 25 mM Tris-HCl buffer containing 10 mM of $MgCl_2$. Protein concentrations of membrane suspensions were determined with the BCA^R Protein Assay (Pierce, Rockford, IL), using BSA as standard.

Binding Assays. Increasing amounts (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 24 ng) of ^{125}I-oLH or ^{125}I-hCG were

incubated in triplicate for 20 to 22 h with testicular membranes equivalent to 1.5 mg of protein and assay buffer for a final volume of 500 μl per tube. The assay buffer was 25 mM Tris-HCl (pH 7.5) containing 10 mM of $MgCl_2$ and 1 mg per ml of BSA. None specific binding (NSB) was determined in the presence of 1 μg or 0.5 μg of unlabeled hCG or oLH per tube, respectively.

Binding of hormone and receptor was stopped by addition of 2 ml of cold assay buffer and centrifugation at 3,000 x g for 20 min at 4°C. Radioactivity was determined in the pellets (counts bound) and supernatants (counts free) in a Packard 5160 auto-gamma scintillation spectrometer with an efficiency of 58%.

Maximum binding capacity of each labeled hormone preparation used in the assays was assessed by incubating 0.06 to 0.5 ng of each preparation with an excess of sheep testicular membrane preparations and were 17.2% for ^{125}I-oLH and 21.3% for ^{125} I-hCG. All binding assays were performed with a single preparation of each labeled hormone.

Estimation of Receptor Characteristics. Receptors binding capacity and dissociation constants (K_d) were estimated by Scatchard plot analysis using the computer program ENZFITTER (Elsevier-BIOSOFT, Cambridge, UK). For the purpose of these analyses, counts of radioactivity specifically bound were obtained by subtracting the NSB counts

from the counts bound (counts in the pellets). Specifically bound radioactivity was transformed into ng of hormone using the specific activity and the equivalence of 1 μCi = 2.2 x 10^6 dpm. Free hormone was calculated by subtracting the amount (ng) of hormone specifically bound from the total amount (ng) of hormone added to the assay tube. Molar concentrations of free and bound hormone were estimated using the molecular weights for oLH and hCG given above.

Statistical Analysis

Data for seminal characteristics, body weight, scrotal circumference, testes weight, testis tone, testis volume, testicular histomorphological characteristics, oLH and hCG receptor characteristics and testosterone concentrations were analyzed by separate analyses of variance using the General Linear Model procedure of SAS (SAS, 1987). Means were compared by the PDIFF procedure of SAS (SAS, 1987). The model for the analyses of characteristics other than testosterone concentrations included only line. A preliminary analysis of variance was performed for testicular histomorphological characteristics with line, animal within line, location (top, middle or bottom of the testis) and line by location in the model. Location did not influence ($P<.10$) any histomorphological characteristic, therefore observations from the top, middle and bottom of the testis for each

histomorphologic characteristic were pooled.

Data for testosterone secretion per h per g of testicular parenchyma were analyzed by a split-split-plot design for each gonadotropin, oLH or hCG. For the analysis of testosterone secretion after hCG stimulation, data of Trials 1 and 2 were pooled because a preliminary analysis showed that trial did not influence ($P<.10$) testosterone secretion. The observations of testosterone concentrations were the averages of the 4 replicates for the combinations of dose by time within each animal. For the analyses the main plot was line, the sub-plot included dose and dose by line interaction and the sub-sub-plot included time, time by line, time by dose and time by dose by line interactions. The error term to test line was animal within line and the error term to test dose and the interaction of dose by line was animal within line by dose.

Testosterone secretion per h per g of testicular parenchyma was also analyzed using a model that included both gonadotropins in a split-split-plot design. The main plots were line, gonadotropin and line by gonadotropin, which were tested with animal within line by gonadotropin as the error term. The sub-plot included dose, line by dose, gonadotropin by dose and line by gonadotropin by dose. These effects were tested with the animal within line by gonadotropin by dose as the error term. The sub-sub-plot included time and the interactions of line by time, time by gonadotropin, time by

dose, line by time by dose, time by gonadotropin by dose and time by gonadotropin by line. Doses 0 and 13 ng for oLH were not included in the analysis because dose 0 was not tested within each gonadotropin and there was no dose of hCG equivalent to 13 ng of oLH.

Total testosterone secretion per g of testicular parenchyma was calculated by summing testosterone concentrations at each h of incubation. Total testosterone secretion for each gonadotropin was analyzed by analysis of variance for a split-plot design. The main plot was line and the sub-plot included dose and the interaction of line by dose. Line was tested with animal within line as the error term. This characteristic was also analyzed with a model that included gonadotropin. The main plots included line, gonadotropin and the line by gonadotropin interaction, which were tested with animal within line by gonadotropin as the error term, and the sub-plot included dose and the interactions of line by dose, gonadotropin by dose and line by gonadotropin by dose. Proportions of rams whose testicular parenchyma exhibited stimulation to gonadotropin were analyzed by contingency Chi-square (Steel and Torrie, 1960). Rams were considered "responders" if they exhibited an increase in testosterone by the second, third or fourth h compared to the first h of incubation. Rams that did not respond using this criterion were not included in the analysis of testosterone secretion.

Capacities and K_d's of oLH and hCG binding to their receptors were compared within each line by paired t-tests using MSUSTAT (MSUSTAT, 1985). Correlations of total testosterone secretion with receptors and histomorphological characteristics and testis weight, and those of total testosterone secretion per Leydig cell with number of receptors per Leydig cell were calculated using the CORR procedure of SAS (SAS, 1987).

RESULTS

Seminal Characteristics

Percentage of progressively motile sperm was higher ($P<.05$) in HL and CL rams compared to LL rams and did not differ ($P>.10$) between HL and CL rams (Table 1). Semen volume, percentages of live and dead sperm, sperm concentration per mm^3 and total ejaculated sperm did not differ among lines ($P>.10$).

Table 1. Least squares means of seminal characteristics of Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates or from a random-bred control line (CL).

Line	Semen volume (ml)	Sperm motility (%)	Live sperm (%)	Normal sperm (%)	Sperm cells per mm^3 ($x10^6$)	Total sperm cells ($x10^9$)
LL (n=3)	1.0	43.3[a]	57.5	72.8	4.150	4.657
HL (n=6)	1.0	70.0[b]	63.4	80.8	4.008	4.169
CL (n=6)	1.2	68.3[b]	66.4	80.4	5.127	4.608
SE[c]	0.29	22.4	16.4	16.7	1.125	2.0

[a,b]Means with different superscripts within each column differ ($P<.05$).
[c]Pooled standard error.

Scrotal Circumference and Gross Testicular Characteristics

Scrotal circumference did not differ ($P>.1$) among lines

in both trials (Tables 2 and 3). Paired testes weight tended (P<.09) to be heavier in HL rams than in LL rams while paired testes weight of CL rams was intermediate between weights of the other two lines in Trial 1 (Table 2). In Trial 2 left testis weight did not differ (P>.10) among lines (Table 3). Tones of the right and left testis did not differ (P>.10) among lines (Table 3). Testicular tone was evaluated as a measurement of testicular hardness or turgidity.

Scrotal circumference was positively correlated (P<.01) with left testis weight (Trial 2) in the LL and CL rams, but only moderately correlated in the HL rams. Left testis weight and body weight were not correlated (P>.10) in the HL, however the correlations between these two characteristics were moderately positive in the LL an CL rams (Table 4).

Table 2. Means for scrotal circumference (SC), left testis weight (LT), right testis weight (RT) and paired testes weight (PTW) of Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-breed control line (CL) in Trial 1.

Line	n	SC (cm)	LT (g)	RT (g)	PTW (g)
LL	7	24.8	85.2[b]	86.5	171.8[b]
HL	7	26.7	104.6[a]	105.1	209.7[a]
CL	9	25.6	92.3[b]	93.7	186.3[b]
SE[c]		1.6	14.8	16.8	11.4

[a,b]Means with different superscripts differ (P<.09).
[c]Pooled standard error.

Table 3. Least squares means for scrotal circumference (SC), left testis weight (LTW) and testes tones of Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL) in Trial 2.

Lines	n	SC (cm)	LTW (g)	Left testis tone	Right testis tone
LL	8	26.2	94.6	1.7	1.6
HL	7	25.2	88.1	1.6	1.7
CL	13	26.6	91.8	1.6	1.6
	SE[b]	1.9	23.6	0.18	0.14

[b]Pooled standard error.

Table 4. Correlation coefficients for testis weight (TW), body weight (BW) and scrotal circumference (SC) of Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL).

	LL		HL		CL	
Trait	TW	BW	TW	BW	TW	BW
SC	0.93**	0.16	0.68	0.50	0.83**	0.77**
TW		0.40		-0.07		0.59

**P<.01

Histomorphology of Testicular Parenchyma

Testicular parenchyma of LL and CL rams tended to have lower (P<.08) volume percentages of Leydig cells than HL rams, whereas, volume percentage of vascular tissue was higher (P<.05) in the LL rams than in HL and CL rams. Volume percentages of vascular tissue did not differ (P>.10) between

HL and CL rams. Volume percentages of seminiferous tubules, interstitium or components of the interstitium other than Leydig cells and vascular tissue did not differ (P>.10) in rams among these lines (Table 5).

Table 5. Least squares means for testis volume and volume percentages of seminiferous tubules (STUB), interstitium (INTERS), vascular tissue (VASCT), Leydig cells and other components of the testicular interstitium (OCOMP) of Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL).

Lines	Testis volume (cm^3)	STUB	INTERS	VASCT	Leydig cells	OCOMP
LL (n=8)	91.0	80.7	19.3	4.6[c]	2.3[a]	12.5
HL (n=6)	85.2	81.6	18.4	3.6[d]	2.7[b]	12.0
CL (n=7)	86.5	82.2	17.8	3.6[d]	2.2[a]	11.8
SE[e]	24.8	3.4	3.4	0.8	0.5	2.7

[a,b]Means with different superscript differ (P<.08).
[c,d]Means with different superscripts differ (P<.05).
[d]Pooled standard error.

Seminiferous tubule diameter did not differ among rams of these lines (P>.10). Least square means were 169.6, 178.2 and 182.2 μm and the number of seminiferous tubules measured to evaluate this characteristic were 158 ± 76, 160 ± 31 and 149 ± 26 for the LL, the HL and the CL rams, respectively.

Volume of Leydig cell nucleus, total volume of Leydig

cells in the testis, number of Leydig cells per g of testicular parenchyma and total number of Leydig cells per testis did not differ (P>.10) among lines. Even though Leydig cell diameter did not differ (P>.10) among lines, Leydig cell volume was higher (P<.08) in HL rams relative to LL and the CL rams, respectively (Table 6).

Table 6. Least squares means for Leydig cell characteristics in Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL).

Line	Leydig cell diameter (μm)	Leydig cell volume (μm^3)	Leydig cell nuclei volume (μm^3)	Total volume of Leydig cells (cm^3)	Number of Leydig cells per testis ($x10^6$)	Number of Leydig cells per g testis ($x10^3$)
LL (n=8)	12.2	943.5[a]	207.5	2.0	2,129	23,240
HL (n=6)	12.7	1083.6[b]	226.5	2.3	2,210	24,887
CL (n=7)	12.0	917.5[a]	205.9	1.9	2,015	23,813
SE[c]	0.7	151.8	28.3	0.6	574	6263

[a,b]Means with different superscripts differ (P<.08).
[c]Pooled standard error.

Testicular oLH/hCG Receptors

The Scatchard analyses for binding of both oLH and hCG to testicular membrane receptors indicated the presence of only one set of receptors. Only for three rams was it found that oLH binding could be fitted to a "one ligand, two

binding-group" model. The K_d's for the oLH receptor did not differ (P>.10) among lines, but the K_d for the hCG receptor was higher (P<.06) in the CL relative to LL and HL rams. Values of K_d's did not differ (P>.10) between LL and HL rams.

Capacities of the receptors for both gonadotropins did not differ (P>.10) among lines when it was expressed as a function of mg of membrane protein or whole testis (Table 7). The oLH receptor capacity per g of testicular parenchyma tended to be higher (P<.07) in HL rams compared to LL and CL rams. Similarly, number of oLH receptor sites per Leydig cell tended to be higher (P<.07) in HL rams than in rams of the other lines. Differences in capacity of the oLH receptor among lines were not observed for the hCG receptor (Table 7).

Capacity, regardless of how it was expressed, and K_d of the LH binding were higher (P<.05) than the corresponding values for hCG binding, within each line (Table 7). Values were approximately 150% higher for LH than for hCG in capacity per g of testicular parenchyma and number of receptor sites per Leydig cell (Table 7).

In Vitro Testosterone Secretion

oLH-Stimulated Testosterone Secretion

There was a line by time interaction (P<.08) for oLH-stimulated testosterone secretion per g of testicular

Table 7. Least squares means for LH and hCG testicular receptors characteristics of Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL).

Line	K_d (x 10^{-12} M)		Capacity per mg of membrane protein (fM)		Capacity per g of parenchyma (fM)		Capacity per testis (pM)		Receptor sites per Leydig cell	
	GTH*		GTH		GTH		GTH		GTH	
	oLH	hCG	oLH	hCG	oLH	hCG	oLH	hCG	oLH	hCG
LL (n=8)	87.3	12.9[a]	45.3	17.2	383.2[c]	147.1	34.8	13.3	10,206[c]	3,847
HL (n=7)	82.5	13.8[a]	62.6	23.2	554.4[d]	208.7	47.7	18.5	15,287[d]	5,686
CL (n=8)	133.8	26.1[b]	48.1	19.9	378.8[c]	157.6	34.8	13.6	9,115[c]	3,703
SE[e]	88.6	11.4	23.5	11.8	172.1	102.2	16.7	9.2	4,803	2,666

*Means differ (P<.05) between gonadotropic hormones (GTH), LH and hCG, within lines for each characteristic.
[a,b]Means with different superscripts differ (P<.06).
[c,d]Means with different superscripts within each column differ (P<.07).
[e]Pooled standard error.

parenchyma per h of in vitro incubation indicating that the lines of rams showed different patterns of testosterone secretion over time (Figure 1). Testosterone secretion in HL and CL did not change between 1 and 2 h, whereas testosterone secretion in LL rams increased from 1 to 2 h. Between 2 and 3 h testosterone secretion decreased in LL and CL rams but did not change in HL rams. Testosterone secretion between 3 and 4 h did not differ in LL and CL rams but decreased in HL rams. Testosterone concentrations did not differ among lines at 1 h of incubation but at 2 h testosterone was higher in LL rams than in HL and CL rams and it did not differ between HL and CL rams. At 3 and 4 h, testosterone was higher in LL and HL rams than in CL rams. Testosterone concentrations at these hours did not differ ($P>.10$) between LL and HL rams.

Patterns of testosterone secretion per g of testicular parenchyma per h of in vitro incubation were also influenced ($P<.01$) by the dose of oLH by time interaction (Figure 1). This interaction was due primarily to the fact that testosterone secretion over time did not change ($P>.10$) in flasks that recieved either the 0 or 0.13 $ng \cdot ml^{-1}$ dose of oLH, while testosterone secretion for doses 1.3, 13 and 130 $ng \cdot ml^{-1}$ showed and apparent increase ($P<.08$) by 2 h and a decrease ($P<.08$) by 4 h. Testosterone secretion for each h increased ($P<.05$) as dose of oLH increased from 0.13 $ng \cdot ml^{-1}$ up to dose 13 $ng \cdot ml^{-1}$. Dose 130 $ng \cdot ml^{-1}$ did not stimulate ($P>.10$) more testosterone secretion than 13 $ng \cdot ml^{-1}$. Line by dose and line

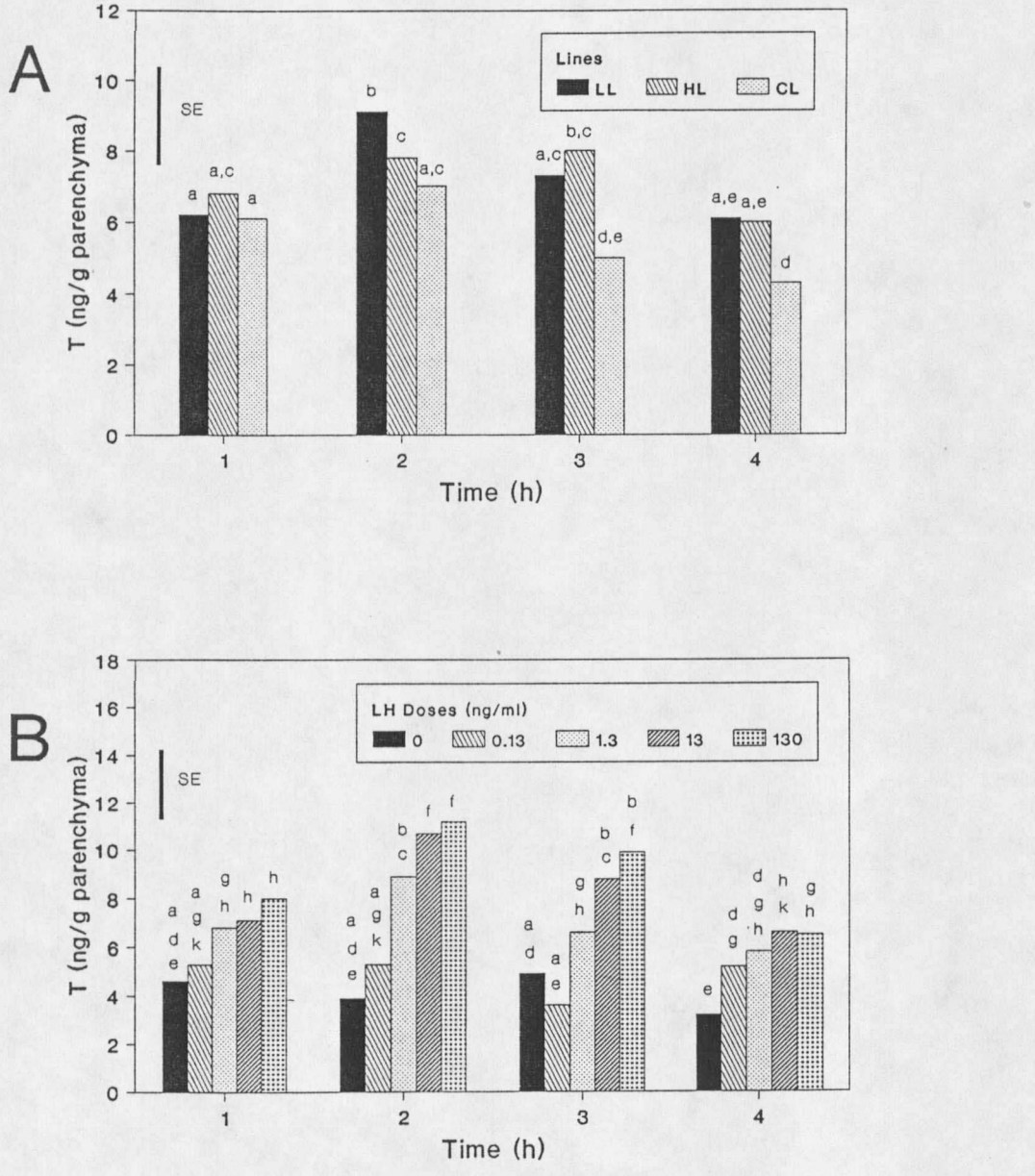

Figure 1. Line by time (A) and dose by time (B) interactions for in vitro testosterone (T) secretion after stimulation by oLH in Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL). Bars without common letters differ (P<.10).

by dose by time interactions did not influence (P>.10) oLH-stimulated testosterone secretion per h.

hCG-Stimulated Testosterone Secretion

The line by time interaction influenced (P<.05) hCG-stimulated testosterone secretion per g of testicular parenchyma per h (Figure 2). Low line and HL rams secreted more testosterone at 2 h of incubation compared to the 1 h. Concentrations of testosterone at 3 h did not differ relative to 2 h but decreased by 4 h in LL and HL rams. Contrary to the similar patterns of testosterone secretion over time between LL and HL rams, CL rams showed a different pattern. Testosterone secretion in these rams did not change during the first 3 h and decreased by the fourth h. During every h of incubation HL rams secreted greater concentrations of testosterone than LL rams, with the exception of 3 h when testosterone secretion between HL and LL did not differ.

The dose of hCG by time interaction influenced (P<.05) hCG-stimulated testosterone secretion per h and is depicted in Figure 2. Testosterone secretion for dose 0 $IU \cdot ml^{-1}$ did not change during the first 3 hours and decreased by the fourth h. Patterns of testosterone secretion for 0.0025 $IU \cdot ml^{-1}$ and for 0.025 $IU \cdot ml^{-1}$ were similar; testosterone secreted increased from 1 h to 2 h, did not change by 3 h and then decreased by 4 h. A different pattern was found for 2.5 $IU \cdot ml^{-1}$,

testosterone concentration increased from 1 h to 2 h, decreased by 3 h and decreased again by 4 h. Testosterone secretion after hCG stimulation within each h, increased as dose of hCG increased from 0 to 2.5 $IU \cdot ml^{-1}$.

Trial and other two- or three-way interactions of combinations of line, trial, dose of hCG and time, did not alter ($P>.10$) testosterone secretion per g of tissue per h in vitro.

Patterns of testosterone secretion per h for equi-molar doses of oLH and hCG did not differ ($P>.10$) at each h or over time between type of gonadotropin.

Total Testosterone Secretion

Trial did not influence ($P>.10$) total testosterone secretion during incubation per g of parenchyma after hCG stimulation. Therefore, data of total testosterone secretion of both trials were pooled and equi-molar doses of oLH and hCG were then analyzed.

Total oLH- and hCG-stimulated testosterone secretion did not differ ($P>.10$) between gonadotropins at any dose and increased as dose of gonadotropin increased (Table 8). Line did not affect ($P>.10$) total testosterone secretion (Table 9) and there was no ($P>.10$) line by gonadotropin interaction.

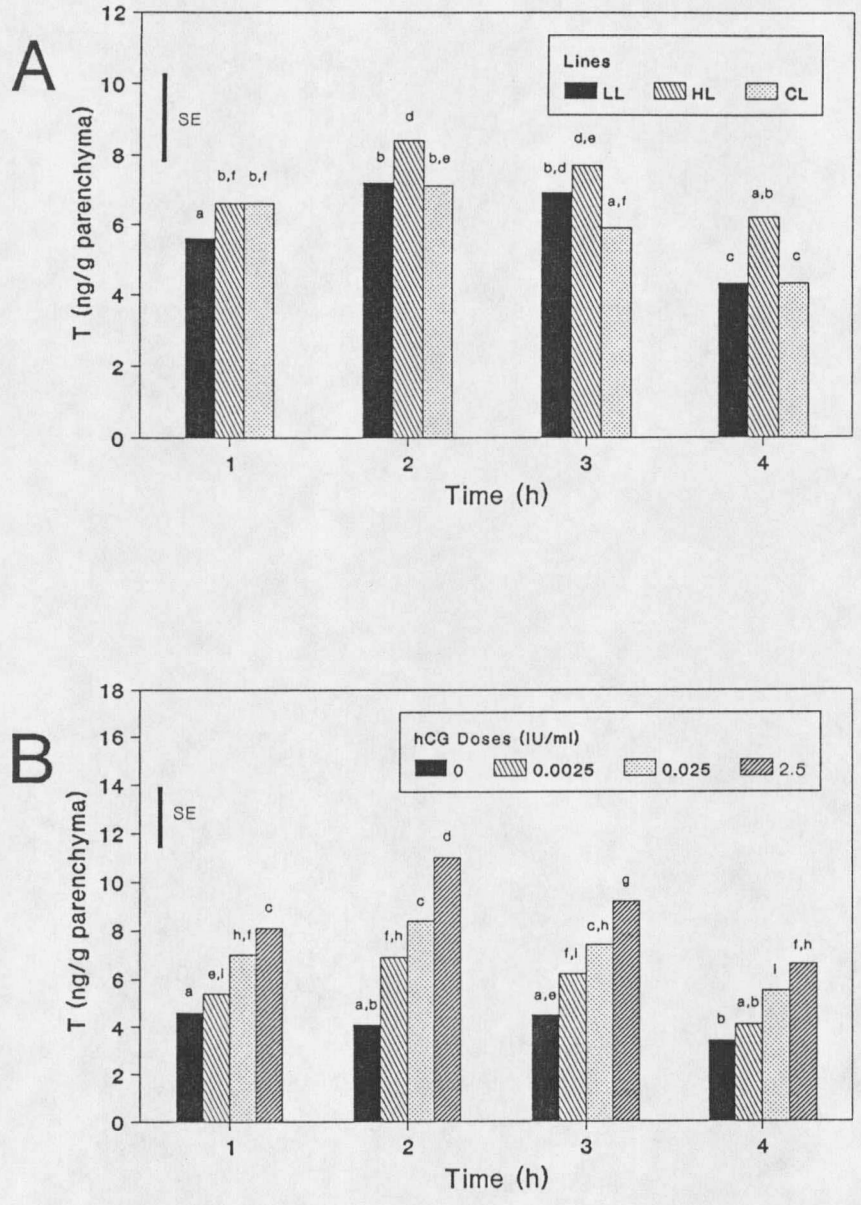

Figure 2. Line by time (A) and dose by time (B) interactions for in vitro testosterone (T) secretion after stimulation by hCG in Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL). Bars without common letters differ (P<.10).

Table 8. Least squares means for total testosterone (T) secretion per g of testicular parenchyma in vitro after stimulation of equi-molar doses of the gonadotropins LH or hCG in Rambouillet rams.

Dose of gonadotropin (pM)	Total oLH-stimulated T secretion (ng/g parenchyma)[d]	Total hCG-stimulated T secretion (ng/g parenchyma)[e]
4.6	19.5[a]	22.5[g]
46	28.1[b]	28.3[b]
4600	35.6[c]	34.9[c]
SE[f]	5.7	5.7

[a,b,c]Means with different superscripts differ (P<.10).
[d]n=20
[e]n=39
[f]Pooled standard error.

Table 9. Least squares means for total oLH- and hCG-stimulated testosterone (T) secretion per g of testicular parenchyma in vitro in Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL).

Line	oLH-stimulated total T secretion (ng/g parenchyma)[a]	hCG-stimulated total T secretion (ng/g parenchyma)[b]
LL	30.5	26.8
HL	29.7	32.3
CL	23.0	26.6
SE[c]	23.6	23.6

[a]LL, n=6; HL; n=6, CL; n=8.
[b]LL, n=12; HL; n=12, CL; n=15.
[c]Pooled standard error.

Correlations of Total Testosterone Secretion with Other Testicular Characteristics. Correlation coefficients for total testosterone secretion per g of parenchyma during the 4-h incubation with total testosterone secreted per Leydig cell were generally high and positive for each dose of oLH (Table 10) and hCG (Table 11) within each line.

Correlations of total testosterone secretion for only one or two doses of gonadotropin were found with receptor sites per Leydig cell in CL rams, with gonadotropin capacity per g of parenchyma in HL rams and with hCG receptor capacity per mg of protein in HL rams. No relationships were found between total testosterone secretion with number of Leydig cells per g of parenchyma, LH receptor capacity per mg of protein and testis weight.

Total testosterone secreted per Leydig cell was positively correlated ($P<.05$) with number of receptor sites per Leydig cell at intermediate doses of gonadotropin used to stimulate the testicular tissue only in HL rams, whereas no relationship between these characteristics was found in the other two lines (Table 12).

Table 10. Correlation coefficients of total oLH-stimulated testosterone (T) secretion with other testicular characteristics in Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL).

Trait	Dose of LH (ng/ml)	Lines		
		LL	HL	CL
Total T secreted per Leydig cell	0	0.87*	0.72	0.77*
	0.13	0.92**	0.85*	0.83*
	1.3	0.84*	0.90*	0.96**
	13	0.89*	0.84	0.94**
	130	0.84*	0.89*	0.92**
Leydig cells per g of parenchyma	0	0.55	0.20	-0.31
	0.13	0.65	-0.22	-0.39
	1.3	0.42	-0.44	-0.53
	13	0.54	0.16	-0.73
	130	0.39	0.20	-0.64
Receptor sites per Leydig cell	0	-0.25	0.26	-0.79*
	0.13	-0.13	0.60	-0.60
	1.3	-0.20	0.76	-0.18
	13	-0.26	0.33	-0.36
	130	-0.22	0.63	-0.18
Capacity per g of parenchyma	0	0.14	0.80*	-0.51
	0.13	0.36	0.79	-0.26
	1.3	0.08	0.84*	-0.07
	13	0.13	0.74	-0.24
	130	0.03	0.81	-0.15
Capacity per mg of protein	0	0.13	0.74	-0.36
	0.13	0.33	0.62	-0.01
	1.3	0.09	0.62	-0.05
	13	0.14	0.77	-0.18
	130	0.04	0.73	-0.17
Testis weight	0	-0.17	0.09	0.21
	0.13	-0.40	0.29	0.28
	1.3	-0.08	0.35	0.58
	13	-0.16	-0.01	0.50
	130	-0.02	0.10	0.64

*P<.05
**P<.01

Table 11. Correlation coefficients of total hCG-stimulated testosterone (T) secretion in vitro with other testicular characteristics in Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL).

Trait	Dose of hCG (IU/ml)	Lines		
		LL	HL	CL
Total T secreted per Leydig cell	0	0.87*	0.72	0.77*
	0.0025	0.88*	0.83	0.80*
	0.025	0.86*	0.78	0.95**
	2.5	0.86*	0.89*	0.96**
Leydig cells per g of parenchyma	0	0.55	0.20	-0.31
	0.0025	0.36	0.05	-0.32
	0.025	0.37	0.04	-0.73
	2.5	0.44	-0.09	-0.73
Receptor sites per Leydig cell	0	-0.25	0.26	-0.79*
	0.0025	-0.61	0.37	-0.89**
	0.025	-0.58	0.44	-0.60
	2.5	-0.37	0.40	-0.66
Capacity per g of parenchyma	0	0.14	0.80*	-0.51
	0.0025	-0.28	0.70	-0.33
	0.025	-0.25	0.79	-0.36
	2.5	-0.04	0.24	-0.57
Capacity per mg of protein	0	0.13	0.74	-0.36
	0.0025	-0.21	0.79	-0.20
	0.025	-0.22	0.82*	-0.29
	2.5	-0.02	0.48	-0.51
Testis weight	0	-0.17	0.09	0.21
	0.0025	0.14	0.23	0.34
	0.025	0.12	0.29	0.46
	2.5	-0.10	-0.09	0.65

*P<.05
**P<.01

Table 12. Correlation coefficients of total testosterone secretion per Leydig cell after stimulation by different doses of oLH or hCG in vitro with number of oLH and hCG receptor sites per Leydig cell in Rambouillet rams from lines selected for low (LL) or high (HL) reproductive rates and from a random-bred control line (CL).

Dose of LH (ng/ml)	Lines		
	LL	HL	CL
0	-0.14	0.83	-0.56
0.13	0.01	0.83	-0.50
1.3	0.06	0.92*	-0.29
13	-0.10	0.76	-0.39
130	0.03	0.82	-0.30
Dose of hCG (IU/ml)			
0	-0.14	0.83	-0.56
0.0025	-0.55	0.81	-0.54
0.025	-0.54	0.88*	-0.43
2.5	-0.32	0.75	-0.51

*P<.05

DISCUSSION

The effects of selection for or against female reproductive rate on some testicular characteristics in Rambouillet sheep were evaluated in this study. The only seminal trait that was influenced by selection for reproductive rate in females was percentage of progressively motile sperm. Rams from ewes selected for low reproductive rate had approximately 30% less progressively motile sperm than rams from ewes selected for high reproductive rate or rams from unselected ewes.

All other characteristics that are usually used for determination of semen quality did not appear to be altered by selection applied upon female reproductive rate. These results may be a reflection of the fact that lines did not differ in testicular weight, volume percentage of the testis occupied by seminiferous tubules and diameter of seminiferous tubules. Furthermore, rams from these lines did not differ in epididymal sperm reserves (Curry, 1990).

The relationship between female reproductive rate and sperm motility has not been investigated and there were no reports in the literature concerning semen quality of rams from lines of different prolificacy. The mechanisms by which selection against female reproductive rate decreased the percentage of progressively motile sperm in LL Rambouillet rams in this study are unknown and probably constitute a

complicated process because of the numerous factors that are involved in the regulation of sperm motility (more than 20 factors were listed by Eddy, 1988).

Only 38% of LL rams responded to training for semen collection, thus results for semen quality for these lines should be considered preliminary until more extensive trials are performed. However, it is interesting to note that the poor response to training of LL rams may indicate a deficiency of libido in rams of this line. In fact, Tulley and Burfening (1983) evaluated libido in these lines of Rambouillet sheep and reported that LL rams had lower libido than HL rams.

Scrotal circumference and testicular weight have been extensively studied in relation to their relationship with different female reproductive rate (e.g., prolificacy or ovulation rate) exhibited by some breeds and lines of sheep. Because scrotal circumference and testis diameter are relatively easy to measure, both have been considered ideal traits for the purpose of indirect selection for reproductive rate in females based on selection applied to male characteristics.

In our study, scrotal circumference was not influenced by line. This result is consistent with that of Curry (1990) but differs from results of Tulley and Burfening (1983) who found that LL rams had larger scrotal circumference than CL rams. Both experiments employed rams from the same lines of Rambouillet sheep that were used in the present study. Thus,

selection for or against female reproductive rate in these lines did not appear to alter scrotal circumference of male offspring even though it has caused an increase in ovulation rates (1.67 and 1.25, respectively) and litter size (1.43 and 1.04, respectively) in HL ewes compared to LL ewes (Shoenian and Burfening, 1990).

Several investigators have reported positive correlations between scrotal circumference and (or) testis diameter with ovulation rate (Land, 1973; Land and Carr, 1975) or litter size (Knight, 1984) in breeds of sheep of different prolificacy. Finish Landrace and Tasmanian Merino ewes with ovulation rates of 2.9 and 1.1, respectively (Wheeler and Land, 1973) were evaluated in the studies by Land (1973) and Land and Carr (1975). The difference in ovulation rates between these breeds is 1.8 which is higher than the difference of 0.42 between our HL and LL ewes. It is possible that the divergence in ovulation rate that has been achieved by selection between these lines of Rambouillet sheep is not large enough to induce a clear difference in scrotal circumference between lines.

Another important consideration is the assumption that scrotal circumference and ovulation rate and litter size may not be highly genetically correlated characteristics in these lines. Therefore, differences in scrotal circumference among these lines may not have been expressed because selection has been applied over a relatively short period of time (19

years). This assumption is consistent with results of Haley et al. (1990) who used testicular diameter, adjusted for body weight at 6, 10 and 14 week of age, as selection criteria to form 2 lines of Finn-Dorset sheep with high or low testicular size. This selection process did not produce differences in ovulation rate or litter size between ewes from lines in which rams had been selected for high or low testicular diameters.

There was a tendency for the HL rams to have heavier paired TW than LL rams due to a difference in left testicular weight between lines. In Trial 2, left testicular weight did not differ among lines. Curry (1990) found that neither right, left, nor paired testicular weights differed among rams from these same lines after 13 years of selection. The finding that left testicular weight in one trial differed among lines and did not differ among lines in another trial could have been a matter of chance.

Like testis weight, testis volume did not differ among lines in Trial 2. This result was expected due to the fact that testis weight did not differ among lines and the same value of specific gravity was used to calculate testicular volume for all animals.

Unlike our results, Land and Carr (1975) found that testicular weight differed between breeds that differed in prolificacy. Scrotal circumference also differed between these breeds and one would expect a difference in this characteristic because of the high positive correlation of

scrotal circumference with testicular diameter and weight in ram lambs (Notter et al., 1981) and mature rams (Lino, 1972). A high positive correlation between scrotal circumference and testis weight was also found in our study and supports the idea that measurements of scrotal circumference yields an estimate of testicular diameter and weight in these lines of sheep.

Hochereau-de Reviers et al. (1990) reported lighter testicular weight in Romanov compared to Ile de France rams even though Romanov ewes had higher ovulation rates than Ile de France ewes. This is contrary to the result of Land and Carr (1975) for other breeds that differ in prolificacy. The discrepancy between the results of Hochereau-de Reviers (1990) and Land and Carr (1975) may be due to the fact that testicular weight of Ile de France and Romanov rams were not corrected for body weight which was higher in Ile de France than in Romanov rams at castration. This is an important consideration because it may be that the factors that control body weight could influence testicular weight simultaneously and be more important in determining testicular weight than factors involved in the development of different prolificacy among breeds.

In our study, body weight was highly positively correlated with scrotal circumference only in CL rams to the same degree as that reported by Braun at al (1980) for Rambouillet sheep. There is no apparent explanation for the

lack of correlation between body weight with scrotal circumference in the HL and LL rams, except to suggest that selection has eliminated the relationship between these characteristics by limiting the variation in one or the other.

Histomorphological characteristics of the testicular parenchyma of rams from lines of sheep that differ in reproductive rate have not been studied previously. Selection for or against female reproductive rate altered certain components of the testicular interstitium: volume percentages of Leydig cells, volume percentage of vascular tissue and Leydig cell volume. All other histomorphological characteristics were not influenced by selection.

High line rams had a greater volume percentage of the interstitium occupied by Leydig cells than LL or CL rams. Since the volume percentages of the testicle that included the interstitium did not differ among lines there must have been a compensatory decrease in either the volume percentage of vascular tissue or "other components" of the interstitium in HL rams. Concomitantly, LL rams would have had to compensate by either an increase in the volume percentage in vascular tissue or "other components". We found that the volume percentage occupied by the "other components" did not differ among lines therefore the higher volume percentage of Leydig cells observed in HL rams was caused by selection for one or more factors that induced an increase in volume percentage of Leydig cells in the testicle. The higher volume percentage of

vascular tissue found in LL rams was caused by selection for any factor(s) that can increase vascular volume in the testicle. Thus, selection for female reproductive rate in these lines of Rambouillet sheep produced an increase in percentage of volume of the testicle occupied by Leydig cells, while selection against female reproductive rate produced an increase in the volume percentage of the testicle occupied by vascular tissue without changing the histomorphology of other structures of the interstitium.

The difference in volume percentage of the testicle occupied by Leydig cells between HL and LL or CL rams may have been due to differences in either number of Leydig cells per unit of mass of parenchyma or volume occupied by single Leydig cells. We found that rams from these lines did not differ in number of Leydig cells per g of parenchyma but that HL rams had larger Leydig cells than rams from the other lines. It appears that the different volumes of the testicle occupied by Leydig cells found in these lines may have been actually produced by an increase in Leydig cell volume in HL rams as a result of selection for increase in female reproductive rate in this line.

Our knowledge of the genetic and ontological factors that control the multiplication and development of Leydig cells is not sufficient to provide a satisfactory explanation for the change in Leydig cell volume observed in HL rams. However, there is the possibility that the gonadotropin LH may be

involved in this process because of its major role in the control of Leydig cell function (Brady, 1951; Hall and Eik-Nes, 1962; Connell and Eik-Nes, 1975). Chronic treatments of rats with high doses of hCG, a gonadotropin that has LH-like activity, produced an increase in number and volume of Leydig cells (Christensen and Peacock, 1980). However, studies in boars demonstrated that the rapid increase in Leydig cell volume during pubertal transition was not accompanied by comparable changes in serum LH (Allrich et al., 1982; Lunstra et al., 1986).

The physiological significance of changes in Leydig cell volume is not well understood. Hochereau-de Reviers et al. (1979) and Lunstra and Shanbacher (1988) reported that the number of Leydig cells decreased dramatically by experimentally-induced cryptorchidism in rams. Furthermore, Lunstra and Shanbacher (1988) found that Leydig cell volume was greater in cryptorchid rams than in intact rams. Another interesting finding of their study was that testosterone concentrations in peripheral and spermatic vein serum of cryptorchid rams did not differ from those in intact rams. The maintenance of normal testosterone concentrations with lower numbers but larger Leydig cells in cryptorchid rams may indicate that size of Leydig cells was an important factor that influenced the amount of testosterone secreted by cryptorchid rams. Based upon these data it would appear that HL rams could secrete more testosterone because of larger

Leydig cells whereas LL and CL rams with smaller Leydig cells may secrete lower amounts of testosterone.

Testosterone synthesis and secretion by Leydig cells is regulated by LH. This action is mediated by membrane proteins known as receptors. Binding of LH to its receptor initiates a cascade of biochemical events that results in the synthesis and secretion of testosterone. Two parameters that have been used to characterize the interaction between LH and its receptor are the dissociation constant (K_d), a measure of the "bindability" of LH to its receptor, and capacity or number of receptor sites. We included both LH and hCG to stimulate testosterone secretion by the parenchymal tissue because Sairam et al. (1988) reported that the LH receptor of the ram testicle discriminates between LH and hCG. Our experiment was designed to test the hypotheses that K_d's and numbers of receptor sites for LH and hCG do not differ among lines and that K_d's and receptor site numbers do not differ for LH and hCG.

Within each line, K_d's for receptor binding of oLH were approximately six-fold higher than K_d's for hCG and binding capacities were approximately three-fold higher for oLH than for hCG. These results agree in part with those of Sairam et al. (1988) who found higher binding capacities for oLH than for hCG but similar K_d's for both gonadotropins in testicular preparations from rams.

In contrast to these results are results reported by

Huhtaniemi and Catt (1981) for rats. They found similar binding capacities for oLH and hCG but lower K_d for hCG than for oLH. In fact they observed that binding of oLH in the rat fit a model with two different binding (receptor) sites: one that had high affinity (low K_d) that represented about 20% of the oLH receptors, and the other with low affinity (high K_d) that represented 80% of the oLH receptor. In our study, examination of Scatchard plots revealed that binding data for hCG fit exclusively a "one ligand, one-binding site" model and, for the most part, binding data for oLH did likewise with the exception of three rams whose data appeared to fit a "one ligand, two-binding site" model. There are few data to support the notion that there are two different types of receptor molecules for oLH in rams. Yarney and Sanford (1989) noted that a small number of rams in their study yielded binding data for oLH that appeared to fit a "one ligand, two-binding site" model.

Values of K_d for oLH and hCG were approximately 4 and 20 times lower, respectively, than the values reported by Sairam et al. (1988). The hCG receptor capacities per g of tissue were comparable between these two studies but our results of oLH receptor capacities were about 3 times lower. Values of LH capacities per testis were comparable with those reported by Hochereau-de Reviers et al. (1990), about 6 times lower than the values of Yarney and Sanford (1989) and 15 times higher than those reported by Barenton et al. (1983). Number

of LH receptor sites per Leydig cell were higher than the number (900 ± 200) found by Barenton et al. (1983) in two-year-old rams. Different breeds of sheep, season, procedures for the radioreceptor assays and in some cases age of the rams could be factors for discrepancies among studies.

Dissociation constants for hCG were larger for CL rams than for HL and LL rams. Although K_d's for oLH were not statistically different they were approximately 58% larger in CL rams than in either HL or LL rams. We do not know or understand the biological significance of this result but it would appear from these data that selection either for or against reproductive rate in females has changed the LH/hCG receptor in testicles of rams in such a way that it allows these gonadotropin (LH or hCG) molecules to bind more efficiently. This is a novel and unique finding for large animals and there are no data in the literature concerning this interesting phenomenon.

Binding capacity per g of testicular parenchyma and receptor sites per Leydig cell for oLH were higher for HL rams than for LL or CL rams. Binding capacity of testicular receptors expressed in different ways and whether it was for oLH or hCG was numerically higher for HL rams than for either LL or CL rams. These results may indicate that selection for increased reproductive rate in female Rambouillet sheep has increased the number of available receptor sites on Leydig cells to which a oLH or hCG molecule might bind.

Physiologically this would mean that Leydig cells of HL rams may be more efficient in synthesis and secretion of testosterone since they have a greater number of receptors to bind LH.

How selection for female reproductive rate has increased the number of receptors is not known. One explanation may involve circulating LH concentrations. LH concentrations were found to be correlated with female prolificacy (Thimonier and Pelletier, 1972; Land and Carr, 1975, Carr and Land, 1975, Haley et al., 1990). Tähkä (1986) showed that LH participates in controlling the number of its own receptor in Leydig cells. The differences in LH receptors among lines in our study could have been influenced by differences in LH concentrations. However, we do not know if this was the case because we did not determine LH concentrations in these rams.

So the question is: Are cellular and molecular changes observed in these lines manifested in the function of Leydig cells, namely testosterone secretion? We attempted to answer this question using an in vitro rather than an in vivo approach for the following reasons: 1) to examine testosterone secretion under controlled environmental conditions, and 2) avoid possible interacting factors associated with metabolic processes and hormones in individual animals. Doses of oLH and hCG used in this experiment were pre-determined in preliminary trials so that they would yield a minimum, intermediate and maximum stimulation of

testosterone by testicular parenchyma in vitro.

All three lines of rams showed different patterns of testosterone secretion after oLH stimulation in vitro. Patterns of HL and LL were different because LL rams secreted higher testosterone concentrations by the second hour relative to the first and third hours of incubation, whereas HL rams secreted concentrations of testosterone that did not differ during the first three hours. Patterns for the HL and LL rams differed from the patterns of CL rams because CL rams did not show an increase by the second hour and had a decrease by the third and again by the fourth hour. Unlike patterns of testosterone observed after oLH stimulation, patterns of testosterone after hCG stimulation of HL and LL rams did not differ but CL rams showed a different pattern than HL and LL rams. High line rams secreted higher concentrations of testosterone than LL and CL rams by the first, second and fourth hours of incubation.

The interactions found in our study indicate that selection has altered the manner in which Leydig cells of these rams responded to gonadotropin stimulation in vitro. The mechanisms whereby selection, either for or against reproductive rate in females, has changed the ability of the testicle to respond to oLH in vitro is not clear. One might have expected that the rank for these lines in regard to this response would be HL, LL and CL because HL rams had higher volume percentage of Leydig cells, larger Leydig cells and oLH

receptor capacity than CL or LL rams. On the other hand, LL rams would perform better than CL rams because of their higher testicular vascularity and higher gonadotropin receptor affinity. Instead, LL rams ranked similar to or higher than HL rams for at least one hour of incubation after oLH stimulation, however, after hCG stimulation, HL rams ranked higher than LL rams during three hours of incubation. As expected CL were usually the lowest, even though not always significant, in testosterone secretion.

Total testosterone secretion was positively correlated to total testosterone secretion per Leydig cell within line but no relationship was found with receptor sites per Leydig cells and testicular weight. Thus, testicular weight is not a good indicator of steroidogenic capacity in rams of these lines.

Levels of testosterone secretion for each gonadotropin within line, did not correspond with the levels of oLH and hCG receptor capacities found in this study. Although the capacity of the LH receptor was higher than the capacity of hCG receptor, total oLH- and hCG-stimulated testosterone secretion did not differ in two out of three equi-molar doses of gonadotropin (see Table 8). Similarly, HL and LL rams did not differ in oLH-stimulated testosterone secretion per h or in total oLH-stimulated testosterone secretion, regardless of the facts that HL rams had more LH receptor capacity per g of parenchyma and per Leydig cell than the other lines.

It is difficult to find a reasonable explanation for the

discrepancies between characteristics of testicular LH receptors and the testosterone response of testicular parenchyma to stimulation by oLH or the LH-like hormone hCG. The in vitro technique used in this study was considered adequate to evaluate gonadotropin-stimulated testosterone secretion by the ram testicular parenchyma. Significant dose responses were obtained in total testosterone secretion and in testosterone secretion per h, particularly during the first, second and third hours of incubation. Trial or interactions of trial with other factors did not influence hCG-stimulated total testosterone secretion or testosterone secretion per h which demonstrated that the in vitro technique was repeatable. To the best of my knowledge this was the first study in which an in vitro technique was used to evaluate testicular capacity to secrete testosterone in rams.

An explanation for the discrepancies between characteristics of the testicular LH/hCG receptor and testosterone secretion may be related to the "spare receptor" hypothesis (Catt et al., 1979). Included in this hypothesis is the postulate that only a small number of receptors is required for a maximum biological response to be exhibited by the target tissue. In our experiment, the possible formation of more oLH-receptor than hCG-receptor complexes in rams of each line and more oLH-receptor in HL rams than in LL rams inferred from the number of LH receptors determined in the binding assays, did not produce any increase in testosterone

secretion.

Another factor that could be used to explain these discrepancies is the higher affinity of hCG relative to oLH or, in other terms, the faster dissociation of the oLH-receptor complexes. Considering capacities and K_d of the oLH and hCG receptors together, it may be stated that when the same concentration of oLH or hCG were present oLH was able to bind more receptors on the Leydig cells than hCG did. Because of the high affinity of the latter for its receptor, the parenchymal tissue somehow compensated for the endocrine response of Leydig cells to hCG making it equivalent to the response exhibited after oLH stimulation.

The importance of hormone affinity to its receptor for a particular cellular response should be more evident at lower than at high doses of hormone. Testosterone concentrations did not differ after stimulation of testicular parenchyma with 0 or 0.13 ng of oLH, but unlike oLH, the equi-molar dose of hCG, 0.0025 IU, stimulated secretion of more testosterone during the second and the third hours than the 0 dose did. The differences at these hours increased the total testosterone secretion of this low concentration of hormone for hCG (see Table 8). Because of the lower capacity of the hCG receptor than the LH receptor, the higher stimulation exhibited after hCG may be explained, at least partially, by the high affinity of hCG to its receptor which, according to Hutaniemi and Catt (1981), contributes to the well-known high bioactivity of hCG.

Discrepancies like those described herein were found in rats in which a massive increase in responsiveness of the pituitary gland to GnRH that occurred during the LH surge was not accompanied by an increase in GnRH receptors (Clayton et al., 1980; Savoy-Moore et al., 1980). Indeed, current knowledge on the relationship between the magnitude of the hormone-receptor interaction and the corresponding biological response is not complete enough to encompass all different amplitudes in responsiveness of a target tissue.

An important concept to keep in mind for interpreting this kind of data is that many biochemical steps occur before the hormone-receptor binding can be translated into testosterone synthesis and secretion in Leydig cells. One (or perhaps more than one) of these factors may be more important in eliciting a particular cellular response to a hormone than the actual number of binding sites that are thought to be receptors.

In conclusion, oLH and hCG bind to a single set of testicular receptors, but these receptors appears to have higher capacity and lower affinity for oLH than for hCG. However, equi-molar doses of both gonadotropins stimulated similar testosterone secretion in vitro by testicular parenchyma of rams. Selection for or against female reproductive rate in these lines of Rambouillet sheep applied for approximately 19 years did not alter, semen volume, sperm concentration, total number of sperm cells per ejaculate,

percentages of live and dead sperm, percentages of normal and abnormal sperm cells scrotal circumference, testicular weight, testicular volume, testicular tone, Leydig cell diameter, Leydig cells nucleus volume, total volume of Leydig cells in the testis, number of Leydig cells in the testis, number of Leydig cells per g of testicular parenchyma, volume percentages of seminiferous tubules and interstitium, volume percentage of structures of the interstitium other than Leydig cells and vascular tissue; receptor capacity for hCG, receptor capacity for oLH per mg of membrane protein and per testis, and total testosterone secretion after stimulation of LH or hCG in vitro.

Selection against reproductive rate in females, decreased percentage of progressively motile sperm and increased the percentage of the testicle occupied by vascular tissue in males. On the other hand, selection for increased reproductive rate in females increased the percentage of the testicle occupied by Leydig cells, volume of Leydig cells, receptor capacity for oLH per g of testicular parenchyma, and number of receptors for oLH per Leydig cell. Selection for or against female reproductive rate in these lines of Rambouillet sheep increased the affinity of the testicular hCG receptor and changed the patterns of testosterone secretion by the testicular parenchyma after stimulation of LH or hCG in vitro.

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APPENDICES

APPENDIX I

EFFECT OF TIME BETWEEN ORCHIECTOMY AND PARENCHYMAL TISSUE RINSING ON IN VITRO SECRETION OF TESTOSTERONE

This trial was performed to determine the effect of elapsed time between orchiectomy and the beginning of tissue rinsing on in vitro gonadotropin-stimulated testosterone secretion by testicular parenchyma of rams.

Two periods of time (which will be considered treatments) were tested, 60 min and 150 min. Procedures used for orchiectomy, parenchymal tissue handling and mincing, incubations and sampling were the same as those described by Berardinelli et al.(1989) and summarized in the Materials and Methods of this thesis. Three doses of oLH were used to stimulate the tissue; 0, 0.13 and 1.3 $ng.ml^{-1}$ of ME 199. The incubation period lasted 5 h.

Accumulated concentrations of testosterone increased from 0 to 5 h of incubation for every dose in both treatments. Within each treatment, testosterone concentrations increased as dose of LH increased at 3 h and 5 h of incubation. The accumulated concentration of testosterone by 5 h were numerically higher in the 150 min than the 60 min treatment for dose 0 $ng.ml^{-1}$. However, concentrations of testosterone for 0.13 and 1.3 $ng \cdot ml^{-1}$ of LH were higher in the 60 min than in the 150 min treatment (Figure 3).

It was hypothesized that LH already present in the tissue bound its receptors and change from a "loose" to a "tight" form as time increased between orchiectomy and rinsing, making it more difficult to separate the hormone from the receptors by rinsing. Therefore, less free receptors

probably existed in the 150 min samples to participate in the exogenous stimulation of testosterone secretion.

The grater levels of testosterone found at 0 dose of LH in the 150 min than in the 60 min treatment indicated that the tissue was still in satisfactory condition 150 min after orchiectomy and that the lower levels of testosterone found in the 150 min samples after the stimulation of doses higher than 0 was not due to deterioration of tissue produced by the time elapsed from orchiectomy to first rinsing.

In conclusion, oLH-stimulated testosterone secretion decreased when time from orchiectomy to the beginning of tissue rinsing increased from 60 min to 150 min. An unknown factor influenced the 150 min samples to secrete more testosterone than 60 min samples when 0 dose of oLH was used to stimulate the tissue. It could be possible that more number of LH-receptor complexes were formed in 150 min than in 60 min samples which stimulated more testosterone secretion in 150 min than in 60 min samples.

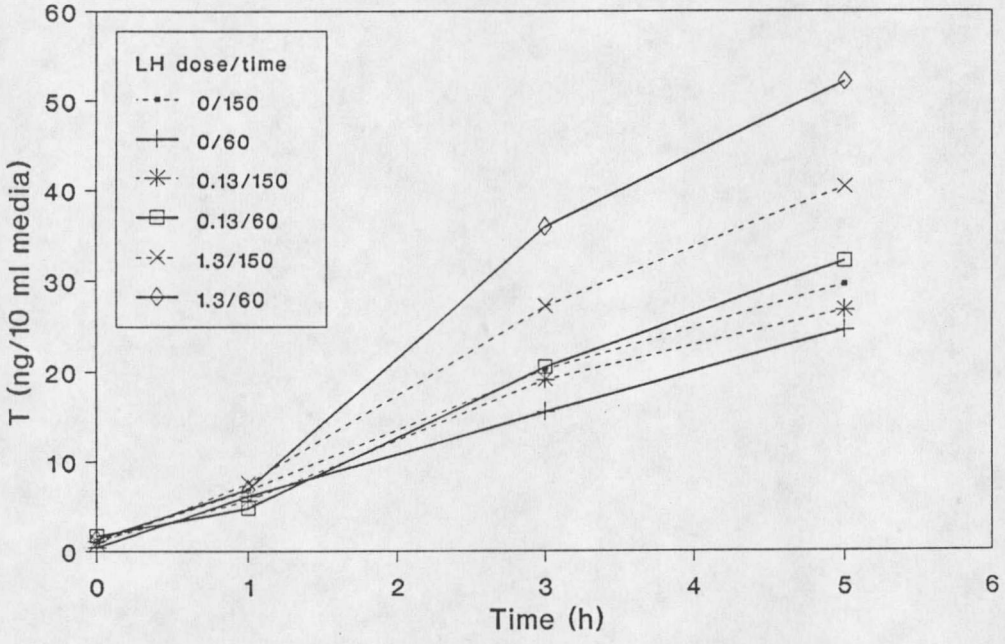

Figure 3. Effect of time (60 or 150 min) from orchiectomy to first tissue rinse on in vitro testosterone (T) secretion after stimulation by 0, 0.13 or 1.3 ng of oLH per ml of tissue culture media.

APPENDIX II

TESTICLE PERFUSION, FIXATION AND EMBEDDING

Testis Perfusion and Fixation

Reagents. All reagents were purchased from Polysciences, Inc., Warrington, PA.

Cacodylate acid sodium salt ($C_2H_6AsNaO_2.3H_2O$).

Glutaraldehyde, E.M. grade (70% and 8% purified).

Formaldehyde (40%).

Osmium tetroxide, E.M. grade (OsO_4, 4% purified).

Araldite Epoxy Resin, Grade 502.

Dodecenylsuccinic anhydride, DDSA (resin hardener).

2-4-6-Tri(dimethylaminomethyl)phenol, DMP-30 (resin accelerator).

Propylene oxide.

Solutions. The following solutions were prepared in glass distilled water and stored at 4°C when necessary.

Perfusion Buffer Solution; Cacodylate buffer, 0.1M, pH 7.4; 21.4 g of cacodylate acid sodium salt was diluted in water up to 1000 ml, pH was adjusted with 1N HCl.

Buffered Perfusion Fixative; 3% glutaraldehyde, 1% formaldehyde in 0.075 M cacodylate buffer; 60 ml of 70% purified glutaraldehyde, 255 ml of water and 35 ml of 40% formaldehyde were added to 1,050 ml of 0.1 M cacodylate buffer, pH was adjusted to 7.4 with 1 N NaOH or 1 N HCl.

Buffered 2% Glutaraldehyde Fixative; 2% glutaraldehyde in 0.075 M cacodylate buffer; 20 ml of 8% purified glutaraldehyde

was mixed with 60 ml of 0.1 M cacodylate buffer, pH was adjusted to 7.4 with 1 N HCl or 1 N NaOH.

Buffered 1% Osmium Fixative; 1% OsO_4 in 0.075 M cacodylate buffer, pH 7.4; 10 ml of 4% purified OsO^4 was mixed with 30 ml of 0.1 M cacodylate buffer, pH 7.4.

Luft Araldite Resin Mixture; 75 g of DDSA resin hardener was added to 100 g of Araldite Epoxy Resin, Grade 502 and mixed slowly and thoroughly, then 3 ml of DMP-30 was added and mixed as before.

Perfusion and Fixation Procedure. The right testis of each ram was perfused at room temperature immediately after orchiectomy and separation of epididymis. The testis was suspended approximately 30 cm high from a ring stand using suture placed through the connective tissue that attaches the epididymis to the testicular capsule in the area of the caput and the cauda epididymis and then anchoring the suture to the ring stand. This was done to avoid compression of blood vessels during perfusion.

A major branch of the testicular artery was located in the area of the caput epididymis and then a 23 G needle was inserted 1 cm into the vessel. In few cases it was necessary to tie the needle with suture. The needle was connected with a piece of tubing to a 60 ml plastic syringe. Perfusion buffer solutions were perfused into the artery by a syringe pump (Model 251-1, Sage Instruments, Inc., White Plains, NY)

at a flow rate of 5 $ml \cdot min^{-1}$.

The perfusion buffer was perfused into the artery for approximately 3 min to clear blood from the vascular system. This step ended when clear buffer started issuing from the testicular vein at the severed end of the spermatic cord. Then, without interrupting the flow, perfusion was continued with buffered perfusion fixative solution for approximately 30 min or until no soft (unfixed) areas were detected by palpation of the hardened testicle.

After perfusion, the fixed testicle was cut with a new razor blade into 3 parts; top, middle and bottom. From each part, four 2 x 2 x 4 mm pieces were cut at random and placed in pairs in 5 ml vials containing 3ml of buffered 2% glutaraldehyde fixative. Vials were capped and placed on a rotator (Model No 76, TechniLab Instruments, Inc., Pequannock, NJ) set at slow speed. This postperfusion fixation was performed overnight at 4°C. Vials were then removed from the rotator and placed on ice. Fixative solution was removed from each vial and replaced by an equal volume of 0.1 M cacodylate buffer. Vials were let stand on ice for 10 min and then the rinse buffer was removed and the pieces of tissue resubmerged in buffered 1% OsO_4 fixative. Fixation with OsO_4 continued under rotation for approximately 4 h at 4°C.

Tissue Embedding

After fixation with buffered OsO_4, tissue was rinsed twice for 10 minutes each at 4°C with 0.1 M cacodylate buffer. Dehydration was performed at 20°C by resubmerging the tissue in 50, 70, 80, 95, 100, and 100% ethanol, 10 min each and then twice in propylene oxide for 2 min. After the propylene oxide was removed, a mix of half propylene oxide and half Luft Araldite Mixture was added and the vials placed on the rotator for 60 min. The mix of propylene oxide and Luft Araldite was then replaced by 100% Luft Araldite mixture and the vials placed uncapped on the rotator for 60 min. Pieces of tissue were removed from the vials and transferred to a flexible silicone rubber mold containing 100% Luft Araldite mixture in 5 x 7 by 4 mm (W x L x D) cavities (Polysciences, Inc., Warrington, PA). Resin mixture was polymerized by putting the molds in oven for 24 h at 45°C, followed by another 24 h at 60°C.

APPENDIX III

HISTOMETRIC TECHNIQUES FOR EVALUATION OF TESTICULAR PARENCHYMA

Tissue Sectioning

Five thin (1 μm) sections, suitable for high resolution light microscopy, were cut from each block of embedded tissue using an ultramicrotome and then mounted on a microscope slide.

Tissue Staining

Sections were stained with toluidine blue and basic fuchsin following a modified procedure from Hoffmann et al. (1983) described bellow.

Stains. Stains were obtained from SIGMA, St. Louis, MO.

Toluidine Blue O, Certified (C.I. 52040).

Pararosaniline, Certified (C.I. 42500; Basic Fuchsin).

Solutions. Solutions were prepared with glass distilled water.

Toluidine Blue, 4 g of toluidine blue O and 4 g of sodium borate 10-Hydrate (J. T. Baker Chemical Co., Phillipsburg, NJ) in 100 ml of distilled water. Sodium borate was dissolved first in distilled water and then toluidine blue was added. The solution was double filtered using No 4 filter paper (Whatman Ltd., England) and stored at room temperature in a dark bottle.

Basic Fuchsin; 0.2 g of pararosaniline in 200 ml of distilled water. Stain was dissolved by heating the solution at 100°C. The solution was double filtered and stored in the same manner as the toluidine blue solution.

Sodium borate; 1.3 g of sodium borate in 200 ml of distilled water. This solution was used within 4 h after preparation.

Procedure. Slides were placed on a hot plate at 70°C for about 10 sec and then flooded with toluidine blue solution for 30 sec. Slides were allowed to cool slightly for about 10 sec before excess of stain was washed off with distilled water. Slides were dried at room temperature and placed again on the hot plate (70°C). After about 10 sec, slides were flooded for 1 min with a fresh mix of equal parts of sodium borate and basic fuchsin solutions. Slides were removed from the hot plate and allowed to cool slightly. Excess stain was washed off with distilled water and the slides dried at room temperature. A cover glass was mounted onto each slide using immersion oil (Zeiss, W. Germany) and the edges of the cover glass sealed with piccolyte mounting media.

Photomicrography

Two tissue sections from each slide were chosen at random and examined under a light microscope (Zeiss, W. Germany).

The microscope was fitted with a SLR 35mm automatic photographic camera (Model OM-2, Olympus, Japan) which was used to take photomicrographs of tissue sections. Each section was photographed at 125X magnification with the microscope light set at 5 and a blue (No 12) and orange O(G) filters set in the light path to enhance contrast. The purpose of these pictures was to obtain two dimensional configurations of seminiferous tubules and interstitium within a relatively large area of the testicular parenchyma.

Details of two kinds of representative areas (one simple and one complex) of interstitium within each large area (where 125X pictures were taken) were obtained by taking two photomicrographs at 1250X magnification, with the microscope light set at 6 and only the blue filter. All photomicrographs were taken with the camera in automatic setting using Kodak Plus-X Pan film (ASA 125). Each photomicrograph was identified automatically with a number at the time it was taken by a data-back recording instrument fitted to the camera. Additionally, photomicrographs of a 1 mm scale with 100 divisions (10 μm each) were obtained at 125 and 1250X and used for standardizing area measurements on the photomicrographs.

Negatives were developed following standard procedures for black and white film. Enlarged prints (5 x 7 inch, Kodak Ektamatic SC, Fs photographic paper) were obtained from negatives using a Beseler-Dichro enlarging easel. Development

of prints was performed using a rapid print processor (Spiratone Print-all™, Model LS-200).

Histometry

Areas occupied by different kinds of testicular parenchymal structures on the photomicrographs were digitized by tracing on a Kurta IS/ONE® digitizer and computed by using the area measurement feature of the program Sigma-Scan™. The program was calibrated with a 0.25 mm^2 area for the measurements on the 125X photomicrographs and with a 2,500 μm^2 area for the measurements on the 1250X photomicrographs. The photomicrograph of the 1 mm scale at equal magnification was used in each case to draw the calibration areas.

Photomicrograph Area. Total area covered by each 125X photomicrograph or the area of interstitium on the 1250X photomicrograph.

Area of Seminiferous Tubules. Sum of all sectional areas (including the incomplete ones along the borders of the photomicrograph) of seminiferous tubules in the 125X photomicrographs.

Area of Interstitium. Total area of the photomicrograph minus the area of seminiferous tubules.

Cross Sectional Areas of Seminiferous Tubules. Areas of round sections of seminiferous tubules.

Area of Vascular Tissue. Sum of the areas of blood vessels (including capillaries) and lymphatic vessels (including lymphatic sinusoids) in the 1250X pictures.

Area of Leydig Cells. Sum of the areas of Leydig cell sections with and without nuclei.

Cross Sectional Areas of Leydig Cells. Areas of Leydig cells with well defined nuclei.

Area of Other Components. Total area of interstitium measured on the 1250X photomicrographs minus the sum of areas for vascular tissue and Leydig cells.

Volume Percentage of Seminiferous Tubules. Mean of areas of seminiferous tubule expressed as a percentage of the photomicrograph area of 24 sections from the top, middle and bottom of a testis. In this case and in the other volume percentages, the area of a particular structure expressed in percentage was assumed to be equivalent to the percentage of the volume (volume percentage) of the testicle occupied by that particular structure.

Volume Percentage of Interstitium. Mean of areas of interstitium expressed as a percentage of the photomicrographs areas of 24 sections from the top, middle and bottom of a testicle.

Volume Percentage of Vascular Tissue. Mean of the percentages of the area occupied by vascular tissue for 48 photomicrographs times the volume percentage of interstitium of a testicle.

Volume Percentage of Leydig Cells. Mean of the percentages of the area occupied by Leydig cells for 48 photomicrographs times the volume percentage of interstitium of a testicle.

Volume Percentage of "Other Components". Mean of the percentages of the area occupied by "other components" for 48 photomicrographs times the volume percentage of interstitium of a testicle.

Volume of the Testicle. Specific gravity (1.04) times testicular weight.

Total Volume of Leydig Cells. Volume percentage of Leydig cells times the volume of a testicle.

Number of Leydig Cells per Testis. Total volume of Leydig cells divided by the average volume of the Leydig cells of a testicle.

Number of Leydig Cells per Gram of Testicular Parenchyma. Total number of Leydig cells per testicle divided by testicular weight.

APPENDIX IV

IODINATION AND DETERMINATION OF SPECIFIC ACTIVITY OF GONADOTROPINS

Iodination

Gonadotropins, oLH and hCG, were iodinated by the lactoperoxidase method. Information about the origin of these hormones are given in the text (page 40).

Reagents. The following reagents were used:

Iodination grade hCG, diluted in water 10 $\mu g \cdot 20\ \mu l^{-1}$ solution.

Iodination grade oLH, diluted in water 10 $\mu g \cdot 20\ \mu l^{-1}$ solution.

Iodine-125, sodium iodine in aqueous solution, 2m Ci, specific activity: 17.4 Ci/mg (NEN).

Lactoperoxidase (SIGMA).

Hydrogen peroxide 30% solution (Fisher).

Bovine serum albumin , BSA, RIA grade (SIGMA).

Sodium Phosphate Monobasic, monohydrate, FW = 138 (Baker Chemical Company, Phillipsburg, NJ).

Sodium Phosphate Dibasic, FW = 142 (SIGMA).

Sodium Chloride (SIGMA).

Solutions. Solutions were prepared with glass distilled water.

Phosphate Buffer Saline (PBS), pH 7.5 (PO_4 = 0.5 M, NaCl = 0.14 M).

Iodine-125, 2 mCi in 195 μl of PBS.

Lactoperoxidase, 100 μg/ml in water; 10 mg of lactoperoxidase was diluted up to 100 ml with water, divided into 2 ml aliquots and kept frozen.

Hydrogen peroxide, 1/30,000 solution, 10 μl of 30% hydrogen peroxide was diluted in 300 ml of water.

BSA, 1 mg/ml; 10 mg of BSA diluted in 10 ml of PBS.

Procedure. The same procedure was used for oLH and hCG and was performed at room temperature. One mCi of ^{125}I was added to the reaction vial containing 10 μg of gonadotropin. Then, 10 μl of the lactoperoxidase solution was added followed by 10 μl of the hydrogen peroxide solution. The vial was agitated gently and the reaction allowed to run for 3 min. The reaction was stopped by adding 500 μl of BSA solution. The reaction mixture was immediately transferred to a sephadex G-75 column equilibrated with 25 mM Tris-HCl (pH 7.5) containing 10 mM of $MgCl_2$ and 1 $mg \cdot ml^{-1}$ of BSA for separation of the iodinated gonadotropin from free ^{125}I.

Percentage of ^{125}I Incorporation Based on Trichloroacetic Acid Protein Precipitation

Reagents. All reagents were obtained from SIGMA Chemical Company, St. Louis, MO.

Tris (hydroxymethyl) aminomethane.

Tris (hydroxymethyl) aminomethane hydrochloride.

Ethylenediaminetetraacetic acid, EDTA.

Bovine Serum Albumin, BSA, RIA grade.

Trichloroacetic acid (TCA).

Solutions. All solutions were prepared in glass distilled water.

Buffer Tris HCl, 25 mM, pH 7.5 plus .001 M EDTA and 0.5 mg of BSA per ml.

TCA, 60% in water.

Procedure. A small volume of each fraction to be tested was diluted with buffer Tris-HCl to have a concentration of approximately 15,000 cpm per 200 μl. Two hundred μl of these dilutions were pipetted in duplicate into 12 x 75 mm assay tubes followed by 50 μl of TCA solution. Tubes were vortexed for about 5 sec and then centrifuged at 3,500 x g for 25 min at 4°C. The supernatants were poured into another set of tubes and the radioactivity determined in both the pellets and the supernatants.

Total counts of radioactivity added to the tubes were obtained by summing the counts in the supernatant and pellet. Percentage of ^{125}I incorporated into the hormone was obtained by calculating the percentage of radioactivity in the pellet related to the total radioactivity in the tube. Fractions with at least 60% of incorporation were pooled and used in the radioreceptor assay.

Specific Activity of the Labeled Hormone Preparation

Total counts per min of the pool of labeled hormone was determined and then transformed into desintegrations per min (dpm) by dividing by 0.5755 (57.55% was the efficiency of the gamma counter). Total dpm of the pool was divided by 2.22 x 10^6 dpm.μCi^{-1} to obtain the total activity of the preparation in μCi. To calculate the activity corresponding to the labeled hormone (specific activity), the total activity was multiplied by the percentage of incorporation of radioactivity in the hormone. The specific activity per μg of hormone was calculated based upon 90% of recovery of hormone after loses during iodination, purification, TCA precipitation and other non-specific factors.