THE EFFECT OF GROWTH HORMONE ON TESTOSTERONE SECRETION IN PERFUSED RABBIT TESTES

By

DAN EDWARD GOODWIN

Doctor of Veterinary Medicine

Oklahoma State University

Stillwater, Oklahoma

1955

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1972

THE EFFECT OF GROWTH HORMONE ON TESTOSTERONE SECRETION IN PERFUSED RABBIT TESTES

Thesis Approved:

Larry Ewing
Thesis Adviser

Claude Desjardins

Roger J. Panciera

George V. Odell

D. R. Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

I am grateful to Doctor L. L. Ewing, my graduate committee chairman, for his guidance and assistance throughout the course of this study. I am also indebted to Doctors Claude Desjardins, Roger Panciera, and George Odell, members of my graduate committee, whose helpful suggestions and compassionate attitudes helped to sustain me during the study. Special thanks are extended to the National Institutes of Health for financial support (Training Grant GM-1119-06) and for the gift of hormones used in the perfusion experiments.

Finally, I acknowledge with deep gratitude the forbearance of my wife and children during this sojourn in a strange new world.

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LITERATURE REVIEW

Introduction

The testis serves two distinct but related functions in the reproductive life of adult mammals: (1) the exocrine function of producing spermatazoa, and (2) the endocrine function of secreting hormones -- principally androgens. Testosterone is the principle androgenic hormone secreted by the testis in most mammals. Testosterone promotes spermatogenesis and growth and development of the prostate, seminal vesicles and penis. Testosterone influences the differentiation of the hypothalamus and adult reproductive behavior patterns in many species. The hormone is protein anabolic and brings about nitrogen retention, thus influencing the development of body musculature.

These varied actions of testosterone suggest that fluctuations in the body content of the steroid may result in profound changes in reproductive function, body growth and sexual behavior. Prostatic hypertrophy and Klinefelter's syndrome are examples of clinical conditions characterized by altered testosterone levels.

Testosterone may arise from sites other than the testis and before the influence of the testicular steroid can be placed in proper perspective, it is necessary that other sites be identified. A major objective of this review will be to identify sources of testosterone and to evaluate their relative contributions to the total body testosterone pool.

Lipsett and Korenman (176) were cognizant of the multiple origins of testosterone when they reported that entry of testosterone into the blood could not be expressed adequately by the term "secretion". Because of this, "secretion rate" was defined to mean the rate of entry of a hormone into the blood as a result of glandular secretions, and "production rate" as the total rate of entry of the hormone into the blood from all sources.

Testosterone production follows a diurnal pattern in some species, exhibits seasonal variation in others and is altered by aging, ejaculation, social stress, alterations in light-dark regime, blood flow through testes and increased testicular temperature. The fact that testosterone production is influenced by such a wide variety of signals argues for a complex control system. Thus, the second objective of this review will be to elucidate factors that are involved in the regulation of testosterone production.

Sources of Testosterone

Gonadal

Castration of roosters, as in other species, results in an alteration of masculine psychic and physical characteristics. Berthold demonstrated that atrophied combs of capons grew to normal proportions in response to intra-abdominal testicular grafts (168). Capons with grafts displayed normal sexual behavior and their sex accessories were comparable to those of normal males. He concluded that testes release something into the blood that maintains male behavior and secondary sex characteristics.

A nearly simultaneous achievement was that of F. Leydig who in 1850

discovered the cells within the testicular interstitium that bear his name (163). While it was his opinion that the cells were simply connective tissue cells containing fat and pigment, Bouin and Ancel in 1903 (164) presented evidence that the cells were possibly the source of the masculinizing hormone of the testes. They based their argument on the fact that cryptorchid domestic animals may exhibit masculine attributes despite defective spermatogenesis, and they reported that the intertubular tissue was well developed in undescended testes of dogs, horses, and pigs, although the tubules were atrophied. Inasmuch as Leydig cells had the structural attributes of gland cells, were a regular component of the testis, and were well preserved in animals exhibiting well developed secondary sex characters but deficient in tubular elements, they argued that these cells must be the site of production of testicular androgen. While this early work provided strong evidence that the Leydig cells were capable of producing testosterone, no evidence was presented that would argue absolutely against production of the steroid by other cell types.

The damaging effect of radiation on the testis has been noted in many animal species. Witschi, et al. (165) reported that exposure to x-rays resulted in many cases in complete degeneration of germ cells without affecting the endocrine system as evidenced by the normal development and persistence of secondary sex characters. While this observation supported the evidence that the Leydig cell was the source of the male sex hormone, it was not conclusive for some spermatogonia and Sertoli cells are radioresistant (128) and thus cannot be excluded as sources of the hormonal steroid.

Huggins and Pazos (166) presented evidence that interstitial cell

tumors in dogs are derived from Leydig cells and that these tumors secrete androgen as evidenced by enlargement of the prostate glands of affected dogs. This evidence appears to be presumptive for other cell types are present and further, neoplastic cells may not function in a physiologic manner. Christensen and Mason (167) provided the first direct biochemical evidence that androgens are produced in interstitial tissue. They separated interstitial tissue from tubular tissue of normal rat testes by dissection with jewelers forceps. The two tissues were incubated in separate flasks with progesterone-4-^{14}C and the resulting androgens analyzed. Both the interstitial tissue and the seminiferous tubules were capable of transforming progesterone to 17α-hydroxyprogesterone, 4-androstene-3, 17-dione and testosterone, although the interstitial tissue was considerably more active than the tubules. (The specific activity of ^{14}C in interstitial tissue was 40 to 148 times greater than in the tubules.) These results confirmed previous evidence that the interstitial tissue is the principle source of testicular androgens, but also showed that seminiferous tubules were capable of significant androgen biosynthetic activity in vitro.

It appears that the case for Leydig cells as a site of production of testosterone is strong but that other cell types, especially Sertoli cells, cannot be ruled out as additional gonadal sources of the hormone.

The strongest evidence against the testis serving as a unique source of testosterone is the demonstration of the hormone in plasma of females (171). On the other hand, the evidence seems irrefutable that the testis is the major source of testosterone in males. Gandy and Peterson (185) reported a marked fall in plasma testosterone levels in 14 men following orchidectomy. Mean plasma levels before surgery were

0.58 µg/100 ml and 24 hours after surgery they had declined to 0.04 µg/100 ml. This dramatic change suggested that most of the testosterone had arisen from the testes.

Extra-Gonadal Sources of Testosterone

Tissues other than the testis have the ability to transform steroid precursors into testosterone and the main sequence of biosynthetic steps from acetate through cholesterol and pregnenolone to the adrenocortical and gonadal hormones can be traced. The important concept has emerged that all of the organs that synthesize steroid hormones, viz., adrenal cortices, testes, ovaries and placenta, possess the same enzyme systems. Although the enzymes that catalyze particular transformations at particular times may predominate in specific steroid hormone-producing tissues, the others are not necessarily completely absent (1). In normal men it appears that testes produce 95% of the total body testosterone (183). While extra-gonadal sites produce only small amounts of testosterone they do possess the potential to produce large amounts should homeostatic mechanisms responsible for the regulation of testosterone secretion be upset by experimental or pathologic conditions. A review of literature would not be complete without a discussion of all known sites of testosterone production; thus, evidence will be presented for adrenal and epididymal secretion of testosterone and peripheral conversion of testosterone precursors to testosterone.

Adrenal Gland. The adrenal gland possesses the potential to produce large amounts of testosterone. For instance, congenital adrenal hyperplasia, the most common cause of virilization in the human female (169), is characterized by abnormally low plasma cortisol levels and

excessively high androgen levels. The lowered cortisol levels have been attributed to an inborn error of metabolism characterized by a deficiency of the enzyme 17β-hydroxylase. The circulating level of cortisol is a major determinant of the secretion of ACTH by the anterior pituitary gland. In all types of congenital adrenal hyperplasia, there is a reduced production of this key hormone. The lack of negative feedback of cortisol on the hypothalamo-hypophysial axis results in adrenal cortical hyperplasia and increased function. Steroids in the biosynthetic sequence preceding the metabolic block are produced in excessive quantities. Since androgen production diverges from the main stream of corticosteroid biosynthesis early in the sequence, most cases of congenital adrenal hyperplasia produce excessive androgen with the resulting clinical denominators of virilization and excessive early growth.

Plasma testosterone concentration in the male range or higher has been observed in congenital adrenal hyperplasia of women. Adrenal suppression by corticoid replacement therapy lowers the plasma testosterone levels to normal female values (170).

That the adrenal gland was the source of testosterone in congenital adrenal hyperplasia was indicated by experiments demonstrating significantly higher testosterone levels in the plasma of ovariectomized women compared with ovariectomized-adrenalectomized women suffering from this syndrome (171). Incubation studies have confirmed that the normal human adrenal can synthesize testosterone, dehydroepiandrosterone (DHEA), and Δ^4-androstenedione from various precursors (172,173).

That the adrenal of the castrate rat was an androgen source was clearly demonstrated by Bardin and Peterson (174). The concentrations of testosterone and androstenedione in adrenal venous blood were

compared to those found in peripheral venous blood. Testosterone concentrations were similar in the adrenal and peripheral venous blood from intact rats. Three days after orchidectomy, testosterone was present in adrenal venous blood while it was undetectable in peripheral venous blood. These data suggested that little if any testosterone was secreted by the adrenal in the intact animal; however, castration facilitated testosterone secretion. Androstenedione secretion was also stimulated by castration.

It seems apparent that the adrenal gland is capable of producing large amounts of testosterone in disturbed physiologic states. It seems equally apparent that it is a minor source of the hormone in the normal male of some mammalian species.

Epididymis. The epididymis has been added recently to the list of tissues that may have the capacity to synthesize testosterone. Baille, et al. (186) first demonstrated steroid metabolizing enzymes in epididymides of hamsters by histochemical techniques. They reported that various amounts of 11β-hydroxysteroid dehydrogenase, 16β-hydroxysteroid dehydrogenase, 3α-hydroxysteroid dehydrogenase and 16α-hydroxysteroid dehydrogenase enzymes were located in specific zones of epididymides.

The first direct evidence for the presence of steroids in the mammalian epididymis was provided by Frankel and Eik-Nes (179) who found both testosterone and DHEA in the rabbit epididymis. Inano, et al. (180) reported the in vitro metabolism of pregnenolone and progesterone to testosterone by epididymal homogenates of rats. Hamilton, et al. (181) showed that the mouse epididymis and ductus deferens could incorporate acetate-1-^{14}C into cholesterol in vitro. In a later paper, the same group reported that the epididymis and ductus deferens of the rat

were capable of synthesizing cholesterol and testosterone from acetate *in vitro* (182).

Frankel and Eik-Nes (129) have provided evidence that argues against epididymal synthesis of testosterone. They incubated tissue slices of rabbit epididymis in media that contained either acetate, cholesterol, pregnenolone, progesterone or other substrates. They reported no testosterone synthesis at any time regardless of the substrate used and concluded that it was reasonable to assume that epididymal testosterone originates in the testis.

While there is disagreement about the ability of the epididymis to synthesize testosterone, it is possible that the disagreement may be due to species differences since positive reports derive from experiments with rat epididymides and the negative report from experiments with rabbit epididymides. It is concluded that the question of epididymal synthesis of testosterone is unresolved. The final answer must await results of experiments that are designed to test the ability of epididymides of various species to synthesize testosterone *in vivo*.

Peripheral Conversion. There is considerable evidence that testosterone can be produced peripherally from other steroids.

The liver appears to be the most active extra-gonadal tissue involved in the synthesis of testosterone from other steroid precursors. Klempien, *et al*. (175) showed that the dog liver perfused with DHEA synthesized testosterone. Lipsett and Korenman (176) reported that radioactive testosterone and its conjugate testosterone glucuronide were isolated from human hepatic vein blood after intravenous administration of labeled DHEA. The same authors reported that other tissues, such as human prostate, rabbit skeletal muscle, and guinea pig kidney

could synthesize testosterone but believed that their contribution as compared to the liver was probably small. They observed that since DHEA and androstenedione were secreted by the adrenal cortex and ovary, they would indirectly augment the plasma pool of testosterone by peripheral conversion.

Chapledaine, et al. (177) concluded on the basis of radioactive tracer studies in man that androstenedione could be secreted by both the testes and adrenals, whereas testosterone appeared to be secreted only by the testes. By use of ^{14}C and ^{3}H labeled testosterone and androstenedione, Horton and Tait (178) were able to demonstrate the interconversion of testosterone and androstenedione in the human. They concluded that 60% of the testosterone in the female is derived from androstenedione in blood by peripheral conversion whereas less than 0.1% of the androstenedione was derived from testosterone. In the male, almost the opposite was true. Thirty-six percent of the androstenedione was derived from testosterone while less than 0.3% of the testosterone was derived from androstenedione.

Paulsen (183) reported that the combined contribution of extra-gonadal sites to the plasma testosterone pool is less than 5% of that secreted by the testis in man, except when adrenal cortical hyperplasia or an adrenal cortical tumor is present. That peripheral conversion is a minimal source of testosterone was indicated by Baird, et al. (184) when they reported that the secretion rate of testosterone in man makes up almost the entire blood production rate of testosterone.

It seems clear that testosterone may arise from a number of sources in male animals. Although it appears that Leydig cells of the testis are the principle gonadal source of testosterone the Sertoli cells have

not been excluded as possible sources. In any event the testis apparently produces about 95% of the total body testosterone with only minimal contributions being made by the adrenal gland or by peripheral conversion. The epididymal contribution of testosterone to the body pools is unresolved.

Control of Testosterone Secretion

Testosterone secretion by the testis is influenced by exteroceptive stimuli such as light, temperature, and social stress; and interoceptive stimuli such as blood flow through the testis and gonadotrophic hormone levels in the plasma. Several chemical messengers probably act singularly or in concert in a permissive, inductive, or inhibitory fashion to regulate testosterone secretion according to the needs of the total organism.

A review of all the factors involved in control of testis function is beyond the scope of this discussion and hence it will be narrowed to include only those factors that regulate testosterone secretion. To be considered specifically are the influences of signals that emanate from the hypothalamus, anterior pituitary, pineal, pancreas, testis, adrenal and thyroid gland.

Hypothalamus

Introduction. It is now firmly established that the central nervous system (CNS) regulates practically all the functions of the anterior pituitary gland. Since direct nervous connections from the hypothalamus to the anterior pituitary are lacking, it is generally accepted that this regulation is exerted through the release, into the pituitary

portal vessels, of specific mediators (187). Whatever the modality of exteroceptive stimuli effective in provoking the secretion, or in causing the inhibition of the secretion, of gonadotrophic hormones, it is clear that the hypothalamus is concerned in organizing the appropriate response. It is largely within the hypothalamus that integration of neural and humoral stimuli takes place (188).

Morphology. The hypothalamus is located on the floor of the brain and is bounded cranially by the edge of the optic chiasma, laterally by the optic tracts, and caudally by the cranial edge of the mammillary bodies. The hypothalamus is further divided into a pars medialis and a pars lateralis according to the saggital planes. All the nuclei of importance for the hypothalamo-hypophysial relationships are in the medial region (189). The smooth rounded base of the hypothalamus is termed the tuber cinereum and its central region the median eminence, from which the pituitary stalk descends (190).

Neuron pathways leaving or entering the hypothalamus are very numerous. In addition to differentiated bundles of nerve fibers, they include many isolated scattered fibers connecting the hypothalamic nuclei with one another and with other parts of the central nervous system. The possibilities for connections with other parts of the central nervous system are virtually unlimited (191). Thus a morphologic basis exists for the reciprocal functional relationship that exists between the CNS and the reproductive system.

The shortest but most clearly defined bundle of efferent hypothalamic fibers originates in the supraoptic nucleus and the paraventricular nucleus and leads via the hypophysial stalk to the neurohypophysis. In addition to this neural connection there is a vascular connection --

the hypophysial portal system (189). This vascular connection was described by Popa and Fielding in 1930 (192). The direction of the circulation in the portal system was at first a matter of controversy until Green and Harris (193) demonstrated by direct observation that the blood flowed from the hypothalamus to the hypophysis.

Releasing and Inhibitory Factors. Credit for discovery of the significance of the portal system goes to Harris (194) who postulated that hypothalamic factors were carried by means of the portal system from the hypothalamus to the hypophysis. It is now generally accepted that the hypothalamic nerve tracts release various neurotransmitter agents into the primary plexus in the median eminence and that these agents are carried to the anterior pituitary gland where they exert specific actions on the various types of glandular cells to excite or inhibit their secretory rate (195). The median eminence is the key link in neuroendocrine relationships and is represented by the region where hypothalamic nerve tracts come into contact with the primary plexus of the portal vessels (195).

By means of ingenious experiments including hypothalamic stimulation and lesioning, pituitary stalk transection, and pituitary transplantation it has been demonstrated that the hypothalamus contains distinct substances which regulate the release of anterior pituitary hormones. Full structures of all the substances have not yet been elucidated and there are relatively few data as to their measurement in the peripheral circulation. Nevertheless, various physiological and biochemical studies indicate their important physiological role and support the concept that some of these are hormones (196).

Evidence for the existence of a luteinizing hormone releasing

factor (LHRF) and a follicle stimulating hormone releasing factor (FSHRF) was reported by McCann, et al. (197). Schally, et al. (196) identified and separated the two factors and described the biological properties of each in both in vitro and in vivo experiments. More recently (1971), Schally, et al. (154) reported that a single hypothalamic substance was responsible for the release of both FSH and LH. They reported that LHRF was obtained in apparently a homogeneous state from extracts of 165,000 pig hypothalami and furthermore that the isolated LHRF had FSHRF activity which appeared to be intrinsic to the LHRF. The amino acid composition of the LHRF/FSHRF was determined by acid hydrolysis to be: His 1, Arg 1, Ser 1, Glu 1, Pro 1, Gly 2, Leu 1 and Tyr 1. A few nanograms of the purified LHRF caused the release of both FSH and LH in vivo and in vitro from rat pituitaries. They concluded that this polypeptide appeared to represent the hypothalamic hormone which controls the secretion of both LH and FSH from the anterior pituitary. While the evidence presented by this group argues strongly for a single gonadotrophin releasing factor the proof is not absolutely conclusive for they further reported that while the product appeared to be homogeneous, due to the limited amount of material available, many conventional methods of proof of purity were inapplicable.

Talwalker, et al. (198) reported that when rat anterior pituitary (AP) was incubated for two hours, 169% more prolactin was found in the combined medium and AP than in non-incubated AP. When AP was incubated together with homogenate or acid extract of rat hypothalamus, prolactin levels in the medium and AP were markedly decreased (36-75%), indicating inhibition of prolactin synthesis and release. These in vitro findings suggest that the hypothalamus contains a factor(s) which inhibits

synthesis and release of prolactin. Schally, et al. (196) extended these findings when they demonstrated that the in vivo injection of pig hypothalamic extracts inhibited depletion of prolactin from the AP of rats.

Kamberi, et al. (204) observed the effect of infusion via a hypophyseal portal vessel of a crude hypothalamic extract on gonadotrophin release in male rats. Infusion during a 30 minute period caused a steady rise in the plasma concentration of LH and FSH and a decline in the prolactin level. Soon after the cessation of infusion the plasma concentration of LH and FSH fell and prolactin rose. Thus it was confirmed that the hypothalamus contains substances that stimulate the release of FSH and ICSH and inhibit the release of prolactin.

Integrative Function. Halasz (199) studied the influence of the hypothalamus upon reproduction. By transplanting AP tissue into various regions of the hypothalamus of rats, he determined that only one hypothalamic area was capable of maintaining the normal structure and function of the AP. He labeled the area, located in the medial basal hypothalamus, the "hypophysiotrophic" area. He isolated the hypophysiotrophic area surgically so that all afferent neural connections were interrupted. The deafferentiated region was left in contact with the pituitary by the unbroken pituitary stalk. Neural isolation of the medial basal hypothalamus did not result in appreciable changes in gonadotrophic hormone secretion in male rats (200) but altered it in female rats (201) by interfering with ovulation. Subsequent experiments with partial deafferentiation of the "hypothalamic island" allowed location of other central nervous system areas that influenced the anterior pituitary via the medial basal hypothalamus. This series of experiments

suggested to Halasz that there may be two hypothalamic levels involved in the neural control of the anterior pituitary. (1) One level seems to be represented by the medial basal hypothalamus (the hypophysiotrophic area) which produces the releasing and inhibiting factors essential for the anterior lobe of the pituitary. This area may be responsible by itself for maintaining the basal secretion of pituitary hormones and appears to be able to produce and release the "hypophysiotrophic" substances in the absence of neural elements. (2) The second control level is considered to be the nervous structures located outside the medial basal hypothalamic area that are involved in the neural control of the anterior pituitary. This level consists of several structures including various hypothalamic areas outside the hypophysiotrophic area, the limbic system, midbrain reticular formation and the cerebral cortex. The work of Halasz is important for it suggests mechanisms whereby the hypothalamus can mediate both hormonal and nervous signals that result in secretion of AP hormones, e.g., the hypophysiotrophic area is sensitive to (1) hormones that are produced by the gonads, and (2) nervous impulses arising in the central nervous system in response to environmental influences such as light, sexual stimuli and nutritional state. Thus, the hypothalamus serves an integrative function with respect to the internal and external environment of the organism.

Richlein (190), Whalen (203), and Davidson and Bloch (202) reported that the male hypothalamus promotes a tonic gonadotrophic secretion from the AP in contrast to the female hypothalamus which contains an intrinsic neural mechanism that results in a surge of LH secretion at estrus. While the concensus appears to favor tonic ICSH secretion from the male

AP, a recent report by Bolt (152) indicates that ICSH secretion in adult male sheep may be cyclic. He measured plasma ICSH levels at approximately 4 hour intervals during a 53 hour period and reported that the levels varied from 0.21 mμg/ml to 12.8 mμg/ml and that at least three separate surges of ICSH secretion occurred during the test period.

Testosterone "feedback" upon the hypothalamus inhibits the release of hypothalamic factors that in turn regulate the secretion of anterior pituitary hormones. The specific mechanisms of control will be considered in the anterior pituitary section of this review.

In summary, this discussion has presented evidence supporting the concept that the hypothalamus plays a key role in mediating both environmental and hormonal influences on the secretion of gonadotrophins and prolactin by the anterior pituitary. Several experiments indicate that the hypothalamus has a net stimulating effect on the secretion of FSH and ICSH and a net inhibitory effect on the secretion of prolactin. The pathway by which the hypothalamus exercises its control over the anterior pituitary has been shown to be the hypophysial portal system of vessels. The vessels originate in the median eminence and carry capillary blood to the anterior pituitary. These vessels provide a route by which neurohumors secreted in the median eminence might trigger a release of FSH and ICSH or inhibit release of prolactin from the anterior pituitary. This leads to the concept of neurohumoral control of the anterior pituitary and thus provides a mechanism for control of secretion of testosterone by the testis.

Pituitary Gland

Introduction. Endocrine studies of the pituitary gland were hampered for many years because of the surgical difficulties attendant upon removing the organ without injury to the brain. In 1910, Aschner worked out a technique for performing hypophysectomies in dogs and in 1926, P. E. Smith developed a similar technique for ablating the gland in rats and other laboratory rodents (1). Thus, it became possible to study the functions of the pituitary gland in classical endocrine experiments including ablation and transplantation. That the gland was important in reproductive processes was readily apparent for after hypophysectomy of adult rats, the testes and ovaries became nonfunctional and failed to produce mature germ cells or sufficient quantities of gonadal hormones to maintain the functional status of the accessory sex organs (1).

In this literature review, attention will be focused on the role of the anterior pituitary in regulating the secretion of testosterone from mammalian testes. The actions of the pituitary hormones; ICSH, FSH, prolactin and GH will be considered.

Embryology and Gross Morphology. Many references contain descriptions of pituitary gland embryology and morphology (1,2,3,4,5). The pituitary gland, or hypophysis, is a compound gland of ectodermal origin, arising from two different sources. One part, the neurohypophysis, arises from the ventral floor of the diencephalon and remains connected to the hypothalamus throughout life by means of its stalk. The glandular portion of the hypophysis, the adenohypophysis, stems from oral ectoderm as Rathke's pouch, which is an outgrowth from the roof of the mouth. This outgrowth meets the embryonic neural portion of the gland and then

loses its connection with the oral epithelium.

The adenohypophysis is described as consisting of 3 parts: the pars tuberalis, the pars intermedia, and the pars distalis (often referred to as the anterior pituitary or AP). The neurohypophysis is also divided into 3 parts: the median eminence of the tuber cinereum, the infundibular stem, and the infundibular process (neural or posterior lobe). The median eminence and infundibular stem are collectively referred to as the infundibular or neural stalk, whereas the hypophysial stalk includes the neural stalk plus the sheath portions of the adenohypophysis, the pars tuberalis.

The well-protected hypophysis is one of the most inaccessible organs of the body, being located in the sella turcica, a depression in the sphenoid bone. The gland is encapsulated by the dura mater, but the hypophysial stalk penetrates the dura through the diaphragma sella. The neurohypophysis is characterized by its rich innervation of hypothalamic origin; whereas the adenohypophysis is characterized by rich vascularization.

Adenohypophysis. The glandular portion of the pituitary is made up of irregular masses and columns of epithelial cells which are supported by a delicate framework of connective tissue. The groups and columns of cells are separated by sinusoids. Two main types of cells are evident. One type does not show any conspicuous stainable cytoplasmic granules and these are referred to as chromophobes. The other contains cytoplasmic granules which take up stains readily and are called chromophiles. The latter are considered to be secretory in nature. The chromophiles may be further divided, according to the stainability of their granules, into alpha cells (acidophiles) and beta cells

(basophiles).

FSH and LH are both secreted by beta cells (1). The cells which secrete FSH and LH undergo hypertrophy and degranulate following castration. Large cytoplasmic vacuoles also form, and the nucleus is compressed against the cell membrane to form a "signet ring" or castration cell. The cells which secrete FSH are located near the periphery of the AP, whereas those which secrete LH are located centrally. Prolactin is secreted by alpha cells (1) and in most species these cells increase during pregnancy and lactation. GH is also secreted by alpha cells and by noting differences between granule size and shape it is possible to distinguish between cells that secrete prolactin and those that secrete GH.

Interstitial Cell Stimulating Hormone (ICSH). ICSH (also known as luteinizing hormone or LH) is a carbohydrate containing protein (6). The molecular weight of ovine ICSH is 28,000 to 30,000. The human hormone has a molecular weight of about 26,000.

Atrophy is the most frequently mentioned change in Leydig cells after hypophysectomy (27). In hypophysectomized mice, it was found that testicular grafts of anterior pituitary tissue repair the atrophic tubules and the involuted Leydig cells (8). In addition, it was observed that injection of anterior pituitary extracts into testes of guinea pigs resulted in pronounced stimulation and hypertrophy of the Leydig cells (7). Thus it is clear that the anterior pituitary gland contains a substance(s) that exercises control over Leydig cells.

Maintenance, initiation, or restoration of production of androgen, as indicated by weight, structure, and secretory activity of the several glands and ducts of the genital system, particularly the prostate glands

and the seminal vesicles, by administered ICSH has been demonstrated chiefly in the rat (27). Examination of this action in other species has not been extensive but has been reported in such widely diverse animals as the frog (30) and the monkey (31). Similar evidence of the stimulation of secretion of androgen by various gonadotrophin preparations such as pregnant mare's serum (PMS), human chorionic gonadotrophin (HCG), extracts of hypophysis and hypophyseal implants has been observed by many investigators and is usually taken to be a manifestation of action of an interstitial cell stimulating component of these preparations (27).

The biological evidence that ICSH provokes testosterone secretion has been supported by direct measurement of steroid compounds produced in response to hormone injections. Brinck-Johnson and Eik-Nes (32) administered single intravenous injections of HCG or ICSH into heparinized, anesthetized dogs and observed a significant increase in the level of testosterone and androstenedione in spermatic vein blood. The high levels suggested augmentation of synthesis rather than mere release into the blood. That synthesis did in fact occur in response to ICSH, was demonstrated by Eik-Nes (33) when infusion of ICSH through the spermatic artery in dogs promptly increased the level of testosterone in the blood of the spermatic vein and increased the incorporation of tritiated acetate into testosterone.

In vitro studies have supported the in vivo evidence that ICSH stimulates testosterone synthesis and release. A single injection of ICSH or HCG into hypophysectomized rats increased the conversion of cholesterol to testosterone and androstenedione by homogenates of the testes of treated animals (34). Ewing and Eik-Nes (46) demonstrated

the biosynthesis of testosterone in perfused rabbit testes *in vitro*. They reported that addition of HCG or ICSH to the perfusion medium resulted in increased testosterone secretion and alterations in rates of incorporation of acetate-1-^{14}C and cholesterol-7α-^{3}H into testosterone.

Immunologic studies have added to the evidence that ICSH stimulates androgen production. Mougdal and Li (35) reported that antiserum produced in rabbits in response to ICSH from ovine hypophyses reduced the weight of the ventral prostate in immature rats in a period of 4 days, suggesting inhibition or inactivation of endogenous ICSH. In a similar study with adult rats, Wakabayashi and Tamaoki (36) reported that active immunization of rats to ovine ICSH given with Freund's adjuvant led to atrophy of the testes, prostate glands and seminal vesicles.

It is well established that ICSH stimulates the Leydig cells to synthesize and secrete testosterone. How does the hormone accomplish this action? In describing concepts of hormone action, Sutherland, *et al*. (10) reported that when a hormone (first messenger) reaches its target cell, it interacts with a specific portion of the cell and as a result of this interaction, there occurs an increased or decreased rate of production within the cell of a second messenger which mediates the action of the hormone. The change in the level of the second messenger so alters cellular function by changing an enzyme activity, membrane permeability, or other process, that the cell responds in a characteristic way to the hormone. Cyclic adenosine mono-phosphate (AMP) is the only recognized second messenger, but other similar agents may exist. The formation of this nucleotide from adenosine tri-phosphate (ATP) in biological systems is catalyzed by the enzyme adenyl cyclase, which is present in all animal cells examined except non-nucleated erythrocytes

and, in at least some tissues, is located in the plasma membrane. Three criteria are listed for establishing a role for cyclic AMP as a mediator of hormone action: (1) The hormone should stimulate the formation of cyclic AMP *in vitro* in broken cell preparations of the target tissue. (2) The levels of cyclic AMP in the intact tissue should change in response to the hormone. (3) The effect of the hormone should be reproduced by cyclic AMP. These 3 criteria seem to have been met for ICSH.

The action of ICSH has been shown to be mediated through cyclic AMP in bovine corpus luteum tissue by Marsh (9). He reported that the hormone brought about a striking increase (as much as 100 fold) in endogenous cyclic AMP of incubating slices of corpora lutea. He stated that the results supported the conclusion that ICSH acts by stimulating the adenyl cyclase system. Kuehl, *et al.* (11) have reported that ICSH is capable of increasing cyclic AMP levels in testicular slices from both normal and hypophysectomized rats. They reported also that ICSH stimulated adenyl cyclase in isolated seminiferous tubules and suggested that the action of ICSH in the testes was not restricted to the Leydig cells but it also played a role in conjunction with FSH in the growth of the epithelium of the seminiferous tubules. Connell and Eik-Nes (12) demonstrated that cyclic AMP will increase the production of testosterone in rabbit testes slices.

How is ICSH secretion regulated? It has been clearly demonstrated in the rat that a decrease in gonadal steroid production in either the male or female results in enhanced rates of synthesis and release of LH as evidenced by rises in plasma and pituitary levels of LH (13,14,15). Earlier work also demonstrated that administration of gonadal steroids to the castrate rat inhibits release and synthesis of LH (13,15).

It is now well established that the pituitary gland is regulated by two different types of feedback mechanisms (16). The first, which might be called the "classic" feedback system, was discovered several years ago; in this system the controlling (inhibiting or activating) signals are the hormones produced by the peripheral target glands (adrenal cortex, gonads, thyroid). The second, discovered more recently, is usually referred to as the "short", "auto", or "internal" feedback mechanism; in this system the controlling signals are the pituitary hormones themselves.

The location of the feedback receptor for gonadal steroids appears to be in both the hypothalamus and anterior pituitary for the female, while in the male it appears to be confined to the hypothalamus (17). Implants of testosterone propionate in the median eminence region, but not in the pituitary, resulted in testicular and accessory sex gland atrophy in the dog (18) and the rat (19,20). In the rat, different areas of the hypothalamus have been carefully mapped, and the effect is well localized to the basal medial hypothalamic region (19). The problem of whether median eminence testosterone implants act merely via diffusion of the steroid to the pituitary has been investigated by Smith and Davidson (20). Testosterone implants were placed in the median eminence region of hypophysectomized rats with renal pituitary transplants that maintained testicular structure and spermatogenesis. Four weeks later, significant decreases in testicular weight and inhibition of spermatogenesis were noted. Since prostates were inhibited rather than stimulated by the implants, the results were not due to systemic release of testosterone.

It has proven difficult to find an approach to the localization of

the hypothalamic receptors which respond to decreases in circulating testosterone by producing an increase in gonadotrophin output. Davidson and Bloch attacked the problem in a novel way (26). They implanted pellets of cyproterone, an antiandrogen that blocks the action of testosterone on the adult reproductive system, into the median eminence region of male rats. They observed significant stimulation of seminal vesicles, ventral prostates and occasionally testes. After extensive mapping experiments that involved the pituitary, median eminence, and various other regions of the brain, they concluded that the same region -- the basal medial hypothalamus -- which responds to increases in plasma testosterone by decreasing gonadotrophin secretion, also responds to decreases in plasma testosterone by initiating increases in gonadotrophin secretion.

The regulatory effects of testosterone on ICSH secretion were reflected in a dose response curve reported by Davidson (17). He showed a negative correlation between administered testosterone and plasma ICSH in adult orchidectomized rats. Plasma ICSH declined in a linear fashion in response to increasing doses of testosterone propionate. The effective range of testosterone propionate doses extended from 6.2 to 100.0 µg/100 gm body weight/day. Despite the well established negative feedback effects of changes in blood androgen level as is seen in castration and administration of pharmacological doses of androgen, Davidson and Bloch (26) have reported that it is still not clear what role variations in circulating androgen within the physiological range play in the normal regulation of gonadotrophin secretion.

The existence of a "short" feedback mechanism for the control of ICSH secretion was proposed by Sawyer and Kawakami (21) and by Kawakami

and Sawyer (22) on the basis of their observations that, in estrogen primed ovariectomized rabbits, the EEG was influenced by endogenous gonadotrophins released by coitus. Further evidence for short feedback was provided by the demonstration that placement of small amounts of ICSH in the median eminence of normal or castrated rats of both sexes resulted in a decrease in pituitary and plasma ICSH levels (23,24). Desjardins (37) determined the concentration of ICSH and FSH in the hypophyses and plasma of orchidectomized mice after injecting ICSH or FSH on days 1, 3, and 6. Exogenous ICSH reduced the concentration of hypophyseal and plasma ICSH, but FSH release was not affected in the same animals. Similarly, exogenous FSH reduced the concentration of hypophyseal and plasma FSH, but ICSH release was not affected. This evidence suggested that exogenous ICSH and FSH could suppress their respective secretions specifically and argues against the concept of a single hypothalamic substance regulating the secretion of both FSH and ICSH as proposed recently by Schally, et al. (154). McCann, et al. (25) have observed that while accumulated data indicated that auto-feedback of ICSH does exist, it appeared to be inoperative except at upper physiological levels of ICSH secretion.

It is concluded that the anterior pituitary gland is the source of ICSH and that the hormone stimulates Leydig cells to synthesize and secrete testosterone. While the mechanism of action of ICSH has not been conclusively resolved it appears that cyclic AMP is an integral part of the mechanism. The regulation of ICSH secretion is under the direct control of the hypothalamus. A hypothalamic releasing factor exerts the control by way of the hypothalamo-hypophysial portal system. Synthesis and secretion of the releasing factor is governed by (1)

"classic" feedback of testosterone, and (2) "internal" feedback of ICSH.

Follicle Stimulating Hormone (FSH). It has been generally agreed that the chief function of FSH is to stimulate spermatogenesis while that of ICSH is to stimulate steroidogenesis by the Leydig cells. In recent years, however, evidence has accumulated that argues against clearly defined roles for the gonadotrophic hormones and the concept has emerged that FSH may not be as distinctively involved in male reproduction as it is in female reproduction.

FSH is a water soluble glycoprotein. Molecular weights ranging from 30,000 to 67,000 have been reported for the hormone (1). Roos (38) reported that human pituitary FSH has a molecular weight of 41,000. Although considerable progress in the purification of FSH has been reported, there seems to be no biologically pure preparation available. The principle active contaminant of FSH is ICSH (39).

Woods and Simpson (40) reported that FSH was unable to stimulate Leydig cells in hypophysectomized rats except at dose levels that contained sufficient contaminating ICSH to stimulate Leydig cells in the absence of FSH. They stated that it was clear that in the male ICSH was the primary anterior pituitary hormone responsible for the seminiferous epithelium as well as the Leydig tissue, inasmuch as FSH was ineffective in its absence. Means and Hall (41) reported that while FSH stimulated the incorporation of labeled amino acids into testicular protein of 20 day old rats, the hormone had no effect on the incorporation of amino acids into protein of testes from sexually mature animals. Lostroh (42) suggested that certain of the synthetic systems that develop in the immature testes under the influence of FSH and ICSH, once established, persist and show little specific hormone dependency.

Clermont and Harvey (43) concluded that the role of FSH in the adult male rat, if it has any, has yet to be elucidated.

Hamberger and Steward (44) measured oxygen uptake in isolated interstitial cells from testes of 30 day old rats. In all instances, the oxygen uptake of the interstitial cells, incubated in a medium containing succinate as substrate, raised when ICSH (50 µg/ml) was added to the incubation medium. In contrast, the addition of FSH (50 µg/ml) to the medium was without measurable effect on the oxygen consumption of the cells. Connell and Eik-Nes (12) reported that FSH stimulated testosterone production in rabbit testes slices. They speculated that the response was probably due to ICSH contamination and suggested that a synergistic action might exist between the two hormones.

The significance of a synergistic relationship between FSH and ICSH was emphasized by Greep (45) when he observed recently that separation of the gonadotrophin complex into pure fractions of FSH and ICSH was now mainly of academic interest for it seemed certain that physiologically the gonadotrophins act only in concert.

One of the earliest reports that indicated a possible synergism between FSH and ICSH was by Greep, et al. (28) who reported in 1936 that in both hypophysectomized and normal immature male rats, FSH augmented the effect of ICSH on secondary sexual structures. They observed that when both FSH and ICSH were administered the accessory structures attained a larger size than would have been produced by an equivalent amount of ICSH acting alone. It seems apparent that contaminating ICSH in the FSH preparation could not be excluded as an additional source of ICSH, sufficient in itself to produce an additive effect.

Parlow and Reichert (29) reported that FSH at dose levels

predetermined to have no effect on prostate weight of hypophysectomized rats when injected alone, produced a significant augmentation of the prostatic response when combined with ICSH. Simpson, et al. (47) demonstrated that doses of HCG, which were "sub-minimal" or "marginal" for maintenance of interstitial cells in hypophysectomized male rats, were increased in effectiveness by doses of FSH far below those having any effect on interstitial cells alone. Woods and Simpson (40) noted the importance of ICSH synergism when they reported that none of the hormones of the anterior pituitary gland could maintain the testicular tubules of hypophysectomized male rats unless ICSH was present, nor could any combination of anterior pituitary hormones repair the testes after post-hypophysectomy regression unless ICSH was present.

Lostroh (42) observed the effects of FSH plus ICSH when administered to male rats 2 months after hypophysectomy performed at 28 days of age. A daily dose of 3 µg of FSH, a dose that iself stimulated neither androgen secretion nor repair of the germinal epithelium, promoted a 40-fold increase in the weights of the sex accessory glands when given together with 100 µg of ICSH; ICSH alone produced only an 11-fold increase in these organ weights.

Johnson and Ewing (48) demonstrated conclusively that FSH augments ICSH stimulated testosterone secretion by rabbit testes perfused in vitro with an artificial medium. They reported that the synergistic effect of FSH upon ICSH stimulated testosterone secretion was not due to ICSH contamination since testes were maximally stimulated by saturating levels of ICSH. Their experimental system permitted the effects of FSH and ICSH on testosterone secretion to be examined without interference from extra-gonadal signals.

In conclusion, it appears that while the direct role of FSH in spermatogenesis is not clear, the evidence is conclusive for an FSH-ICSH synergism that augments the production of testosterone from ICSH stimulated Leydig cells. The positive effects of testosterone on spermatogenesis are well known and it seems reasonable to speculate that the effects of FSH on spermatogenesis may be indirect, mediated through testosterone.

Prolactin. Prolactin is a protein hormone with a molecular weight of about 25,000 (1). The hormone functions in the female rat and mouse to promote the secretion of progesterone by corpora lutea (1) and is needed in addition to LH and FSH to maintain functional corpora lutea in pregnant hamsters (59).

Until very recently the most dramatic effects of prolactin in males were recorded for avian species, e.g., Riddle and Bates (50) reported that the hormone induced rapid involution in the mature testes of ring doves. According to Riddle (52), prolactin prevents the release of gonadotrophins from the pituitary and has a number of extra-gonadal effects in some birds which are involved indirectly with reproduction. Among them are the formation of pigeon milk by crop glands of birds in the order Columbiformes. The hormone is also required for the formation of the so-called incubation or brood patch, which is formed on the breast of a number of avian species that incubate their eggs by setting on them.

The effects of prolactin in male mammals are not well defined, but evidence is accumulating that indicates a possible physiologic role in reproduction. Early studies assigned the hormone a synergistic role with testosterone in promoting growth of accessory sex organs. Meites

and Nicoll (53) reported that large doses of prolactin synergise with androgens in stimulating the growth of prostate glands of male rats. Antliff, et al. (54) studied the role of prolactin in stimulating epithelium of seminal vesicles of castrated guinea pigs. The hormone had no effect on seminal vesicles of castrates but together with "subminimal" testosterone propionate caused significant increases in weight as well as height of epithelium. Gunn, et al. (55) reported that in mature hypophysectomized rats, the amount of ^{65}Zn taken up by the dorsolateral prostate is a more sensitive indicator of androgen activity than the weight of the gland. They used this system to test the effects of prolactin and reported (56) that the hormone caused an augmentation of testosterone activity on uptake of ^{65}Zn in the hypophysectomized, castrated rat, indicating an effect of prolactin on the prostate that was not mediated by the testes.

In addition to prolactin-testosterone synergism, there is evidence that prolactin effects a direct, non androgen-dependent stimulation of the reproductive system of the male mouse. Bartke and Lloyd (149) reported that pituitary homografts under the kidney capsules of mice that had been castrated, castrated and adrenalectomized, or castrated and hypophysectomized resulted in an increase in seminal vesicle weights. They concluded that prolactin, secreted by the grafts, was responsible for the seminal vesicle stimulation.

The most significant effects of prolactin in male reproduction appear to be due to a possible synergism with gonadotrophic hormones to augment the production of testosterone by the testis. Woods and Simpson (40) reported that prolactin potentiates the action of ICSH and FSH on the reinitiation and/or maintenance of spermatogenesis in the

hypophysectomized rat. It appears that their evidence was indirect for the possibility exists that prolactin-testosterone synergism rather than prolactin-gonadotrophin synergism could have influenced spermatogenesis. Pituitary dwarf mice are sterile although females may mature and mate, and males produce living spermatazoa (57). Bartke (58) has reported that various histological, cytochemical, electron microscopic, and bioassay studies indicated that in dwarf mice, growth hormone, thyrotrophic hormone, and prolactin were absent or deficient, whereas FSH and LH were produced. He reported that he provided a constant supply of prolactin to male dwarf mice by grafting hypophyses from normal mice into their renal capsules and observed that of 7 male dwarf mice so treated, 6 became fertile. Though the results implied a prolactin-gonadotrophin synergism the experiment could be criticized for two reasons: (1) abnormal animals were used, and (2) pituitary substances other than prolactin could have been responsible for the improved fertility. Bartke and Lloyd (150) administered ovine prolactin daily for 28 days to intact male dwarf mice and reported that this treatment stimulated spermatogenesis and increased seminal vesicle weights. They concluded that the observed stimulation of spermatogenesis was most likely due to elevated testicular androgen production indicating that, in the male dwarf mouse, prolactin may be involved in pituitary regulation of Leydig cell function. To elucidate the mechanism of this action Bartke (151) treated hypophysectomized male dwarf mice daily for 28 days with either prolactin, ICSH, prolactin + ICSH, testosterone propionate, or prolactin + testosterone propionate. Treatment with ICSH or testosterone propionate caused partial restoration of spermatogenesis. Addition of prolactin to testosterone propionate treatment had no effect on spermatogenesis,

however, in animals given prolactin + ICSH the yield of spermatogenesis was considerably greater than in animals given ICSH alone. These results were interpreted to mean that prolactin stimulated spermatogenesis in dwarf mice by potentiating the action of ICSH rather than by acting directly on the seminiferous epithelium or potentiating the effect of testosterone. It was concluded that prolactin appeared to act synergistically with ICSH to increase the production of androgenic hormones. This elegant experiment provided strong evidence that prolactin-ICSH synergism occurred, but was weakened, as were previous experiments, because the experimental animals were genetic dwarfs. That weakness, however, was overcome when the same group of researchers conducted an experiment with normal male rats. Thus Hafiez, et al. (110) injected hypophysectomized adult male rats subcutaneously twice daily for 3.5 days with 0.9 percent saline, 200 µg ovine prolactin, 5 µg ovine ICSH, or 200 µg ovine prolactin + 5 µg ovine ICSH. They reported that plasma testosterone levels in the saline and prolactin treated rats were generally unmeasurable whereas administration of ICSH increased testosterone levels to 1.42 mµg/ml and prolactin + ICSH increased the levels to 4.63 mµg/ml which approximated testosterone levels in intact adult males (5.34 mµg/ml). They concluded that the results provided evidence for a synergistic action of prolactin and ICSH on the biosynthesis of testosterone.

In summary, it appears that prolactin may deserve to be assigned an important role in reproductive processes of male animals. Evidence has been presented that suggests three possible effects of the hormone: (1) independent stimulation of accessory sex organ growth, (2) synergism with testosterone to promote accessory sex organ growth, and (3)

synergism with ICSH to augment secretion of testosterone from the testis. While the first two effects cannot be excluded, the evidence appears to favor a prolactin-ICSH synergism. Strong evidence has been presented to argue for prolactin-ICSH synergism but until the synergism has been demonstrated in an isolated testis preparation, the evidence must be considered presumptive.

Growth Hormone. For many years the principle function of growth hormone was thought to be the promotion of somatic growth. Evidence has accumulated in recent years suggesting that the hormone has a much broader activity and the concept has emerged that growth hormone creates a more beneficial environment for other agents and thereby acts as a biological synergist (61).

Growth hormone is a protein hormone, whose chemical and physical nature has been described by Evans, et al. (62). Molecular weight varies from 21,500 to 48,000; materials of lowest molecular weight are found in primates while those from sheep and cattle are of highest molecular weight. Human growth hormone possesses lactogenic in addition to growth promoting activity, whereas in the non-primate these two functions are mediated by separate substances.

The effect of growth hormone on male reproductive processes has been investigated by many workers. While there is disagreement in some instances about the purity of the hormone used, there seems to be general agreement that it synergises with testosterone and limited agreement that it synergises with ICSH. Huggins, et al. (63) revealed that when GH was administered concurrently with testosterone to young hypophysectomized castrated male rats, the two substances operated synergistically to promote growth of the accessory reproductive organs.

The observation of Grayhack and Scott (64) that prostates of hypophysectomized rats were less responsive to testosterone than those of non-hypophysectomized rats indicated that another pituitary hormone might be acting synergistically with the androgen. Boccabella (60), testing the ability of testosterone propionate to restore spermatogenesis in 70 day hypophysectomized rats, administered testosterone propionate either separately or in combination with bovine GH. He reported that while testosterone alone could restore spermatogenesis to a limited extent, the addition of GH appeared to augment the effect significantly.

In a study designed to elucidate hormonal factors that influence the bio-assay for ICSH, Lostroh, et al. (66) reported a strain difference among rats in their response to bovine GH. They found that the administration of GH concurrently with ICSH had no influence on the prostatic response of hypophysectomized Long-Evans rats. On the other hand, in hypophysectomized Sprague-Dawley rats, GH, when administered with ICSH, caused a significantly greater increase in ventral prostate weight than ICSH alone. In light of these findings and in view of the fact that the prostatic response to exogenous testosterone was enhanced when GH was administered with the androgen, they concluded that GH probably synergised with both ICSH and testosterone.

Randolph, et al. (67) reported that bovine growth hormone maintained testicular weight as well as spermatogenic activity in hypophysectomized mice. They believed that the response was due to ICSH contamination for periodate treated and chymotrypsin treated preparations of GH were ineffective in ameliorating the degenerative changes in the testes that ensued following hypophysectomy, although they retained their capacity to stimulate body and skeletal growth. They reported

that it was in combination with ICSH that GH exerted its characteristic effect; a synergistic action of the two hormones in stimulating growth of the ventral prostate and seminal vesicles. ICSH alone was incapable of stimulating normal growth of the ventral prostate and seminal vesicles whereas either ICSH plus GH or testosterone propionate stimulated growth of the organs parallel to that observed in the normal animal.

Lostroh (42) evaluated the effects of certain hormones on weight and histologic structure of testes and accessory sex organs of immature hypophysectomized Sprague-Dawley rats. She reported that GH given together with FSH and testosterone increased the testicular weight 57% over that obtained with the other two hormones (FSH and testosterone), but did not selectively advance germ cell development.

Woods and Simpson (40) have assessed the action of each of the pituitary hormones in the maintenance of the reproductive system of the hypophysectomized 40 day male rat. They reported that low doses of FSH injected with ICSH increased testicular size and development. Doses of GH and prolactin, having no effect on the reproductive system alone, increased the response to ICSH and also further augmented the response to ICSH with FSH. Other anterior pituitary hormones, thyrotrophin and adrenocorticotrophin, had no influence on the reproductive system given alone or with the gonadotrophins. In repair of the testes after post-hypophysectomy regression, ICSH alone repaired Leydig cells but caused no more than a slight repair of testicular tubules even at high doses. FSH alone was without effect in repair but synergised with ICSH to induce tubular differentiation and augmentation of accessory organ growth. Addition of either growth hormone or prolactin to the combination of ICSH with FSH increased the degree of stimulation; simultaneous

injection of all four hormones was even more effective, and was the only treatment in which sperm were formed in all animals. It was reported that extracts of rat pituitaries caused similar development indicating presumably that the four hormones (of sheep origin) in combination, were capable of maintaining normal function of the male rat reproductive system.

In conclusion, it appears that the anterior pituitary hormones FSH, ICSH, prolactin and GH all may play some role in regulating testosterone secretion. The experiments of Johnson and Ewing (48) demonstrated conclusively that FSH synergises with ICSH to augment testosterone secretion by ICSH stimulated testes. Direct evidence for similar functions of prolactin and GH is not yet available.

Pineal Gland

A relationship between the pineal gland and certain reproductive functions in mammals has been suspected for several years. For instance, it has been shown that, in female rodents, pinealectomy causes precocious puberty, ovarian hypertrophy and constant estrous (68). These effects of pinealectomy can be reversed by the administration of pineal extracts and thus it is suggested that the pineal gland exerts an inhibitory influence on the gonad.

The anatomy and histology of the pineal gland has been discussed by several authors (69,71,135). The gland is attached to the posterior end of the roof of the third ventricle. Usually the gland is conical or oval in shape and its base lies above the posterior commissure. In the rabbit the pineal gland consists of a terminal pyriform expansion which is connected to the brain by a long, narrow stalk. In the rat

the terminal part of the gland becomes separated by rupture from the stalk and in the adult animal the organ is connected with the brain by blood vessels and sympathetic nerves. The pineal gland is composed of secretory cells called pinealocytes, which form numerous cytoplasmic processes that end at the walls of blood vessels or join with each other to form plexuses.

Wurtman (70) described the mammalian pineal organ as a neuro-endocrine transducer; it responds to an input of neural information (impulses from its sympathetic nerves) by releasing hormonal messages into the blood stream and/or cerebrospinal fluid. Its place in the hierarchy of biological communication systems thus differs from that of such "classic" glands as the pituitary and the thyroid (which receive their instructions via portal or arterial blood) and is similar to that of the adrenal medulla, the supraoptic region of the hypothalamus, and the median eminence.

The mammalian pineal gland is unusually rich in such biogenic amines as serotonin, noradrenalin, and histamine, as well as the enzymes that synthesize and metabolize these physiologically active compounds (71). A characteristic indole, melatonin, is highly localized in the pineal; the enzyme, hydroxy-indole-o-methyl transferase (HIOMT), responsible for its production is found nowhere else in the mammal (71). HIOMT transfers the methyl group of S-adenosylmethionine to the hydroxyl group of N-acetylserotonin, forming melatonin and S-adenosylhomocysteine (49). Since HIOMT is essential for, and appears to be rate-limiting to, the synthesis of melatonin, the factors controlling HIOMT are of extreme importance in pineal physiology (49). Activity of HIOMT was increased by extended darkness in rats (72) and was higher during the daily dark

period in rats (73) and monkeys (74). Sectioning the superior cervical ganglia abolished the effect of photoperiod on HIOMT, suggesting the mediation of the sympathetic nervous system in light-regulated control of pineal function (71).

The results of many investigations have confirmed that the pineal gland can act as a mediator of light-induced reproductive changes in laboratory animals and domestic birds (49). Light deprivation, either by removal of the eyes or by subjecting animals to "short days", biochemically and physiologically activates the pineal gland to secrete a reproductive antagonist (76). Desjardins, et al. (80), have reported that light deprivation in hamsters decreases testicular glucose metabolism and peripheral testosterone concentration.

Seibel, et al. (117) reported that bilateral enucleation of the eyes of male hamsters resulted in a significant decrease of testicular and seminal vesicle weight. When bilaterally enucleated males were parabiotically joined to normal males or females the antigonadal substance of the pineal significantly depressed testicular weight of males and lowered the uterine weight of females. The authors concluded that these results confirmed the presence of a pineal antigonadotrophic substance and indicated that this substance was released into the systemic circulation in order to reach its target organ.

A pineal-gonadal relationship in male hamsters has been demonstrated by Reiter (75). He reported that unless adult hamsters are either pinealectomized or superior cervical gangliectomized, blinding is followed by involution of the reproductive organs, indicating a fall in the production, release, or action of gonadotrophins. The atrophic reproductive organs of the eyeless hamsters were actively inhibited as

evidenced by the fact that if 8-week blinded hamsters were pinealectomized, the testes grew to the adult condition within 7 to 8 weeks. As with pinealectomy, superior cervical gangliectomy of blinded hamsters with involuted gonads initiated regeneration of the reproductive system, the pattern of which was similar to that following pineal removal. The author suggested that these data emphasized the important role that the sympathetic nervous system plays in determining the synthesis and/or secretion of the pineal reproductive antagonist.

The mechanism of action of pineal substances has been studied by several investigators with equivocal results. Ellis (130) measured the effects of melatonin and serotonin on in vitro bioconversions of isotopically labeled precursors into androgens by testis tissue from the rat and on the disappearance of testosterone from media containing testis tissues. Both melatonin and serotonin suppressed synthesis of androgens (with melatonin 500 times more effective) and both speeded the disappearance of testosterone; analysis of metabolites suggested that each compound stimulated a different catabolic enzyme. While this report indicates a direct effect of pineal substances upon testicular tissue, other reports favor an indirect effect mediated by the hypothalamo-hypophysial axis. Fraschini (77) studied the pineal gland and its influence on the control of FSH and ICSH secretion. Following pinealectomy of sexually mature male rats, he reported that testicular weight and accessory sex organ weight was enhanced. He suggested that pinealectomy exerted a stimulatory influence on the release of both FSH and ICSH. Measurements of FSH and ICSH content of the pituitary glands twelve days following pinealectomy revealed that both gonadotrophins were present at levels more than twice as great as those in controls.

Thus, he reported that pinealectomy stimulated the synthesis of both FSH and ICSH and concluded that the pineal gland normally inhibits the synthesis as well as the release of both FSH and ICSH.

Melatonin has been proposed as "the" pineal hormone (78). In order to validate the hypothesis and to clarify at the same time whether melatonin was able to inhibit the secretion of FSH and ICSH, Motta, et al. (79) administered the compound daily for 21 days to 30-day-old male rats. Testicular weights were not modified, but prostates and seminal vesicles were significantly atrophied following treatment. They reported that melatonin significantly reduced the secretion of ICSH (decrease of the weights of prostate and seminal vesicles) but did not alter the secretion of FSH (normality of testis weights). It appeared to them that melatonin could not be the only hormone manufactured in the pineal gland and that the existence of other pineal principles, specially devoted to the control of FSH secretion must be postulated.

In light of the strong evidence against a distinctive function for FSH in spermatogenesis (discussed previously in this review) it would seem that the interpretation regarding the failure of melatonin to inhibit FSH secretion as judged by the normality of testis weights might be questioned. However, because melatonin administration failed to counteract the increased testis weights reported for pinealectomy (77), it is apparent that melatonin is not the only pineal hormone involved in the pineal-gonad relationship.

Fraschini (77) reported in male rats that median eminence implants of melatonin and a pineal indole derivative, 5-hydroxytryptophol, inhibited specifically the secretion of ICSH, whereas implants of serotonin and the indole derivative, 5-methoxytryptophol, inhibited

specifically the secretion of FSH. These observations confirm those of Motta, et al. (79), who reported that melatonin reduced ICSH secretion without affecting FSH secretion.

These reports all serve to indicate that the hypothalamus in some way exerts dual control over the secretion of FSH and ICSH in contrast to the observations of Schally, et al. (154). Perhaps it is reasonable to speculate that both groups are correct; e.g., pineal influences and gonadal influences may exert their regulatory actions upon the hypothalamus by separate and distinct pathways.

Reiter (75) has summarized the current knowledge of pineal-gonad relationships. He concluded that the pineal gland is definitely an organ of internal secretion and that it could play a major role in regulating reproductive functions in some mammals; that several substances, namely, melatonin, serotonin, 5-methoxytryptophol and 5-hydroxytryptophol are synthesized within the pineal gland and are possible pineal hormones; that it is likely that these substances are secreted by the pineal gland and act on the hypothalamus and/or other parts of the brain where they exert their influence on gonadotrophin metabolism, and finally that the physiological importance of the pineal-gonadal relationship remains the subject of future studies.

Pancreas

The mammalian pancreas is a compound gland consisting of exocrine and endocrine tissues. The exocrine constituent secretes pancreatic juice into the duodenum. The endocrine constituent is contained within the Islets of Langerhans and secretes insulin and glucagon into the blood. The effects of insulin on testosterone secretion will be

examined in this review.

Insulin is essentially an anabolic hormone and its main actions favor the formation and storage of materials that are essential for growth. A reduction in insulin secretion results in the development of diabetes. That insulin deficiency may affect male reproduction has been shown by clinical observation in diabetic men. Klebanow and MacLeod (82) reported that erectile and ejaculatory impotence was a frequent finding in diabetic male patients. Horstmann (83) has observed that the most prominent disturbance in sex function of diabetic men was a lack of libido and a decrease in sexual potency. He reported that the excretion of urinary gonadotrophins was normal but that the excretion of 17-ketosteroids was extraordinarily low in diabetic men between twenty and fifty years.

A constant supply of carbohydrate, or more specifically, glucose, is considered to be an essential requirement for the proper functioning of the testis (81). Insulin and glucagon are involved in the regulation of blood glucose levels -- insulin reduces blood glucose concentration while glucagon causes an elevation in the concentration of blood glucose (1). Thus it is apparent that biological avenues exist whereby pancreatic hormones might influence testosterone secretion by the Leydig cells.

Experimental diabetes may be produced by the injection of alloxan or by sub-total pancreatectomy (1), thus animal models may be used to study the effects of insulin deficiency. Hunt and Bailey (85) have reported that severe alloxan diabetes in young male rats resulted in failure of descent of the testes, failure of development of the germinal epithelium and castrate-type accessory organs. Treatment with insulin corrected all the deleterious effects of diabetes on the reproductive

system. In diabetic animals not receiving insulin, treatment with chorionic gonadotrophin resulted in hypertrophy of the interstitial cells and some development of accessory structures but did not bring about testicular descent nor onset of spermatogenesis. Treatment with testosterone stimulated accessory structures but did not affect testicular descent. Because the testes in the diabetic animals responded to chorionic gonadotrophin they believed that diabetes likely had no effect on the ability of the testis to synthesize testosterone. Because it had been established previously that protein synthesis was abnormal in diabetes (1) and that malnutrition had an adverse effect on reproductive organs as a result of diminished pituitary gonadotrophin secretion, the authors suggested that the effects of alloxan diabetes on the rat reproductive tract might be produced by pituitary gonadotrophin deficiency resulting from protein malnutrition.

Mancini, et al. (87) reported that hypoglycemia induced by either insulin or tolbutamide results in injury to the seminiferous tubules and edema of the interstitium of adult rats. Chatterjee (84) reported that alloxanized rats show a marked degree of testicular atrophy and degeneration and seminal vesicle atrophy. That fertility is affected by insulin deficiency was shown by Foglia (86) who reported that after removal of 95% of the pancreas in 3 month old rats, hyperglycemia ensued and fertility decreased. The decrease in fertility was related to the level of hyperglycemia and was progressively more marked. Testicular changes varied from slight change in the seminiferous epithelium up to complete disappearance of the epithelium in animals with prolonged hyperglycemia.

The foregoing reports serve to demonstrate that the testis is dependent upon a ready supply of glucose. While the specific mechanism of

of action of insulin is not known, there is evidence that it acts in some undetermined manner to promote the transfer of glucose across cell membranes (1). It follows then that insulin deficiency may interfere with glucose transport to cells of the testes and therefore result in metabolic damage that is manifested by atrophy and degeneration. Free and Van Demark (131) demonstrated the incorporation of glucose carbons into volatile fatty acids by testis tissue from rats, rabbits, and chickens. It seems then, that insulin deficiency might also result in decreased steroidogenesis and a consequent reduction in testosterone secretion that would be manifested in atrophy of testes and accessory sex glands.

While reports of the effects of insulin deficiency on the testis are numerous, specific effects of insulin on the normal testis have apparently not been studied. The action of the hormone in other systems argues for a possible insulin role in testosterone secretion, e.g., insulin may act in concert with other hormones as demonstrated by Lockwood, et al. (132) when they showed that insulin, hydrocortisone and prolactin, acting synergistically, selectively stimulated in vitro synthesis of milk proteins by the pregnant mouse mammary gland. Hjalmarson, et al. (133) reported that insulin increased the uptake of amino acids in perfused rat hearts from both hypophysectomized and normal rats. Mayne, et al. (134) reported that insulin promoted RNA formation in vitro in mammary gland explants of pregnant mice. Thus it appears that insulin could be involved in the regulation of testosterone secretion by one or more of the following interrelated avenues: (1) increased utilization of glucose by the testis; (2) increased conversion of glucose to steroids; (3) increased rate of enzyme protein synthesis

and gonadotrophin protein synthesis; or (4) synergism with gonadotrophic or other hormones.

Gonad

Hormones arising in the testes may act to influence the secretion of testosterone by Leydig cells. Albert (90) reported that the mammalian testis produces androgens and estrogens. Vigdoff, et al. (92) postulated that in addition to the well known steroid hormones, the testis produced a water soluble hormone, "inhibin". The origin, effect upon testosterone secretion by Leydig cells, and mechanism of action for each of the testicular hormones will be examined.

Androgens. As previously discussed, it is generally agreed that the Leydig cells within the interstitial tissue of the testis produce androgens. Shay, et al. (88) reported that small doses of androgen in the mature intact rat caused atrophy of the testis because of suppression of gonadotrophins. Large doses of androgen had the same suppressing effect but this was overridden by a direct stimulating effect of androgen on the testis and atrophy did not occur. In both instances the Leydig cells were atrophic. van Tienhoven (51) reported that androgens can maintain spermatogenesis in the rat after hypophysectomy. Boccabella (60) reported that testosterone propionate can reinitiate and restore spermatogenesis in hypophysectomized rats whose testes have undergone severe regression.

In adult men suffering from oligospermia, testicular depression by testosterone was followed by a return, after withdrawal, to pre-treatment functional levels or, in about half of the men, to function superior to pre-treatment levels. This increase was termed a "rebound

phenomenon" (93) and has been widely used in the treatment of infertility.

A similar response to testosterone has been observed in bulls. Meinecke (101) reported that the injection of testosterone propionate for a period of 18 weeks to Hereford bulls resulted in a 50% decrease in the volume of the testes and a reduction in spermatogenesis. While the number and size of the Leydig cells and the number of germinal epithelial cells declined, the Sertoli cells increased in size. After cessation of testosterone injections the testis volume increased to a level that was slightly greater than the pre-injection volume.

Albert (90) reported that testosterone treatment in the human male results in disappearance of Leydig cells, atrophy of the tubules, arrest of spermatogenesis and pronounced hyalinization of the basement membrane. He concluded that the suppressive effect of androgen resulted from inhibition of pituitary gonadotrophin as evidenced by measurement of urinary gonadotrophin before, during, and after use of testosterone. While the mechanism of gonadotrophin inhibition had been assumed to be a direct effect of androgen on the pituitary, Albert (90) noted that Paulsen (94) had shown that the use of testosterone, while reducing urinary gonadotrophin, increased the amount of urinary estrogen 20-fold. He reasoned that as estrogen was by far the most powerful suppressant of gonadotrophin secretion known, it was possible that the atrophy of the testis observed during testosterone therapy in man may be caused by estrogen.

In a discussion of unilateral compensatory hypertrophy of the gonad that occurs after hemicastration, Johnson (95) reported that while the mechanisms for the compensatory growth were undefined, one theory

suggested that the loss of a gonad resulted in a decrease in circulating steroids which reduced their negative feedback effect on the hypothalamo-hypophysial axis and consequently triggered an increased outflow of gonadotrophins. An alternative view was that the remaining gonad was stimulated by more gonadotrophin because of a decrease in the rate of utilization. With either of these possibilities the amount of circulating gonadotrophin should increase over the normal intact level. That gonadotrophins did increase was demonstrated in unilaterally castrated rats joined parabiotically to hypophysectomized male or female rats. Circulating levels of both FSH and LH increased before any obvious gonadal compensatory growth had occurred.

Forchielli, et al. (96) reported that there is increasing interest and realization of the possible importance of steroid hormone inhibition at the site of biosynthesis as a part of a negative control mechanism, which in essence could constitute a local mechanism with the possibility that each and every step along the entire biosynthetic route could be vulnerable to inhibition by its respective product. The following observations lend credence to this concept.

Ichii, et al. (97) demonstrated that pregnenolone and progesterone inhibited the rate of side-chain cleavage of cholesterol by a soluble enzyme system of the bovine corpus luteum. Forchielli, et al. (96) reported that the addition of testosterone to an incubation medium containing an immature rat testis mitochondrial preparation (source of cholesterol side-chain cleaving enzyme) markedly inhibited the side-chain cleavage of cholesterol. Inano, et al. (162) found that testicular 20α-hydroxysteroid dehydrogenase decreases the synthesis of androgens by competing for the substrate 17α-hydroxyprogesterone with the

formation of 17α, 20α-dihydroxypregn-4-en-3-one which is a competitive inhibitor of the desmolase (lyase) responsible for removing the side-chain of 17α-hydroxyprogesterone.

In summary, it appears that testosterone may participate in regulating its own secretion by a dual mechanism: (1) feedback to the hypothalamo-hypophysial axis and/or (2) inhibition of intra-testicular steroidogenic enzyme activity.

Estrogens. Estrogens have been isolated from the testes of a number of species including man (51). In the horse the concentration of estrogen in spermatic vein blood is about 20 times higher than that in peripheral blood (98). Hall (91) reported that the testes are responsible for more than two-thirds of the production of estrogens in adult men.

The cellular source of estrogen production may be the Leydig cell. Leach, et al. (98) arrived at this conclusion on the basis of the following evidence: (1) Leydig cell tumors are often associated with high estrogen secretion; (2) in cases in which the Leydig cells were the only elements present in the testes, estrogen excretion was higher than in castrates and only slightly lower than in normal men; and (3) chorionic gonadotrophic injection in men stimulated the Leydig cells, increased estrogen excretion and necrosis of the seminiferous tubules and caused no observable changes in the Sertoli cells. That Sertoli cells may also be a source of estrogen has been long recognized, for dogs with Sertoli cell tumors manifest signs of feminization. Affected animals may show enlargement of the teats, flaccidity of the penile sheath, atrophy of the penis, attractiveness to other dogs and adoption of the female posture for micturation (99). Pierrepoint, et al. (99) provided direct evidence that a Sertoli cell tumor from a dog can synthesize estrogen.

In a comparison between tumor tissue and normal testis tissue incubates the conversion rate of dehydroepiandrosterone to estrone or estradiol -17β in tumor tissue was 300 times as great as it was in normal tissue.

Sohval (100) reported that large amounts of free estrogen are excreted in the urine of certain men with cirrhosis of the liver particularly when gynecomastia is present. The damaged liver is unable to inactivate circulating estrogens and the resultant increase in circulating estrogens exerts a suppressive effect on the secretion of pituitary gonadotrophins, resulting in degenerative and atrophic changes in the seminiferous tubular epithelium.

Albert (90) reported that natural and synthetic estrogens in all forms induce atrophy of the male gonad. He described testicular changes that occur in response to estrogens in rats, hamsters, and man. In rats the Leydig cells and germinal epithelium are atrophic and only spermatocytes, spermatogonia, and Sertoli cells remain. In hamsters, atrophy of the germinal epithelium is almost complete; only a few spermatocytes remain in addition to Sertoli cells. In man the atrophy proceeds so that only the Sertoli cells remain in the tubules; even these cells may disappear with the induction of peri-tubular hyalinization and sclerosis.

Oštádalová, et al. (89) reported that gonadotrophins given on the 22-24th day of life to normal male rats, induced marked growth of seminal vesicles and prostate glands as a result of stimulated androgen secretion, whereas in rats receiving estrogens on the 5th day after birth this effect did not occur. Histochemical studies of Leydig cells from both groups of rats disclosed a reduction of 3-beta-ol-steroid dehydrogenase, beta-hydroxybutyrate dehydrogenase, and nonspecific esterase in estrogen treated animals. The authors concluded that estrogen caused a

metabolic disturbance in Leydig cells which resulted in their inability to respond adequately to stimulation by gonadotrophic hormones.

The powerful inhibitory action on gonadotrophin secretion exerted by median eminence implants of estrogen in the hypothalamus of male animals is well known (187) and Gomes (49) suggested that all of the testicular effects claimed for in vivo estrogen treatment can be explained by this mechanism. He concluded that the separation of direct and indirect effects of estrogen on the testis in the intact animal in vivo appeared to have not been resolved.

Inhibin. It has been postulated that the germinal epithelium secretes a hormone specifically responsible for feedback inhibition of the secretion of FSH. McCullagh (102) reported in 1932 that aqueous testicular extracts which could have contained no more than an insignificant amount of the prostate regenerating hormone, prevented cellular changes from appearing in the pituitary gland after castration of rats and also completely inhibited the hyperfunction of the pituitary gland. Based on this observation and other evidence the author concluded that the testicle secreted a water soluble hormone that had not been recognized previously, one function of which was the control of the pituitary gland.

Vigdoff and Hill (103) reported that a water soluble extract of frozen bull testes injected subcutaneously into intact rats produced atrophy and degeneration of the prostates, seminal vesicles and testes. They believed the deleterious changes were due to an inhibitory effect produced on the anterior pituitary by the extract which interfered with or stopped the production of male hormone in the testes.

Taria and Tarkhan (104) conducted parabiotic experiments that permitted the estimation of circulating gonadotrophic activity in

experimental rats. They reported that in normal rats with intact testes there was almost no release of FSH or ICSH. FSH release from the hypophysis of cryptorchid rats was almost equal to that released in castrates, but ICSH release was much less in cryptorchids. They reasoned that the cryptorchid testes, like the scrotal ones inhibited the release of ICSH, but unlike the scrotal testes did not inhibit the release of FSH. Since cryptorchidism results in degeneration of spermatogenic epithelium but not Leydig cells they suggested that the spermatogenic epithelium liberated a hormone which inhibited the release of FSH from the adenohypophysis while the Leydig cells liberated a hormone which inhibited the release of ICSH.

A possible relationship between the Sertoli cell of the germinal epithelium and FSH was demonstrated by Castro, et al. (105). Electron microscopic studies of testis from rats injected with ferritin-labeled FSH demonstrated a predominant accumulation of the ferritin particles in vesicles and dense bodies of the Sertoli cell cytoplasm. After the injection of ferritin-labeled LH the ferritin molecules appeared predominantly in the same organelles of immature Leydig cells, in peritubular structures, and in lesser amount in vesicles of Sertoli cells.

Johnsen (106) evaluated urinary gonadotrophin and 17-ketosteroid levels in addition to testicular biopsies from men and concluded that the testicular-hypophysial feedback mechanism in man depended on a very late stage in spermatogenesis. He suggested that the cytoplasm which was split off from the spermatid immediately before liberation of the mature spermatazoon produced the hypophysial inhibitor.

A comparison of patterns of changes in ICSH, FSH, and "total" gonadotrophins in orchidectomized and cryptorchid rats led Steinberger

and Duckett (107) to suggest the hypothesis that the Leydig cell secretions influenced the release of FSH from the pituitary while germinal epithelium influenced the production of this hormone.

Voglymar and Mattner (108) showed that removal of the tubular products by cannulation of the vasa efferentia in sheep leads to a surprisingly large testicular hypertrophy in response to unilateral orchidectomy. The possibility arises that tubular secretions in sheep affect the pituitary after re-entering the blood stream from the vasa efferentia. Setchell (109) observed the rete testis fluid would be a logical pathway for the feedback of information to the pituitary about the intensity of spermatogenesis and would probably be the most reasonable place to search for the inhibitory substance that might fill the role. He further observed that conclusive proof of the existence of inhibin in rete testis fluid would hinge on the demonstration of increased plasma concentrations of FSH or other pituitary hormones after diversion of rete testis fluid from its normal course into the epididymis.

Hall (91) reviewed the evidence for the existence of inhibin and concluded that it must be assumed, until compelling evidence to the contrary appears, that inhibin is an artifact and that if the secretion of FSH is regulated in this manner the hormone responsible is likely to be an estrogen.

In summary, it is apparent that the products of the testis, androgens, estrogens, and perhaps "inhibin", exert an inhibitory influence on the secretion of testosterone. The main control mechanism appears to be mediated by the hypothalamo-hypophysial axis.

Adrenal Gland

The relationship between the adrenal and the testis is most conveniently discussed by subdividing the discussion into two parts. One part deals with the hormones of the adrenal cortex and the other part with the hormones of the adrenal medulla.

Adrenal Cortex. That stress may affect the testis via the adrenal cortex has been demonstrated by Flickinger (111,112). When chickens were grouped in such a way that a social or peck order could be established among roosters, the subordinate birds exhibited delayed testicular maturation and delayed spermatogenesis accompanied by degenerative changes in the testes. He described the anatomical changes in the testes of White Leghorn cocks as influenced by group numbers, population density, and social status. Numbers of roosters per group and social rank within the group, previously related to adrenal size, were highly correlated with testis size and morphology. Increased group size resulted in smaller testes which were attributable to degenerative changes in the germinal epithelium of subordinate cocks. Testes in these birds were consistently smaller and occasionally completely atrophic. Similar changes were produced in testes of individually caged birds by the injection of ACTH for 14 days (113), thus implicating the adrenal and its response to stress in the deleterious effects of social rank on the testes of the chicken.

Christian, et al. (114) showed that increasing size of populations of mice resulted in adrenal hypertrophy, inhibition of reproductive maturation and loss of reproductive function in adults. Weights of accessory sex organs declined with increased population density, and response to exogenous gonadotrophin was suppressed. They were able to inhibit

maturation of male mice with ACTH injections further implicating the adrenal as a mediator of gonad response to social interaction.

Desjardins (115) studied the effect of social rank on the metabolism and biochemical constituents of mouse testes. He reported that testes of unranked and dominant mice metabolized carbohydrates and amino acids similarly but that the emotional stress of subordination markedly depressed testicular carbohydrate and amino acid metabolism. The adrenal glands of subordinate mice were heavier than those of dominant and unranked animals.

Ewing, et al. (116) conducted an investigation to test the effects of competition stress on the testes of rabbits. They reported that stress for 10 days did not have any significant effect on testis morphology or size but did cause significant alterations in in vitro metabolic activity of testis and kidney cortex slices.

The common steroid nucleus upon which the structures of adrenal corticosteroids and sex hormones are based, the similar pathways utilized for the synthesis of these compounds, the several common precursors and enzymes used in the biosynthesis of progestational, androgenic, estrogenic, and cortical steroids and the foregoing observations of several investigators all provide evidence that the adrenal gland exerts an influence on testosterone secretion by the testis.

Conclusive evidence for the mechanism(s) by which the adrenal exerts its influence has not been presented. The bulk of the evidence available has derived from clinical observations of human patients suffering from adrenal hyperplasia or adrenal hypoplasia.

As previously discussed, congenital adrenal hyperplasia is characterized by an excessive production of adrenal androgens that presumably

feedback onto the hypothalamo-pituitary axis to decrease the production of gonadotrophins necessary for gonad function. Adrenal insufficiency may be either primary (lesions confined to the adrenal gland) or secondary (lesions initially present in the anterior pituitary gland with secondary involvement of the adrenal). Clinical and pathological investigations (137,138) have revealed that infection, infarction, hemorrhage and tumors in either the anterior pituitary or adrenal gland are common causes of adrenal insufficiency. Loss of libido, impotence and reduced fertility occur in the disease and are much more likely to occur in secondary insufficiency where the anterior pituitary gland is involved. It seems reasonable to attribute the gonadal defects to interference with gonadotrophin secretion by the anterior pituitary lesion. It is obvious that complicating factors (infarction, infection, etc.) may confound specific endocrine studies in clinical patients.

Controlled experiments in both man and animals have contributed to an understanding of adrenal-testis relationships. In metabolic clearance rate studies of testosterone in man, Rivarola, et al. (139) showed that ACTH decreased the plasma levels of testosterone in man. These workers suggested that the increased adrenal secretion of androgens and/or estrogens in response to ACTH could inhibit the output of pituitary ICSH and therefore lower testosterone production by the testis. Liptrap and Raeside (118) conducted similar experiments in boars and suggested a different mechanism of action for ACTH. They reported that either ACTH or hydrocortisone was effective in reducing plasma ICSH levels. ACTH was ineffective in this respect in adrenalectomized boars, but hydrocortisone was equally effective in intact boars or castrated animals suggesting that adrenal corticoids directly inhibited the

hypothalamo-hypophysial axis. Additional evidence that corticoids rather than androgens were responsible for the inhibition was indicated by the observation that adrenal androgens and estrogens are produced in very small quantities, if at all, by boars. Yet another mechanism of action of ACTH on testis function was proposed by Chatterjee (84,119) who observed that both alloxan and chlorpromazine produced testicular degeneration in rats by an inhibition of pituitary gonadotrophins. He reported that in both instances the adrenal cortex was hyperactive due to excessive secretion of pituitary ACTH. Cortisone prevented the harmful testicular effects of the drugs and he reasoned that its mechanism of action was by feedback onto the hypothalamus to prevent excessive secretion of ACTH which had competitively inhibited the secretion of gonadotrophins. To recapitulate, 3 different mechanisms have been offered by these reports to explain an inhibition of testosterone secretion by ACTH: (1) promotion of adrenal androgen or estrogen secretion with feedback effects reducing ICSH production; (2) promotion of adrenal corticoid secretion with feedback effects reducing ICSH production; and (3) direct competitive inhibition of ICSH secretion by ACTH.

An interesting adrenal-gonad relationship has been described by Wasserman and Eik-Nes (140). They observed that when testes of normal anesthetized dogs were infused with the animals own adrenal venous blood (oxygenated) via the spermatic artery, the secretion of testosterone was higher than by testes infused with the animals own peripheral blood. The augmentation was probably due to increased biosynthesis of testosterone since the tissue concentration of the hormone at the end of the infusion was highest in testes infused with adrenal venous blood. As they were unable to demonstrate testosterone in the adrenal venous

blood, they concluded that the increased testosterone synthesis was due to biotransformation of some secretion product of the adrenal gland to testosterone. Attempts to identify the adrenal steroids responsible were not conclusive. The model used in these experiments -- infusing adrenal venous blood directly into the testis -- does not permit a conclusive statement as to the physiological importance of reutilization of blood borne steroids by the animal, but does demonstrate that such metabolism can take place. Moreover, it indicates that the adrenal gland may influence the testis directly, rather than through hypothalamic or pituitary mediated signals. A possible influence of adrenal catecholamines cannot be excluded in these experiments for it has been demonstrated that infusion of epinephrine or norepinephrine via the spermatic artery of the dog testis will result in increased secretion of testosterone (125).

Experiments that depend on the response of accessory sex organs as a criterion for evaluating cortisol stimulated testosterone secretion may need to be re-evaluated in light of the observations of Klaiber, et al. (141). They reported that the androgenic activity of testosterone propionate, as measured by its ability to increase the weights of accessory sex organs of castrated immature rats, was significantly augmented by the simultaneous administration of cortisol. The effects of testosterone propionate plus cortisol was greater than the additive effects of the two steroids administered alone. The mechanism by which cortisol achieved this augmentation was unknown, but it was suggested that it may have resulted from competition between cortisol and testosterone for binding sites in certain tissues resulting in an increased unbound androgen available for physiologic action in the accessory sex organs.

That competition between cortisol and testosterone for binding site does, in fact, occur in several tissues was demonstrated by Parra and Reddy (136) who reported that simultaneous administration of cortisol and testosterone-4-^{14}C to rats resulted in a decrease in the concentration of radioactivity in kidney, muscle, heart, lung and skin similar to that observed when the testosterone -4-^{14}C was diluted with an equal amount of nonlabeled testosterone. These experiments serve to illustrate two points: (1) the imprecision of accessory sex organ response as a criterion for evaluating specific testosterone stimulation; and (2) the possibility that cortisol may compete with testosterone for binding sites in the hypothalamus and thereby raise the threshold for testosterone feedback resulting in an increase in testosterone secretion by the testis.

In conclusion, the adrenal cortex is capable of influencing the testis by exercising effects that may either increase or decrease testosterone secretion. The mechanisms that mediate the adrenal effects are not clearly defined, but it appears that the following explanations all have merit and that specific physiologic situations or species differences may dictate the mechanism that is engaged to accomplish a certain objective. Mechanisms by which testosterone secretion by the testis may be decreased include the following hypothalamus-pituitary mediated effects characterized by increased ACTH secretion in response to lowered secretion of adrenal corticoids: (1) ACTH stimulation of adrenal androgen or estrogen production with consequent feedback of the steroid to the hypothalamus to reduce ICSH secretion and testosterone secretion; (2) ACTH stimulation of adrenal corticoids, rather than androgen or estrogen, with feedback of the corticoids to the

hypothalamus to reduce ICSH secretion and testosterone secretion; and (3) direct ACTH competitive inhibition of ICSH secretion and a consequent decrease in testosterone secretion.

Mechanisms by which increased testosterone secretion by the testis may be influenced by the adrenal cortex are: (1) biotransformation of adrenal steroids to testosterone within the testis; and (2) competition between cortisol and testosterone for hypothalamic binding sites resulting in a raised threshold for testosterone feedback and a consequent increased secretion of testosterone by the testis.

Adrenal Medulla. The cells of the adrenal medulla elaborate epinephrine and norepinephrine often referred to collectively as catecholamines (1). All the metabolic steps involved in the formation of these hormones from the amino acid tryosine have been elucidated and proceed as follows: tyrosine → dopa → dopamine → norepinephrine → epinephrine (120). Tepperman (121) described the effects of epinephrine and norepinephrine on blood vessels. Epinephrine, especially, constricts cutaneous and renal arterioles, but dilates skeletal muscle vessels and the coronary vessels. Norepinephrine is the more potent general vasoconstrictor, being at least 50% more powerfully hypertensive than epinephrine.

Although changes in the testes produced by hormones of the adrenal medulla have not been studied extensively, most available reports indicate that exogenous catecholamines are deleterious to the testis. That the adrenal medulla is not essential for normal function of the testis was indicated by Morales and Hotchkiss (148) who reported that no significant changes in testicular histologic morphology were observed in testes of adrenalectomized dogs and men maintained on corticosteroids. Thus it appears that an excess of medullary hormones produces damage to

the testis while the absence of the hormones has no effect.

Evidence for damaging effect of the catecholamines on testis function has derived from several studies. Waites and Setchell (123) reported that the adrenal medulla releases catecholamines in response to many stressful situations: hypoxia, reduced barometric pressure, hypoglycemia, hyperthermia and reserpine injections; and further that these stresses are known to be associated with testicular dysfunction. Van Demark and Baker (142) studied the effect of daily injections of epinephrine on semen production in bulls. They reported that epinephrine caused a reduction in semen volume and sperm concentration. Ewing, et al. (116) reported that twice daily injections of 1 mg doses of epinephrine into rabbits did not have any significant effect on testis morphology or size, but did cause significant alterations in in vitro metabolic activity (increased oxygen uptake and decreased glucose uptake) of testis and kidney cortex slices. On the other hand, Van Demark and Boyd (147) observed that epinephrine injections produced testicular atrophy in sexually mature rabbits. Thus, while there is disagreement about the nature of the insult, there is general agreement that exogenous catecholamines may produce testicular damage.

Different mechanisms have been proposed to explain the dysfunction produced by the catecholamines. The proposed mechanisms fall into two broad categories: (1) interference with adrenal and testicular steroid metabolism mediated by the hypothalamus and anterior pituitary; and (2) interference with testosterone secretion from the testis by intratesticular vascular changes.

Vernikos-Danellis and Marks (144) reported that epinephrine induced release of ACTH in normal human subjects. Their results were so

consistent that they proposed the measurement of epinephrine induced ACTH changes as a clinical test of pituitary function. These results were compatible with the observations of Armstrong and Hansel (145) who reported that epinephrine injections into rabbits caused highly significant increases in adrenal weights and histologically detectable hypertrophic changes in adrenal cortices. Segre, et al. (146) studied the effects of epinephrine on metabolic clearance and production rates of cortisol in man. They reported highly significant increases in cortisol production in response to epinephrine infusion. In a similar study Levin, et al. (143) reported that epinephrine significantly decreased plasma concentration and production rate of testosterone in men. It seems apparent that epinephrine can decrease testosterone secretion by stimulating ACTH increases which may in turn act by the possible mechanisms outlined in the adrenal cortex section of this review.

It seems reasonable to assume that catecholamines may depress testosterone secretion by altering blood flow to the testis, for Eik-Nes (122) reported that over a considerable range the rate of blood flow in the spermatic artery of the anesthetized, heparinized dog determined the rate of secretion of testosterone by the testis. Setchell, et al. (124) reported that infusions of epinephrine and norepinephrine into the testicular artery in rams, in doses calculated to produce physiological concentrations, caused varying but usually intense local vasoconstriction. With the reduction in blood flow, it is presumed that a corresponding reduction in blood borne nutrients would reduce the supply of substrates to Leydig cells resulting in a diminution of testosterone secretion. This rationale is consistent with the observation of Ewing, et al. (116) who reported that epinephrine decreased glucose uptake by

incubated rabbit testis slices.

Isoproterenol (isopropyl norepinephrine) is a catecholamine closely similar to epinephrine and norepinephrine. It is present in the adrenal medulla in small amounts and is possibly secreted into the blood along with the other medullary hormones (126). Eik-Nes (125) reported that when isoproterenol was infused into the spermatic artery of the dog testosterone secretion was stimulated and in infusion experiments of 15 min duration the effect of isoproterenol on testosterone secretion was similar to that following gonadotrophin infusion. He observed that testes infused with isoproterenol had higher tissue levels of testosterone than control testes and thus concluded that isoproterenol promoted both synthesis and secretion of testosterone. He noted, however, that since doses of isoproterenol used in the experiment were high the observed effects on testosterone secretion may have had little to do with the physiological situation in the testis. This is an important observation for this report indicated an <u>increase</u> in testosterone secretion as opposed to the general concensus that catecholamines bring about a <u>decrease</u> in testosterone secretion.

In summary, it appears that adrenal medullary hormones may depress the secretion of testicular testosterone by promoting increases in ACTH secretion which in turn stimulate androgen production from the adrenal and/or testis with consequent feedback inhibition of ICSH from the anterior pituitary. In addition, the medullary hormones may depress testosterone secretion by intra-testicular vasoconstriction with a consequent reduction in substrate uptake by Leydig cells.

Thyroid Gland

Gross changes in the thyroid gland have been associated with abnormal reproductive processes for many years (49). There seems to be no concensus, however, about the specific relationships between the thyroid and testis as evidenced by the vast body of literature that is filled with widely divergent reports of the effects of hypothyroidism and hyperthyroidism. The nature of the mechanisms by which thyroid hormones may influence Leydig cell secretion of testosterone are difficult to evaluate because of the multiple sites at which throid hormones may act to alter physiological processes which, in turn, may directly or indirectly alter testicular function. In view of the conflicts in the literature reviewed and the difficulty encountered in separating multiple effects from specific effects of thyroid hormone, only general conclusions can be drawn about a thyroid-testis relationship. Hamolsky (127) reported recently that the following general concepts have emerged as valid: (1) Thyroid hormone may exert direct action upon testis function by [a] altering specific biochemical mechanisms within the testis, [b] acting on some fundamental energy-controlling process in all tissues of the body, or [c] modulating the sensitivity of the testis to its pituitary trophic hormones. (2) Thyroid hormone may exercise an indirect effect on the testis via a stimulatory or inhibitory effect on the anterior pituitary synthesis and release of gonadotrophins.

In summary, it appears that the thyroid gland may exert an influence on testosterone secretion by the testis, but the mechanisms and direction of the influence are not clear.

Summary and Conclusions

The Leydig cells of the interstitial tissue of the testis appear to be the principle source of testosterone in domestic and laboratory animals. The adrenal gland is capable of synthesizing testosterone while the liver and some other tissues may transform non-androgenic steroid precursors into testosterone. The contribution by tissues other than the testis to the testosterone pool of the normal male appears to be minimal, and likely does not exceed 5% of the total pool.

The regulation of testosterone secretion is complex and under the immediate control of a neuroendocrine mechanism located within the hypothalamus and anterior pituitary gland. In addition to trophic hormones of the anterior pituitary, hormones arising from the pineal, pancreas, adrenal gland, thyroid and testis are all capable of exerting an effect on testosterone secretion by Leydig cells. ICSH appears to be the most potent hormonal force acting on Leydig cells and clearly FSH synergises with ICSH to augment testosterone synthesis and secretion. Because GH has demonstrated ability to enhance the effect of several hormones it seems logical that it may increase testosterone secretion by ICSH stimulated testes. While ICSH-GH synergism has been reported, conclusions have been based on indirect evidence provided by observed changes in testosterone target tissues. If ICSH-GH synergism is to be confirmed it seems necessary to demonstrate a quantifiable increase in testosterone secretion rate by the ICSH stimulated testis in response to GH administration. Accordingly, an experiment has been designed to test for ICSH-GH synergism in the *in vitro* perfused rabbit testis.

In order that hormonally stimulated testosterone secretion rates obtained by *in vitro* procedures may be put in proper perspective, it is

apparent that comparisons must be made with normal testosterone production rates *in vivo*. As the testosterone production rate of rabbits has not been determined and/or published an experiment was designed to measure it.

The specific objectives of this study are to answer the following questions; (1) what is the testosterone production rate for the rabbit; and (2) does GH synergise with ICSH to augment the secretion of testosterone from the perfused rabbit testis?

CHAPTER II

MATERIALS AND METHODS

Materials

Many of the reagents and much of the equipment used were common to all parts of the investigation. Accordingly, materials will be listed in general except where specific assignments were made to certain experiments.

Reagents

Most of the solvents were "nanograde" in quality (Mallinckrodt Chemical Works, St. Louis) and included dichloromethane, ether, benzene, acetone, toluene, chloroform, ethanol and methanol. Ether and dichloromethane were further purified by glass distillation. Water used in the procedures was glass distilled two times, ether washed, and finally redistilled. Heptafluorobutyric anhydride was obtained from Peninsular Chemresearch, Gainseville, Florida. Sodium hydroxide (0.1N) was freshly prepared by mixing 0.4 gm pellets (analytical grade, Mallinckrodt) in 100 mls distilled water. Scintillation fluid was made up of 15.14 gm of 2, 5-diphenyloxazole (PPO), and 0.1514 gm of 1,4-his-1-(5 phenoxazolyl)-benzene (POPOP) in 3.79 ℓ of spectral quality toluene. The mixture was allowed to equilibrate 24 hours before use.

Hormones

1. Growth Hormone (ovine), NIH-GH-S9. Fresh hormone solutions were prepared immediately before use by dissolving GH in 0.9 percent NaCl in H_2O (w:v) at pH 10.4.

2. Luteinizing Hormone (ovine) NIH-LH-S16. Physiological salt solutions (0.9 percent NaCl in H_2O; w:v) containing 1 mg LH/ml were divided into 1 ml aliquots and kept frozen (-40°C) until thawed immediately prior to dilution and injection. Four weeks was maximum storage time.

Both GH and LH were obtained from the Endocrinology Study Section, National Institutes of Health, Bethesda, Maryland. The specification sheet that accompanied the growth hormone reported the mean relative potency of contaminating LH activity as equal to 0.025 mμg LH/mμg GH. The sheet accompanying the LH reported that it had not been assayed for GH activity.

3. Testosterone (17β-hydroxyandrost-4-ene-3-one) and 20β-hydroxyprogesterone (20β hydroxypregn-4-ene-3,20dione) obtained from Steraloids, Inc., Pawling, N. Y., were recrystallized to their constant melting points and dissolved in ethanol at a concentration of 1 mg/ml solvent. Testosterone -1,2-^{3}H and testosterone -4-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. and purified via thin layer chromatography. Purity was checked by a radiochromatographic scan and identity confirmed by the use of authentic standards in adjacent lanes on the thin layer plates. They were stored in ethanol at 5°C and their purity was redetermined at two to four month intervals.

Silica Gel

Silica gel (Silicar TLC-7GF, Mallinckrodt) was washed three times with dilute acetic acid over a scintered glass filter, followed by three rinses in boiling triple distilled water, and finally washed two times with boiling methanol. It was dried 24 hours at 110°C. Thin layer chromatography (TLC) plates were poured (0.25 mm thickness) with slurry containing 33 gm silica gel in 78 ml distilled water.

Anesthetic

Pentobarbital sodium injection, U.S.P. was obtained from Haver-Lockhart Laboratories, Kansas City, Missouri.

Artificial Medium for Perfusion Experiments

The artificial medium was composed of Krebs-Ringer bicarbonate buffer (pH 7.4), bovine albumin powder-fraction V (3% w/v) obtained from Armour Pharmaceutical Co., Chicago, glucose (1 mg/ml), washed bovine red blood cells added to a hematocrit of 25% (blood was obtained from a Holstein steer approximately 10 months of age), crystalline penicillin G potassium, U.S.P. (1000 units/ml) and crystalline dihydrostreptomycin sulfate, U.S.P. (1 mg/ml). The antibiotics were obtained from E. R. Squibb and Sons, New York. The artificial medium was stored in graduate cylinders at 5°C and was used within 4 days of preparation.

Glassware

Laboratory glassware was rinsed immediately after use, soaked in detergent solution, brushed and placed in chromic acid overnight. Following ten tap water rinses, the glassware was again soaked in detergent

solution, rinsed in tap water, and placed in dilute hydrochloric acid. After rinsing ten times with tap water, ten times with distilled water and two times with nanograde methanol, the glassware was air-dried and covered until used.

Equipment

1. Perfusion Apparatus. The perfusion apparatus originally described by Van Demark and Ewing (220) and modified by Ewing and Eik-Nes (221) was used with the following modifications. Silastic tubing was substituted for rubber tubing, the New Brunswick pump was replaced by a Harvard Sigma motor peristaltic pump and a Sage micro pump was added. The latter facilitated the infusion of hormones directly into the arterial cannula within a wide range of concentrations. After each perfusion the apparatus was washed in a detergent solution, rinsed in tap water and then distilled water and air dried. It was then wrapped in heavy paper, autoclaved and stored until its next use. Two apparati were available and thus it was possible to perfuse paired testes simultaneously. The perfusions were conducted in a walk-in constant temperature room maintained at 36.5^{o}C.

2. Radiochromatogram scanner (Packard, Model 7201).

3. Selectra Series 5000 Barber-Coleman gas chromatograph equipped with a Model 5120 electron capture detector.

4. Mass Spectrometer-Gas Chromatograph, LKB-9000, Prototype (Karolinska Institute, Stockholm, Sweden).

5. Cahn Electronic Balance (Cahn Instrument Co., Paramount, Calif.).

6. Model UV International Centrifuge.

Animals

New Zealand White male rabbits were obtained from Redwood Game Farms, Salt Lake City, Utah. They were housed in air conditioned, light controlled (10:14, L:D) quarters and provided water ad lib and 6 oz/day Purina Rabbit Checkers. The animals used in the experiments were 6-12 months of age and had been housed in the animal quarters for at least two weeks prior to use.

Methods

Testosterone Assay Method

The method used to quantify testosterone in this study was adapted from methods described by Brownie, et al. (222) and Kirschner and Coffman (223).

Preparation of Extraction Tubes. Fifty µl of tritium labeled testosterone solution (testosterone-1,2-^{3}H, 50 Ci/mM) were pipetted into 80 ml extraction tubes and evaporated to dryness under a stream of nitrogen. This quantity of the solution contained approximately 15,000 dpm and 0.04 mµg testosterone. Fifty microliters of the isotope solution was also pipetted into scintillation vials which served to monitor losses through the method thus permitting the calculation of testosterone recovery rate.

Extraction. Samples were extracted two times with 50 ml of ice cold dichloromethane. The extracts were transferred to 80 ml conical centrifuge tubes and evaporated to dryness under nitrogen.

Alkaline Wash. Fifteen milliliters of ether was added to each tube followed by 5 ml of 0.1N sodium hydroxide. The tubes were gently inverted

for 60 seconds and then centrifuged. The alkaline aqueous phase was removed and the remaining ether phase washed one time with 15 ml water. The tubes were recentrifuged and the water was removed. The washed ether extracts were evaporated to dryness under nitrogen and the residue concentrated in the tube tip with benzene:acetone (1:1, V:V).

Chromatographic Isolation of Testosterone. Thin layer chromatography (TLC) plates were marked into nine lanes (approximately 2 cm wide), 8 for samples and one for standard testosterone. The samples were spotted on the plates in benzene. The plates were then developed in toluene: chloroform:methanol:H_2O (120:60:20:1, V:V). The R_f of testosterone in this solvent system was approximately 0.6. The areas corresponding to authentic testosterone were eluted. Elution was accomplished by scraping the silica gel from the plates and placing it in 35 ml conical centrifuge tubes. The silica gel was extracted two times with a benzene:water partition system using 2 ml of benzene with 8 drops of water. After vortexing and centrifugation the benzene fractions were placed in 12 ml conical centrifuge tubes and evaporated to dryness under a nitrogen stream.

Formation of the Heptafluorobutyrate Derivative of Testosterone. One-tenth milliliter of a 2% solution of heptafluorobutyric anhydride in hexane and tetrahydrofuran was added to each of the sample tubes. The tubes were sealed, vortexed, and incubated in a 60°C water bath for 30 minutes. After incubation the samples were evaporated to dryness under nitrogen and the residue concentrated in the tube tips with benzene: acetone (1:1, V:V).

Chromatographic Isolation of Testosterone Heptafluorobutyrate (THFB). The residue was dissolved in benzene and spotted on TLC plates. The

plates were developed in benzene:ethyl acetate (175:25, V:V). In this solvent system THFB had an R_f of approximately 0.4. The area corresponding to authentic THFB was scraped off and placed in 35 ml conical centrifuge tubes and extracted two times with the benzene:water partition system. The benzene extracts were placed in 12 ml conical centrifuge tubes and evaporated to dryness under nitrogen.

Preparation of Samples for GLC and Liquid Scintillation Counting. One milliliter benzene was added to each of the dried 12 ml centrifuge tubes and vortexed. A 0.2 ml aliquot was removed and placed in a scintillation vial. Depending upon the estimated quantity of testosterone in the sample (based on the intensity of the spots on the TLC plates when visualized under ultra-violet light) from 0.1 to 0.5 ml of the sample was transferred to a 2 ml conical centrifuge tube for GLC quantification. Fifty millimicrograms of 20β-OH progesterone HFB were added to each of the tubes to serve as an internal standard. The scintillation vials and the 2 ml tubes containing their respective aliquots were evaporated under a stream of nitrogen. The portions of the samples remaining after the GLC and scintillation aliquots had been removed were pooled for structural confirmation studies.

Gas Liquid Chromatography. A Selectra Series 5000 Barber-Coleman gas chromatograph equipped with a Model 5120 electron capture detector was used to quantify the heptafluorobutyrate derivative of testosterone. Samples were applied in three to ten µl of benzene. A U-shaped glass column (3 feet long, 4 mm internal diameter), silanized with a 5% solution of dimethyl-dichlorosilane in toluene and packed with Gas-Chrom Q (80 to 100 mesh) coated with 3% OV-225 (Applied Science Laboratories, Inc.), was used to effect separation of steroids. Column bath

temperature was 200°C. Pure, extra dry nitrogen was used as a carrier gas with an outlet velocity of approximately 100 ml/minute. The detector bath temperature was 240°C.

The electron capture detector was operated in the DC mode and voltage was adjusted to give a standing current that was 60% of that obtained at the plateau of the voltage input curve. The detector bath temperature was 240°C. Sensitivity and linearity of detector response to standard HFB derivatives of testosterone and 20β-OH progesterone were ascertained each day of data collection on GLC. Samples were not run unless the response was linear over the range of expected THFB concentration. Peak size was calculated by multiplying one-half the height (measured by a perpendicular line from the vortex to the base) by the base.

Monitoring Testosterone Loss Through the Method. Testosterone loss prior to GLC was determined by measuring the loss of testosterone -1,2-^{3}H added to the 80 ml extraction tubes. The 0.2 ml sample aliquots in scintillation vials and the scintillation vials containing the 50 µl isotope aliquots for monitoring losses were evaporated to dryness under nitrogen. Fifteen milliliters of scintillation fluid was added to each vial. The samples were counted in a three channel Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003, equipped with a ^{137}Cs Automatic External Standard device. The liquid scintillation spectrometer operated at an efficiency of 41% for tritium with a background of approximately 15 cpm. Ratio of background cpm to sample cpm varied with the activity of the samples, but usually fell within a range of 0.003 to 0.03. Quenching was minimal and uniform between samples; therefore it was disregarded as a significant factor in calculations.

Each sample was counted for three periods of ten minutes each. Appropriate background and standard vials were included each time unknown samples were counted.

Adjustment for losses incurred during quantification by GLC was made by the internal standard (50 mμg 20β-OH progesterone HFB) added to each sample as outlined by Horning, et al. (224).

Calculation of Testosterone Concentration. The amount of testosterone in the samples was calculated according to a formula used by Stabenfeldt (225). The formula follows:

$$\text{Testosterone (m}\mu\text{g/sample)} = R \times C \times U \times A \times 0.596 \times X$$

where: $R = \dfrac{\text{cpm testosterone-1,2-}^{3}\text{H added to plasma}}{5 \times \text{cpm in 20\% aliquot obtained prior to GLC}}$;

$C = \dfrac{\text{peak area (cm}^2\text{) of 10 m}\mu\text{g 20}\beta\text{-OH progesterone HFB standard}}{\text{peak area (cm}^2\text{) of 10 m}\mu\text{g THFB standard}}$;

$U = \dfrac{\text{peak area (cm}^2\text{) of THFB in sample}}{\text{peak area (cm}^2\text{) of 20}\beta\text{-OH progesterone HFB in sample}}$;

$A =$ mμg 20β-OH progesterone HFB added as internal standard;

$0.596 = \dfrac{\text{molecular weight testosterone}}{\text{molecular weight THFB}}$;

$X = \dfrac{1}{\text{GLC Aliquot}}$.

THFB Structural Confirmation Studies. The pooled sample remainders that accumulated during the perfusion experiments were held at 5°C until the conclusion of the study. They were combined in an 80 ml conical centrifuge tube and the benzene solvent evaporated under nitrogen. The residue was transferred in benzene to a TLC plate which was developed in benzene:ethyl acetate (175:25, V:V). The THFB spot, identified by standard THFB in an adjacent lane was scraped off and placed in a 35 ml

conical centrifuge tube. A similar amount of silica gel in a blank lane was scraped off and placed in a 35 ml conical centrifuge tube. Silica gel in both tubes was extracted two times with the benzene:water partition system described previously. The benzene extracts were evaporated under nitrogen and the residue transferred to a TLC plate which was developed in the same benzene:ethyl acetate (175:25, V:V) solvent system. The procedure was repeated once again so that the silica gel blank and the THFB sample were purified in a total of 3 chromatographic procedures using the same solvent system each time. The THFB sample, the silica gel blank sample, and a sample of authentic crystalline THFB were submitted to the Oklahoma State University Mass Spectrometry Laboratory for mass spectral determination. The mass spectrometric operation has been described by Waller (226).

Measurement of Testosterone Metabolic Clearance Rate (MCR) and 24 Hour Production Rate

The methods employed in this study were adapted from those described by Horton, et al. (227), Southren, et al. (228) and Rhynes (229). As a basis for the discussion which follows it should be kept in mind that the major factors which control the concentration of testosterone in plasma are: (1) the rate at which it is produced and secreted into the plasma; and (2) the rate at which it is removed or cleared from the plasma. It seems logical then that if one assumes during the course of the 50 minute study that the concentration of plasma testosterone remains constant and that the production rate of testosterone remains constant, then the rate at which testosterone is removed from the plasma must be equal to the rate at which it is produced.

Preparation of Tritiated Testosterone for Injection. Testosterone -1,2-^{3}H (44.92 Ci/mM) and testosterone -4-^{14}C (58.80 mCi/mM) were purchased from New England Nuclear Corporation, Boston, Massachusetts. Radiochemical purity of the isotopes was determined by radiochromatographic scanning. Isotope identity was confirmed by TLC procedures that permitted comparison of R_f values with those of authentic testosterone-^{12}C.

Tritiated testosterone was diluted with 10% ethanol in normal saline so that each 2 ml dose to be injected contained approximately 40 x 10^6 dpm (18 μCi) radioactivity and 113 mμg testosterone. Duplicate 50 μl aliquots of the diluted testosterone -1,2-^{3}H were placed in scintillation vials to be counted in order that the exact amount of radioactivity in the injected material could be determined.

Preparation of Animals. Rabbits were removed from cages and restrained on their backs in a V trough. Animals that were excited by this procedure were rejected. Five animals were used in the study.

Injection of Tritiated Testosterone and Collection of Blood Samples. A 3 1/2 inch, 20 ga short bevelled syringe needle attached to a 20 ml glass syringe containing 1 ml of heparin solution (3000 units heparin in physiological salt solution [0.9 percent NaCl in H_2O; w:v]) was inserted into the heart by entering the skin at the notch formed between the xiphoid cartilage and the left rib cage. The heparin solution was injected after which 20 ml of blood were collected for the determination of plasma levels of endogenous testosterone. The 20 ml syringe was disconnected from the needle, which remained in place in the heart, and a 5 ml disposable plastic syringe containing 2 ml of tritiated testosterone solution was attached. The isotope was injected and the syringe

flushed twice with blood to remove residual radioactivity. A timer was started as the isotope was injected (0 time). The needle was removed from the heart and the rabbit was returned to an upright position in the trough where he remained for the duration of the experiment. Three milliliter blood samples were collected from the marginal ear vein at approximate intervals of 5, 10, 15, 30, 40, and 50 minutes after the injection of the radioactive testosterone. The ear vein blood samples were collected by a vacuum method described by Hoppe, et al. (230). Approximately 30 seconds were required for the collection of each sample and the sample collection time was recorded as the midpoint between the time blood started flowing and the time vacuum was released. The blood samples were kept in an ice bath and at the conclusion of the collection period all were centrifuged and the plasma removed and frozen (-40°C) until assays could be conducted. Zero time plasma samples (collected immediately before the injection of the tritiated testosterone) were assayed for testosterone concentration by use of the method described previously.

Extraction of Testosterone -1,2-^{3}H From Plasma Samples for Calculation of Disappearance Curves. Ten microliters of cold testosterone -^{12}C (1 µg/µl) were pipetted into 80 ml extraction tubes. The tubes were vortexed and dried under nitrogen. One hundred microliters of testosterone -4-^{14}C solution (58.8 mCi/mM) containing approximately 9,000 dpm were pipetted into the dried tubes after which they were dried again under nitrogen. Similar duplicate aliquots of testosterone -4-^{14}C were pipetted into scintillation vials. These vials were used to monitor losses through the method and thus permitted the calculation of recovery rate. One milliliter aliquots of plasma were measured into each of the

previously prepared extraction tubes. Each sample was extracted one time with 50 ml dichloromethane by stirring for two minutes, equilibrating for 30 minutes and restirring for 2 minutes. The samples were centrifuged and the dichloromethane fraction was separated from the extracted plasma and transferred to 80 ml conical centrifuge tubes.

Chromatographic Isolation of Testosterone. The dichloromethane was evaporated under nitrogen and the residue concentrated in the bottom of the tube with benzene:acetone (1:1, V:V). The tube contents were spotted on a TLC plate with benzene. The plate was developed in benzene:methanol (175:25, V:V). Areas of silica gel containing testosterone were visualized under ultra-violet light, scraped from the plates and extracted with benzene:water partition. The eluate was evaporated under nitrogen and the residue concentrated with benzene:acetone and spotted on a second TLC plate with benzene. The second TLC plate was developed in benzene:ethyl acetate (110:100, V:V). Areas of silica gel containing testosterone were scraped from the plate and extracted by benzene:water partition. The eluate was placed in scintillation vials and evaporated to dryness under nitrogen. Fifteen milliliters of scintillation fluid was added to each vial. Radioactivity was determined in the binary-labeled samples by counting in a Packard Tri-Carb Liquid Scintillation Spectrometer adjusted to separate ^{3}H cpm from ^{14}C cpm.

Binary-Label Scintillation Counting. The binary-labeled samples in this study contained the internal standard testosterone-4-^{14}C and testosterone -1,2-^{3}H recovered from the plasma samples. The Packard Tri-Carb Spectrometer operated at approximately 25% efficiency for ^{3}H and 56% efficiency for ^{14}C with binary-label settings. Background in both

channels was similar (10-15 cpm). The ratio of background to sample varied from 0.002 to 0.02. Each sample was counted for three periods of 10 minutes. Appropriate background and standard vials were included each time samples were counted. Quench correction was made by the automatic external standardization method utilizing ^{137}Cs as a radiation source.

Determination of ^{3}H and ^{14}C DPM Present in Samples After Purification. The calculations necessary to separate the radioactivity attributable to each isotope in the binary labeled samples and to determine dpm of ^{3}H and ^{14}C have been described by Williams, et al. (231) and are set out below. Counting efficiencies were read from a graph by applying the external standard quench ratio value printed out by the scintillation counter.

(1) Subtraction of Background Counts:

$$Ip - k_1 = Iq \quad ,$$

$$IIp - k_2 = IIq \quad ,$$

(2) Determination of ^{14}C Counts in Window I:

$$\frac{IIq \times y_1}{y_2} = R \quad ,$$

(3) Determination of ^{3}H D.P.M.:

$$\frac{(Iq - R)}{y_3} \times k_3 = D_t \quad ,$$

(4) Determination of ^{14}C D.P.M.:

$$\frac{IIq}{y_2} \times k_3 = D_c \quad ,$$

where

Ip = cpm in window I;

IIp = cpm in window II;

Iq = cpm in window I with background counts subtracted;

IIq = cpm in window II with background counts subtracted;

k_1 = background counts in window I;

k_2 = background counts in window II;

k_3 = 100;

y_1 = ^{14}C counting efficiency in window I;

y_2 = ^{14}C counting efficiency in window II;

y_3 = ^{3}H counting efficiency in window I;

D_t = ^{3}H d.p.m. (uncorrected for loss through the method);

D_c = ^{14}C d.p.m. (uncorrected for loss through the method); and

R = ^{14}C c.p.m. in window I.

Determination of ^{3}H and ^{14}C DPM Present in Samples Before Purification. Losses of radioactivity occurred through the extraction and TLC procedures applied to the plasma samples of the production rate study. Correction was made for these losses by assuming that testosterone -1,2-^{3}H behaved similarly to testosterone -4-^{14}C during the extraction and TLC procedures. Therefore:

$$\text{Percent testosterone -4-}^{14}\text{C} = \frac{\text{dpm testosterone -4-}^{14}\text{C recovered}}{\text{dpm testosterone -4-}^{14}\text{C added as internal standard}} \times 100$$

and

$$\text{dpm testosterone -1,2-}^{3}\text{H in 1 ml plasma sample} = \frac{\text{dpm testosterone -1,2-}^{3}\text{H in sample after purification}}{\text{percent testosterone -4-}^{14}\text{C recovered in sample}}$$

Determination of Rate of Disappearance of Injected Testosterone -1,2-^{3}H From Plasma. By using the values for ^{3}H dpm in each of the serial plasma samples it was possible to calculate the percent of the injected testosterone -1,2-^{3}H remaining in the plasma at specified time intervals. The following formula was used:

$$\text{Percent injected testosterone -1,2-}^{3}\text{H recovered in 1 ml plasma sample} = \frac{\text{dpm testosterone -1,2-}^{3}\text{H in 1 ml plasma sample}}{\text{dpm testosterone -1,2-}^{3}\text{H injected in 2 ml intracardial dose}} \times 100 .$$

A curve that described the disappearance of the isotope was constructed by plotting percent recovery of injected tritiated testosterone versus time of sample collection on semilogarithmic graph paper.

Construction of Slopes Depicting Disappearance of Testosterone -1,2-^{3}H From Plasma. The value for the radioactivity in each plasma sample was plotted as the logarithm of the percentage of dose injected per milliliter of plasma against time after injection. The disappearance curve consisted of two distinct slopes. The first slope was plotted from the 5, 10 and 15 minute values and the second from the 30, 40, and 50 minute values. Because two distinct slopes were apparent, two exponentials were required to describe the disappearance of testosterone. In each instance the line of best fit for each portion of the curve was obtained by the method of least squares. Figure 1 in this section represents a composite calculated testosterone disappearance curve for the five rabbits of this study and is included for the sake of illustrating the methods used in calculating metabolic clearance rate, volumes of distribution and metabolic constants.

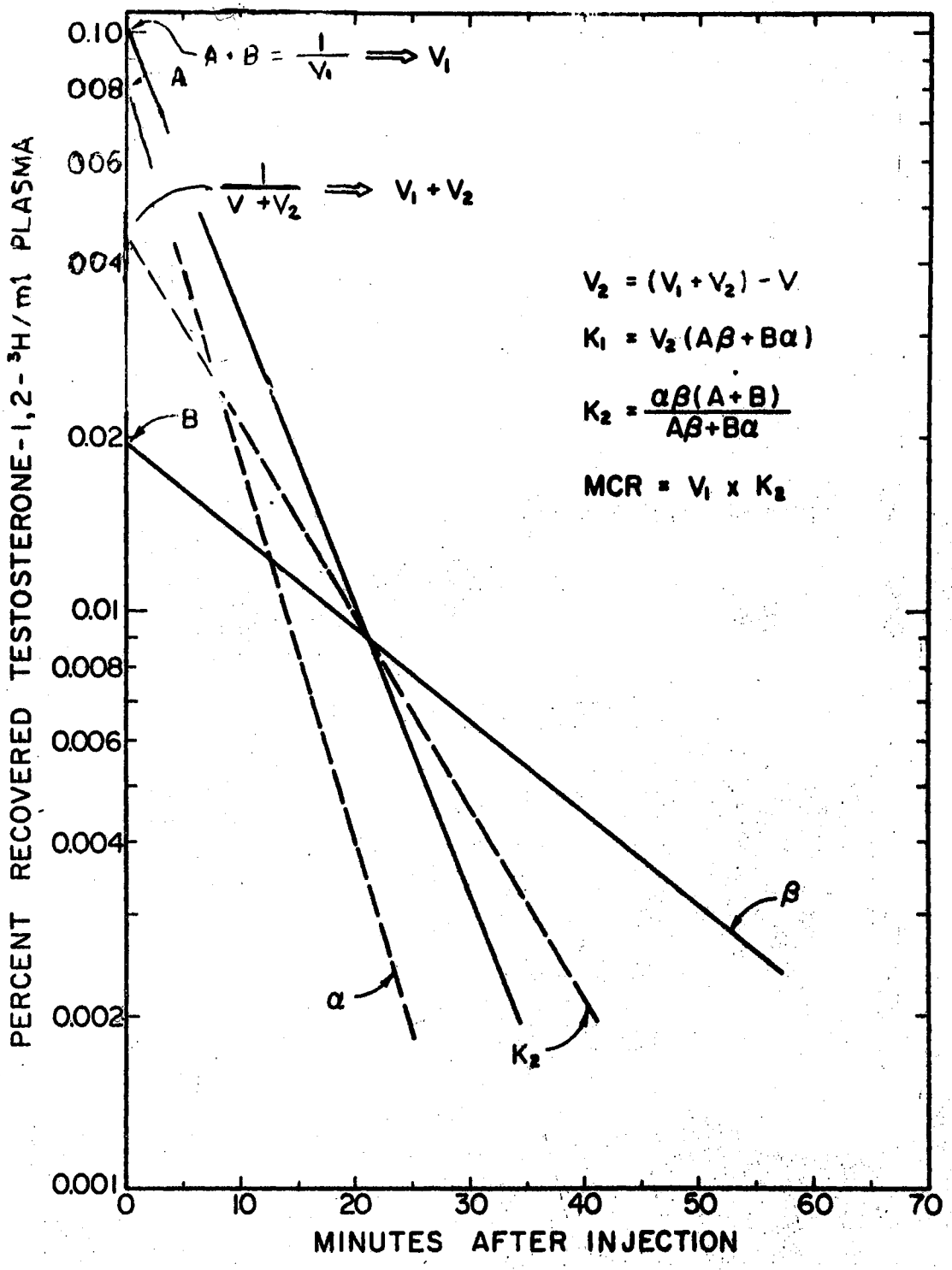

Figure 1. Mean Disappearance Curve of Testosterone -1,2-^{3}H in Plasma of Five New Zealand White Male Rabbits

Transport and Metabolism of Testosterone -1,2-^{3}H. The model and calculations used to describe the transport and metabolism of testosterone are those used by Tait and his co-workers (232) for the metabolism of aldosterone. Additional information was obtained from reports by Southren, et al. (228), Horton, et al. (227) and Rhynes (229). In Figure 1, A + B is the intercept on the ordinate of the initial part of the curve. The final curve has a slope β and an intercept B. Since the initial curve is influenced by the final curve, a third curve is constructed by subtracting the extrapolated radioactivity contributed by the final curve from the measured values and plotting the corrected concentrations semilogarithmically. This curve has slope α and intercept A. The metabolic clearance rate (MCR), defined as the volume of blood cleared of testosterone in unit time, is then obtained by integrating the area of these two curves. As production rate of testosterone is the product of MCR x peripheral testosterone concentration, sufficient information is available at this point for the determination of testosterone production rate. However, with additional calculations, information about distribution and metabolism of testosterone in the rabbit can be elucidated.

Tait, et al. (232) have reported that a reasonable model for the transport and metabolism of a steroid in man consists of an inner pool (V_1) which, for steroids weakly bound to plasma proteins, probably includes the plasma, extra-cellular fluid and the liver; and an outer (tissue) pool (V_2). The radioactive concentration in the outer pool is lower than that in the inner pool for some time after injection. These concentrations then become equal when that in the outer pool is maximal. From this time on the radioactive concentration in the outer pool is

greater than in the inner. The final slope of the plasma concentration (β) is a result of the combination of the effects of metabolism and transport. It will be a flatter slope than would be expected from the effects of metabolism alone because continuous transport occurs from the outer pool to the inner pool. Transport between the pools can be described by the rate constant, K_1. Metabolism is assumed to be negligible in the outer pool. If all the measured radioactivity is due to testosterone, then K_1 equals the fraction of the injected dose per milliliter of plasma transported to the outer pool per unit time; K_2 equals the fraction of the injected dose per milliliter of plasma metabolized in the inner pool per unit time.

After an intracardial injection of testosterone -1,2-^{3}H into a rabbit, the radioactive hormone is distributed rapidly into the inner pool. The inner pool volume (V_1) is equal to body testosterone in rapid equilibrium, divided by plasma concentration. V_1 can be calculated by determining the reciprocal of the A + B intercept ($\frac{1}{A + B}$). After the initial distribution the radioactive hormone moves more slowly into a larger total volume ($V_1 + V_2$). This volume multiplied by the blood concentration of testosterone is equal to the total body content of non-isotopic testosterone. $V_1 + V_2$ can be calculated from the formula appearing at the end of this discussion and alternatively by determining the reciprocal of the intercept on the ordinate obtained by extrapolating, using the slope K_2, from the point where the intial and final disapperance curves intersect. The half-life of the radioactive hormone in the inner and outer pools may be determined by either consulting the constructed graphs or by calculation.

The metabolic and transport behavior of the injected radioactive

testosterone was determined in this study by the following calculations:

(1) The slopes of the initial (α) and final (β) curves were expressed in units per day:

$$\text{slope} = \frac{2.3 \times (-\log X) \times 1400}{t}$$

where

$$X = \frac{\%\ \text{dose per ml at 10 min}}{\%\ \text{dose per ml at 0 min}};$$

t = 10 minutes;

1440 = minutes per day.

(2) The half-life of testosterone -1,2-^{3}H in the pools was expressed in minutes:

$$T\,\frac{1}{2} = \frac{0.6923\ (1440)}{\text{slope}}$$

where

slope = α or β

$0.6923 = 2.3\ (\log 2)$.

(3) Metabolic Clearance Rate (MCR) was expressed in liters of plasma per day:

$$\text{MCR} = \frac{\alpha\beta}{A\beta + B\alpha}$$

where

A = ordinate intercept for line with slope α;

B = ordinate intercept for line with slope β.

(4) Testosterone production rate (TPR) was expressed in mg per day:

$$TPR\ (mg/day) = MCR \times i$$

where

i = concentration of endogenous testosterone in peripheral plasma expressed as mg/ℓ.

(5) Pool volumes were expressed in liters:

$$V_1 = \frac{1}{A + B}$$

$$V_2 = \frac{\beta^2 A + B\alpha^2}{(A\beta + B\alpha)^2} - V_1 \quad .$$

(6) Rate constants were expressed in units/day:

$$K_1 = V_2(A\beta + B\alpha)$$

$$K_2 = \frac{\alpha\beta(A + B)}{A\beta + B\alpha} = \frac{MCR}{V_1} \quad .$$

Perfusion Methods

Rabbits were anesthetized by intravenous injection of pentobarbital sodium solution (64.8 mg/ml). Scrotal, perineal, and abdominal areas were clipped closely and ethanol was applied to the surgical site. Testes were removed and their spermatic arteries cannulated by the technique described previously (220). After cannulation of the spermatic artery the testis was flushed with 0.25 M sucrose from a 1 liter infusion bottle suspended approximately 1.5 m above the cannulation surface.

The testis was then placed in the organ chamber and the spermatic artery was attached to the inflow syringe. The organ was perfused for 30 minutes before the first collection of venous effluent.

The first testis was removed about 5 minutes after the initial injection of anesthetic. As about 15 minutes were required for cannulation of the spermatic artery and placement in the organ chamber, the contralateral testis was removed approximately 20 minutes after the induction of anesthesia.

With the exception of testes used to determine non-stimulating growth hormone levels (to be described) all testes in the various experiments were perfused with artifical medium alone for the first 2 hours after the initial 30 minute equilibration period. This allowed the dissipation of residual gonadotrophins from within the testes and permitted the establishment of a base level for testosterone secretion rate in the unstimulated testis. Arterial inlet pressure was maintained at approximately 120 mm Hg in all experiments. The glucose concentration of the perfusate was maintained by the hourly injection of decreasing amounts of 100 mg percent glucose solution.

Six hourly samples of venous effluent were obtained from each testis. Venous effluent accumulated in 35 ml conical centrifuge tubes that were held in an ice bath. The effluent was collected at hourly intervals and centrifuged for 20 minutes at 1,200 RPM. The plasma was removed, the red cells washed once with 5 ml 0.9% saline and recentrifuged. The wash was collected, combined with the plasma in 80 ml extraction tubes containing a tracer isotope, and kept frozen (-40^{o}C) until the samples could be assayed for testosterone. A water blank and an "arterial" sample of artificial medium were prepared at the same

time and placed in isotope treated extraction tubes. Each of the extraction tubes contained 12-14 ml of material and a typical set of assay samples consisted of 1 water blank, 1 "arterial" sample, and 6 hourly venous samples. The frozen samples were thawed at room temperature in preparation for extraction.

Protocol of Perfusion Experiments

(1) Five testes were perfused for the entire 6 hour perfusion period with artificial medium. The purpose of the experiment was to determine the testosterone secretory response of the unstimulated testis and thus establish a control or base level of testosterone secretion.

(2) Two testes were perfused with growth hormone in increasing concentrations. After the first hour of perfusion with artifical medium alone GH was infused by the Sage Micro Pump into the arterial cannula so that the concentration in the perfusion medium entering the testis was 10, 25, 100, 1000, and 5000 mμg/ml for the second, third, fourth, fifth and sixth hours of perfusion, respectively. The objective of this experiment was to determine the maximum amount of GH that could be infused into the testis without stimulating the secretion of testosterone.

(3) Four testes were perfused with the non-stimulating level of GH (10 mμg/ml) that was selected in Experiment 2. The GH was injected into the perfusate reservoir after the 2nd hour of perfusion. Thus the testis was exposed to a relatively constant level of hormone for the ensuing 4 hours of perfusion. The purpose of this experiment was to confirm that the non-stimulating level of GH would not stimulate testosterone production during an entire 4 hour perfusion period.

(4) Four testes were perfused with low levels of ICSH (5.0 mμg/ml

of perfusion medium) added after 2 hours of perfusion. The purpose of this experiment was to determine the response of the testis to ICSH at a dose level that would contain only very small amounts of contaminating gonadotrophins or other hormones.

(5) Fourteen testes were perfused with high levels of ICSH (8 of the testes were stimulated with 8000 mμg/ml and the remaining 6 with 30 mμg/ml of medium) added to the perfusion medium after 2 hours of perfusion. The purpose of this experiment was to determine the response of the testis to levels of ICSH that would presumably saturate the testis by binding with all available testosterone producing cellular elements.

(6) Four testes (contralateral to the four testes used in Experiment 4) were perfused with a low level dose of ICSH (5 mμg/ml) combined with a non-stimulating dose of GH (10 mμg/ml). The hormone preparations were mixed together and injected into the perfusate reservoir after the second hour of perfusion. The purpose of this experiment was to test for synergism between ICSH at doses capable of stimulating testosterone secretion (yet containing only very small amounts of contaminating hormones) and GH at doses which, alone, were incapable of stimulating testosterone secretion.

(7) Eight testes were perfused with saturating levels of ICSH (100 mμg/ml) combined with GH (200 mμg/ml) in the same proportion as that used in Experiment 6. The hormones were mixed together and added to the perfusate reservoir after the second hour of perfusion. The purpose of this experiment was to determine if GH could synergise with ICSH to elevate the testosterone secretion rate from testes maximally stimulated with ICSH.

At the conclusion of each perfusion the testis was removed from the organ chamber, trimmed free of epididymis and surrounding fat and weighed.

CHAPTER III

RESULTS

The capacity of the testis to synthesize and secrete testosterone is well documented (163,164,166,167,168,185). However, the mechanisms regulating testosterone biosynthesis and secretion are not fully understood. For example, it appears that several hormones, including ICSH (32,33,34), FSH (48), prolactin (110) and GH (40,42,66,67), may enhance testosterone secretion in various species. Growth hormone's role in regulating testosterone secretion is supported only by indirect evidence.

The specific objectives of the study were: (1) to determine the *in vivo* testosterone production rate in rabbits; and (2) to test the hypothesis that growth hormone (GH) synergises with interstitial cell stimulating hormone (ICSH) to increase testosterone secretion rate in perfused rabbit testes. The phrase "testosterone production rate" (TPR) will be used throughout the report to refer to the *in vivo* testosterone production experiments whereas "testosterone secretion rate" (TSR) will be reserved for the *in vitro* perfusion experiments.

Validation of Techniques

Since TPR and TSR determinations require the accurate quantification of testosterone in blood and artifical medium it was necessary to confirm the radiochemical purity of testosterone isotopes used for the

production rate studies, to verify the validity of TLC purification procedures used in the production rate study and to confirm the identity of testosterone isolated and quantified from biological samples.

Evidence for Radiochemical Purity of Labeled Testosterone

Testosterone -1,2-^{3}H was used as a standard for monitoring losses in the determination of testosterone concentration in venous effluent samples collected in the perfusion studies and in peripheral plasma samples collected at "zero time" in the production rate studies. In addition, the isotope was injected into rabbits and its rate of disappearance from blood used in the calculation of MCR in the production rate study. Testosterone -4-^{14}C was used as an internal standard for monitoring losses of testosterone -1,2-^{3}H in blood samples subjected to purification procedures for the calculation of MCR. Radiochemical impurities in the isotopes would result in errors in the determination of MCR in the production rate study and in the calculation of testosterone recovery rates in both the production rate and secretion rate studies.

Inspection of the testosterone -1,2-^{3}H and testosterone -4-^{14}C scans (Figures 2 and 3) disclosed that both compounds migrated discretely on TLC plates developed in benzene:ethyl acetate (110:100, V:V). The R_f for both isotopes was 0.27 and the peaks in radioactivity occurred at the same R_f value (0.27) as authentic testosterone -^{12}C developed in the same solvent system in adjacent lanes on the TLC plates. Approximately 98% of the radioactivity in both isotopes was located in the major peaks. It was concluded that the isotopes were radiochemically pure testosterone and were thus used without further purification steps.

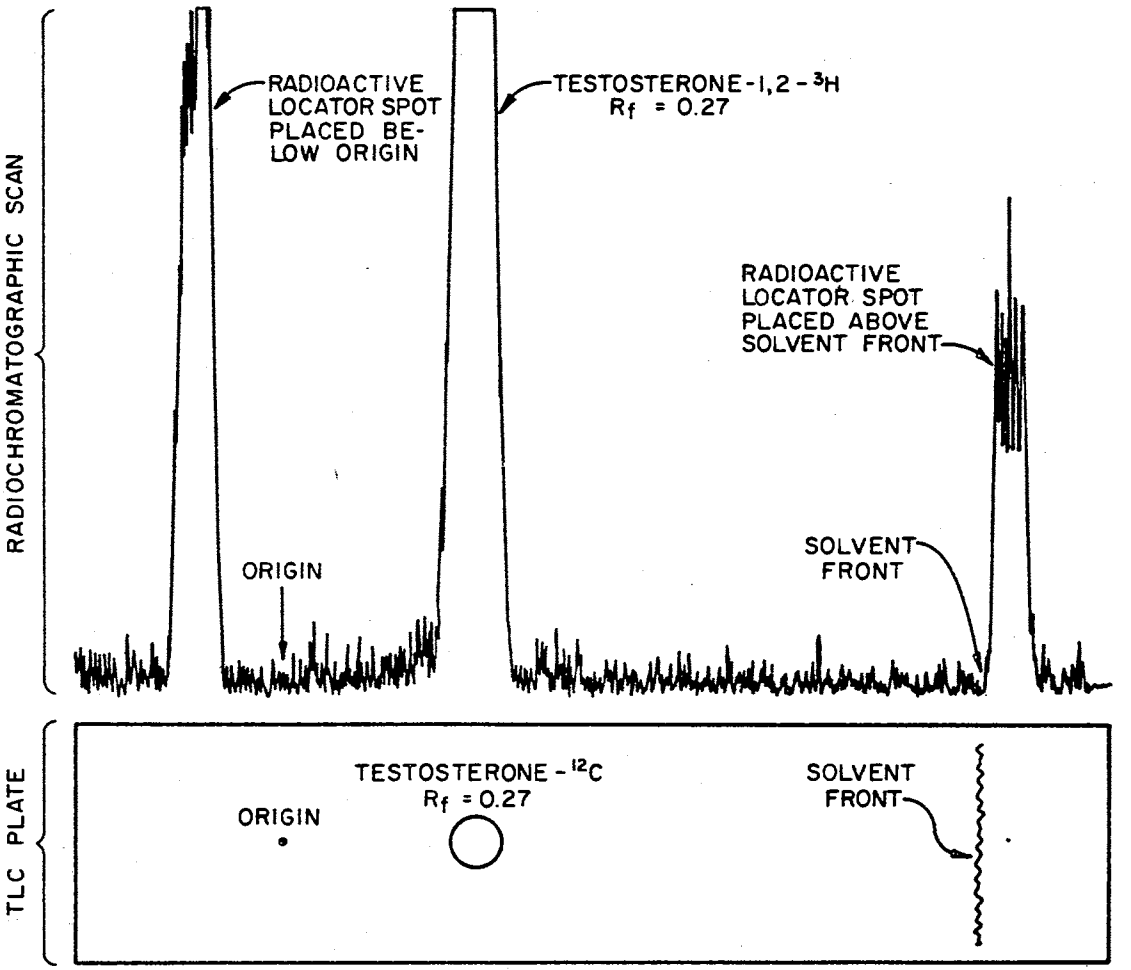

Figure 2. Radiochromatographic Scan of Testosterone -1,2-^{3}H and Concurrent Thin Layer Chromatography of Authentic Testosterone -^{12}C. The solvent system was benzene:ethyl acetate (110:100, V:V).

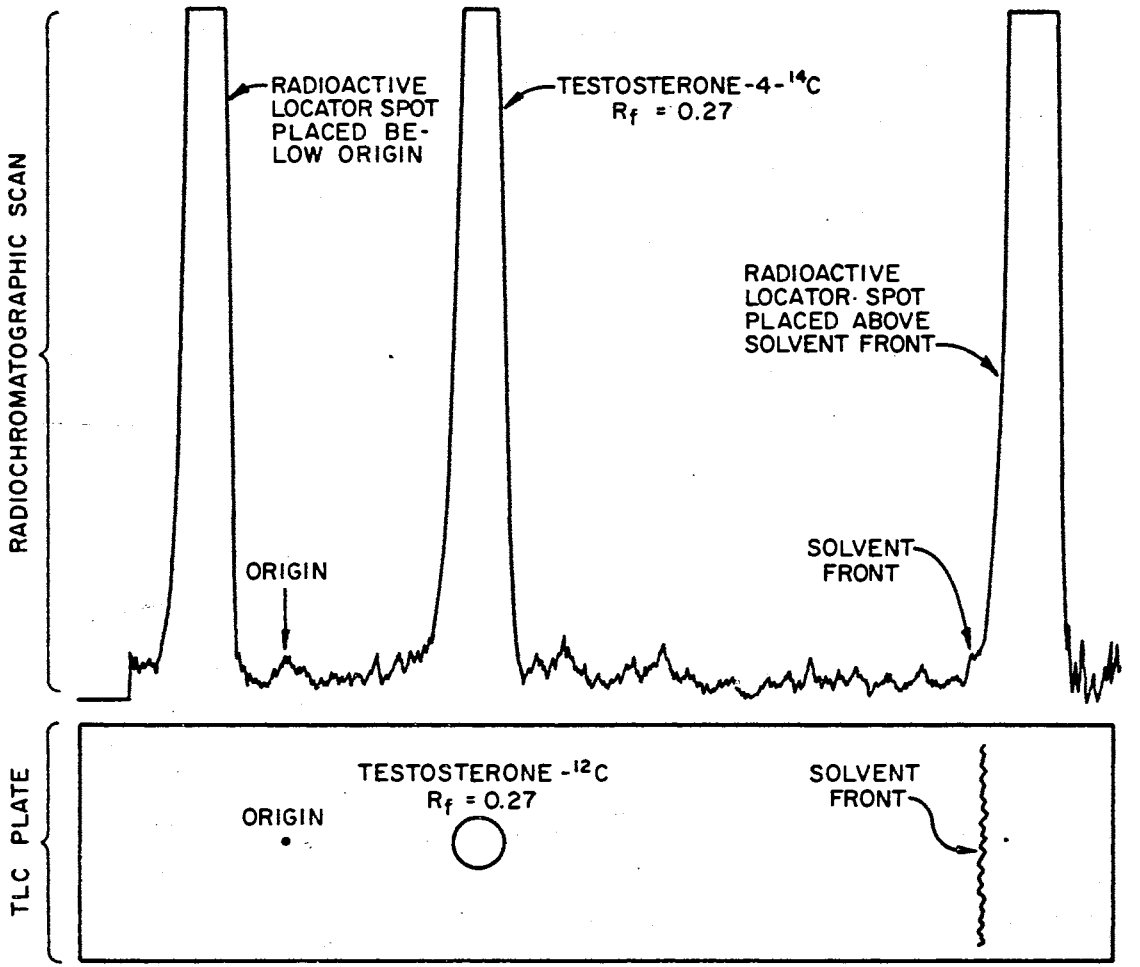

Figure 3. Radiochromatographic Scan of Testosterone -4-^{14}C and Concurrent Thin Layer Chromatography of Authentic Testosterone -^{12}C. The solvent system was benzene:ethyl acetate (110:100, V:V).

Evidence for Validity of Purification Procedures Used in the Testosterone Production Rate Study

Though the tritiated testosterone injected into the rabbits was radiochemically pure at the time of injection, metabolic activity *in vivo* resulted in transformation of the testosterone into other compounds with a consequent transfer of the tritium label to these compounds and a concomitant increase in tritium labeled substances that were not testosterone. It is apparent then that the tritium label of the injected isotope would no longer serve to identify testosterone exclusively. Tritium could exchange for the hydrogen of water molecules present in reagents used in purification procedures resulting in another source of error that would confound the results. To avoid errors that might arise from these two sources (metabolic biotransformation and tritium-hydrogen exchange), a preliminary experiment was conducted as follows: (1) Aliquots from serial plasma samples obtained from a rabbit that had been injected with tritiated testosterone were pooled in a test tube containing testosterone -4-^{14}C as an internal standard, extracted with dichloromethane and developed in three successive TLC systems. (2) A solution of testosterone -1,2-^{3}H, testosterone -4-^{14}C and cold carrier testosterone in 1 ml of H_2O was extracted with dichloromethane and developed in the same three TLC systems with the plasma sample.

The results of the experiment (Table I) disclosed that the ^{14}C:^{3}H ratio of the plasma sample increased between the first and second chromatography, but remained stable between the second and third chromatography. This suggested that the ^{14}C and ^{3}H, migrating with the same R_f in the second TLC system as authentic testosterone ^{12}C, was isotopic testosterone. Therefore, purification by two TLC systems was considered

TABLE I

$^{14}C:^{3}H$ IN: (1) POOLED ALIQUOTS OF BLOOD PLASMA COLLECTED VIA THE MARGINAL EAR VEINS FROM A RABBIT INJECTED WITH TESTOSTERONE -1,2-^{3}H, AND (2) A SOLUTION OF TESTOSTERONE -1,2-^{3}H AND TESTOSTERONE -4-^{14}C DISSOLVED IN WATER[1]

TLC	$^{14}C:^{3}H$	
	(1) Plasma	(2) Testosterone Solution
#1	0.31	0.32
#2	0.37	0.32
#3	0.37	0.32

[1]All samples were purified in 3 ascending TLC systems. Solvent systems were: TLC #1) 175 ml benzene, 25 ml methanol; TLC #2) 110 ml benzene, 100 ml ethyl acetate; TLC #3) 1 ml H_2O, 20 ml methanol, 60 ml chloroform and 120 ml toluene.

sufficient to provide accurate results in the testosterone production rate study. The $^{14}C:^{3}H$ ratio of the testosterone solution in water remained constant in the three successive TLC systems suggesting that tritium did not exchange with hydrogen. These data support the conclusion that tritium-hydrogen exchange would not confound the purification procedures in the production rate study.

Evidence for Identity of Testosterone Assayed in the Perfusion Experiments

Accurate quantification of testosterone in biological systems required that the testosterone be isolated in a highly purified state. Preliminary evidence indicated that the material quantified in the perfusion experiments was testosterone since the R_f value in the first TLC system was similar to that of authentic testosterone standard. Furthermore, the R_f value of the heptafluorobutyrate derivative of the eluate from the first TLC system was similar to authentic testosterone heptafluorobutyrate standard in the second TLC system. The retention time on a 3 foot glass column in GLC was similar to authentic testosterone heptafluorobutyrate standard. Mass spectrometric studies confirmed these observations since the molecular weights were identical and the fragmentation patterns similar for the material isolated from venous effluent and for authentic testosterone heptafluorobutyrate (Figure 4). Thus, it was concluded that the material assayed via the method of Brownie, et al. (222) was the heptafluorobutyrate derivative of testosterone.

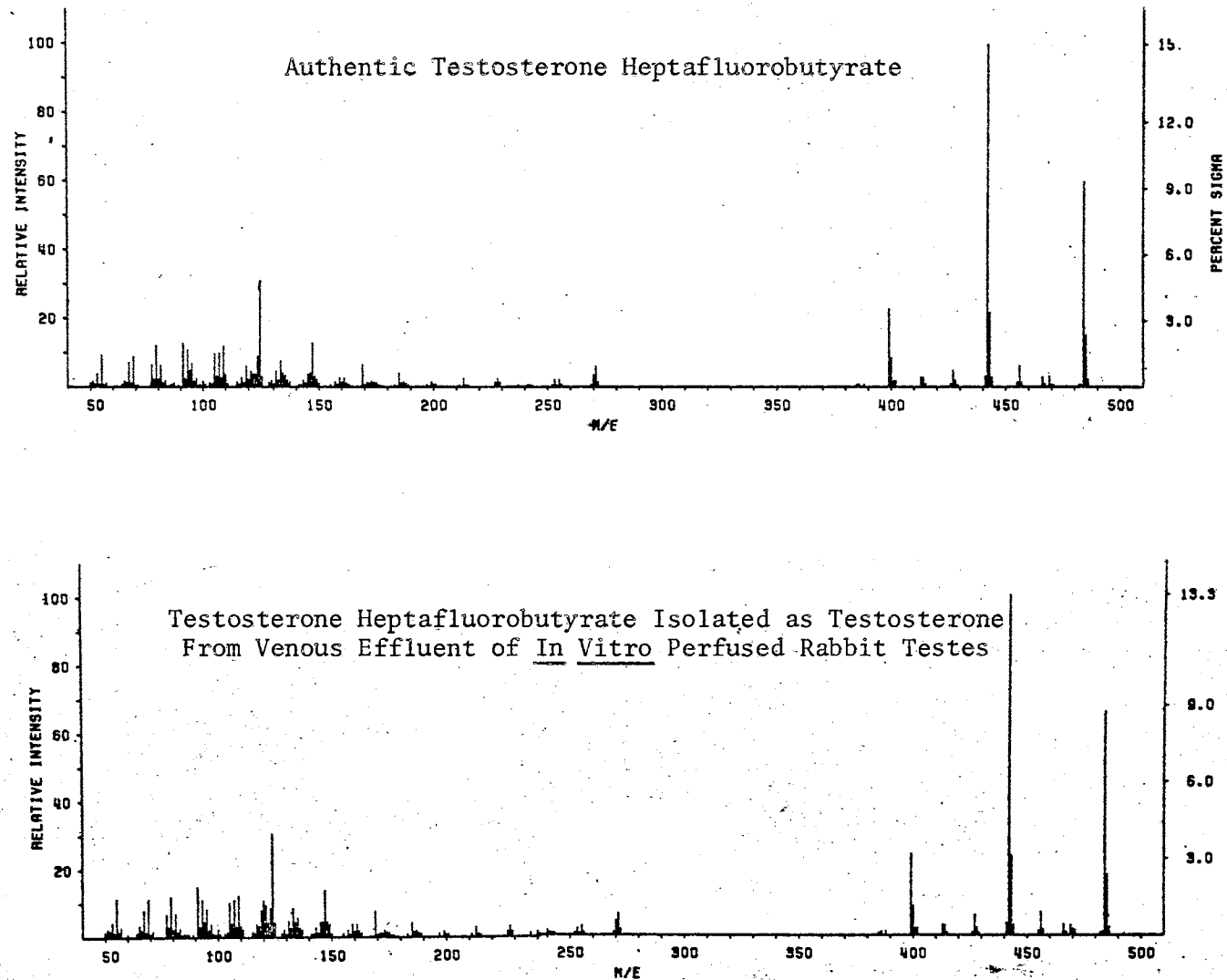

Figure 4. Mass Spectra of Testosterone Heptafluorobutyrate

Correlation of Inherent Experimental Variables With TSR and Artificial Medium Flow Rate of Perfused Rabbit Testes

At the conclusion of the perfusion experiments it was possible to examine the influence that various experimental factors exercised in altering TSR responses by the perfused testes. It was important that these factors be identified so that allowances could be made for them in interpretation of the experimental results.

It was determined that: (1) Order of testis removal from the rabbits did not influence TSR. See Table VI in Appendix. (2) Origin of testis (left vs. right) did not influence TSR. See Figure 15 in Appendix. (3) Variations in testis weight did not influence artificial medium flow rate through the testes (Figure 16 in Appendix) or TSR (Figure 17 in Appendix). (4) Variations in artificial medium flow rate through the testes did not influence TSR. See Figure 18 in Appendix. (5) Artificial medium flow rate through the testes was not altered by the addition of ICSH or GH to the artificial medium. See Table VII in Appendix.

Thus, it was concluded that none of the foregoing inherent experimental variables would confound the interpretation of data derived from the in vitro perfusion experiments.

Determination of In Vivo Testosterone Production Rate

Elucidation of the factors controlling testosterone secretion requires a model system that allows investigation of the effects of hormones or other substances either singly or in combination without interference from extrinsic signals. This cannot be accomplished by studying regulation of testosterone secretion in intact animals. However, the

in vitro perfusion apparatus meets this need and should permit experiments to be conducted that allow one to establish *in vitro* conditions that will stimulate the isolated perfused rabbit testis to produce testosterone at the same rate as the normal *in vivo* rabbit testis. Toward this end the *in vivo* testosterone production rate was determined in five New Zealand White male rabbits by procedures described by Horton, *et al.* (227), Southren, *et al.* (228) and Rhynes (229). Testosterone production rate is defined as the total rate of entry of testosterone from all sources into the general circulation and as extra-gonadal testosterone sources contribute less than 5% of the total body testosterone (183), the *in vivo* testosterone secretion rate by testes is taken to be 95% of the testosterone production rate.

Disappearance of Tritiated Testosterone From Plasma

Isotopic testosterone injected into the circulation of a rabbit mixes with the animal's endogenous testosterone. The rate of metabolism and transport of testosterone -1,2-^{3}H then reflects the rate of metabolism and transport of endogenous testosterone. Collection of serial blood samples at timed intervals after the injection of testosterone -1,2-^{3}H allows the experimenter to monitor the disappearance of the isotope from the peripheral circulation. These data can be used to calculate MCR, production rate, transport constants and metabolism constants for testosterone.

Testosterone -1,2-^{3}H injected intracardially disappeared rapidly from peripheral blood. The percent recovery of the isotope per ml of plasma at 5, 10, 15, 30, 40, and 50 minutes after injection is plotted in graphical form for each of the five rabbits in Figure 5. There was

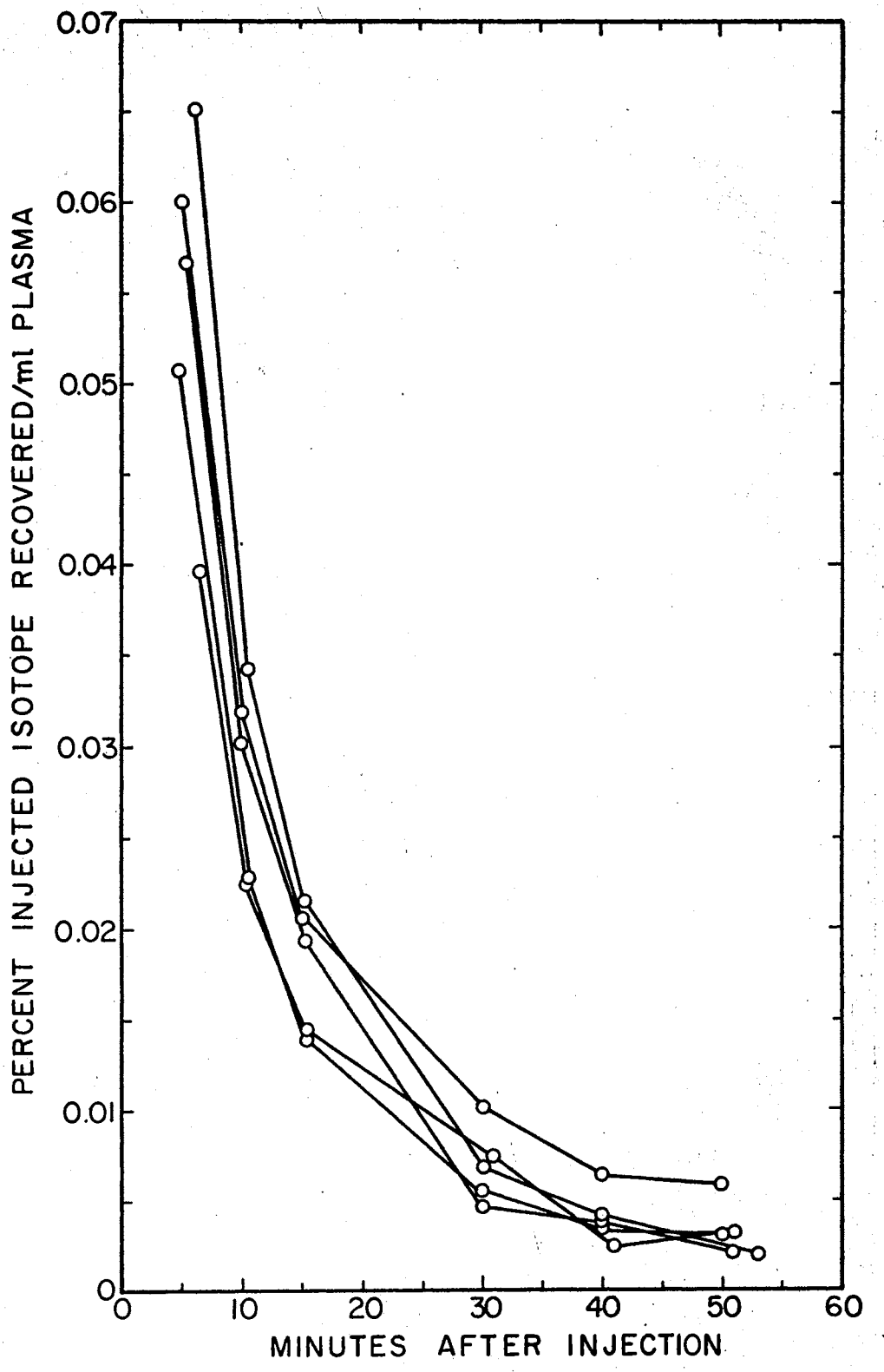

Figure 5. Disappearance of Testosterone -1,2-^{3}H (44.92 Ci/mM) From Plasma of Five New Zealand White Male Rabbits. The isotope was injected intracardially and blood collected at timed intervals from marginal ear veins.

an early very rapid decline in plasma isotope concentration followed by a period of slower decline. Five minutes after injection only 6.35% of the isotope was present in the plasma and 50 minutes after injection 0.37% was present (Table II). A remarkable similarity in isotope disappearance was observed for all the animals. The mean recovery rate of the internal standard, testosterone -4-^{14}C, upon which the disappearance curves were based was 55.5 $\pm$ 2.45%. Thus, approximately one-half of the testosterone in the samples was lost during the extraction and TLC procedures. The presence of the internal standard permitted accurate calculation of testosterone -1,2-^{3}H in each of the plasma samples.

Metabolic Clearance Rate of Testosterone

The disappearance curves displayed two distinct slopes, thus it was necessary to construct two regression lines to describe the disappearance of testosterone. The regression lines were calculated for each of the five rabbits and mean values used to construct the graph that is labeled Figure 6. Line A + B is the regression line that describes the disappearance of the isotopic testosterone during the first 15 minutes after injection. Line B with slope β describes the disappearance of the isotope during the 30 to 50 minute period after injection. Since line A + B is influenced by line B, a third line (A) is constructed by subtracting the extrapolated radioactivity contributed by line B from the measured values and plotting the corrected concentrations logarithmically. This yields line A with slope α. By integrating the area of line A and line B, metabolic clearance rate (MCR) was determined and the values for individual rabbits recorded in Table III. MCR is defined as the volume of blood cleared of testosterone in unit time and in this

TABLE II

PERCENT OF INJECTED TESTOSTERONE -1,2-^{3}H PRESENT IN PLASMA[1] OF NEW ZEALAND WHITE MALE RABBITS AT TIMED INTERVALS AFTER INJECTION

Minutes After Injection					
5	10	15	30	40	50
6.35%[2]	3.57%	2.00%	0.73%	0.52%	0.37%
±0.56	±0.37	±0.16	±0.09	±0.08	±0.07

[1]Plasma volume calculated on the basis of 30.17 ml of plasma/kg body weight in albino rabbits as described by Armin, et al. (233).

[2]Mean value ± SEM for 5 rabbits.

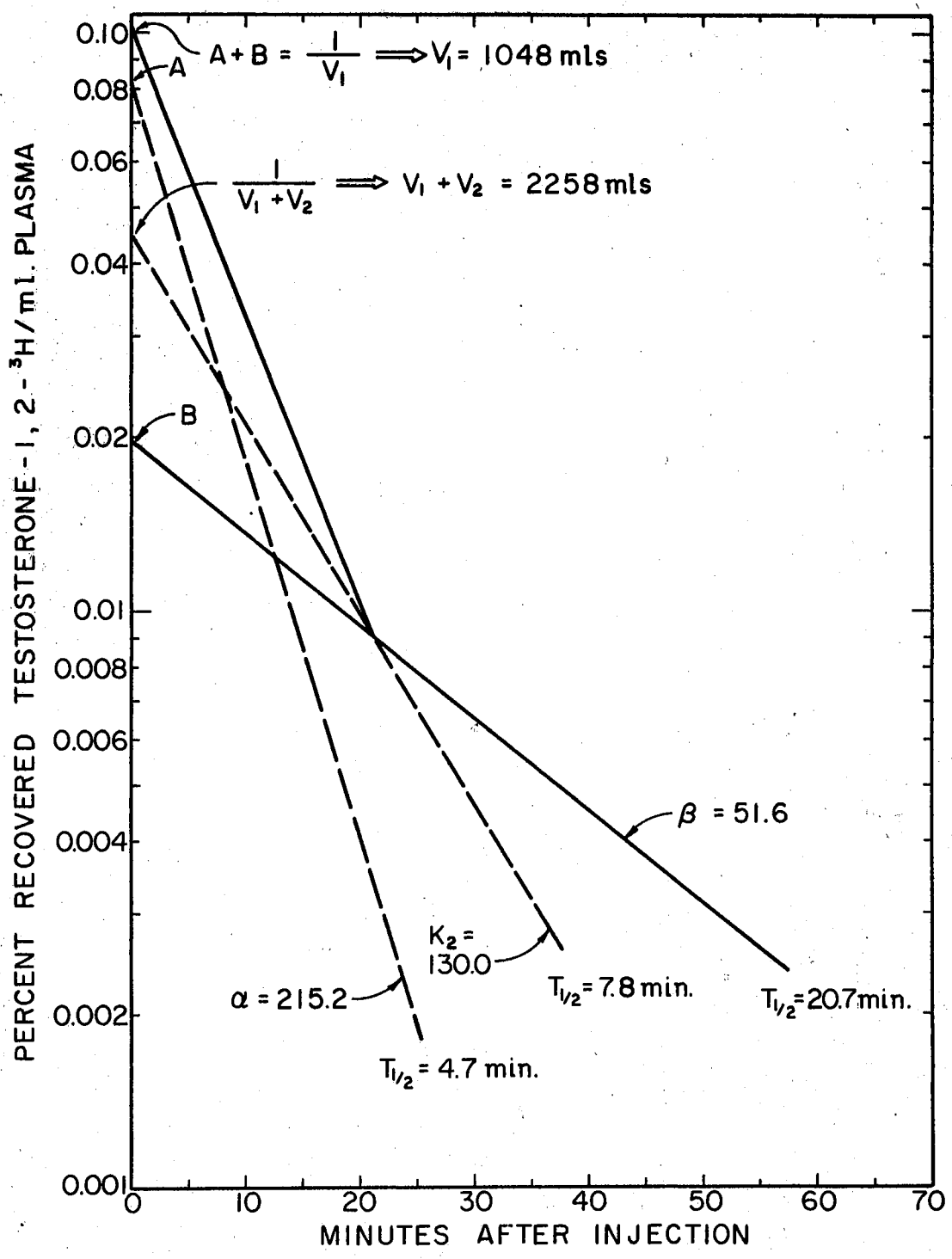

Figure 6. Regression Lines Describing Disappearance, Volumes of Distribution and Half-Life of Testosterone -1,2-^{3}H (44.92 Ci/mM) in New Zealand White Male Rabbits. The lines were constructed from mean values for five animals.

TABLE III

WEIGHT, PERIPHERAL TESTOSTERONE CONCENTRATION, METABOLIC CLEARANCE RATE (MCR) AND TESTOSTERONE PRODUCTION RATE (TPR)[1] OF FIVE NEW ZEALAND WHITE MALE RABBITS

Animal	wt. (lbs)	Peripheral testosterone (mg/ℓ plasma)	MCR (ℓ/24 hrs)	TPR (mg/animal/24 hrs)
#18	8.12	0.00555	105.0	0.581
#19	7.62	0.00266	166.0	0.443
#20	8.75	0.00143	114.0	0.163
#21	8.00	0.00397	135.0	0.537
#22	8.50	0.00201	151.0	0.304
Mean	8.20	0.00312	134.2	0.406
SEM	±0.20	±0.00074	± 11.3	±0.077

[1]TPR = MCR x peripheral testosterone concentration.

study the mean MCR was 134 liters/24 hours.

The additional data recorded in Figure 6 and Table III will be discussed in subsequent sections.

Plasma Testosterone Levels and In Vivo Testosterone Production Rate

Since the amount of testosterone leaving the plasma in the steady state is equal to the amount entering, the product of the MCR and the concentration of testosterone in plasma must be the testosterone production rate. In order to calculate production rate, it was necessary to determine the plasma concentration of testosterone for each of the rabbits. The determinations were made by assaying 20 ml blood samples that were collected immediately prior to the isotope injection. The mean plasma testosterone concentration was 0.00312 $\pm$ 0.00074 mg/liter. The values for individual animals are recorded in Table III.

By multiplying MCR x plasma concentration of testosterone a mean TPR of 0.406 $\pm$ 0.077 mg/rabbit/24 hours was obtained for this study (Table III). This means that the male rabbit produces testosterone in the testes, the adrenals, possibly the epididymides and by peripheral conversion at a combined rate of approximately 0.4 mg/24 hours. Assuming that 95% of the total production is represented by testosterone that is secreted by the testes then approximately 0.38 mg/24 hours is the in vivo testicular testosterone secretion rate for young, sexually mature, New Zealand White male rabbits.

Transport and Metabolism of Testosterone -1,2-^{3}H

Although the primary objective of the in vivo study was to determine the production rate of testosterone the data collected permitted

the elucidation of transport and metabolic behavior of testosterone in the rabbit. As this information has not been documented for this species and as its elucidation may contribute to a broader understanding of factors that regulate testosterone secretion it is included here.

The first part of this discussion will deal with general concepts after which specific results of the study will be presented. Tait, et al. (232) reported that a reasonable model for the transport and metabolism of a radioactive steroid in man consists of an inner pool (V_1) which, for steroids weakly bound to plasma proteins, probably includes the plasma, extra-cellular fluid and the liver; and an outer (tissue) pool (V_2). The radioactive concentration in the outer pool is lower than that in the inner pool for some time after injection. These concentrations then become equal when that in the outer pool is maximal. From this time on the radioactive concentration in the outer pool is greater than in the inner pool. The final slope of the plasma concentration (β) is a result of the combination of the effects of metabolism and transport. It will be a flatter slope than would be expected from the effects of metabolism alone because continuous transport occurs from the outer pool to the inner pool. Transport between the pools can be described by the rate constant, K_1. Metabolism is assumed to be negligible in the outer pool. If all the measured radioactivity is due to testosterone, then K_1 equals the fraction of the injected dose per ml of plasma transported to the outer pool per unit time; K_2 equals the fraction of the injected dose per ml of plasma metabolized in the inner pool per unit time.

Figure 6 depicts the disappearance, volumes of distribution and half-life of the injected tritiated testosterone. After the

intracardial injection of testosterone -1,2-^{3}H the radioactive hormone was distributed rapidly into the inner pool. The inner pool volume (V_1) is equal to body testosterone in rapid equilibrium, divided by plasma concentration. V_1 was calculated by determining the reciprocal of the A + B intercept ($\frac{1}{A + B}$). After the initial distribution the radioactive testosterone moved more slowly into a larger total volume ($V_1 + V_2$). This volume multiplied by the blood concentration of testosterone was equal to the total body content of non-isotopic testosterone. $V_1 + V_2$ was calculated by determining the reciprocal of the intercept on the ordinate obtained by extrapolating, using the slope K_2, from the point where the initial (A + B) and final (B) disappearance curves intersected. V_2 was determined by subtracting V_1 from ($V_1 + V_2$). The half-life of the radioactive hormone in the inner and outer pools was determined by using the slopes of lines A and B, respectively, were T 1/2 = $\frac{0.6923(1440)}{\text{slope}}$.

The values for pool sizes, total body testosterone and transport and metabolic constants for individual rabbits are given in Table IV. It appears that testosterone is distributed into a total space of about 2 liters with the inner and outer spaces contributing almost equal volumes to the space. Fifty-four percent (K_1 = 153) of the injected isotope was transported to the outer pool leaving 46% (K_2 = 130) in the inner pool to be metabolized. The mean total non-isotopic body testosterone was calculated to be 6.35 µg/rabbit (774 mµg/lb of body weight). The intracardial tritiated testosterone dose contained 113 mµg testosterone, thus the dose injected represented less than 2% of the total body testosterone and presumably did not significantly disturb endogenous steroid dynamics. In view of the 24 hour production rate of

TABLE IV

VOLUMES OF DISTRIBUTION, TRANSPORT AND METABOLIC CONSTANTS AND TOTAL BODY NON-ISOTOPIC TESTOSTERONE FOR FIVE NEW ZEALAND WHITE MALE RABBITS

Animal	V_1	V_2	V_1+V_2	K_1	K_2	Total Body Testosterone mμg/lb
	mls			units/24 hrs		
18	998.0	968.0	1,966.0	67.97	104.89	1,343.0
19	1,290.0	689.0	1,969.0	55.74	129.02	690.0
20	728.0	3,018.0	3,746.0	561.61	156.78	612.0
21	1,116.0	336.0	1,452.0	24.55	121.17	720.0
22	1,107.0	1,038.0	2,145.0	57.04	136.51	507.0
Mean	1,048.0	1,210.0	2,258.0	153.0	130.0	774.0
SEM	±93.0	±469.0	±389.0	±102.0	±8.6	±147.0

406 µg/24 hours, it is apparent that the total body testosterone of 6.35 µg must turn over many times each day. Calculations disclosed that the total body testosterone was replaced every 22.5 minutes.

The values for testosterone -1,2-^{3}H half-life in the various pools are given in Table V. The half-life for the isotope in the inner pool was 4.67 minutes which reflects the rapid metabolism in that pool. The half-life for the isotope in the outer tissue pool was much greater, 20.75 minutes, which demonstrates that metabolic activity was much slower in that space and tends to confirm the observations of Tait, et al. (232), e.g., that the disappearance of testosterone from the outer pool is likely due to transport back into the inner pool rather than to metabolism.

In summary, these results show that young sexually mature male rabbits clear 134 liters of plasma completely and irreversibly of testosterone in a 24 hour period. The peripheral blood concentration of testosterone in the rabbits was 3.12 mµg/ml. Assuming that MCR and blood concentration of testosterone remain constant, then it follows that these rabbits must produce testosterone from all body sources at a rate of 406 µg/24 hours to maintain the steady state (TPR = MCR x peripheral testosterone concentration). Transport and metabolic studies disclosed that the mean total endogenous body testosterone present in the rabbits was 6.35 µg and further that 44% of this testosterone was located in an inner pool (plasma, liver, etc.) of 1048 mls where its half-life was short (T 1/2 = 4.67 min) because of rapid metabolic activity. The remainder of the total body testosterone (56%) was "stored" in an outer pool (tissue) of 1210 mls where its half-life was prolonged (T 1/2 = 20.75 min). Thus, testosterone remains in the outer pool much longer

TABLE V

HALF-LIFE OF TESTOSTERONE -1,2-^{3}H IN VARIOUS POOLS OF NEW ZEALAND WHITE MALE RABBITS

Animal	V_1	V_2	$V_1 + V_2$
18	4.81[1]	28.17	9.50
19	4.32	17.11	7.73
20	4.25	13.70	6.36
21	5.35	18.83	8.23
22	4.62	25.96	7.30
Mean	4.67	20.75	7.82
SEM	±0.20	±2.73	±0.52

[1]minutes.

and its ultimate disappearance presumably results from transport back into the inner pool rather than from metabolism in the outer pool.

In Vitro Testosterone Secretion by Perfused Rabbit Testes Stimulated by ICSH and GH

This section contains the results of experiments designed to test the hypothesis that GH synergises with ICSH to augment testosterone secretion by isolated perfused rabbit testes.

The TPR studies reported in the previous section disclosed that the male rabbit secretes approximately 0.38 mg of testosterone/24 hours from the testes. Assuming that the mean testis weight of the rabbits used in the production rate studies were similar to the mean weight of 47 testes (2.4 gm) used in the perfusion studies, then the testosterone secretion rate *in vivo* may be expressed as 3,333 mμg/gm of testis/hour. The ICSH-FSH synergism experiments of Johnson and Ewing (48) disclosed that perfused rabbit testes stimulated with 8 μg ICSH ml of artificial medium secreted 521 mμg testosterone/gm of testis/hour. The addition of 4 μg FSH/ml of artificial medium to the perfusate containing the ICSH resulted in a two-fold increase in TSR to 1,022 mμg/gm of testis/hour. Since the ICSH-FSH combination was unable to stimulate testosterone secretion to a level comparable to *in vivo* TSR, this suggests that some other hormonal factor plays a role in stimulating testosterone secretion.

Since GH is reported to exert a permissive or synergistic effect on the action of several hormones (40,42,60,63,64,65,66,67) in various species it seems appropriate to test the augmentation of ICSH stimulated TSR by GH. The *in vitro* perfused rabbit testis preparation lends itself to experiments testing the effects of various hormonal stimuli on testosterone secretion because it allows the investigator to examine the

effects of specific hormones without interference from signals arising from without the testis. The results of individual experiments will be presented in graphical form in the following discussion and in tabular form in Table VIII of the Appendix.

Response of Perfused Testes to Artificial Medium

Five testes were perfused with artificial medium to determine basal testosterone secretion. Mean testosterone secretion declined steadily during the first 2 hours of perfusion (Figure 7). The testosterone secretion rate at the end of the first hour was 191.8 mμg/gm testis/hour, and by the end of the sixth hour had declined to 88.2 mμg/gm testis/hour. These results demonstrate that the artificial medium lacked factor(s) responsible for maintaining testosterone secretion by perfused rabbit testes.

Effect of GH Administered in Graded Doses Upon TSR by Perfused Rabbit Testes

This experiment was designed to test the effect of different concentrations of GH upon TSR by perfused testes. The objective of the experiment was to select a GH concentration that would not result in increases in TSR. GH was infused into the arterial cannula of two testes by the Sage Micro Pump at a rate sufficient to maintain its concentration at 10, 25, 100, 1,000, and 5,000 mμg/ml of artificial medium in the spermatic artery for hours 2, 3, 4, 5, and 6 respectively of the perfusion period (Figure 8). Infusions of 10, 25, and 100 mμg/ml of GH did not affect TSR. In contrast, infusion of 1,000 and 5,000 mμg/ml of GH increased TSR. These results show that 10 mμg GH/ml of artificial medium was incapable of stimulating testosterone secretion.

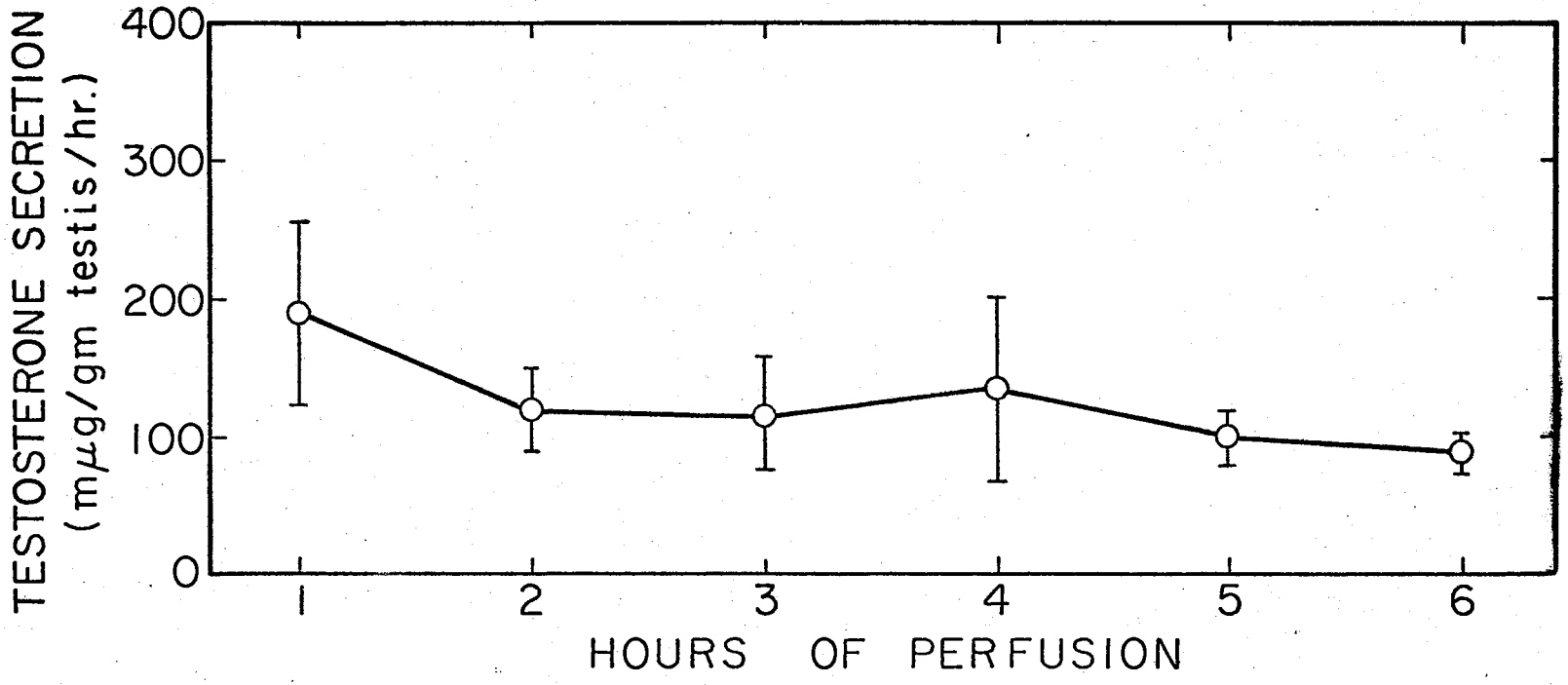

Figure 7. Testosterone Secretion Rate of Rabbit Testes Perfused With Artificial Medium. I = SEM.

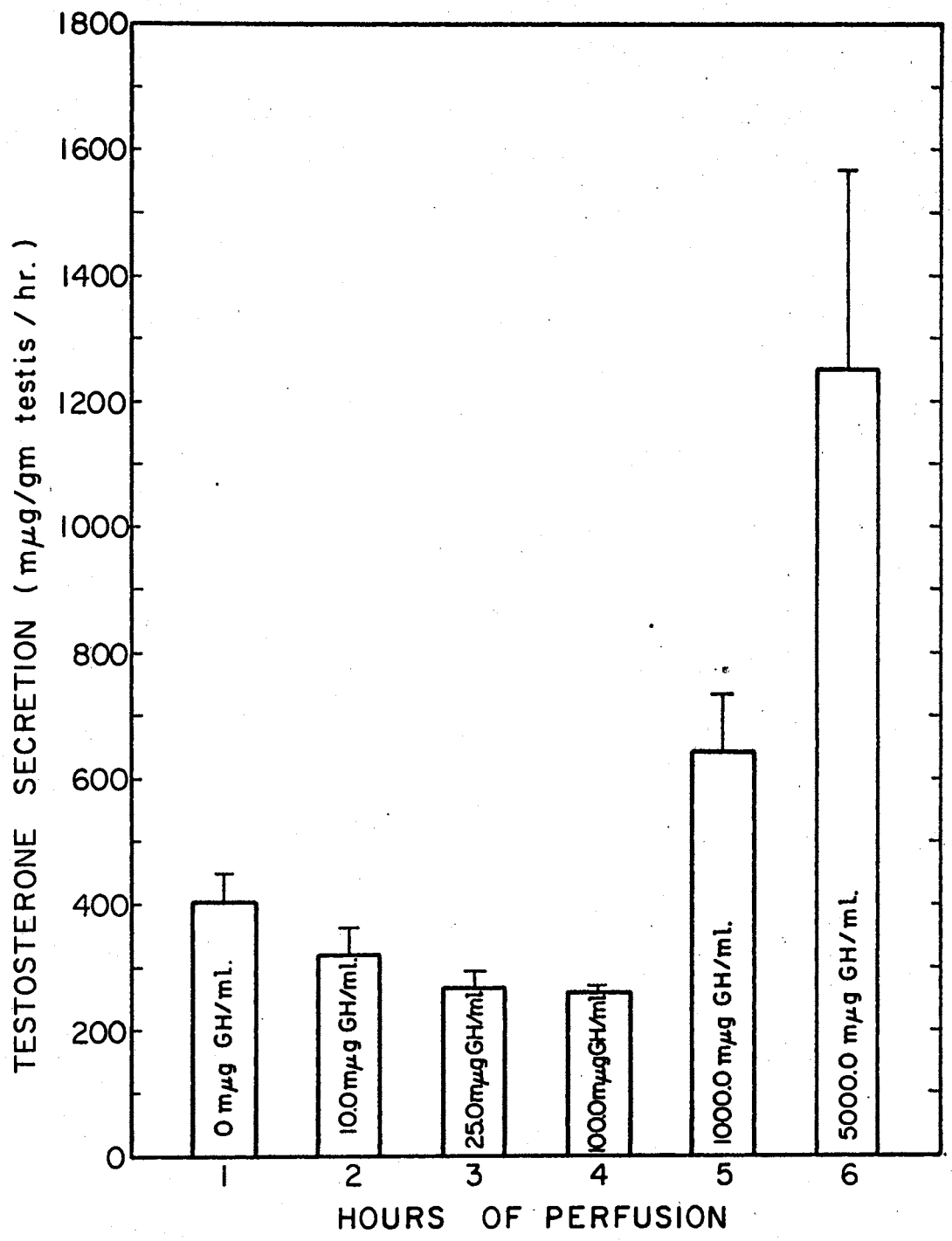

Figure 8. Testosterone Secretion Rate of Perfused Rabbit Testes in Response to the Administration of Growth Hormone in Graded Doses. I = SEM.

Effect of Non-Stimulating Levels of GH Upon TSR by Perfused Rabbit Testes

The purpose of this experiment was to demonstrate that a level of GH that approximated physiological levels in various species had no effect on TSR by perfused rabbit testes. Because 10 mμg GH/ml of medium failed to stimulate testes to secrete testosterone in the previous experiment and because this concentration is within the physiological range reported for blood plasma of cattle (205), pigs (206), baboons (155) and man (156), this dose level was selected as the non-stimulating dose to use in conjunction with low levels of ICSH for a synergism test. It was necessary, however, to confirm that testes exposed to 10 mμg GH/ml of artificial medium for 4 hours of perfusion did not secrete testosterone in an amount greater than that secreted by testes perfused with artificial medium alone. Accordingly, four testes were treated with 10 mμg GH/ml artificial medium after 2 hours of perfusion and TSR measured for the next 4 hours (Figure 9). The response of the testes was almost identical to that of testes perfused with artificial medium alone (Figure 7). Testosterone secretion declined initially and then plateaued at a level of approximately 100 mμg/gm of testis/hour. This suggested that 10 mμg GH/ml of artificial medium did not influence testosterone secretion by perfused rabbit testes.

Effect of Low Level ICSH (5 mμg/ml of Artificial Medium) Upon TSR by Perfused Rabbit Testes

The purpose of this experiment was to determine the response of perfused testes to ICSH at a concentration that contains very small amounts of contaminating gonadotrophins or other hormones. Also, 5 mμg ICSH/ml of artificial medium would provide a hormone level that was

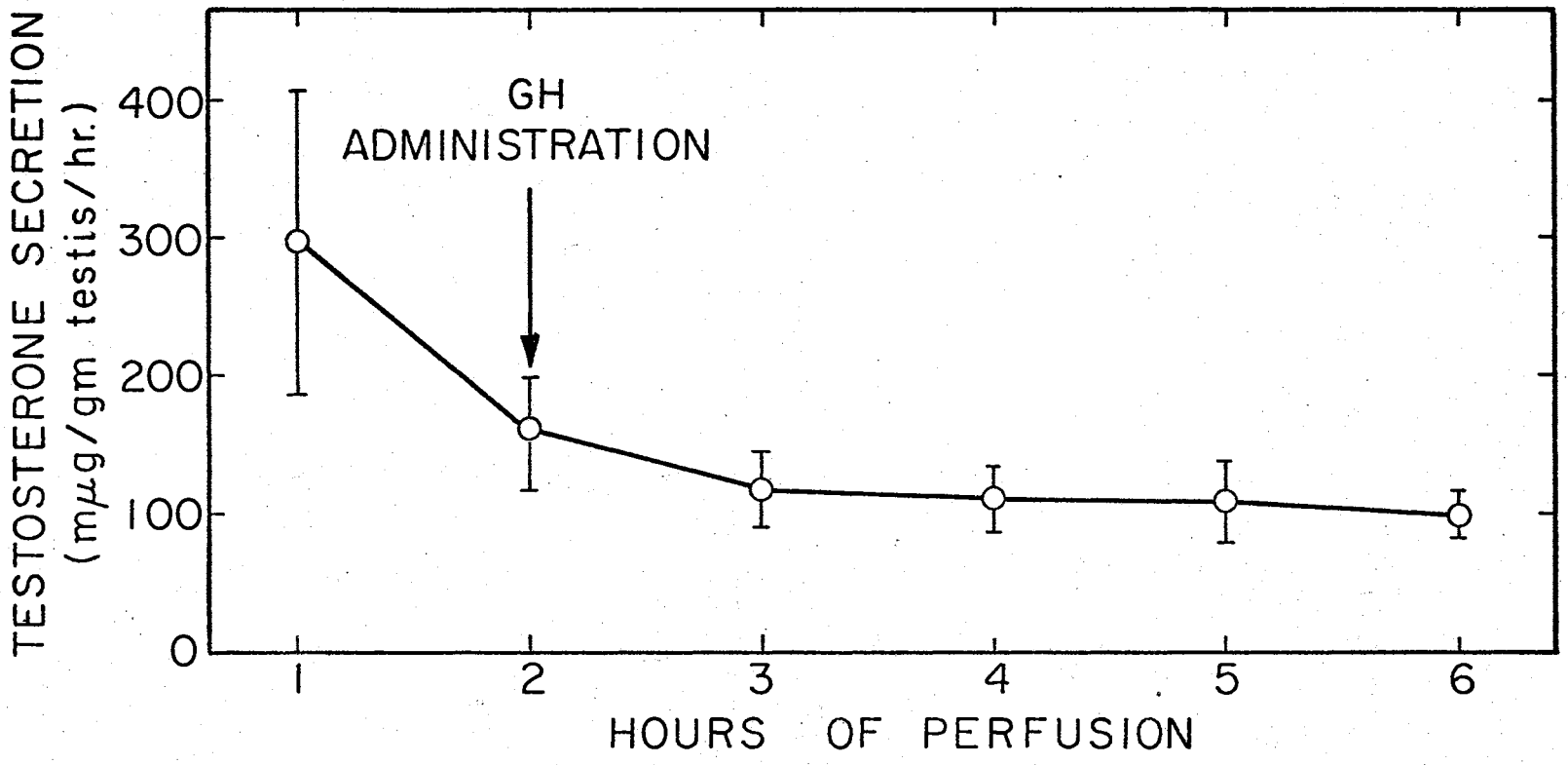

Figure 9. Testosterone Secretion Rate of Perfused Rabbit Testes in Response to 10 mμg Growth Hormone/ml of Artificial Medium. I = SEM.

similar to the physiologic ICSH level (4.5 mμg/ml plasma) reported for the male rabbit by Desjardins and Ewing (153) and would therefore presumably result in a "physiologic" response.

The administration of 5 mμg ICSH/ml of artificial medium after two hours of perfusion resulted in a prompt increase in testosterone secretion (Figure 10). The secretion rate appeared to be sustained in almost linear fashion throughout the remainder of the perfusion period and during the last hour of perfusion 792.4 mμg of testosterone per gm of testis was secreted.

Effect of High Levels of ICSH (30 mμg and 8,000 mμg/ml of Artificial Medium) Upon TSR by Perfused Rabbit Testes

The purpose of this experiment was to measure the response of perfused testes to levels of ICSH that would presumably saturate all available testosterone producing cellular elements of the testes.

The testes responded promptly to the administration of either 30 mμg or 8,000 mμg ICSH/ml of artificial medium. Testosterone secretion rose sharply during the first 2 hours after ICSH stimulation, then tended to level off during the next 2 hours indicating that saturation of testosterone producing cellular elements within the testis was occurring. As the testes responded similarly to both dose levels of ICSH (Figure 11) the data were combined and mean values determined for the construction of the line representing the response of testes to saturating levels of ICSH (Figure 12). Mean testosterone secretion rate for the final hour of perfusion was 879.2 mμg/gm testis/hour.

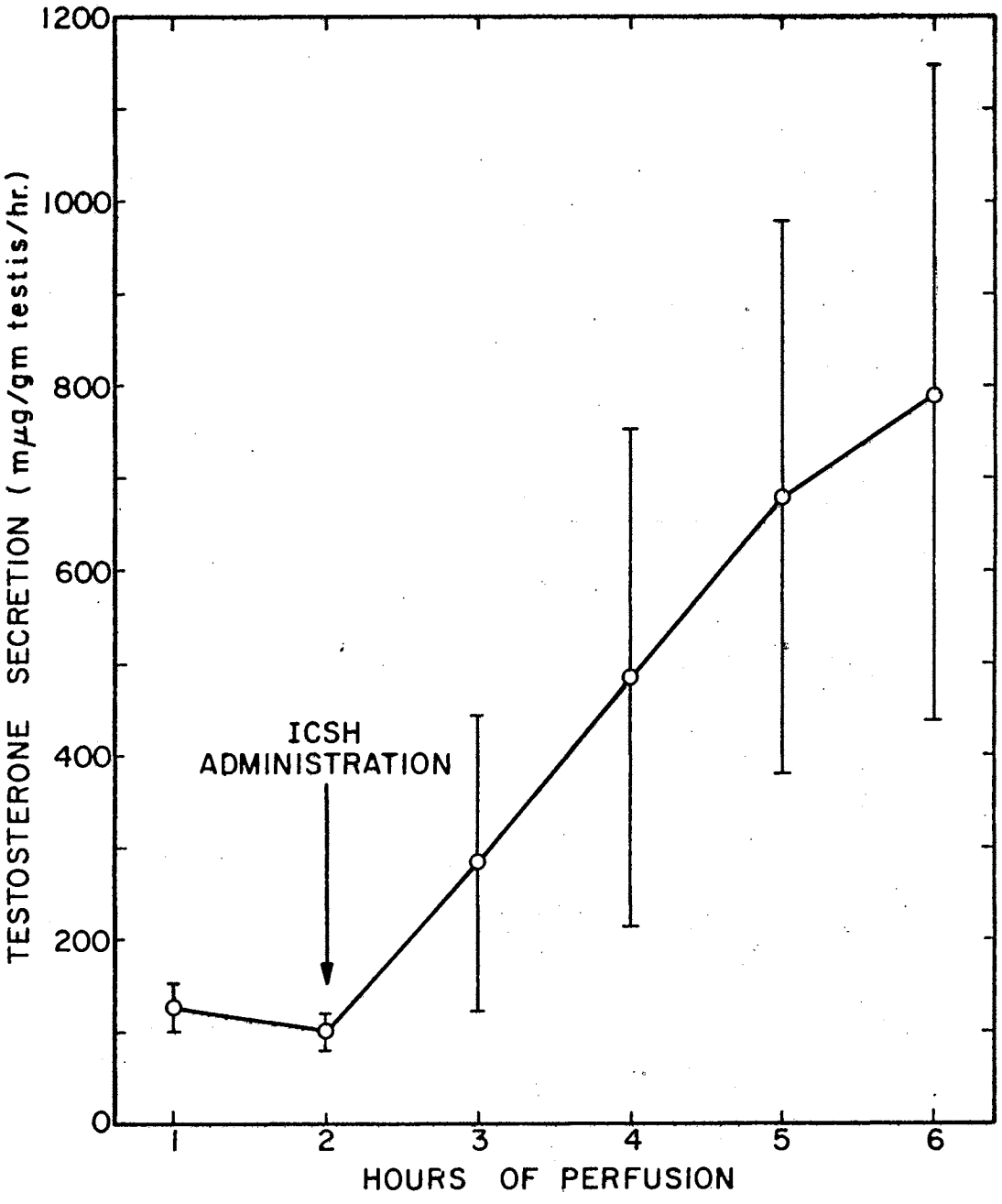

Figure 10. Testosterone Secretion Rate of Perfused Rabbit Testes Stimulated With 5 mμg ICSH/ml of Artificial Medium. I = SEM.

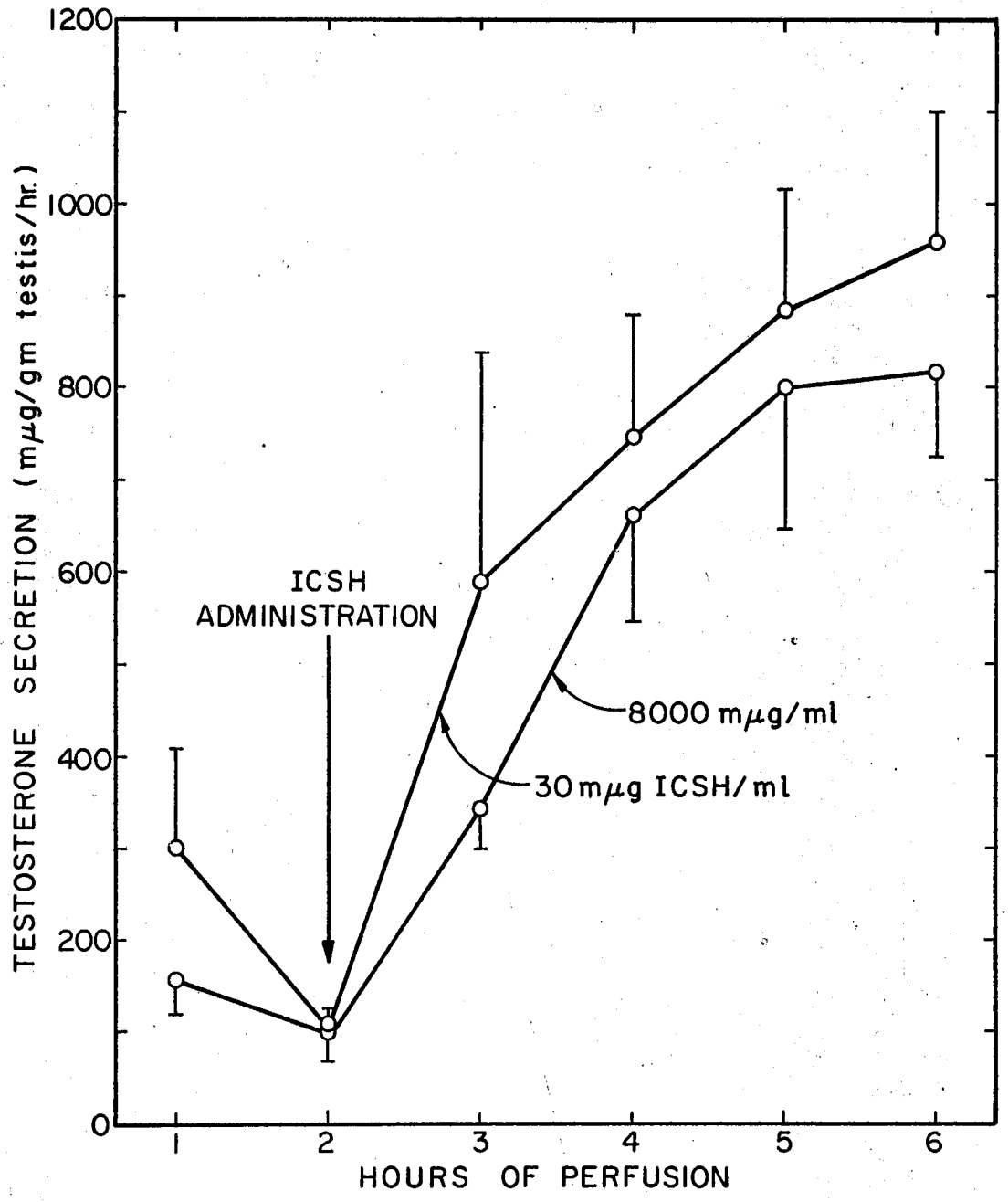

Figure 11. Testosterone Secretion Rate of Perfused Rabbit Testes in Response to Two Different Saturating Levels of ICSH. I = SEM.

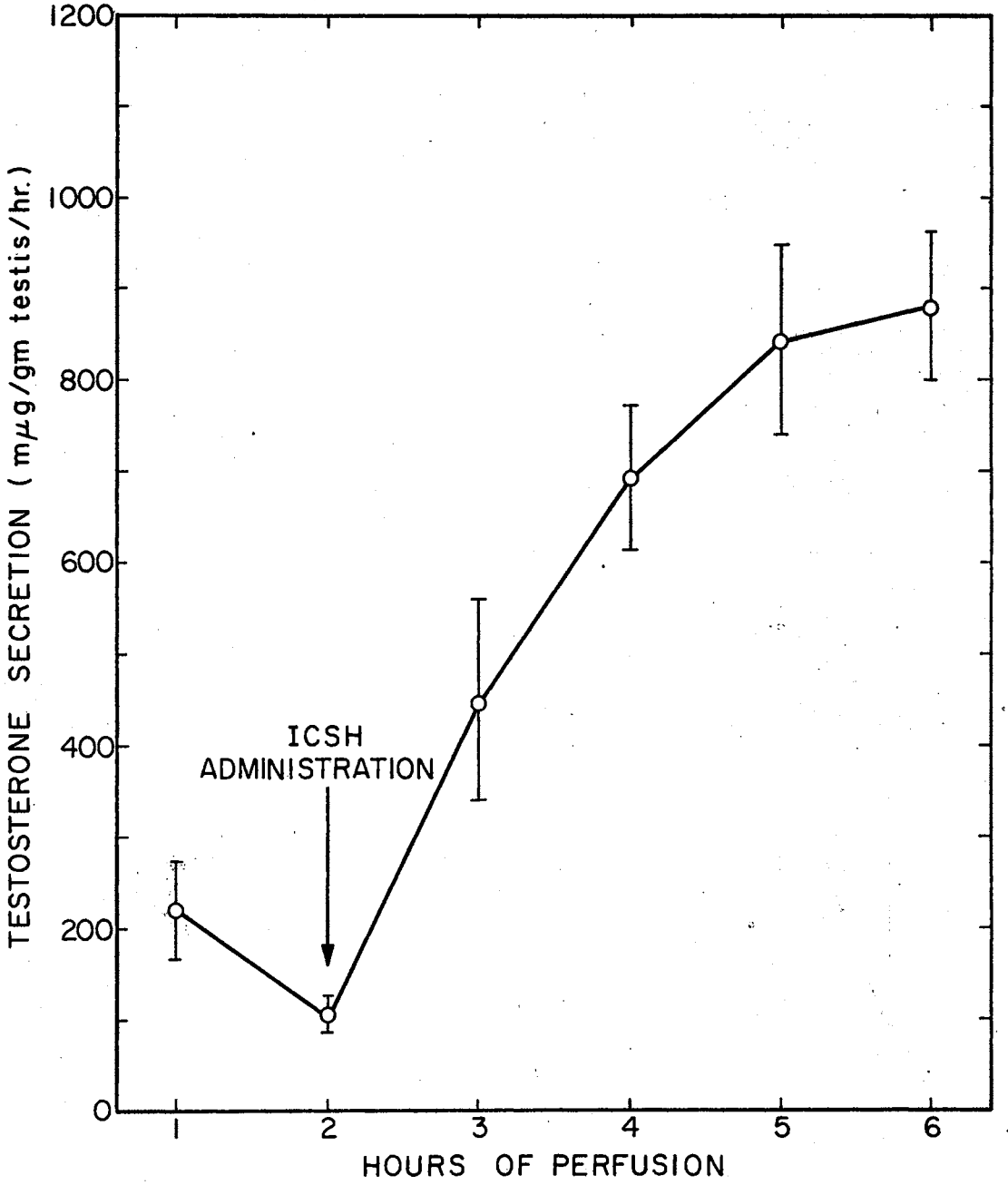

Figure 12. Mean Testosterone Secretion Rate of Perfused Rabbit Testes Stimulated With 30 or 8,000 mμg ICSH/ml of Artificial Medium. I = SEM.

Effect of 5 mμg ICSH/ml of Artificial Medium Combined With 10 mμg GH/ml of Artificial Medium Upon TSR by Perfused Rabbit Testes

The purpose of this experiment was to test for ICSH-GH synergism at "physiologic" levels of both hormones. Testes were perfused with 5 mμg ICSH/ml of medium plus 10 mμg GH/ml of medium. Testes stimulated with the hormone combination responded similarly to those receiving 5 mμg ICSH/ml of medium alone (Figure 13). Synergism was not apparent.

Effect of Saturating Levels of ICSH (100 mμg/ml of Artificial Medium) Combined With 200 mμg GH/ml of Artificial Medium Upon TSR by Perfused Rabbit Testes

The purpose of this experiment was to evaluate the TSR response of perfused testes stimulated with GH and saturating levels of ICSH. The ICSH:GH ratio was held at 1:2 as in the previous experiment. Thus, the concentration of both hormones was 20 times greater than "physiologic" concentrations and presumably, hormone binding sites would be fully occupied and the TSR response maximal.

Because the amount of testosterone secreted by the *in vitro* perfused testis was similar at 30 and 8,000 mμg ICSH an intermediate value of 100 mμg ICSH/ml of artificial medium was selected arbitrarily as the saturating dose to use in combination with 200 mμg growth hormone/ml for the synergism test. The testosterone secretion rate of testes stimulated after the second hour of perfusion with 100 mμg ICSH and 200 mμg GH/ml of artificial medium was similar to that of testes stimulated with saturating levels of ICSH alone. The response curve was almost parallel with but slightly below that of the saturating ICSH curve (Figure 14).

Two hundred millimicrograms of GH/ml of artificial medium contained

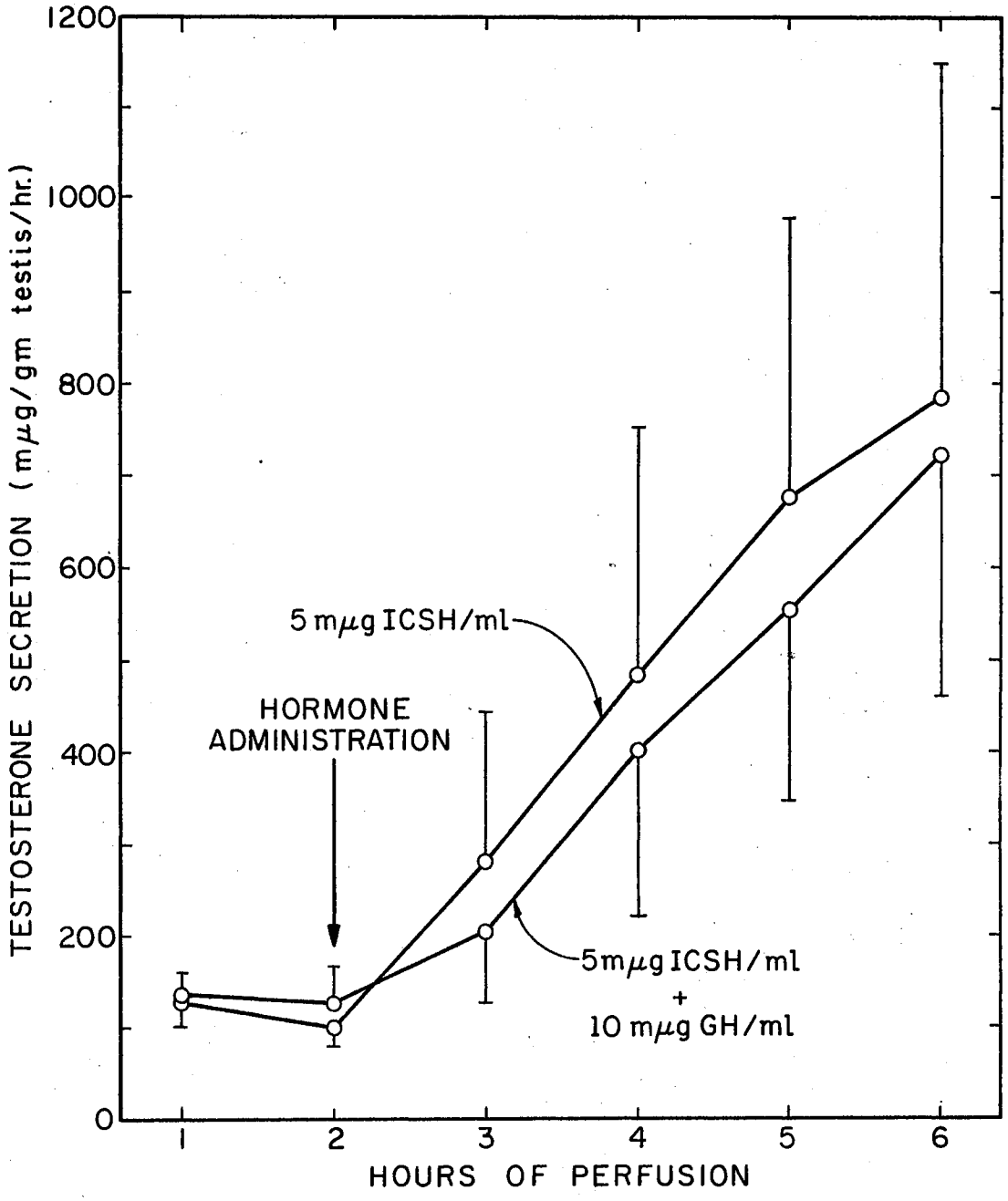

Figure 13. Testosterone Secretion Rate of Perfused Rabbit Testes in Response to ICSH or to ICSH + GH. I = SEM.

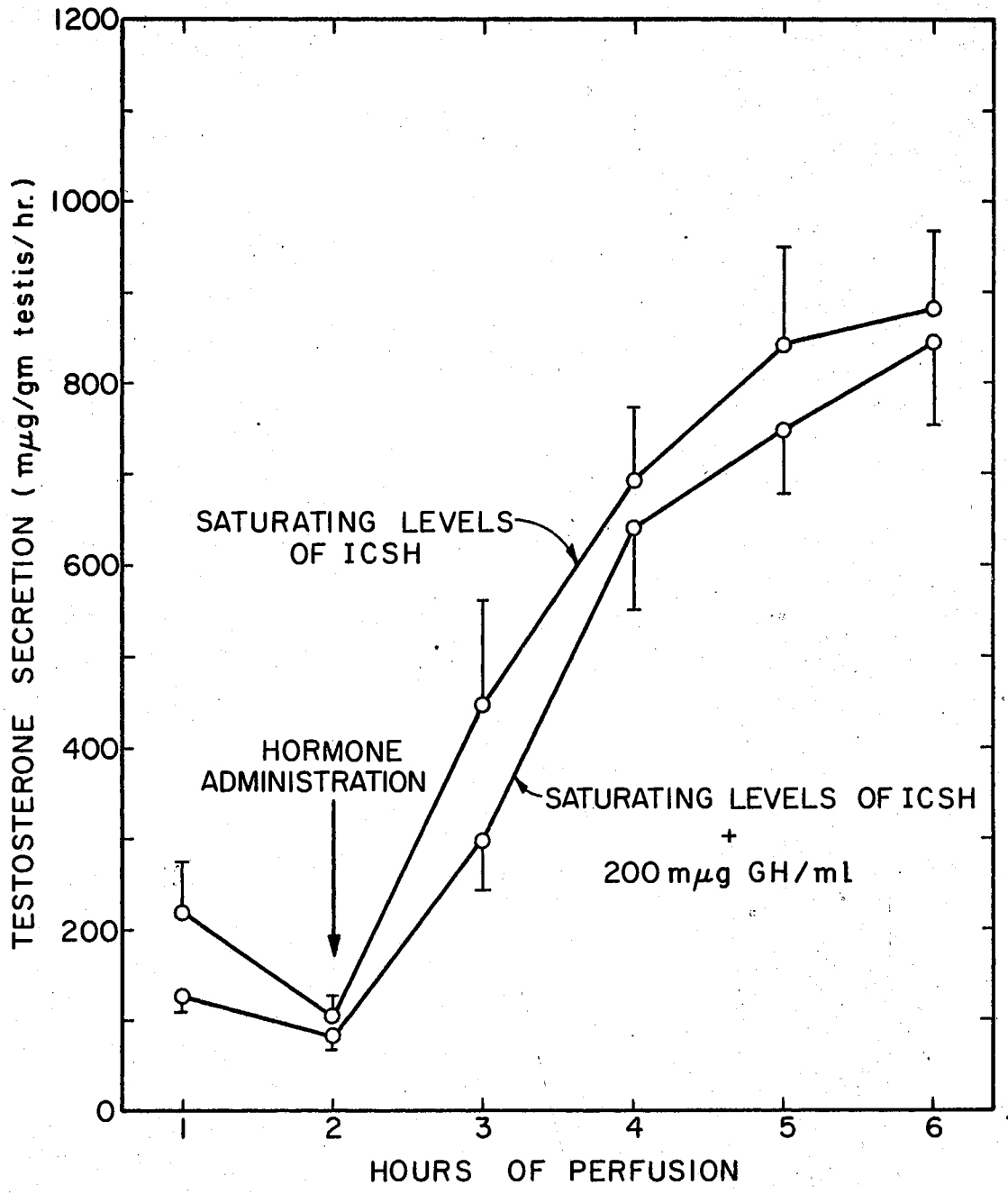

Figure 14. Testosterone Secretion Rate of Perfused Rabbit Testes in Response to a Saturating Level of ICSH Alone (100 mμg/ml of Artificial Medium) or in Combination With 200 mμg Growth Hormone/ml of Artificial Medium. I = SEM.

sufficient contaminating ICSH (5 mμg/ml) to stimulate testosterone secretion in the absence of other sources of ICSH. However, with binding sites fully occupied by ICSH and maximal stimulation already occurring in response to 100 mμg ICSH/ml it was reasoned that the influence of the additional ICSH contained in the GH preparation was not significant. It was concluded that growth hormone did not augment the secretion of testosterone from testes maximally stimulated with ICSH.

In summary, rabbit testes perfused in vitro with either artificial medium alone or with artificial medium containing 10 mμg GH/ml of medium, secreted testosterone at a basal level of approximately 100 mμg/gm of testis/hour. Perfused testes stimulated with either low "physiologic" levels or high "saturating" levels of ICSH responded by increasing TSR eight to ten-fold. Neither "physiologic" nor "saturating" levels of ICSH combined with GH at a ratio of 1:2 increased TSR above the rates seen with ICSH alone. It was concluded that GH does not synergise with ICSH to augment the secretion of testosterone from in vitro perfused rabbit testes.

CHAPTER IV

DISCUSSION

Metabolic clearance rate, defined as the volume of plasma completely cleared of a substance in unit time, can be used to characterize the overall metabolism of testosterone. The metabolic clearance rate can be calculated from the disappearance curve in plasma of labeled testosterone following a single injection of the isotope into the circulation. Since the amount of testosterone leaving the plasma in the steady state is equal to the amount entering, the product of the metabolic clearance rate and the concentration of testosterone in plasma is equal to the production rate of testosterone. Thus, testosterone production rate is defined as the quantity of testosterone entering the blood from all sources in unit time and is composed of testosterone that is secreted from endocrine organs in addition to that originating from other steroids by peripheral conversion. In men, less than 5% of the total body testosterone is derived from extra-gonadal sources (183).

With the MCR defined as volume of blood cleared completely and irreversibly of testosterone in unit time, the blood flow through a hypothetic metabolic organ (in this instance, the entire rabbit) which completely and exclusively extracts the testosterone is under consideration. Let the concentration of steroid in the afferent supply of such an organ be equal to i. Then the concentration in the efferent supply equals zero. By application of the Fick Principle, MCR x i = the rate

of irreversible clearance of steroid, and in the steady state this will equal the rate of steroid irreversibly entering the circulation from all sources, or the production rate. The mean MCR for the rabbits in this study was 134.2 ℓ/day (Table III). When this value is adjusted on a body weight basis it becomes 16.4 ℓ/lb/day, a value that is similar to the value of 13.6 ℓ/lb/day reported for Hereford bulls by Rhynes (229), but much higher than the value reported for man by Horton, et al. (227). Assuming body weights of 150 lbs, the mean MCR for five normal male subjects in their study was 6.4 ℓ/lb/day.

The mean plasma testosterone concentration for the five rabbits was 0.00312 mg/ℓ (3.12 mμg/ml). This value is very close to the 3.26 mμg/ml level reported for Hereford bulls and similar to the values in other animals (rat, dog, monkey, man) that vary from 3-10 mμg/ml (229). The mean testosterone production rate (MCR x plasma testosterone concentration) in the rabbit was determined to be 0.406 mg/24 hours (Table III). Rhynes (229) reported a value of 44.26 mg/24 hours for the bull and Horton, et al. (227) reported 6.5 mg/24 hours for the human male. Assuming weights of 1,000 lb for bulls and 150 lb for men, the production rates of testosterone are remarkably similar -- rabbit, 2.05 μg/lb/hour; bull, 1.86 μg/lb/hour; and man, 1.80 μg/lb/hour. This indicates that the production rate of testosterone is closely related to body weight and may mean that protein anabolic effects of the steroid are of primary importance to the organism, e.g., the distinctive skeletal muscle mass characteristic of maleness may require much larger amounts of testosterone for maintenance than does the testis for spermatogenesis or the brain for male behavior patterns.

The data derived from the testosterone production rate study

allowed the determination of additional parameters that serve to illustrate the dynamic behavior of testosterone in the rabbit. As transport and metabolic activities of testosterone have not been documented for this species, a discussion of their significance is warranted. There was an initial rapid distribution of the injected testosterone -1,2-^{3}H into a space of 1048 ml (V_1) and then a more gradual spread of radioactivity during the course of about 30 minutes into a calculated total volume of distribution ($V_1 + V_2$) of 2,258 ml. This indicated an outer pool volume of distribution (V_2) of 1,210 ml. Fifty-four percent (K_1) of the injected isotope was transported to the outer pool leaving 46% (K_2) in the inner pool to be metabolized. The rapid metabolism of testosterone in the inner pool was reflected in the short half-life (4.67 min). Testosterone in the outer pool had a half-life of 20.75 min and as metabolism in the outer pool is considered to be negligible, the disappearance of testosterone from that pool probably represents transport back into the inner pool. The half-life for testosterone in the combined pools ($V_1 + V_2$) was 7.82 min and as this T 1/2 is much closer to T 1/2 in the inner pool (V_2) it serves to illustrate that metabolic activity in V_1 had a much greater influence on the disappearance of the steroid than did transport in V_2.

Total body testosterone is calculated by multiplying the total volume of distribution ($V_1 + V_2$) times plasma concentration. Noteworthy in this regard is that both the total volume of distribution and plasma concentration of testosterone varied widely among the five rabbits in this study (Tables III and IV). For individual rabbits these two parameters tended to be related inversely and these relationships permitted the calculation of total body testosterone values that were

remarkably similar for four of the rabbits (Table IV). Thus, there is an indication that plasma testosterone values are dependent upon the total volume of distribution of testosterone. Expressed another way, it might indicate that the rabbit contains a rather constant amount of total testosterone and that shifts in distribution between the various compartments could be responsible for the differences observed in plasma testosterone levels of these rabbits. It also seems reasonable to suspect that changes in compartment size may influence dynamic temporal changes in plasma testosterone levels of individual animals as recorded for men (228) and bulls (229). As the plasma volume of the rabbit is contained in a space of approximately 100 ml as opposed to a total distribution space ($V_1 + V_2$) of 2,200 ml then it is apparent that small changes in the total space would result in large changes in the plasma concentration of testosterone.

Assuming that 95% of the total testosterone produced by the rabbit arises in the testis as reported for man by Paulsen (183), and further that the mean weights of testes of the rabbits used in the in vivo study were equal to those of the perfused testes (2.4 gm), then the calculated mean secretion rate of the in vivo testes is 3,333 mμg/gm of testis/hour. The addition of 5 mμg ICSH/ml of artificial medium after two hours of perfusion resulted in a prompt increase in TSR and during the last hour of perfusion the mean TSR was approximately 800 mμg/gm of testis/hour (Figure 10). The 5 mμg ICSH dose level was significant for it is very similar to ICSH values that have been reported for male rabbits, e.g., Desjardins and Ewing reported that plasma levels of ICSH in normal intact New Zealand White male rabbits was 4.5 mμg/ml (153). Johnson and Ewing (48) have reported an approximate two-fold increase

in testosterone secretion from ICSH stimulated testes that were treated with FSH. By extrapolation, it appears then, that FSH combined with physiologic levels of ICSH (5 mμg/ml) would have increased the TSR from 800 mμg to 1,600 mμg/gm of testis/hour. Comparing this value with the in vivo value of 3,333 mμg/gm of testis/hour, it is obvious that there may be additional hormonal factors involved in the regulation of testosterone secretion.

Hormones that have been reported to increase testosterone secretion by synergising with the gonadotrophins are growth hormone (40,42,66,67) and prolactin (40,58,110,150,151). Other hormones that may increase testosterone secretion by indirect mechanisms are: (1) adrenal cortical hormones that may [a] inhibit ACTH secretion by the anterior pituitary and thus result in increased secretion of ICSH (84,118,119,139), [b] be transformed into testosterone within the testis (140), or [c] compete with testosterone for hypothalamic binding sites (136) and thus raise the threshold for testosterone feedback resulting in increased testosterone secretion by the testis; (2) insulin, through anabolic effects at the testicular level that may result in increased utilization of glucose (1), increased conversion of glucose to steroids (131), or increased rate of enzyme protein synthesis (133). Another avenue by which insulin may exert an effect is at the gonadotrophin level by synergism, for Lockwood, et al. (132) have shown that insulin, hydrocortisone and prolactin act synergistically to stimulate the synthesis of milk proteins by the pregnant mouse mammary gland. Based on the foregoing evidence it was concluded that growth hormone, prolactin, adrenal cortical hormones and insulin may all act to promote testosterone secretion by the rabbit testis.

Of the four hormones, growth hormone appeared to hold the most promise as an ICSH synergist in the in vitro perfusion system because of: (1) reports suggesting that its mechanism of action was independent of extra-gonadal substances which would be absent from the perfusion system; (2) its reputation as a biological synergist in other organ systems; and (3) specific reports of ICSH-GH synergism in augmentation of testicular testosterone secretion (40,42,66,67). Thus growth hormone was selected as the hormone that would most likely make the greatest contribution toward ameliorating the disparity in testosterone secretion between the in vitro and in vivo systems.

The validity of the perfusion apparatus as a test system was substantiated by: (1) the isolation of testosterone heptafluorobutyrate from venous effluent as confirmed by mass spectrometric studies that revealed that the isolated steroid had the same molecular weight and a similar fragmentation pattern as authentic testosterone heptafluorobutyrate (Figure 4); and (2) the successful demonstration of FSH-ICSH synergism in augmenting testicular testosterone secretion in the same system by Johnson and Ewing (48). The accuracy of specific perfusion procedures was confirmed by demonstrating that: (1) testosterone secretion rate was not influenced by order of testis removal from the rabbits after the induction of anesthesia, thus testes remaining in situ for 20 minutes before removal responded similarly to testes removed 15 minutes earlier (Table VI in Appendix); (2) testosterone secretion rate was not influenced by origin of the testis (left vs. right) and thus either testis could be used without bias (Figure 15 in Appendix); (3) testosterone secretion rate was not influenced by testis weight (Figure 17 in Appendix) or artificial medium flow rate (Figure 18 in

Appendix), nor was artificial medium flow rate influenced by testis weight (Figure 16 in Appendix). Therefore, these common experimental variables were discounted in the interpretation of data.

Elucidation of the role of GH in testosterone secretion has been hindered by the presence of ICSH contaminants in GH preparations. This problem was circumvented in two ways: first, experiments were conducted with GH concentrations (non-stimulating) that failed to stimulate testosterone secretion when administered alone; secondly, experiments were conducted in which GH was tested for synergism in the presence of ICSH concentrations (saturating) that produced maximum testosterone secretion in the perfused testis.

It seems clear that 10 mμg GH/ml of artificial medium does not stimulate testosterone secretion for the secretion curve seen with that level was almost identical to that of testes perfused with the artificial medium alone (Figures 7 and 9). The effect of larger doses appears to be positive (Figure 8) until it is realized that contaminating ICSH is present at levels which alone can stimulate testosterone secretion, i.e., with a 200 mμg dose of GH, 5 mμg of contaminating ICSH is present (based on NIH assay report that accompanied the GH preparation), an amount which promoted marked testosterone secretion in this study (Figure 10). The 10 mμg GH dose level was selected because it did not stimulate testosterone secretion and in addition because it approaches the concentration found in plasma of several species, e.g., Irvin and Trenkle (205) reported recently that the plasma GH level in yearling male beef cattle was 12.9 mμg/ml and Machlin, et al. (206) reported that the basal level of GH in the pig was 5.8 mμg/ml plasma. GH levels of 5 to 10 mμg/ml of plasma have been reported for fasting conscious

adolescent male baboons (155) and mean peak values of 11.8 mμg/ml plasma were recorded for 10 adult men (156).

Low levels of ICSH (5 mμg/ml) in the perfusion media resulted in an eight-fold increase in testosterone secretion. The responsiveness of the testis to ICSH was reflected in the increased testosterone concentration seen in the sample collected one hour after ICSH was added to the perfusion media; thus, the onset of increased secretion must have occurred very soon after ICSH stimulation. The response to this ICSH level was significant for it appears that 5 mμg ICSH/ml of blood may be well within the physiologic range of several species. Monroe, et al. (216) reported that sera from adult male rats had ICSH levels that varied from less than 12.0 up to 22.5 mμg/ml while Gay and Midgley (219) observed serum levels in adult male rats that were usually less than 5.0 mμg/ml. Niswender, et al. (217) reported ICSH values of 2.0 mμg/ml of plasma in intact male sheep and Hartree (218) reported 2.3 mμg ICSH/ml of plasma for normal human males. Of special importance is the recent report of Desjardins and Ewing (153) who observed ICSH concentration to be 4.5 mμg/ml of plasma in male rabbits.

The response of the testes to 30 mμg ICSH/ml was similar to the response seen with 8,000 mμg ICSH/ml (Figure 11). The similarity of responses demonstrated that saturation of ICSH cellular binding sites occurred at the 30 mμg/ml level. One hundred millimicrograms ICSH/ml was selected arbitrarily as the saturating level to be employed in the synergism test. By combining 200 mμg GH with 100 mμg ICSH/ml of perfusion media the same 2:1 ratio that was used in the low level synergism test was preserved. Thus the cellular sites within the testes were presented the same qualitative combination of hormones; difference in quantity was

the only variation.

Growth hormone failed to augment the secretion of testosterone from perfused rabbit testes stimulated with either low or high levels of ICSH (Figures 13 and 14). Rather than augmentation there appeared to be a tendency for GH to inhibit the secretion of testosterone. In view of the literature (40,42,66,67) that has reported an ICSH-GH synergism in vivo and the original hypothesis put forth for this study, e.g., that GH synergises with ICSH to augment the secretion of testosterone from perfused rabbit testes, it becomes necessary to explain the failure to confirm the hypothesis. The discussion will be divided into two parts. First to be considered will be possible reasons for the failure to demonstrate synergism and second, evidence will be presented to substantiate the experimental results obtained, e.g., GH does not synergise with ICSH to augment the secretion of testosterone from the testis.

Possible Reasons for Failure to Demonstrate Synergism

Of necessity, this discussion will be speculative. At the outset it must be established that growth hormone is a normal constituent of the rabbit pituitary gland and that ovine GH is biologically active in the rabbit. Solomon and Greep (207) reported that crude extracts of whole pituitaries obtained from rabbits had significant growth hormone potencies when measured by the tibia test assay in hypophysectomized rats. Thus, it is concluded that rabbit testes in vivo are exposed to potent growth hormone in circulating blood. The evidence for biological activity of ovine GH in rabbits is indirect. A survey of literature failed to disclose reports of specific usage of ovine GH in rabbits, however, three reports were found that alluded to positive biological

effects of GH (species origin of the hormone was not identified) in the rabbit, e.g., Milman (157) reported that GH causes the development of glycosuria in young rabbits; Abelove and Paschkis (159) reported that Wilson Laboratories' GH produced hyperglycemia, traces of glycosuria and slight weight gain in rabbits and finally, Meites (158) reported that rabbits receiving prolactin alone showed an intense lactation but when Armour GH was added to the prolactin treatment there was a marked inhibition of lactation. Presumably, the Wilson and Armour GH preparations were obtained at slaughter from either bovine or ovine pituitary glands. Bovine and ovine growth hormones are antigenically similar (160) and by inference their biological activities are similar. The foregoing evidence is admittedly indirect, but taken together the reports suggest that ovine GH is capable of exercising biological activity in the rabbit and it is believed that failure to demonstrate synergism between ICSH and GH of ovine origin in perfused rabbit testes was not due to species specificity of the GH preparation.

Although ovine GH is presumably biologically active in the rabbit, the possibility seems to exist that there might be some physiological peculiarity that would preclude a specific synergism between GH and ICSH. For instance, Lostroh, et al. (66) reported that GH has no influence on the prostatic response of hypophysectomized Long-Evans rats to ICSH, whereas a significant enhancement in response was seen in hypophysectomized Sprague-Dawley rats.

Does ICSH-GH synergism require an intermediate that is present in vivo but absent in the perfusion system? Fell (208) reported that growth hormone does not have a direct effect on chick skeletal rudiments in in vitro culture and suggested that the hormone may produce its

effect on the skeleton in vivo by indirect means. A similar dichotomy between the in vivo and in vitro action of GH on an isolated system has been described in relation to the production of aldosterone by adrenal tissue in vitro. Venning and Lucis (161) found that treatment of hypophysectomized rats with monkey GH increased the production of aldosterone by their adrenals in vitro, without significantly influencing the secretion of corticosterone. The plasma of hypophysectomized rats treated with GH, when added to the incubation medium, had a similar effect on the adrenals of both normal and hypophysectomized animals, but the addition of various growth hormone preparations in vitro was ineffective. These observations suggested to these investigators that GH may stimulate the secretion of an "aldosterone-stimulating hormone", perhaps by the kidney. This explanation is compatible with the suggestion put forth above for failure to demonstrate ICHS-GH synergism in this study, e.g., perhaps an intermediate hormone or other substance present in vivo but absent in the perfusion system is necessary for the manifestation of synergism. This concept is in keeping with the observations of Lukens and McCann (209) who reported that studies of hypophysectomized-depancreatized cats suggested that the presence of insulin was essential for protein anabolism and that an increased secretion of insulin was needed if maximal response to administered GH was to be obtained. In view of these findings one might reason that the presence of insulin in the artificial medium perfusate would increase the effectiveness of GH in the perfused rabbit testes.

Other possible essential factors that would be present in vivo, but perhaps absent, or available only in limited quantities in the perfusion system would be numerous vitamins. McHenry (210) reported that

GH increased the load on enzyme systems for which various vitamins were needed as co-enzymes and Houssay (211) observed in hypophysectomized rats that folic acid and vitamin B_{12} enhanced the action of GH on body weight and on the weight of liver, kidney and many other organs.

Is it possible that the effects of GH on carbohydrate metabolism in the perfused testis may have interfered with testosterone secretion? Goodman (212) reported that 3 hours after the intravenous injection of 100 µg of GH to hypophysectomized rats there was a diminution in glucose uptake by diaphragm muscle in vitro. There was no evidence of increased mobilization of free fatty acids from adipose tissue of the same animals. In light of this observation and the report of Ewing and Eik-Nes (221) that glucose uptake by the perfused rabbit testis is related positively to testosterone secretion, it appears that a mechanism may exist in the perfusion system that would explain an inhibition of testosterone secretion by GH.

It seems plausible that a different effect could have resulted from a sequential addition of hormones to the perfusion medium. Best (213) reported that in studies with depancreatized dogs, where protein anabolic effects of GH and insulin were determined, that a certain amount of residual insulin was necessary for demonstrating the protein anabolic effect of GH. When GH was added to the dose of insulin, i.e., the two given together, there was no augmentation of the effect produced by either substance alone, whereas when GH followed insulin an augmentation was observed. In the perfusion experiments, the addition of GH after ICSH may have been a more logical method for stimulating Leydig cells.

Finally, inspection of Figures 13 and 14 discloses that the curves representing ICHS-GH combinations had a tendency to continue upward

whereas the ICSH curves exhibited a greater tendency to plateau. Thus, the possibility exists that more than 4 hours exposure of testes to ICSH-GH combinations is necessary for the manifestation of synergism.

Evidence Against ICSH-GH Synergism

The foregoing discussion has been built around the premise that ICSH and GH synergise in vivo to stimulate the Leydig cells to increase their secretion of testosterone. It is pertinent at this point to cite evidence for a mechanism that would allow for increased androgen output in vivo in response to GH in the absence of additional stimulation of Leydig cells. Lostroh and Li (65) reported that GH (treated with chymotrypsin to digest contaminating ACTH) promoted a significant increase in adrenal weight of the hypophysectomized, castrated male rat and further that 17-β-OH-progesterone, 11-desoxy-17-OH-corticosterone and 11-dehydro-corticosterone, all products of the adrenal cortex, exerted androgenic effects on the ventral prostate. Moreover, the authors reported that GH was involved in the restoration of sex accessories that had undergone post-hypophysectomy atrophy. The hormone manifested its action histologically in a general improvement in the secretory epithelium and connective tissue stroma of the accessory structures. These effects are consistent with the observation of Li (214) who reported that it was reasonable to assume that GH created the necessary and sufficient environment for other biological agents to exercise the full scope of their functions. Thus it seems reasonable to speculate that the increased androgenic activity reported for ICSH-GH administration in vivo could conceivably have been brought about by the dual effects of GH on the adrenal gland and the sex accessories.

A critical evaluation of the literature reports (40,42,66,67) that have supported the premise that GH synergises with ICSH in vivo to augment the secretion of testosterone from Leydig cells discloses that in each instance the basis for synergism was stimulation of growth in accessory sex structures. It seems clear that the synergism described may have been between GH and testosterone rather than between GH and ICSH. That synergism may exist between GH and testosterone has been demonstrated by several investigators (60,63,64).

A recent report has provided strong evidence that argues against a synergism between GH and ICSH at the Leydig cell level. DeKretser, et al. (215) injected twenty day old rats intraperitoneally with either human ICSH (10 μg) or human growth hormone (10 μg) labeled with ^{125}I. The localization of these hormones 1-2 hours after injection was examined under the light microscope after autoradiography. Major sites of localization of labeled ICSH were the interstitial cells of the testis and the proximal convoluted tubules of the kidney. Some hormone was also present in adipose tissue, hepatic parenchymal cells, the mesothelial lining of the peritoneum and underlying macrophages. ICSH was not localized in seminiferous tubules. Growth hormone was localized principally in the proximal convoluted tubules of the kidney with some hormone present in liver, adipose tissue and the adrenal cortex. There was no evidence of GH localization in the adrenal medulla or the testis. The presence of GH in the adrenal cortex is consistent with the observation of Lostroh and Li (65) discussed previously. The presence of ICSH in Leydig cells and the absence of GH in the same cells provides further evidence that ICSH-GH synergism does not occur at that site.

Strong evidence against ICSH-GH synergism has been provided by the

perfusion experiments reported here. That the system is fully capable of demonstrating synergism was illustrated by the FSH-ICSH synergism experiments of Johnson and Ewing (48). Inherent within the system was the capacity to test for direct effects of ICSH and GH on Leydig cells, a characteristic absent in in vivo experiments that were dependent upon indirect evidence for synergism.

In summary, the arguments against ICSH-GH synergism are: (1) evidence that the reported ICSH-GH synergism in in vivo experiments could have been due to GH-testosterone synergism and/or an additional production of androgenic steroids from the adrenal gland in response to GH; (2) the failure to demonstrate synergism in a proven in vitro system that permitted specific measurement of direct effects of GH and ICSH on Leydig cells; and (3) radioautographic evidence that GH does not localize in the testis. Finally, while the aforementioned speculative reasons for the failure to demonstrate ICSH-GH synergism cannot be disregarded, it appears that the evidence against the synergism is far stronger.

The failure to demonstrate ICSH-GH synergism in this study strengthens the argument for an ICSH-prolactin synergism in the regulation of testosterone secretion. In that regard it is interesting to note that ICSH alone in studies with hypophysectomized rats by Hafiez, et al. (110) elevated plasma testosterone levels to a level which was one-fourth of that observed in intact male rats (1.42 and 5.34 mμg/ml). This response to ICSH agrees exactly with that seen in this study when the testosterone secretion rate of ICSH stimulated perfused rabbit testes is compared with that of in vivo rabbit testes (792 and 3,333 mμg/gm/hour). The addition of prolactin to ICSH treatment resulted in

a three-fold elevation of plasma testosterone levels in hypophysectomized rats (110), while the addition of FSH to ICSH treatment resulted in a two-fold increase in testosterone secretion rate of perfused rabbit testes (48). Taken together the results of the rat and rabbit experiments suggest that the addition of FSH to prolactin-ICSH combinations or of prolactin to FSH-ICSH combinations would result in increased testosterone secretion from the testes of both species. Thus it seems clear that a complex of hormones regulate testosterone secretion and while the influences of insulin and adrenal cortical hormones deserve investigation it would appear that prolactin may exert a major effect in combination with FSH and ICSH and should be investigated first. The advantages of the perfusion system in testing for direct effects of hormones on the testis have been alluded to previously and it should prove to be especially valuable in sorting out the effects of hormones singly or in combination. Finally, while this study failed to demonstrate a specific ICSH-GH synergism in augmenting testosterone secretion, it seems possible that GH may increase testosterone secretion by either synergising with other hormones or by promoting the secretion of an intermediate hormone. It deserves further investigation with various hormone combinations and especially as a component of a complex of hormones that includes ICSH, FSH, prolactin, insulin and cortisol.

CHAPTER V

SUMMARY AND CONCLUSIONS

The specific objectives of this study were to answer the following questions: (1) What is the in vivo testosterone production rate of the rabbit? (2) Does GH synergise with ICSH to augment the secretion of testosterone from the perfused rabbit testis?

Metabolic clearance rate (MCR) of testosterone was determined in five young sexually mature New Zealand White male rabbits by monitoring the disappearance from plasma of a single intracardial injection of tritium labeled testosterone. Plasma levels of endogenous testosterone were determined by gas liquid chromatography. Twenty-four hour production rate, half-life, metabolic constants, transport constants and volumes of distribution were calculated for testosterone.

The mean MCR for testosterone in this study was 134.2 ℓ/24 hours. The mean plasma concentration of testosterone was 3.12 mμg/ml. Twenty-four hour production rate of testosterone was calculated to be 0.406 mg/24 hours. Fifty-four percent of the tritium labeled testosterone dose was transported to an outer theoretical compartment of 1,210 ml and the remainder was metabolized in an inner theoretical compartment of 1,048 ml. The half-life of testosterone in the outer compartment was 20.75 minutes and in the inner compartment was 4.67 minutes. Mean total body concentration of non-isotopic testosterone was calculated to be 774 mμg/lb of body weight.

To test whether GH synergises with ICSH to augment the secretion of testosterone from Leydig cells, 41 testes from young sexually mature New Zealand White rabbits were perfused with an artificial medium for 6 1/2 hours in an *in vitro* perfusion system that was maintained in a walk-in constant temperature room heated to 36.5°C. Experimental treatments were imposed by adding to the artificial medium: (1) nothing; (2) 10 mμg GH/ml; (3) 5 mμg ICSH/ml; (4) 30 mμg ICSH/ml; (5) 8,000 mμg ICSH/ml; (6) 10 mμg GH/ml + 5 mμg ICSH/ml; or (7) 200 mμg GH/ml + 100 mμg ICSH/ml. Testosterone levels in hourly venous effluent samples were determined by gas liquid chromatography. Artificial medium flow rate through the testes was determined each hour. Testis weights were obtained at the conclusion of each experiment.

The results of these studies showed that perfused testes responded promptly to either low or high levels of ICSH by increasing testosterone secretion approximately eight-fold. The addition of GH to artificial medium containing either low or high levels of ICSH did not augment testosterone secretion. Although other investigators (40,42,66,67) reported ICHS-GH synergism *in vivo*, arguments developed against it as a result of this study and a review of pertinent references include: (1) failure of *in vivo* experiments to confine the action of GH to the testis; (2) evidence that the reported synergism could have been due to the action of GH on the adrenal gland (65); (3) evidence that the reported synergism could have been due to GH-testosterone synergism (60,63,64); (4) radioautographic evidence that GH does not localize in the testis (215); and finally (5) failure to demonstrate ICSH-GH synergism in the perfusion experiments reported here where specific measurements of direct effects of GH + ICSH on the isolated testis were

possible.

A comparison between *in vitro* and *in vivo* testosterone secretion rates disclosed that perfused testes stimulated by ICSH secreted testosterone at approximately one-fourth the *in vivo* rate. This disparity was interpreted to mean that factors other than ICSH are needed for optimal testosterone secretion by the testis.

Final conclusions based on the study and a selected literature review are: (1) the testosterone production rate for young sexually mature New Zealand White male rabbits is 0.406 mg/24 hours; and (2) GH does not synergise with ICSH to augment testosterone secretion from the perfused rabbit testis and moreover the evidence seems strong that ICSH-GH synergism does not occur *in vivo*.

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APPENDIX

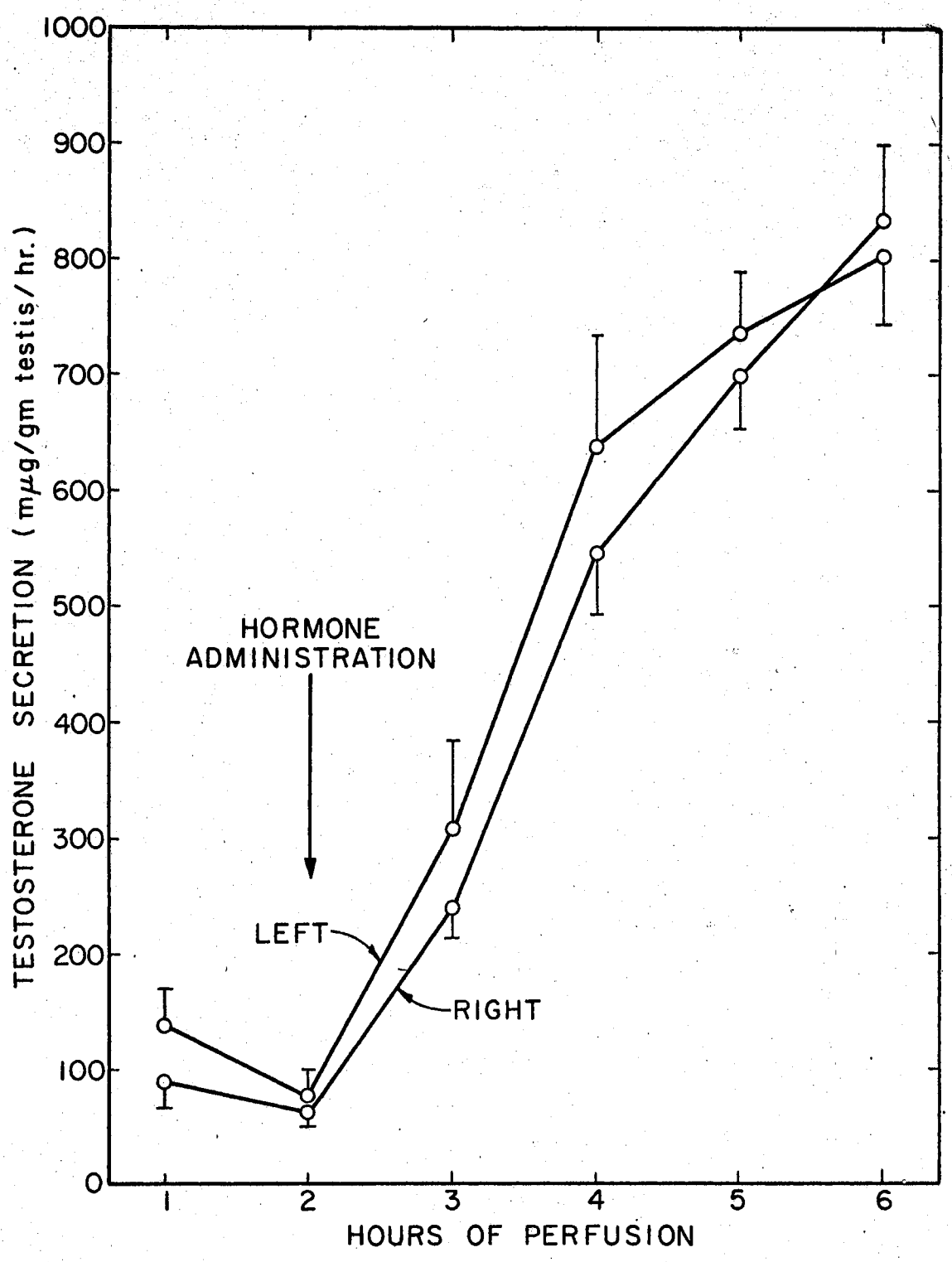

Figure 15. Response of Perfused Paired Rabbit Testes to Same Treatment. Each line represents 3 testes; treatment was 100 mμg ICSH/ml of artificial medium plus 200 mμg growth hormone/ml of artificial medium. I = SEM.

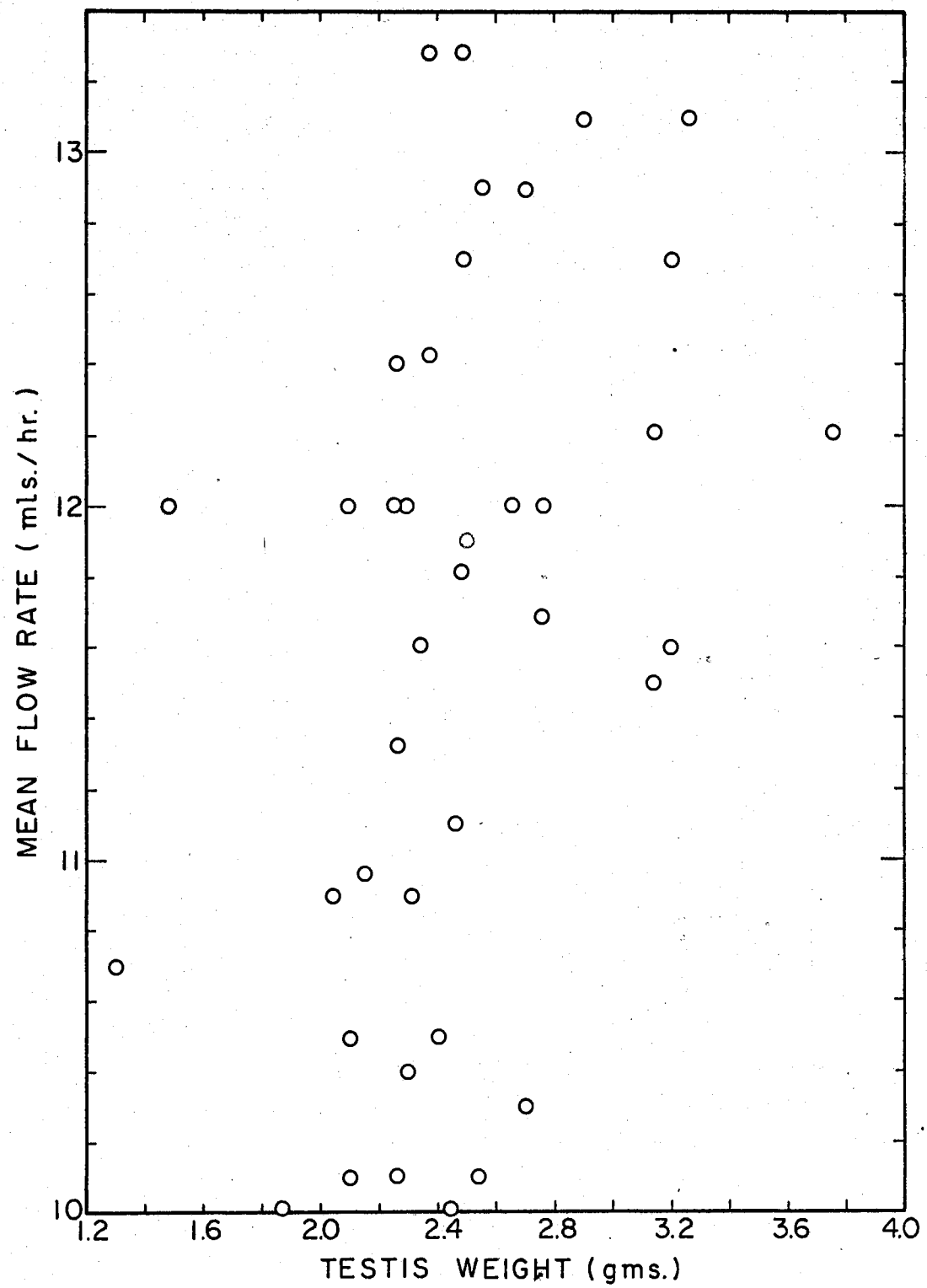

Figure 16. Correlation Between Testis Weight and Mean Artificial Medium Flow Rate of Perfused Rabbit Testes

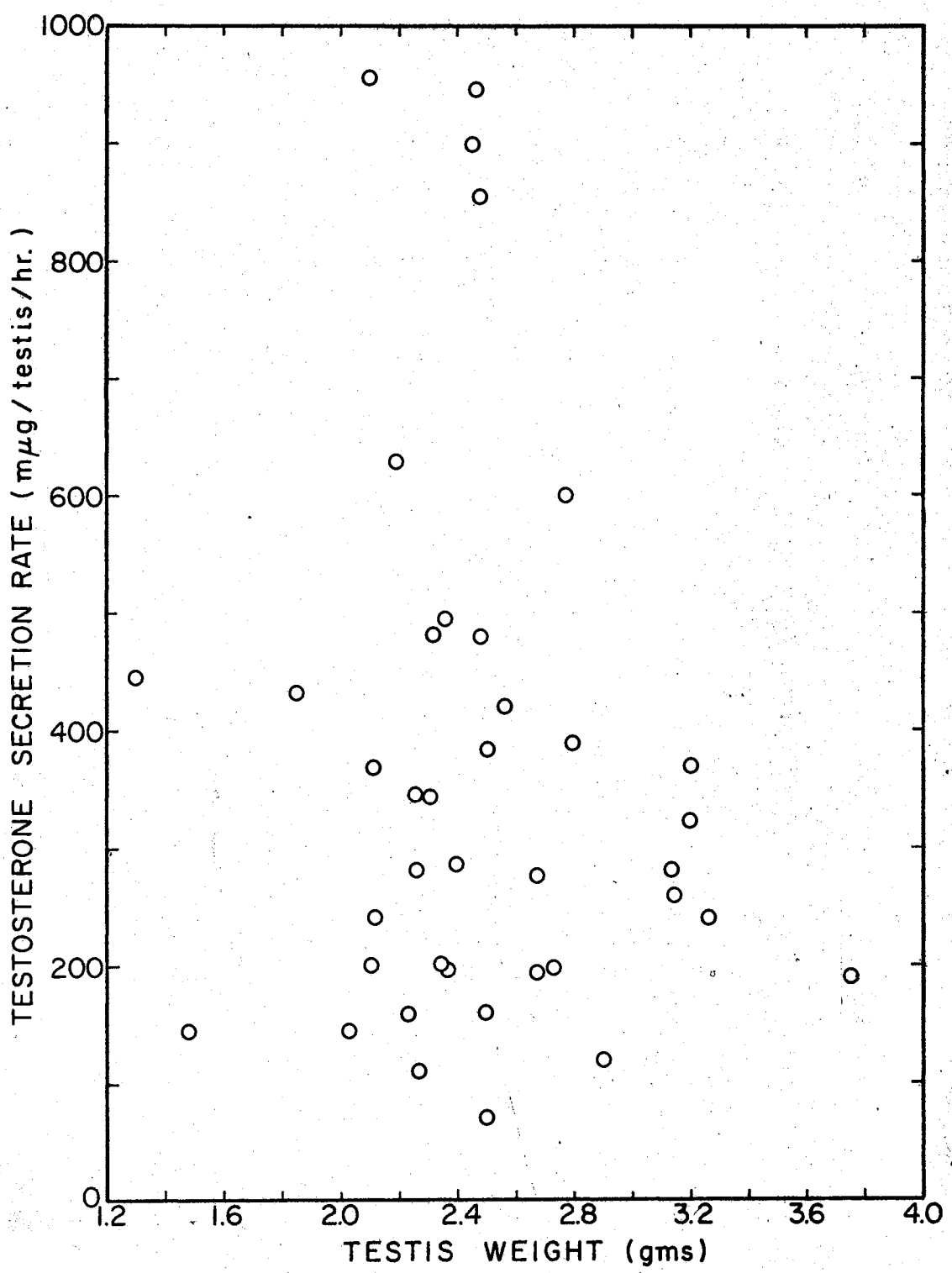

Figure 17. Correlation Between Testis Weight and Testosterone Secretion Rate of Perfused Rabbit Testes

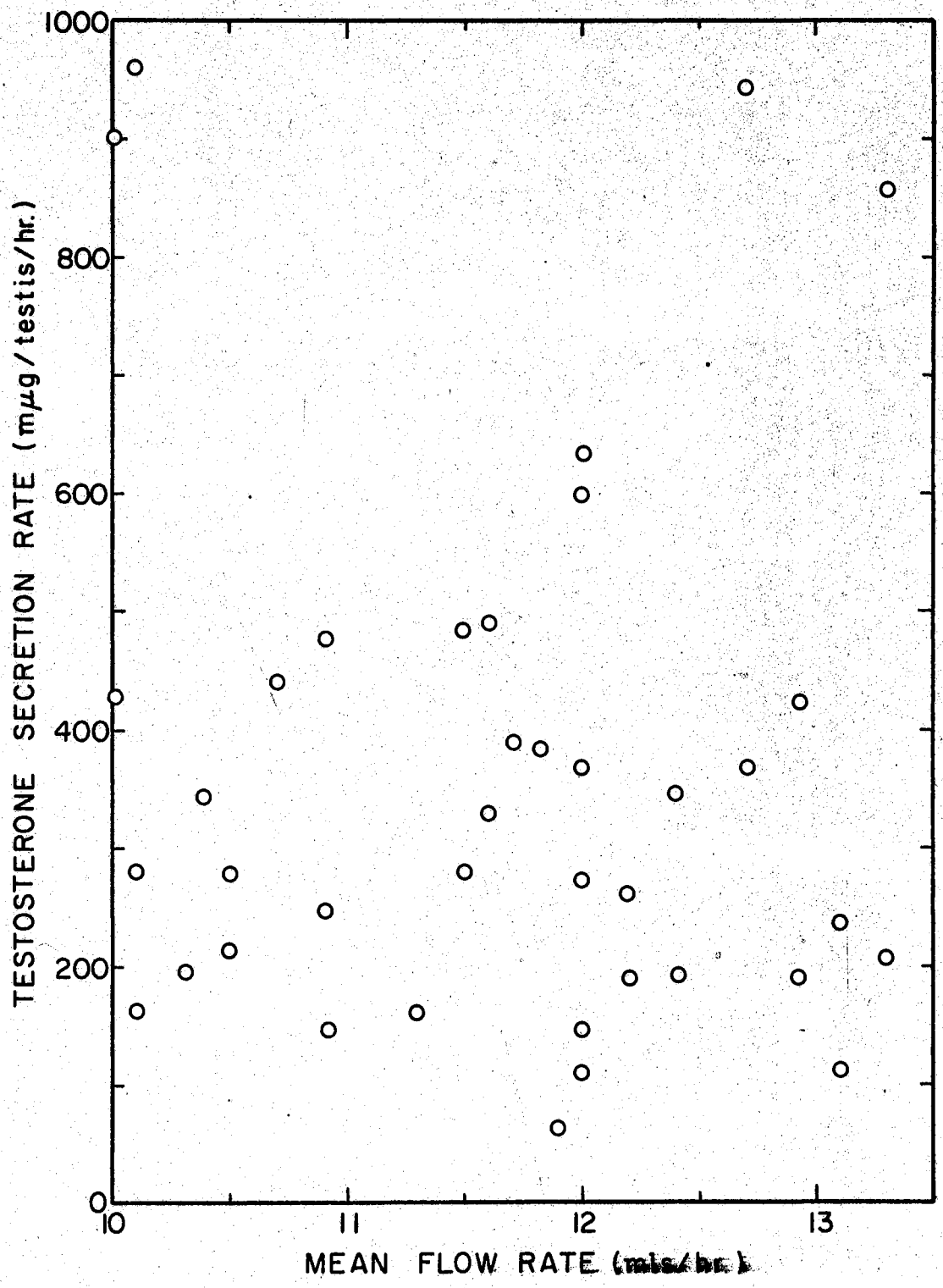

Figure 18. Correlation Between Mean Artificial Medium Flow Rate and Testosterone Secretion Rate of Perfused Rabbit Testes

TABLE VI

EFFECT OF ORDER OF TESTIS REMOVAL FROM ANESTHETIZED RABBITS UPON SUBSEQUENT TSR OF 14 PAIRS OF PERFUSED TESTES

	Time of Removal After Induction of Anesthesia[1]	
	5 min	20 min
TSR[2]	151.3 ± 28.2[3]	161.6 ± 31.6

[1] Intravenous pentobarbital sodium to effect.

[2] Mean values for first 2 hours of perfusion with artificial medium.

[3] mμg testosterone/gm of testis/hour ± SEM.

TABLE VII

MEAN ARTIFICAL MEDIUM FLOW RATE FOR 47 PERFUSED RABBIT TESTES[1] STIMULATED AFTER 2 HOURS WITH ICSH AND/OR GH

Hours of Perfusion						
0	1	2	3	4	5	6
11.40[2]	11.70	11.58	11.41	11.36	11.21	11.20
0.16[3]	0.15	0.14	0.14	0.14	0.16	0.16

[1]Mean testis weight = 2.39 gm ± 0.07 SEM.

[2]Flow rate in ml/hour.

[3]± SEM.

TABLE VIII

EFFECT OF VARIOUS TREATMENTS ON TESTOSTERONE SECRETION RATE OF PERFUSED RABBIT TESTES

Treatment	No. of Testes	Hours of Perfusion					
		1	2	3	4	5	6
Artificial Medium Alone	5	191.8[1] ± 65.6	119.9 ±30.0	115.5 ± 40.0	134.9 ± 71.4	99.9 ± 17.3	88.2 ± 14.1
GH in Graded Doses	2	408.7 ± 53.6	322.5 ±44.5	268.6 ± 28.6	262.0 ± 8.0	644.5 ± 94.7	1253.0 ±323.1
GH; 10 mμg per ml	4	300.5 ±110.4	160.8 ±43.5	118.5 ± 26.4	112.4 ± 24.4	111.8 ± 28.2	98.0 ± 17.3
ICSH; 5 mμg per ml	4	128.4 ± 30.0	103.2 ±22.3	280.7 ±159.3	484.1 ±270.1	677.3 ±299.6	792.4 ±350.1
ICSH; 30 mμg per ml	6	303.1 ±110.0	112.6 ±17.3	590.6 ±255.1	743.4 ±132.2	887.6 ±136.7	958.3 ±142.4
ICSH; 8000 mμg per ml	8	155.9 ± 46.9	100.2 ±33.1	340.6 ± 44.7	657.9 ±113.5	803.1 ±155.8	819.8 ± 93.8
ICSH; 5 mμg per ml + GH; 10 mμg per ml	4	134.6 ± 24.4	130.8 ±42.4	204.8 ± 81.8	401.7 ±175.4	555.8 ±213.3	725.7 ±264.1
ICSH; 100 mμg per ml + GH; 200 mμg per ml	8	126.4 ± 17.3	77.8 ±10.0	297.7 ± 51.9	640.2 ± 94.8	746.7 ± 69.2	843.5 ± 95.3

[1]Testosterone (mμg/gm of testis/hour). Each value represents the mean ± SEM.

VITA

Dan Edward Goodwin

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EFFECT OF GROWTH HORMONE ON TESTOSTERONE SECRETION IN PERFUSED RABBIT TESTES

Major Field: Physiology

Biographical:

Personal Data: Born January 4, 1928 at Durham in Roger Mills County, Oklahoma, the son of Mr. and Mrs. R. L. Goodwin.

Education: Attended elementary and high school at Durham, Oklahoma, graduating in 1946; studied pre-veterinary medicine at Panhandle State College, Goodwell, Oklahoma in 1950-1951; received the degree of Doctor of Veterinary Medicine from Oklahoma State University, Stillwater, Oklahoma in May, 1955; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in May, 1972.

Professional Experience: General veterinary practice, Chelsea, Oklahoma, 1955-1957; Resident Veterinarian, Kermac Angus Ranch, Poteau, Oklahoma, 1957-1963; Resident Veterinarian, Winrock Farms, Morrilton, Arkansas, 1963-1968.