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INHIBIN-LIKE ACTIVITY
IN
BULL SEMINAL PLASMA

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THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

November 1980
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ACKNOWLEDGMENTS

I thank:

Dr Wayne Watkins for introducing me to inhibin and for his guidance, my fellow PhD students Vernon Choy, Lloyd Moore and Shiva Reddy for their friendship, moral support, and helpful criticism, and other members of the Postgraduate School for a congenial work-place.

I am indebted to:

the N.Z.Dairy Board Artificial Breeding Centres at Newstead, Hamilton, and Awahuri, Palmerston North, for supplying bull seminal plasma, the N.I.A.M.D.D. and its distribution officer Dr A.F.Parlow for providing radioimmunoassay reagents, and

the staff of the Surgical laboratory at Green Lane Hospital - particularly Mr Richard Elliott, Hillary, Kim, Robyn and Gordon - and of the Animal Laboratory at Middlemore Hospital - especially Mrs Mary Hyde - for breeding and caring for my rats.

I also thank:

Dr John Bass of Ruakura Animal Research Centre, Hamilton for his interest and for undertaking experiments beyond my means,

Dr K.P.McNatty of Wallaceville Animal Research Centre, Upper Hutt, and the Westfield Freezing Co., Auckland, for providing biological samples, and

Dr John France for arranging the assay of testosterone.

Messrs John Urry and Ian Foot did the photographic work; Mrs Patricia James did the typing.

That I started this thesis I attribute to my parents, who taught me to find out things for myself.

That I finished I attribute to Deborah, my wife, who supported me patiently and bore with me when I was absent in body or in spirit.

This thesis is dedicated to Deborah.
**ABBREVIATIONS**

In addition to chemical symbols, abbreviations for S.I. units, and journal abbreviations of Index Medicus, the following abbreviations have been used:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABP</td>
<td>androgen-binding protein</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>B&lt;sub&gt;0&lt;/sub&gt;</td>
<td>maximum binding</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bSP</td>
<td>bull seminal plasma</td>
</tr>
<tr>
<td>bSP-E</td>
<td>bull seminal plasma-extract</td>
</tr>
<tr>
<td>bSVF</td>
<td>bull seminal vesicle fluid</td>
</tr>
<tr>
<td>bSVF-E</td>
<td>bull seminal vesicle fluid-extract</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>αChym</td>
<td>α chymotrypsinogen</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
</tr>
<tr>
<td>CM</td>
<td>carboxymethyl</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>Cyt C</td>
<td>cytochrome C</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>g</td>
<td>acceleration due to gravity</td>
</tr>
<tr>
<td>G-75,100,200</td>
<td>Sephadex G-75,100,200 gels</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine monophosphate</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotrophin</td>
</tr>
<tr>
<td>hSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>K&lt;sub&gt;av&lt;/sub&gt;</td>
<td>fraction of stationary volume available for diffusion</td>
</tr>
<tr>
<td>LH</td>
<td>luteinising hormone</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinising hormone-releasing hormone</td>
</tr>
<tr>
<td>log MW</td>
<td>logarithm of molecular weight</td>
</tr>
<tr>
<td>min.</td>
<td>minute</td>
</tr>
<tr>
<td>Myo</td>
<td>myoglobin</td>
</tr>
<tr>
<td>NIAMDD</td>
<td>National Institute of Arthritis, Metabolism and Digestive Diseases</td>
</tr>
</tbody>
</table>
no., n

oA.
oFF

OFF-E

p

PAGE

PAGIEF

pH

PM 10

r

RNA

SHBG

TCA

TEMED

Tris

UM 05

v

V_e

V_o

V_t

vBSP

vBSP-E

w

XM 100

number

ovalbumin

ovine follicular fluid

ovine follicular fluid-extract

probability

polyacrylamide gel electrophoresis

polyacrylamide gel isoelectrofocusing

-logarithm (hydrogen ion concentration)

Amicon PM 10 membrane

regression coefficient

ribonucleic acid

sex hormone-binding globulin

trichloroacetic acid

N,N,N',N'-tetramethylethylenediamine

tris(hydroxymethyl) aminomethane

Amicon UM 05 membrane

volume

elution volume

void volume

total column volume

seminal plasma from vasectomised bulls

extract of seminal plasma from vasectomised bulls

weight

Amicon XM 100 membrane
SUMMARY

Testicular function is stimulated from the pituitary gland by the gonadotrophic hormones FSH and LH. The testis in turn regulates gonadotrophin secretion by negative feedback. The agents of feedback are steroids, and in addition, probably a protein hormone, which has been given the name inhibin.

A method capable of detecting inhibin-like activity in bull seminal plasma (bSP) was developed. Administration of bSP at the time of castration to five-week old male rats inhibited the post-castration rise of serum concentrations of FSH and LH otherwise seen 24 h later. The degree of inhibition depended on the dose; 0.5 ml bSP or the equivalent amount of bSP-extract always suppressed FSH and LH to levels typical of intact rats. The rats' sensitivity to 0.2 ml bSP varied with the time of year, possibly reflecting seasonal changes in the onset of puberty.

The time-course of action of bSP-extract was studied in intact rats. Serum FSH was suppressed 12, 24 and 48 h after a single injection, but not at 3 or 6 h. LH was suppressed at 6, 12, 24 and possibly 48 h.

The activity in bSP was proteinous - activity was lost after heating bSP-extract at 67°C for 1 h, and after incubation with pepsin. The activity appeared to associate either with itself or with other components of bSP. The apparent molecular size of the activity on gel exclusion chromatography decreased when the ionic strength of the eluting buffer was increased, when the pH was changed from 7.6 to 4.0, and when 4 M urea was present. In the presence of 4 M urea its molecular weight lay between 15 000 and 35 000 daltons. The activity bound to CM cellulose equilibrated with 4 M urea - 0.05 M sodium acetate, pH 4, but could be eluted by increasing the sodium concentration of the buffer. If the proteins that were co-eluted are used as a guide, the active factor would have an isoelectric point of between 4.7 and 5.4.

The presence of activity in seminal plasma from vasectomised bulls meant the activity could not have come from the testis by way of the vas deferens. The seminal vesicles seemed the likely source. An experiment was designed to see if the activity came from the testis by an indirect route, or whether it could be secreted in the absence of testes. The
Results were inconclusive.

The suppression of both serum FSH and LH was problematic, since inhibin has been hypothesised to preferentially inhibit FSH secretion. The dual action was not a peculiarity of the strain of rats used - both Sprague-Dawley and Brattleboro rats responded in the same way, - nor was it peculiar to bSP - an extract of rat testes, presumably containing endogenous rat inhibin, also suppressed both gonadotrophins. In intact rats bSP-extract could always suppress FSH, but only sometimes did it affect LH. LH suppression occurred during periods of greater sensitivity on the part of the acutely castrated rats.

While bSP-extract suppressed FSH and LH equally in acutely castrated rats, testosterone propionate selectively suppressed LH. Small doses of bSP-extract and testosterone that had little effect separately acted synergistically to suppress FSH and LH to levels found in intact rats, while combinations of larger doses had little further effect. This selective interaction suggested how inhibin and testosterone might together regulate blood concentrations of FSH and LH in the male rat.

Although acutely castrated rats responded to bSP, chronically castrated rats did not. It was hypothesised that testosterone might maintain the rat's sensitivity to inhibin, and that this might be the mechanism underlying the synergism between the two hormones. Capsules containing testosterone were implanted subcutaneously in rats at the time of castration, and were withdrawn seven days later. However, only very high doses of testosterone maintained responsiveness to bSP-extract.
1.1 ORIENTATION

The testis has two main functions, production of sperm and sex hormones. Both functions require the pituitary for stimulus; its agents are the gonadotrophic hormones. The testis in turn produces hormones that regulate gonadotrophin secretion. This feedback loop dominates the complex interplay of hormones that serves to ensure normal testicular function.

The subject of this thesis is inhibin - a hormone, different from the sex steroids, that was proposed to be responsible for the testis's feedback on the pituitary. The inhibin hypothesis was put forward in 1932, a time when reproductive endocrinology was advancing rapidly, and when many theories were being offered. The concept was soon overshadowed when sex steroids themselves were shown to inhibit gonadotrophin secretion. The purification, isolation, and chemical synthesis of steroids were well within the capabilities of contemporary chemistry and occurred with extraordinary speed. In contrast, inhibin appeared to be a protein and protein chemistry was rudimentary. Any work with inhibin meant using crude and often toxic extracts, so that the meaning of the results was sometimes questionable. Not only was work on inhibin difficult, but as the actions of steroids were unveiled, the very need for inhibin waned.

However, forty years later it had become clear that steroids could not completely account for testicular control of gonadotrophin secretion. The concept of inhibin, albeit somewhat changed, was resurrected.

This chapter introduces the setting in which the inhibin must now be placed and the setting for the work in this thesis. The information in the following pages comes mainly from the rat, the species most studied, and indeed used in this thesis. The rat has been widely adopted as a model, at first approximation, for mammals in general and man in particular.
Figure 1.1 Components of the hypothalamus-pituitary-testis axis
See the text for elaboration. (From a) Cross (1972), b) Clermont & Huckins (1961), and c) Fawcett (1975).
1.2 HORMONAL CONTROL OF REPRODUCTION IN THE MALE

1.2.1 Hypothalamus-pituitary-testis Axis

The testis is under control of specific hormones secreted by the pituitary gland, which itself is controlled by hormones from the hypothalamus. The hypothalamus, pituitary, and testis constitute an axis, in which control is exerted up and down the chain. Other hormones and nerve signals contribute to testicular function, but their action is largely mediated by the hypothalamus and pituitary.

The hypothalamus is located at the base of the brain, and forms the floor and part of the walls of the third ventricle. It is composed of nuclei - areas of different morphology. The posterior pituitary, the neurohypophysis, is an outgrowth of the hypothalamus. It has three distinct regions: the median eminence which runs into the hypothalamus, the infundibular stem, and the infundibular process or pars nervosa. The anterior pituitary, the adenohypophysis, is derived not from the brain but from ectoderm from the primitive mouth cavity. It too has three regions: the pars tuberalis which embraces the infundibular stem, the pars distalis which contains the bulk of the hormone-secreting cells, and the small pars intermedia sandwiched between the pars distalis and the posterior pituitary gland.(Bloom & Fawcett, 1962)

Nerve cells in the hypothalamus send axons to the pars nervosa and to the median eminence. Hormones secreted from the axons in the median eminence enter a plexus of capillaries which carry them, via the portal vessels, to the venous sinusoids of the pars distalis (Green & Harris, 1947) (Figure 1.1a). Here they exert their actions on the release of the anterior pituitary hormones. Few veins from the adenohypophysis drain directly to the general circulation, so it is possible that some blood drains into the neurohypophysis and thence to the brain (Bergland & Page, 1979). This would allow hormones secreted by the adenohypophysis to reach the hypothalamus.

The testis in mammals is packed with long convoluted tubules, the seminiferous tubules, which usually open at both ends into the rete testis (Figure 1.1b). The tubules are bounded by several layers of tissue: innermost a noncellular membrane, then a layer of smooth muscle-like myoid
cells, then another noncellular layer, and outermost a layer of cells that in the rat is equivalent to the epithelium lining lymphatic spaces. There are no blood vessels in the tubules, so nutrients must diffuse through these layers to reach the cells within. The tubules are lined inside with Sertoli cells which are attached to the basement membrane (Figure 1.1c). Embedded in the Sertoli cells and drawing nourishment from them are germ cells in various stages of development. The germ cells are divided, in order of maturation, into type A spermatogonia, intermediate spermatogonia, type B spermatogonia, primary spermatocytes, and after meiosis secondary spermatocytes and spermatids. The spatial distribution of the germ cells depends on their stage of development, from resting spermatogonia at the periphery of the tubule to spermatids with tails protruding into the lumen. Sertoli cells are joined to each other, above the spermatogonia but below the spermatocytes, by specialised junctions. Substances from the blood can diffuse between cells only up to the junctions. As a corollary, spermatocytes and spermatids are exposed only to substances that have first traversed the Sertoli cell. The junctions are probably the site of the blood-testis barrier. (Fawcett, 1975; Setchell, 1978)

Between the tubules is interstitial tissue. This contains the steroid-producing Leydig cells, as well as blood vessels and lymph vessels. The amount of interstitial tissue and its fine structure varies among species (Fawcett et al., 1973). Tubules and interstitial tissue are enclosed in a fibrous capsule, the tunica albuginea. In man and in animals commonly used in endocrine experiments — rat, ram and bull — the testes are suspended from the abdomen in a scrotum. This allows the testes to be maintained at below body temperature, an essential condition for spermatogenesis in these species (Waites, 1970). Retention of the testes within the abdomen in these species is called cryptorchidism.

1.2.2 The Gonadotrophins

The gonadotrophins are proteins made up of two peptide chains. The α-chain is the smaller (96 amino acids in the bovine) and is common to follicle-stimulating hormone (FSH) and luteinising hormone (LH). The β-chain is larger (120 amino acids in the bovine) and confers the gonadotrophin's identity (Sairam & Papkoff, 1974). The similar structures of FSH and LH are explained by their evolution from a single ancestral
molecule (Licht et al., 1977). FSH and LH have carbohydrate attached to their protein backbone. Although not essential for biological action, the carbohydrate slows their metabolism and hence lengthens their biological half-life (Vaitukaitis et al., 1976). The peptide chains are transcribed from DNA and so have constant composition, whereas the carbohydrate is added by enzymes after the peptide chains have been synthesised.

The gonadotrophins are synthesised by a class of cells in the anterior pituitary, the gonadotrophs. The classical description of separate FSH- and LH-producing cells was incorrect; the two types of cells originally distinguished on the basis of their morphology both produce FSH and LH (Moriarty, 1975). Although most cells contain FSH and LH (Nakane, 1970; Herbert, 1975; Purandare et al., 1978), about ten percent stain only for FSH (Pelletier et al., 1976). It has been suggested that this basic cell type (the basophils) undergoes cycles in its secretion, from producing thyroid-stimulating hormone (also a glycoprotein, with an α-chain identical to that of FSH and LH) to predominantly LH, to mainly FSH (Yoshimura et al., 1977). The gonadotrophs are located in the anterior and ventral parts of the pars distalis (Nakane, 1970), and in the pars tuberalis (Osamura & Watanabe, 1978). It is possible that some portion of the pars tuberalis remains after hypophysectomy.

1.2.3 The Effect of Gonadotrophins on the Testis

It is now recognised that the hormonal requirements for establishing spermatogenesis - at puberty or after degeneration of the germinal epithelium following hypophysectomy - differ from those for maintaining spermatogenesis once it has begun.

1.2.3.1 Sexual Maturation

LH binds to specific receptors on the Leydig cells, and, through a series of metabolic events that probably include the activation of adenyl cyclase, it stimulates steroidogenesis (Dorrington & Fritz, 1974; Cooke et al., 1979). LH prompts conversion of cholesterol to pregnenolone, a set of enzyme-mediated reactions occurring in the mitochondria of the cell (Christensen, 1975). The subsequent conversion
of pregnenolone to androstenedione and thence to testosterone takes place in the smooth endoplasmic reticulum and is not dependent on LH.

FSH binds only to Sertoli cells. Binding sets in motion a cascade of intracellular events: activation of adenyl cyclase, activation of protein kinases, the phosphorylation of proteins, RNA synthesis in the cell nucleus, synthesis of ribosomal RNA, culminating in prolonged synthesis of proteins (Steinberger, 1975; Means, 1975; Means et al., 1976; Dorrington & Armstrong, 1979). FSH exerts a general effect on protein synthesis and is probably responsible for the growth of the seminiferous tubules. By the 18th day of age in the male rat, the Sertoli cells cease to multiply and thereafter grow only in size. Around day 20 the tubules develop a lumen, the blood-testis barrier forms, spermatogenesis reaches an adult rate with the first cells undergoing meiosis (Ramaley, 1979), and by day 24 the general trophic effects of FSH have ceased (Means et al., 1976).

Testosterone is a corequisite for normal development. Since Sertoli cells have little ability to produce testosterone (de Jong et al., 1974), testosterone must diffuse from the interstitial tissue through the lymphatic spaces into the tubules.

In the immature rat FSH stimulates the production of 5α reductase and 3α hydroxy-steroid dehydrogenase. As a result the tubules readily convert testosterone to dihydrotestosterone and 5α androstane-3α,17β-diol (Steinberger & Ficher, 1971) and these are the major steroids leaving the testis until about day 35 (Podestá & Rivarola, 1974; Moger, 1977). Older rats retain this ability, but the amount of testosterone converted hardly changes as the Leydig cells increase their output.

FSH also induces aromatases which convert testosterone to estrogens (Dorrington et al., 1978a). The Leydig cells have estrogen receptors (Brinkman et al., 1972), and estrogens may act to inhibit testosterone synthesis (Chen et al., 1977; Saez et al., 1978) and so prevent precocious puberty. Tubular aromatases are lost by day 30 (Dorrington et al., 1978a), but interstitial tissue acquires the ability to aromatise androgens. This is dependent on LH and is not lost with age (Valladares & Payne, 1979).

FSH also increases the testis's sensitivity to LH during

a Welsh & Wiebe (1978)
sexual maturation (Odell & Swerdloff, 1976), possibly by inducing LH receptors as it does in the ovary (Channing, 1975). The rapid rise in the number of LH receptors parallels the rise in serum FSH between 20 and 35 days (Ketelslegers et al., 1978). How FSH exerts its effect on the Leydig cells is unknown. Estradiol has been suggested as the agent, but testicular sensitivity to LH increases when FSH-induced estradiol production disappears (Bartke et al., 1978).

1.2.3.2. Adulthood

In adulthood LH functions as it did before maturation, stimulating synthesis of androgens.

FSH still binds to the Sertoli cell, but it unable to stimulate cyclic AMP accumulation as much as before (Dorrington & Armstrong, 1979; Steinberger et al., 1978). This seems partly due to an increase in intracellular phosphodiesterase, an enzyme which metabolises cyclic AMP. Administration of 1-methyl-3-isobutylxanthine, an inhibitor of the enzyme, enables FSH to increase intracellular cyclic AMP and to activate protein kinase in older rats (Means et al., 1976). In the immature rat FSH suppresses the enzyme, so maturation may promote an isoenzyme unresponsive to FSH (Monn et al., 1972). This may be produced by the germ cells, since it has been reported absent in rats whose germ cells had been killed off by fetal irradiation (Means et al., 1976). On the other hand, Heindel et al. (1978) indicate age brings a defect in the cells' synthetic mechanism, not just more efficient hydrolysis of cyclic AMP.

In the adult FSH continues to stimulate production of androgen-binding protein (ABP). The time-course of synthesis is shorter than for the general protein synthesis seen during sexual development, and a different intracellular process seems to be involved (Means et al., 1976). Although both FSH and testosterone are able to increase ABP (Hansson et al., 1975; Dym et al., 1977), the two hormones might not be interchangeable. Tindall et al. (1978) found FSH stimulated ABP synthesis, while testosterone stopped its metabolism.

1.2.3.3 Modulation of the Effects of FSH and LH by Other Hormones

In hypophysectomised rats prolactin and growth hormone can
enhance the effect of LH on testosterone production and spermatogenesis (Woods & Simpson, 1961; Bartke, 1971). It has been suggested that these hormones may, by different mechanisms, help maintain LH receptors (Zipf et al., 1978), although other mechanisms are possible (Bartke et al., 1978). The effect is probably direct, since the interstitial tissue possesses receptors for prolactin (Charreau et al., 1977). In contrast, too high a concentration of prolactin in the blood is antigonadotrophic, but here evidence points to action on the pituitary or hypothalamus (Grandison et al., 1977; McNeilly et al., 1978).

1.2.3.4 Control of Fertility by the Control of Gonadotrophins

Spermatogenesis is clearly dependent on gonadotrophins - it ceases after hypophysectomy and it can be restored by administration of FSH and LH (Smith & Engle, 1927; Zondek & Aschheim, 1927). LH exerts its action through producing testosterone. Indeed, very high concentrations of testosterone or dihydrotestosterone can alone maintain spermatogenesis in hypophysectomised rats (Nelson, 1937; Boccabella, 1963; von Berswordt-Wallrabe & Mehring, 1975; Ahmad et al., 1975; Chowdhury & Steinberger, 1975; Harris et al., 1977), although apparently not in rams (Courot et al., 1979). However under normal conditions, FSH and testosterone are both believed to have roles. The hormonal requirements of individual stages of spermatogenesis have been sought by a variety of methods, including administration of hormones to hypophysectomised animals (Woods & Simpson, 1961; Lostroh, 1969; Chowdhury, 1979), administration of antisera to FSH and LH (Raj & Dym, 1976; Chemes et al., 1979), and by culturing seminiferous tubules in the presence of hormones (Steinberger & Steinberger, 1974). Spermatogonia transform into spermatids in the absence of hormones but testosterone is essential for diakinesis and the reductive division of meiosis of spermatocytes, and FSH and testosterone both seem required for full maturation of spermatids. FSH and testosterone may also bolster the number of germ cells by preventing their degeneration (Mills & Means, 1972; Russel & Clermont, 1977; Sivelle et al., 1978; Chowdhury, 1979).

FSH may exert its synergistic action with testosterone (Woods & Simpson, 1961) by producing ABP (Hansson et al., 1975). In this way FSH could increase the intratubular concentration of testosterone, the seemingly irreducible factor needed for spermatogenesis. (Although very
recently, even this has been challenged. Cunningham & Huckins (1979) found FSH could maintain spermatogenesis, to some extent, in rats which had very low testicular concentrations of testosterone.

Overall, testosterone has a biphasic effect on spermatogenesis. While larger doses exert a direct stimulus, small doses inhibit sperm production by suppressing gonadotrophin secretion (see next section) (Walsh & Swerdloff, 1973). In fact, suitable doses of testosterone and estradiol administered together can totally prevent sperm formation in the rat but at the same time maintain normal blood concentrations of testosterone and estradiol! (Ewing et al., 1977). It has been proposed that a similar nett effect might be achieved by selective suppression of FSH secretion, with the important difference that the testis would be supplying the usual amount of peripheral androgen.

1.2.4 Control of Gonadotrophin Secretion

Gonadotrophin secretion is controlled basically in two ways - by stimulus from the hypothalamus and by inhibition from the testis. Although control can be dissected into many aspects, for instance the action of hypothalamic releasing factors on the pituitary, or of testosterone on the hypothalamus, it is well to remember that these components are highly integrated.

1.2.4.1 Hypothalamic Stimulus

LH-releasing activity in the hypothalamus was first demonstrated by McCann et al. (1960). Intensive biochemical work over the next ten years was rewarded by the isolation of the factor and elucidation of its structure (Schally et al., 1971a; Amoss et al., 1971). Luteinising hormone-releasing hormone (LHRH), as it was called, promotes synthesis of the gonadotrophins as well as their release (Liu et al., 1976), although synthesis and release are not necessarily related (Liu & Jackson, 1978, 1979).

LHRH is released in pulsatile fashion (Carmel et al., 1978; Schuiling & Gnodde, 1976), which is reflected in pulsatile release of LH (and FSH, although masked by this hormone's longer half-life (Bogdanove & Gay, 1969)). The frequency as well as the amplitude of the pulses is important. A change in frequency may constitute the hypothalamic
mechanism for initiating spermatogenesis in seasonal breeders (Lincoln, 1978). If the pulses are correctly spaced self-priming occurs, so that the second pulse releases more LH than the first (Aiyer et al., 1974). Different biochemical processes are involved in priming than in release; priming requires protein synthesis (Pickering & Fink, 1979), and it may be that intermittent stimulation allows replenishment of LHRH receptors (Belchetz et al., 1979). In any case, intermittent stimulation of the pituitary is essential for normal FSH and LH release; the pituitary becomes refractory to constant infusion of LHRH (Belchetz et al., 1979).

Since LHRH released both FSH and LH, Schally et al. (1971b) proposed it was the only hypothalamic releasing hormone for the gonadotrophins. Indeed, LHRH accounts for most, if not all, FSH-releasing activity in the hypothalamus (Schally et al., 1976). The isolated report of a separate FSH-releasing hormone (Johansson et al., 1975; Fuchs et al., 1979) has not been confirmed by others. However there is abundant evidence that the hypothalamus can control FSH and LH separately. The ratio of FSH and LH concentrations in blood changes according to which hypothalamic areas steroids are implanted (Danguy et al., 1977), nerves are cut (Martini, 1975), electrical stimulation is applied (Kimura & Kawakami, 1978; Chappel & Barraclough, 1976), or neurotransmitters or their antagonists are injected (Löfström et al., 1977; Beck et al., 1978; Dobson & Narayana, 1978; Borrell et al., 1979; Piva et al., 1980). Many of these results have been cited as evidence for separate FSH and LH releasing hormones. But recently Barraclough et al. (1979) have proposed an alternative explanation - that large, short-lived pulses of LHRH would cause secretion of mainly LH, while prolonged exposure of the pituitary to low doses of LHRH would release mainly FSH. They proposed that hypothalamic nuclei differ in the time-course of their delivery of LHRH and hence their effect on pituitary FSH and LH secretion. Experiments bear out the hypothesis (Wise et al., 1979). Barraclough et al. (1979) assumed that the pituitary contained two types of gonadotrophs which differed in their sensitivity to LHRH. Interestingly, Denef et al. (1978, 1980) have reported that the gonadotroph population was heterogeneous in its response to LHRH and in the relative amounts of FSH and LH released.
1.2.4.2 Pituitary Feedback

LH may feedback directly on its own secretion. Evidence comes from castrated rabbits, in which injection of human LH caused a selective fall in the blood concentration of the rabbit's native LH (Patritti-Laborde & Odell, 1978). The site of feedback is probably the hypothalamus, since the presence of LH has not been reported to inhibit further LH release from cultured pituitary cells. LH is known to stimulate hypothalamic L-cystine arylamidase, which breaks down LHRH (Kuhl & Taubert, 1975). LHRH may even influence its own secretion within the hypothalamus (Hyyppa et al., 1971), but perhaps in its capacity as a neurotransmitter rather than as a releaser of gonadotrophins.

1.2.4.3 Feedback by Testicular Steroids

Removal of the testes increases gonadotrophin output. This was first noticed from pituitary hypertrophy (Ficherä, 1905) with the formation of "castration cells" (Hoskins, 1911). Later it was shown that concentrations of gonadotrophins rose in urine and in blood, and that injection of testicular steroids reversed these effects. Accordingly it was proposed that testicular steroids controlled gonadotrophin secretion by negative feedback (Moore & Price, 1932), a concept that has become firmly established.

Testosterone is the predominant steroid secreted by the testis of all mammalian species studied, although small amounts of the intermediates in the biosynthetic pathway from pregnenolone to testosterone leak into circulation. The testis also secretes some estradiol and dihydrotestosterone, and more is formed outside the testis from metabolism of testosterone. (Brooks, 1975; Jeffcoate, 1975) Testosterone, dihydrotestosterone and estradiol are ultimately the steroids involved in controlling gonadotrophins. Cells in the pituitary and hypothalamus have specific receptors that bind testosterone/dihydrotestosterone and estradiol (Naess et al., 1975, 1977), and binding to these receptors is the first and prerequisite step for the steroid to have hormonal action.

Steroids have long been known to act on the hypothalamus and pituitary, but their relative effects at the two sites have only recently been untangled. Work with pituitary cell cultures reveals purely the
steroid's action on the pituitary, while the effect on the hypothalamus can be inferred from comparing the responses of the pituitary culture and of the whole animal.

When pituitary cells from male (or female) rats are cultured in the presence of estradiol they become more sensitive to LHRH, so that a short incubation with LHRH elicits a greater release of FSH and LH (Drouin et al., 1976). Testosterone has a very different effect - it decreases the LH response to LHRH but leaves the FSH response unchanged (Drouin & Labrie, 1976). Testosterone seems to achieve its differential effect by stimulating the synthesis of FSH. This complies with the earlier in vivo observations that testosterone increased the pituitary content of FSH (Kingsley & Bogdanove, 1973; Chowdhury & Steinberger, 1976; Steinberger & Chowdhury, 1977). Despite its elegance, the pituitary cell culture might not reveal the whole complexity of what happens in vivo, for although prior exposure to estradiol indeed augmented LH release when LHRH was given to rats (Arimura & Schally, 1971; Verjans & Eik-Nes, 1976), estradiol seems to have a biphasic effect, at first decreasing the response to LHRH (Libertun et al., 1974; Vilchez-Martinez et al., 1974).

In addition, pulsatile stimulation by LHRH may be essential for the pituitary to exhibit some of its responses to steroids. In rats given phenobarbital to block endogenous LHRH release, dihydrotestosterone did not affect the LH response to a constant infusion of LHRH. However, the steroid lessened the amount of LH released by half-hourly injections of LHRH (Nansel & Trent, 1979). The frequency of the LHRH pulses is also important; when LHRH was injected hourly, instead of half-hourly, dihydrotestosterone no longer had an effect.

Cyclic AMP, cyclic GMP, and prostaglandins have all been implicated as intracellular messengers for LHRH-induced gonadotrophin release (Drouin et al., 1978; Nakano et al., 1978; Naor et al., 1978a; Nair et al., 1979). The relative importance of the different pathways may depend on steroids (Naor et al., 1978b), but the mechanism of action of steroids in attenuating the cell's response to LHRH is not yet clear (see Labrie et al., 1978a).

Estradiol acts on the hypothalamus as well as the pituitary. Although small doses can increase serum FSH and LH in castrated rats, larger doses inhibit the secretion of both gonadotrophins (Swerdluff & Walsh, 1973; Swerdluff et al., 1973; Eldridge et al., 1974). Since
estrogens increase the pituitary's sensitivity to LHRH, estradiol must exert its inhibitory action by suppressing hypothalamic secretion of LHRH. A similar argument has been advanced for hypothalamic inhibition by androgens. Dihydrotestosterone lowers serum LH in castrated female rats at much smaller doses than are needed to reduce the LH response to LHRH (Labrie et al., 1978a). Within the hypothalamus testosterone can be aromatised to estrogens, or reduced to dihydrotestosterone (Naftolin et al., 1972). While there has been much debate as to how much of testosterone's action is actually exercised by its estrogen metabolites (Loriaux et al., 1977), the two steroids do have separate and independent effects. In man, infusion of testosterone lowers the frequency of episodic LH release, but increases the amplitude of the LH peaks. Estradiol leaves frequency unchanged but reduces amplitude (Santen, 1977). It seems, therefore, that the two steroids inhibit the hypothalamus by different mechanisms.

Steroids also influence gonadotrophins secretion by indirect methods.

Testosterone treatment increases the molecular size of FSH made by the rat; the larger molecule lasts longer before being metabolised and has higher biological activity (Bogdanove et al., 1974; 1975). Estradiol does the opposite (Chowdhury & Steinberger, 1977). This could be achieved by changing the carbohydrate portion of the molecule. LHRH enhances glucosamine incorporation into LH just before its release, and the biological potency of the molecule is changed by steroids (Mukhopadhyay et al., 1979). Pleomorphism is also shown by monkey FSH and LH. The larger molecule again has a longer life, but in this species androgens promote the formation of a smaller molecule (Peckham & Knobil, 1976). It is interesting that in the rat the qualitative change opposes the quantitative change - testosterone decreases the amount of LH secreted but enhances its potency.

The hypothalamus and pituitary contain L-cystine arylamidase, an enzyme that degrades LHRH (Kuhl & Taubert, 1975; Griffiths & Kelly, 1979). Both testosterone and estradiol increase the pituitary concentration of the enzyme in male rats (Kuhl et al., 1977).

In the blood steroids bind to proteins; only the portion unbound is free to bind to receptors and exert its hormonal effect. In man about 60% of testosterone is bound to a specific sex hormone-binding globulin
(SHBG), 39% is bound to albumin, leaving only about 2% free (Anderson, 1974). Estradiol does not appear to bind to SHBG under physiological conditions (Vigersky et al., 1979). Rats lack a SHBG so a larger proportion of the total testosterone in the blood is free to act. The production of SHBG is under hormonal control; estradiol increases SHBG, testosterone decreases it. Thus estrogen will damp the action of androgen by sequestering testosterone, while testosterone will enhance its own availability. SHBG and albumin also buffer the concentration of free testosterone in blood from the fluctuations in testosterone output that arise from episodic LH secretion. This may mean that a single injection or infusion of testosterone is not too unphysiological.

There is also a miscellany of effects that inhibit the action of gonadotrophins on the testis and thereby influence testicular feedback. Estrogen directly inhibits LH-stimulated testosterone secretion (Hsueh et al., 1978; Saez et al., 1978). The rat testis makes a small molecular weight (1400 daltons) factor that prevents FSH from binding to its receptor (Reichert & Abou-Issa, 1977). LHRH can decrease testicular responsiveness to LH by hypophysectomised rat (Hsueh & Erickson, 1979), perhaps by decreasing the number of LH receptors. Blood concentrations of LHRH are so low that a direct hypothalamic effect is unlikely, but a local mechanism might operate.

1.2.4.4 Are Steroids Sufficient to Explain Testicular Feedback?

Testosterone suppresses FSH and LH in castrated rats, but LH is usually suppressed to a greater extent at a particular dose (Swerdloff & Walsh, 1973; Eldridge & Mahesh, 1974; Verjans & Eik-Nes, 1976; 1977), while estradiol suppresses FSH and LH equally in acutely castrated rats (Swerdloff & Walsh, 1972). Testosterone and estradiol have similar selectivity in man (reviewed by Setchell et al., 1977). Although many steroids have been tested, none selectively suppresses FSH (Swerdloff et al., 1973), nordo combinations of testosterone and estradiol (Swerdloff & Walsh, 1973; Gay & Dever, 1971).

And yet damage to the germinal epithelium often leads to a preferential rise in the blood concentration of FSH. Several treatments have been used in an attempt to impair spermatogenesis without affecting the Leydig cells, including x-irradiation, cryptorchidism, scrotal heating, ligation of the efferent ducts, alcohol, busulphan, α-chlorohydrin,
hydroxyurea, and vitamin A deficiency (see Setchell, 1978). Sometimes treatment increases FSH alone in rats (Gomes et al., 1973; Morris & Jackson, 1978b; van Thiel et al., 1979; Huang & Hembree, 1979), but more often both FSH and LH become elevated (Amatayakul et al., 1971; Swerdloff et al., 1971; Debeljuk et al., 1973; Bain & Keene, 1975; Gupta et al., 1975; Gomes & Jain, 1976; Rich & de Kretser, 1977; Aafjes et al., 1978; Main et al., 1978; Morris & Jackson, 1978a). Similar effects are seen in man and domestic animals (reviewed by Setchell & Main, 1974; Setchell et al., 1977). In all these cases the rise in FSH and LH was less than after castration, and compared to castration, FSH rose more than LH. Serum concentrations of testosterone were often unchanged, which was at first taken as evidence that the Leydig cells continued to function normally. More recent work has shown that the morphology of the Leydig cell changes, and that the cells responded abnormally to LH (de Kretser et al., 1979; Rich et al., 1979; Damber et al., 1978). Although infliction of damage to the germinal epithelium alters Leydig cell function, it is clear that the relative changes in the serum FSH and LH levels cannot be accounted for by disturbed steroid feedback. Another testicular factor must be involved; it has been called "inhibin".

1.3 SOME ASPECTS OF HORMONAL CONTROL OF REPRODUCTION IN THE FEMALE

Inhibin was postulated to explain gonadal control of gonadotrophin secretion in the male, but inhibin-like activity has also been discovered in the female (Hopkinson et al., 1975; 1977a; de Jong & Sharpe, 1976), and much recent work has used follicular fluid as a source of inhibin-like activity. This brief excursion into the vast field of hormonal control of reproduction in the female considers only those aspects pertinent to inhibin (as far as known), and they are introduced by analogy to the male.

The ovary contains follicles, at different stages of development, embedded in the stroma. Primordial follicles consist of germ cells surrounded by a single layer of granulosa cells. Germ cells stop proliferating around birth, but every day some of the primordial follicles begin developing. Follicular growth involves a change in the shape of the granulosa cells and their multiplication. As they grow, the granulosa cells become bounded by a basement membrane, and outside the membrane, layers of theca cells arise from the stroma. Most follicles cease to grow at some stage and become atretic, but a few enlarge and
develop a space in their centre - the antrum - filled with fluid secreted by the granulosa cells. In the rat, the few follicles that continue to grow may be those just at the right stage of development to be boosted by the gonadotrophin surges of the previous ovulation. (Bloom & Fawcett, 1962; Richards et al., 1978)

The stroma consists of several cell types, including interstitial cells that resemble Leydig cells, but most numerous are thecal cells left from degenerated follicles. These have receptors for LH and synthesise progesterone and androgens (androstenedione and testosterone) from cholesterol.

The granulosa cells resemble immature Sertoli cells in many ways: they have the same embryological origin, they are enclosed by a basement membrane so have no direct blood supply, they are the gateway for substances reaching the germ cells, they secrete fluid and synthesise proteins, they are the only cells to have receptors for FSH, and they aromatise androgens under the stimulus of FSH. But there are important differences. The granulosa cells do not make androgen-binding protein, and they can synthesise progesterone from cholesterol - a set of reactions stimulated by FSH and augmented by testosterone. They also have receptors for estradiol. Most importantly, the granulosa cell evolves under the influence of hormones to acquire receptors for LH and prolactin. It starts with FSH stimulating the conversion of androgens, made by the theca and interstitial cells, to estradiol. FSH and estradiol together increase the granulosa cell's sensitivity to FSH, which further increases the production of estradiol. Estradiol is a mitogen that promotes proliferation of granulosa cells and so the follicle grows. Later, FSH and estradiol evoke LH receptors. (Ross & Lipsett, 1978; Dorrington & Armstrong, 1979; Dorrington et al., 1978b)

High concentrations of peripheral estrogen, supplied by the dominant follicle(s), trigger a surge of LH. LH stops the proliferation of the granulosa cells, releases the ovum from the follicle, and causes the ruptured follicle to differentiate into a corpus luteum. The transformed granulosa cells and the adjoining theca cells now secrete progesterone as their main product (Richards, 1979).

Ovarian steroids exert negative feedback on gonadotrophin secretion in a manner qualitatively the same as the same steroids do in the male (Labrie et al., 1978a). However, unlike androgen and estrogen
in the male, progesterone and estrogen can act synergistically (McPherson et al., 1975; Goodman, 1978). Although low concentrations of estrogen exert negative feedback, prolonged high concentrations have a positive effect which results in the LH surge preceding ovulation (Fink, 1979a). Positive feedback probably has three component mechanisms: increased LHRH secretion, increased pituitary responsiveness to LHRH induced by estrogens, and increased pituitary responsiveness from self priming by LHRH (Labrie et al., 1979; Fink, 1979b). In some species, including the rat, pre-exposure to estrogen changes progesterone's effect from negative to positive feedback on LH secretion. Positive feedback depends on a "cyclic centre" in the hypothalamus that recognises that estrogen concentrations have been elevated for a certain period. In rodents this centre can be permanently turned off by perinatal exposure to androgens or estrogens, but this is not the case in humans, since prolonged estrogen treatment can induce positive feedback in men (Stearns et al., 1973).

1.4 THE INHIBIN HYPOTHESIS

The concept of negative feedback on gonadotrophin secretion by steroids and the concept of inhibin were both proposed in 1932. The first flourished while the second languished for forty years. Why?

1.4.1 The Context of the Inhibin Hypothesis

Experimental endocrinology was born with Berthold's (1849) discovery that transplantation of testes from a cockerel to a capon redeveloped the wattles and comb in the capon. By the 1920s the existence of gonadal hormones and their stimulation of the secondary sexual characteristics were well known. Reproductive endocrinology entered a golden age around 1925-1935, with the discovery of the pituitary's control of the gonads and a glimpse of the agents of control - the gonadotrophins, the isolation, identification, and chemical synthesis of the sex steroids, and the concept of negative feedback by gonadal hormones on gonadotrophin secretion (Greep, 1974; 1978).

Clear demonstration of the pituitary's role awaited a technique of hypophysectomy that avoided brain damage (Smith, 1926). Ablation of the pituitary in adult animals caused the Leydig cells and seminiferous tubules to regress, and they could be restored by daily pituitary implants.
In younger animals, hypophysectomy prevented sexual maturation, whereas pituitary implants caused precocious puberty (Smith & Engle, 1927; Zondek & Aschheim, 1927). The different effects on the ovary of gonadotrophic activity from different sources, particularly urine from pregnant and menopausal women, led to the concept of two distinct factors - a luteinising hormone and a follicle-stimulating hormone. Partial separation of the two activities in pituitary extracts was accomplished in 1931 (Fevold et al., 1931), but the existence of two pituitary gonadotrophins was not firmly accepted by all until the mid 1930s. Isolation of LH (ovine) was finally achieved in 1959 (Squire & Li, 1959), and FSH in 1964 (Papkoff et al., 1964). In contrast, the chemistry of lipids was well developed in the 1920s, and the sex steroids were quickly purified. By 1936, estradiol, testosterone, and several other steroids had been isolated and their chemical structure worked out.

Differential control of the androgenic and spermatogenic functions of the testis was suggested by Smith and his co-workers around 1934 (Smith & Leonard, 1934; Smith et al., 1934), and in 1937 Greep & Fevold found that preparations of FSH stimulated the seminiferous tubules and LH the Leydig cells in hypophysectomised rats.

In 1932 Moore & Price published their classic paper on the interrelationship between gonad and pituitary. They concluded that the inhibitory effects of estrogens on the male reproductive tract and of androgens on the female tract were caused by the steroid inhibiting gonadotrophin secretion, and not due to direct antagonism.

1.4.2 The Inhibin Hypothesis

In 1923 Mottram & Cramer had observed changes in the morphology of the pituitary, typical of those seen after castration, in animals whose germ cells had been depleted by irradiation. Since the Leydig cells and accessory reproductive system appeared normal, these changes were attributed to the absence of a secretion from the seminiferous tubules. Further evidence for such a secretion was provided by Martins & Rocha (1931) from a series of experiments using rats in parabiosis. A castrated male caused prostatic hypertrophy in its normal partner, and injection of aqueous testicular extracts prevented the effect. (Proteins cross from the circulation of one rat to the other while steroids do not.)
From these and his own experiments, McCullagh (1932) established that:

1) castration causes two distinct changes - atrophy of the prostate and hypertrophy of the pituitary,

2) oil-soluble extracts from the testis (steroids) prevent atrophy of the prostate following castration,

3) similar oil-soluble hormones, in doses sufficient to prevent prostatic atrophy, do not prevent pituitary hypertrophy,

4) aqueous extracts of the testis do not regenerate the prostate, but do prevent pituitary hypertrophy, and

5) destruction of the germinal epithelium causes pituitary hypertrophy without affecting the prostate.

McCullagh concluded that the testis produced two hormones - andro tin, an oil-soluble hormone responsible for maintenance of the accessory sex glands, and inhibin, a water-soluble hormone which acted on the pituitary.

1.4.3 Evolution of the Inhibin Hypothesis until 1976

Moore & Price (1932) not only showed that oil-soluble testicular hormones maintained the accessory sex glands, but also that these hormones very likely inhibited gonadotrophin secretion. McCullagh (1932) had admitted that it was possible that andro tin might reverse pituitary hypertrophy at a dose higher than was needed to prevent atrophy of the prostate. This was subsequently shown, and taken as evidence that a single testicular hormone was responsible for control of the pituitary and the accessory sex glands (Nelson, 1937).

While McCullagh & Walsh (1935), McCullagh & Schneider (1940), Vidgoff et al., (1939), and Vidgoff & Vehrs (1940) presented further evidence for ant gonadotrophic activity in aqueous extracts of testes, others could not find any activity (Nelson & Gallagher, 1935; Rubin, 1941). Other evidence questioned the need for inhibin. Spermatogenesis could be maintained by androgens alone, and cryptorchidism was shown to eventually decrease the weights of the accessory sex glands, so it was argued that the appearance of castration cells after cryptorchidism could arise solely from lack of androgens (Nelson, 1937).

It is not clear when the proposed role for inhibin changed from
The growth of research on inhibin is illustrated by the number of publications indexed under "inhibin" and "folliculostatin" (an alternative name for inhibin in the female) in Biological Abstracts and Bioresearch Index between 1970 and 1979. There were no entries before 1970.
negative feedback on gonadotrophins in general to feedback on FSH specifically. There is no mention of separate control of the two hormones by Nelson (1937) or Johnsen (1964). The distinction was undoubtedly aided by the obvious action of testosterone on LH secretion, and the development of bioassays capable of measuring FSH and LH activity in urine and later in blood. Between 1940 and 1970 much evidence accumulated to suggest that the germinal epithelium controlled the secretion of gonadotrophins, and FSH in particular, and that androgens and estrogens were not totally responsible (reviewed by Setchell & Main, 1974; Setchell et al., 1977). The idea that damaged tubules failed to utilise gonadotrophins and remove them from the blood was a short-lived alternative to the inhibin hypothesis (Heller & Nelson, 1948).

The discovery of nonsteroidal activity in bull sperm (Fachini et al., 1963), human seminal plasma (Franchimont, 1973) and ovine rete testis fluid (Setchell & Sirinathsinghji, 1972) renewed interest in inhibin. The study was aided by the advent of radioimmunoassays for FSH and LH, which were a great improvement over bioassays in their sensitivity, specificity and convenience. The activity from bull sperm was partially purified and identified as a peptide (Lugaro et al., 1969, 1973). Doses as small as 100 ng injected into the third ventricle of the hypothalamus suppressed serum FSH in castrated male rats and reduced the hypothalamic content of FSH-releasing activity (Lugaro et al., 1974). LH was unaffected. Protein extracts from bull and human seminal plasma suppressed only serum FSH when given to castrated male rats, and antisera to the extracts increased serum FSH when given to male rats (Franchimont et al., 1975a, 1975b). Rete testis fluid also suppressed FSH, but not LH, in intact and castrated male rats (Setchell & Jacks, 1974), and extracts of bovine testes selectively lowered plasma FSH in wethers (Lee et al., 1974).

Thus by the beginning of 1976, inhibin-like activity had been shown in the testis, rete testis fluid, and semen, and there was a theory on how the activities in these tissues and secretions were related. Setchell & Sirinathsinghji (1972) had suggested that inhibin might be secreted into the lumen of the seminiferous tubules and carried through the rete testis to the epididymis, where it would be absorbed and transferred to the blood. The presence of activity in seminal plasma was attributed to incomplete absorption in the epididymis (Franchimont et al., 1975b).
Figure 1.3 The reproductive tract of the bull (From Frandson, 1965).
This was the state of the art when this thesis began. Since then the study of inhibin has grown enormously (Figure 1.2). It has been encouraged because of inhibin's potential role in controlling male fertility - by suppressing spermatogenesis (and hence fertility) without affecting androgen production (and hence libido), and accordingly much of the research on inhibin has been funded by the World Health Organisation (World Health Organisation, 1976). Work published after 1975 is covered in Chapter 8.

1.5 ACCESSORY SEX GLANDS OF THE MALE

The testis provides the sperm, but nearly all the other constituents of seminal plasma come from the glands of the accessory reproductive tract.

1.5.1 Anatomy

The gross anatomy of the reproductive tract of the bull is shown in Figure 1.3. The testis is connected to the epididymis by the efferent ducts (see Figure 1.1b). Most of the fluid arriving from the testis is absorbed in the head of the epididymis (Setchell, 1970). The tail of the epididymis runs into the vas deferens. The vas deferens - enveloped in a layer of smooth muscle-, the testicular nerves, blood vessels and lymph vessels constitute the spermatic cord; the life-line to the testicle. Each vas deferens enlarges at its distal end into an ampulla and the ampullae and the pair of seminal vesicles empty into the urethra. Surrounding the urethra is the prostate, and more caudal, the bulbourethral glands (Frandsen, 1965). The portion of the tract from efferent ducts to ejaculatory ducts (linking the seminal vesicles with the urethra) is derived from the Wolffian duct during embryogenesis; the prostate and the rest of the genital duct arise from the urogenital sinus (Moore, 1974). The whole of the reproductive tract requires testosterone for its differentiation, development and maintenance (Neumann et al., 1969; Cavazos, 1975).

Not all species have a full complement of glands - the dog, for instance, lacks seminal vesicles, and the form of the glands can vary - the seminal vesicles are lobated in the bull, but hollow sacs in the horse (Frandsen, 1975). Function too can vary - citric acid in human seminal plasma comes mainly from the prostate, while the seminal vesicles are the
<table>
<thead>
<tr>
<th>Table 1.1</th>
<th>Some proteins from seminal plasma, and their physicochemical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>seminal plasmin</td>
<td>(bull seminal vesicle)</td>
</tr>
<tr>
<td>haemolytic factor</td>
<td>(bull seminal vesicle)</td>
</tr>
<tr>
<td>plasminogen activator</td>
<td>(human)</td>
</tr>
<tr>
<td>protease inhibitor</td>
<td>(human, boar)</td>
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<tr>
<td>prostatic binding protein/prostatein</td>
<td>(rat prostate)</td>
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<tr>
<td>uteroglobin-like protein</td>
<td>(rabbit seminal vesicle)</td>
</tr>
<tr>
<td>antispermaticogenic/antiembryonic substance</td>
<td>(bull seminal vesicle)</td>
</tr>
<tr>
<td>gonadotrophin-like protein</td>
<td>(human)</td>
</tr>
<tr>
<td>inhibin-like proteins</td>
<td>(human and bull)</td>
</tr>
</tbody>
</table>
main source in the bull (Mann, 1975).

Severence of the vas deferens is commonly called vasectomy. If the rest of the spermatic cord is left intact, vasectomy has no apparent ill effect on the testicular production of sperm or sex steroids, nor on the functioning of the accessory reproductive tract. However, inflammation, secondary to infection, or autoimmunity against the germinal epithelium, caused by leakage of sperm from the vas deferens, can impair spermatogenesis (Neaves, 1975).

1.5.2 Secretions

The glands and ducts of the male reproductive tract are secretory (Cavazos, 1975). The secretions are tailored to the needs of the spermatoza, and include nutrients (Mann, 1975), factors to induce maturation in the epididymis (Orgebin-Crist et al., 1975; Orgebin-Crist & Jahad, 1979), and stabilisers and metabolic inhibitors to enable storage of sperm (Harrison, 1978). Upon ejaculation the sperm are exposed to other substances that render them fertile and aid their transport to the ovum. Ejaculation mixes the contents of the different glands, combining, for instance, enzymes and substrates for coagulation and liquification of semen (Mann, 1975).

Seminal plasma contains proteins with biological activity. Some clearly affect the sperms' well-being and function. These include acrosome stabilisers and decapacitation factors (Fritz et al., 1976; Harrison, 1978); inhibitors of oxidative metabolism (Trifunac & Bernstein, 1976); and a protein which confers upon sperm the ability to swim forward (Brandt et al., 1978). The role of other proteins can only be surmised. These include (references in Table 1.1) uteroglobin-like protein - also seen in early pregnancy in some species; a very abundant sex steroid-binding protein (distinct from ABP) in the rat prostate; seminal plasma - a bacteriocide that also inhibits RNA and protein synthesis in sperm; and an antispermatic substance (related or identical to seminal plasmin?) that inhibits sperm production and development of embryos, and reduces overall body weight.

Seminal plasma also contains proteins with hormonal activity. These include inhibin-like activity in human and bull seminal plasma, which is capable of suppressing FSH concentrations in rats (Franchimont et al.,
1975a), and of inhibiting the hCG-induced increase in ovarian weight in immature rats (Chari et al., 1976). Human seminal plasma has also been reported to contain hCG-like activity that increases ovarian weight in hypophysectomised rats (Smith et al., 1978). The two activities are not mutually exclusive, since Chari et al.'s (1976) assay incorporated a maximal dose of hCG. The pituitary hormones FSH, LH and prolactin are present in human seminal plasma in amounts comparable to blood (Dericks-Tan et al., 1977).

It is not known whether hormones in seminal plasma exert a local effect on the glands and ducts they occupy. But the high concentrations of androgens (Ganjam & Amann, 1976; Purvis et al., 1976) - which systemically have a trophic effect on the accessory reproductive glands, - and prostaglandins (Kelly et al., 1978) - which stimulate the smooth muscle that participates in ejaculation, - suggests that some hormones do have a local effect. The seminal vesicles and prostate have receptors for prolactin (but not FSH or LH), and recently prolactin has been shown to directly affect the metabolism of sperm (Sheth et al., 1978).

1.6 AIMS OF THIS THESIS

This thesis aimed

1) to develop a convenient method for detecting and measuring inhibin-like activity,
2) to confirm the presence of inhibin-like activity in bull seminal plasma,
3) to locate the activity's tissue of origin, and its route into seminal plasma,
4) to purify and isolate the active factor, and
5) to determine the factor's physicochemical properties.

The pure material was to be used as a source of inhibin-like activity

6) to explore inhibin's real effect on gonadotrophin secretion.

In practice the activity was only partially purified, so the term "antigonadotrophic activity" has been applied to the FSH- and
LH-inhibiting ability of bull seminal plasma extracts. Its relationship to the inhibin-like activities described by others is discussed in Chapter 8.
CHAPTER 2 MATERIALS AND METHODS

2.1 RATS

2.1.1 Origin

Rats derived from the Sprague-Dawley strain were bred at the Surgical Laboratory, Green Lane Hospital, and at the Animal Laboratory, Middlemore Hospital, Auckland. Brattleboro rats came from a colony kept at the Animal Laboratories, School of Medicine, University of Auckland. These were descended from members of a litter of a pure strain of Long-Evans hooded rats from Brattleboro, Vermont, U.S.A., which had exhibited a spontaneous mutation for diabetes insipidus along with an independent mutation for albinism (Valtin et al., 1962).

2.1.2 Housing and Care

The rat rooms at Green Lane and Middlemore had outside windows so the rats were exposed to a natural photoperiod. A room temperature of 21°C was aimed at, but actual temperature varied between 15 and 25°C. Food and water were always available. Rats bred at Middlemore were transferred to Green Lane at least two days before use. All experiments were approved by the Animal Ethics Committee of Green Lane and National Women's Hospitals.

2.1.3 Castration Technique

Briefly, the testes were excised through a scrotal incision under aseptic conditions while the rats were anaesthetised with penthrane (methoxyflurane) and nitrous oxide.

The testes were manipulated into the scrotum, the scrotum was washed with antiseptic solution, a trans-scrotal incision was made, and one testis exposed. The spermatic cord was clamped above the pampiniform plexus, and cut below the clamp. After 2-3 min. the clamp was removed and the other testis excised. The wound was closed by a single loose suture. Castration took 8-10 min.
2.1.4 Allocation of Rats to Treatment Groups

Rats from the same litter (bred at Green Lane) and rats from the same batch (Middlemore) were distributed among the various control and treatment groups of an experiment as evenly as possible. Once the allocation had been made for the litter or batch as a whole, littermates were randomly assigned to particular groups. Rats with small testes and those greatly different in size from their littermates were excluded.

2.1.5 Administration of Test Substances

Test substances were administered immediately after castration, or under light penthrane anaesthesia in the case of intact rats. There were two exceptions. In early experiments with chronically castrated rats, injections were given without anaesthesia, and in experiments following Nandini et al.'s (1976) method, injections were given under ether anaesthesia.

Water-soluble substances, dissolved in 0.5 or 0.1 ml saline, and bull seminal plasma, diluted to 0.5 ml with saline, were injected intraperitoneally. Testosterone propionate (Sigma Chemical Co., St Louis, Missouri, U.S.A.) was dissolved in a small amount of ethanol and this solution mixed with peanut oil so that the rats received 1 ml/kg body weight (BW). The oil solution was injected subcutaneously. Testosterone (Sigma Chemical Co.) was packed into capsules which were inserted subcutaneously. Rats assigned to control groups received saline, peanut oil, or an empty capsule as appropriate.

The capsules were prepared as by Aafjes et al. (1978). Silastic tubing, 2 mm internal diameter and 4 mm external diameter (cat.no.602-304, Dow Corning Corp., Midland, Michigan, U.S.A.) was cut into lengths 5 mm longer than the column of testosterone required. One end was sealed with a 2.5 mm plug of Silastic Silicon Type A adhesive (cat.no.890, Dow Corning Corp.), and testosterone was packed to the desired length. The inside wall was washed with a drop of acetone, and the 2.5 mm length of tube remaining was sealed with adhesive. The testosterone content of the first batch of capsules was checked by weighing them before and after packing. The amount of testosterone averaged 1.93 mg/mm, close to 2.5 mg/mm reported for the same sized tubing by Aafjes et al. (1978). There was little variation in packing density; coefficients of variation
of capsules containing 5, 10 and 15 mm testosterone were 4.6, 5.5 and 1.8%.

The capsules were washed in three changes of phosphate-buffered saline at room temperature during the 24 h before implantation to avoid an initial surge of steroid when the capsules were implanted into the rats (Kincl & Rudel, 1971). Fur was clipped from the nape, the patch swabbed with antiseptic solution, and a 3 mm incision made across the skin. A pocket was made beneath the skin by pushing in a closed pair of forceps. The capsule, previously washed in antiseptic solution, was inserted. The wound was left open - tension of the skin kept the capsule in place. Capsules were removed under penthrane anaesthesia.

2.1.6 Blood Sampling

Decapitation without anaesthesia was chosen as the method to collect blood. This avoids the changes in blood hormone concentrations that anaesthesia can cause, and if done promptly forestalls the effects of disturbance stress (Döbler et al., 1976).

The animals were stunned with a blow to the back of the neck, decapitated, and trunk blood was collected through a funnel into a plain glass tube. The blood was left to clot for 24 h at 4°C, was then centrifuged, and the serum was decanted and stored at -20°C. All rats were killed within 8 min. of removing the cage from the rat room and within 30s of handling. Thus bleeding was probably rapid enough to avoid the effect disturbance stress has on blood gonadotrophin concentrations, since Döbler et al. (1977) found that FSH and LH concentrations were only slightly raised 5 and 10 min. after disturbance, although they were significantly elevated after 15 min. As a precaution, the killing order of the rats in each cage was carefully randomised.

When sequential blood samples were required, rats were anaesthetised within 1-2 min. with chloroform or ether, and 300 μl blood withdrawn from the heart using a syringe previously rinsed with heparin solution. The blood was put into tubes containing heparin, kept at 4°C for 1-2 h, and the plasma collected after centrifugation.
2.1.7 Pituitary Samples

Whole pituitaries were dissected out within 5 min. of decapitation, snap-frozen on dry-ice, and stored at -20°C. Each pituitary was homogenised in 100 µl 0.01 M phosphate-0.15 M NaCl, pH 7.6, at 4°C. The homogenate was made up to 1 ml with the buffer, centrifuged at 3000g, 20 min., 4°C, and 500 µl of the supernatant was decanted and stored at -20°C. The protein content of the supernatant was determined in duplicate 100 µl samples by the method of Lowry et al. (1951), using bovine serum albumin (BSA) (Sigma Chemical Co.) as the standard. The coefficient of variation of samples and standards throughout the protein assay was 2%.

2.2 MEASUREMENT OF FSH AND LH

2.2.1 Introduction

The concentrations of FSH and LH in rat sera and pituitary extracts were measured by radioimmunoassay. In a radioimmunoassay the hormone in the sample and a trace amount of added radioactively-labelled hormone (tracer) compete for binding sites on antibody molecules. Thus the proportion of radioactivity bound to antibody will depend on the amount of hormone in the sample. The antibody molecules are separated from unbound hormone, and the amount of radioactivity associated with the antibody fraction is determined. Commonly, the antibody is precipitated by adding an antibody to the antibody - the "second antibody method". The amount of radioactivity precipitated in the absence of added hormone is termed maximum binding (B₀), and is often expressed as a percentage of the total amount of radioactivity added. Radioactivity precipitated in the absence of antibody is called background.

2.2.2 Reagents

The following reagents were provided by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), National Institute of Health, U.S.A., under its Rat Pituitary Hormone Distribution Programme.

FSH for iodination, FSH-I-3, two batches potency 150x NIH-FSH-S1
<0.002x NIH-LH-S1
FSH antisera, FSH-S-6, 7 and 9  
FSH reference preparation, FSH-RP-1  
potency 2.1x NIH-FSH-S1  
LH for iodination, LH-I-4, two batches  
potency 1x NIH-LH-S1  
<0.04x NIH-FSH-S1  
LH antisera, LH-S-3 and 4  
LH reference preparation, LH-RP-1  
potency 0.03x NIH-LH-S1

The second antibody initially used was donkey anti-rabbit  
precipitating serum (RD 17, lot K1953B) purchased from Wellcome Reagents  
Ltd., Beckenham, England. Later antisera against rabbit γ-globulin were  
raised in a wether.

A γ-globulin-rich fraction was extracted from normal rabbit  
serum by ammonium sulphate precipitation. The method of Stelos (1967)  
was altered by precipitating with 35% saturated ammonium sulphate instead  
of with 33% to increase yield, and by eliminating the washing of the  
ic precipitate with 40% ammonium sulphate to curb loss. Polyacrylamide  
electrophoresis showed strongly staining globulin bands and a weak  
albumin band.

At fortnightly intervals, an emulsion of 5 mg of the preparation  
dissolved in 3 ml saline and 3 ml Freund's complete adjuvant (Commonwealth  
Serum Laboratories, Melbourne, Australia) was injected subcutaneously at  
several sites into the back of a wether. Blood samples were taken at the  
same time. Precipitating antibodies were detected in the antisera by  
immunodiffusion in agar (Ouchterlony, 1968), and like the Wellcome reagent,  
the antisera showed several bands at higher concentrations and one band at  
lower concentrations. Antisera were assessed in the FSH and LH  
radioimmunoassays. The plot of the amount of radioactivity precipitated  
versus the antiserum dilution varied over the immunisation period, both  
in its position along the dilution axis (titre), and in its shape. Some  
antisera showed a sharp peak, others a plateau. Only antisera showing a  
plateau centred at a working dilution of 1:8 or greater were used.

2.2.3 Assay Procedure

The iodination method and incubation conditions for the  
radioimmunoassays followed the outline provided by NIAMDD.
2.2.3.1 Iodination

Iodine-125 was attached to FSH and LH by the chloramine-T method of Greenwood et al. (1963).

To 2 μg FSH-I-3, or LH-I-4, in 20 μl 0.01 M phosphate-0.15 M NaCl, pH 7.6 were added in turn -

- 25 μl 0.5 M phosphate, pH 7.6,
- 5-10 μl carrier-free Na$^{125}$I solution (Radiochemical Centre, Amersham, England) containing 500-1000 μCi,
- 15 μg chloramine-T in 10 μl 0.05 M phosphate, pH 7.6, and following a 40-60 s reaction time,
- 32 μg sodium metabisulphite in 25 μl 0.05 M phosphate, pH 7.6, and
- 100 μl 0.006 M KI-0.023 M sucrose.

Iodinated hormone was separated from products of small molecular weight, including unreacted iodine, by chromatography of the reaction mixture on a 12 ml column of Sephadex G-75. Fractions were evaluated by measuring their maximum binding, binding in the presence of FSH or LH, and background under normal assay conditions.

The proportion of radioactivity incorporated into the hormone and the degree of damage the hormone sustained were assessed by chromatoelectrophoresis (Berson et al., 1956; London et al., 1967) of a sample of the reaction mixture of Whatman 3 MM paper (W & R.Balston Ltd, Maidstone, England). The specific activity was calculated as the (proportion of radioactivity associated with protein)$\times$ (amount of radioactivity added)/(2 μg). Estimates so obtained agreed well with measurements made by the more accurate "self-displacement" method (Morris, 1976).

For FSH, a 60 s reaction time gave tracer with an average specific activity of 137 μCi/μg (range 101-185, n = 10 iodinations) with 500 μCi, and 188 μCi/μg (175-204, n = 3) with 1000 μCi. Since doubling the amount of iodine increased specific activity only 37%, 500 μCi was routinely used.

For LH, reaction time was shortened to 40 s to minimise damage to the hormone. Specific activity averaged 56 μCi/μg (41-69, n = 5). Later, for unknown reasons, iodination damage decreased, and by increasing the amount of chloramine-T to 25 μg the specific activity rose to 103 Ci/μg (82-129, n = 6). LH showed some vagrancy in its ability to be iodinated. Two solutions of LH-I-4(2) showed adequate incorporation
but much damage, while a third solution of the same material was very robust to iodination damage. Subtle differences in the dissolution of a hormone may contribute to its stability on iodination.

After one year's storage in solution, FSH-I-3(1) and LH-I-4(1) did not iodinate as well. Maximum binding was low, and fell sharply with the fraction number off the G-75 column, although the sensitivity of the assays was unchanged. Consequently, after the receipt of the second batches of hormone, FSH-I-3(2) and LH-I-4(2), fresh solutions were made up every six months. Maximum binding (average, range, no. assays) was for FSH-I-3(1): 19% (9-34, n = 29), FSH-I-3(2): 34% (23-47, n = 15), LH-I-4(1): 26% (17-43, n = 26), LH-I-4(2): 33% (29-38, n = 7). Background averaged 1.1% (0.7-2.1) for FSH, and 1.0% (0.8-3.2) for LH.

2.2.3.2 Assay Procedure

The assays were set up as follows. Reference preparations were added at 10-1000 ng for FSH-RP-1, and 1-100 ng for LH-RP-1, dissolved in 1% BSA-0.01 M phosphate-0.15 M NaCl-0.01% NaN₃, pH 7.6 (assay buffer). For sera, 100 and 200 μl of each sample were assayed, for pituitary extracts, 50, 1000 and 200 μl of 1:100 dilution of the original extract was assayed for FSH, and 10, 20 and 50 μl of the 1:100 dilution for LH. The volume of each tube was made up to 500 μl with assay buffer. 100 μl of tracer, made up in 0.1% BSA-0.01 M phosphate-0.15 M NaCl-0.01% NaN₃, pH 7.6, and containing about 2 x 10⁸ cpm/μl, was dispensed to each tube.

FSH antisera were diluted to 1:2500, LH antisera to 1:10 000, in 0.05 M EDTA-0.01 M phosphate-0.15 M NaCl-0.1% NaN₃, pH 7.6, containing 0.5-0.75% rabbit serum, and 200 μl of this solution was added to each tube. Tubes without references or samples provided maximum binding; tubes with rabbit serum but without antiserum provided background.

After being mixed with a vortex stirrer, the tubes were incubated for 24-48 h at room temperature. 200 μl of second antibody solution was then added, and the tubes were left for 20-24 h. The tubes were centrifuged at 4000 g, 20 min., 4°C, the supernatant was decanted, and the radioactivity in the precipitate counted. A standard curve of the amount of radioactivity bound against the quantity of reference preparation was drawn by hand, and the amount of hormone in the samples read off.
2.2.4 Increasing the Sensitivity of the LH Radioimmunoassay

Serum concentrations of LH in intact rats were found to be near or below the sensitivity of the radioimmunoassay when the procedure in section 2.2.3.2 was used. One method that may increase sensitivity is preincubation of hormone (references and samples) with antibody before addition of tracer (Rodbard et al., 1971). Under these conditions the tracer must "displace" the hormone from the antibody. (This is a macroscopic view. At the molecular level the tracer can only bind to the antigenic site if the site is free; the dissociation rate as well as the association rate of hormone and antibody become important.) The rate at which a reaction approaches equilibrium, as well as the position of equilibrium, depends on temperature. Although others have used preincubation or incubations at 4°C to increase the sensitivity of the NIAMDD LH assay (Eldridge et al., 1974; Griffiths et al., 1975; Collins et al., 1978), no systematic study of choosing the best conditions has been published. Accordingly, the effects of preincubation and temperature were studied.

Preincubation for 24 h increased sensitivity (binding at 90% B₀) approximately three-fold, and for 48 h six-fold, in the experiment shown in Figure 2.1a. Longer times had little further effect. Increasing the incubation time from 24 to 48 h diminished the increase in sensitivity conferred by preincubation. Presumably the longer the incubation with tracer, the more time to reapproach equilibrium.

Figure 2.1 Improving the sensitivity of the LH radioimmunoassay.
a) The sensitivity of the LH assay was increased by preincubating samples with antibody before addition of tracer. The conditions were:
   no preincubation and 24 h incubation (●), 24 h preincubation and 24 h incubation (△), 24 h preincubation and 48 h incubation (▲), 48 h preincubation and 24 h incubation (■), 48 h preincubation and 48 h incubation (○). Means ± standard deviation of triplicate measurements.
b) Characteristics of the modified LH assay, showing increased sensitivity with a preincubation of 72 h (●) compared with no preincubation (●), and the parallel displacement of tracer by the reference preparation (●), a serum sample (○), and a pituitary extract (▲). The reference preparation was measured in triplicate, the serum and the pituitary extract in duplicate.
Incubation at 4°C left sensitivity largely unchanged but increased maximum binding by 30%. In further experiments, preincubations of 24 and 48 h at 4°C both increased sensitivity three-fold, which was not as much as a 48 h preincubation at room temperature. Combinations of preincubations at 4°C or room temperature with incubations the other way around were inferior to room temperature throughout. In all, inclusion of steps at 4°C offered no advantages as far as increasing sensitivity.

The LH assay was therefore modified by incorporating a preincubation of 48-72 h before 24 h incubation with tracer. For experiments with castrated rats, 50, 100 and 200 μl of each serum sample were assayed; for experiments with intact rats alone, 100 and 200 μl were assayed.

2.2.5 Characterisation and Quality of the Radioimmunoassays

The NIAMDD rat FSH and LH radioimmunoassay reagents and procedures have been widely used and are well characterised (Monroe et al., 1968; Swerdloff et al., 1971). Hence this section considers only the quality of the assays as used in this thesis. The principles for assessing properties and quality of radioimmunassays have been reviewed by Midgley et al. (1969) and Rodbard (1974).

2.2.5.1 Specificity and Validity

Specificity is freedom from interference by other substances so that only the hormone intended is measured. An assay is valid if hormone in the reference preparation and samples behaves in the same way. One indication that the required hormone is indeed being measured and that the sample composition is not affecting its measurement is by demonstrating superimposable dose-response curves for samples and reference preparation. Superimposable curves are parallel to each other when hormone content is plotted on a logarithmic scale.

Samples of rat serum, plasma and pituitary extracts gave curves parallel to the FSH-RP-1 standard. Similarly, serum, plasma and pituitary extracts were parallel to LH-RP-1 with the original and preincubation procedures (Figure 2.1b). FSH-RP-1 and FSH-I-3 gave parallel curves, as did LH-RP-1 and LH-I-4.
Table 2.1  Between-radioimmunoassay variation indicated by the quality control sera A, B and C.

(mean concentration (µg/l), coefficient of variation (%), numbers of assays)

<table>
<thead>
<tr>
<th></th>
<th>FSH</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>1825, 12.4 (10)</td>
<td>1025, 19.5 (10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 2</td>
<td>919, 10.4 (24)</td>
<td>1138, 13.0 (27)</td>
<td>601, 11.4 (27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 3</td>
<td>871, 10.2 (6)</td>
<td>1125, 6.2 (6)</td>
<td>637, 10.9 (6)</td>
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<td>328, 16.6 (9)</td>
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<td>Set 2, normal</td>
<td>155, 10.0 (7)</td>
<td>247, 14.4 (9)</td>
<td>77, 14.3 (8)</td>
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<td></td>
<td>preincubation</td>
<td>106, 12.2 (4)</td>
<td>191, 13.5 (4)</td>
<td>62, 13.3 (4)</td>
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</tr>
<tr>
<td>Set 3, preincubation</td>
<td>100, 8.6 (9)</td>
<td>184, 8.5 (8)</td>
<td>62, 12.2 (9)</td>
<td></td>
<td></td>
</tr>
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</table>
2.2.5.2 Sensitivity

Sensitivity is defined as the smallest amount of hormone that can be distinguished from no hormone, and it is usually taken as the lower 95% confidence limit of maximum binding. When 9-15 tubes for maximum binding were run throughout the FSH and LH assays, the limit averaged 96% (range 93-97).

In practice, sensitivity was chosen as 90% $B_o$ for the 200 μl serum sample and 95% $B_o$ for the 100 μl sample. If either the 200 or 100 μl sample showed binding above these limits the sample was considered to have a concentration below the assay's sensitivity. For the LH assay without preincubation, 90% $B_o$ ranged over 3-6 ng LH-RP-1, while with preincubation it was 0.7-1.4 ng, giving sensitivities of 15-30 μg/l and 3-7 μg/l respectively. For FSH, 90% $B_o$ was 10-36 ng FSH-RP-1, giving a sensitivity of 50-180 μg/l. No FSH samples were below the sensitivity of the assay, and few were below 300 μg/l.

2.2.5.3 Reproducibility

An assay is reproducible if estimates of a hormone concentration are the same upon repeated measurement of the sample. Degree of reproducibility was assessed by running quality control samples in each assay. Three sets of samples were used over the three years of this thesis. The second and third sets were made up by pooling sera from previous experiments to provide - A: a general pool, C: a low FSH (<600 μg/l) and low LH (<180 μg/l) pool, and B: a high FSH and high LH pool. The dividing lines fell midway along the ranges of FSH and LH concentrations seen in most experiments with castrated rats.

In each assay, six replicates of 100 μl A were assayed to give an estimate of within-assay variation, and B and C were assayed at the usual volumes to estimate between-assay variation (Table 2.1). The overall within-assay coefficient of variation was 5% for both assays, ranging between 1 and 12% for individual assays. Strictly these estimates apply only to that particular part of the standard curve, which was 50% $B_o$ for the FSH assay and for the LH assay with preincubation. In practice they probably apply to the region of 20-70% $B_o$ (Rodbard, 1974), where most of the samples fell. The between-assay coefficient of variation
averaged 11% for FSH and 12.5% for LH. To reduce error, all samples from a particular experiment were assayed together. Assays with quality control values too high were rejected and the samples in them reassayed.

Curiously, LH estimates were 20-30% lower when the preincubation step was included. The reason for this was not investigated.

Many intact rats had LH concentrations near the sensitivity of the radioimmunoassay, where precision is lower than in the middle regions. Indeed concentration estimates from the 200 and 100 µl volumes of the same sample often differed by 20-30%, implying within-assay coefficients of variations of 14-20%. However, the variation in these estimates was dwarfed by the variation between animals in the same experimental group; LH concentrations often spanned from below 5 µg/l to above 50 µg/l.

To check that the low concentrations of LH apparent in intact rats were actually due to LH and not to nonspecific effects of serum, LH-free serum should have been tested. In its place, sera was obtained from 21 intact rats which had been injected with 10 mg testosterone propionate/kg BW. This dose exerted maximal negative feedback on LH secretion (see Section 7.4.2) so that these sera should contain very little LH. All 21 sera showed binding greater than 93% B₀ at 200 µl and 96% B₀ at 100 µl, so that none had detectable LH concentrations. These samples were pooled, and included in every subsequent assay; the apparent LH concentration was always below assay sensitivity.

2.3 REPRESENTATION AND ANALYSIS OF FSH AND LH CONCENTRATIONS

2.3.1 Representation

Serum FSH and LH concentrations were assumed to be log-normally distributed, because of the wide range of concentrations encountered (the arithmetic mean minus two standard deviations would often have been negative) and because the spread of values about their average increased as the average value increased. Treating data in this way puts more emphasis than does the normal distribution on low values, an important consideration when one is looking for suppression. It also lessens the impact of the occasional rat that, unlike fellow members of its treatment group, does not respond. Accordingly, each group is represented by the geometric mean and its 67% confidence interval, which are analogous to the
arithmetic mean ± standard error of the mean for normally distributed data. The hormone concentrations are expressed in terms of the reference preparations FSH-RP-1 and LH-RP-1.

When more than half the members of a group had LH concentrations below the radioimmunoassay's sensitivity, individual concentrations are shown. Otherwise LH concentrations below assay sensitivity were assigned a value equal to the sensitivity and the geometric mean was calculated.

Occasionally very high concentrations of LH were observed (see Figure 3.8). If these were both greater than 500 µg/l and above the 95% confidence interval of the log-transformed data for the rest of the group, they were omitted. Very high concentrations were probably caused by some extraordinary process so they cannot be considered as part of the ordinary distribution of hormone concentrations.

2.3.2 Analysis

In all but one instance (see Figure 7.8) statistical analysis was applied to a priori comparisons, that is, to test the questions the experiment was designed to answer. Most commonly the one-tailed Student's t-test was applied to log-transformed data to see if treatment suppressed FSH or LH concentrations. The one-tailed Mann-Whitney test (Snedecor & Cochran, 1967) was used when the data could not be assumed to be log-normally distributed. In practice this was when the LH concentrations of two groups of intact rats were compared, and for castrated rats, when more than half the LH concentrations of one group were below the assay sensitivity.

When several pair-wise comparisons are made in an experiment the probability of "finding" a difference when it is not really present increases with the number of comparisons. One remedy is to limit the overall error rate for a family of comparisons. However what should constitute a family is debatable (see Dunnett, 1970). For instance, in analysing a gel exclusion chromatography, are control groups (e.g., those receiving a dose of bull seminal plasma-extract known to be active, or a recombination of fractions) and the set of groups receiving individual fractions separate families? Or, should FSH and LH data belong to the same or separate families? To bypass this problem, experiments were designed where possible to look for trends, rather than pick out individual
differences, and test substances were screened at doses that were predicted to cause a clear response. The number of comparisons within an experiment was kept to a minimum.

A posteriori comparisons (Figure 7.8) were tested by one way analysis of variance.

2.4 BIOLOGICAL MATERIALS

All biological materials were stored at -20°C.

2.4.1 Bull seminal plasma

Seminal plasma from intact bulls (bSP) was supplied by the N.Z. Dairy Board Artificial Breeding Centres at Newstead, Hamilton (batch #1), and Awahuri, Palmerston North (batches #2 to 7). Batch #1 was a pool from 20 young bulls of proven fertility, and batches #2 to 7 were each pools from 5 bulls. Sperm had been removed and the plasma had been passed through 5 or 8 μm filters.

Seminal plasma from vasectomised bulls (vbSP) came from the same sources. Batch #1, from Newstead, was a pool from 5 bulls, while batches #2 to 7, from Awahuri, were from individual bulls, 2 ejaculates each. All bulls had been vasectomised over a year before semen collection. They had shown good fertility, but had not been used for breeding for genetic reasons.

2.4.2 Ejaculate from Androgen-treated Steers

These samples were kindly provided by Dr J.J.Bass, Ruakura Animal Research Centre, Hamilton. Six steers were injected twice, a fortnight apart, with testosterone esters, and were electroejaculated on two successive days a fortnight after the second injection. The animals were slaughtered, their seminal vesicles removed, and the absence of testes confirmed.

2.4.3 Bull Seminal Vesicle Fluid

Bull seminal vesicles were collected from the chain at the
Westfield Freezing Company's works, Westfield, Auckland, and put on ice. Within 3 h the tissue was cut into slices and the fluid extruding was scooped up on the lip of a cooled beaker. The fluid was yellowish red, viscous, and contained few sperm or red blood cells. It was centrifuged at 10 000 g, 20 min, 4°C, and the supernatant - bull seminal vesicle fluid (bSVF) - was snap-frozen.

2.4.4 Ovine Follicular Fluid

Two samples of ovine follicular fluid (oFF) from two Dorset ewes were provided by Dr K.P. McNatty, Wallaceville Animal Research Centre, Upper Hutt.

2.4.5 Rat Testes

Testes from five-week old rats were placed in ice-cold saline immediately after their excision. They were frozen within 3 h of collection.

2.5 METHODS OF EXTRACTION, PURIFICATION AND ANALYSIS OF BIOLOGICAL MATERIALS

2.5.1 Extraction of bSP and oFF by ethanol precipitation

Protein was extracted from bSP, and oFF, by addition of cold ethanol to a concentration of 86% (v/v) (Franchimont et al., 1975a).

The bSP was thawed at room temperature, but subsequent steps were done at 4°C. To 1 volume of fluid was added 6.1 volumes of cold ethanol over 15 min., the mixture being stirred constantly. After a further 15-30 min. stirring, the precipitate was recovered by centrifugation at 3000 g, 10 min., 4°C, then dissolved in water and freeze-dried. The fluffy white powder obtained was stored at -20°C.

The oFF was thawed, and the small amount of cryoprecipitate removed by centrifugation before ethanol extraction. After extraction as above, the precipitate was washed with five changes of five volumes of cold acetone to remove steroids (Daunre et al., 1977). The precipitate was dissolved in water and freeze-dried.

Ethanol precipitation was also used to fraction bSP. On an analytical scale, ethanol was added to 0.5 ml aliquots of bSP to a
concentration of 10, 20, ..., or 90% (v/v). On a preparative scale, ethanol was added to 5 ml aliquots of bSP to a concentration of 30, 50 or 86% (v/v). The mixtures were centrifuged and ethanol was added to the supernatants to bring the concentration to 86% (v/v). Precipitates were recovered as before.

2.5.2 Extraction of Rat Testes

Rat testes were extracted by the method Keogh et al. (1976) used for bovine testes. Stored testicles were thawed and the testis dissected free from the epididymis and fat pad. The batch of testes was weighed and an equal weight of cold 0.01 M phosphate-0.15 M NaCl, pH 7.6, added. The batch, cooled on an ice bath, was homogenised for 60 s in a Virtis homogeniser (The Virtis Co., Gardiner, New York, U.S.A.). The homogenate was filtered through cheesecloth to remove large debris, then centrifuged at 27 000g, 30 min., 4°C. The supernatant was extracted twice with three volumes of diethylether. Residual ether was removed from the aqueous phase by rotary evaporation before the extract was dialysed and freeze-dried.

2.5.3 Denaturation of Protein

Extracts of bSP and vbSP were heated and exposed to pepsin to denature protein.

For heat denaturation, 400 mg bSP-extract (#1-5), or 230 mg vbSP-extract (#1-1) was dissolved in 10 ml water and heated on a water bath at 67°C for 1 h. The solutions were then snap-frozen and stored at -20°C.

For pepsin digestion, 400 mg bSP-extract, or 230 mg vbSP-extract (same batches as above), was dissolved in 5 ml water. 2 M glycine and 2 M HCl were added to buffer the solution at pH 2, and they provided an ionic strength of 0.2 when the solution was diluted to its final volume of 10 ml. Pepsin (Sigma Chemical Co.) was added at 1 mg per 50 mg of extract. The solutions were incubated at 37°C, 24 h, then neutralised with 2 M NaOH, snap-frozen, and stored at -20°C.

2.5.4 Dialysis

Dialysis was routinely used to remove small molecular weight substances, mainly salts and urea, from protein solutions. Solutions, up
to 500 ml at a time, were placed in Visking 16/32 tubing (Visking Co., Chicago, Illinois, U.S.A.) and dialysed at 4°C against at least four changes of 5 l distilled water, each change lasting 8-24 h. The number of changes was sufficient to ensure that the final salt or urea content of each sample was less than 1 mg.

2.5.5 Ultrafiltration

Ultrafiltration separates molecules on the basis of size. Small solutes and the solvent are forced through a porous membrane by hydraulic pressure (to form the expressate), thus concentrating large solute molecules (in the retentate). When solute is added to the retentate during ultrafiltration, the process is called diafiltration. Diafiltration was used to extract and fractionate bSP, and to remove salts and urea from column fractions. In the latter case, diafiltration was continued until the salt or urea content of the fraction was less than 1 mg.

Equipment was obtained from the Amicon Corp., Lexington, Massachusetts, U.S.A. A model 202 cell was fitted with UM 05, PM 10 or XM 100 membranes, which have nominal molecular weight cut-offs of 500, 10,000 and 100,000 daltons respectively. Hydraulic pressure was provided by oxygen-free compressed nitrogen, and all ultrafiltration took place at 4°C.

2.5.6 Gel Exclusion Chromatography

2.5.6.1 General

Gel exclusion chromatography separates molecules primarily on the basis of molecular size. The rate at which a molecule passes through the gel depends on the relative time spent in the stationary and moving phases. The stationary phase consists of solvent within various sized pores in the gel matrix; therefore the smaller the molecule the greater the proportion of stationary phase accessible to it, and the slower its journey. The following terms are used - \( V_t \): the total volume occupied by the gel, \( V_e \): the elution volume of a substance, \( V_o \): the void volume, which is the elution volume for a substance too large to enter the largest pores in the gel.

Sephadex G-75, G-100 and G-200 gels were obtained from Pharmacia
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<th>Column Characteristics for Gel Exclusion and Ion Exchange Chromatographies</th>
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<tr>
<td></td>
<td>Column Size (ml)</td>
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<tr>
<td><strong>Gel Exclusion</strong></td>
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<tr>
<td>Chromatography</td>
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<tr>
<td></td>
<td>160</td>
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<td>470</td>
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<td></td>
<td>2300</td>
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<td>16</td>
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Fine Chemicals, Uppsala, Sweden. Preparative chromatographies, the fractions of which were screened for antigonadotrophic activity, were performed at 4°C, while analytical runs were often done at room temperature. The dimensions, flow rates and volume per collection tube of the columns are listed in Table 2.2. The maximum sample loading was 0.5 mg/ml of gel, and the volume of the sample solution was 2% V_t. The absorbance of the effluent was measured at 260 or 280 nm, and the contents of appropriate tubes were pooled to form fractions.

Fractions were prepared for freeze-drying as follows. For the first preparative chromatography in a particular buffer, the fractions were concentrated and diafiltered against water using a UM 05 membrane. When it was known that the antigonadotrophic activity had a molecular weight greater than 10 000 daltons in that buffer, fractions were concentrated and diafiltered with a PM 10 membrane, or else concentrated then dialysed. The details for the various buffers -

0.01 M ammonium acetate: fractions were freeze-dried without treatment.
0.1 M ammonium acetate: fractions were concentrated to 100 ml with a PM 10 membrane, diafiltered until the salt concentration was 0.02 M, diluted two-fold with water, then freeze-dried.
0.05 M sodium acetate: fractions were initially concentrated to 100 ml and diafiltered with a UM 05 membrane. Later they were dialysed.
4 M urea-containing buffers: fractions were initially concentrated to 100 ml and diafiltered with a UM 05 membrane. Later a PM 10 membrane was used. Sometimes for subsequent ion exchange chromatography, fractions were concentrated to 20-50 ml, diafiltered against 6 volumes of the ion exchanger's starting buffer, and then concentrated to 20 ml, all with a PM 10 membrane.

The recovery of starting material from the fractions was 88-100% by weight, unless stated otherwise. The retention of material during re-equilibration for ion exchange chromatography was evaluated by monitoring absorbance at 280 nm, and it was always greater than 95%.

2.5.6.2 Molecular Weight Estimation

On Sephadex gels the elution volume of a protein is proportional to the logarithm of the protein's molecular weight (log MW) over much of the fractionation range (Andrews, 1964; 1965). Moreover, the distribution
coefficient $K_{av} = (V_e - V_o)/(V_t - V_o)$ is independent of protein concentration, column size and column packing (Whitaker, 1963).

The G-75 and G-200 columns were calibrated by running the following proteins as standards:

- Human serum albumin (hSA) (Sigma Chemical Co.), 68 000 daltons (a),
- Ovalbumin (oA) (Sigma Chemical Co.), 43 000 daltons (a),
- α-Chymotrypsinogen, Bovine Pancreas, Type II (α-Chym) (Sigma Chemical Co.), 25 700 daltons (a),
- Myoglobin, Whale Skeletal Muscle (Myo) (Calbiochem, San Diego, California, U.S.A.), 17 800 daltons (b),
- Cytochrome C, Horse Heart, Type III (Cyt C) (Sigma Chemical Co.), 12 400 daltons (b),

where values (a) are from Weber & Osborn (1969) and (b) from Andrews (1964). Blue dextran 2000 (Pharmacia Fine Chemicals) was used to indicate the void volume. The rabbit γ-globulin fraction described in section 2.2.2 was used in the calibration of the G-200 column. Some glycoproteins show anomalous behaviour on Sephadex gels, for instance, human γ-globulin with molecular weight 160 000 daltons has an apparent molecular weight of 205 000 daltons on G-200 chromatography (Andrews, 1965). Accordingly, the rabbit γ-globulin was assigned a value of 205 000 daltons for the purpose of column calibration.

For the 2.31 G-75 column equilibrated with 0.01 M ammonium acetate, pH 7.6, $V_e$ versus log MW was linear for Myo, α-Chym, and oA, with a regression coefficient $r = 0.997$ ($n = 7$), while hSA was just off the linear part of the curve. Cyt C had an apparent molecular weight of 17 000 daltons, which was unexplained. Making the buffer 0.15 M in NaCl did not change the elution volumes, and similar elution volumes were observed with 0.1 M ammonium acetate, pH 7.6, ($r = 0.975$, $n = 3$).

A 160 ml column equilibrated with 4 M urea-0.1 M ammonium acetate, pH 7.6, was calibrated instead of the 2.31 column to economise on urea. Myo, α-Chym, oA, and hSA all fell on the linear part of the $V_e$ versus log MW curve ($r = 0.996$, $n = 5$), and Cyt C almost did, with an apparent molecular weight of 14 000 daltons. The elution profiles of bSP extracts on the 160 ml and 2.31 columns were very similar so that calibration of the profile on the smaller column served to calibrate that on the larger one.
The 160 ml G-200 column was calibrated with Myo, hSA, and the rabbit γ-globulin preparation ($r = 0.998, n = 3$).

2.5.7 Ion Exchange Chromatography

An ion exchanger has charged functional groups, for instance the negatively charged carboxymethyl (CM) group. The mobile counter ion, which initially provides electroneutrality, can be exchanged for other ions with higher affinity for the carboxymethyl group. Substances with only slightly greater affinity have their passage through the ion exchange column retarded, while those of much greater affinity are bound, and can only be displaced by an increase in the ionic strength of the counter ion, or a change in the pH of the eluent.

Whatman CM 32 cellulose (V. & R. Balston Ltd.) was precycled, equilibrated, and packed into columns according to the manufacturer's instructions. Column dimensions, flow rates and fraction sizes appear in Table 2.2. Preparative chromatographies were done at 4°C.

Columns were developed in two ways.

In the first, termed starting buffer elution, the column was equilibrated and eluted with the same buffer. Sample loading was less than 1 mg/ml of gel, and the sample volume was less than 4% $V_t$.

In the second, termed gradient elution, the sodium concentration of the eluent was gradually increased. The NaCl gradient was usually 15 mM/$V_t$. Larger loadings and sample volumes were possible. Equally good resolution was achieved with loadings of 0.5 and 2 mg/ml of gel, but resolution was impaired at 5 mg/ml.

Urea and salt were removed from fractions by dialysis for analytical runs. For preparative chromatography, the fractions were concentrated to 50 ml on a PM 10 membrane, then dialysed. Later, to curb possible loss on dialysis, the duration of each dialysis change was shortened from 8-24 h to 2 h.

2.5.8 Polyacrylamide Gel Electrophoresis

Polyacrylamide electrophoresis (PAGE) was used to estimate the degree of homogeneity of protein samples and sometimes to identify their components. In PAGE, molecules migrate through a polyacrylamide gel
matrix because of an applied electric field. Their mobility depends on their net charge and their size relative to the pore size of the matrix. A gel with an acrylamide concentration of 7% (w/v) was chosen since it best separated those proteins in bSP that had mobilities similar to that of Chari et al.'s (1978) "bSP-inhibin". Gels were prepared by the method of Davies (1964), but without sample or spacer gels (Clarke, 1964).

The following solutions were used:

A: the gel buffer, 0.5 M HCl-3 M Tris(hydroxymethyl)aminomethane (Tris)-0.015 M N,N,N',N'-tetramethylethylenediamine (TEMED),

B: the catalyst, 0.006 M ammonium persulphate, and

C: the monomer, 4.5 M acrylamide-0.05 M methylene-bisacrylamide.

The gelling mixture was made by mixing A, B, C, and water in the proportions 7:4:5:16, it was deaerated, poured to form cylindrical gels (70 mm x 4.5 mm) or slabs (70 mm x 70 mm x 3 mm), covered with a layer of water and left to polymerise.

The electrode buffer was 0.384 M glycine-0.0495 M Tris, with \(10^{-7}\) M bromophenol blue in the anodic buffer to indicate the ion front during electrophoresis. Samples were dissolved in 15 µl electrode buffer, 2 µl 3 M sucrose was added to increase the density, and this solution was deposited onto the gel. Electrophoresis at 340V, was stopped when the ion front had migrated 70 mm. The cathodic buffer, which bathed most of the gel length, was cooled by reticulation through an ice bath. After electrophoresis, the gels were fixed in 0.75 M trichloroacetic acid (TCA) for 45 minutes, stained in 0.025% Coomassie Brilliant Blue R-250 (Mann Research Labs, Becton, New York, U.S.A.) -0.75 M TCA, and destained in several changes of 0.75 M TCA (Chrambach et al., 1967).

2.3.9 Polyacrylamide Gel Isoelectric Focusing

Polyacrylamide gel isoelectric focusing (PAGIEF) was used to indicate the degree of homogeneity of protein samples, and to provide an estimate of the isoelectric point of the proteins. In an electric field, the ampholytes form a pH gradient, which is protected from disturbance by the gel matrix. The charge a protein carries is a function of pH, and in an electric field superimposed on a pH gradient proteins migrate to the pH where they have no net charge - their isoelectric point.
Proteins were focused on cylindrical gels (70 mm x 4.5 mm or 60 mm x 3 mm) of 8% polyacylamide, with pH 3.5-10 or pH 4-6 Ampholine (LKB-produckter, Brommal, Sweden) providing the pH gradient. The gelling solution was prepared by mixing 0.5 ml solution A (see Section 2.5.8), 1.4 µl TEMED, 40 µl Ampholine, 0.48 ml water, and 1 ml solution C (see Section 2.5.8) containing the sample. The mixture was deaerated, poured into a tube, and left to polymerise.

The anodic buffer was 0.18 M H₂SO₄, the cathodic buffer 0.25 M NaOH. Voltage was increased as current fell so that power expended was 100 mW per larger tube and 50 mW per smaller tube. Focusing was stopped when the current had been steady for 30 min. at the highest voltage of 350V. The gels were stained for 24 h in 0.04% Coomassie Brilliant Blue G-250 (Sigma Chemical Co.)-0.35 M perchloric acid, and stored in 0.005% Coomassie Brilliant Blue-0.35 M perchloric acid (Reisner et al., 1975).

Isoelectric points were determined using the larger gels. Two gels with sample and three blank gels were focused together, the gel lengths measured, and the gels with the sample stained. The blank gels were cut into 3.3 mm slices, the slices were placed in 1.5 ml CO₂-free water in sealed tubes, and the tubes were gently shaken overnight to extract the ampholyte. The pH of the extracts was measured, and plotted against gel length. A regression line was fitted to the linear part of the curve.
CHAPTER 3 DETECTION OF ANTIGONADOTROPHIC ACTIVITY

3.1 INTRODUCTION

Central to the study of a hormone is the development of a method of detecting the hormone's presence. Since inhibin would, by definition, regulate FSH secretion by negative feedback, an obvious method would be the demonstration of lowered concentrations of FSH in the blood after administration of the hormone. The measurement of both FSH and LH would be useful, because preferential suppression of FSH would be the hallmark of inhibin.

The rat has many advantages as a test animal. It is small, its reproductive physiology is comparatively well understood, and rats were readily available, as were radioimmunoassays for measuring FSH and LH in rat blood. The male was the obvious sex. The castrated animal allows greater scope for detecting suppression of serum FSH and LH, since concentrations are higher than in the intact animal due to the removal of the testes - the source of endogenous feedback agents.

3.2 Chronically Castrated Rats

Chronically castrated adult male rats had been used by Franchimont et al. (1975a) to detect inhibin-like activity in bull and human seminal plasma, and by Setchell & Jacks (1974) to do the same in ovine rete testis fluid. Since bSP was also the source material in this study, Franchimont et al's (1975a) regimen was tried, although it was slightly modified by castrating the rats three instead of two weeks beforehand (Setchell & Jacks, 1974) by giving daily instead of twice daily injection. Doses of up to 1 ml bSP per day for 2 days failed to suppress FSH (Figure 3.1a), although the higher doses left the rats subdued and increased their urine output. Administration was subcutaneous rather than intraperitoneal through inexperience in injecting unanaesthetised rats.

Treatment was extended to three and four days in the second experiment, since the results of Setchell & Jacks (1974) had indicated greater suppression of FSH by ovine rete testis fluid on the third, fourth and fifth days of injection. Again no suppression of FSH, or LH, was observed (Figure 3.1b)
3.3 Acutely Castrated Rats

3.3.1 Suppression of the 24h Post-castration Rise of Serum Gonadotrophins

At the time of the experiments with chronically castrated rats, two reports were published describing the use of acutely castrated, 35-day old, male rats to detect inhibin-like activity (Davies et al., 1976; Nandini et al., 1976). Davies et al. (1976) stated that long-term castrated rats were unsatisfactory because the nature of the response to inhibin seemed to change with time after castration. A modified version of Davies et al's (1976) assay was tried.

Male rats were castrated and injected immediately afterwards. A dose of 0.5 ml bSP suppressed the rise in serum FSH and LH concentrations otherwise seen 24h after castration, both in adult and prepubertal animals (Figure 3.2). Five-week old rats offered a practical advantage over adults; the shorter the time between weaning and use the better, since cage space rather than breeding rate limited the number of animals available. Although intraperitoneal and subcutaneous injection worked equally as well, fluid accumulated under the skin when bSP (but not saline) was injected subcutaneously.

The degree to which FSH and LH concentrations were suppressed depended on the dose of bSP (Figure 3.3a).

The duration of treatment was extended to 48h in case the sensitivity of the method could be improved by exposing the rats to bSP for longer. The opposite appeared to be true (Figure 3.3a & b). This introduced the question of the time-course of bSP's action on FSH and LH.

Figure 3.1 Effect of bSP on serum FSH and LH in chronically castrated adult male rats.

Three weeks after castration, adult male rats (220-350g) were

a) injected subcutaneously with saline or 0.1-1.0 ml bSP (#1) daily for two days, then bled by decapitation 24h after the second injection, or

b) injected subcutaneously with saline or 0.2-0.5 ml bSP daily for

i) three or ii) four days, then bled by decapitation 4 h after the last injection.

Geometric means and their 67% confidence intervals.

Inside FSH bars; no.per group.
specifically, whether treatment for some period shorter than 24h would achieve greater sensitivity. Rats were castrated, injected, and sequential blood samples taken at 0, 3, 6, 12, 24 and 48h. The groups that received 0.2 ml and 0.5 ml equivalent of bSP-extract suffered high mortality rates - by 15h all had died, whereas none of the saline-treated rats died over the 48h. The experiment was repeated with similar results. However, two other experiments six months apart from each other provided a partial answer (Table 3.1).

Table 3.1 Serum FSH and LH 6h after castration in two experiments six months apart (Geometric means and their 67% confidence intervals).

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<th>Expt.</th>
<th>Condition</th>
<th>FSH (µg/l)</th>
<th>LH (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>saline</td>
<td>764 (727-804)</td>
<td>225 (196-259)</td>
</tr>
<tr>
<td></td>
<td>0.5 ml bSP</td>
<td>734 (695-776)</td>
<td>190 (153-235)</td>
</tr>
<tr>
<td>2</td>
<td>saline</td>
<td>572 (520-626)</td>
<td>&lt;30 - 46</td>
</tr>
</tbody>
</table>

a n = no. per group
b range

In the first experiment, LH concentrations 6h after castration were typical of those seen at 24h while FSH concentrations were lower. In the second experiment, both FSH and LH were typical of intact rats. Thus the rate at

Figure 3.2 Suppression of serum FSH and LH in acutely castrated male rats by bSP.

Immediately after castration, adult male rats were injected a) subcutaneously or b) intraperitoneally, and 35-38-day old rats were injected c) intraperitoneally with saline or 0.5 ml bSP (#1). Rats were bled by decapitation 24h after castration.

Geometric means and their 67% confidence intervals.

Inside FSH bars: s/n; n no. allotted per group, s no. surviving.

Significantly lower than the control group: * p <0.05, ** p <0.005.
a

- Treatment
- 24h
- Castration bleeding

b

- 24h
- 24h

Serum FSH (μg/l)

- 1500
- 1000
- 500
- 0

- 0.7 0.4 0.4 0.4 0.5 0.5 0.5 0.8

Serum LH (ng/l)

- 200
- 100
- 0

- 0 0.1 0.2 0.5

- 0 0.1 0.2 0.5

bSP (ml per injection)

- **
- *

- **
- **
- **
Figure 3.3  Effect of one and two days treatment with bSP on serum FSH and LH in acutely castrated five-week old rats.

Immediately after castration, rats were injected with saline or 0.1-0.5 ml bSP (# 1). 24h later, a) half were bled by decapitation, while b) the other half received a second injection and were bled by decapitation after another 24h.

Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05, ** p <0.005.
which serum LH (and possibly FSH) rises after castration varies. Moreover, bSP had not exercised its antigonadotrophic effect by 6h. Bleeding later that 6h after castration or much before 24h would be inconvenient in a routine method, so the treatment time of 24h was not changed. Later 24h was found to an optimum time for detecting the suppression of serum FSH and LH that followed a single injection of bSP-extract into intact rats. (see Section 7.3.3).

3.3.2 Other Designs

Nandini et al., (1976) described a method for detecting inhibin-like activity in an ovine testis extract in which rats were castrated on the evening of day 35 and injected 12-24h later. Blood samples were taken just before injection and 6h later. The preinjection sample served as a control, so only the response to the extract was measured; the response to castration itself did not contribute. The value of this can be seen in Figure 3.4. Five-week old rats were castrated, injected 24h later, and bled 0, 24, 30 and 48h after castration. Although no significant difference was detected between serum FSH levels of the saline and bSP treated groups (Figure 3.4), bSP had indeed suppressed FSH concentrations 6h after injection, as can be seen from the percent change in FSH (Figure 3.4b). This model, however, has some disadvantages; the main one is that it requires two blood samples per rat (with twice as many samples for radioimmunoassay) - one of which is taken under anaesthesia, with its consequent stress, and is necessarily of small volume.

Figure 3.4  FSH-suppressing activity of bSP measured by the system of Nandini et al. (1976).

Five-week old male rats were castrated and 24h later injected intra-peritoneally with saline (s/n = 10/10) or 40 mg bSP-extract (#1-5) (s/n = 6/10).

a) FSH levels of saline (——) and bSP-extract (----) treated groups.

b) Change in FSH concentrations upon treatment, taking the 24h concentration of each rat as 100%.

Geometric means and their 67% confidence intervals (a), and means ± standard error of the mean (b). n no. allotted per group, s no. surviving. Significantly lower than the saline treated group: *p <0.05.
Figure 3.5  Maximal suppression of FSH by bSP-extract in acutely castrated five-week old rats.

The bSP-extract (#1-5) was given at doses equivalent to 0.25 and 0.5 ml bSP. Geometric means and their 67% confidence intervals, and individual LH concentrations. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05, **p <0.005.
3.3.3 Design Adopted

The following method was adopted for routine screening for antgonadotrophic activity. Male rats five weeks old (32-39 days) were castrated, usually between 9 am and 1 pm, and immediately injected intraperitoneally with a solution of the substance to be tested. Between 23 and 25 h later the rats were bled by decapitation, and the FSH and LH concentrations in the blood measured by radioimmunoassay.

3.4 CHARACTERISATION OF THE METHOD ADOPTED

3.4.1 Dose-response

The degree serum FSH and LH levels were suppressed clearly depended on the quantity of bSP given. This is illustrated several times throughout this thesis (Figures 3.3a, 6.1, 6.4, 7.1 and 7.10, and Table 7.2). Dose-response was usually steep. Often a particular dose caused little or no suppression, but twice as much lowered FSH and LH to near intact levels. Occasionally a shallower dose-response was observed (Figure 6.1). It is possible that there was a maximal response, at least of FSH (Figure 3.5). Evidence was scanty and arose by chance; systematic investigation was hindered by the toxicity of larger doses of bSP (see Section 3.5.1).

3.4.2 Reliability

The experience with one batch of bSP and its extracts (#1-1 to 1-8) over a period of three years (8/1976-6/1979) is summarised in Table 3.2. Of the 21 experiments, 16 had a group that received 0.5 ml bSP, or 40 mg bSP-extract, and 12 had a group that received 0.2 ml, or 20 mg bSP-extract. For each experiment, the geometric mean hormone concentration of the bSP treated group was expressed as a percentage of the geometric mean concentration of the castrate control group. The mean and range of these percentages are tabulated, along with the proportion of treated groups that had means significantly lower than that of their respective control group. As a guide to the degree of suppression achieved, also shown are the hormone levels of intact rats, expressed in the same way, in 14 experiments (some of the 21 included) covering the same period.
Table 3.2  Response of acutely castrated five-week old rats to one batch of bSP (#1) over a three year period.

Hormone levels as a percentage of castrate control levels, and the degree of significance of their suppression.

<table>
<thead>
<tr>
<th></th>
<th>FSH</th>
<th></th>
<th></th>
<th>LH</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml bSP, or 40 mg bSP-extract</td>
<td>44.8</td>
<td>0/16</td>
<td>1/16</td>
<td>15/16</td>
<td>22.0</td>
<td>0/16</td>
</tr>
<tr>
<td></td>
<td>(34-66)</td>
<td></td>
<td></td>
<td>(13-31)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 ml bSP, or 20 mg bSP-extract</td>
<td>7/12</td>
<td>1/12</td>
<td>4/12</td>
<td>7/12</td>
<td>4/12</td>
<td>1/12</td>
</tr>
<tr>
<td></td>
<td>(46-96)</td>
<td></td>
<td></td>
<td>(26-96)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>intact rats</td>
<td>41.9</td>
<td>0/14</td>
<td>1/14</td>
<td>13/14</td>
<td>19.5</td>
<td>0/13</td>
</tr>
<tr>
<td></td>
<td>(33-52)</td>
<td></td>
<td></td>
<td>(10-37)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\) The geometric mean hormone concentration was expressed as a percentage of the geometric mean of the castrate control group for each experiment. The mean and range of these percentages is shown.

\(b\) The proportion of experiments in which the geometric mean hormone concentration was significantly lower than that of the castrate control group.

\(- p <0.05 \quad * p <0.05 \quad ** p <0.005\)
A dose of 0.5 ml bSP, or its equivalent, consistently suppressed serum FSH and LH to levels typical of intact rats. On the other hand, the effect of 0.2 ml bSP varied.

3.4.3 Sensitivity

The sensitivity of the detection method lay somewhere between 0.1 ml and 0.5 ml of the first batch (#1) of bSP. A dose of 0.1 ml, or the equivalent amount of bSP-extract, significantly suppressed serum FSH in only one, and LH also in one, out of eight experiments, while 0.5 ml, or equivalent, always suppressed FSH and LH (Table 3.2).

A dose of 0.2 ml bSP, or equivalent, had a variable effect. The cause could lie with the extract - the potency of bSP might change during storage; partial and variable inactivation might occur during extraction of bSP so that different preparations had different potencies; or the potency of the extracts might diminish with age. Alternatively, the sensitivity of the rats to the antigonadotrophic activity in bSP might change.

Of the 12 experiments listed in Table 3.2 that included a group receiving 0.2 ml bSP, or equivalent, seven took place within the span of a year and used the same preparation of bSP-extract (#1-8) (Table 3.3). The response within this subgroup fluctuated rather than changed in a monotonic way, which indicated the source of variation was within the rats (Figure 3.6).

The average body weight of the rats in the seven experiments was not uniform, and there was some correlation between this variable and the mean FSH concentration (as a percentage of the castrate control level) of the groups receiving 20 mg of the extract (regression coefficient \( r = 0.806, n = 7 \)). The probability that this relationship was spurious is less than 0.05 (Two-tailed Student's t-test). In contrast, there was no correlation between an individual's body weight and serum FSH concentration. (For the rats in the control groups, \( r = 0.032, n = 49 \); for the rats given 20 mg bSP-extract, \( r = 0.111, n = 45 \).) It is possible that a common factor influences average body weight and sensitivity to the antigonadotrophic activity of bSP-extract.
Table 3.3  Fluctuations in the sensitivity of acutely castrated five-week old rats to bSP-extract (#1-8).

<table>
<thead>
<tr>
<th>Date and duration of experiment</th>
<th>Hormone levels as a percentage of castrate control levels&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Body weight&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FSH</td>
<td>LH</td>
</tr>
<tr>
<td></td>
<td>Dose of extract</td>
<td>Dose of extract</td>
</tr>
<tr>
<td></td>
<td>40mg</td>
<td>20mg</td>
</tr>
<tr>
<td>19/4 - 3/5/1978</td>
<td>41 *&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76</td>
</tr>
<tr>
<td>16/8 - 6/9</td>
<td>60 **</td>
<td>42 *</td>
</tr>
<tr>
<td>11/10- 1/11</td>
<td>52 **</td>
<td>24 *</td>
</tr>
<tr>
<td>22/11- 6/12</td>
<td>47 **</td>
<td>74 *</td>
</tr>
<tr>
<td>20/12-27/12</td>
<td>65 **</td>
<td>78</td>
</tr>
<tr>
<td>31/1-28/2/1979</td>
<td>55 *&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102</td>
</tr>
<tr>
<td>30/5 -13/6</td>
<td>83</td>
<td>98</td>
</tr>
</tbody>
</table>

<sup>a</sup> The geometric mean hormone concentration is expressed as a percentage of the geometric mean of the control group for that experiment.

<sup>b</sup> Mean ± standard deviation, no, rats in control and bSP-extract treated groups.

<sup>c</sup> Significantly lower than the control group: * : p < 0.05, ** : p < 0.005.
Figure 3.6 Fluctuating sensitivity of acutely castrated five-week old rats to antigonadotrophic activity of 20 mg bSP-extract.

Eight experiments between 2/1978 and 6/1979 included a group that received 20 mg bSP-extract (#1-6 or 1-8). The geometric mean hormone concentration of each group is expressed as a percentage of the geometric mean of the control group for that experiment (▲). (Data from Table 3.3.). Hormone concentrations of intact rats are represented in the same way (▼).
3.5 PROBLEMS

3.5.1 Toxicity of bSP

Bull seminal plasma was toxic to rats, especially acutely castrated animals. (Table 3.4).

Table 3.4 Toxicity of bSP - incidence of death 1-24h after injection of bSP into five-week old rats.

<table>
<thead>
<tr>
<th>Condition</th>
<th>no. dying/no. treated</th>
<th>mortality rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acutely castrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline</td>
<td>17/423</td>
<td>0.04</td>
</tr>
<tr>
<td>bSP-extract, 20 mg</td>
<td>11/86</td>
<td>0.13</td>
</tr>
<tr>
<td>40 mg</td>
<td>45/161</td>
<td>0.28</td>
</tr>
<tr>
<td>Intact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline</td>
<td>1/172</td>
<td>0.006</td>
</tr>
<tr>
<td>bSP-extract, 20 mg</td>
<td>0/18</td>
<td>0</td>
</tr>
<tr>
<td>40 mg</td>
<td>0/33</td>
<td>0</td>
</tr>
<tr>
<td>60 mg</td>
<td>2/10</td>
<td>0.20</td>
</tr>
<tr>
<td>150 mg</td>
<td>4/6</td>
<td>0.67</td>
</tr>
</tbody>
</table>

The proportion of acutely castrated rats treated with 40 mg bSP-extract that died ranged from 0 to 0.6 in different experiments, but the cause of the variation was not uncovered. bSP itself seemed more toxic than extracts prepared by ethanol precipitation or diafiltration. The rats afflicted were lethargic and hypothermic, but not in pain; warming or administration of oxygen did not arrest their decline. Death in the saline treated rats arose mainly from intestinal prolapse through the scrotal wound.

Animals dying within one hour of castration were excluded from the experimental results. Their death was attributed to the effects of anaesthesia; early mortality was more prevalent when the rats had respiratory infections.
Figure 3.7  Serum concentrations of FSH in intact rats of different ages.

Geometric means and their 67% confidence intervals.
Inside FSH bars: no. per group.
3.5.2 Changes in Serum Gonadotrophins with Maturation

Of the many studies plotting serum gonadotrophin levels throughout maturation in the male rat, most show that FSH rises sometime after day 20 to reach a peak about day 35 (see Section 8.1.3 for references). The subsequent fall in FSH is thought to be due to implementation of negative feedback by inhibin, and hence the choice of 35-day old rats to detect inhibin-like activity. Some studies show a sharp and high FSH peak between days 32 and 39—the age range of the rats used in this study. If serum FSH concentrations are an indication of the rats' sensitivity to inhibin, or its potential response to inhibin, then inclusion of rats of different ages could lead to widely variable and seemingly erratic responses to bSP.

In practice this problem did not arise. The rats here exhibited only a slight change in serum FSH concentrations between days 32 and 38 (Figure 3.7). Moreover, only rats of accurately known age were used in the first year of experiments. They showed no age related trends, whether treated with saline or 0.5 ml bSP. Nor were the differences in sensitivity to 0.2 ml bSP related to age.

3.5.3 Changes in the 24h Post-castration Rise of Serum FSH and LH

It was soon apparent that the serum concentrations of LH 24h after castration varied, and that the variation followed a temporal, possibly seasonal, pattern. Within an experiment such variation would spread the hormone concentrations of the control group, masking differences between control and treated groups and so impairing the usefulness of the method. The duration of any experiment was limited to four weeks to lessen this effect.

The magnitude of the 24h post-castration rise of LH continued to vary over a three year period (Figure 3.8). In the figure the contribution of between-radioimmunoassay variation has been reduced by two strategies. In the first, the original data was scaled by dividing $A_i$ by $\bar{A}$, where $A_i$ was the mean concentration of the quality control sample $A$ in the original assay, and $\bar{A}$ the average of all $A_i$'s. In the second, 200 µl of serum from each animal in the group was pooled, and the pools assayed (in triplicate) in one radioimmunoassay. The two methods gave similar
hormone patterns, although the first showed more variation. In Figure 3.9 the patterns have been smoothed by taking the running median. Smoothing reveals long-term changes free of the overlying "static" of random variation, but it also rejects isolated high and low values which may nevertheless be true.

Very high concentrations of LH were occasionally seen in rats in control or treated groups (Figure 3.8). These values appeared to follow a cyclic pattern, being present during spring and summer - times of higher than average LH levels in castrated rats.

Figure 3.8  Mean serum FSH and LH concentrations in five-week old rats 24h after castration over a three year period.

Plotted are the (arithmetic) mean serum concentrations of FSH and LH of the castrate control groups from all the experiments done over a three year period (▲, △). FSH concentrations of intact rats are also shown (■, □)

The influence of between-radioimmunoassay variation has been reduced in two ways.

a) The means measured from the original radioimmunoassays were scaled by a factor derived from the estimates of the quality control sample A, as described in the text (▲, ■).

b) For each group, 200 µl of serum from each animal was pooled. The hormone concentration in the pool will be the arithmetic mean hormone concentration of the group. The pool samples were assayed together (▲, □).

Also shown is the monthly incidence of very high LH concentrations (>500 µg/l, and above the 95% confidence interval of the rest of the group), which were occasionally seen in control or treated groups.
Figure 3.9 Running median of the mean serum FSH and LH concentrations in five-week old rats 24h after castration.

The data in Figure 3.8 have been smoothed by plotting the running median of the means.
CHAPTER 4  EXTRACTION AND PARTIAL PURIFICATION OF ANTIGONADOTROPHIC ACTIVITY IN bSP

4.1 INTRODUCTION

This chapter reports attempts to isolate the antigonadotrophic activity from bSP. Methods were chosen that would not only purify the activity, but also help elucidate its physicochemical properties. The properties inferred are presented separately in Chapter 5. The choice of fractionation methods was restricted by the sensitivity of the method for detecting antigonadotrophic activity; gel filtration and ion exchange chromatoographies with their large sample capacities were ideal, whereas isoelectric focusing, although of high resolution and valuable for determining isoelectric points, would have required too much of the expensive ampholyte.

Most of this work was carried out with the first batch of bSP, of which 0.5 ml or the equivalent amount of extract always suppressed FSH or LH in the detection method (see Section 3.4.2). Accordingly, fractions were screened at doses which were calculated to contain at least as much activity as 0.5 ml bSP. For instances, doses equivalent to 1 ml bSP (i.e., the amount of fraction derived from 1 ml bSP) were screened after chromatography of bSP-extract, in case the activity was equally divided between two fractions. If one of those fractions was rechromatographed, dose was equivalent to 2 ml bSP if enough material was available.

When the procedure was first used, the fractions were recombined and screened to ensure the activity was not lost under the new conditions. When a buffer was used for the first time, salts or urea were removed by diafiltration with a UM 05 membrane so that substances with molecular weight greater than about 500 daltons were retained. Subsequently, a PM 10 membrane, or dialysis, was used when it had been shown that the activity had a molecular weight greater than 10 000 daltons.

4.2 EXTRACTION

Initially protein was extracted from bSP by adding cold ethanol to a concentration of 86% (v/v) - the method of Franchimont et al. (1975a).
The precipitate was redissolved in water and freeze-dried. This solution was liable to thaw; prior evacuation, at 4\(^{\circ}\)C, to remove residual ethanol lessened but did not eliminate the problem. To circumvent any inactivation that might occur by exposure to ethanol or by thawing, diafiltration using a PM 10 membrane was tried as an alternative extraction method. Gel exclusion chromatography (Section 4.3.3) had already shown that the activity had a molecular weight much greater than the nominal cut-off of the PM 10 membrane. By diafiltering 100 ml bSP against 11 water, the membrane-permeable components of bSP were reduced 10 000-fold.

The antagonadotrophic activity of bSP was retained in the 86% ethanol precipitates and PM 10 retentates. Table 4.1 lists the bSP-extracts used in this thesis.

Table 4.1

<table>
<thead>
<tr>
<th>Batch of bSP</th>
<th>Preparation</th>
<th>Method of extraction</th>
<th>Yield (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-1 to 1-10</td>
<td>ethanol precipitation</td>
<td>77.6 - 84.7</td>
</tr>
<tr>
<td></td>
<td>1-11</td>
<td>PM 10 diafiltration</td>
<td>69.0</td>
</tr>
<tr>
<td>2</td>
<td>2-1 and 2-2</td>
<td>ethanol precipitation</td>
<td>57.0 &amp; 58.8</td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>PM 10 diafiltration</td>
<td>51.8</td>
</tr>
<tr>
<td>3</td>
<td>3-1</td>
<td>PM 10 diafiltration</td>
<td>34.6</td>
</tr>
<tr>
<td>4</td>
<td>4-1</td>
<td>&quot;</td>
<td>61.3</td>
</tr>
<tr>
<td>5</td>
<td>5-1</td>
<td>&quot;</td>
<td>60.8</td>
</tr>
<tr>
<td>6</td>
<td>6-1</td>
<td>&quot;</td>
<td>72.5</td>
</tr>
<tr>
<td>7</td>
<td>7-1</td>
<td>&quot;</td>
<td>48.1</td>
</tr>
</tbody>
</table>

Ethanol precipitation reduced the testosterone content of bSP (#1) from 1.1 ng/ml to 0.18 ng per 80 mg bSP-extract. Extraction of steroids with diethyl ether was not feasible; a gel formed between the aqueous and ether layers, and it did not separate either on prolonged standing or centrifugation.
4.3 PRELIMINARY FRACTIONATION

4.3.1 Fractional Ethanol Precipitation

The 0-86% ethanol precipitate of bSP contained the antigonadotrophic activity. Could ethanol precipitation be more selectively used to purify the activity? In an analytical experiment, 10, 20, ..., 90% (v/v) ethanol extracts of bSP were prepared. Half the maximum possible yield was precipitated at an ethanol concentration of 30%, three quarters at 50%. Three aliquots of 5 ml bSP were used to prepare 30%, 50% and 86% (v/v) ethanol precipitates, and more ethanol was added to the supernatants of the first and second aliquots to form 30-86% and 50-86% precipitates. All were screened for antigonadotrophic activity (Figure 4.1).

From the suppression of FSH, it appeared that the activity was precipitated mainly upon an increase in ethanol concentration from 30 to 50%. The LH response indicated all preparations had some activity. However, none of the fractions had as much activity as the 0-86% precipitate, implying that ethanol fractionation offered little scope for concentrating the activity.

4.3.2 Gel Exclusion Chromatography on G-100 eluted with 0.01M Ammonium Acetate, pH 7.6.

Since inhibin-like activity from several sources (bull testis: Lee et al., 1974; ram testes: Moodbidri et al., 1976; ovine rete testis fluid: Davies et al., 1976; bSP: Franchimont et al., 1975a) was considered to have a molecular weight less than 100 000 daltons but probably greater than 5000 daltons, G-100, with a fractionation range of 4000-150 000 daltons,

Figure 4.1 Fractionation of bSP by ethanol precipitation.

Ethanol was added to bSP to a concentration of 30, 50 or 86% (v/v) and the precipitates recovered. More ethanol was added to the 30 and 50% supernatants to bring them to 86% (v/v) ethanol. Precipitates were screened for antigonadotrophic activity. Dose was equivalent to 0.5 ml bSP. Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: * p < 0.05, ** p < 0.005.
was chosen to fractionate bSP-extract (Figure 4.2). The eluent 0.01M ammonium acetate, pH 7.6, was chosen because it could be freeze-dried directly.

The activity was retained upon chromatography (from the recombination of the fractions), and was present in the fraction N-I+II - the void volume and its shoulder. This fraction contained 56.3% of the material applied to the column. The void volume contained a small amount of precipitate (3% of the starting material by weight), which formed after the sample solution had drained into the column.

4.3.3 Diafiltration with an XM 100 Membrane

The XM 100 membrane retains substances with molecular weight greater than 100,000 daltons, and thus XM 100 diafiltration seemed a convenient way of separating the large amount of high molecular weight material in bSP-extract, evident from the large G-100 void volume peak, from the antogonadotrophic activity, which, by analogy with the inhibin-like activity in other sources, could be expected to have a molecular weight of less than 100,000 daltons.

Total recovery, retentate plus expressate, averaged 99.5%. A known amount of BSA was added to the expressates to aid their freeze-drying. The activity was retained after diafiltration, and seemed more present in the retentate, although the expressate also exhibited activity (Figure 4.3).

Figure 4.2 Chromatography of bSP-extract on G-100 eluted with 0.01M ammonium acetate, pH 7.6.

Three batches of 300 mg bSP-extract (#1-3 and 1-4) were chromatographed on a 470 ml column of G-100 eluted with 0.01M ammonium acetate, pH 7.6. The effluent was divided into fractions N-I to V, which were screened for antogonadotrophic activity. The dose of the starting material (bSP-extract), a recombination of the fractions, and N-V was equivalent to 0.5 ml bSP; the dose of N-I to N-V was equivalent to 1.0 ml bSP. Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p < 0.05, ** p < 0.005.
Figure 4.3  Fractionation of bSP-extract by diafiltration with an XM 100 membrane.

Batches of 400 mg bSP-extract (#1-5) dissolved in 150 ml 0.01M ammonium acetate, pH 7.6, were diafiltered using an Amicon XM 100 membrane against 3-5 volumes of the same buffer. Expressate and retentate were screened for antigonadotrophic activity. Dose was equivalent to 0.5 ml bSP. Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05, ** p <0.005.
4.4 GEL EXCLUSION CHROMATOGRAPHY ON G-75

Chromatography on G-100 (Section 4.3.2) and diafiltration on an XM 100 membrane (Section 4.3.3) indicated that the antigonadotrophic activity in bSP had a molecular weight in the vicinity of 100 000 daltons in 0.01 M ammonium acetate buffer at pH 7.6. Yet Franchimont et al. (1975a) had clearly shown that the inhibin-like activity in bSP resided in the second peak on G-100 chromatography in 0.05 M sodium acetate, pH 4.

If the behaviour of the activity differs according to the conditions of chromatography, then this trait could be used to purify the activity. This section explores the apparent molecular weight of the activity at different ionic strengths and pHs, and in the presence of a dissociating agent, 4 M urea. The gel matrix chosen was G-75, for although G-200 has a wider fractionation range its resolution is poorer (Andrews, 1965).

4.4.1 Effect of Ionic Strength

Chromatography of bSP-extract with 0.01M ammonium acetate, pH 7.6, on G-75 (Figure 4.4) gave results similar to those from chromatography on G-100. The activity was in the void volume, fraction N-I, which contained 53.6% of the starting material. Some LH-suppressing activity was present in the salt volume, which was screened at a higher dose than before.

With eluent ten times more concentrated, namely 0.1M ammonium acetate, pH 7.6, the elution profile was slightly different (Figure 4.5), and there was no precipitate when the sample solution was drained into the column. Fractions for screening were made narrower. Activity was present in the void volume, and, possibly to a greater extent, in the

Figure 4.4 Chromatography of bSP-extract on G-75 eluted with 0.01M ammonium acetate buffer, pH 7.6.

1 g bSP extract (#1-5) was chromatographed on a 2.31 column of G-75 eluted with 0.01M ammonium acetate, pH 7.6. The effluent was divided into fractions N-I to V, which were screened for antigonadotrophic activity. The dose of starting material was equivalent to 0.5 ml bSP; the dose of the fractions was equivalent to 1.0 ml bSP.

Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05, ** p <0.005.
Figure 4.5  Chromatography of bSP-extract on G-75 eluted with 0.1M ammonium acetate, pH 7.6.

1 g bSP-extract (# 1-6) was chromatographed on a 2.3 l column of G-75 eluted with 0.1M ammonium acetate, pH 7.6. The effluent was divided into fractions N'-I to VI, of which I to IV were screened for antigonadotrophic activity. Dose of starting material was equivalent to 0.5 ml bSP; the dose of the fractions was equivalent to 1.0 ml bSP.

Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05, **p <0.005.
Figure 4.6  PAGE of fractions from chromatographies N and N'.

a) Fractions N-I to IV (see Figure 4.4),
b) Fractions N'-I to VI (see Figure 4.5).
Figure 4.7  Effect of different buffer concentrations on the elution profile of bSP-extract on G-200.

a) 70 mg bSP-extract (# 1-8) was chromatographed on a 160 ml column of G-200 eluted with 0.05M ammonium acetate, pH 7.6. The column was then re-equilibrated by washing with 0.1M ammonium acetate, pH 7.6, and

b) 70 mg was chromatographed with the new buffer. The determination of the molecular weight scale is described in Section 2.5.6.2. The void volume of the G-75 column in Figures 4.4 and 4.5 corresponds to a molecular weight greater than 150 000 daltons.
adjacent fraction. These fractions, N'-I and N'-II, contained 45.5% and 7.4% of the starting material.

The PAGE patterns of corresponding fractions from the two chromatographies were very similar (Figure 4.6). The differences in the elution profiles near the void volume were amplified by chromatography on a G-200 column (Figure 4.7).

4.4.2 Effect of pH

The elution profile with 0.05M sodium acetate, pH 4 (Figure 4.8) was quite different from the profiles obtained with the ammonium acetate buffers of pH 7.6, but similar to the profile reported by Franchimont et al. (1975a). Not all of the bSP-extract dissolved in 0.05M sodium acetate, pH 4, at the usual sample concentration of 1g/50 ml; 24.1% of the starting material was insoluble.

Activity was retained on chromatography, and although possibly present in several fractions was mainly localised in A-II. This corresponds to the second peak in the pattern reported by Franchimont et al. (1975a).

Interestingly, the profile resembled that of the G-200 chromatography of bSP-extract in 0.1M ammonium acetate, pH 7.6 (Figure 4.7b), and the PAGE patterns of matching peaks were very similar. The PAGE patterns for the G-75 fractions appear in Figure 4.10.

Figure 4.8 Chromatography of bSP-extract on G-75 eluted with 0.05M sodium acetate, pH 4.

1 g bSP-extract (#1-5) was added to 50 ml 0.05M sodium acetate, pH 4, and the insoluble material removed. The supernatant was chromatographed on a 2.3 l column of G-75 eluted with the same buffer, and the effluent was divided into fractions A-I to IV. The starting material and a recombination of the fractions were screened for antgonadotrophic activity at a dose equivalent to 0.5 ml bSP, while fractions I to IV and the pH 4 precipitate were screened at a dose equivalent to 1.0 ml bSP.

Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. rats allotted, s no. surviving. Significantly lower than the control group: *p <0.05, ** p <0.005.
4.4.3 Effect of Urea

Extract of bSP was chromatographed in 4M urea-0.1M ammonium acetate, pH 7.6, after material insoluble at pH 4 had been removed (Figure 4.9). The sample was prepared in this way because in the previous experiment (Section 4.4.2) the pH 4 precipitate had a higher mortality rate than the other fractions. But this was not the case here.

The antigonadotrophic activity in bSP had previously been shown to survive chromatography in 4M urea-0.1M ammonium acetate buffer. The activity resided mainly in fraction AU-IV, which contained 29.3% of the material applied to the column. A second experiment in which the bSP-extract was dissolved in the elution buffer (without pretreatment at pH 4) gave a similar profile and distribution of activity, although the results were not as clear because of the high mortality rate in the group receiving NU-IV, the fraction equivalent to AU-IV. PAGE patterns of the AU fractions appear in Figure 4.11.

4.4.4 Combinations of G-75 Chromatographies

The elution volume of the antigonadotrophic activity on G-75 chromatography depended on ionic strength, pH, and the presence of urea. However in all buffers except 0.1M ammonium acetate, pH 7.6, the activity

Figure 4.9 Chromatography of bSP-extract on G-75 eluted with
4M urea-0.1M ammonium acetate, pH 7.6.

1 g bSP-extract (# 1-6) was added to 50 ml 0.05M sodium acetate, pH 4, and the insoluble material removed. The supernatant was made 4M in urea, its pH brought to 7.6 with ammonia, and it was chromatographed on a 2.3 l column of G-75 eluted with 4M urea-0.1M ammonium acetate, pH 7.6. The effluent was divided into fractions AU-I to IV, which were screened for antigonadotrophic activity. Dose of starting material was equivalent to 0.5 ml bSP; the dose of the fractions and the pH 4 precipitate was equivalent to 1.0 ml bSP.

Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p < 0.05, **p < 0.005.
Figure 4.10  PAGE of fractions from chromatography A.
Fractions A-I to IV and the pH 4 precipitate (see Figure 4.8).

Figure 4.11  PAGE of fractions from chromatography AU.
Fractions AU-I to IV and the pH 4 precipitate (see Figure 4.9).
appeared in the most abundant fraction, so there was little prospect of purification by a series of G-75 chromatographies.

The most promising combination seemed chromatography with 0.1M ammonium acetate, pH 7.6, then with 0.05M sodium acetate, pH 4 (Figure 4.12). 94.3 mg N'-II, the amount derived from 1.3 g bSP-extract, was added to 50 ml buffer and the insoluble material removed. The supernatant was chromatographed on the same sized column (2.3 l) as before, and the effluent was divided into the same sized fractions as in the N' chromatography (Section 4.4.1). Only 48.6% of the starting material was recovered. FSH-suppressing activity of the fractions was slight, perhaps because of the poor recovery, and it was in the more abundant fractions N'-A-II and N'-A-III. These spanned the same elution volumes as N'-II and N'-III.

4.5 ION EXCHANGE CHROMATOGRAPHY ON CM CELLULOSE

4.5.1 Preparation of "bSP-inhibin"

In 1978, Chari et al. described the isolation and characterisation of a protein fraction from bSP which they called "bSP-inhibin". This section duplicates and extends their purification.

Initially, their steps were copied on an analytical scale. 75.2 mg bSP-extract was chromatographed on a 160 ml column of G-75 eluted with 4M urea-0.05M sodium acetate, pH 4, and the fraction corresponding to AU-IV was chromatographed on a 16 ml column of CM cellulose equilibrated and eluted with the same buffer. The profile of absorbance at 280 nm was very similar to that of Chari et al. (1978), although the elution volume of the

Figure 4.12 Chromatography of N'-II on G-75 eluted with 0.05M sodium acetate, pH 4.

94.3 mg N'-II (see Figure 4.5) was chromatographed on a 2.3 l column of G-75 eluted with 0.05M sodium acetate, pH 4. The effluent was divided into fractions N'-A-I to V, of which II to V were screened for antigonadotrophic activity at a dose equivalent to 2.0 ml bSP. Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: * p <0.05.
Figure 4.13  Ion exchange chromatography of AU-IV on CM cellulose eluted with 4M urea-0.05M sodium acetate, pH 4.

48.6 mg AU-IV (see Figure 4.9) was chromatographed on a 16 ml column of CM cellulose eluted with 4M urea-0.05M sodium acetate, pH 4. The fraction CM-VI corresponded to "bSP-inhibin" of Chari et al. (1978).

Figure 4.14  PAGE of AU-IV and CM-VI.

See Figure 4.13. The two major components of CM-VI have been labelled A and B.
"Inhibin" peak on the CM column was 6.6V_t instead of 1.2V_t. PAGE of material from the "bSP-inhibin" peak showed a similar pattern of two bands here labelled A and B, with A the faster running. When AU-IV was substituted as the starting material for CM chromatography, very similar absorbance and PAGE patterns were obtained (Figures 4.13 and 4.14). Of the AU-IV applied to the column, 18% was recovered.

On a preparative scale, AU-IV was chromatographed on a 250 ml column (chromatographies CM_c and CM_d), and NU-IV on a 250 ml column connected in series with a 50 ml column (chromatographies CM_e and CM_f). The elution volumes for the "bSP-inhibin" peaks were 4.3, 4.4, 6.1 and 5.4V_t for CM_c, CM_d, CM_e and CM_f. On the larger columns the leading shoulder seen in Figure 4.13 emerged as a distinct peak, CM-V (see Figure 4.18), although the electrophoretic pattern of the material in it was almost identical to that in the following and major peak, CM-VI (see Figure 4.19). Yields of CM_c,d-V and CM_c,d-VI as a percentage of the starting amount of bSP-extract were 0.34% and 3.34%.

The fraction CM-VI had two bands on PAGE and two bands on PAGEIF over the pH range of 3.5-10. The isoelectric points were determined by PAGEIF using pH 4-6 ampholyte. (The gradient was linear between pHs 4.1 and 5.8 with regression coefficient r = 0.9990, n = 11.) The isoelectric points of the two bands were 4.70 (average of 4.68 and 4.71) and 4.88 (average of 4.85 and 4.90).

It is possible that A and B might be separated by ion exchange chromatography at a pH closer to the isoelectric point of one of the components. The optimum pH was investigated by chromatography CM-VI on columns of CM cellulose which were equilibrated and eluted with 4M urea-0.05M sodium acetate buffers of different pH. The fractions were dialysed and freeze-dried, and samples submitted to PAGE and PAGIEF. The elution volumes of the peaks containing A and B were plotted against the pH of the buffer (Figure 4.15). Components A and B were completely separated at pH 4.6 (chromatographies denoted CM(4.6)) (Figures 4.16 and 4.17). Identical results came from using NU-IV or CM-VI as starting material. As expected, the faster running band on PAGE was the more acidic of the two components and was the first to be eluted from the CM columns.
Figure 4.15  Elution volumes of components A and B of CM-VI on CM cellulose columns eluted with buffers of different pH.

2 mg batches of CM-VI (see Figure 4.13) were chromatographed on 16 ml columns of CM cellulose eluted with 4M urea-0.05M sodium acetate buffers of pH 3.8, 4.2, 4.6 and 4.75. Fractions were dialysed and freeze-dried, and samples submitted to PAGE to determine which peak contained component A (▲) and which contained B (▲). Elution volumes of the peaks are plotted against pH of the buffer.
Figure 4.16  Ion exchange chromatography of NU-IV on CM cellulose eluted with 4M urea-0.05M sodium acetate buffer of pH 4.6. 205.8 mg NU-IV was chromatographed on 50 and 250 ml columns in series eluted with 4M urea-0.05M sodium acetate, pH 4.6. Fractions were denoted CM(4.6)-I to V.

Figure 4.17  PAGE of fractions from the CM(4.6) chromatography of NU-IV. See Figure 4.16.
Table 4.2  Antigonadotrophic activity in the "bSP-inhibin" preparation $CM_{c,d}-V + VI$ (Geometric means and their 67% confidence intervals).

<table>
<thead>
<tr>
<th></th>
<th>FSH (μg/l)</th>
<th>LH (μg/l)</th>
<th>s/n$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1308 (1226-1396)</td>
<td>130 (101-166)</td>
<td>5/5</td>
</tr>
<tr>
<td>$CM_{c,d}-V + VI$ 0.36 mg</td>
<td>1321 (1262-1385)</td>
<td>195 (160-231)</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>1454 (1326-1594)</td>
<td>134 (109-165)</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>1340 (1215-1477)</td>
<td>148 (113-194)</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>1185 (1131-1241)</td>
<td>162 (127-208)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

$^a$ n no. allotted per group,  s no. surviving
4.5.2 Assessment of "bSP-inhibin"

The fractions CM$_{e,f}$-V and VI were combined and screened for antgonadotrophic activity, but no suppression of FSH or LH was detected (Table 4.2).

The recovery of AU-IV or NU-IV from the CM columns was never more than 20%, which suggested that most of the sample remained bound to the column. To catch this material, a 50 ml column was put ahead of the 250 ml column in the chromatographies CM$_e$ and CM$_f$. After elution as usual, the 50 ml column was disconnected and washed with buffer containing 1M sodium chloride. The fractions obtained were called CM-B-I to III. Yields, as a percentage of the starting amount of bSP-extract, were:

- NU-IV: 36% (calculated)
- CM$_{e,f}$-V: 1.1
- CM-VI: 3.7
- CM-B-I: 0.3
- II: 13.1
- III: 0.1

combined to form CM-E: 4.8%
combined to form CM-B: 13.5%

Thus about half the NU-IV applied to the column was recovered; very little additional material was eluted by a subsequent wash with buffer containing 4M sodium chloride. The PAGE patterns of the fractions appear in Figure 4.19.

Antigonadotrophic activity was localised in the bound fraction CM-B, rather than in the eluted fraction CM-E (Figure 4.18).

The PAGE pattern of CM-VI resembled the two-band patterns of N-IV, N'-V, and A-III. Accordingly, these fractions and CM(4.6)-I and V were submitted to PAGE on the same slab gel so their patterns could be compared exactly. The two major bands had identical mobilities in all these samples.

4.5.3 Ion Exchange Chromatography with a Salt Gradient

The material that bound to the CM column was fractionated by applying a salt gradient (Figure 4.20a). The profiles demonstrated the heterogeneity of the material and showed that preliminary G-75 chromatography was not necessary (Figure 4.20b).
Accordingly, three batches of 270 mg bSP-extract (#1-8) were chromatographed (chromatographies CM$_h$, CM$_i$, CM$_j$) on 50 ml columns of CM cellulose eluted initially with 4M urea-0.05M sodium acetate, pH 4, and then, after the passage of 12V$_t$, with the same buffer containing an increasing concentration of sodium chloride. The elution profile was similar to that of Figure 4.20b, with slight loss of resolution. Fraction sizes for CM$_{h-j}$ appear in Figure 4.20b. The fractions were screened at a dose equivalent to 1.0 ml bSP, but no activity was detected (Table 4.3). However now was any activity detected in a recombination of the fractions at a dose equivalent to 0.5 ml bSP. Since the recovery of the starting material from the column averaged only 59.3%, it was possible that the dose was too low. In case activity had been lost when the urea and salts were removed, dialysis changes were later shortened from 8-24h to 2h.

In the next experiment, bSP-extract (#2-2) was partially purified by G-75 chromatography before CM cellulose chromatography to increase the potency of the sample and so reduce the number of runs needed to produce enough material for screening. Unfortunately, the activity resided solely in N'-I for the second batch of bSP (see Section 5.3.2) so loading was greater than planned and resolution suffered (Figure 4.21). Whether due to a different sample or reduced dialysis time, 73.2% of the starting material was covered.

The activity resided in the main peak, CM$_i$-IV.

Figure 4.18  Ion exchange chromatography of NU-IV on CM cellulose at pH 4, followed by washing with salt solution.

NU-IV (prepared from bSP-extract #1-9, see Section 4.4.3) was chromatographed on 50 and 250 ml columns in series eluted initially with 4M urea-0.05M sodium acetate, pH 4. The 50 ml column was then eluted with the same buffer containing 1M sodium chloride. Fractions CM-E and CM-B were screened for antigonadotrophic activity at doses equivalent to 1.0 and 2.0 ml bSP.

Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05, **p <0.005.
Figure 4.19  PAGE of the fractions from the chromatography CM_e,f.

See Figure 4.18. The original fractions from the chromatography were combined to form CM-E (CM-V + VI) and CM-B (CM-B-I - III).

Figure 4.20  Ion exchange chromatography of NU-IV and bSP-extract on CM cellulose in the presence of a salt gradient.

a) 10.8 mg NU-IV (see Section 4.4.3) and
b) 32.3 mg bSP-extract (#1-8) were chromatographed on 16 ml columns of CM cellulose eluted initially with 4M urea-0.05M sodium acetate, pH 4, and then with the same buffer containing an increasing concentration of sodium chloride. The comparable positions of fractions of the preparative chromatographies CM_h-j and CM_i are indicated.
Table 4.3  Antigonadotrophic activity in the fractions CM_{h-j} to V 
(Geometric means and their 67\% confidence intervals).

<table>
<thead>
<tr>
<th></th>
<th>FSH (\mu g/l)</th>
<th>LH (\mu g/l)</th>
<th>s/n$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline</td>
<td>1351 (1283-1366)</td>
<td>151 (135-168)</td>
<td>8/8</td>
</tr>
<tr>
<td>bSP-extract, 20 mg</td>
<td>1087 (945-1251)</td>
<td>119 (102-138)</td>
<td>6/7</td>
</tr>
<tr>
<td></td>
<td>875 (745-1027)$^{**b}$</td>
<td>42 (21-84)</td>
<td>5/7</td>
</tr>
<tr>
<td>CM_{h-j-I}, 11.6</td>
<td>1068 (967-1179)*</td>
<td>115 (99-132)</td>
<td>5/6</td>
</tr>
<tr>
<td>II, 6.4</td>
<td>1263 (1189-1342)</td>
<td>97 (73-129)</td>
<td>6/6</td>
</tr>
<tr>
<td>III, 10.9</td>
<td>1175 (1150-1200)*</td>
<td>138 (121-157)</td>
<td>6/6</td>
</tr>
<tr>
<td>IV, 5.7</td>
<td>1195 (1059-1348)</td>
<td>98 (76-126)</td>
<td>6/6</td>
</tr>
<tr>
<td>V, 12.4</td>
<td>1063 (953-1185)*</td>
<td>95 (78-116)*</td>
<td>6/6</td>
</tr>
<tr>
<td>II+III+IV+V, 17.3</td>
<td>1162 (1047-1290)</td>
<td>123 (110-137)</td>
<td>5/6</td>
</tr>
</tbody>
</table>

$^a$ n no. allotted per group, s no. surviving.

$^b$ Significantly lower than the control group:
* p < 0.05, ** p < 0.005.
The isoelectric points of the proteins in CM$_1$-IV were determined by PAGIEF. (The pH gradient between 4.1 and 5.8 was linear, with regression coefficient $r = 0.9970$, $n = 13$.) CM$_1$-IV had at least ten components, and they had isoelectric points between 4.7 and 5.4 (Figure 4.23a). PAGIEF on small gels (Figure 4.23b) also showed ten or more bands. The sharp bands on PAGIEF contrasted with the indistinct PAGE pattern (Figure 4.22).

The active fraction CM$_1$-IV can be separated into two peaks (see Figure 4.20). 494.7 mg N'-I (from bSP-extract #2-2) was chromatographed on a 50 ml column of CM cellulose. After the passage of several volumes of 4M urea-0.05M sodium acetate, pH 4, the sodium concentration of the buffer was increased to 80 mM. When the absorbance at 280 nm had reached baseline again, a sodium chloride gradient was instituted until the sodium concentration reached 140 mM. The two peaks eluted, CM$_{-1}$-III and IV, had PAGE and PAGIEF patterns identical to those of CM$_{-j}$-III and IV. The two fractions were screened for antigonadotrophic activity at the same doses as the CM$_1$ fraction, with each rat also receiving 100 μg testosterone propionate/kg BW to increase the sensitivity of the detection method (see Section 7.5.1). No activity was observed (Table 4.4).

Figure 4.21 Ion exchange chromatography of N'-I on CM cellulose in the presence of a salt gradient.

355 mg N'-I (prepared from bSP-extract #2-2) was chromatographed on a 50 ml column of CM cellulose eluted initially with 4M urea-0.05M sodium acetate, pH 4, and then with the same buffer containing an increasing concentration of sodium chloride. The effluent was divided into fractions CM$_1$-I to V, which were screened for antigonadotrophic activity at a dose equivalent to 1.3 ml bSP (first batch of bSP, which was equivalent to 2.0 ml of the second batch of bSP). Geometric means and their 67% confidence intervals, also individual LH concentrations for the group receiving CM(4)$_{-j}$-III. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05.
Table 4.4  Antigonadotrophic activity in the fractions $C_{m}^{-\text{III}}$ and IV (Geometric means and their 67% confidence intervals).

<table>
<thead>
<tr>
<th></th>
<th>FSH (μg/l)</th>
<th>s/n$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline$^{a}$</td>
<td>1267 (1181-1358)</td>
<td>6/6</td>
</tr>
<tr>
<td>$C_{m}^{-\text{III}}$</td>
<td>13.8 mg 1257 (1149-1376)</td>
<td>5/6</td>
</tr>
<tr>
<td>$C_{m}^{-\text{IV}}$</td>
<td>8.0 mg 1150 (1007-1314)</td>
<td>6/6</td>
</tr>
</tbody>
</table>

$^{a}$All animals were also given 100 μg testosterone propionate/kg BW to increase the sensitivity of the detection method

$^{b}$n no. allotted per group, s no. surviving
Figure 4.22  PAGE of fractions of the CM$_1$ chromatography.

See Figure 4.21.

Figure 4.23  PAGIEF patterns of CM$_1$-IV.

a) 200 µg CM$_1$-IV (see Figure 4.21) was focused on a 70 mm x 4.5 mm gel. The pH scale was obtained by measuring the pH in other gels run at the same time.

b) 20 and 60 mg CM$_1$-IV focused on 60 mm x 3 mm gels.
5.1 INTRODUCTION

Since the antigonadotrophic activity in bSP was only partially purified, its physicochemical properties could only be inferred from the way it behaved in the various fractionation steps. Hence most of the properties described here are tentative.

5.2 PROTEINOUS NATURE

A proteinous nature was indicated by the precipitation of the activity by ethanol (see Section 4.2). Organic solvents have long been used to extract protein from solutions (Kaufman, 1971).

The nature of the activity was examined further by heating bSP-extract - for proteins are heat labile, although some more so than others -, and by incubating the extract with pepsin - an enzyme which cleaves to certain amino acid linkages. With both treatments the activity was lost (Figure 5.1a). The loss on pepsin digestion was not merely due to prolonged heating at 37°C nor exposure to a low pH, because the activity was retained when bSP-extract was subjected to the same conditions but in the absence of pepsin itself (Figure 5.1b).

5.3 MOLECULAR WEIGHT AND ASSOCIATIVE PROPERTIES

5.3.1 Studies on the First Batch of bSP

Information on the molecular weight of the activity came from gel exclusion chromatography on G-75 columns eluted with various buffers (see Section 4.4). The chromatograms, calibrated for molecular weight, appear in Figure 5.2. Despite the wide fractions taken, the elution volume of the activity clearly depended on the buffer: the molecular weight was greater than 50 000 daltons in the ammonium acetate buffers of pH 7.6, it appeared to be less than 70 000 but greater than 25 000 daltons in sodium acetate, pH 4, and it fell between 15 000 and 35 000 daltons in the presence of urea. (Although the fraction AU-IV covered 5000 - 35 000 daltons (Figure 5.2d), activity was also present in NU-IV, which did not
Figure 5.1 Effect of heat and pepsin on the antigonadotrophic activity in bSP.

a) Solutions of bSP-extract (#1-5) were heated at 67°C for 1h or incubated with pepsin.

b) A solution of bSP-extract (#1-6) was incubated at 37°C at pH 2 for 24h - the conditions for pepsin incubation - but without pepsin. Dose was equivalent to 0.5 ml bSP.

Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05, ** p <0.005.
include the trailing peak seen in AU-IV and so covered 15 000-35 000 daltons.)

The differences can be explained by self-association or by association of the active factor with other components in the bSP-extract. High concentrations of urea should eliminate such interactions, and indeed the molecular weight was lowest in the buffer containing 4M urea.

A large portion of the material in bSP changed its apparent size in the same way as the activity. The active factor is probably a small fraction of this, since with 0.1M ammonium acetate, pH 7.6, as buffer the activity was present in the trailing edge of the void column while most of the associating material was in the void volume itself.

In 0.05M sodium acetate, pH 4, the degree of association depended on the loading of the column (Figure 5.3). The less material applied the more the absorbance profile resembled that obtained with 4M urea. Further experiments showed it was indeed the loading, not the sample

Figure 5.2 Effect of different buffers on the elution volume of the anticonadotrophic activity in bSP upon G-75 chromatography.

Batches of 1g bSP-extract (from the first batch of bSP) were chromatographed on 2.3 l columns of G-75 eluted with -

a) 0.01M ammonium acetate, pH 7.6,
b) 0.1M ammonium acetate, pH 7.6,
c) 0.05M sodium acetate, pH 4, and
d) 4M urea-0.1, ammonium acetate, pH 7.6.

Molecular weight scales were obtained by running proteins of known molecular weight as described in Section 2.5.6.2. Profiles a, b, c and d were taken from Figures 4.4, 4.5, 4.8 and 4.9. Profiles b and d were distorted so their molecular weight scale because the same as that of profile a.

Fractions which suppressed serum FSH and LH are shaded:

<table>
<thead>
<tr>
<th></th>
<th>p &lt;0.05</th>
<th>p &lt;0.005</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
concentration, that was important. (Differences between the profiles in Figures 5.2c and 5.3a had two sources. Firstly, 24.1% of the bSP-extract was insoluble at pH 4 for Figure 5.2c, whereas 34.9% was insoluble for Figure 5.3. Secondly, better resolution was achieved on the 2.3 l column than on the 160 ml column.)

5.3.2 Differences between Batches of bSP

Extracts prepared from the first and second batches of bSP were chromatographed on G-75 and G-200 columns eluted with 0.1M ammonium acetate, pH 7.6 (Figure 5.4). The two batches showed peaks at the same elution volumes, but the relative sizes of the peaks differed. The G-75 chromatographies were preparative, and identical fractions were taken. Although PAGE patterns of corresponding fractions were very similar (Figure 5.5) the distribution of antigonadotrophic activity was different (Table 5.1). With the first batch of bSP, both N'-I and II were active - with perhaps more activity in N'-II, while with the second batch only N'-I was active. Was the difference due to different proportions of associating proteins in the two batches of bSP?

5.4 ISOELECTRIC POINT

The isoelectric point of the activity must be greater than 4 since the activity bound to CM cellulose at that pH. When the active fraction CM₁-IV was focused on a pH gradient, its components lay between pHs 4.7 and 5.4 (see Section 4.5.3). The isoelectric point of the active factor(s) can be expected to be similar to the isoelectric points of the other components of the fraction, since PAGIEF of fractions off other CM columns (e.g. CM₉₋₃) had shown that the higher the salt concentration, the higher, on average, the isoelectric points of the protein eluted. The

Figure 5.3 Effect of loading on the elution profile of bSP-extract on G-75 eluted with 0.05M sodium acetate, pH 4.

200 mg bSP-extract (#1-9) was added to 10 ml 0.05M sodium acetate, pH 4. The insoluble material was removed and the supernatant dialysed and freeze-dried. a) 29.4 mg, b) 15.2 mg, and c) 6.0 mg of the supernatant was dissolved in 3.5 ml of the same buffer and chromatographed on a 160 ml column of G-75.
Figure 5.4 Profiles of extracts prepared from different batches of bSP on G-75 and G-200 chromatography.

Extracts prepared from the first batch of bSP were chromatographed on a) G-75 and c) G-200 columns eluted with 0.1M ammonium acetate, pH 7.6. Similarly, extracts from the second batch of bSP were run on the same b) G-75 and d) G-200 columns. The G-75 column was 2.3 l, and was loaded with 1 g (#1-6) and 1.3 g (#2-2); the G-200 column was 160 ml, and was loaded with 69.7 mg (#1-8) and 80.0 mg (#2-2).
Figure 5.5  PAGE patterns of N'-I and II prepared from the first and second batches of bSP.

See Figures 5.4a and 5.4b.
relationship is not exact because the pH at which a protein is eluted from an ion exchanger depends on the protein's nett charge at pHs close to its isoelectric point as well as the isoelectric point itself.

5.5 PAGE PATTERN

PAGE patterns of active fractions had a common feature: a diffuse band, or bands, that migrated 45-60% as fast as the ion front on 7% gels. The mobility of this band was similar to that of the two bands in fractions N-IV, N'-V, A-III, and CM-VI.

Table 5.1 Antigonadotrophic activity in N'-I and II prepared from different batches of bSP (Geometric means and their 67% confidence intervals).

<table>
<thead>
<tr>
<th></th>
<th>FSH (µg/l)</th>
<th>LH (µg/l)</th>
<th>s/n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First batch of bSP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1 saline</td>
<td>1148 (1032-1277)</td>
<td>163 (136-196)</td>
<td>9/9</td>
</tr>
<tr>
<td>N'-I, 37 mg</td>
<td>810 (743-884)**c</td>
<td>109 (84-141)</td>
<td>7/8</td>
</tr>
<tr>
<td>N'-II, 6.2 mg</td>
<td>676 (591-775)**</td>
<td>81 (60-111) *</td>
<td>8/8</td>
</tr>
</tbody>
</table>

| **Second batch of bSP** |                     |                    |      |
| Expt 2 saline  | 1448 (1317-1592)    | 168 (144-196)      | 7/7  |
| N'-I, 37 mg    | 705 (583-872)**     | 54 (31-96) *       | 4/6  |
| N'-II, 6.2 mg  | 1096 (918-1311)     | 117 (62-222)       | 5/5  |

| Expt 3c saline | 1280 (1137-1443)    |                    | 7/7  |
| N'-II, 5.6 mg  | 1045 (874-1249)     | 11.2 mg 1123 (996-1266) | 4/4  |

\( ^a \) All animals also received 100 µg testosterone propionate/kg BW to increase the sensitivity of the detection method.

\( ^b \) n no. allotted per group, s no. surviving

\( ^c \) Significantly lower than the control group: * p <0.05, ** p <0.005
CHAPTER 6 ORIGIN OF ANTIGONADOTROPHIC ACTIVITY IN bSP

6.1 SEMINAL PLASMA FROM VASECTOMISED BULLS

Seminal plasma pooled from vasectomised bulls (vbSP) suppressed serum FSH and LH in a dose related way (Figure 6.1). The potencies of individual samples were comparable to the potency of the first batch of bSP (Figure 6.2). For all samples of vbSP, a dose of 0.5 ml or equivalent always suppressed FSH and LH to near-intact levels.

The activity in vbSP was precipitated by 86% (v/v) ethanol; yields were 41.6 mg/ml for the batch of vbSP pooled from five bulls (vbSP #1-1), and ranged from 59.1 to 73.8 mg/ml for the samples from individual bulls (vbSP #2-1 to 5-1). (Table 6.1)

The antigonadotrophic activity in vbSP-extract was destroyed by heating at 67°C for 1 h, and by digestion with pepsin (Figure 6.3).

6.2 BULL SEMINAL VESICLE FLUID

Bull seminal vesicle fluid (bSVF) was extracted by diafiltration with a PM 10 membrane. The yield was 82.6 mg/ml, and the testosterone content of the extract was 15.5 ng/80 mg.

Like the extracts of bSP and vbSP, bSVF-extract suppressed FSH and LH in a dose-dependent manner, and a dose equivalent to 0.5 ml bSVF brought about near-intact levels (Figure 6.4).

Heating the extract destroyed its activity.

6.3 SEMINAL FLUID FROM ANDROGEN-TREATED STEERS

Although the presence of activity in vbSP and bSVF argues against a testicular origin, it did not exclude the possibility that the activity was transported indirectly from the testes to the seminal vesicles. The detection of activity in animals whose seminal vesicle function had been maintained (by treatment with androgens) in the absence of the testes would negate this alternative hypothesis.
Figure 6.1  Antagonadotrophic activity in seminal plasma from vasectomised bulls

Seminal plasma pooled from five vasectomised bulls (vbSP #1) was screened for antagonadotrophic activity at doses of 0.1-0.5 ml. Geometric means and their 67% confidence intervals. Inside the FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: * p <0.05, ** p <0.005.
Figure 6.2  Antagonadotrophic activity in seminal plasma samples from individual vasectomised bulls.

Samples of vbSP from four individual bulls (vbSP #2 to 5) and bSP pooled from 20 intact bulls (bSP #1) were screened for antagonadotrophic activity as doses of a) 0.5 ml and b) 0.2 ml. Geometric means and their 67% confidence intervals, and individual concentrations. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: *p <0.05, **p <0.005.
Table 6.1  Some properties of seminal fluid from vasectomised bulls and androgen-treated steers.

<table>
<thead>
<tr>
<th>ejaculate volume(s) (ml)</th>
<th>yield of ethanol precipitate (mg/ml)</th>
<th>Na(^+) concentration (mM)</th>
<th>K(^+) concentration (mM)</th>
<th>group</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>b</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bull (^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>68.2 (#2-1)</td>
<td>140</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>8.7</td>
<td>71.7 (#3-1)</td>
<td>105</td>
<td>81</td>
</tr>
<tr>
<td>4</td>
<td>4.3</td>
<td>59.1 (#4-1)</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>7.9</td>
<td>73.8 (#5-1)</td>
<td>117</td>
<td>33</td>
</tr>
<tr>
<td>steer (^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11.9, 5.6</td>
<td>4.97</td>
<td>139, 140</td>
<td>18, 18 A</td>
</tr>
<tr>
<td>2</td>
<td>7.1</td>
<td>3.55</td>
<td>133</td>
<td>27 A</td>
</tr>
<tr>
<td>3</td>
<td>2.0, 1.4</td>
<td>2.77</td>
<td>136, 67</td>
<td>21, 103 B</td>
</tr>
<tr>
<td>4</td>
<td>9.2</td>
<td>3.70</td>
<td>84</td>
<td>90 B</td>
</tr>
<tr>
<td>5</td>
<td>11.2, 5.4</td>
<td>4.87</td>
<td>140, 141</td>
<td>17, 22 A</td>
</tr>
<tr>
<td>6</td>
<td>7.0</td>
<td>2.56</td>
<td>51</td>
<td>147 B</td>
</tr>
</tbody>
</table>

\(^{a}\)mean of two ejaculates

\(^{b}\)individual ejaculates
Figure 6.3 Effect of heat and pepsin on the antigonadotrophic activity in vbSP-extract.

Solutions of vbSP-extract (#1-1) were heated at 67°C for 1 h or incubated with pepsin, and then screened for antigonadotrophic activity at a dose equivalent to 0.5 ml vbSP.

Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: * p <0.05, ** p <0.005
Figure 6.4  Antigonadotrophic activity in an extract of bull seminal vesicle fluid, and the effect of heat.

An extract of bSVF was screened for antigonadotrophic activity at doses equivalent to 0.13-0.5 ml bSVF, and a solution of the extract previously heated at 67°C for 1 h was screened at a dose equivalent to 0.5 ml. Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the control group: * p <0.05, ** p <0.005
Figure 6.5  PAGE patterns of bSP, vbSP, bSVF, and seminal fluid from androgen-treated steers.
Six adult steers (castrated at birth) were treated with testosterone esters for four weeks and then electroejaculated. It was obvious that the treatment had failed to revive seminal vesicle function—the ejaculates were thin, not viscous like bSP, vbSP, and bSVF; their protein content was low (Table 6.1); their electrophoretic patterns, and hence protein composition, were very different to those of bSP, vbSP, and bSVF (Figure 6.5); and the seminal vesicles themselves were small (less than 20 g).

The ejaculates were divided into two groups, A - those with low potassium concentrations, and B - those with high potassium. Ethanol extracts of samples of group A freeze-dried to fluffy white powders; extracts of B formed brown granular powders. Pooled samples of A and B were screened for antigonadotrophic activity, but none was detected (Table 6.2). Doses were necessarily low - nearly all the material obtained was used in the screening.

Table 6.2 Antigonadotrophic activity in seminal fluid from androgen-treated steers.
(Geometric means and their 67% confidence intervals).

<table>
<thead>
<tr>
<th></th>
<th>FSH (µg/l)</th>
<th>LH (µg/l)</th>
<th>s/n^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline</td>
<td>1397 (1331-1467)</td>
<td>123 (105-120)</td>
<td>6/6</td>
</tr>
<tr>
<td>bSP-extract, 20 mg</td>
<td>1199 (1091-1317)</td>
<td>113 (105-120)</td>
<td>5/6</td>
</tr>
<tr>
<td>(#2-2) 40 mg</td>
<td>766 (629-934)^b</td>
<td>25 (14-43)*</td>
<td>4/6</td>
</tr>
</tbody>
</table>

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>seminal fluid from androgen-treated steers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract A, 10 mg</td>
<td>1440 (1344-1543)</td>
<td>116 (96-141)</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>1479 (1393-1571)</td>
<td>125 (100-156)</td>
<td>6/6</td>
</tr>
<tr>
<td>Extract B, 10 mg</td>
<td>1557 (1451-1671)</td>
<td>144 (118-175)</td>
<td>6/6</td>
</tr>
</tbody>
</table>

^a n no. allotted per group, s no. surviving
^b Significantly lower than the control group: * p <0.05;
  ** p <0.005
CHAPTER 7 PROPERTIES OF THE ANTIGONADOTROPHIC ACTIVITY IN bSP IN MALE RATS

7.1 INTRODUCTION

So far the expression of bSP's antigonadotrophic activity in male rats has been used only as a tool for purifying, localising, and elucidating the physicochemical properties of the activity. It has not been studied in its own right. Study of the physiological properties (or perhaps more correctly, the pharmacological properties) of the activity was prompted by two practical concerns. Was the activity truly inhibin-like? And, how could the detection method be made more sensitive? These questions crystallised into a chain of four major hypotheses, which provide the structure for the chapter. Despite the prosaic reasons for the questions, the answers go further to suggest a place for inhibin in the control of serum FSH and LH in the male rat.

7.2 CASTRATED RATS

The first hypothesis was that bSP contained an inhibin-like substance. Suppression of serum FSH, but not LH, in a test animal would support this.

7.2.1 Effect of bSP in Castrated Rats

The hypothesis was tested by giving bSP, or extracts or fractions of it, to chronically and acutely castrated male rats. The experimental designs and the reasons for their choice are presented in Chapter 3.

bSP and its extracts always suppressed FSH and LH together in acutely castrated rats. When the dose-response was studied, serum concentrations of FSH and LH were on average equally sensitive to suppression, and a dose which suppressed FSH to intact levels invariably suppressed LH to near-intact levels also (see Tables 3.2 and 7.2 and Figure 3.6). The same was true for antigonadotrophic activity from the related sources of vbSP and bSVF (see Figures 6.1, 6.2 and 6.4). Moreover, the FSH- and LH-suppressing activities were never separated when bSP-extract was fractionated by a variety of methods (see the figures of Chapter 4).
Figure 7.1 Effect of bSP-extract on serum FSH and LH in acutely castrated rats from two strains - Sprague-Dawley and Brattleboro.

In contemporary experiments, bSP-extract (#1-6 and 1-7) was screened for antigonadotrophic activity in acutely castrated Sprague-Dawley rats (open bars) and Brattleboro rats (shaded bars). Geometric means and their 67% confidence intervals, and individual LH concentrations. Inside FSH bars: s/n, n no. allotted per group, s no. surviving.
So although bSP did contain antgonadotrophic activity, it did not preferentially suppress serum FSH in the detection method chosen. Was the difference between the observed and expected degree of selectivity for FSH due to the detection method, or the material itself?

7.2.2 Influence of the Strain of Rat

It is possible that the strain of rat used here - Sprague-Dawley, inbred for many generations - responded differently to the rats used by others. Accordingly, the response to bSP-extract was studied in rats of two widely separated strains: local Sprague-Dawleys, and Brattleboros - a strain derived from Long-Evans hooded rats. The relative degree of suppression of FSH and LH and the dose-response appeared identical in the two strains (Figure 7.1).

7.2.3 Comparison of bSP-extract and Rat Testis-extract

An alternative explanation for not seeing selective suppression of FSH was that the antgonadotrophic activity in bSP might have different properties than inhibin. This was tested by comparing the response of acutely castrated rats to bSP-extract and to an extract of rat testes, which would presumably contain the rat's own inhibin. Both extracts appeared to suppress FSH and LH (Figure 7.2). Because one rat in each group failed to respond (these were heavier than their litter-mates) suppression was not statistically significant by the Mann-Whitney test. The antgonadotrophic activity in the rat testis-extract was elusive; other experiments detected none or only a small amount, even at doses up to 100 mg. The inconsistency was probably due to the variable sensitivity of the detection method (see Section 3.4.3); the experiment in Figure 7.2 took place when sensitivity was very great. Although there was no direct evidence that a protein was responsible for the activity, steroids were unlikely candidates for the extract had been washed with diethyl ether and its testosterone content was only 0.3 mg/60 mg.

7.2.4 Comparison of Acutely Castrated Rats and Pituitary Cell Cultures

Differences between detection methods and between inhibin-like activities from different sources was further investigated by comparing
Figure 7.2  Antagonadotrophic activity in bSP-extract and rat testis-extract.

bSP-extract (#1-2) and rat testis-extract were screened for antagonadotrophic activity at doses equivalent to 0.2 ml bSP and 2.5 g wet weight of testes. Individual FSH and LH concentrations.
(μg/ml culture medium)
the effects of bSP-extract and ovine follicular fluid (oFF)-extract in acutely castrated male rats and in pituitary cell cultures (Figure 7.3). The cell cultures were performed by Drs G. Erickson and A. Hseuh of the Department of Reproductive Medicine, University of California, San Diego. The two samples of oFF had been extracted with 86% (v/v) ethanol, and the precipitates washed with acetone; yields were 48.9 mg/ml (#1-1) and 50.1 mg/ml (#2-1).

In pituitary cells cultured from ovariectomised rats (Figure 7.3a and b), small doses of bSP-extract (0.5 and 5 μg/ml) had little effect on FSH or LH secretion. Larger doses of the extract and its derivative CM-B (25 μg/ml) increased FSH secretion, and to a lesser extent LH secretion. In contrast, the oFF-extracts selectively suppressed FSH, with #1-1 being more potent than #2-1.

In cultures from intact males (Figure 7.3c), CM-B may have suppressed FSH secretion slightly, but it had a greater effect on LH secretion. In comparison, oFF-extract had more effect on FSH than on LH.

Two differences emerged. The first was between activities from...
Table 7.1  Antigonadotrophic activity in oFF-extract$^a$ and bSP-extract$^b$
(Geometric means and their 67% confidence intervals).

<table>
<thead>
<tr>
<th></th>
<th>FSH (μg/l)</th>
<th>LH (μg/l)</th>
<th>s/n$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline</td>
<td>1452 (1313-1604)</td>
<td>200 (173-230)</td>
<td>8/8</td>
</tr>
<tr>
<td>bSP-extract, 40 mg</td>
<td>586 (505-679)**$^d$</td>
<td>26 (25-27)**</td>
<td>5/8</td>
</tr>
<tr>
<td>oFF-extract, 1 mg</td>
<td>1370 (1284-1451)</td>
<td>228 (186-279)</td>
<td>7/7</td>
</tr>
<tr>
<td>2.5 mg</td>
<td>1320 (1202-1449)</td>
<td>224 (195-258)</td>
<td>7/7</td>
</tr>
<tr>
<td>5 mg</td>
<td>1346 (1260-1438)</td>
<td>274 (232-324)</td>
<td>7/7</td>
</tr>
</tbody>
</table>

$^a$ oFF-extract (64% #1-1, 36% #2-1).

$^b$ bSP-extract (#1-6)

$^c$ n no. allotted per group, s no. surviving.

$^d$ Significantly lower than the saline control group:

*p < 0.05,  **p < 0.005.
different sources: material derived from bSP had a greater effect on 
LH (relative to its effect on FSH) than did oFF-extract. The second was 
between detection methods: pituitary cells from males appeared more 
sensitive to the LH-suppressing activity of oFF-extract than did cells 
from ovariectomised rats. (The latter difference could conceivably be 
due merely to greater sensitivity of the male cells - a quantitative, not 
qualitative, distinction.)

The response to bSP-extract and oFF-extract was also measured 
in acutely castrated rats (Table 7.1). Although 40 mg bSP-extract 
suppressed FSH and LH to intact levels, doses of up to 5 mg oFF-extract 
(containing 3.2 mg of the more potent #1-1) had no effect. The 
sensitivities of the two detection systems to the two samples are 
tabulated below.

<table>
<thead>
<tr>
<th></th>
<th>bSP-extract</th>
<th>oFF-extract (#1-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acutely castrated rats</td>
<td>&lt; 40 mg</td>
<td>&gt; 3.2 mg</td>
</tr>
<tr>
<td>pituitary cell culture, ovariectomised rats</td>
<td>&gt; 25 μg/ml</td>
<td>&lt; 0.5 μg/ml</td>
</tr>
</tbody>
</table>

According to acutely castrated rats, oFF-extract (#1-1) cannot be more 
than 40/3.2=12.5 times as potent as the bSP-extract. On the other hand, 
from the cultures, the same oFF-extract is more than 25/0.5=50 times as 
potent as the bSP-extract. The apparent contradiction implies different 
potencies in the two systems - the cultures were more sensitive to oFF-
extract, the castrate rats to bSP-extract.

Thus there were differences between detection methods - both 
between cell cultures and acutely castrated rats and between cells 
cultured from male and ovariectomised rats, and there were differences in 
the properties of the antigonadotrophic activities in bSP- and oFF-extracts.

7.3 INTACT RATS

The second hypothesis was that intact rats might respond 
differently to the antigonadotrophic activity in bSP than do castrate rats. 
Earlier experiments had suggested that sensitivity fell with time after 
castration (see Section 3.2 and 3.3); if this occurred rapidly then intact 
rats might be superior to castrates for the detection of antigonadotrophic 
activity. A second prospect was a qualitative difference, in particular
Table 7.2  Suppression of FSH and LH in acutely castrated and intact rats by bSP-extract (# 1-8)
(Geometric means and their 67% confidence intervals).

<table>
<thead>
<tr>
<th>bSP-extract (mg)</th>
<th>FSH (μg/l⁻¹)</th>
<th>LH (μg/l⁻¹)</th>
<th>s/n</th>
<th>FSH (μg/l⁻¹)</th>
<th>LH (μg/l⁻¹)</th>
<th>s/n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1283 (1192-1382)</td>
<td>168 (130-214)</td>
<td>6/6</td>
<td>667 (590-754)</td>
<td>18 (14-23)</td>
<td>6/6</td>
</tr>
<tr>
<td>20</td>
<td>1240 (1200-1280)</td>
<td>124 (105-152)</td>
<td>6/7</td>
<td>563 (538-589)</td>
<td>17 (12-24)</td>
<td>6/6</td>
</tr>
<tr>
<td>40</td>
<td>605 (516-709)**b</td>
<td>29 (18-46)**</td>
<td>6/7</td>
<td>476 (429-528)*</td>
<td>17 (11-26)</td>
<td>6/6</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1319 (1086-1602)</td>
<td>143 (97-212)</td>
<td>6/6</td>
<td>533 (518-548)</td>
<td>21 (17-28)</td>
<td>9/9</td>
</tr>
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<td>10</td>
<td>1344 (1158-1559)</td>
<td>151 (124-183)</td>
<td>6/8</td>
<td>473 (438-511)</td>
<td>22 (16-28)</td>
<td>6/6</td>
</tr>
<tr>
<td>20</td>
<td>726 (600-880)*</td>
<td>35 (22-57)*</td>
<td>7/8</td>
<td>430 (390-474)*</td>
<td>12 (11-13)*</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* n no. allotted per group, s no. surviving.
**b Significantly lower than control group, * p <0.05, ** p <0.005.
selective suppression of FSH.

7.3.1 Sensitivity

Intact rats appeared as sensitive as castrate rats to the FSH-suppressing activity of bSP-extracts; a significant lowering of serum FSH levels 24h after injection was detected at the same dose in both types of animals in two experiments (Table 7.2), although the dose itself differed between the two experiments.

7.3.2 Variable Suppression of LH

LH was suppressed in intact rats in only one of the two experiments in Table 7.2. Figure 7.4 collates data from all experiments in which intact rats received bSP-extract; only the groups showing significant suppression of FSH have been included. (Some of these experiments appear complete elsewhere.) The groups in which LH was suppressed did not necessarily receive higher doses of bSP-extract, nor did they show uniformly greater FSH suppression. Instead, the pertinent factor appeared to be when the experiment was done. Those in which LH was suppressed took place when acutely castrated rats were more sensitive to the antigonadotrophic effect of bSP-extract, and conversely, the experiments without suppression occurred at times of relative insensitivity on the part of acutely castrated rats (Figure 7.5).

7.3.3 Time-course of Action

An alternative explanation for variable suppression of LH in intact rats (and one postulated before all the experiments presented in Section 7.3.2 had taken place) was variation in the time-course of action of the antigonadotrophic activity. This possibility arose from results of Baker et al. (1976), who had found that intact rats receiving bovine testis extracts had significantly lower concentrations of serum LH at 24h, but of FSH at 48 and 72h. The time-course of action is also of interest in its own right. Intact rats were the choice for this study; the response of castrates would have been complicated by diminishing sensitivity to bSP-extract and rising baseline concentrations of FSH and LH. Pituitary concentrations of FSH and LH were also measured in case relative changes
Figure 7.4  Variable suppression of serum LH in intact rats given bSP-extract.

In six experiments, five-week-old rats were injected with saline or bSP-extract and were bled 24h later. Only doses which significantly suppressed serum FSH are shown. Geometric means and their 67% confidence intervals for FSH, and individual concentrations for LH. Inside FSH bars: no. per group. Significantly lower than control groups: *p < 0.05, **p < 0.005 for FSH, and exact probabilities for LH.
Figure 7.5  Suppression of serum LH in intact rats treated with bSP-extract - when it occurred.

a) Time of occurrence and "p" values for the six experiments in Figure 7.4

b) On the same scale, the sensitivity of acutely castrated rats to 20 mg bSP-extract (from Figure 3.6).
in pituitary and serum levels gave a clue to the site of feedback.

Rats were injected with saline or bSP-extract at 9-10 am, and killed 3-48h later (Figure 7.6). Each time had its own control group to remove the contribution of circadian rhythms and of stress from anaesthesia and injection. Serum FSH was suppressed 12, 24 and 48h after injection, LH at 6, 12, 24 and possibly 48h. Monitoring hormone levels 24h after injection seems optimal for detecting the antigonadotrophic activity in bSP. With blood sampling at 24h there seems little chance of missing LH suppression; the alternative explanation for variable suppression of LH in intact rats can be dismissed. Pituitary concentrations of FSH and LH seemed not to change. (Although the probability that the mean concentrations of pituitary LH in the control and treated groups at 24h were the same was only 0.054, the probability of finding at least one "significant" difference (p <0.05) among five independent tests (here control versus treated groups at 3, 6, 12, 24 and 48h) is 0.23.)

7.4 COMPARISON WITH TESTOSTERONE

The third hypothesis was that bSP-extract and testosterone had different relative effects on FSH and LH. Although preferential suppression of LH by testosterone is widely accepted, it had to be demonstrated in the detection method used here.

7.4.1 Castrated Rats

Testosterone propionate suppressed serum FSH and LH in acutely castrated rats, with the hormone levels being related logarithmically to

Figure 7.6 Time-course of the antigonadotrophic activity of bSP-extract in intact male rats.

Five-week old rats were injected with saline (open bars) or 26 mg bSP-extract (#2-3) (shaded bars). They were bled and their pituitaries removed 3, 6, 12, 24 or 48h later. Untreated rats were bled at 0h. Geometric means and their 67% confidence intervals for pituitary FSH and LH, and serum FSH; individual concentrations for serum LH. Inside FSH bars: no. per group. Significantly lower than the control groups: exact probabilities.
Figure 7.7  Response of acutely castrated rats to testosterone propionate.

Five-week old rats were given 0, 70, 150, 300 or 600 µg testosterone propionate/kg BW immediately after castration, and were bled 24h later. Geometric means and their 67% confidence intervals, and individual LH concentrations for the intact group. Inside FSH bars: no. per group. Significantly lower than the control group: *p <0.05, **p <0.005.
I = \text{testosterone propionate (µg/kg B.W.)}

\text{c)}

\begin{align*}
\text{serum testosterone (µg/l)} & \\
\text{testosterone propionate (µg/kg B.W.)} & \\
\text{300} & \text{intact}
\end{align*}
the dose (Figure 7.7). LH was suppressed preferentially: a dose of 70 µg/kg body weight (BW) suppressed LH to midway between castrate and intact levels, whereas 150 µg/mg BW was needed to do the same for FSH. However, LH concentrations showed greater variation than FSH concentrations so that a statistically significant suppression was more observable for FSH. To lessen the impact of such variation, the experiment was repeated with more animals per group.

In the second experiment, dose-response changed during the experiment (9-20/8/1979) (Figure 7.8a). In the first week 300 µg testosterone propionate/kg BW lowered FSH to intact levels, whereas 600 µg/kg BW was needed in the fourth week. For LH the shift was more striking: sensitivity fell four- to eight-fold between the first and

Figure 7.8 Fluctuating sensitivity of acutely castrated rats to testosterone propionate.

a) Five-week old rats were given 0, 70, 150, 300, 600 or 1200 µg testosterone propionate/kg BW immediately after castration, and were bled 24h later. The experiment spanned four weeks; rats from the first week are shown in the first column of each triad (heavily shaded), those from the second and third weeks were combined and appear in the second column (lightly shaded), and those from the fourth week in the third column (unshaded).

b) Response of acutely castrated rats to 300 µg testosterone propionate four weeks after the end of the experiment in a). Two solutions were tested - that used in a) (denoted "old") and one freshly prepared (denoted "new").

c) Serum testosterone concentrations of the intact rats and the rats receiving 300 µg testosterone propionate/kg BW in Figure 7.8a. The method of the testosterone radioimmunoassay included extraction of the samples with diethyl ether. When solutions of testosterone propionate were treated in the same way as serum, they showed a curve parallel to the standard curve but displaced, such that 1 mg/l testosterone propionate appeared to contain 1.4 µg/l testosterone. Such slight interference allowed the measurement of serum testosterone in the presence of testosterone propionate.

Geometric means and their 67% confidence intervals, also individual testosterone concentrations. Inside FSH bars: no. per group.
Figure 7.9  Responses of acutely castrated and intact rats to testosterone propionate.

Five-week old rats were given 0, 0.1, 1.0 or 10 mg testosterone propionate/kg BW and bled 24h later. In experiment a), rats were castrated immediately before injection, in b) they were left intact. Geometric means and their 67% confidence intervals, and individual LH concentrations. Inside FSH bars: no. per group. Significantly lower than intact control: *p <0.05, **p <0.005.
fourth weeks. Analysis of variance confirmed (p < 0.05) that there were differences in the weekly response of FSH to 300 μg testosterone propionate/kg BW, and of LH to 70, 300 and 600 μg/kg BW. Serum concentrations of testosterone of the rats receiving 300 μg/kg BW and of the intact rats are shown in Figure 7.8c.

The change originated in the rats; it was not caused by degradation of the testosterone propionate solutions. Four weeks after the end of the experiment, 300 μg/kg BW suppressed FSH and LH to the same extent as it had done in the first week; and the original testosterone propionate solution and a freshly prepared solution were equally effective (Figure 7.8b).

7.4.2 Intact Rats

The response of acutely castrated rats to a wide range of testosterone propionate doses is shown in Figure 7.9a. Even large doses failed to suppress FSH below intact levels. In contrast, testosterone propionate did suppress FSH in intact rats, although to a limit (Figure 7.9b). The different responses of castrate and intact rats to large doses was not just due to between-experiment variation. It was seen in two other experiments: one where the rats received 1 mg/kg BW (see Table 7.3), the other where the dose was 10 mg/kg BW (not shown).

7.5 INTERACTIONS WITH TESTOSTERONE

Experiments in previous sections had shown that bSP-extract and testosterone propionate had different relative effects on serum FSH and LH. These prompted the fourth hypothesis - that testosterone and the antgonadotrophic activity of bSP (as an analogue of inhibin) might interact, and in particular, act synergistically to suppress serum FSH in male rats.

7.5.1 Effect of Testosterone on the Dose-response to bSP-extract

The first experiment looked at the effect of testosterone propionate on the dose-response of acutely castrated rats to bSP-extract (Figure 7.10). In the absence of the steroid, 10 mg bSP-extract suppressed FSH slightly, while 20 mg suppressed FSH to near intact levels.
Although 100 μg testosterone propionate/kg BW had no effect on FSH by itself, it acted synergistically with 10 mg bSP-extract to suppress FSH to near intact levels. However this small dose of testosterone propionate only slightly enhanced the effect of 20 mg bSP-extract. A larger dose of 500 μg/kg BW which by itself suppressed FSH to intact levels had little combinative effect with bSP-extract.

LH levels appeared to follow the same pattern as FSH, although the pattern was blurred by the greater spread of LH concentrations and the greater (although in this experiment statistically insignificant) suppression of LH by 100 μg testosterone propionate/kg BW. In a second experiment, the dose of testosterone propionate was reduced to 30 μg/kg BW (Figure 7.11). Even at this small dose it interacted synergistically with bSP-extract to suppress FSH and LH.

Large doses of testosterone propionate (1 and 10 mg/kg BW) suppressed FSH further in intact than in castrated rats (see Section 7.4.2 and Figure 7.9). A possible explanation was that the testes enhanced the effect of testosterone by producing bSP-extract-like activity (endogenous inhibin?) which interacted with the steroid. An experiment was designed to test this hypothesis (Table 7.3). As before, 1 mg testosterone propionate/kg BW lowered FSH further in intact than in castrated rats, and 40 mg bSP-extract suppressed FSH to near intact levels in castrated rats. However, co-administration of 1 mg testosterone propionate/kg BW and 40 mg bSP-extract to castrated rats had no more effect than the two treatments had separately, and the FSH level attained was still higher than that of intact

Figure 7.10 Suppression of FSH and LH in acutely castrated rats by bSP-extract and testosterone propionate given separately and together.

Five-week old rats were castrated and given 0, 10 or 20 mg bSP-extract (#1-8) and 0, 100 or 500 μg testosterone propionate/kg BW. The rats were bled 24h later.

Geometric means and their 67% confidence intervals, and individual LH concentrations. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the appropriate control group: * p <0.05, ** p <0.005.
Figure 7.11  Suppression of FSH and LH in acutely castrated rats by bSP-extract in the presence and absence of a small amount of testosterone propionate

As in Figure 7.10, but with 0 or 30 μg testosterone propionate/kg BW. Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted per group, s no. surviving. Significantly lower than the appropriate control group: † p = 0.06, *p <0.05, **p <0.005.
Table 7.3  Suppression of FSH in acutely castrated and in intact rats by larger doses of bSP-extract (#2-2) and testosterone propionate - no combined effect  
(Geometric means and their 67% confidence intervals).

<table>
<thead>
<tr>
<th>group</th>
<th>status</th>
<th>testosterone propionate (mg/kg BW)</th>
<th>bSP-extract (mg)</th>
<th>s/n&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FSH (μg l&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>intact</td>
<td>0</td>
<td>0</td>
<td>6/6</td>
<td>644 (607-684)</td>
</tr>
<tr>
<td>2</td>
<td>intact</td>
<td>1.0</td>
<td>0</td>
<td>6/6</td>
<td>377 (358-395) **&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>castrated</td>
<td>1.0</td>
<td>0</td>
<td>7/7</td>
<td>465 (437-495) **&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>intact</td>
<td>0</td>
<td>40</td>
<td>6/6</td>
<td>490 (449-533) *</td>
</tr>
<tr>
<td>5</td>
<td>castrated</td>
<td>0</td>
<td>40</td>
<td>5/7</td>
<td>526 (422-632)</td>
</tr>
<tr>
<td>6</td>
<td>castrated</td>
<td>1.0</td>
<td>40</td>
<td>6/7</td>
<td>444 (427-461) **&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> n no. allotted per group, s no. surviving

<sup>b</sup> Significantly lower than the intact control group (Group 1): *p <0.05, **p <0.005,
Significantly different from intact group treated with testosterone propionate (Group 2):  † p <0.05.
rats receiving testosterone propionate alone.

7.5.2 Does Testosterone Maintain the Sensitivity of Castrated Rats to bSP-extract?

Earlier experiments (see Sections 3.2 and 3.3 and Figures 3.1 and 3.3) had indicated that male rats lost their sensitivity to the antigonadotrophic activity of bSP soon after castration. The testis must make some substance(s) which maintains the rat's ability to respond to bSP-extract. Is it testosterone? Indeed the synergism between low doses of testosterone and bSP-extract in acutely castrated rats could be explained by such a mechanism.

The question was investigated in the following way. Rats were castrated at five weeks old, and at the same time a capsule containing testosterone (or an empty capsule for controls) was implanted subcutaneously. Such capsules release a steady amount of hormone over long periods. After a week the capsule was removed - in effect a "pseudo-castration" - and the rats were treated with saline or bSP-extract as usual.

The first step was finding the size of capsule that reproduced the serum testosterone concentrations of intact rats (Figure 7.12). However the testosterone concentrations attained were erratic. The presence of detectable concentrations in the castrate control group and the incidence of concentrations >5 μg/l in all groups (and mostly in rats from the same litters) implied that some rat sera interfered in the measurement of testosterone. Instead, FSH and LH levels were taken as a guide to the desirable capsule size. The degree of suppression of FSH and LH depended on the length of the capsule although there was wide

Figure 7.12 Suppression of FSH and LH in acutely castrated rats by subcutaneous capsules containing testosterone.

Five-week old rats were castrated and silastic capsules, packed to a length of 5, 10 or 20 mm with testosterone, were implanted subcutaneously. The rats were bled 8 days later and serum concentrations of FSH, LH and testosterone measured. Individual concentrations are shown. For testosterone, rats from the same litter have the same symbol.
castration & capsule implantation

b

capsule implantation

castration bleeding

castration bleeding

a 7 days treatment 24h

c

treatment

capsulation implantation

a b c

serum FSH (µg/l)

serum LH (µg/l)

0 bSP-E 0 bSP-E 0 bSP-E intact rats

(40mg) (40mg) (40mg) rats
Figure 7.13  Loss of sensitivity to the antigonadotrophic activity in bSP-extract with time after castration - effect of testosterone.

Five-week old rats were either castrated (a & b), or left intact (c), and at the same time empty capsules (a & c) or capsules containing 10 mm testosterone (b) were implanted subcutaneously. Seven days later the capsules were removed and the rats in c) were castrated. The rats were then given saline or 40 mg bSP-extract (#2-2) and were bled 24h later. Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, n no. allotted, s no. surviving. Significantly lower than the appropriate control: *p <0.05, **p <0.005.
variation at non-maximal doses. Variation arose from differences between litters; rats from the same litter exhibited similar sensitivity. A capsule length of 10 mm was chosen because it suppressed FSH and LH to concentrations comparable to those of intact rats.

Capsules with 10 mm testosterone did not prevent the loss of sensitivity to bSP-extract that developed after castration (Figure 7.13). Nevertheless, 40 mg bSP-extract did suppress FSH slightly and significantly (p < 0.05) in the testosterone treated rats in both the experiment in Figure 7.13 and in another (not shown). Consequently, different sized capsules were tried in case the dose of testosterone was wrong. Capsules containing 2.5 mm testosterone had no effect, while a capsule containing 20 mm testosterone at least partially retained the rat’s ability to respond to bSP-extract (Figure 7.14).

Figure 7.14  Loss of sensitivity to the antigonadotrophic activity in bSP-extract with time after castration - effect of different doses of testosterone.

Five-week old rats were either castrated (a & b) or left intact (c), and at the same time capsules containing a) 2.5 mm or b) 20 mm testosterone were implanted subcutaneously. Seven days later the capsules were removed, and the rats in c) were castrated. The rats were then given saline or 40 mg bSP-extract (#2-2) and were bled 24h later. Geometric means and their 67% confidence intervals. Inside FSH bars: s/n, no. allotted per group, s no. surviving. Significantly lower than the appropriate control: *p <0.05.
8.1 CHOOSING A METHOD FOR DETECTING INHIBIN-LIKE ACTIVITY

8.1.1 Requirements for an Assay

Detection of inhibin-like activity rests on observing the essential property of inhibin - its ability to inhibit gonadotrophin secretion, or according to more recent definitions, its ability to selectively inhibit FSH secretion. Early investigators looked for inhibition of some gonadotrophin-dependent process, such as maintenance or hypertrophy of ovarian, uterine, or testicular weights (Martins, 1930; Martins & Rocha, 1931; McCullagh & Walsh, 1935). Although such bioassays have an easily measured endpoint, their specificity can be questioned, especially when the treatment also reduces body weight. The advent of radioimmunoassays for FSH and LH allows direct measurement of the concentrations of these hormones in blood. Reduction of serum FSH, but not LH, on administration of a test substance into an animal would allow unambiguous detection of inhibin-like activity.

8.1.2 Chronically Castrated Male Rats

Adult rats castrated two to three weeks beforehand were used to detect inhibin-like activity in human and bull seminal plasma by Franchimont (1973) and Franchimont et al. (1975a), and in ovine rete testis fluid by Setchell & Jacks (1974). Castration increases serum FSH and LH several-fold by removing endogenous feedback, allowing suppression to be more easily observed. Serum LH concentrations in intact male rats are near or below the sensitivity of most radioimmunoassays. Franchimont et al.'s (1975a) method was tried initially since bSP was the source material here. However doses of up to 1 ml per day for two days failed to suppress FSH. This dose had some side-effects so higher doses could not be tested. Since the data of Setchell & Jacks (1974) indicated treatment needed to run three days or more to have a maximum effect, 0.2 or 0.5 ml was injected daily for three or four days. FSH and LH were still unaffected. Chronically castrated rats are now recognised as being comparatively insensitive (Davies et al., 1976; Franchimont et al.,...
8.1.3 Acutely Castrated Male Rats

At the time of the unsuccessful experiments with chronically castrated rats, two papers were published describing the use of acutely castrated, 35-day old, male rats (Nandini et al., 1976; Davies et al., 1976). A very simple procedure was tried. Male rats were castrated, injected immediately afterwards, and bled 24h later. A dose of 0.5 ml bSP inhibited the post-castration rise of FSH and LH in both adult and five-week old rats. Preliminary experiments indicated a similar dose-response for both ages. These experiments however took place at different times, and the sensitivity of five-week old rats at least can depend on when an experiment is done. Five week-olds were chosen instead of adults as a matter of convenience.

Nandini et al. (1976) had chosen 35-day old rats because of the changes in serum FSH that occur during sexual maturation. Like many others, they found FSH levels rose after day 20 and reached a peak around day 35 (Eldridge et al., 1974; Negro-Vilar et al., 1973a; Gupta et al., 1975; Lee et al., 1975; Mackinnon et al., 1976; Payne et al., 1977; Ketelslegers et al., 1978). LH concentrations do not show this change. The subsequent fall in FSH has been attributed to the onset of negative feedback by a factor from the germinal epithelium, since FSH does not fall in cryptorchid rats (Swerdlhoff et al., 1971; Gupta et al., 1975). Some studies show a less pronounced peak, with a definite fall not occurring until 50-60 days of age (Ojeda & Ramírez, 1972; Krueger et al., 1974; Döhler & Wuttke, 1975; de Jong & Sharpe, 1977; Piacsek & Goodspeed, 1978). The fall of FSH could arise from increased production of inhibin or increased sensitivity to inhibin. A recent in vitro study indicates that the pituitary's response to inhibin increases between 33 and 60 days of age, while the testis's output remains unchanged between 18 and 90 days (Steinberger, 1979). Similarly, de Jong et al. (1978, 1979b) found that the degree of FSH suppression in response to a large dose of bovine follicular fluid was greater at 35 and 75 days than at 15 days. Both these studies report a change in response to a large, perhaps maximal, dose of inhibin-like activity. An increase in maximal response does not necessarily imply increased sensitivity. In fact, if the dose-responses to bovine follicular fluid reported by de Jong et al. (1978)(in Figure 3) are replotted as dose against percentage of maximal response, for each age, it appears
that only half as much follicular fluid is needed to achieve 50% maximal suppression in 15-day old male and female rats as in 75-day old adults. This is an apparent decrease in sensitivity with age.

If the changes in serum FSH around five-weeks of age reflect changing sensitivity to inhibin, then the use of rats of slightly different ages could lead to wide variation in the response to inhibin-like activity. In practice this was not a problem. Serum FSH changed only slightly between 32 and 38 days in the rats studied here (at least when tested), and there were no age-related trends in the magnitude of the post-castration rise of FSH or LH, nor in the degree of suppression achieved by 0.5 ml bSP.

Although the procedure was simple and convenient, it was not necessarily optimal. Two aspects were considered - the duration of treatment, and the timing of blood collection. Extension of treatment from one to two days did not confer any advantage, indeed sensitivity seemed impaired. The loss of sensitivity with time, illustrated by chronically castrated rats, might start within 1-2 days of castration. Study of the time-course of FSH and LH suppression in acutely castrated rats was hampered by the extreme toxicity of bSP when sequential blood samples were taken. A later study on intact rats showed FSH to be suppressed 12, 24 and 48h after injection of bSP-extract, and LH at 6, 12, 24 and possibly at 48h. The castrated rat would probably show a similar time-course but perhaps curtailed by loss of sensitivity with time after castration. Bleeding later than 6h or much before 24h would be inconvenient in a routine method, so sampling at 24h after castration was retained.

8.2 PROPERTIES OF THE METHOD FOR DETECTING INHIBIN-LIKE ACTIVITY

8.2.1 Dose-response

The dose-response to the antgonadotrophic activity in bSP was usually steep. Often a particular dose had no effect while twice the amount completely suppressed the post-castration rise of FSH and LH. There was evidence for a maximal response, at least for FSH. Occasionally the dose-response was not so steep - in these cases the degree of suppression seemed proportional to the logarithm of the dose.
The steep dose-response curve implies high precision - that is, a small error in estimating the potency of an unknown. But the limited range means that an unknown would have to be assayed at several concentrations to be sure it fell on the "linear" part of the dose-response curve. For this reason, the system was largely used to detect the presence or absence of activity at a particular dose.

Several investigators have used acutely castrated five-week old rats to detect inhibin-like activity, and some have given material at different doses. Franchimont et al. (1977) and Nandini et al. (1976) also found steep dose response curves for FSH, and Nandini et al. (1976) observed a maximal response. However, the slope of the dose-response curve may depend on the source of inhibin-like activity. Bovine testicular extracts gave a steep curve, but porcine follicular fluid a shallow curve (Rush & Lipner, 1979); fractions of rete testis fluid a steep curve (Franchimont et al., 1977), but fractions of human seminal plasma a shallow curve (Franchimont et al., 1979). The relationship between dose and response is not always simple. Davies et al. (1978b) found that suppression of FSH by bSP-extract increased gradually as the dose was increased from 1 to 30 mg, but declined thereafter. Paradoxical rises of FSH have been reported with some preparations (Hudson et al., 1979). Since all these investigators used different regimens for injecting and bleeding, a critical comparison is impossible. The method used here had a steep dose-response in common with some, but escaped the complications attending others.

8.2.2 Variability

The detection method was reliable - 0.5 ml bSP or 40 mg bSP-extract always suppressed FSH and LH to near-intact levels. Two sorts of between-experiment variation were seen.

The first to be noticed was variation in the LH concentrations of saline-treated rats. It is not known whether this arose from changes in the rate at which LH rose after castration or from changes in the onset of the LH rise, nor whether it reflected the LH levels that would ultimately have been attained. The only comparable report is that the magnitude of the post-castration rise in rainbow trout depended on the season (Billard et al., 1977). Although the changes here appear periodic,
the pattern is not strictly circannual.

Occasionally very high concentrations of LH were detected in castrated or intact rats. Their incidence was cyclic; they occurred in spring and summer when the post-castration rise of LH was higher than average. Billard et al. (1977) found some trout had extremely high gonadotrophin concentrations, but only in December, the period when gonadotrophins were most elevated after castration. Very high LH concentrations have been observed in a small percentage of prepubertal male and female rats (MacKinnon et al., 1976, 1978). They were attributed to episodic release of LH, and their incidence could be abolished if the rats were mildly stressed 30-60 min, beforehand. In man, episodic release of LH, mainly at night, signals puberty and causes sexual maturation (Boyar et al., 1975).

The second type of between-experiment variation was a change in the sensitivity of the rats to the activity in bSP. This is discussed in the next section.

Within-experiment variation was not measured - that is, the same sample at the same dose was not given to two groups in one experiment. But we can assume little variation because of the random but balanced allocation of rats among the experimental groups, and because at least four, and usually six, rats were in each group.

8.2.3 Sensitivity

A dose of 0.2 ml bSP or 20 mg bSP-extract only sometimes suppressed FSH and LH. The variation could not have arisen solely from differences between bSP-extracts, also contributing were changes in the inherent sensitivity of the rats to the antigonadotrophic activity in bSP. It was the position of the dose-response curve that changed, not the slope.

One possible cause for an increase in sensitivity would be accelerated sexual maturation. As already outlined (Section 8.1.3), serum concentrations of FSH rise between about 20 and 35 days of age in the male rat and fall thereafter. If the fall is indeed due to the rats becoming more sensitive to inhibin (de Jong et al., 1978, 1979b; Steinberger, 1979), then early maturation would be accompanied by greater sensitivity to inhibin at five weeks of age.
The changing sensitivity of gonadotrophin secretion to testosterone with maturation is well documented. Acutely castrated rats become less sensitive to testosterone propionate injections or testosterone released from subcutaneous capsules sometime between 26-31 and 51-60 days (Negro-Vilar et al., 1973b; Smith et al., 1977; Negri & Gay, 1976; Nazian & Mahesh, 1979). Serum testosterone begins to rise about day 35, and despite a four-fold increase over the next 20 days, serum LH actually rises slightly (Ketelslegers et al., 1978).

The sensitivity of five-week old rats to testosterone did change. The effectiveness of the steroid fell progressively between the four, weekly instalments of one experiment, but had returned to its initial level after another four weeks. In intact controls, serum LH and serum testosterone were both lower in the first week of the experiment than in subsequent weeks. Here is evidence that the rate of sexual maturation of the rats used for this thesis did vary.

The changes in sensitivity to bSP-extract did not fluctuate randomly, but followed a temporal pattern. Seasonal changes in the onset of maturation have not only been observed in feral rodents (Christian, 1961), but even in rats housed under constant laboratory conditions. Ramaly & Bunn (1972) found that vaginal opening and first ovulation in rats was 5-6 days earlier in summer than in winter, and Mock et al., (1975) have observed summer/winter differences in the maturational pattern of testosterone in male rats. A delay, once a year, in the testosterone surge usually seen at two months of age manifested as a seasonal surge in three-month old animals (Mock & Frankel, 1978b). The pattern of sensitivity to bSP-extract here is fragmentary, but different responses in February 1978 and 1979 challenge, but do not rule out, a circannual rhythm. Sensitivity to 20 mg bSP-extract fell between April and May 1977, and rose between January and March 1980 - consistent with the changes in 1979. The rats were exposed, to a limited extent, to changes in environmental temperature, which could have modulated the effect of photoperiod. These results may still be relevant to rats housed under constant conditions, since environment seems to cue, not generate, circannual reproductive rhythms in the rat (Mock & Frankel, 1978b). The changes in sensitivity to bSP-extract and the changes in the post-castration rise of LH did not seem to be aligned.

It is possible that the variation was not linked to maturation.
Mock & Frankel (1978a) have found seasonal differences in serum FSH, LH, and prolactin in adult male rats. Although the decrease in sensitivity to testosterone was accompanied by increased serum LH and testosterone—consistent with quickened sexual maturation—there was no comparable decrease in serum FSH in intact rats when the sensitivity to bSP-extract increased. Other experiments showed that in the acutely castrated rat, near-intact levels of FSH could be maintained by a range of doses of bSP-extract and testosterone, alone or combined. Thus the impact of a change in sensitivity to inhibin might be buffered.

Lack of an inhibin standard makes it difficult to compare the sensitivity of different detection methods. However, two groups have used acutely castrated rats to measure activity in extracts of bSP. Davies et al. (1978b) injected 35-40 day old rats at 0, 6, and 20 h after castration, and bled them at 22-23 h. Thirty milligrams of ethanol precipitate of bSP suppressed FSH by 50%—a maximal dose. In comparison, Franchimont et al. (1977) method could detect activity in about 4 mg of bSP-extract (calculated from data in Chari et al. (1976, 1978) and Franchimont et al. (1977)). In their assay, material is injected intravenously 14 h after castration and the rats are bled 2 h later.

8.2.4 Specificity

The detection method is not specific; steroids and possibly other substances also lower serum gonadotrophins in acutely castrated rats. Although inhibin is hypothesised to selectively inhibit FSH secretion, its actual behaviour in this test system will remain unknown until some factor is isolated and defined as inhibin. Whether the dual suppression of FSH and LH by bSP can be accepted as inhibin-like will be discussed later.

Any method that measures inhibition of a response is open to nonspecific interference. There are, however, several lines of evidence to suggest that the suppression of FSH and LH by bSP and its extracts was due to hormonal activity. The response was lost with time after castration, so that even doses which caused noticeable side effects left FSH and LH unchanged in chronically castrated rats. This shows that the action cannot be due to, say, an enzyme in bSP that destroys circulating gonadotrophins. There was a maximal effect on FSH, and there were subtle interactions between bSP-extract and testosterone. Heat destroyed the
activity, so the response was not just a reaction to foreign protein. The specific activity could be increased (e.g., fraction N-11, Figure 4.5).

bSP was toxic to some extent. However it was later found that the incidence of death after injection of 0.5 ml bSP or 40 mg of extract was completely abolished if the spermatic cords were tied at castration. This suggests that the toxic effect was due to some substance that prevented clotting, and thus the sealing of the cut blood vessels. bSP is known to contain plasminogen activators (Åstedt et al., 1976), which convert plasminogen to plasmin - a thrombolytic enzyme. Haemolytic proteins may have contributed to mortality in intact rats that received a high dose of bSP-extract (Kysilka, 1973, 1975).

8.3 DETECTION METHODS OF OTHERS

Rats in various endocrine states - male or female, intact or castrated - acutely or chronically-, have been used to detect inhibin-like activity. Nearly always the test substance is injected intraperitoneally or subcutaneously.

In intact adult male rats the suppression of FSH is slight because any response is additional to endogenous feedback (Setchell & Jacks, 1974; Baker et al., 1976; Lee et al., 1977; Franchimont et al., 1977).

Chronically castrated males have been used successfully by many groups (Setchell & Jacks, 1974; Franchimont et al., 1975a; Moodbidri et al., 1976; Lee et al., 1977; Le Lannou & Chambon, 1977a; Thakur et al., 1978). Typically adult rats are castrated at least two weeks in advance, given multiple injections over two or more days, and bled 4-24 h after the last injection. Intact rats are also treated for two days or more.

Acutely castrated five-week old male rats have been widely adopted, although the injection regimen and time of bleeding varies. In Nandini et al.'s (1976) model B, rats are injected 12-24 h after castration, and bled at injection (to provide a control) and 6 h later. This method was tried here, but it was not as good as the method adopted - 6 h treatment was probably too short a time for the activity in bSP to be efficacious. Franchimont et al. (1977) injected rats intravenously 14 h after castration and bled them 2 h later. Several methods incorporate multiple injections, some beginning at castration and lasting 24 h or less (de Jong & Sharpe, 1976; Davies et al., 1978b; Chari et al., 1978), some covering several
days (Hopkinson et al., 1975, 1977a). de Jong et al. (1978) castrated their rats two days before treatment.

Female rats have been popular for detecting activity in follicular fluid. Intact rats, immature (Setchell & Jacks, 1974; Franchimont et al., 1977; de Jong et al., 1978) or adults at various stages of the estrous cycle (Marder et al., 1977; Schwartz & Channing, 1977; Lorenzen et al., 1978a; De Paolo et al., 1979b), rats castrated in metestrous (Marder et al., 1977; Lorenzen et al., 1978a) or in proestrous (Batta et al., 1978), and rats chronically castrated (Welschen et al., 1977; Campbell & Schwartz, 1979) have all been used. Unlike systems using male rats, female rats have usually received only one injection, or two closely spaced injections, and have been bled 8-10 h later.

Rodents other than rats have been tried, including acutely castrated mice (Lee et al., 1977; Hudson et al., 1979), long-term ovariectomised mice with or without a maximal dose of estrogen (Bronson & Channing, 1979), and intact and ovariectomised hamsters (Chappel, 1979; Chappel & Selker, 1979).

The only non-rodent to be studied in detail is the sheep. Extracts of bovine testes and ovine rete testis fluid have been infused into wethers (Lee et al., 1974; Keogh et al., 1976; Baker et al., 1976; Cahoreau et al., 1979) and cryptorchid rams (Blanc & Dacheux, 1976; Blanc et al., 1978; Cahoreau et al., 1979).

One alternative to directly measuring serum concentrations of FSH is to use hCG-primed rats or mice as a bioassay for endogenous FSH secretion. Exogenous hCG (LH-like) and endogenous FSH act synergistically to increase ovarian and uterine weight in immature animals (Lamond & Emmens, 1959). Such assays have been developed by Chari et al. (1976) and Ramasharma et al. (1979) to monitor purification of inhibin-like activity. Although their easily measured endpoint makes them convenient to use, specificity cannot be proved, and not everyone can make them work (Davies et al., 1978b; de Jong et al., 1979b; Hudson et al., 1979).

The inhibition of FSH secretion from pituitary cells in culture is likely to eclipse other methods for the routine detection of activity. Pituitary cell cultures are precise, sensitive, and reproducible. Typically cells are cultured for 2-3 days and then incubated for 1-3 days in the presence of the test substance. A 4-6 h incubation with LHRH may follow. Generally, inhibin-like activity lowers basal secretion of FSH.
Table 8.1  Comparative sensitivities of different detection systems for inhibin-like activity  
(Different arbitrary units for each study).

<table>
<thead>
<tr>
<th>Authors (&amp; inhibin preparation)</th>
<th>Suppression of post-castration rise of FSH (Five-week rat adult mouse)</th>
<th>Suppression of FSH (intact rat wether monkey)</th>
<th>Inhibition of hCG-augmentation (rat ovary mouse uterus)</th>
<th>In vitro (hemi-pituitary culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hudson et al. (1979) (many)</td>
<td>&gt;300</td>
<td>&gt;4</td>
<td>&gt;12 000 70 000 10 000</td>
<td>&gt;140 200-3000</td>
</tr>
<tr>
<td>Lee et al. (1977) (bT-E)</td>
<td>1-2</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>de Jong et al. (1978) (off-E)</td>
<td>500-1000</td>
<td></td>
<td></td>
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<tr>
<td>Davies et al. (1978a) (ortf-E)</td>
<td>1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Franchimont et al. (1978) (ortf-fractions)</td>
<td>50-70</td>
<td></td>
<td></td>
<td>0.006-0.013</td>
</tr>
<tr>
<td>Charri et al. (1978) (&quot;bSP-inhibin&quot;)</td>
<td>7</td>
<td></td>
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<tr>
<td>Ramasharma et al. (1979) (ot-fraction)</td>
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<tr>
<td>This thesis (bSP-E)</td>
<td>1</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>(bSP-E)</td>
<td>&lt;800</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(off-E)</td>
<td>&gt;3200</td>
<td></td>
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</tbody>
</table>

α bT-E: bovine testis extract; off-E: ovine follicular fluid extract; ortf-E: ovine rete testis fluid extract; ot: ovine testis; bSP-E: bull seminal plasma extract.
only, and LHRH-induced secretion of FSH and LH. Details of in vitro methods and the insights they provide into the mode of action of inhibin are discussed later. Akin is the use of hemipituitaries instead of dispersed cells (Davies et al., 1978a; Cahoreau et al., 1980). Inhibin-like activity also reduces cellular content of FSH (de Jong et al., 1978; Franchimont et al., 1978). Scott & Burger (1979a) have recently developed an assay exploiting this property.

How do these diverse methods compare? For those using rats the studies of de Jong et al. (1978, 1979b) and Melschen et al. (1979) offer a guide. A high dose of bovine follicular fluid suppressed FSH further (as a percentage) in 35 and 70 day olds than in 15 and 25 day olds, male and female, intact or castrated. FSH was lowered more in females at each age. Similar conclusions can be drawn from the complex data of Lorenzen et al. (1978b). Thus for males, five-week old animals are the choice. Females seem better than males, although the scope of suppression may depend on the source of activity as well as the sex of the animal (Rush & Lipner, 1979). In this thesis, intact and acutely castrated males appeared equally sensitive.

Hudson et al. (1979) have compared the sensitivity of different detection systems. The ratios they obtained generally accord with the results of others (Table 8.1). However, for Hudson et al. (1979), some methods did not work well; FSH suppression in intact and acutely castrated rats, inhibition of hCG-augmented ovarian weight, and inhibition of FSH secretion by hemipituitaries were all characterised by shallow dose-response curves and high within-experiment variation. Once again, the sensitivity may depend on the identity of the material. The relative potencies of ovine rete testis fluid and testicular lymph were 70 by cell culture, but 5 by the mouse uterine assay (Hudson et al., 1979). Similarly, the ovarian weight assay was reported to be about 6000 times as sensitive as the acutely castrated rat for activity from rete testis fluid (Franchimont et al., 1978), but only 7 times as sensitive for "bSP-inhibin" (Chari et al., 1978). From the results in this thesis, the relative sensitivities of acutely castrated rats and cell cultures were different for bSP-extract and for ovine follicular fluid.

Only the hCG-augmentation assays and pituitary cell cultures are suitable for quantifying activity. In these assays parallelity to the dose-response curve gives some measure of specificity. But only the
reduction of cellular concent of FSH appears truly specific for inhibin-like activity. In this assay steroids have little effect or actually increase FSH content (Scott & Burger, 1979b; Labrie et al., 1978b).

8.4 PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF THE ANTIGONADOTROPHIC ACTIVITY IN bSP

8.4.1 Extraction and Proteinous Nature

Protein was precipitated from bSP by adding ethanol - the method of Franchimont et al. (1975a). The method has the disadvantage of not extracting low molecular weight activity from ovine rete testis fluid and human seminal plasma (Franchimont et al., 1977), and it extracted only half the activity from ovine testicular lymph and rete testis fluid (Baker et al., 1978). There was no evidence here that bSP-extract had less activity than bSP, but this must be qualified, since dose-response to bSP and bSP-extract was tested at different times, and the sensitivity of acutely castrated rats can change.

Ethanol precipitation was also used to fractionate bSP. However, the activity was precipitated over a wide range of ethanol concentrations, although perhaps more between 30-50% than outside this range. Baker et al. (1978) found a similar spread of activity when ammonium sulphate was added to ovine testicular lymph and rete testis fluid.

Interest in the steroid content of bSP was confined to whether steroids contributed to the observed antigenadotrophic activity. The testosterone concentration of bSP (#1) was 1.1 µg/l, similar to 1.15 µg/l reported by Eiler & Graves (1977), but less than 2.8 µg/l by Ganjam & Amann (1976). The latter authors found all steroids, except testosterone, in higher concentrations in bSP than in peripheral blood, particularly estradiol which was 5-10 times as high. The minimum dose of testosterone propionate needed to significantly suppress serum FSH in acutely castrated rats was 30-100 µg/kg BW, 5000 times the testosterone content of 0.5 ml bSP, and 30 000 times the amount in 40 mg bSP-extract. For estradiol, about 1 µg/kg BW was necessary to suppress FSH, a dose much in excess of the amount in 0.5 ml bSP (from the values of Eiler & Graves, 1977; Ganjam & Amann, 1976). In addition, testosterone and estradiol are less potent than their esters 24 h after injection, because of their shorter half lives.
Steroids in high concentrations do occur in some sources of inhibin-like activity, particularly testis extracts and follicular fluid. In this study, preparations of rat testes were washed with diethyl ether, and ethanol precipitates of ovine follicular fluid with acetone.

Precipitation with ethanol indicated the activity in bSP was proteinous. This was confirmed by the loss of activity after heating bSP-extract at 67°C for 1 h, and after digestion with pepsin. Inactivation by heat or proteases has been commonly used to show the protein-nature of inhibin-like activities. The temperature required to cause inactivation varies. Nandini et al. (1976) heated ovine testicular extracts to 55°C, de Jong et al. (1980) heated bovine follicular fluid at 55°C for 30 min, and Lorenzen et al. (1978a) porcine follicular fluid at 60°C for 30 min., and all found activity retained. In contrast, heating at 60°C for 1 h destroyed activity in human seminal plasma (Franchimont et al., 1979), and in ovine rete testis fluid (Baker et al., 1980), and heating at 56°C for 30 min, reduced the activity in bovine follicular fluid (Sato & Ishibashi, 1978). The variability is important, because although Keogh et al. (1976) found that McCullagh & Walsh's (1935) extraction method, which included heating to 65°C, destroyed the activity in bovine testis extracts, it need not have been so for McCullagh & Walsh. Temperatures of 80-100°C always destroy activity.

8.4.2 Gel Exclusion Chromatography - an Indication of Molecular Weight

The activity had a high molecular weight when bSP-extract was dissolved in 0.01M ammonium acetate of pH 7.6. It eluted in the void volume on Sephadex G-100 and G-75, and was largely retained by an XM-100 membrane. Increasing the buffer concentration to 0.1M changed the elution profile of bSP-extract on G-200; a new peak arose at 180 000 daltons. On G-75 chromatography with this buffer, the activity was present not only in the void volume but also in its trailing shoulder.

On elution with 0.05M sodium acetate buffer of pH 4, the activity was no longer in the void volume but in a subsequent fraction, a large peak spanning 25 000-70 000 daltons. Addition of 4M urea to either the ammonium acetate or sodium acetate buffers changed the elution profile dramatically - most of the material, and the activity, was eluted in a large peak covering 15 000-35 000 daltons. The effect of urea on the
elution pattern of bSP-extract was reversible.

The elution profiles and the positions of the activity upon chromatography in 0.05M sodium acetate, with and without urea, were very similar to those reported by Franchimont et al. (1975a) and Chari et al. (1978) for the same starting material subjected to similar procedures.

The changes in elution volumes, and hence apparent molecular weight, are probably caused by different degrees of aggregation of the active protein(s), either with itself or with other proteins in bSP. Such interactions are decreased by an increase in ionic strength, or by a change in pH towards the isoelectric point of the proteins. (Most of the proteins in bSP have isoelectric points between 4.7 and 5.4. See Sections 4.5.1 and 4.5.2, and Georgiev, 1978.) A high concentration of urea prevents non-covalent association. The large proportion of material that changed molecular weight in the presence of urea, and the heterogeneity of the material (by electrophoresis and isoelectric focusing) shows that many proteins in bSP aggregate.

Association between proteins could explain three other observations. The first: the activity in bSP was precipitated over a wide range of ethanol concentrations. Different degrees of association would give the activity different physical properties. The second: the elution pattern of bSP-extract in 0.05M sodium acetate, pH 4, depended on the amount put on the column. Heavy loading and hence a high concentration of protein within the gel would promote association, which would manifest as more material of higher molecular weight. The third: the molecular size of the activity in 0.1M ammonium acetate, pH 7.6, differed between batches of bSP. After G-75 chromatography of an extract of the first batch of bSP, activity appeared in the void volume peak and its shoulder. With the second batch, activity was only present in the void volume. Although the composition of the two batches seemed identical from electrophoresis, G-200 chromatography revealed that the relative abundance of the components differed. The degree of association would depend on the quantity of associating proteins. Since both extracts had similar amounts of activity, here is evidence that association is not between the active molecules themselves, but between the active factor and other proteins.

The dependence of molecular weight on the identity of the buffer suggested that the activity might be purified by a sequence of gel exclusion
chromatographies, each with a different buffer. This was thwarted because a large portion of the material changed molecular weight in the same way as the activity, and because different batches of bSP behaved slightly differently under the same conditions.

Activity of different molecular size has also been observed in extracts from human seminal plasma and ovine rete testis fluid. Chromatography of human seminal plasma on G-200 revealed three peaks of activity, with molecular weights of 100 000, 45 000, and 15 000 daltons (Scott & Burger, 1980). Ovine rete testis fluid has been studied more thoroughly. Davies et al. (1976, 1978a & b, 1979b) have found activity of 80 000-100 000, 15 000-30 000 and <5000 daltons.

The highest fraction generated low molecular weight activity upon rechromatography, while all the activity had molecular weight less than 5000 daltons in the presence of 4M urea. Similar behaviour has been reported by Franchimont et al. (1978). On G-200 chromatography at pH 4, activity was of high and low molecular weights. Chromatography of the high molecular weight fraction generated activity with molecular weight of less than 5000 daltons, as well as leaving some activity of high molecular weight (RTF13). Chromatography of RTF13 again produced low molecular weight activity.

Unlike human seminal plasma or ovine rete testis fluid, the activity in bSP seemed confined mainly to one molecular size under any particular condition. This could have arisen from the way the fractions were screened, at a dose just large enough to be sure of detecting activity where it was most abundant.

8.4.3 Ion Exchange Chromatography - an Indication of Isoelectric Point

Active material from bSP bound to CM cellulose when the buffer was 4M urea-0.05M sodium acetate of pH 4. When the ion exchanger was eluted with buffer containing an increasing concentration of sodium chloride, the activity was eluted in a fraction that contained much of the material that bound to the column. This fraction had at least ten components, all with isoelectric points between 4.7 and 5.4.

This behaviour contrasts with that reported by Chari et al. (1978). Using the same buffer and starting material prepared in the same way, these authors found the activity slowly passed through a CM cellulose
A similar peak was observed in this study, although it had an elution volume of 4.3-6.6 Vₑ (depending on the chromatographic run) rather than 0.8-1.2 Vₑ. This material and bSP-inhibin had similar electrophoretic patterns - two closely spaced bands migrating about half as fast as the ion-front. These two components, A and B, had isoelectric points of 4.70 and 4.88, and could be separated on CM cellulose eluted with a urea buffer of pH 4.6.

A careful comparison of electrophoretic patterns indicated that components A and B were consistently present in a peak centred at 18 000 daltons whenever bSP-extract was chromatographed on G-75, no matter whether the buffer was 0.01 or 0.1M ammonium acetate, pH 7.6, or 0.05 M sodium acetate, pH 4. That is, while the molecular size of the activity varied according to the buffer, the molecular weights of A and B did not. The electrophoretic pattern of the active fraction from the CM column was an indistinct smear with similar mobility to A and B. Interestingly, the two bands of bSP-inhibin appear enveloped in a smear.

Is the different behaviour due to different substances, or subtle differences in chromatographic conditions? The similar behaviour on Sephadex gels, with and without urea, suggests the activities observed here and by Chari et al. (1978) have at least some properties in common. The ion exchanger used by Chari et al. (1978) was Whatman's CM 11, which has a lower capacity for binding ions than the CM 32 used here. A different capacity can alter the selectivity of an ion exchanger. Unfortunately, CM 11 has been withdrawn by the manufacturers so the question could not be resolved.

8.5 A COMPARISON OF THE PHYSICOCHEMICAL PROPERTIES OF INHIBIN-LIKE ACTIVITIES FROM VARIOUS SOURCES

Table 8.2 lists the properties of inhibin-like activity from various tissues and secretions. Many of the reports are abstracts or short communications and so lack supporting evidence, for others the properties are secondary to the major theme of the paper, hence the data should be interpreted with caution. Some molecular weights are reported as the size of the active fraction, others as the midpoint of the fraction. Determination by gel exclusion chromatography usually implies the activity itself was measured, whereas SDS-polyacrylamide gel electrophoresis determines the molecular weight of the most (or only) visual component of
Table 8.2  Physicochemical properties of inhibin preparations.

<table>
<thead>
<tr>
<th>Source</th>
<th>Authors</th>
<th>Molecular weight (daltons)</th>
<th>Isoelectric point</th>
<th>Carbohydrate</th>
<th>Associative properties</th>
<th>minimum purity (no. components)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bull seminal plasma</td>
<td>This thesis</td>
<td>15 000-35 000</td>
<td>4.7-5.4</td>
<td>+</td>
<td></td>
<td>10^e</td>
</tr>
<tr>
<td></td>
<td>Franchimont et al. (1977)</td>
<td>18 000</td>
<td>5&lt;6.5</td>
<td>-</td>
<td>+</td>
<td>2^e</td>
</tr>
<tr>
<td></td>
<td>Chari et al. (1978)</td>
<td>21 500</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Chari (1977)</td>
<td></td>
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<tr>
<td></td>
<td>Sairam et al. (1978, 1980b)</td>
<td>15 000</td>
<td>&gt;8.5</td>
<td>-</td>
<td></td>
<td>2^e</td>
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<tr>
<td></td>
<td></td>
<td>18 000</td>
<td></td>
<td></td>
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<tr>
<td>human seminal plasma</td>
<td>Franchimont et al. (1978, 1979)</td>
<td>&lt;5000</td>
<td></td>
<td></td>
<td></td>
<td>1^e</td>
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<td></td>
<td>Thakur et al. (1978)</td>
<td>19 000</td>
<td>&lt;8.3^e</td>
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<td></td>
<td>Vaze et al. (1979)</td>
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<td></td>
<td>Scott &amp; Burger (1980)</td>
<td>&gt;100 000</td>
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<td></td>
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<td>45 000</td>
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<td></td>
<td></td>
<td>15 000</td>
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<tr>
<td>bull sperm</td>
<td>Lugaro et al. (1973)</td>
<td>&lt;10 000</td>
<td></td>
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<td>9</td>
</tr>
<tr>
<td>ovine rete testis fluid</td>
<td>Davies et al. (1976; 1978a &amp; b; 1979b)</td>
<td>90 000</td>
<td>&lt;8^d</td>
<td>+</td>
<td></td>
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<tr>
<td></td>
<td>Franchimont et al. (1976, 1977, 1978)</td>
<td>&quot;high molecular weight&quot;</td>
<td>&lt;8^d</td>
<td>+</td>
<td></td>
<td>3^e</td>
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<td></td>
<td></td>
<td>&lt;5000</td>
<td></td>
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<td></td>
<td>Baker et al. (1976)</td>
<td>10 000-20 000</td>
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<td></td>
<td>Baker et al. (1978, 1980)</td>
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<td>5-8.8^e,d</td>
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<tr>
<td></td>
<td></td>
<td>10 000</td>
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<td></td>
<td></td>
<td>many^e</td>
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<td>Species</td>
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<tr>
<td>bovine testis</td>
<td>Cahoreau et al. (1979)</td>
<td>&gt;160 000&lt;sup&gt;g&lt;/sup&gt;</td>
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<td></td>
<td>Lee et al. (1974)</td>
<td>&lt;100 000&lt;sup&gt;u&lt;/sup&gt;</td>
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<td></td>
<td>Keogh et al. (1976)</td>
<td>10 000-70 000&lt;sup&gt;g&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Baker et al. (1976)</td>
<td>15 000-100 000&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
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<tr>
<td></td>
<td>Lee et al. (1977)</td>
<td>500-3000&lt;sup&gt;g&lt;/sup&gt;</td>
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</tr>
<tr>
<td>ovine testis</td>
<td>Moodbidri et al. (1976)</td>
<td>&lt;5000&lt;sup&gt;g&lt;/sup&gt;</td>
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<td></td>
<td>Sheth et al. (1979)</td>
<td>1400-1500&lt;sup&gt;g&lt;/sup&gt;</td>
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<sup>a</sup>by binding to CM cellulose or Sephadex
<sup>b</sup>by binding to DEAE cellulose or Sephadex
<sup>c</sup>by PAGE
<sup>d</sup>by gel exclusion chromatography
<sup>e</sup>by PAGIEF
<sup>f</sup>by sodium decyl sulphate PAGE
<sup>g</sup>by ultrafiltration or dialysis
the fraction. A guide to the isoelectric points has been gleaned from pH of absorption to ion exchangers, or from the direction the component(s) of the active fraction migrated upon electrophoresis. Note that the pH within the gel during electrophoresis may be different from the pH of the gelling buffer and electrode buffer (Lewis et al., 1979). Purity has usually been assessed by the number of bands on electrophoresis, which may sometimes be misleading. The fraction with activity off the CM column here showed a smear covering perhaps two bands, but isoelectric-focusing revealed at least ten components. Sheth et al. (1979) fractionated ovine testis extract on G-75; the three peaks each showed only one band on electrophoresis, but each band had identical mobility.

Human and bull seminal plasma contain activity of 15 000-20 000 daltons. Activity of higher molecular weight can be attributed to association of the active factor with other proteins in semen (Franchimont et al., 1977; Chari et al., 1978). The activity in bSP studied here and by Chari et al. (1978) was clearly acidic, yet Sairam et al. (1978, 1980b) have reported the isolation of a basic protein - it did not migrate into the gel on electrophoresis at pH 8.5, and it was rich in basic amino acids. It is unlikely that the rigours of purification could change the isoelectric point of a protein so much. Human seminal plasma also seems to contain a separate factor with molecular weight less than 5000 daltons (Franchimont et al., 1978, 1979). Exposure of bSP fractions to urea did not produce activity with so low a molecular weight.

Ovine rete testis fluid contains a low molecular weight factor than can either polymerise or bind to other proteins in rete testis fluid (Franchimont et al., 1978; Davies et al., 1978b). But again, perhaps more than one factor is present, since Baker et al. (1980) have found activity with molecular weight greater than 10 000 daltons even in the presence of dissociating agents such as 8M urea and 8M guanidine HCl.

Testicular extracts too seem heterogeneous, with activity greater than 10 000 daltons (bovine: Baker et al., 1976; ovine: Shashidhara Murthy et al., 1979, 1980; rats: Steinberger & Steinberger, 1976, 1977; Eddie et al., 1978) and less than 5000 daltons (ovine: Moodbidri et al., 1976; Sheth et al., 1979).

Inhibin-like activity in follicular fluid is generally agreed to be greater than 10 000 daltons (human: Chari et al., 1979; Batta et al., 1978; bovine: de Jong & Sharpe, 1976; Sato & Ishibashi, 1978;
Dobos et al., 1979; porcine: Lorenzen et al., 1978a; Lagacé et al., 1979), although smaller molecular weight activity with a different spectrum of biological properties has been reported by de Jong et al. (1979b) and Daume et al. (1979b).

The activity of 60,000-90,000 daltons in ovine rete testis fluid (Baker et al., 1980) and bovine follicular fluid (de Jong et al., 1980; Dobos et al., 1979) appears to be, in part, a glycoprotein, which does not dissociate in urea. The activity of about 20,000 daltons in bSP (Chari 1977; Sairam et al., 1980b) and ovine testis extracts (Shashidhara Murthy et al., 1980) did not have carbohydrate residues.

We can tentatively conclude that there are several distinct types of protein with inhibin-like activity - glycoprotein(s) of 60,000-90,000 daltons, proteins of about 20,000 daltons, perhaps acidic and basic, and peptide(s) smaller than 5000 daltons. How similar chemically are the various active factors? Immunological crossreactivity gives some indication. Antisera raised against the 20,000 dalton activities of human and bull seminal plasma crossreact with material in bovine and porcine follicular fluids (Vaze et al., 1979; Sairam et al., 1978, 1980b), but not with the small molecular weight activity of the ovine testis.

The small molecular weight activity in ovine rete testis fluid also seems dissimilar to the 20,000 dalton activity of bull and human seminal plasma (Franchimont et al., 1977). Antiserum against a bull seminal plasma fraction seemed to neutralise endogenous rat inhibin (Franchimont et al., 1975b), and an antisera to the 20,000 dalton species from the ovine testis neutralised endogenous monkey inhibin (Shashidhara Murthy et al., 1980).

How can different investigators looking at the same tissue or secretion find activity with different properties, for instance, acidic and basic proteins in bSP? Different detection procedures may be responsible - in this study fractions with the greatest gross activity were uncovered, more exact methods could pursue the fractions with the greatest specific activity.

Although the activity from several sources has been purified to apparent homogeneity, few of the preparations have been physically or chemically characterised beyond their molecular weight. None of the purifications have, as yet, been duplicated by another laboratory. Biological potencies are difficult to compare without a common standard or bioassay. Nevertheless, several preparations are active at doses of
10-100 µg in acutely castrated, prepubertal male rats ("bSP-inhibin" of Chari et al., 1978; a bSP fraction of Sairam et al., 1978; a human seminal plasma fraction of Franchimont et al., 1979; an ovine rete testis fluid fraction of Franchimont et al., 1978, and a human follicular fluid fraction of Chari et al., 1979).

8.6 ORIGIN OF THE ANTIGONADOTROPHIC ACTIVITY IN SEMINAL PLASMA

The concept of inhibin arose from the behaviour of aqueous extracts of bull testes (Martins & Rocha, 1931; McCullagh, 1932; McCullagh & Walsh, 1935). Subsequently, inhibin-like activity has been found in testicular extracts from several species (ovine: Nandini et al., 1976; Moodbidri et al., 1976; bovine: Keogh et al., 1976; human: Chari et al., 1977). Elevated gonadotrophin levels attending damage, disease, or experimentally induced defects in the seminiferous tubules further pinpointed the activity's origin (see Setchell & Main, 1974; Setchell et al., 1977). Although there have been many attempts to correlate increased FSH levels with decreased numbers of some particular stage of the germinal line, Sertoli cells cultured by themselves release activity that selectively inhibits FSH secretion of pituitary cells (Steinberger & Steinberger, 1976; de Jong et al., 1978; Labrie et al., 1978b). Inhibin-like activity has also been observed in ovine rete testis fluid (Setchell & Sirinathasinghji, 1972; Setchell & Jacks, 1974; Franchimont et al., 1976; Baker et al., 1976; Blanc & Dacheux, 1976), rat epididymal extracts (Le Lannou & Chambon, 1977a), and in human and bovine seminal plasma (Franchimont, 1973; Franchimont et al., 1975a; Davies et al., 1978b).

The presence of activity in these tissues and fluids has given rise to a theory of how inhibin might enter the general circulation. Inhibin would be synthesised by the Sertoli cells and secreted into the lumen of the seminiferous tubules. Because inhibin is a protein, the blood-testis barrier could restrict its passage from the tubule into blood or lymph (Setchell & Waites, 1975). Instead, it would be transported within the intratubular fluid through the rete testis to the head of the epididymis, where it would be absorbed along with most of the rete testis fluid (Setchell & SirinathSinghji, 1972). The presence of activity in seminal plasma has been attributed to incomplete absorption...
at the epididymis (Franchimont et al., 1975b). This hypothesis was tested by looking for activity in seminal plasma from vasectomised bulls.

The seminal plasma from vasectomised bulls had antigonaladotrophic activity with similar properties and in a comparable amount - within the limitations of the detection method - to that in seminal plasma from normal bulls. Since the activity cannot have arrived from the testis by way of the vas deferens, it cannot be inhibin that has escaped absorption from the rete testis fluid. The presence of activity in fluid from the seminal vesicles suggests that these glands are the source.

These experiments do not rule out an indirect route from the testis to the seminal vesicles. Indeed there is evidence that the testis can supply the prostate and seminal vesicles with steroids by retrograde flow of blood from the deferential vein into the vessels supplying the accessory glands (Pierrepont et al., 1975). Steroids have been shown to be transferred, by way of the pampiniform plexus, from the testicular vein to the testicular artery, which supplies the epididymis and vas deferens (Amann & Ganjam, 1976; Free & Jaffe, 1978). The other step, retrograde flow, is only surmised.

An experiment was designed to see if the antigonaladotrophic activity in bSP was derived from the testis, or whether it could be produced by the seminal vesicles. Ideally, bulls would have been castrated and injected with testosterone to maintain secretion of the accessory glands. In the rat at least, testosterone appears to promote protein synthesis in general rather than the synthesis of particular proteins by the seminal vesicle (Higgins et al., 1976). Because bulls were not available, steers (castrated at birth) were used. The small size of the seminal vesicles, the low protein concentration of the ejaculate, and the very different electrophoretic pattern of the proteins indicated that the androgen treatment had not restored normal accessory gland secretion, so the lack of antigonaladotrophic activity was not unexpected.

The question of where the activity in seminal plasma ultimately comes from has been partially answered by Scott, Burger & Quigg (1980), who have accurately measured concentrations of inhibin-like activity in human seminal plasma by using an in vitro assay. Men with azoospermia caused by obstruction of the vas deferens had normal blood concentrations of FSH and testosterone, but only half the normal concentration of inhibin-like activity in their seminal plasma. Vasectomised men were similar.
Hence both the testis, by way of the vas deferens, and the accessory glands contribute inhibin-like activity to human seminal plasma. In patients with germinal cell failure FSH was elevated, while the concentration of inhibin-like activity was ten times lower than normal. This was despite normal testosterone concentrations, and, apart from the lack of sperm, apparently normal ejaculates. It therefore appears that the inhibin-like activity secreted by the accessory glands is derived from the testis.

8.7 INHIBIN'S ROUTE INTO GENERAL CIRCULATION

The importance of the rete testis fluid as a vehicle in the transport of inhibin from the testis into general circulation is in doubt. On one hand, Le Lannou et al. (1979) have evidence that the rat epididymis stores and releases inhibin-like activity. FSH levels were more elevated after removal of the testis and epididymis than after removal of the testis alone. (This was not found by Davies et al., 1979b.) The activity appeared to come from the testis via the rete testis fluid, since there was no activity in the epididymis when the efferent ducts had been ligated three days beforehand. On the other hand, there is evidence that the activity in rete testis fluid does not participate in the negative feedback on FSH. Cannulation of the efferent ducts of rams did not increase FSH or LH (Walton et al., 1979; Blanc et al., 1979; Davies et al., 1979b), and ligation of the efferent ducts in rats left FSH unchanged for three days - the subsequent rise could be attributed to increased pressure within the tubules and the resulting disorganisation of the germinal epithelium (Main et al., 1978; Collins et al., 1978; Davies et al., 1979b).

There is another way inhibin could reach the circulation. The Sertoli cells could secrete it from their basal surface into the testicular lymph or blood, as well as from their luminal surface into the tubule. Although concentrations of activity in ovine testicular lymph are low compared to those in rete testis fluid, the lymph has a greater secretion rate (Setchell et al., 1977). Even so, simple calculations show that considerable activity is secreted into rete testis fluid. (Taking inhibin concentrations of 0.8 and 137U/mg protein for ovine testicular lymph and rete testis fluid (Eddie et al., 1979), flow rates of 10 and 1.5 ml/h for the average testis (Setchell, 1970), and protein concentrations of 46 and 2.1 mg/ml (Cowie et al., 1964; Mann, 1975), 368U/h is secreted into
testicular lymph and 432U/h into rete testis fluid.) The inhibin concentration in testicular blood is unknown.

Inhibin-like activity has been detected in the female, in ovarian extracts and in follicular fluid (bovine: Hopkinson et al., 1977a; de Jong & Sharpe, 1976; procine: Welschen et al., 1977; Marder et al., 1977; human: Daume et al., 1977; Chari et al., 1979; Batta et al., 1978; hamster: Chappel, 1979; mouse: Bronson & Channing, 1978). The source is the granulosa cell (Erickson & Hseuh, 1978), which is analogous in function to the Sertoli cell. In the female, the activity seems to enter circulation by way of the ovarian vein (De Paolo et al., 1979a; Channing et al., 1980).

8.8 PHYSIOLOGICAL PROPERTIES OF ANTIGONADOTROPHIC ACTIVITY IN bSP - A COMPARISON WITH INHIBIN-LIKE ACTIVITY FROM OTHER SOURCES

8.8.1 FSH/LH Selectivity

Bull seminal plasma and its extracts suppressed FSH and LH equally in acutely castrated rats. Since a variety of fractionation techniques, exploiting different molecular properties, failed to separate the FSH- and LH-suppressing activities, it is likely that the dual action is an intrinsic property of the active factor(s). In intact rats LH suppression only sometimes accompanied FSH suppression, its incidence seemed linked to the sensitivity of the rats to bSP-extract.

Inhibin, of course, has been hypothesised to inhibit only FSH secretion, so how "inhibin-like" is the activity in bSP? Whether a material affects LH or not could depend on the detection system or the material itself.

In chronically castrated rats, material considered to contain inhibin has almost always suppressed only FSH (Setchell & Jacks, 1974; Lugaro et al., 1974; Franchimont et al., 1975a, Moodbidri et al., 1976; Le Lannou & Chambon, 1977a; Thakur et al., 1978; Sheth et al., 1979; Campbell & Schwartz, 1979). In contrast, FSH and LH are often both suppressed with acutely castrated rats. The response runs from FSH only (bovine testis extract: Nandini et al., 1976; bovine follicular fluid: de Jong et al., 1978, 1979b; human seminal plasma fraction hSP 3.4: Franchimont et al., 1979), to a slight effect on LH at higher doses
(bovine testis extract: Rush & Lipner, 1979; Lee et al., 1977; human seminal plasma fraction hSP₃: Franchimont et al., 1979; ovine rete testis fluid: Franchimont et al., 1977; Davies et al., 1978b), to apparently equal effects on FSH and LH ("aged" bSP fraction: Chari et al., 1978; bSP: Davies et al., 1978b; human follicular fluid fraction: Chari et al., 1979).

Generally FSH alone is suppressed in intact rats (bull and human seminal plasma: Franchimont et al., 1975a; follicular fluids: Schwartz & Channing, 1977; de Jong et al., 1978, 1979b; De Paolo et al., 1979b; Hoffmann et al., 1979), although Baker et al. (1976) have reported suppression of LH but at a different time to that of FSH. In sheep, rete testis fluid suppresses LH as well as FSH, but again the time course differs (Blanc et al., 1978; Cahoreau et al., 1979). Non-specific suppression of LH has been observed in intact and chronically castrated rats; sometimes all fractions of a bovine testis extract lowered LH although only some affected FSH (Lee et al., 1977).

Thus material considered to contain inhibin-like activity sometimes lowers serum LH as well as FSH, particularly in acutely castrated rats. Wide variations in LH concentrations, arising from episodic release of LH and a short half life, might mask LH suppression, particularly at low doses of activity.

The source of the activity also determines FSH/LH selectivity. Human seminal plasma fraction hSP₃ suppressed FSH and LH in vivo, whereas the adjacent fraction hSP₃-4 affected only FSH (Franchimont et al., 1979). Ovine rete testis fluid appeared to have a greater relative effect on LH in vitro than hSP₃ (Franchimont et al., 1978). bSP-extract suppressed LH whenever it suppressed FSH, while lower doses of ovine rete testis fluid suppressed FSH alone in acutely castrated rats (Davies et al., 1978b).

Of the various sources of activity, bSP seems to affect LH most. The data from pituitary cell cultures in this thesis support this contention. Ovine follicular fluid suppressed FSH more than LH, while a fraction of bSP had the reverse effect. The behaviour of the follicular fluid was comparable to that of culture medium from granulosa cells tested in the same system (Erickson & Hseuh, 1978).

The degree of selectivity of bSP may depend on its treatment. Freshly prepared "bSP-inhibin" suppressed only FSH in acutely castrated rats, but after storage at -20°C for six weeks, the extract's potency was reduced and it suppressed both gonadotrophins (Chari et al., 1978).
This observation could be pertinent; every sample of bSP used here had been stored at -20°C for at least four weeks before being used.

While bSP-extract appeared to suppress FSH and LH equally, testosterone propionate preferentially suppressed LH. The greater sensitivity of LH secretion to androgens in in vivo experiments of similar design is well established (Swerdloff et al., 1973; Swerdloff & Walsh, 1973; Eldridge & Mahesh, 1974), and has been explained by selective desensitisation of LHRH-induced LH secretion by androgens coupled with hypothalamic inhibition of LHRH (Labrie et al., 1978a). Another difference between the actions of bSP-extract and testosterone propionate was the steeper dose-response of the bSP-extract. A similar observation has been made by Eddie et al. (1979), who used pituitary cells to measure inhibition of LHRH-induced secretion of FSH and LH by androgens and a preparation of ovine rete testis fluid.

8.8.2 Time-course of Action

FSH was suppressed in intact rats within 12h of a single injection of bSP-extract, and levels remained lower than normal for more than 48h after injection. LH suppression occurred more rapidly - levels were lower by 6h-, and it may not have lasted so long. The shorter half-life of LH may be partly responsible.

In comparison, bovine testicular extracts suppressed FSH within 3h in acutely castrated rats (Nandini et al., 1976). The loss of effect between 24 and 36h may have been compounded by loss of sensitivity with time after castration. Fractions of ovine rete testis fluid and bull and human seminal plasma suppressed FSH in acutely castrated rats within 2h of intravenous injection (Franchimont et al., 1977, 1979; Chari et al., 1978). The time-course of action of follicular fluid can be mapped from those experiments utilising a single injection of material. There is a latency period of more than 3h, FSH is lower by 5h, suppression is greatest about 10h, it generally lasts until at least 14h, but is lost by 24h (Welschen et al., 1977; de Jong et al., 1978; Campbell & Schwartz, 1979; De Paolo et al., 1979b; Hoffmann et al., 1979).

Thus the time-course of action of the various inhibin-like activities differs. This is not unexpected, considering their different physicochemical properties (see Section 8.6).
the intraperitoneal space, the rate of uptake by the target organ, and the rate of clearance from the blood all depend on the physical properties, and could differ for foreign and native proteins. Indeed, extracts of hamster ovaries acted more quickly and for a shorter time in hamsters than did porcine follicular fluid (Chappel, 1979).

When LH is suppressed, its suppression often occurs before that of FSH. In rats given bovine testicular extracts, FSH was suppressed at 24, 48 and 72h after the beginning of treatment (although suppression at 24h was not statistically significant, it was of the same magnitude as at the later times), but LH was suppressed only at 24h (Baker et al., 1976). Similarly, injection of ovine rete testis fluid immediately stopped episodic release of LH in cryptorchid rams (Blanc et al., 1978; Cahoreau et al., 1979). The LH peaks were abolished for a short period (2-18h), and had often resumed by the time FSH fell (at 5-10h).

8.8.3 Mechanism of Action

There was no direct evidence in the experiments of where or how bSP-extract acted to suppress serum FSH and LH. Pituitary concentrations of the gonadotrophins in intact rats remain unchanged after injection of bSP-extract. Others have observed the same lack of effect (ovine rete testis fluid: Davies et al., 1979b; bSP fraction: Franchimont et al., 1975b; bovine follicular fluid: de Jong & Sharpe, 1976; rat epididymal extract: Le Lannou & Chambon, 1977b). In contrast, androgens increase pituitary FSH content in vivo (Kingsley & Bogdanove, 1973; Chowdhury & Steinberger, 1976; Steinberger & Chowdhury, 1977).

The use of pituitary cells in culture to measure inhibin-like activity has provided information on the site and mechanism of action. Activity, mainly from follicular fluid and Sertoli cell culture media, inhibits basal release of FSH only (Steinberger & Steinberger, 1976, 1977: de Jong et al., 1978, 1979a; Erickson & Hseuh, 1978; Labrie et al., 1978b; Lagacé et al., 1979; De Paolo et al., 1979a; Channing et al., 1980). The slight effects on LH that are sometimes observed may be caused by different factors (de Jong et al., 1979b; Demoulin et al., 1979). Unlike its effect on basal secretion, activity inhibits both FSH and LH secretion induced by LHRH. Sensitivity for LH is lower and the dose response shallower than for FSH (Baker et al., 1976; Eddie et al., 1978, 1979; Franchimont et al., 1978; de Jong et al., 1978, 1979a; Labrie et al.,
1978b; Lagacé et al., 1979). This behaviour has led de Jong (1979) to suggest that inhibin selectively inhibits FSH release, and secondly, decreases the sensitivity of the gonadotroph to LHRH.

There is also considerable evidence from in vivo experiments that inhibin-like activity blocks the action of LHRH. Bovine testis extracts and porcine follicular fluid reduced the LHRH-induced rise of FSH, but not LH, in male rats (Rush & Lipner, 1979), and porcine follicular fluid had the same effect in female rats whose endogenous LHRH secretion was blocked by phenobarbital (De Paolo et al., 1979b). Decreased response of LH to LHRH has been noted. Whether or not it is observed may depend on the design of the experiment - fractions of bull and human seminal plasma injected intraperitoneally over two days reduced only the FSH rise induced by LHRH, while intravenous injection 1h before administration of LHRH reduced the subsequent rises of FSH and LH (Franchimont et al., 1975a). Indirect evidence also points to the pituitary. Cryptorchid men have an exaggerated FSH response to LHRH (Bramble et al., 1975). In rats, this hypersensitivity was reduced by follicular fluid but not by steroids (Hopkinson et al., 1979b).

Inhibin-like activity inhibits the synthesis of FSH as well as its release (Chowdhury et al., 1978). Cellular depletion of FSH takes longer to occur than does inhibition of its release (Scott & Burger, 1979b, 1979c), which might explain why changes in pituitary concentrations of FSH have not been seen after injection of inhibin-like activity into rats.

Like steroids, inhibin may also act on the hypothalamus. Intraventricular injection of an extract of bull sperm (Lugaro et al., 1974) and intraperitoneal injection of rat epididymal extract (Le Lannou & Chambon, 1977b) both reduced hypothalamic content of FSH-releasing activity without changing LH-releasing activity. These experiments suggest separate releasing hormones for FSH and LH.

Unveiling the mechanism of action is confounded by the presence of more than one factor in some extracts. de Jong et al. (1979b) have evidence for a low molecular weight species in bovine follicular fluid and Sertoli cell culture medium that releases LH in the absence of LHRH, but inhibits FSH and LH secretion in the presence of LHRH - possibly an LHRH antagonist with some intrinsic LHRH activity. Similar activity has been found in rat ovarian extracts (Ying & Guillem, 1979). Low molecular weight activity in bovine and human follicular fluid has been
reported to prevent FSH from binding to the FSH receptor (Daume et al., 1979a & b), as has the 1500 dalton factor from ram testes (Moodbidri et al., 1980). Their relationship to FSH-receptor inhibitors already described in rat testes (Reichert & Abou-Issa, 1977), human serum (Reichert et al., 1977) and bovine follicular fluid (Darga & Reichert, 1978) is unknown. It is difficult to see how this property could give rise to inhibin-like activity. If FSH exerts direct negative feedback on its own secretion (Hirono et al., 1970) then interference of feedback should increase, not decrease, FSH secretion.

Also problematic is Chari et al.'s (1978) observation that freshly prepared "bSP-inhibin" lowered only serum FSH, but that stored material suppressed FSH and LH. How could a slight change in the active molecule lead to a different effect unless the molecule acted by a different mechanism? This raises the possibility of different effectors for FSH and LH suppression. The observation needs checking especially to ensure that the activity changes, not the rats. (Recall the incidence of LH suppression in intact rats given bSP-extract.)

8.8.4 Interaction with Steroids

Testosterone is an important regulator of FSH secretion in the male rat. Immunisation against testosterone elevates serum FSH as well as LH (Nieschlag et al., 1975; Hillier et al., 1975; Gay & Kerlan, 1978; Main et al., 1977). Do testicular steroids act independently of inhibin, or are their actions integrated?

Small doses of testosterone propionate and bSP-extract that had little effect alone acted synergistically to suppress FSH and LH to near the levels seen in intact animals. Larger doses of testosterone propionate and bSP-extract that by themselves suppressed FSH and LH to near intact levels had little further effect when combined.

Hopkinson et al. (1975, 1977a) gave dihydrotestosterone to increase the sensitivity of acutely castrated rats to inhibin-like activity, and synergistic interaction between testosterone and inhibin-like activity has been briefly reported by Hopkinson et al., (1977b). In their experiment acutely castrated rats received 500 µg testosterone propionate/kg BW a dose sufficient to suppress FSH almost to intact levels and LH to below intact levels. Small doses of a fraction of human
seminal plasma suppressed FSH significantly only in the testosterone-treated rats.

Although there was no evidence of synergism at higher doses in the experiments in this thesis, large doses of testosterone propionate suppressed FSH more in intact rats than in castrated rats. This suggested that the testis was enhancing testosterone's feedback on FSH. A subsequent experiment indicated the greater suppression in the intact rat was not due to testicular contribution of bSP-extract-like activity. The administered testosterone might have interacted with other testicular hormones, or it may have been metabolised by the testis to a more potent feedback agent.

The ability of rats to respond to bSP-extract diminished within 48h of castration. If responsiveness falls within 24h, then the observation of synergism between testosterone and bSP-extract could possibly be due to testosterone maintaining sensitivity to inhibin. To test this possibility, rats were castrated and treated with testosterone for a week before their response to bSP-extract was tested. Testosterone was supplied by subcutaneous capsules, which are known to maintain steady blood concentrations of the steroid over long periods (Damassa et al., 1976). Although the concentration of testosterone in the blood is proportional to the size of the capsule (Moger, 1976; Damassa et al., 1976), the effectiveness of a particular length of capsule in lowering FSH and LH varied between rats. Damassa et al. (1976) also found some rats more sensitive than others. The experiments showed that high doses of testosterone were needed to maintain sensitivity to bSP-extract, indicating this was not the mechanism of testosterone's action in the experiment showing synergism. It is nevertheless interesting that testosterone could maintain some sensitivity to bSP-extract. The active agent may be a steroid that can be metabolised from testosterone peripherally, but under normal conditions comes mainly from the testis.

An understanding of how testosterone and inhibin-like activity interact may come from in vitro studies. A recent abstract reports that activity from cultured Sertoli and granulosa cells inhibits the stimulatory effect of steroids on basal and LHRH-induced FSH secretion (Massicotte et al., 1979).
8.8.5 Is the Antigonadotrophic Activity in bSP "Inhibin-like"?

The antigonadotrophic activity in bSP was not exceptionally different from other activities considered to be inhibin-like. Other materials suppress LH as well as FSH, and studies with pituitary cell cultures give insight into how FSH secretion alone could be inhibited under some conditions, but FSH and LH secretion under other conditions.

The differences in physiological properties of activity from different sources - FSH/LH selectivity, time-course of action, and perhaps mechanism of action - cannot all be attributed to differences in method. Without doubt, there are different hormones, each with its own spectrum of properties and its own chemical composition. The gonadotrophins themselves provide an analogy. Some have follicle-stimulating activity, others luteinising activity. Some molecules have only one of these functions (e.g., human pituitary FSH or LH), while others have both (e.g., pregnant mare serum gonadotrophin), and the physical properties may differ according to their tissue of origin (e.g., pituitary and chorionic gonadotrophins). In addition there are other substances which are gonadotrophic but not gonadotrophins (e.g., estrogen acting on immature ovaries and uterus).

The inhibins can be considered a class of hormones. But how shall we define "inhibin-like"? McCullagh (1932) defined inhibin as a water-soluble factor from the testis which restored normal pituitary morphology in castrated rats without exerting androgenic effects on the accessory sex glands. In the meantime, with the discovery of two gonadotrophins and the recognition of testosterone's negative feedback on LH secretion, inhibin became described as a factor that specifically regulated FSH (Setchell & Main, 1974). de Jong (1979) has gone further to suggest that the term inhibin should be reserved for a water-soluble factor which specifically inhibits FSH release from the pituitary in the absence of LHRH. This is a tight definition which excludes bSP-extract. (witness the inhibition of FSH and LH secretion from cultured pituitary cells by a fraction of bSP-extract). If selectivity for FSH is a criterion, then the results of Chari et al. (1978) indicate that a slight change, wrought by storage at -20°C, could turn inhibin into not-inhibin.

The distinctive interaction between bSP-extract and testosterone suggests that a hormone with similar activity could participate in the
control of FSH and LH secretion in the male rat. The exogenous material must act via some biochemical pathway. We could define inhibin not by the details of its effects, which might depend on the endocrine status of the recipient or the regimen of administration, but by the mechanism through which it acts. If we assume there is an endogenous "inhibin", then we could call any substance that acted by the same route as inhibin. This is in some respects a tautology, but one which is made anyway. In any discussion on the role of inhibin we assumed there is an inhibin and that our tools of investigation, bSP-extract or follicular fluid for instance, act like inhibin.

8.9 ROLES FOR INHIBIN

8.9.1 Feedback on Gonadotrophin Secretion in the Male

The interaction between bSP-extract and testosterone propionate suggests how inhibin and testosterone could participate in the control of serum concentrations of FSH and LH in the male rat. If bSP-extract acts like inhibin, and if inhibin and testosterone are the major agents of gonadotrophin feedback, then there would be overlap with some degree of independence in the control of FSH and LH. The experiment in Figure 7.10 suggests that the removal of inhibin would lead to an equal rise of FSH and LH (Group 5 to Group 4), while removal of testosterone would cause a proportionally greater rise of LH than FSH (Group 5 to Group 2), similar to the effect of castration (Group 5 to Group 1). Indeed selective damage to the germinal epithelium and by implication removal of inhibin often results in comparable rises of FSH and LH (Amatayakul et al., 1971; Gomes & Jain, 1976; Rich & de Kretser, 1977; Hopkinson et al., 1978; Main et al., 1978; Hopkinson et al., 1979a), which is often interpreted as a preferential rise in FSH when compared to the effect of castration (Main et al., 1978). However, the relative changes in the levels of FSH and LH observed after removal of inhibin or testosterone would depend on the amounts of inhibin and testosterone present initially (compare Group 5 to Group 2, with Group 6 to Group 3), even though normal FSH levels (and to a narrower extent LH levels) might be maintained by different concentrations of endogenous inhibin and testosterone (compare Group 10 with Groups 3, 5, 6, 7, 8 and 9).
The experiment in Figure 7.10 illustrates only a potential role. Joint control of FSH and LH by testosterone and inhibin has been proposed from indirect evidence (human: Rosenfield et al., 1977; rat: Collins et al., 1978; Main et al., 1978). Main et al. (1978) have calculated, from the comparative rises of FSH and LH after castration and after damage to the germinal epithelium, that inhibin contributes about one third the total testicular feedback on gonadotrophin secretion. However, if synergism occurs, then simple apportioning of control between the two hormones is impossible. Interaction between inhibin and testosterone could explain why FSH can rise to castrate levels after administration of antiserum to either testosterone (Nieschlag et al., 1975; Hillier et al., 1975; Main et al., 1977) or inhibin (Thakur et al., 1978; Vaze et al., 1979; Franchimont et al., 1975b).

The importance of inhibin in controlling fertility, and therefore its potential as a contraceptive agent, depends on whether FSH, or how much FSH, is necessary for spermatogenesis to occur normally. While FSH is essential for sexual maturation in the rat (see Dorrington & Armstrong, 1978; Raj & Dym, 1976), its importance in the nature animal is equivocal. Sufficiently large doses of androgen can maintain and restore fertility in hypophysectomised adult rats (Nelson, 1937; Boccabella, 1963; von Berswordt-Wallrabe & Mehuring, 1974; Chowdhury & Steinberger, 1975; Harris et al., 1977), but spermatogenesis may not be quantitative (Chowdhury, 1979). In any case, these experiments are far removed from the delicate interplay of hormones in the intact rat (Woods & Simpson, 1961; Bartke et al., 1978).

Complete removal of FSH by passive immunisation in bonnet monkeys left testicular size and histology unchanged, but reduced the quality of the sperm (Murty et al., 1979). Similar treatment did not affect germ cell numbers or fertility in rats (Dym et al., 1979). Sheep, on the other hand, appear to need FSH to complete spermatogenesis (Courot et al., 1979). It is therefore not unexpected that long term administration of inhibin-like activity had little effect on the fertility of adult rats (Davies et al., 1979a), although it did delay sexual maturation in immature rats (de Jong et al., 1978).

Inhibin's main role might be to allow stimulation of spermatogenesis through inhibin's absence or lack of effect, before puberty, before the breeding season, or after impairment of total sperm production, such as occurs upon hemicastration or damage to the germinal epithelium.
This would tie in with the observation here, and elsewhere, that suppression of FSH to intact levels in acutely castrated rats is easy, but that further suppression is more difficult.

8.9.2 Feedback on Gonadotrophin Secretion in the Female

Steroids cannot fully account for feedback on FSH in the female rat. After unilateral ovariectomy FSH rises without concomitant changes in LH or estradiol (Welschen et al., 1978). Even high doses of estrogens, which suppress LH to below intact levels, cannot restore normal FSH levels in ovariectomised rats or mice, nor can combinations of estrogen and progesterone (Bronson, 1976; Bronson & Channing, 1978). However, ovarian transplants or injections of porcine follicular fluid can further suppress FSH in rats or mice receiving a maximal dose of estrogen (Bronson & Channing, 1978; Campbell & Schwartz, 1979).

The preovulatory surge of FSH, but not LH, in rats can be blocked by porcine follicular fluid (De Paolo et al., 1979b; Hoffmann et al., 1979). The second surge of FSH, triggered by LH, can also be blocked by porcine follicular fluid (Schwartz & Channing, 1979). These authors suggested that the preovulatory surge of LH impairs inhibin's feedback on FSH, thus allowing a second surge of FSH. Luteinisation of the granulosa cells could reduce their production of inhibin. The presence of more inhibin-like activity in ovaries on the day of proestrus has been used to advance a similar theory for the hamster (Chappel, 1979).

These experiments show only what inhibin might do. Changes in inhibin-like activity in ovarian venous plasma have been studied by De Paolo et al. (1979a). The concentration fell midway through proestrus and rose towards the end of estrus - thus the activity in the blood varied inversely with the concentration of FSH. In addition, Shander et al. (1980) have recently shown that an ovulatory dose of LH can indeed reduce the inhibin-like activity in ovarian venous plasma.

Evidence of a role for inhibin in other species is more limited. Welschen et al. (1977) found higher concentrations of activity in medium and large antral follicles of cows. Since serum FSH was negatively correlated with the number of large antral follicles, the follicular content of activity could reflect the amount of activity in circulation (Welschen et al., 1978). Follicular concentration might be proportional
to the number of granulosa cells, since in the rat even undifferentiated cells secreted activity in vitro (Erickson & Hseuh, 1978). However Lorenzen et al. (1978a) found the opposite relationship between follicular size and the concentration of activity in pigs.

In ovariectomised ewes, judicious administration of estradiol and progesterone (by implanting and then removing subcutaneous capsules at certain times) could duplicate concentrations of LH associated with a normal estrous cycle (Goodman et al., 1980). The regimen left FSH elevated. Inhibin may be less important in primates; estradiol and progesterone by themselves can maintain normal FSH and LH levels in castrate monkeys (Plant et al., 1978). Moreover, even large doses of porcine follicular fluid were not as effective as steroids (Hodgen, 1980). Nevertheless, inhibin may still play some part. Activity was detected in human follicular fluid collected from the follicular phase but not in fluid from the luteal phase (Chappel et al., 1980).

8.9.3 A Local Effect

It has been proposed that inhibin might act on the testis itself, although no evidence has yet been presented (Main et al., 1979; Main & Davies, 1979). The testis does produce other protein factors that act locally - meiosis — inducing and preventing factors in the fetal testis (O & Baker, 1976; Byskov, 1978), and Müllerian-inhibiting substance during sexual differentiation (Blanchard & Josso, 1974). The ovary releases factors into follicular fluid which can inhibit and stimulate oocyte maturation (Tsafiriri et al., 1976; Gwathin & Andersen, 1976; Ledwitz-Rigby & Rigby, 1979).

The role of inhibin-like activity in seminal plasma is enigmatic. The indirect transport of activity from the testis to the accessory glands suggests a purpose, but it may only be the by-product of transport of some other testicular factor, such as testosterone. Many hormones are present in seminal plasma, some in very different concentrations to that in blood; for instance, FSH concentrations are 100 times greater in bSP than blood (Sairam et al., 1980a). Their presence could be incidental, although the possibility of a local role is inviting. Recently prolactin has been reported to enhance fructose metabolism and adenyl cyclase activity in sperm (Sheth et al., 1978).
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APPENDIX

Measurement of Testosterone

Testosterone was measured by radioimmunoassay using the methods and reagents of the World Health Organisation Matched Reagent Programme.

A small amount of tritiated testosterone was added to 500µl sample, and after 15 min this solution was extracted once with 5ml diethyl ether. The mixture was cooled in an acetone-dry ice bath to freeze the aqueous phase. The ether phase was decanted, and blown dry under a stream of nitrogen. The extract was dissolved in 2ml 0.1 M phosphate-0.15 M NaCl-0.1% gelatin-0.01% merthiolate, pH 7.4 (assay buffer), and 500µl aliquots were assayed. The incubation mixture consisted of the sample or standards, tracer in 100µl buffer, and antibody in 100µl buffer. The antiserum was raised against testosterone-3- (O-carboxymethyl) -oxine BSA in a sheep, and was used at a final dilution of 1:210000. After 18 h incubation at 4°C, 200µl of 0.063% charcoal- 0.0063% dextran suspension was added to each tube. The tubes were left standing for 15 min at 4°C, and then centrifuged at 500g, 5 min, 4°C. The radioactivity in the supernatant was counted.

The assay had a sensitivity (90% B₀) of 4-9pg, and showed crossreactivity of 14% with dihydrotestosterone, 6% with 5α-androstane-3α, 17β-diol, 2.1% with androst-5-ene-3β,17β-diol, and 0.8% with androstenedione.