INHIBIN

ITS ROLE IN THE REGULATION OF THE PITUITARY-TESTIS AXIS

DE ROL VAN INHIBINE IN DE REGULATIE VAN DE HYPOFYSE-TESTIS AS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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door

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geboren te Wisch

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VOORWOORD

Het inhibine-onderzoek, beschreven in dit proefschrift, is voltooid op het moment dat relevante publicaties van onderzoeksgroepen uit verschillende werelddelen zijn verschenen. Deze hebben het bestaan van het eiwithormoon inhibine, waaraan lange tijd werd getwijfeld - zoals bij onderwijs aan studenten Geneeskunde - bevestigd.
Via arbeidsintensieve methoden om inhibine-activiteit vast te stellen, is in deze dissertatie getracht een beeld te verkrijgen van de regulatie en de betekenis van inhibineproductie in een mannelijk proefdiermodel (rat). Nader onderzoek betreffende de werking van inhibine op het doelorgaan en de regulatie op gen-niveau, is gewenst.
Het moge duidelijk zijn, dat bij het tot stand komen van een proefschrift goede samenwerking een vereiste is.
Ik hecht eraan waardering uit te spreken voor de voortdurende interesse van mijn promotor Prof. Henk van der Molen in het inhibine-onderzoek en hem te bedanken voor het heldere, kritische commentaar tijdens het schrijven. Zijn suggesties waren onontbeerlijk.
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ABBREVIATIONS

ABP	androgen binding protein
α-MSH	α-melanocyte stimulating hormone
bFF	bovine follicular fluid
bPcas	bovine plasma from a castrated cow
cAMP	adenosine cyclic-3':5'-monophosphate
(c)DNA	(complementary) deoxyribonucleic acid
dbcAMP	N^6-2^1-O-dibutyryl adenosine cyclic-3^1:5-monosphosphate
DHT	dihydrotestosterone
EDF	erythroid differentiation factor
EDL	efferent duct ligation
EGF	epidermal growth factor
DPP-C	deca penta plegic gene-complex
FRP	follicle-stimulating hormone releasing protein
FSH	follicle-stimulating hormone, follitropin
hCG	human chorionic gonadotrophin
IGF(-I,-II)	insulin-like growth factor (-I,-II)
(k)Da	(kilo) Dalton
LH	luteinizing hormone, lutropin
LH-RH	LH-releasing hormone
MEM	Eagle's minimum essential medium
MIS	Müllerian inhibiting substance
MIX	3-isobutyl methylxanthine
(m)RNA	(messenger) ribonucleic acid
MW	molecular weight
n	number of determinations
oRTF	ovine rete testis fluid
oTLP	ovine testicular lymph protein
pFF	porcine follicular fluid
PMSG	pregnant mare serum gonadotrophin
RIA	radioimmunoassay
SCF	Sertoli cell factor
S.E.M.	standard error of the mean
TGF(-β)	transforming growth factor (-β)
TSH	thyroid stimulating hormone
u, U	units
v/v	volume/volume
w/v	weight/volume

CONTENTS

PART ONE

PART TWO

PART THREE

PART ONE

1

GENERAL INTRODUCTION

1.1 Introduction and scope of this thesis

The endocrine and exocrine functions of the male gonads, the testes, are regulated by gonadotrophic hormones which are secreted by the pituitary gland. Two separate gonadotrophic hormones have been recognized: luteinizing hormone (LH) which influences Leydig cell function, and follicle-stimulating hormone (FSH) which affects the function of the seminiferous tubules. The secretion of gonadotrophins is stimulated by a hypothalamic factor, luteinizing hormone-releasing hormone (LH-RH) and can be inhibited by steroid hormones which are secreted by the testes.
The existence of another hormone which is produced by the seminiferous tubules in the testis and also influences the pituitary gland in males has been suspected for many years (Mottram & Cramer, 1923). This principle appeared to be a *non* steroidal factor and has been called "inhibin" by McCullagh (1932). The reality and significance of the inhibin concept has been much debated and has received increasing attention during the past ten years (see *chapter 2*). A schematic diagram on the interactions between stimulating and inhibiting substances in the regulation of the hypothalamic-pituitary-testicular axis is shown in figure 1.1.
The aim of the investigations, described in this thesis was to examine the regulation of inhibin production in and inhibin secretion from testicular tissue and to investigate the physiological significance of this protein hormone in male reproduction.
Chapter 2 provides a broad outline of the history, assay and nature of inhibin.
Peripheral levels of FSH change in a characteristic way during development of the male rat, and this stimulated us to study the testicular content of inhibin in rats of various ages. The results of these studies were related to endogenous gonadotrophin levels in order to investigate at which age inhibin might play a role in pituitary FSH regulation. These studies have been described and discussed in *chapter 3* and *appendix paper 1.*
In vitro experiments were performed to show which cells within the testis are involved in the production and secretion of inhibin and which factors can affect inhibin production (*chapter 4* and *appendix paper 2*).
In *chapter 5* and *appendix papers 3 and 4*, a number of *in vivo* experiments has been described, which involve manipulation of testicular and pituitary function with the objective of

ascertaining the age at which inhibin plays a major role in the regulation of pituitary FSH secretion.
Finally, in *chapter 6* the significance of inhibin in the regulation of male fertility is discussed.

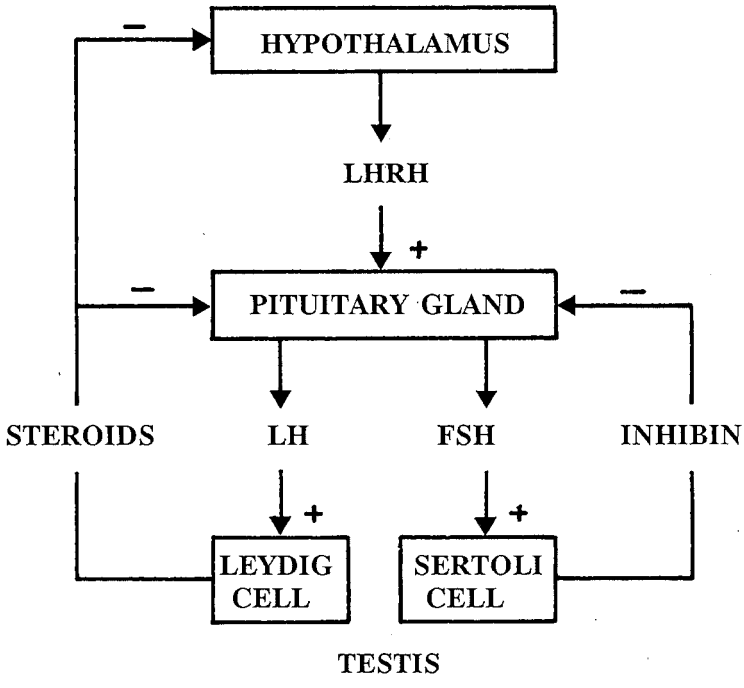

Fig. 1.1 Schematic representation of the hypothalamic - pituitary - testicular axis. Steroids produced by Leydig cells within the testis act on hypothalamic - pituitary components of the axis to suppress LH and FSH secretion by the pituitary gland. In addition, inhibin is secreted by testicular Sertoli cells and exerts direct specific inhibitory effects on the FSH secretion by the pituitary gland.

1.2 Gonadotrophic cells of the pituitary gland

The pituitary gland consists of at least two parts, an anterior lobe, the adenohypophysis and a posterior lobe, the neurohypophysis, while in some species an intermediary lobe can be discerned.
The adenohypophysis or anterior pituitary gland synthesizes several (glyco)-protein hormones, which are secreted into the peripheral circulation. The glycoprotein hormones

consist of two *non* identical subunits, α and β. These subunits are encoded by separate genes in the pituitary cells (see review: Chin, 1985). Within a given species, the α-subunits of FSH and LH have identical amino acid sequences and are often produced in excess, while the β-subunit is considered to be hormone specific (Pierce & Parsons, 1981). The association between an α-subunit and a β-subunit results in a biologically active dimer. In numerous studies it was attempted to identify and classify the cells responsible for the secretion of FSH and LH by the pituitary gland. The fact that FSH and LH share a common α-subunit has led to initial difficulties in the immunological identification of specific secretory cell types. With the purification and sequencing of the structure of FSH and LH, antisera to the entire hormone or its α- and β-subunits have been developed. Localization studies with labelled antisera against the β-subunits of FSH or LH have also shown that both antisera can bind to the same cell type within the pituitary tissue, the gonadotroph (Kofler, 1982; Childs *et al.*, 1985). On the other hand, subtypes of pituitary gonadotrophs which contain only LH or FSH may also exist (Moriarty, 1976; Childs *et al.*, 1983).
Steroid hormones can suppress peripheral concentrations of gonadotrophins indirectly by an inhibitory action on the hypothalamic secretion of LH-RH or directly, by an action on the pituitary gland (see section 1.4 and Fig. 1.1). Until recently, little was known about the biochemical mechanisms of the effects of steroid hormones on the biosynthesis of FSH and LH. The technique of cell free translation of messenger ribonucleic acids (mRNAs) from pituitary glands and the use of complementary deoxyribonucleic acids (cDNAs) in RNA blot hybridization assays, made it possible to study the effects of steroids at the level of pituitary mRNAs that encode for FSH and LH subunits. (Alexander & Miller, 1982; Counis *et al.*, 1983). The high LH secretion in long term castrated rats is associated with increased concentrations of α and β mRNAs for LH. From these studies, it appears that concentrations of mRNA for the β-subunit are the rate-limiting factor in the synthesis and secretion of LH (Papavasiliou *et al.*, 1986). Furthermore, administration of 17β-oestradiol to castrated animals reverses the effect of gonadectomy and results in levels of the mRNAs of gonadotrophin subunits comparable to the levels present in intact animals (Alexander & Miller, 1982; Counis *et al.*, 1983). These data indicate that in adult animals the pituitary concentrations of mRNAs for FSH are also regulated by the gonadal sex steroids.

1.3 The testis

The testis is composed of two main compartments: the seminiferous tubules containing the spermatogenic epithelium, and the interstitial compartment containing Leydig cells, blood vessels and lymphatics embedded in a connective tissue matrix (Fig. 1.2).
Spermatogenesis is the process of development of spermatozoa from immature germ cells. This process takes place within the seminiferous tubules and has been characterized in mammals on basis of structural characteristics of different spermatogenic cell types (rats: Clermont, 1972; Clermont & Perey, 1957). In the rat, early spermatogenic cells (gonocytes) divide actively in the foetal gonad until day 17 of gestation. They do not proliferate further

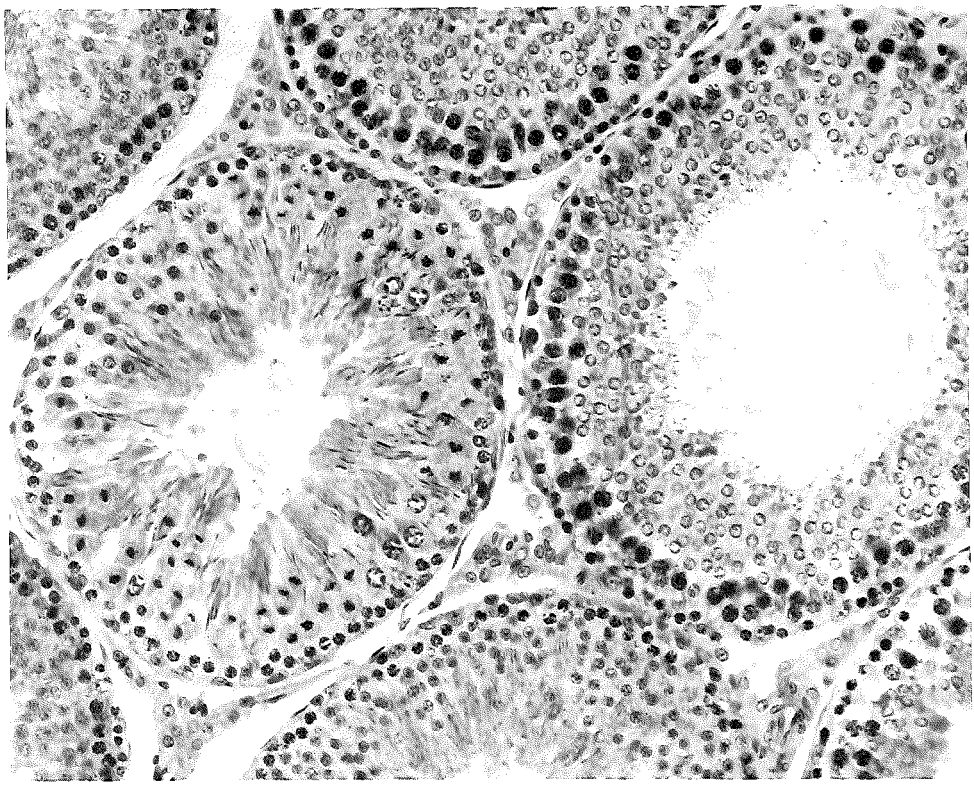

Fig. 1.2 Cross section of normal testicular tissue from an adult rat demonstrating round seminiferous tubules containing Sertoli cells and developing spermatogenic cells. A tubular lumen is present and outside the tubules, interstitial tissue where Leydig cells can be seen (HE-staining; x340).

until the end of the first postnatal week (Beaumont, 1960). At this time they divide to form spermatogonia. Differentiating spermatogonia divide to form spermatocytes which enter the process of meiosis, resulting in the formation of haploid cells, the spermatids. These cells do not divide further but undergo a complicated process of maturation which results in formation of spermatozoa. The latter cells leave the testis via the rete testis and are stored in the epididymis. During the foetal and prepubertal period, the supporting cells of the seminiferous tubules, the Sertoli cells (Sertoli, 1865), divide (Orth, 1982 and 1984). Tight junctions between adjacent Sertoli cells are formed between day 16-20 of postnatal life in the rat. This phenomenon is closely related to the formation of a tubular lumen and to the cessation of the proliferative activity of the Sertoli cell population. Several morphological

and functional changes occur in the Sertoli cell during sexual maturation, but a fixed number of Sertoli cells is present in the adult testis. The tight junctions prevent extracellular access of compounds with high molecular weight into the adluminal compartment of the seminiferous tubules (Setchell, 1967), but the entry rate of substances across the cell membrane is high when the lipid solubility is high (Waites & Gladwell, 1982). The existence of this so-called "blood-testis barrier" has been deduced from the inability of radio-opaque tracers to permeate into the seminiferous epithelium (Vitale *et al.*, 1973). In this way the seminiferous epithelium is divided by the blood-testis barrier into a basal and an adluminal compartment. Spermatogonia and young spermatocytes reside in the basal compartment which is in contact with the blood and lymph circulation, while further developed spermatogenic cells, such as older spermatocytes and spermatids, are present in the adluminal compartment, and are directly influenced only by secretions from the Sertoli cells. The Sertoli cell extends from the base of the epithelium to the tubular lumen and its lateral cytoplasmic processes surround the differentiating spermatogenic cells.
According to Leblond and Clermont (1952) fourteen well defined associations of cells or developmental stages of the seminiferous epithelium can be found in the rat, which follow each other in a regular fashion: the spermatogenic wave. The whole process of spermatogenesis is a cyclic phenomenon, during which Sertoli cell function may be modified by the influence from the neighboring spermatogenic cells, in addition to its regulation by hormones (Parvinen, 1982). Because of the intimate relationship between spermatogenic cells and Sertoli cells, it is generally assumed that the control of spermatogenic differentiation is mediated by the Sertoli cell.

1.4 The hypothalamic-pituitary-testicular axis

Secretory products of the hypothalamus, pituitary gland and testes can have stimulatory or inhibitory effects on the functions of the components of the hypothalamic-pituitary-testicular axis, as shown in a simplified form in figure 1.1.
The hypothalamus maintains and regulates pituitary-gonadal function. Nerve endings of hypothalamic neurons release a variety of small peptides into the portal capillary system which links the hypothalamus with the anterior lobe of the pituitary gland. These hypothalamic peptides regulate the secretion of hormones from endocrine cells in the pituitary gland. A number of these peptides have been purified, their amino acid sequences are known and chemical synthesis has been achieved. This includes the purification and characterization of LH-RH by Guillemin (1972) and Schally *et al.* (1972). This releasing factor is secreted in a pulsatile way from the hypothalamus into the pituitary portal system and is bound by specific receptors on the gonadotrophs of the anterior pituitary gland. This process results in stimulation of gonadotrophin secretion.
The basis for the elucidation of pituitary-gonadal interaction had already been established at the beginning of this century by a number of investigators, who found a relationship between the morphology of pituitary cells and the functioning of the gonads. These investigators

observed that removal of the gonads resulted in an increase in size of a population of pituitary cells. The cytoplasm of these cells became enlarged and vacuolized, and these "castration cells" could easily be recognized in the pituitary gland of castrated animals. In 1923, Mottram and Cramer showed that irradiation of testes in young male rats resulted in an increase of the number of such castration cells in the pituitary gland, while the seminiferous tubules were heavily damaged. On basis of these results, these authors postulated "an internal secretion of the seminiferous epithelium, which controls pituitary function", the first indication that the seminiferous epithelium secretes a factor which might exert a negative feedback action on the pituitary gland.

Swerdloff *et al.* (1971) measured plasma FSH and LH by radioimmunoassay methods during sexual maturation in the male rat and indicated that a dynamic pituitary-gonadal control system exists in the immature animal. These authors performed experimental bilateral cryptorchidism and castration at the age of 21 days and observed that the control of pituitary FSH and LH release differed in relation with these alterations in gonadal function.

Furthermore, levels of LH and FSH were measured by radioimmunoassay in plasma and pituitary tissue of foetal and newborn rats (Chowdhury & Steinberger, 1976). Levels of plasma FSH and LH in male foetuses were high on day 16 of foetal life and dropped afterwards. Pituitary FSH and LH were first detected in male foetuses on day 17 of gestation. In contrast, no detectable gonadotrophin levels were present in female rats until the day of birth. In this regard Chowdhury and Steinberger (1976) suggested that testosterone production by foetal testes is important to start sexual differentiation in the male and is under control of the foetal pituitary, but they did not consider the possible involvement of *non* steroidal factors, like inhibin.

The role of secretory products from the pituitary gland in the regulation of testicular function was already described by Smith (1930), who performed both hypophysectomy and replacement therapy with pituitary extracts in rats, and observed the importance of pituitary gland secretions in testicular physiology.

Pituitary LH secretion is thought to be negatively controlled by gonadal steroids such as testosterone, but the relative contributions of inhibin and steroids in the control of pituitary FSH secretion remain to be determined. McCullagh (1932) was able to prevent the appearance of castration cells in the pituitary glands of castrated rats by injection of an aqueous extract prepared from bulls' testes, but more recent studies in adult rhesus monkeys and rats demonstrated that implantation of silastic capsules, filled with testosterone into castrated animals, can suppress the high castration levels of FSH to normal levels (Plant *et al.*, 1978; Decker *et al.*, 1981). These authors argued that the contribution of inhibin in the regulation of FSH secretion in these animals must be relatively small. The observation that a relatively fast increase of plasma FSH and not of LH occurred after castration of prepubertal, but not of adult male rats (Hermans *et al.*, 1980) led to the suggestion that inhibin could be more important to suppress pituitary FSH secretion in immature animals when compared with the situation in adult males. More information on the role of inhibin in the regulation of the pituitary secretion of FSH under physiological and experimental conditions was obtained in the investigations described and discussed in this thesis.

2

THE INHIBIN CONCEPT

2.1 Historical background

Already in 1923 Mottram and Cramer suspected the existence of a hormone which is produced by the seminiferous tubules in the testis and influences the pituitary gland. In their studies with rats morphological changes were observed in the anterior lobe of the pituitary gland after spermatogenesis had been severely damaged by exposure of the testes to radium, whereas the function of interstitial cells was apparently unaffected. The interrelationship between pituitary gland and gonads in mammals was reported also by several other authors (Martins & Rocha, 1931; Moore & Price, 1932). In 1932, McCullagh was able to prevent morphological changes in the pituitary glands of castrated rats by injection of an aqueous testicular extract, while atrophy of the accessory sex organs was not reversed. Injections of an organic solvent extract of testes prevented atrophy of these androgen dependent organs but did not influence the appearance of "castration cells" in castrated male rats. On basis of these results, McCullagh (1932) suggested the existence of two testicular hormones: "androtin", present in benzene extracts of the testes, which was held responsible for the development and maintenance of the function of the seminal vesicles and prostate, and "inhibin", a water soluble factor which could reverse the appearance of castration cells in the pituitary gland of castrated animals. The isolation and characterization of "androtin" - now testosterone - was achieved by David *et al.* (1935), but attempts to identify the hypothetical substance "inhibin" did not succeed. Moreover, due to technical difficulties, these finding on the effect of inhibin could not be reproduced by other authors, and the inhibin hypothesis was abandoned. For instance, Nelson & Gallagher (1935) prevented the appearance of castration changes in the pituitary gland by treatment with steroid containing testicular extracts, whereas they did not obtain any effect with aqueous testicular extracts. The fact that the amount of steroids required for the inhibition of the appearance of castration cells after gonadectomy also caused excessive stimulation of accessory sex organs was explained by postulating differences in the sensitivity of accessory sex organs and pituitary gland for androgens. In addition, Rubin (1941) observed that inorganic testicular extracts were toxic to rats. He suspected that the suppression of pituitary gonadotrophin secretion, obtained after injection of watery extracts of testes, might be due to these *non* specific effects. The fact that aqueous testicular extracts were able to disturb the oestrous cycle of female rats was

ascribed to similar aspecific effects, although it was considered also as evidence for a role of inhibin in the regulation of the pituitary-gonadal axis in female animals (McCullagh & Schneider, 1940).

The development of bioassays for the measurement of urinary concentrations of gonadotrophins offered a new possibility for investigation of the control of gonadotrophin secretion in men. A positive correlation was observed between urinary concentrations of FSH and degree of spermatogenic damage (Klinefelter *et al.*, 1942; Heller & Nelson, 1945; del Castillo *et al.*, 1947; McCullagh & Schaffenburg, 1952), while normal FSH concentrations were reported when Leydig cells were damaged or absent (McCullagh & Schaffenburg, 1952). The finding that men with severe damage of the seminiferous tubules often had increased levels of FSH but normal androgen production was thought to be the result of the *non* utilization of FSH by their gonads (the "utilization" hypothesis, Heller & Nelson, 1948; Heller *et al.*, 1952). These authors suggested that intact spermatogenic epithelium utilized the FSH produced by the pituitary gland and that damage of spermatogenic cells decreased the FSH utilization with a resulting increase of concentrations of FSH in the urine. When this hypothesis had been rejected on the basis of the increased pituitary levels of FSH after castration of rats, which could not be explained by the utilization hypothesis (Howard *et al.*, 1950), the identification of an increasing number of testicular steroids seemed to offer new possibilities to explain separate regulatory mechanisms for the secretion of pituitary FSH and LH. At that time, the existence of inhibin could not be demonstrated directly and some investigators thought of a role for oestrogens in the regulation of FSH secretion (McCullagh & Schaffenburg, 1952; Sherins *et al.*, 1982; see also reviews by Baker *et al.*, 1976, and Setchell *et al.*, 1977).

The interest in the inhibin hypothesis was renewed following the observations of Setchell & Sirinathsinghji (1972), who found a decrease of the uterine weight in human chorionic gonadotrophin (hCG)-treated mice injected with rete testis fluid from rams and boars, and explained this observation on basis of suppression of FSH in these animals by inhibin, present in the rete testis fluid.

The development of more reliable and sensitive bioassay methods for the detection of inhibin activity (section 2.3) led to purification and characterization of inhibin (section 2.4). Discrepancies between the changes of peripheral levels of gonadotrophins in mammals will be discussed first (section 2.2). These data support the inhibin concept which includes the specific suppressive effect on pituitary FSH: inhibin has been defined as ".... a water-soluble, gonadal substance, which exerts a specific inhibition of the release of FSH from the pituitary gland" (de Jong, 1979a), or, later, ".... as a glycoprotein hormone consisting of two dissimilar disulphide-linked subunits which inhibits the production and/or secretion of gonadotrophins, preferentially that of FSH" (Burger *et al.*, 1988). It is not possible to discriminate between "inhibin-like" activity and inhibin activity. However, the term "inhibin activity" will be used throughout this thesis.

2.2 Differential regulation of the secretion of LH and FSH from the pituitary gland

Measurements of FSH and LH, initially by bioassay methods and since the 1970's by specific radioimmunoassays, have provided direct evidence for an LH-independent rise of peripheral concentrations of FSH following damage to the testis or ovary (de Kretser *et al.*, 1972; extensive review by Savoy-Moore & Schwartz, 1980). Using radioimmunoassay techniques it has been shown also that increased levels of FSH are found in men with severe damage of the seminiferous epithelium as may be caused by cryptorchidism, varicocele, irradiation, Klinefelter's syndrome or treatment with cytotoxic drugs (de Kretser *et al.*, 1972; van Thiel *et al.*, 1972; Bramble *et al.*, 1974, 1975). Significantly elevated levels of FSH were found also in patients with "Sertoli-cell-only" syndrome (Nieschlag *et al.*, 1979; Rothman *et al.*, 1982; Bibro *et al.*, 1984) and in cases with azoospermia or oligospermia (Setchell *et al.*, 1977; Bablok *et al.*, 1978).

The association of severe damage of the seminiferous epithelium with elevated levels of FSH has been observed also in rats following a variety of experimental procedures which affect the seminiferous epithelium such as cryptorchidism (Kerr *et al.*, 1979; Risbridger *et al.*, 1981), irradiation (Rich & de Kretser, 1977; Wang *et al.*, 1983), local heating of the testes (Galil & Setchell, 1980, 1981; Main & Setchell, 1980), the use of anti-spermatogenic drugs such as hydroxyurea (Mecklenburg *et al.*, 1975; Rich *et al.*, 1979), vitamin A deficiency (Rich *et al.*, 1979; Huang *et al.*, 1983), and ligation of the efferent ducts (Main & Setchell, 1980; Risbridger *et al.*, 1981). It is important to note that in some of the studies mentioned above, both in men and in rats, a rise of FSH is accompanied by a rise in LH suggesting that a complete dissociation of control of gonadotrophin secretion does not occur. Also, it cannot be excluded that long term damage of spermatogenic cells affects interstitial function directly.

Finally, an observation of differential feedback of LH and FSH secretion in male rats was found within the first 24 h after castration, when changes in secretion patterns of FSH and LH were not parallel (Swerdloff *et al.*, 1971; Hermans *et al.*, 1980). The fast increase of FSH levels could not be suppressed by injection of physiological amounts of testosterone (Hermans *et al.*, 1980).

The differential negative feedback regulation of FSH and LH might be explained in a number of ways: effects of steroids, the existence of a specific FSH-releasing factor, differential effects of changes of LH-RH pulsatility or finally, on basis of the existence of inhibin. It is not likely that steroids play a role in the preferential suppression of FSH, since after long term castration the increased peripheral levels of LH can be suppressed more effectively than those of FSH by injection with several steroids. Supraphysiological doses of testosterone have to be used to suppress FSH in castrated animals to the levels found in intact controls (Verjans *et al.*, 1974).

Secondly, the release of FSH and LH from the pituitary gland is likely to be stimulated by a single hypothalamic-releasing hormone, LH-RH (Guillemin, 1972; Schally *et al.*, 1976; Wise *et al.*, 1979; Elias & Blake, 1981). A number of investigators has claimed evidence for the existence of a separate FSH-releasing hormone (Jutisz *et al.*, 1972; Currie *et al.*, 1977; Fuchs

et al., 1979; Mizunuma *et al.*, 1983), but none of these factors preferentially stimulated the release of FSH (Sandow *et al.*, 1975). Therefore, it is generally accepted that one releasing hormone exists for both gonadotrophins.
Thirdly, Lincoln (1979) indicated that changes in the frequency of LH-RH pulses could have differential effects on secretion of FSH and LH, because of the differences in metabolic clearance rate of the gonadotrophins: LH-RH pulses with a short interval were shown to increase the LH/FSH ratio, while long intervals between the pulses had an opposite effect.
The last possibility *i.e.* an explanation of these discrepancies on basis of the "inhibin concept" presents the most likely explanation for these differences between LH and FSH secretion, especially since inhibin was purified and characterized, and methods for its estimation were established.

2.3 Detection of inhibin activity

In the course of the history of the "inhibin concept" a variety of bioassay methods, both *in vivo* and *in vitro*, have been used to detect inhibin activity in various biological preparations of male and female origin. In order to express inhibin activity, most research groups have used their own reference preparation and until very recently no generally accepted international standard preparation of inhibin activity was available.
During the last years, several different bioassays for inhibin activity have been used, but many assays have been poorly validated, as will be described below. In addition, factors which interfere with pituitary cell function and inhibit production and secretion of FSH might be considered erroneously as inhibin activity. This makes it important to exclude toxic or *non* specific effects on FSH secretion in animals or isolated pituitary tissue or cells.

2.3.1 *In vivo* bioassay methods

In vivo bioassay methods for measurement of inhibin activity are based on either the suppression of organ weight caused by a decrease in the circulating levels of FSH (*indirect* bioassay method) or the suppression of circulating FSH levels in animal models after injection of inhibin containing preparations (*direct* bioassay method) as summarized in Table 2.1.
In early experiments, detection of inhibin activity was based on changes in weight of the ovaries in parabiotic rats (Martins & Rocha, 1931). In this model transfer of gonadotrophins from a castrate adult male to an intact immature female rat resulted in precocious ovarian maturation while metabolism of steroid hormones occurred before they could reach the peripheral circulation of the castrated animal (Baker *et al.*, 1981). When subsequently inhibin containing preparations were injected to the castrated male parabiont, pituitary and ovarian hypertrophy were prevented or inhibited (Fachini *et al.*, 1963; Lugaro *et al.*, 1969, 1973; Baker *et al.*, 1981). This method appears not to be suitable to obtain a dose-response

relationship between the amount of inhibin administered and the reduction of the ovarian weight increase (Lugaro *et al.*, 1973) and no data on the use of this type of detection system as a quantitative assay for inhibin activity have been reported (Baker *et al.*, 1981).

Table 2.1 Precision of *in vivo* bioassay methods used to detect inhibin activity in whole animals

METHOD	PRECISION[a] INDEX (λ)	REFERENCES
Indirect *in vivo* bioassays (ED_{50}>70)[b] :		
- parabiosis of rats	-	Martins & Rocha (1931)
	-	Fachini *et al.* (1963)
	-	Lugaro *et al.* (1973)
- reversed Steelman-Pohley assay		
uterine weight in rat	-	Setchell & Sirinathsinghji (1972)
uterine weight in mouse	-	Ramasharma *et al.* (1979)
	0.104	Murthy *et al.* (1979)
ovarian weight in rat	0.21	Chari *et al.* (1976)
ovarian weight in mouse	-	Sheth *et al.* (1979)
- suppression of compensatory testicular hypertrophy	-	Hopkinson *et al.* (1977)
Direct *in vivo* bioassays (ED_{50}>2)[b] :		
- suppression of FSH in small animals: castrated mouse	-	Hudson *et al.* (1979)
normal rat	2.77	Hudson *et al.* (1979)
castrated rat	-	Nandini *et al.* (1976)
	1.37	Hudson *et al.* (1979)
normal hamster	-	Chappel *et al.* (1979)
- suppression of FSH in large animals: castrated monkey	-	Channing *et al.* (1979)
castrated sheep	-	Keogh *et al.* (1976)
castrated horse	-	Miller *et al.* (1979)

a: λ = precision index defined as the combined standard deviation divided by the slope of the dose response line (Finney, 1964)

b: ED_{50} is defined as the dose resulting in half maximal suppression, expressed in units of inhibin activity as defined by Eddie *et al.* (1979). Data from Hudson *et al.* (1979)

Another method for monitoring changes in the secretion of pituitary FSH uses the involvement of FSH in the human-chorionic gonadotrophin (hCG)-induced augmentation of ovarian weight in immature female rats (Steelman & Pohley, 1953). The reversed "Steelman-Pohley assay" has been widely used in *in vivo* bioassay systems for detection of inhibin activity. In this way, Setchell and Sirinathsinghji (1972), Setchell and Wallace (1972) and Chari *et al.* (1976) have shown that respectively rete testis fluid and follicular fluid have an inhibitory effect on hCG-induced ovarian weight gain suggesting that these fluids contain a substance which inhibits endogenous secretion of FSH, or the action of FSH on the ovary. This assay system provides an indirect evidence of inhibin activity, and shows a poor reproducibility as concluded from the an acceptable high values reported for the precision index λ (Table 2.1). Other groups have found these methods unreliable and difficult to reproduce (Davies *et al.*, 1976; Setchell *et al.*, 1977; Hudson et al, 1979; Franchimont *et al.*, 1979b). This may be due to differences in age of the experimental animals, timing of the injections and the dosage of hCG (Davies *et al.*, 1978). Preparations containing inhibin activity as measured by other methods have been inactive in the ovarian weight assay (Hudson *et al.*, 1979; de Jong *et al.*, 1979a). The presence of FSH-binding inhibitors in gonadal extracts or fluids (Fletcher *et al.*, 1982; Reichert *et al.*, 1982; Sluss & Reichert, 1983; Kalra & Zarabi, 1984; Krishnan *et al.*, 1986) may interfere with the binding of FSH to its receptor in the gonad. Thus, the use of the indirect assay for inhibin activity is limited by its doubtful specificity, and unsatisfactory sensitivity and precision. Reports in which inhibin activity was detected using such indirect *in vivo* methods should be interpreted cautiously. Other investigators have used *in vivo* methods for the detection of inhibin activity by studying the effects on gonadal function after injection of inhibin containing preparations to intact or hemi-orchiectomized, immature or mature, male or female animals. Hopkinson *et al.* (1977) was able to prevent compensatory hypertrophy of the remaining testis after hemicastration in rats by chronic injection of testicular or ovarian extracts. De Jong *et al.* (1978) injected bovine follicular fluid (bFF) into immature male rats for 12 days. In these animals plasma FSH levels were suppressed only during the first four days of treatment and spermatogenesis developed normally but with a slight delay. Prolonged administration of inhibin containing fractions showed also no effect on testicular weight or histology in mature rats (Davies *et al.*, 1979; Baker *et al.*, 1981) and rams (Baker *et al.*, 1981). These discrepant observations may reflect that suppression of FSH by inhibin becomes evident within a few hours after injection, but thereafter levels of peripheral FSH may rebound to levels which are equal to or higher than those in control rats (Nandini *et al.*, 1976; de Jong *et al.*, 1978; Hermans, 1982).

In the direct *in vivo* methods for assessing inhibin activity, reductions of peripheral FSH levels have been measured after injection of inhibin containing preparations. The relatively largest suppression of FSH was observed after injection of inhibin containing preparations into acutely castrated immature male or adult female rats (Davies *et al.*, 1976; Nandini *et al.*, 1976; Franchimont *et al.*, 1979b; de Jong *et al.*, 1978, 1979c), while Davies *et al.* (1976) also reported a suppression of LH when high dosages of rete testis fluid were applied. This kind of *in vivo* bioassay appears to be limited too, as it is poor in precision (Table 2.1) and

specificity. However, injections of inhibin material into large animals such as sheep, cows and monkeys yield more reproducible and dose dependent results (Hudson *et al.*, 1979; review Baker *et al.*, 1981), while the decreased levels of FSH result in effects on the timing of oestrus or number of ova in sheep (Miller *et al.*, 1979). However, large animals are impractical for routine assay of inhibin because large quantities of test material are required. In addition, these detection methods for inhibin have the disadvantage that injections of crude preparations of inhibin from the one species into another species may induce immune responses against the foreign material (Baker *et al.*, 1981; Channing *et al.*, 1982). The specificity of these types of assays is also doubtful because of the possible presence of other factors, such as gonadal FSH-releasing protein or "activin" (Ling *et al.*, 1986; Vale *et al.*, 1986) which may interfere. Although crude or partly purified inhibin preparations may contain such factors it is not clear if activins play a role in the regulation of pituitary function *in vivo*.

2.3.2 *In vitro* bioassay methods

In vitro bioassays for the detection and estimation of inhibin activity make use of cultures of dispersed anterior pituitary cells or short term incubations of whole or halved pituitary glands (Table 2.2). The latter system is less sensitive, possibly because of the very short time of exposure to inhibin containing preparations (Hudson *et al.*, 1979) or a slow diffusion of the active material into the tissue fragments.
In general, *in vitro* assay methods are more sensitive, and more specific, and in the case of cultures of dispersed pituitary cells, a smaller number of experimental animals is used when compared with *in vivo* methods (section 2.3.1). Inhibin activity can be detected by a direct suppression of the production of FSH in and the secretion of FSH from the pituitary cells in culture. A variety of procedures has been used, particularly with respect to the age, sex and species (rat, hamster, rabbit, sheep) of the animals from which the pituitary glands were taken, concentration of cells, composition of culture medium, culture temperature and time of exposure to the test material.
Moreover, several endpoints for the measurement of inhibin activity have been used: suppression of basal secretion of FSH or reduction of the cellular content of FSH as well as inhibition of LHRH-stimulated secretion of FSH (Table 2.2) can indicate the amount of inhibin activity added. Little or no effects on basal secretion or cell content of LH are observed, while in assays involving LH-RH stimulation both FSH and LH are affected. Under the latter conditions dose-response curves are steeper and a better precision index can be obtained when compared with assays using spontaneous release of FSH as an endpoint (de Jong *et al.*, 1979b). In general, a disadvantage of *in vitro* assay systems based on suppression of FSH release or cell content, is the possibility of *non* specific effects which may influence cell viability and metabolism. Therefore, assessment of the specificity of the response observed in this type of bioassay is important. This can be checked by parallelism of dose-response curves of standards and unknown preparations, by measurement of LH and

Table 2.2 Sensitivity (ED_{50}) and precision index of *in vitro* bioassay methods used to detect inhibin activity, based on suppression of FSH in *in vitro* systems with different endpoints.

METHOD	ED_{50}[b]	PRECISION[a] INDEX (λ)	REFERENCES
Incubation of hemipituitary glands:			
- basal FSH release	-	-	Jenner *et al.* (1982)
- LHRH-stimulated FSH release	-	-	Davies *et al.* (1978)
	1.0	0.901	Hudson *et al.* (1979)
Dispersed pituitary cells in culture:			
- *rat*: basal FSH release	0.5	-	Hudson *et al.* (1979)
	0.48	0.09	Robertson *et al.*(1986a)
	-	-	Steinberger & Steinberger(1976)
	-	0.17-0.22	de Jong *et al.* (1979b)
	-	-	Franchimont *et al.* (1979b)
LHRH-stimulated FSH release	-	-	Labrie *et al.* (1978)
	-	0.026-0.28	Eddie *et al.* (1979)
	-	0.048	Hudson *et al.* (1979)
	-	0.07-0.11	de Jong *et al.* (1979b)
	-	0.051-0.120	Scott *et al.* (1980)
	0.65	0.12	Robertson *et al.* (1986a)
FSH cell content	1.2	0.032-0.098	Scott *et al.* (1980)
	0.85	0.14	Robertson *et al.* (1986a)
FSH cell content following LH-RH stimulation	-	0.09	Robertson *et al.* (1986a)
- *hamster*: LHRH-stimulated FSH release	-	-	Chappel *et al.* (1979)
- *rabbit*: basal FSH release	-	-	Goodman (1984)
- *ovine*: basal FSH release	-	-	Huang & Miller (1984)
	0.102	0.076	Tsonis *et al.* (1986)

a: λ = precision index defined as the combined standard deviation divided by the slope of the dose response line (Finney, 1964)

b: ED_{50} is defined as the dose resulting in half maximal suppression, expressed in units of inhibin activity as defined by Eddie *et al.* (1979). Data from Hudson *et al.* (1979)

by assessing the morphological appearance of the pituitary cells in culture (de Jong *et al.*, 1979b; Scott *et al.*, 1980; Baker *et al.*, 1981). To ensure the specificity of the assay Robertson *et al.* (1982) and Scott *et al.* (1982) used the ^{51}Cr cytotoxicity test: the ^{51}Cr release procedure appeared to be more sensitive and specific in assessing cytotoxicity than either morphological evaluation or LH measurements. These authors found parallelism under most conditions but reported that human seminal plasma is highly toxic to pituitary cells in culture. In contrast, preparations of ovine testicular lymph, rat testis and kidney extracts, human peripheral plasma and bovine follicular fluid caused no morphological aberrations and did not affect LH cell content or ^{51}Cr release patterns. A suppression of FSH, consistent with the presence of inhibin was observed with ovine testicular lymph, rat testis extracts and bovine follicular fluid (Robertson *et al.*, 1982). Franchimont *et al.* (1979b) measured prolactin and thyroid stimulating hormone (TSH) in the culture medium as a check on *non* specific toxic effects on the pituitary cells.
In the investigations presented in this thesis inhibin activity was measured using cultures of dispersed rat pituitary cells according to the method described by de Jong *et al.* (1979c), slightly modified by Hermans *et al.* (1982). In this *in vitro* bioassay system the suppression of the secretion of FSH from *non* stimulated pituitary cells obtained from adult male rats is used to detect inhibin activity. The release of LH is not affected systematically by addition of inhibin containing material. In general, samples were assayed at various dose levels to compare the FSH suppression curve with that caused by addition of three different doses of charcoal treated bovine follicular fluid with the arbitrary potency of 1 unit/μg protein. Potencies of the samples were calculated using statistics for parallel line assay (Finney, 1964). In this way a satisfactory precision index (λ) can be obtained (mean value for all assays 0.13; see Materials & Methods sections of *appendix* papers 1,2,3 and 4).

2.3.3 Radioligand assays

In order to find more practical methods for measuring inhibin levels in biological fluids, inhibin assays have been described which are based on the use of inhibin binding principles and radioactively labelled ligands.
Radioimmunoassays have been developed, based on the use of antibodies raised against (partly) purified inhibin-like material from various sources (human seminal plasma inhibin: Frachimont *et al.*, 1977; Sheth *et al.*, 1978; Vaze *et al.*, 1979; bovine follicular fluid: de Jong *et al.*, 1983b; McLachlan *et al.*, 1986a). Other authors have described the use of radioreceptor assays, based on the binding of radioactively labelled inhibin-like preparations to putative inhibin receptors on pituitary cell membranes (Sairam *et al.*, 1981b; Steinberger *et al.*, 1982; Seethalakshimi *et al.*, 1984).
No data on the specificity of radioimmunological methods in studies performed with seminal plasma inhibin have been published (Franchimont *et al.*, 1977; Sheth *et al.*, 1978; Vaze *et al.*, 1979), and no comparisons with validated bioassays were shown. In contrast, correlations between bio- and immunopotencies of several inhibin containing preparations were found

by de Jong *et al.* (1983b) and McLachlan *et al.* (1986a), who used antibodies raised against inhibin activity from partly purified and purified fractions of bovine follicular fluid, respectively.
Recently, antisera raised against synthetic N-terminal amino acid sequences of the trunkated α-chain of inhibin (see section 2.4) have been produced (Rivier *et al.*, 1985, 1986; Bicsak *et al.*, 1986, 1987). The specificity of assays, using these antibodies, in terms of cross-reaction with loose α-subunits of inhibin has not yet been evaluated completely. Finally, monoclonal antibodies against inhibin have been described (Lee *et al.*, 1986; Miyamoto *et al.*, 1986; de Jong *et al.*, 1988).
Antibodies raised against partly purified ovarian inhibin containing preparations were shown to neutralize inhibin activity *in vivo* and *in vitro* (Channing *et al.*, 1982; de Jong *et al.*, 1984). Van Dijk *et al.* (1986) showed that such a conventional antiserum was more effective in neutralizing inhibin activity from ovarian than from testicular sources suggesting a sex related difference in the inhibin molecule within a species. The presence of antibodies against other active principles from follicular fluid cannot be excluded and may interfere in such assays especially in view of the existence of biologically active dimers of the β-subunit of inhibin in ovarian follicular fluid (Ling *et al.*, 1986; Vale *et al.*, 1986). Up until now only very few data on bio/immuno-ratios of inhibins from various sources have been published (McLachlan *et al.*, 1986b).

2.3.4 Concluding remarks on inhibin assays

The use of the enzyme dispersed pituitary cell system for inhibin detection is to be preferred above *in vivo* methods because of a relatively high specificity and sensitivity. In addition, a large number of samples with a wide range of doses can be tested within one *in vitro* bioassay. However, this method is relatively labour intensive and not sensitive enough to measure inhibin levels in peripheral plasma samples. Recently, the development of a more sensitive ovine pituitary cell culture system was reported (Tsonis *et al.*, 1986). Using this system peripheral levels of inhibin could be estimated. It is likely that more sensitive, reliable and well characterized radioimmunoassay systems for the estimation of inhibin will become available within the near future because inhibin has recently been isolated and characterized (section 2.4.3).

2.4 Sources and nature of inhibin

Inhibin activity has been detected in a number of preparations of male and female origin using various assay systems for inhibin. During more recent years attempts to purity and characterize inhibin have been focused mainly on material from testes, from seminal plasma and from ovarian follicular fluid as shown in Table 2.3 (sections 2.4.1, 2.4.2 and 2.4.3). Finally, inhibin activity has been found in placental tissue (section 2.4.4).

Table 2.3 Reported molecular weights of inhibin from various sources of male and female origin

SOURCE	SPECIES	MOLECULAR WEIGHT(S)	REFERENCES
male:			
testis	human	"low"	Krishnan *et al.* (1982)
	bovine	10-70	Baker *et al.* (1976)
	ovine	<1.5	Vijayalakshmi *et al.* (1980)
		20	Moudgal *et al.* (1984)
	rat	50-60	Au *et al.* (1983)
rete testis fluid	ovine	<5; 10-20	Davies *et al.* (1976,1978) Franchimont *et al.* (1978) Baker *et al.* (1976)
		>100	Cahoreau *et al.* (1979)
testicular lymph	ovine	"low"	Baker *et al.* (1976)
testicular lymph after EDL[a]	ovine	<5;20;90	Davies *et al.* (1978)
spermatic vein	bovine	-	Fachini & Ciaccolini (1966)
spermatozoa	bovine	"small"	Fachini *et al.* (1963) Lugaro *et al.* (1974)
seminal plasma	human	5	Franchimont *et al.* (1978)
		19	Sheth *et al.* (1984)
		"low"	Krishnan *et al.* (1982)
		4-10	Ramasharma *et al.* (1984) Seidah *et al.* (1984a,b)
		18	Thakur *et al.* (1981)
		5-10;30;90	Colberg *et al.* (1982)
epididymal homogenates	rat	-	Le Lannou & Chambon (1977)

(continuing Table 2.3)

SOURCE	SPECIES	MOLECULAR WEIGHT(S)	REFERENCES
female:			
ovarian follicular fluid	human	23	Chari *et al.* (1979)
	bovine	32;55;96	Fukuda *et al.* (1986)
		55	Robertson *et al.* (1985)
		31	Robertson *et al.* (1986b)
		65	de Jong *et al.* (1982a)
			van Dijk (1986)
		32;55;65;	Miyamoto *et al.* (1986)
		108;120	Miyamoto *et al.* (1986)
	ovine	<1.5	Vijayalakshmi *et al.* (1980)
		80	Dobos *et al.* (1983)
	porcine	10-12	Rivier *et al.* (1984)
		10-35	Williams *et al.* (1979)
		10-30;43	Sairam *et al.* (1984)
		32	Ling *et al.* (1985)
			Rivier *et al.* (1985)
		32;55;80;	Miyamoto *et al.* (1985)
		100	Miyamoto *et al.* (1985)
		194	Ward *et al.* (1983)

a:EDL = efferent duct ligation

2.4.1 Testicular inhibin

Mottram and Cramer (1923) were the first to suggest that the seminiferous epithelium was able to produce a specific "inhibin-like" hormone, but direct evidence for the site of production of this hormone in the testis came from experiments of Steinberger and Steinberger (1976), who found FSH-suppressing activity in culture medium of isolated rat

Sertoli cells and reported on the association of this activity with a > 12 kDa protein. Earlier data suggested that extracts of spermatozoa (Fachini *et al.*, 1963; Lugaro *et al.*, 1974) may contain inhibin-like activity since such preparations were able to lower plasma FSH using *in vivo* bioassay systems. Other investigators have reported that spermatids are the most likely source of inhibin activity (Franchimont *et al.*, 1972), while experiments performed by Krueger *et al.* (1974) implicated that Sertoli cells and/or spermatogonia are possible sources of testicular inhibin. However, both clinical (see review Setchell *et al.*, 1977) and experimental (de Jong & Sharpe, 1977b; *appendix* paper 2) evidence was obtained against spermatogenic cells as the source of inhibin.
The production of an inhibin-like activity by Sertoli cells was confirmed by several groups (de Jong *et al.*, 1978; Labrie *et al.*, 1978; Le Gac & de Kretser, 1982; Verhoeven & Franchimont, 1983; Bicsak *et al.*, 1987), using different experimental protocols (see chapter 4).
Furthermore, relatively high concentrations of inhibin activity have been detected in rete testis fluid, testicular lymph and homogenates of testicular tissue of different species. A wide range of molecular weights has been reported for testicular inhibin from various sources, probably due to differences in the isolation and assay procedures (Table 2.3). Finally, purification and characterization of testicular inhibin has not yet been achieved completely.

2.4.2 Seminal plasma and prostate inhibin

Several authors have described inhibin-like activity in seminal plasma preparations of human or bovine origin (see Table 2.3). However, Robertson *et al.* (1982) and Scott *et al.* (1982) have shown that seminal plasma proteins cause cytotoxic effects when added to pituitary cells in *in vitro* bioassay systems. After removal of low molecular weight peptides normally present in seminal plasma, inhibin activity was detected in a similar bioassay system and an inverse relationship between levels of seminal plasma inhibin and peripheral FSH was observed in normal and infertile men (Scott *et al.*, 1980; Scott & Burger, 1981). Purification of inhibin-like activity from seminal plasma revealed that it was associated with a protein with a molecular mass of 19 kDa (Chari *et al.*, 1978). Mohapatra *et al.* (1985) found two peptides of 18 and 20 kDa with inhibin-like activity using *in vivo* bioassay system: the 18 kDa protein was able to suppress the postcastration rise of FSH in castrated male and hemicastrated female rats, while the 20 kDa protein prevented binding of FSH to its ovarian receptor. Sairam *et al.* (1981a) also reported the purification of a 18 kDa protein from bovine seminal plasma using the *in vivo* suppression of peripheral FSH. Similar results were reported for human seminal plasma (Vaze *et al.*, 1979; Thakur *et al.*, 1981). A radioimmunoassay for human seminal plasma inhibin has been described using the latter material as immunogen and label (Vaze *et al.*, 1979). Small molecular weight inhibins have been reported to be present in seminal plasma by Ying *et al.* (1981), while Lugaro *et al.* (1984) found 1 kDa proteins with inhibin-like activity in bovine seminal plasma . The latter authors indicate that this material may also act as FSH-binding inhibitor.

Ramasharma *et al.* (1984) and Seidah *et al.* (1984a) reported on a 31 amino acid peptide which was purified from human seminal plasma and exhibited inhibin-like activity *in vivo* and *in vitro*. A synthetic replicate of this 31 amino acid sequence (α-inhibin) was prepared which also showed biological activity *in vitro*: the synthetic peptide inhibited the release of FSH from incubated pituitary glands after addition of LH-RH without affecting LH under the same conditions. However, de Jong *et al.* (1985) reported that this peptide does not suppress the basal release of FSH from dispersed pituitary cells in culture. Bioactivity of this preparation was also tested by the group of Liu *et al.* (1985) using whole pituitaries from 25-day-old male rats: no significant suppression of basal FSH and LH release was observed, while a significant increase of LH release in the presence of LH-RH was found. After injection of this synthetic preparation in test animals also no significant effects were observed by Liu *et al.* (1985).
Furthermore, the presence of another protein with inhibin activity in human seminal plasma has been postulated by Seidah *et al.* (1984b); this 94 amino acid peptide was named β-inhibin. Johansson *et al.* (1984) described the sequence of a peptide identical to β-inhibin and observed that this structure is almost identical to that of a human sperm-coating antigen, which was reported to be produced in prostatic tissue. Partial homology was also found with PDC-109, a peptide of prostatic origin which showed FSH-suppressing activity at high concentrations (Esch *et al.*, 1983). Moreover, it has been shown that PDC-109 has structural similarities with bovine fibronectin and human plasminogen activator (Baker, 1985b). Finally, de Jong & Robertson (1985) suggested that a 48 amino acid peptide of prostatic origin, seminalplasmin, which inhibits the synthesis of mRNA and protein in prokaryotic cells and hepatoma cells (Shivaji, 1984) could play a role in the presumed presence of inhibin in seminal plasma.
In conclusion, seminal plasma preparations apparently show "inhibin-like" properties in a number of less well characterized assay systems. However, according to the most recent definition (section 2.1), this seminal plasma inhibin-like material, which is of prostatic origin (Sheth *et al.*, 1984;Docter *et al.*, 1986), cannot be considered to be inhibin proper. This also casts doubt in reports on the presence of inhibin in gastric juice (Sheth *et al.*, 1982), and in placental tissue (section 2.4.4), which were detected using antibodies against seminal plasma preparations with inhibin-like activity.

2.4.3 Ovarian inhibin

It has been shown by Erickson and Hsueh (1978) that bioactive inhibin is produced and secreted by cultured granulosa cells. Furthermore, granulosa cells obtained from adult female rats have and retain the capacity to secrete inhibin activity under basal conditions in long term cultures (Hermans *et al.*, 1982). Information on the regulation of ovarian inhibin production came from *in vivo* and *in vitro* studies. Androgens stimulated granulosa cell inhibin production, while progesterone inhibited inhibin production and oestrogens had no effect (Henderson & Franchimont, 1981, 1983). Granulosa cells isolated from human

ovarian follicles also secrete inhibin activity in culture and respond to LH and FSH with enhanced inhibin secretion (Channing *et al.*, 1984). Stimulatory effects of gonadotrophins on inhibin production by granulosa cells in culture have also been reported by Henderson *et al.* (1984), while Sander *et al.* (1984) reported no change of inhibin production by granulosa cells in medium with or without 10% fetal calf serum (FCS) in the presence or absence of FSH. Recently, Bicsak *et al.* (1986) showed that granulosa cell inhibin secretion can be stimulated by FSH and LH, but not by prolactin. Lee *et al.* (1982) demonstrated that ovarian and circulating levels of inhibin activity in immature female rats can be increased by pregnant mare serum gonadotrophin (PMSG) or FSH treatment. The presence of inhibin activity in steroid-free ovarian homogenates from cyclic rats has been published by Sander *et al.* (1985), who also observed that ovaries from 13-day-old rats did not contain detectable amounts of inhibin.

Because of the relatively high amount of inhibin activity present in ovarian follicular fluid (de Jong & Sharpe, 1976), many investigators have attempted to purify and characterize inhibin activity from follicular fluid of several species using both *in vivo* and *in vitro* bioassay systems (Table 2.3). Jansen *et al.* (1981) demonstrated an apparent molecular mass of 65 kDa for inhibin activity from bovine follicular fluid.

Recently, much progress on the purification and elucidation of the structure of ovarian inhibin has been made. Robertson *et al.* (1985, 1986b) described the purification of inhibin from bovine follicular fluid and reported a molecular mass of 58 kDa. After reduction this molecule dissociated into two subunits of 43 and 15 kDa, presently called the α- and β-subunit of inhibin. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis the major inhibin activity in bovine follicular fluid appeared to be associated with 32 and 55 kDa molecular mass proteins (Fukuda *et al.*, 1986). The N-terminal amino acid sequence of the 32 kDa bovine follicular fluid inhibin showed homology with the 32 kDa porcine follicular fluid inhibin published by Miyamoto *et al.* (1985). These authors also demonstrated a dissociation of this protein into a 20 (α) and 13 (β) kDa subunit after reduction. A similar subunit structure of the 32 kDa inhibin from porcine follicular fluid was observed by Rivier *et al.* (1985) and Ling *et al.* (1985). Development of monoclonal antibodies which specifically recognize the 20 and 13 kDa subunits of the 32 kDa bovine follicular fluid inhibin by Miyamoto *et al.* (1986) demonstrated that in bovine follicular fluid at least six inhibin forms with apparant molecular mass of 120, 108, 88, 65, 55 and 32 kDa are present. These authors indicated that the 65, 55 and 32 kDa inhibin forms consist of two polypeptide subunits linked by disulphide bridge(s), while a three-subunit model was proposed to explain the high molecular mass forms of inhibin. Several proteolytic cleavage sites may yield multiple inhibin forms. A schematic model of the inhibin molecule is presented in Fig. 2.1.

A relatively large form of inhibin has been described by van Dijk (1986), who found a 68 kDa protein which correlated with biological activity in preparations from bovine or human follicular fluid. After an acid treatment also a 32 kDa form of inhibin was obtained without marked loss of biological activities, while the difference between the 58 kDa (Robertson *et al.*, 1985) and 68 kDa (van Dijk, 1986) has been proposed to be due to a difference in proteolytic cleavage of the α-subunit of inhibin (van Dijk, 1986).

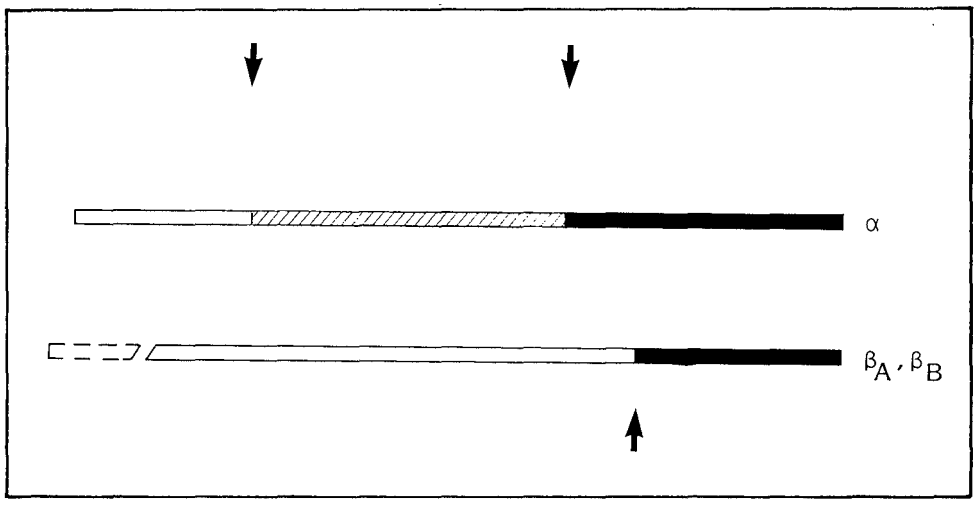

Fig. 2.1 Schematic diagram of the subunit structure of ovarian inhibin.
Black area: purified and partially sequenced subunit
Hatched area: N-terminal extension of the α-subunit
Dashed area: postulated N-terminal extension of the prepro-hormone
White area: extension of subunits, present in their proforms
Arrows: possible cleavage sites
(data from Forage et al., 1986 and Robertson et al., 1986b)

Recently, nucleotide sequences of the cDNAs derived from mRNAs which contain the coding region(s) for the inhibin subunits have been published (Mason *et al.*, 1985, 1986; Forage *et al.*, 1986). From these data, a 30% homology between the amino acid sequence of the β-chain and the C-terminal part of the α-chain becomes apparent. Mason *et al.* (1985) also described that mRNAs for two different β- subunits of inhibin β_A and β_B which are present in the ovary. Davis *et al.* (1986) demonstrated that the mRNA for the α-subunit of inhibin is present in the ovaries of immature female rats and the levels of this mRNA were increased following treatment with PMSG. Similar results were obtained by Mayo *et al.* (1986), who injected pigs with PMSG. Finally, Davis *et al.* (1986) also detected significant levels of inhibin mRNA in the corpora lutea of mature female rats.

2.4.4 Placental inhibin

Inhibin-like activity has been detected in homogenates of human placenta with an *in vivo* bioassay using the suppressive effect on peripheral FSH in castrated, adult male rats (Bandivdekar *et al.*, 1981). Recently, the presence of the mRNA for the α-subunit of inhibin

has been detected in human placenta (Mayo *et al.*, 1986), although Davis *et al.* (1986) could not obtain such results with rat material. Further supporting evidence of the presence of inhibin in placenta came from McLachlan *et al.* (1986b), who demonstrated a dose-dependent suppression of pituitary FSH cell content in an *in vitro* bioassay after addition of human placental extracts. This suppression parallelled that obtained with human follicular fluid. This bioactivity could be neutralized by adding antibodies against ovarian inhibin. McLachlan *et al.* (1986b) also showed the presence of immunoreactive inhibin in placental extracts with a radioimmunoassay. However, the displacement of tracer was not parallel to that caused by human follicular fluid; on basis of these data the authors suggested that differences may exist between placental and ovarian inhibin.

2.4.5 Inhibin related proteins

After elucidation of the molecular structure of inhibin, it became apparent that a number of other proteins which play a role in the regulation of growth or differentiation have a molecular structure related to the β-subunit of inhibin (Massagué, 1987). These include transforming growth factor-β (TGF-β, Mason *et al.*, 1985), a 25 kDa disulfide-linked homodimer originally found in transformed fibroblasts, activin (Ling *et al.*, 1986; Vale *et al.*, 1986), Müllerian inhibiting substance (MIS, Cate *et al.*, 1986), a glycoprotein which causes regression of the Müllerian duct during development in the male embryo, Erythroid Differentiation Factor (EDF), as found in human leukemia cells in culture (Eto *et al.*, 1987), and the decapentaplegic gene complex (DPP-C) transcript from Drosophila (Padgett *et al.*, 1987).
The *in vivo* significance of these proteins is still unknown, except for the role of MIS. However, some of these peptides can modulate gonadal or pituitary function under *in vitro* conditions as described below.
TGF-β is a homodimeric protein. The messenger RNA coding for the subunit precursor is synthesized in various normal and transformed cells (Derynck *et al.*, 1985). The amount of TGF-β mRNA appears to be correlated with the degree of mitotic activity and TGF-β can stimulate cell proliferation or inhibit the mitotic divisions of a particular cell type depending on the culture conditions or the presence of additional growth factors. In addition, TGF-β has been shown to stimulate the basal secretion of FSH by cultured pituitary cells (Ying *et al.*, 1986a), while inhibin from porcine follicular fluid antagonizes this activity of TGF-β. Furthermore, Ying *et al.* (1986b) observed that inhibin and TGF-β have inhibiting and stimulating effects, respectively, on the FSH-mediated oestrogen biosynthesis in a rat granulosa cell culture system. Recently, Dodson & Schomberg (1987) have shown that TGF-β can enhance FSH-stimulated LH receptor induction and progesterone production by cultured granulosa cells in a dose-dependent manner. Their data also suggest that the enhancement of FSH-stimulated differentiation by TGF-β occurs distal to cAMP generation. Knecht *et al.* (1986) have studied the effects of TGF-β on FSH-stimulated LH receptor induction in more detail and demonstrated that TGF-β enhanced the stimulatory

actions of low levels of gonadotrophin on granulosa cells in culture 2-3 fold and inhibited the induction of LH receptors at higher levels of FSH by a similar factor.
During the purification of inhibin from porcine follicular fluid, also fractions which could stimulate the secretion of FSH by cultured pituitary cells have been detected and purified (Vale *et al.*, 1986; Ling *et al.*, 1986). This protein appears to be a homodimer of the β_A-subunit of inhibin (Vale *et al.*, 1986) or a heterodimer of the two forms of the β-subunit as reported by Ling *et al.* (1986). It has been suggested that the various combinations of the translational products of the inhibin genes may have autocrine, paracrine and endocrine roles in ovarian development (Vale *et al.*, 1986; Ling *et al.*, 1986; Dodson & Schomberg, 1987).
Bovine and human MIS showed marked homology with human TGF-β and the β chain of porcine inhibin (Cate *et al.*, 1986). Low amounts of MIS are released into follicular fluid by mature granulosa cells, while Sertoli cells produce MIS not only during the period when Müllerian ducts regress in the male foetus but also during late pregnancy, after birth and even, albeit at a strongly reduced rate, in adulthood (Picard *et al.*, 1986), while MIS and TGF-β have similar growth-inhibiting properties (Cate *et al.*, 1986). Summarizing, the β-subunit of inhibin could represent an important regulatory protein whereas the biological specificity might be attributed to the α chain (Mason *et al.*, 1985). It has been suggested that these dimeric proteins are products of a single gene family with important regulatory functions in the pituitary-gonadal axis (Mason *et al.*, 1985; Cate *et al.*, 1986; Vale *et al.*, 1986; Ying *et al.*, 1986 a,b). These observations suggest that there is some analogy between the subunit structure of inhibin and those of the pituitary glycoproteins (see section 1.2).

2.4.6 Concluding remarks on the nature of inhibin

Many of the earlier reports dealt with male inhibin derived from seminal plasma or testicular extracts, but at present faster progress has been made in the purification and characterization of ovarian inhibin (bovine: Robertson *et al.*, 1985, 1986b; Fukuda *et al.*, 1986; porcine: Rivier *et al.* 1984, 1985, Ling *et al.*, 1985, Miyamoto *et al.*, 1985). From the partial amino acid sequences of inhibin, cloning and DNA sequencing techniques, the structure of ovarian inhibin was elucidated as a glycoprotein consisting of two dissimilar disulphide-linked subunits α and β. The subunits are synthesized on separate genes and levels of the mRNA for the α-subunit of inhibin can be increased by treatment of the animal with gonadotrophins (Davis *et al.*, 1986; Mayo *et al.*, 1986).
It remains to be determined whether the structure of testicular inhibin corresponds with that of ovarian inhibin. Data on the molecular mass of testicular inhibin from ovine rete testis fluid (30 kDa, Baker *et al.*, 1982) and from rat testicular homogenates (50-60 kDa, Au *et al.*, 1983) are in close agreement with data on ovarian inhibin. There are several indications which make it unlikely that the seminal plasma inhibins are related with gonadal inhibin. In addition, since plasma FSH levels in male and female rats are suppressed after injection of inhibin containing preparations of both testicular or ovarian origin (see section

2.3), it is likely that ovarian and testicular inhibin have similar biological activities. However, immunoneutralization of inhibin activity by an antiserum against follicular fluid inhibin in an *in vitro* bioassay system is more effective than when testicular preparations of the same species are added to the system (van Dijk *et al.*, 1986). This phenomenon may be caused both by differences in male and female inhibin or by interference of inhibin-related proteins, which may antagonize inhibin action in the inhibin test system or prevent binding of bioactive inhibin to the antiserum and which may be present in male and female gonadal fluids in different amounts. Further elucidation of the structure of ovarian and testicular inhibin will indicate whether there are differences in the structure and/or processing of male and female inhibins.

3

TESTICULAR CONCENTRATIONS OF INHIBIN IN VIVO

3.1 Introduction

Steinberger & Steinberger (1976) were the first who actually showed that Sertoli cells isolated from testicular tissue are capable of producing an inhibin-like material. More recent studies have confirmed this observation (de Jong *et al.*, 1978; Labrie *et al.*, 1978; Le Gac & de Kretser, 1982; Verhoeven & Franchimont, 1983; *appendix* paper 2 and chapter 4).
There is speculation about the route by which inhibin leaves the testis, since inhibin activity can be detected in rete testis fluid (Setchell & Sirinathsinghji, 1972; Setchell & Jacks, 1974; Baker *et al.*, 1976), testicular lymph (Hudson *et al.*, 1979) seminal plasma (Franchimont *et al.*, 1975; Chari *et al.*, 1978; Davies *et al.*, 1978) and sperm extracts (Lugaro *et al.*, 1974). However, the recently purified seminal plasma inhibins demonstrate no structural or functional relationship with gonadal inhibin (section 2.4.2).
Direct information on the regulation of inhibin secretion by the testis *in vivo* is still lacking, because the currently well accepted bioassay systems are not sufficiently sensitive to detect inhibin activity in the peripheral circulation (see section 2.3). It is still not known how inhibin leaves the adult testis, but it is likely that inhibin can leave the testis freely with the testicular lymph or venous blood before the blood-testis barrier is formed (15-18 days of age: Vitale *et al.*, 1973). However, after this age junctional complexes are present between adjacent Sertoli cells and this physiological barrier will limit the transport from the seminiferous tubular lumen into the surrounding interstitial space (see review Waites & Gladwell, 1982).
In this chapter the regulation of inhibin production in the testis *in vivo* is discussed. Testicular inhibin concentrations at various age are compared. In addition, testicular inhibin contents were compared with corresponding levels of plasma FSH, LH and testosterone in various *in vivo* models.

3.2 Inhibin production in relation to the secretion of pituitary FSH after testicular damage

Earlier studies on testicular inhibin have shown that steroid-free extracts prepared from bovine (Keogh *et al.*, 1976; Nandini *et al.*, 1976), ovine (Sheth *et al.*, 1979) and rat (Davies *et al.*, 1978; Au *et al.*, 1983) testes contain inhibin-like activity. After injection of low doses of such preparations into immature or adult, castrated or cryptorchid animals, a selective suppression of pheripheral FSH was observed, *i.e.* without significant changes of LH secretion. However, *in vivo* (Lee *et al.*, 1976; de Jong *et al.*, 1983a) and *in vitro* (Eddie *et al.*, 1979; Scott & Burger, 1981; de Jong *et al.*, 1983b) both FSH and LH concentrations in peripheral plasma were suppressed when high doses of inhibin were applied.
Au *et al.* (1983) measured inhibin activity in testicular extracts of adult rats: an inverse relationship between testicular inhibin content and plasma FSH was observed after experimental cryptorchidism, whereas peripheral levels of LH were increased and testosterone levels in the circulation were unchanged. The changes of testicular levels of inhibin activity do not indicate a high inhibin secretion and in further studies of Au *et al.* (1984a,b) accumulation of inhibin in seminiferous tubules after unilateral efferent duct ligation (EDL) was measured as an index for testicular inhibin production *in vivo* (Au *et al.*, 1984a). The use of this technique appears to be attractive, although it also has some disadvantages. Firstly, it is rather difficult to perform this technique in young animals. Secondly, data have been published on the bidirectional release of ABP from Sertoli cells, which is under differential control (Gunsalus *et al.*, 1980). Since the EDL technique gives insight only in the amount of inhibin which is secreted into the tubular lumen, use of this model may lead to deceptive conclusions on regulation of inhibin secretion, especially because it is still not known which secretion route of inhibin is important for the regulation of pituitary FSH production.
In several clinical and experimental conditions a selective increase of FSH was found when seminiferous tubule function was disturbed. For example, FSH is increased in men or adult rats, who have damaged seminiferous tubules caused by cryptorchidism, X-irradiation, treatment with anti-spermatogenic drugs, or when seminiferous tubules contain only Sertoli cells (see review by Setchell, 1977; Main *et al.*, 1979; section 3.4). This increase of peripheral FSH is probably due to diminished secretory activity of the Sertoli cell. However, it is not clear if this impairment of Sertoli cell function is due to the absence of spermatogenic cells, to effects mediated by Leydig cells, or to direct effects on Sertoli cell function, which, in turn, can affect spermatogenesis.

3.3 Influence of age on inhibin production

FSH can stimulate testicular development, which starts during the perinatal period in rats (Orth, 1984; chapter 5). For this reason, it might be important to regulate FSH levels rather strictly during this period of life. Furthermore, compensatory hypertrophy after hemicastration only occurs in male rats when the operation is performed relatively early in life.

This phenomenon is thought to be due to increased levels of FSH (*appendix* paper 3) as a result of impaired feedback action by the remaining testis. Similarly, Hermans (1982) postulated that the contribution of inhibin to the regulation of pituitary FSH secretion in male rats may occur only during the prepubertal period, since the relative increase in peripheral FSH after castration decreases gradually at older ages whereas the pituitary responses to exogenous inhibin remain constant. Finally, de Jong & Sharpe (1977b) performed neonatal irradiation and found significantly increased levels of peripheral FSH at 21 days of age, while at all ages a decreased number of Sertoli cells was observed. The finding of similar responses to exogenous inhibin in animals of different ages suggests that the pituitary secretion of FSH is equally sensitive for exogenous inhibin activity at all ages. In contrast, Steinberger (1981) concluded from studies with cultured pituitary cells from rats that the pituitary FSH secretion is very sensitive to inhibin regulation after 33 days of age suggesting that less inhibin is needed to regulate FSH secretion from pituitary cells from older animals.

During development of male rats peripheral concentrations of FSH increase gradually from birth until 4 weeks of age and decrease thereafter (Swerdloff *et al.*, 1971). Since inhibin as well as steroids can be involved in the feedback regulation of pituitary secretion of FSH, and only small amounts of steroids are secreted by immature testis tissue (Swerdloff *et al.*, 1971), it is likely that inhibin in young animals may be more important in the regulation of peripheral FSH levels than in older rats, where increasing concentrations of steroids in blood may play a more important role in the regulation of pituitary FSH secretion (Swerdloff *et al.*, 1971; de Jong & Sharpe, 1977a).

Since it has been suggested that inhibin might play a role in the regulation of pituitary FSH secretion in immature, rather than in adult rats, it was of interest to investigate whether actual levels of inhibin in the testis change with age. In the absence of a well characterized immunological assay system for inhibin, peripheral levels of inhibin can not be measured directly, and physiological studies must rely on the use of specific bioassay methods (chapter 2.3). The approach used for the studies described in this thesis was based on the hypothesis that the amount of inhibin present in the testis may be a parameter for the rate of secretion of inhibin into the circulation.

The results of these estimations have been described in *appendix* paper 1: inhibin activity was already detectable in testes of 1-day-old rats and inhibin content increased with age parallel to the rise in testis weight. Similar results were obtained by Au *et al.* (1986) who measured inhibin levels in testes from rats of 1 to 80 days of age using an *in vitro* bioassay method with ovine testicular lymph as reference preparation. These authors also performed the EDL-method to measure the testicular inhibin production rate in animals with ages between 20 and 80 days, and showed a fast increase of inhibin accumulation between 30 and 70 days of age, after which the levels were sustained. The initial increase of the inhibin production by the testes occurred in parallel with the increase of peripheral FSH, while after day 30 a further increase of inhibin production was characterized by a decrease of peripheral FSH, while relatively low levels of peripheral testosterone were found (Au *et al.*, 1986). These observations will be discussed below together with the experiments presented in this thesis,

which indicate that the age profile of plasma FSH and testicular inhibin can be divided in three stages:

1) before day 21 of age: fast increase of plasma FSH and low amounts of inhibin in the small testis
2) between days 21 and 42 of age: no further rise of plasma FSH, while testicular inhibin content still increases
3) after day 42 of age: decrease of plasma FSH, while testicular inhibin rises further and peripheral testosterone concentrations increase.

The pattern of plasma FSH during development of the rat as observed in these studies agrees with data found earlier by Swerdloff *et al.* (1971) and de Jong & Sharpe (1977a), while significantly lower levels of FSH have been reported by Au *et al.* (1986), as a result of the use of a different rat FSH standard preparation (Au *et al.*, 1983).

Data on inhibin levels in the testis in relation to peripheral FSH concentrations have to be interpreted carefully, because several physiological changes occur throughout development as described in the introduction of this chapter and *appendix* paper 1. Changes of testicular concentrations of inhibin activity as measured in testicular homogenates (*appendix* paper 1) may be caused by the fact, that the weight of the testis initially increases during postnatal development by proliferation of both Sertoli cells and spermatogenic cells while later on only the relative proportion of spermatogenic cells within the tubules becomes larger. The Sertoli cell is the only known source of inhibin in the testis (see also chapter 4). This notion is supported by the observation, that less inhibin is found per mg testicular tissue at older ages. However, the total amount of inhibin present per testis increases with age (*appendix* paper 1). These data suggest that the production of inhibin is influenced by age as has been found earlier for some other Sertoli cell specific secretion products, such as androgen binding protein (ABP) (see chapter 4).

All above mentioned factors are important for interpretation of the results and have to be considered when speculating on the final peripheral concentrations of testicular inhibin which may reach the pituitary gland to regulate the FSH secretion. It is known that peripheral levels of testicular steroids increases after 21 days of age in the rat, as a result of higher levels of LH (de Jong & Sharpe, 1977a). No relationships between plasma LH, testosterone and testicular inhibin content were observed in this study (*appendix* paper 1). The increased peripheral levels of androgenic steroids at 42 and 63 days of age are likely to be responsible for the decrease in plasma FSH.

3.4 Inhibin concentrations in testicular tissue after manipulation of the testicular-pituitary axis

Various treatments resulting in spermatogenic damage cause a relative hypersecretion of FSH: e.g. irradiation (Rich & de Kretser, 1977; Wang *et al.*, 1983; Delic *et al.*, 1986), cytotoxic drugs (Debeljuk *et al.*, 1973; Mecklenburg *et al.*, 1975; Rich *et al.*, 1979), bilateral ligation of the efferent ducts or local heating (Main & Setchell, 1980), and vitamin A

deficiency (Huang *et al.*, 1983). These experimental models have been used to destroy the seminiferous epithelium selectively in studies on the control of FSH secretion from the pituitary gland by the seminiferous epithelium.
Experimental induction of bilateral cryptorchidism leads to a decrease of testicular inhibin content and decreased production of inhibin (Au *et al.*, 1983) as is also the case for ABP (Hagenäs *et al.*, 1976; Jégou *et al.*, 1983). This increase of concentrations of plasma FSH was accompanied by a decrease of testicular inhibin content, while testosterone levels were not measured (Au *et al.*, 1984b). Hence, it is not clear whether inhibin or rather inhibin together with testosterone is involved in the regulation of FSH secretion in these adult animals; Au *et al.* (1984a,b) concluded that testicular inhibin content does not correlate directly with peripheral FSH concentrations.
By this type of treatment both Leydig cell and seminiferous tubule function may be impaired severely for unknown periods. Delic *et al.* (1986) reported that after irradiation of adult rats Sertoli cell function remains damaged as indicated by reduced serum ABP concentrations and increased plasma FSH, while Leydig cell function recovered. Increased levels of FSH, which occur under these experimental conditions can be explained by invoking decreased peripheral inhibin concentrations after damage to Sertoli cells. Most of these studies describe experiments performed in adult animals,in which androgens may play a more important role than inhibin in the regulation of pituitary FSH secretion as suggested by de Jong & Sharpe(1977b) and Hermans *et al.*(1980). This suggestion of a tight coupling between FSH and inhibin in prepubertal animals is also supported by the observation that FSH can stimulate Sertoli cell function (Tindall, *et al.*, 1981) and testis growth in prepubertal animals but not in adult rats. Therefore, manipulation of the testicular-pituitary axis was performed early in life to study developmental changes of several aspects of this axis. Levels of inhibin in testis tissue of treated rats were examined and related with concentrations of peripheral FSH:

1. in rats after *in utero* irradiation in order to destroy spermatogenetic cells (Beaumont, 1960).
2. after removal of one testis on the day after birth (neonatal hemicastration)

In *appendix* paper 1 it is shown that in *in utero* irradiated or in early hemicastrated animals the amounts of inhibin per total testicular mass per rat increased gradually with age as was also found in control rats. Lowest concentrations of inhibin were found in testes from irradiated rats, which also showed lowest testis weights. A significantly decreased level of inhibin activity in testis tissue at young ages leads apparently to significantly increased levels of plasma FSH in the neonatal hemicastration model. At older ages a significantly decreased total testicular inhibin content can apparently exist together with normal plasma FSH values, while also significantly lower plasma testosterone concentrations were found (see *appendix* paper 1, Fig. 1a,b,c). On the other hand, in the prenatal irradiation model normal values of plasma testosterone were observed and the pubertal increase of plasma FSH coincided with increasing amounts of inhibin in the testes. Significantly increased peripheral

concentrations of FSH were found at 42 and 63 days of age. This suggests that the relatively low amounts of inhibin in the testis reflect a lower secretion rate of inhibin in this experimental model.

3.5 Conclusions

The levels of testicular inhibin increase with age during development in normal, hemicastrated or *in utero* irradiated male rats. The relatively low levels of inhibin in the testes of immature animals together with the relatively low plasma FSH levels suggest that most of the inhibin is not stored in the testes at this age but is rather secreted into the circulation to control pituitary FSH secretion. During this period, a relationship between testicular inhibin content and resulting circulating inhibin concentrations may exist. From one week to nine weeks of age larger amounts of inhibin activity can be found in normal as well as in manipulated rat testes. This suggests that either more inhibin is produced or retained and/or that inhibin may rather exert a role within the testis than on the pituitary gland.
No data are available on changes in metabolic clearance of inhibin with age and the reports on changes of pituitary sensitivity for inhibin are contradictory. Nevertheless, it appears from the experiments described in *appendix* paper 1 and this chapter that FSH regulation by inhibin alone is rather important before 6 weeks of age in the rat while thereafter, testicular steroid secretion becomes more important in the regulation of FSH secretion.

4

REGULATION OF INHIBIN PRODUCTION IN VITRO

4.1 Introduction

Sertoli cells can be maintained in primary culture in a chemically defined medium. These cells secrete inhibin for at least 21 days of culture (Steinberger, 1981; *appendix* paper 2). In this chapter, the regulation of inhibin production by Sertoli cells is discussed. Sertoli cell enriched cultures isolated from normal or *in utero* irradiated 21-day-old rat testes were used (section 4.2) and the *in vitro* effects of several factors were investigated (section 4.3). Furthermore, the inhibin production by Sertoli cells, isolated from testes from rats of various ages has been studied (section 4.3.5).

4.2 Sertoli cell cultures

Methods for isolation and culture of Sertoli cells were developed by several research groups (rat: Steinberger & Steinberger, 1976; bull: Smith & Griswold, 1981; pig: Chevalier & Dufaure, 1981; man: Lipschultz *et al.*, 1982). Sertoli cells from immature testes appear to be a good model to study Sertoli cell function *in vitro* since these cells can be easily isolated and maintained in a primary culture (see review Mather *et al.*, 1983). In general, Sertoli cell enriched cultures were prepared using successive digestions with various enzymes (collagenase alone or in combination with trypsin) to separate Sertoli cells from other components of the testis tissue. Probably as a result of the high collagen content of the tubular wall around the seminiferous tubule, treatment with collagenase proved to be very effective as a means to separate tubules from interstitial cells and peritubular myoid cells. In the studies described in this chapter Sertoli cell clusters from immature rat testes were prepared using the methods described in *appendix* paper 2. Two subsequent incubations with collagenase were preferred over a single incubation with this enzyme, because a smaller contamination with interstitial cells was obtained in this way. This treatment resulted in a suspension of tubules, which were separated from interstitial cells and were subsequently dispersed mechanically into smaller fragments or clusters using a Dounce homogenizer. Initially, Sertoli cells were studied in long term studies of which the media were renewed every 2 or 3 days. Sertoli cell cultures maintained for a short period under *in vitro* conditions

were also used to determine effects of various factors on inhibin production (*appendix* paper 2). To obtain Sertoli cell preparations from testes from normal adult rats an incubation with collagenase was followed by incubation with trypsin in order to weaken the connections between the Sertoli cells and between Sertoli cells and the various spermatogenic cells. The clusters of Sertoli cells obtained in these ways were plated out in plastic tissue culture dishes. These cells are still associated with spermatogenic cells when testes from intact animals are used. However, most spermatogenic cells do not survive *in vitro* and disappear after repeated renewal of the culture medium (Fig. 4.1). For some studies Sertoli cell preparations were prepared from spermatogenic cell-depleted rat testes, obtained from animals which were irradiated *in utero*. Such irradiated animals have testes devoid of spermatogenic cells, because the primordial germ cells in the fetal rat testes are very radiosensitive between 19 and 21 days of embryonic life (Beaumont, 1960). At this stage of development, a low dose of radiation (100-150 Rads) is lethal to almost all gonocytes, resulting in subsequent degeneration. The number of Sertoli cells in these prenatally irradiated testes may be higher than in controls at day 21, however (see de Jong & Sharpe, 1977b).

During the first 24 h of culture, Sertoli cells spread from the clusters and form monolayers with epithelial features; the cells do not divide in culture. Sertoli cells in culture can be identified by microscope by the presence of one or more cytoplasmic lipid droplets (Fig. 4.1).

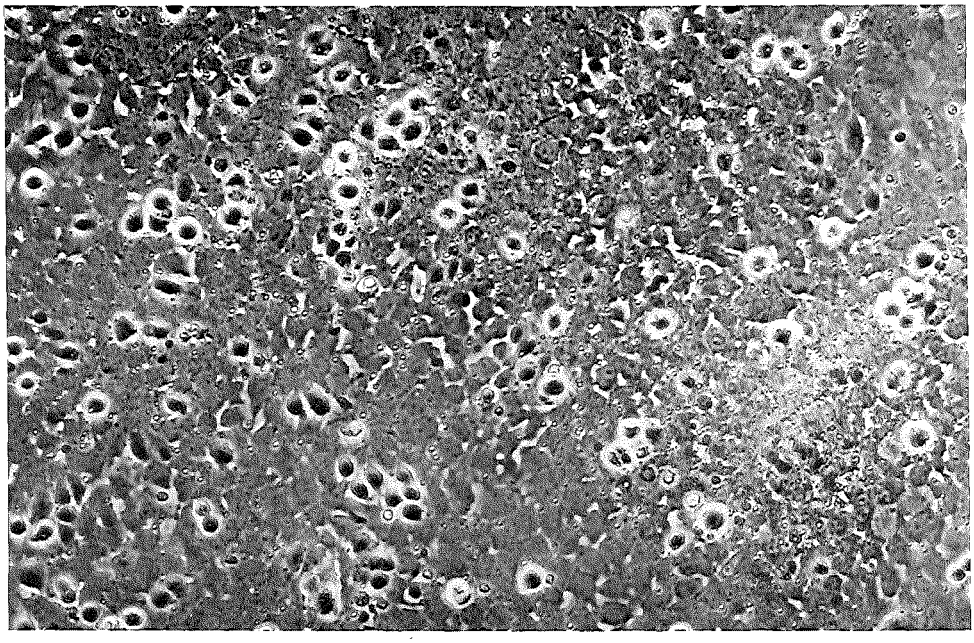

Fig. 4.1 The morphological appearance of Sertoli cells isolated from 21-day-old rats and cultured without serum at 32°C

As summarized in Table 4.1 a large number of factors can affect Sertoli cell function in culture. In addition, these cells can secrete a large number of testis specific products in addition to a number of plasma proteins, which are formed also in the liver (Wright *et al.*, 1981).

Table 4.1 Products of Sertoli cells *in vitro* and factors which influence their secretion

PRODUCTS	FACTORS	REFERENCES
androgen binding protein	FSH, testosterone, insulin, retinol, EGF, spermatogenic cells	Louis & Fritz (1979) Karl & Griswold (1980) Galdieri *et al.* (1984) Rommerts *et al.* (1978) Perez-Infante *et al.* (1986)
inhibin	FSH, testosterone, spermatogenic cells	*Appendix* paper 2 Steinberger (1981) Le Gac & de Kretser (1982) Verhoeven & Franchimont (1983)
transferrin	FSH, testosterone, insulin, retinol	Perez-Infante *et al.* (1986) Skinner & Griswold (1980,82)
lactate	FSH insulin, IGF-I EGF	Jutte *et al.* (1983) Oonk *et al.* (1985) Mallea *et al.* (1986)
Müllerian inhibiting substance	?	Vigier *et al.* (1984,1985) La Quaglia *et al.* (1986)
SCF/IGF-I	?	Bellvé & Feig (1984)
plasminogen activator	FSH	Lacroix & Fritz (1982)
oestradiol	FSH, testosterone	Dorrington & Armstrong (1975) Rommerts *et al.* (1978)
ceruplasmin	?	Wright *et al.* (1981)
testibumin	FSH, testosterone	Cheng & Bardin (1986)
phosphoproteins	FSH, testosterone	Ireland *et al.* (1986)
protein kinase inhibitor	?	Tash *et al.* (1981)
cAMP	α-MSH, FSH	Boitani *et al.* (1986) Rommerts *et al.* (1978)
protein synthesis	FSH, dbcAMP	DePhilip & Kierzenbaum (1982) Wright *et al.* (1981)

The amount of inhibin produced by Sertoli cells in culture can be assessed using its specific suppressive effect on the basal release of FSH from dispersed pituitary cells in culture after addition of aliquots of spent media (Fig. 4.2). In similar experiments it was found that spent media from isolated spermatogenic cells or cultured interstitial cells did not contain any inhibin-like activity *i.e.* addition of these media caused no suppression of pituitary FSH release (data not shown). In the next section the effects of various culture conditions on the inhibin production by rat Sertoli cells *in vitro* will be discussed.

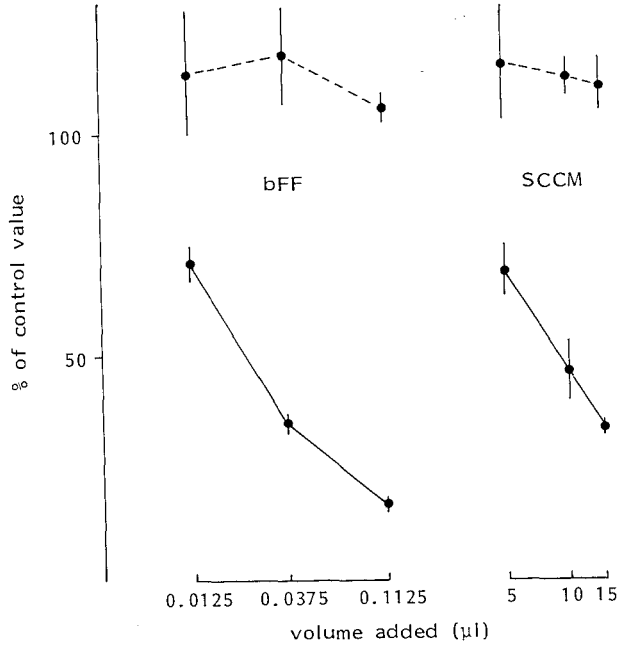

Fig. 4.2 The relative amount of FSH (•——•) and LH (•-----•) in spent media from cultured pituitary cells after addition of various amounts of bFF or SCCM (mean ± S.E.M., n=4).

4.3 Factors regulating secretion of inhibin by cultured rat Sertoli cells

The factors which regulate inhibin production by and secretion from cultured Sertoli cells were studied under defined *in vitro* conditions. These secretion patterns will be compared with data reported by other authors, who studied the regulation of the secretion of inhibin and/or other products of Sertoli cells (Table 4.1).

The amount of inhibin activity secreted into the medium of cultured Sertoli cells is directly correlated with the number of plated Sertoli cells (de Kretser *et al.*, 1983). Similar data have been reported also for granulosa cells in culture (Erickson & Hsueh, 1978; Hermans *et al.*, 1982).

4.3.1 Effect of culture period

Sertoli cells in culture have and retain the capacity for a basal secretion of inhibin (*appendix* paper 2, Fig. 1). However, the amount of inhibin present in spent media changes during culture. A relatively low amount of inhibin activity was found before day 7 of culture whereas a higher level of inhibin production was observed during the second week of culture. These results are in agreement with those of Labrie *et al.* (1978) who observed a higher inhibin production by Sertoli cells in culture between days 5 and 8 than between days 2 and 5. Steinberger (1981) did not observe a change in inhibin secretion when Sertoli cells were cultured for 21 days. However, in the bioassay system for inhibin used by this author a suppression of the basal pituitary secretion of FSH to less than 40% of control levels was observed after addition of the spent medium from the Sertoli cell cultures. Under these conditions an increase of the inhibin production might not have been detected. Hermans *et al.* (1982) reported that granulosa cells also secreted relatively less inhibin during the first 5 days of culture. This may have been due to division and differentiation of the granulosa cells during this initial period of culture. A rather stable production of inhibin was observed thereafter until day 15 of culture, while after day 21 inhibin production steadily decreased towards day 37, probably due to degeneration of the cells. Since Sertoli cells maintain their appearance in culture the *in vitro* changes of inhibin production by Sertoli cells may be caused by elimination of spermatogenic cells during medium changes (see section 4.3.4). Perez-Infante *et al.* (1986) studied ABP and transferrin production by Sertoli cells from rats of different ages as a function of time in culture. They found that the ABP production by Sertoli cells derived from 7- to 10-day-old rats increased with time in culture, while Sertoli cells from older animals produced decreasing amounts of ABP with increasing time of culture. These data agree with observations published by Rich *et al.* (1983), who indicated that the pattern of ABP production during long term culture of Sertoli cells is determined by the total age of the cells (animal + culture age), with increasing amounts of ABP up to 20 days of total age and decreasing amounts thereafter. The decrease of ABP production may reflect the progressive removal of spermatogenic cells as a result of changes of the medium because the presence of spermatogenic cells may stimulate ABP secretion as shown by Galdieri *et al.* (1984), who demonstrated an increase of ABP secretion after addition of spermatocytes, which did adhere to Sertoli cells. In our experiments addition of freshly isolated spermatogenic cells to Sertoli cell monolayers resulted in an inhibition of the inhibin production by 21-day-old Sertoli cells (see section 4.3.4).
The directions of the secretion of ABP and transferrin are opposite under *in vitro* conditions. It appears that the secretions of ABP and transferrin are differentially controlled (Perez-Infante *et al.*, 1986).

4.3.2 Effect of temperature and serum on inhibin production *in vitro*

During postnatal development of the rat the testes descend from an abdominal to a scrotal position. This results in a change of the intratesticular temperature from 37°C to 32°C (Kormano, 1967). It was shown *in vitro* that various cellular processes in Sertoli cells such as protein synthesis, amino acid transport, production of lactate and production of cAMP under influence of hormones occur at a higher rate at 37°C than at scrotal temperature (Rommerts *et al.*, 1980; Hall *et al.*, 1985). On the other hand, spermatogenesis as well as Sertoli cell function are damaged after experimental cryptorchidism as evidenced by a severe impairment of ABP production at 37°C *in vivo* and *in vitro* (Hagenäs *et al.*, 1978). The effect of elevated temperature on inhibin secretion by cultured Sertoli cells was investigated by Steinberger (1980); she observed that after 7 days of culture significantly less inhibin activity was secreted at 38°C when compared with the secretion at 32°C when Sertoli cells from adult normal or cryptorchid rats were used. However, no information was available about the effect of temperature on inhibin secretion by immature Sertoli cells.

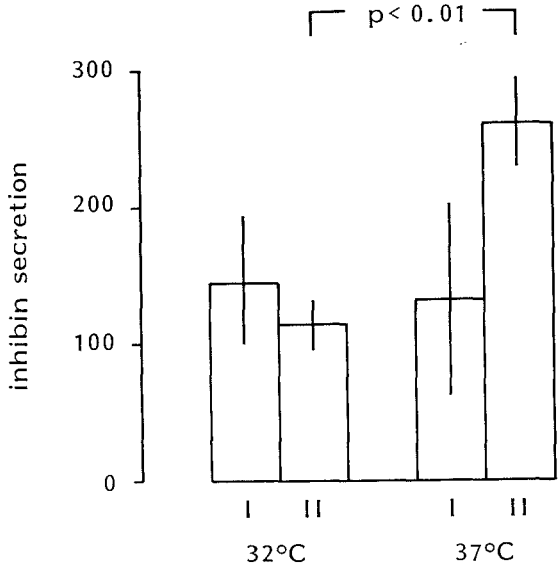

Fig. 4.3 Effect of temperature on inhibin secretion by Sertoli cells during culture period I (day 0-10) and II (day 10-21). Inhibin secretion is expressed as units inhibin activity/day per culture (mean ± S.E.M., n=4-5).

The data summarized in Fig. 4.3 demonstrate that immature Sertoli cells secrete significantly more inhibin activity at elevated temperature after 10 days of culture, while initially similar amounts of inhibin are produced at both temperatures.
In addition, the influence of culture temperature and addition of 5% foetal calf serum (FCS) to the culture medium on inhibin production by Sertoli cells isolated from testes of 21-day-old rats, cultured at 32 or 37°C for a long term period is shown in Fig. 4.4. Every 2 or 3 days spent media were collected and inhibin activity was estimated by bioassay. From these data it is concluded that Sertoli cells release more inhibin activity at elevated temperature in the absence of serum (see also *appendix* paper 2), while the presence of 5% FCS resulted in similar amounts of inhibin at both temperatures. Verhoeven and Franchimont (1983) reported that immature Sertoli cells did not secrete detectable amounts of inhibin in the absence of serum. The final concentration of spent media added to the pituitary cell culture media in the latter study was 50 to 80% as opposed to less than 25% (v/v) in our studies. It may be that in the study by Verhoeven & Franchimont (1983) the function of the pituitary cells, used in the inhibin bioassay, has been affected either by testicular growth factors (see section 6.2). In this respect, Steinberger (1980) reported that the presence of 10% foetal calf serum in the medium of Sertoli cells from 28-day-old normal rats, which had been in culture for 20 days, stimulated inhibin activity in the medium, cell number and protein content by 15-20%. Since Sertoli cells do no longer divide at this age, it is likely that contaminating peritubular myoid cells divided under these conditions; this may have stimulated the inhibin production by Sertoli cells. However, the same author did not find an increase of inhibin production when Sertoli cells were co-cultured with peritubular myoid cells. These data are, unfortunately, difficult to interpret because only one dose of Sertoli cell medium was added to the cultured pituitary cells, which caused maximal suppression of FSH release. On the other hand peritubular myoid cells can stimulate ABP secretion (Hutson & Stocco, 1981; Skinner & Fritz, 1985) and transferrin production (Skinner & Fritz, 1985) by Sertoli cells *in vitro*. With the single collagenase treatment in the preparation of Sertoli cell monolayers for long term cultures, as described in *appendix* paper 2, the presence of 5% FCS during the whole culture period may have stimulated the growth of peritubular myoid cells, which apparently have the capacity to modulate several Sertoli cell functions. Studies using cultures of pure Sertoli cells in the presence or absence of pure peritubular myoid cells are needed to investigate this point further.
In later studies 1% FCS was added during the initial 24 h of culture to promote attachment of the Sertoli cell clusters to the culture dish. Thereafter, serum-free culture conditions were used in order to reduce the proliferative capacity of peritubular myoid cells and to avoid interference of serum factors with Sertoli cell functions. In this way, Sertoli cell cultures from immature rat testes can be kept in culture for at least 3 weeks; their epithelial characteristics as well as inhibin production capacity are preserved during this period (Steinberger, 1980; *appendix* paper 2).

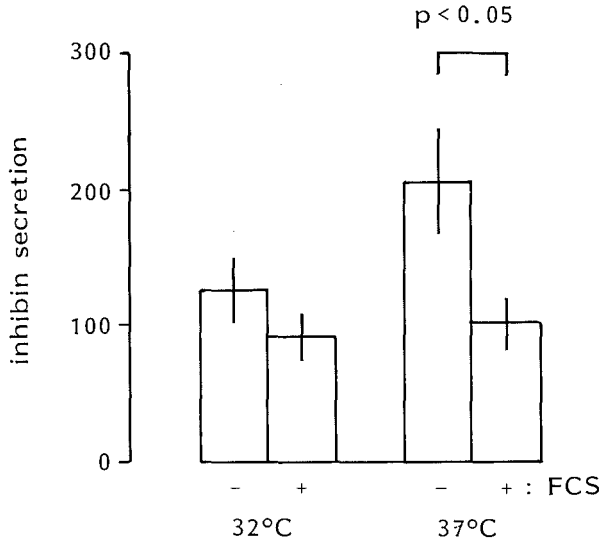

Fig. 4.4 Effect of addition of FCS (5%) to the medium on inhibin secretion by Sertoli cells in *vitro, at different temperatures. Inhibin secretion is expressed as units inhibin activity/day per cultures (means ± S.E.M., n=9).*

4.3.3 Effects of hormones on inhibin production by Sertoli cells *in vitro*

Cultures of isolated rat Sertoli cells provide a good model for the study of the regulation of testicular inhibin production by various hormones. It has been reported that cultured Sertoli cells respond to hormonal stimulation by increased secretion of various proteins (Fritz *et al.*, 1976; Skinner & Griswold, 1980; Ireland *et al.*, 1986). These hormones interact with specific receptors in the Sertoli cell membrane or in the cytosol of the cell (Table 4.2). Receptors for most of these protein and peptide hormones are present in Sertoli cells immediately after isolation of the cells, but β-adrenergic receptors are acquired during primary culture (Kierzenbaum *et al.*, 1985).

Table 4.2 Reported specific receptors in Sertoli cells *in vitro*

RECEPTOR FOR	REFERENCES
FSH	Christensen *et al.* (1977) Means *et al.* (1980)
steroid	
-oestrogen	Christensen *et al.* (1977)
-androgen	Mulder *et al.* (1976) Nakhla *et al.* (1984) Sanborn *et al.* (1975)
-glucocorticoid	Levy *et al.* (1986)
insulin, IGF-I	Borland *et al.* (1984) Oonk *et al.* (1987) Saucier *et al.* (1983)
IGF-II	Borland *et al.* (1984) Oonk *et al.* (1987)
EGF	Chen *et al.* (1984)
retinol	Carson *et al.* (1984) Huggenvik *et al.* (1981)
opiates	Boitani *et al.* (1986) Fabbri *et al.* (1985) Orth (1986)
transferrin	Brown (1985)
β-adrenergic agents*	Heindel *et al.* (1981)
adenosine	Monaco & Conti (1986)

*: acquired *in vitro* (Kierzenbaum *et al.*, 1985)

4.3.3.1 Effects of FSH

The binding of FSH to receptors on the plasma membrane of Sertoli cells results in the activation of adenylate cyclase and in elevation of intracellular levels of cyclic AMP, which, in turn, leads to increased synthesis of RNA and proteins (Means *et al.*, 1976; Tindall *et al.*, 1980).
The stimulation of the secretion of ABP by FSH in immature Sertoli cells *in vitro* has been

established by several authors (Fritz *et al.*, 1976; Perez-Infante *et al.*, 1986), but studies on the effects of FSH on inhibin production yielded conflicting results (see Table 4.3). Verhoeven and Franchimont (1983) could not demonstrate any effect of FSH (10-1000 ng ovine FSH-S13/ml) or PMSG on inhibin concentrations in the culture medium of Sertoli cells after 4 h and 24 h of stimulation, respectively in the presence or absence of 5% fetal bovine serum. In contrast, Le Gac and de Kretser (1982) demonstrated a dose-dependent increase of inhibin activity in the presence of 50-5000 ng ovine FSH- S13/ml. The latter authors added FSH for a period of 48 h to the culture medium and measured FSH cell content in the bioassay for inhibin. This stimulation of inhibin production by FSH confirms the earlier studies by Steinberger (1980,1981) and is in agreement with the concept of a hormone stimulating its target tissue to produce a feedback substance. Recently, Bicsak *et al.* (1987) also found a dose-dependent increase of inhibin production after addition of ovine FSH using 3 day culture periods. These authors detected inhibin with a radioimmunoassay method using an antibody directed against the N-terminal 25 amino acids of the porcine inhibin alpha chain. The FSH-stimulated production of inhibin was augmented by addition of a phosphodiesterase inhibitor and could be mimicked by addition of cholera toxin, forskolin or dibutyryl cAMP, all of which are known to increase intracellular cAMP levels (Bicsak *et al.*, 1987). In addition, Steinberger (1981) demonstrated direct effects of cyclic AMP and of the phosphodiesterase inhibitor 3-isobutyl-methylxanthine (MIX) on the inhibin production by Sertoli cells. In our experiments addition of 500 ng ovine FSH-S13/ml stimulated inhibin production 30-fold and 6-fold at 32 and 37°C, respectively, when compared with control production at the same temperature (Fig. 4.5). In these experiments Sertoli cells were isolated from testes of 21-day-old prenatally irradiated rats and cultured for 3 days in the presence of ovine FSH without serum. Because of cross-reaction of ovine FSH in the radioimmunoassay for rat FSH, which was used in the *in vitro* bioassay for inhibin activity, further experiments on the regulation of inhibin production by FSH were carried out using an experimental design in which FSH was added to Sertoli cells during a 8 h preincubation period. Inhibin was subsequently estimated in the medium collected 24 h after renewal of the medium.

Table 4.3 Effects of hormones on *in vitro* secretion of inhibin by immature Sertoli cells

REFERENCES	FSH	T	DHT
Steinberger (1979,1981)	↑	↑	
Le Gac & de Kretser (1982)	↑		
Verhoeven & Franchimont (1983)	=	↑	↑
Bicsak *et al.* (1987)	↑		=
Appendix paper 2	↑	↓	

↑ : stimulating effect
= : no effect
↓ : inhibiting effect

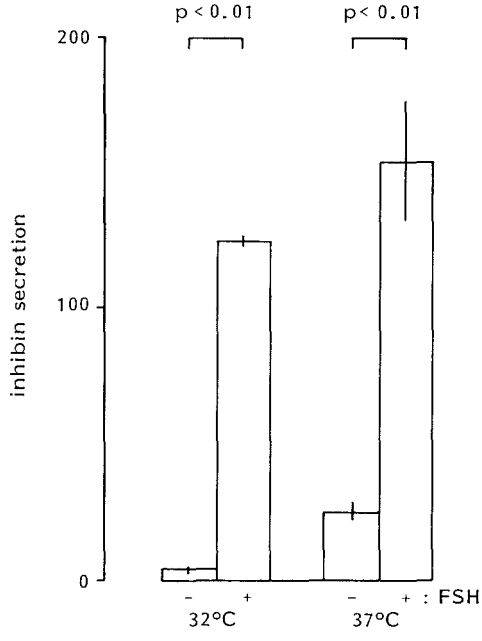

Fig. 4.5 Effect of ovine-FSH on inhibin secretion by Sertoli cells from 21-day-old prenatally irradiated rats during long term incubation experiments at different temperatures. Inhibin secretion is expressed as units inhibin activity/day per culture (mean ± S.E.M., n=2).

In this way, inhibin secretion by Sertoli cells from testes from normal rats can be stimulated by addition of FSH at 32°C, while this increase is reduced at 37°C, at which temperature an increased basal production of inhibin is found (*appendix* paper 2). These results confirm data of Steinberger (1981) who found that Sertoli cells which had been preincubated for two days with ovine FSH (5000 ng/ml) secreted significantly larger amounts of inhibin compared with untreated cells during the following 3 days of culture. These observations show that FSH is involved in the hormonal regulation of inhibin producion by Sertoli cells.

4.3.3.2 Effects of testosterone

The Sertoli cell is also a target cell for testosterone (Steinberger, 1979, Tindall *et al.*, 1980). Addition of testosterone (6.9×10^{-7}M) did not influence the inhibin production by immature Sertoli cells in long term cultures (data not shown). However, the presence of the same concentration of testosterone during an 8 h preincubation period of Sertoli cells in short term culture significantly inhibited the production of inhibin (*appendix* paper 2: Fig. 2). The latter finding is in contrast with results obtained by other investigators. Steinberger (1981) used 10^{-7}M testosterone during a 2-day preincubation period and detected increased amounts of inhibin activity in medium collected after an additional three-day period. Verhoeven and Franchimont (1983) showed stimulation of inhibin production in cultures of immature Sertoli cells in the presence or absence of 5% foetal bovine serum when either testosterone ($0.5\text{-}5 \times 10^{-7}$M) or dihydrotestosterone (DHT, 5×10^{-7}M) was added to the media for 24 h at 32°C. In contrast, Bicsak *et al.* (1987) found no effects of androgens (10^{-7}M) on inhibin production when immature Sertoli cells were cultured for 3 days. However, the latter data were obtained from measurement of inhibin in spent media by RIA, while no data on bioactive inhibin in these samples are available.

4.3.3.3 Effects of combinations of FSH and testosterone

Since FSH or testosterone can modulate the inhibin production by Sertoli cells in culture it was also investigated how these hormones act together in the regulation of inhibin production.
When cultured Sertoli cells were preincubated with ovine FSH-S13 and testosterone, the amount of inhibin which was secreted during the following 24 h was intermediate between the amounts found after preincubation in the presence of each of these hormones alone (*appendix* paper 2: Fig. 2). These results indicate again that FSH and testosterone can exert counteracting effects on the secretion of inhibin *in vitro*. It is likely that FSH is the important modulator of inhibin production in immature male animals, while increased levels of testosterone after 21 day of age in the rat may regulate both the secretion of pituitary FSH and the secretion of testicular inhibin.

4.3.3.4 Effects of insulin

Sertoli cells have receptors for insulin as well as IGF-I (Oonk *et al.*, 1987). Insulin is a growth factor which has been utilized in some serum-free culture systems (Mather *et al.*, 1982) and can bind to IGF-I as well as to specific insulin receptors (Oonk *et al.*, 1987). Evidence that insulin may be involved in the regulation of Sertoli cell activity comes from studies in which insulin and FSH exert trophic effects on glucose metabolism by Sertoli cells in culture (Oonk *et al.*, 1985). Furthermore, after addition of insulin an increased

production of ABP (Karl & Griswold, 1980) and transferrin (Skinner & Griswold, 1982) was detected. A preincubation (8 h) of Sertoli cells with 5 μg/ml bovine insulin (26.8 U/mg Sigma, St. Louis, MO, U.S.A.) suppressed the secretion of inhibin to 30 ± 4% of control volumes (mean ± S.E.M. n=3, $p<0.01$) during the following 24 h when testes from 14 days old rats were used. In Sertoli cells from 21-day-old rats no significant suppression of inhibin secretion was obtained in a similar experiment. In contrast, Bicsak *et al.* (1986) showed that IGF-I enhanced inhibin production in granulosa cells, while insulin was much less effective.

4.3.4 Influence of spermatogenic cells on inhibin production by Sertoli cells in vitro

Steinberger (1980) reported that co-culture of Sertoli cells with spermatogenic cells isolated from 18- or 85-day-old rats did not influence the inhibin production. This conclusion was based on maximal suppression of pituitary FSH release in the *in vitro* bioassay for inhibin, and therefore it is not possible to detect differences between the amounts of inhibin in the various samples. A role of spermatogenic cells in inhibin secretion, however, has been implied by several *in vivo* observations. Peripheral levels of FSH in man are often increased when spermatogenic cells are absent ("Sertoli cell only syndrome") or in oligospermia (see review Setchell *et al.*, 1977). Experiments conducted in hydroxyurea-treated rats by Mecklenburg *et al.* (1975) led to the conclusion that inhibin production by Sertoli cells depends on the interaction with spermatogonia. Depletion of spermatogenic cells caused by cryptorchidism (Risbridger *et al.*, 1981), *in utero* irradiation (Rich & de Kretser, 1977), vitamin A deficiency (Rich *et al.*, 1979; Huang *et al.*, 1983), administration of hydroxyureau (Rich *et al.*, 1979), efferent duct ligation (Main & Setchell, 1980), or local heating (Main & Setchell, 1980) caused a rise in peripheral FSH levels, which is often accompanied by a rise in LH. It has been suggested that such treatments damage Sertoli as well as Leydig cell function (Rich *et al.*, 1977). Therefore, we studied the influence of interaction between spermatogenic cells and Sertoli cells on inhibin secretion *in vitro* in a number of experimental models:

1. Sertoli cells from normal rat testes in culture, which still contain various spermatogenic cells, secrete variable amounts of inhibin during a culture period of 21 days (*appendix* paper 2: Fig. 1). The changes in the amount of inhibin which is secreted during the culture may be due to changes in the number and/or type of spermatogenic cells in such cultures; the number of spermatogenic cells becomes smaller after repeated renewal of the medium. This decrease of spermatogenic cell numbers was accompanied by an increased inhibin secretion.
2. The inhibin secretion by Sertoli cells from normal rat testes is significantly lower than that by Sertoli cells from spermatogenic cell depleted rat testes (51 ± 5 units inhibin/mg protein per 24 h *versus* 90 ± 2; n = 3-6, mean ± SEM), while nevertheless more than 80% of the cells in the former experiment were Sertoli cells as revealed by DNA analysis. Thus, when the amounts of protein per culture are corrected for the presence of spermatogenic cells still more inhibin is secreted by Sertoli cells isolated from testes from *in utero* irradiated rats.

3. The increased basal secretion of inhibin from Sertoli cells isolated from spermatogenic cell depleted rat testes can be stimulated significantly by addition of FSH during a 8 h preincubation period at 37°C, whereas Sertoli cell cultures derived from normal rat testes, which still contain spermatogenic cells on top of the monolayer, are not able to secrete more inhibin at elevated temperatures in the presence of FSH (*appendix* paper 2: Fig. 2).
4. Freshly isolated spermatocytes or spermatids were recombined *in vitro* with Sertoli cell monolayers prepared from *in utero* irradiated rat testes. Adition of spermatogenic cells reduced the amount of FSH-augmented inhibin secretion at 37°C (*appendix* paper 2: Fig. 3). Co-culture of *non* testicular cells (thymocytes) with Sertoli cells did not have any effect on the inhibin secretion. From these observations, it appears that spermatogenic cells can have a specific negative effect on the inhibin secretion *in vitro*. There are other reports on effects of spermatogenic cells on secretion of products from Sertoli cell monolayers in recombination experiments. The addition of spermatogenic cells to Sertoli- cell-only-cultures resulted in an increased ABP production (Galdieri *et al.*, 1984; Le Magueresse & Jégou, 1986). The study from Le Magueresse & Jégou (1986) demonstrated that the production of oestradiol is inhibited by addition of spermatogenic cells *in vitro* while after removal of spermatogenic cells by hypotonic treatment production of oestradiol returned to control values. It is not clear from the latter study which class of spermatogenic cells is responsible for this effect on Sertoli cell activity. These authors suggested that protein factors secreted by spermatogenic cells can influence Sertoli cell function. Recently, Ireland and Welsh (1987) showed that spermatogenic cells are involved in the regulation of Sertoli cell function via mobilization of intracellular calcium and activation of protein kinases resulting in phosphorylation of two specific Sertoli cell proteins as revealed by autoradiograms of two dimensional gel patterns of Sertoli cell phosphoproteins.

Considering all these observations it is clear that spermatogenic cells can influence Sertoli cell functions. There are indications that spermatocytes and/or spermatids decrease the inhibin secretion from Sertoli cells as may also be the case *in vivo* after the age of 20 days.

4.3.5 Influence of stage of development of Sertoli cells on *in vitro* production of inhibin

A number of changes in Sertoli cell function occurs during development of the testes (see review Means *et al.*, 1980). Rich *et al.* (1983) reported that ABP secretion from Sertoli cells *in vitro* increases with increasing age of the donor animals between 7 and 31 days of age. Changes in the amount of inhibin activity secreted from Sertoli cells from rats of various ages have been reported earlier by Massicotte *et al.* (1984). These authors showed a five fold decrease of the inhibin secretion, when results from Sertoli cells from 10-day-old rats were compared with those from 30- to 40-day-old animals.

In chapter 3 and *appendix* paper 1 it was shown that testicular inhibin content increases from

birth to adulthood in the rat. However, after culture of Sertoli cells from rats of various ages an age dependent change in inhibin secretion was found. The decrease of inhibin secretion per 10^6 Sertoli cells after the age of 14 days correlates with the increase of peripheral FSH concentrations during this age period (Fig. 4.6).

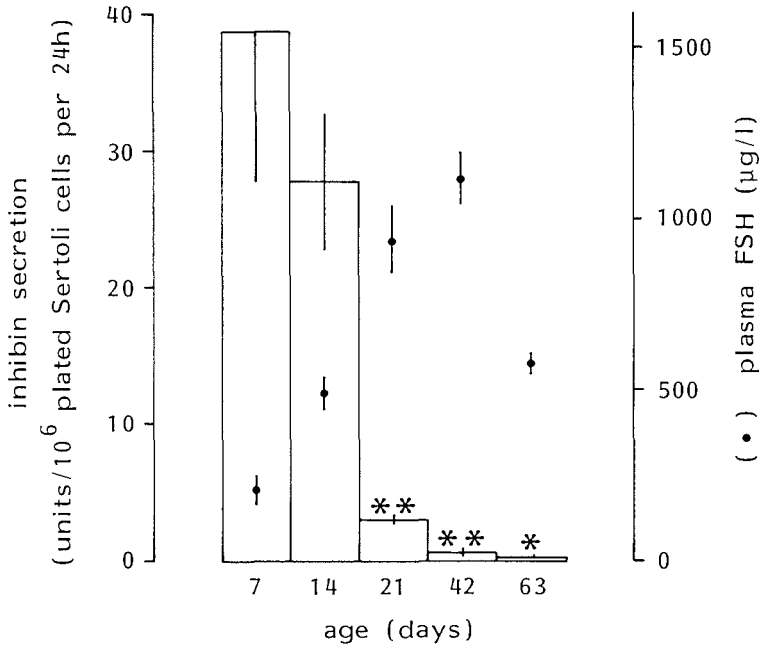

Fig. 4.6 The amount of inhibin activity in culture media of Sertoli cells isolated from testes of normal rats of various ages (bars). The peripheral concentrations of FSH in these animals are indicated by closed circles (mean ± S.E.M.).

Furthermore, the amount of inhibin in the Sertoli cells from 7-day-old rats after culture for 3 days was less than 2% of the amount of inhibin activity secreted into the culture medium. This observation agrees with that of Bicsak *et al.* (1986), who found very low levels of intracellular inhibin in granulosa cells, which were cultured in the presence or absence of FSH. In contrast, Bicksak *et al.* (1987) reported that the addition of FSH to the culture medium of Sertoli cells from 16- to 18-day-old rats increased both cellular and secreted levels of inhibin: approximately one-half to two-third of the amount of inhibin was secreted under the chosen conditions. However, these authors did not estimate intracellular inhibin activity under basal circumstances. Moreover, the experiments on the regulation of the inhibin production performed by Bicsak *et al.* (1986, 1987) were carried out with a radio-immunoassay using an antibody which detects the N-terminal portion of the porcine ovarian

inhibin alpha subunit. It remains to be determined whether inhibin immunoreactivity correlates with bioactivity as measured by bioassay (section 6.2).

4.4 Conclusions

Sertoli cells are the site of inhibin synthesis in the testis. The production of inhibin by Sertoli cells *in vitro* continues for several weeks and is temperature dependent. The presence of serum in the culture medium suppresses inhibin secretion at elevated temperature. Serum may induce attachment and spreading of the cells, but prolonged addition of serum can cause an increase of the number of peritubular myoid cells, which in turn may influence Sertoli cell functions.
Our results indicate, that inhibin production *in vitro* is regulated by FSH and testosterone, and that the age of the animal from which the Sertoli cells are obtained may influence the results. The data from experiments in which insulin was added indicate that insulin may inhibit the secretion of inhibin from immature Sertoli cells. At all ages, the Sertoli cell is capable to secrete inhibin, but the amount of secreted inhibin per Sertoli cell decreases with increasing age. Differences in experimental protocols, used by several research groups, may have led to strinkingly different effects of various hormonal conditions on inhibin production *in vitro*, as summarized in Table 4.3. In addition, spermatogenic cells may have an inhibiting effect on the secretion of inhibin from Sertoli cells.

5

THE ROLES OF FSH AND INHIBIN IN THE REGULATION OF TESTIS SIZE

5.1 General introduction

During sexual development in male animals changes in all components of the hypothalamic-pituitary-gonadal axis result in full maturation of the testicular functions. In male rats, there is a gradual increase of peripheral concentrations of gonadotrophins from birth until day 40 of age concomitant with a rapid increase of testis weight (Odell & Swerdloff 1976; de Jong & Sharpe, 1977a). After this age FSH levels in blood fall, whereas concentrations of LH increase modestly (Swerdloff *et al.* 1971). The further increase in testis weight is due to the increasing numbers of spermatogenic cells. Mature spermatozoa are first found in the testis at 44 days of age (Russell *et al.*, 1987), while the developing rat does not reach full spermatogenic efficiency until about 75 days of age (Robb *et al.*, 1978). The decline in plasma FSH levels after 40 days of age has been ascribed to feedback inhibition by increasing amounts of testosterone in the peripheral circulation (de Jong & Sharpe, 1977a). Growth and maturation of the testes are controlled by pituitary hormones: removal of the pituitary gland causes regression of testicular weight and function, while after replacement of FSH and LH normal testicular weights can be maintained (Hochereau-de Reviers & Courot, 1978; Russell *et al.*, 1987).

The current concensus is that FSH is involved in the maturation and functioning of the Sertoli cells, while LH specifically binds to Leydig cells to promote steroidogenesis (de Kretser *et al.*, 1971). Murphy (1965) demonstrated an increase of testicular weight after a postoperative intratesticular injection of FSH into immature hypophysectomized rats. FSH is able to stimulate various biochemical events in Sertoli cells (see Means *et al.*, 1980; Ireland *et al.*, 1986) and the FSH-mediated response appears to be dependent on the age of the animal: stimulation of testicular protein synthesis was observed in testes of intact rats between 10 and 24 days of age, but not in testes from rats older than 25 days (Means, 1980). Similar results were reported by Steinberger *et al.* (1978), who found a dramatic decrease in cyclic AMP response to FSH between 18 (60 fold increase) and 36 days (10 fold increase) of age when rat Sertoli cells in culture were studied. Numbers of Sertoli cell receptors for FSH rapidly decrease during pubertal development and are low in the adult male rat (Sanborn *et al.*, 1975; Means *et al.*, 1980).

Orth (1984) suggested that FSH may be the major factor in the control of Sertoli cell divisions in developing rat testes.
Inhibin has been shown to be a product of the Sertoli cell (chapter 4) and the inhibin secretory activity of Sertoli cells is stimulated by FSH in immature animals (section 4.3.3.1). We have studied the possible importance of inhibin in the regulation of pituitary FSH secretion in immature rats using the effects of hemicastration and the administration of exogenous FSH on inhibin production. Furthermore, exogenous inhibin was injected into immature rats, while finally experiments on immunoneutralization of endogenous inhibin were performed. Effects of such treatments in prepubertal rats were evaluated at various ages (see also *appendix* papers 3 and 4).

5.2 Effects of neonatal hemicastration on FSH, inhibin and testis size

Partial removal of endogenous inhibin by means of unilateral orchiectomy might give information on the relative contribution of inhibin to the regulation of the pituitary secretion of FSH.
It has been known for a long time that unilateral castration of immature animals results in an increase of the weight of the remaining testis (Lippschutz, 1922). With the development of radioimmunoassays for gonadotrophins, it was found that hemicastration early in life causes increased levels of gonadotrophins, particularly of FSH in the circulation. It was concluded that this might be the reason for the hypertrophic growth of the remaining testis in rats (Cunningham *et al.*, 1978; Hochereau-de Reviers & Courot, 1978; Moger, 1977; Orth,1984) and in rams (Hochereau-de Reviers & Courot, 1978; Waites *et al.*, 1983; Walton *et al.*, 1978). Furthermore, in order to obtain an increase of the weight of the remaining testis, a critical age for performing the operation was observed to be before 45 days of age in the rat, while a constant relative increase of testicular weight was found when rats were hemicastrated between 5 and 20 days of age (Cunningham *et al.*, 1978; Putra and Blackshaw, 1982). The authors showed that the increase of testis weight of hemicastrated rats was correlated with an increase in total seminiferous tubule length and with a larger cross-sectional area of the seminiferous tubules caused by a larger number of spermatogenic cells per Sertoli cell. Because compensatory growth of the remaining testis was also observed after earlier hemicastration, we removed the testis from rats on the day after birth and studied endocrine and testicular growth responses in these animals at prepubertal, pubertal and young adult ages (see *appendix* paper 3). In order to elucidate which mechanism(s) are involved in the regulation of testis growth after this type of operation, pituitary and testicular function were evaluated separately *in vitro*.
Maximal relative compensatory growth of the remaining testis had already been established at 21 days of age. *In vitro*, less inhibin activity was secreted per amount of Sertoli cell protein in the experimental group at 21 days of age. However, more Sertoli cells may have been present in the remaining enlarged testis, which could contribute to a larger production of inhibin per testis. At this age increased levels of pituitary FSH were found *in vivo* (*appendix*

paper 3: Table 1) as well as *in vitro* (*appendix* paper 3: Fig. 1). At older ages, the level of plasma FSH from hemicastrated animals returned to that observed in control rats and comparable release patterns of FSH were noted when hemipituitary glands from control and unilaterally castrated 42- or 63-day-old rats were incubated *in vitro*.
Thus, the defective negative feedback regulation of pituitary FSH secretion after unilateral orchiectomy had not been compensated completely after the age of 21 days despite the presence of an increased number of Sertoli cells in the remaining testis, as was also observed by Putra & Blackshaw (1982) and Hochereau-de Reviers & Courout (1978). At the older ages, the relationship between numbers of Sertoli cells and pituitary FSH secretion becomes less strong (de Jong & Sharpe, 1977b), suggesting that inhibin feedback of FSH is especially important in the prepubertal animals.
Whether there is a decrease in pituitary sensitivity to inhibin with age remains to be determined. Contradictory results were reported by Hermans *et al.* (1980) and Steinberger (1979), while Sheth *et al.* (1980) reported that pituitary sensitivity for inhibin decreased with age as a result of reduction of numbers of inhibin binding sites at the pituitary level. However, these binding studies were carried out with ^{125}I-labelled human seminal plasma preparations, while it has been established that the inhibin-like material from human seminal plasma differs from that which is produced by Sertoli cells (see section 2.4). Hermans *et al.* (1980) found that the relative pituitary response to exogenous inhibin remained constant with age in male rats, although the relative increase in levels of FSH after short term castration decreased with age (Hermans, 1982). In contrast, Steinberger (1979) indicated that anterior pituitary cells isolated from adult rats were considerably more sensitive to inhibin activity Sertoli cell culture media from 29-day-old rat testes than pituitary cells obtained from immature animals.

5.3 Effects of exogenous inhibin on the pituitary-gonadal axis

5.3.1 Introduction

In chapter 2 (section 2.3), it has been discussed that steroid-free inhibin containing preparations selectively suppress peripheral levels of FSH, when injected into immature or adult male or female rats.
In *in vitro* experiments (see chapter 4) it was observed that relatively large amounts of inhibin activity are produced by Sertoli cells from prepubertal testes. Since the relative increase of peripheral FSH concentrations immediately after castration in rats decreases with age at operation, it has been postulated that inhibin is more important in the feedback regulation of pituitary FSH secretion during the prepubertal period, when the Sertoli cell population is still dividing, than during the post pubertal period of life when the Sertoli cell population has been established (Hermans, 1982). If the control of FSH secretion is indeed important for testicular development during this period chronic treatment with inhibin containing preparations in developing rats should induce a reduction in peripheral FSH

concentration and thus an inhibition of testis growth. This hypothesis was tested in the experiments described and discussed in the following sections of chapter 5.

5.3.2 Effects of administration of different doses of bovine follicular fluid on testicular development in rats

Chronic treatment of newborn rats with 0.50 ml charcoal-treated bovine follicular fluid (bFF) per 100 g body weight for 2 weeks caused a *non* significant suppression of testicular weight at 2 weeks of age, while testis weights at one week after cessation of this treatment were also not different from those in control animals (data not shown). When daily injections with bFF were started at a later age (22 days) no significant effects on testicular weights were found after four days (data not shown). Twice daily injections with the same dose of bFF (0.50 ml/100 g body weight) revealed significant suppressive effects on testicular weights at 2 weeks of age, while this difference was not significant when treatment was started one week after birth (Table 5.1). Normal spermatogenesis was observed in cross sectional areas from testes of these animals (not shown).

Table 5.1 Testis weights and hormone levels in male rats after 2 weeks of *twice* daily injections with *0.50* ml charcoal-treated follicular fluid (cbFF), charcoal-treated plasma from castrated cow or saline (contr.) per *100* g body weight, starting on day 1 or 7 of life.
Data are expressed as means ± S.E.M. (n=4-14)

AGE[a]	TREATMENT		TESTES WEIGHT	PLASMA		PITUITARY		INHIBIN (U/paired
	I	II	(mg)	FSH (μg/l)	LH	FSH (μg/gland)	LH	testes)
2	contr.	1+2	83 ± 4	350 ± 27	n.d.§	31 ± 2	n.d.	102 ± 6
	cbFF	1+2	67 ± 4[b]	265 ± 17[b]	n.d.	13 ± 2[b]	n.d.	72 ± 4[b]
3	contr.	2+3	168 ± 12	542 ± 49	29 ± 10	37 ± 2	50 ± 6	142 ± 14
	cbFF	2+3	226 ± 18[c]	595 ± 38	50 ± 19	27 ± 1[b]	69 ± 4[b]	137 ± 19

c and b : P < 0.02 and P < 0.01 respectively when compared with appropriate control (Student's t-test)
§ n.d. : not determined
a : age at autopsy in weeks
I : treatment preparation
II : treatment period in weeks

Table 5.2 Organ weights and hormone levels in male rats after daily injection for 2 or 3 weeks with *1.00* ml charcoal-treated bovine follicular fluid (bFF), charcoal-treated plasma from a castrated cow or saline (contr.) per *100* g body weight, starting the day after birth.
Mean ± S.E.M.

AGE[a]	TREATMENT I	TREATMENT II	No. of rats	TESTES WEIGHT (mg)	PLASMA FSH (μg/l)	PLASMA LH (μg/l)	PITUITARY FSH (μg/gland)	PITUITARY LH (μg/gland)
2	contr.		9	84 ± 3	358 ± 52	54 ± 34	27 ± 2	47 ± 7
	bFF	1+2	6	74 ± 2[c]	342 ± 46	8 ± 1	17 ± 2[b]	43 ± 5
3	contr.		18	246 ± 7	567 ± 48	6 ± 2	55 ± 3	73 ± 6
	bFF	1+2	6	241 ± 9	624 ± 126	38 ± 18	53 ± 11	89 ± 18
	bFF	1+2+3	6	208 ± 5[b]	513 ± 30	42 ± 37	26 ± 3	60 ± 12

c and b : $P < 0.02$ and $P < 0.01$ respectively when compared with appropriate control (Student's t-test)
a : age at autopsy in weeks
I : treatment preparation
II : treatment period in weeks

At 2 weeks of age plasma and pituitary FSH were suppressed by this treatment, and the smaller testes contained less endogenous inhibin activity when compared with controls. In the older group of animals, the pituitary content of FSH was decreased, while LH levels in the pituitary gland were higher than in control animals (Table 5.1). No significant effects on seminal vesicles or prostate weights were detected (data not shown).
Finally, single injections of 1.00 ml bFF/100 g body weight were given each day for 2 or 3 weeks starting on the day after birth. Animals were killed at 2 or 3 weeks of age, 24 h after the last injection or at the age of 9 weeks. Significantly lower testicular weights were observed one day after the last of the injections of bFF when the period of injections lasted for 2 or 3 weeks (Table 5.2), but after cessation of the chronic treatment, these weights returned to values as found in controls at 9 weeks of age (data not shown). No significant effects were observed on plasma gonadotrophin concentrations. However, the pituitary gland contained significant less FSH after a treatment of 2 or 3 weeks. One week after cessation of chronic treatment during 2 weeks with bFF, control values of pituitary FSH were observed in the bFF-treated animals (Table 5.2). Pituitary LH concentrations were not

affected by these treatments: no significant effects were detected on weights of accessory sex organs either (data not shown).

5.3.3 Discussion

In contrast to the short term effects of treatment with inhibin containing preparations on peripheral concentrations of FSH (Hermans, 1982), daily treatment of prepubertal male animals with the same dose (0.50 ml bFF/100 g body weight) failed to suppress peripheral FSH levels for longer periods. This failure of bFF to reduce plasma FSH significantly may be accounted for by the 24 h time interval between the injections. On the other hand, Sarvamangala and Sheth (1984) injected inhibin activity from ram testes subcutaneously into neonatal male rats from day 5 to day 20 of age. Two hours after the last injection, animals were killed and a significant decrease of testicular weight was detected without any effect on the accessory sex organs. Spermatogenesis was affected and reduced plasma FSH levels (to 80% of control) were observed in the inhibin treated rats at 20 days of age. Results from a study of de Jong *et al.* (1978) showed that daily treatment of 21-day-old male rats for up to 12 days with bFF caused a significantly lower testicular weight from day 4 onwards: after this day plasma FSH levels returned to control values. These data suggest that effects of exogenous inhibin on FSH secretion are short lived and compensatory effects due to hypersecretion of FSH as found in female animals (Hermans, 1982) may have occurred. Therefore, twice daily injections were applied in prepubertal rats. This treatment resulted in decreased levels of FSH in the pituitary gland and in peripheral blood, and in a decrease of testicular weight (Table 5.1). However, when the same treatment was started at the age of 1 week, smaller effects were found at 3 weeks of age: although the pituitary gland contained less FSH in bFF-treated animals when compared with levels in controls, peripheral levels of FSH were not affected and normal testicular weights were observed. This indicates that treatment with inhibin has to be started immediately after birth in order to affect testicular weight, as also becomes clear from Table 5.2, where it is shown that it is possible to suppress testicular weight at 3 weeks of age by a 3 week treatment with follicular fluid. Wilkinson *et al.* (1980) reported that pituitary glands from inhibin-treated rats show a high sensitivity to stimulation with LH-RH *in vitro*; after stimulation significantly more FSH is secreted than by control glands. This increased sensitivity might explain the increase of pituitary LH content as shown in Table 5.1. Similarly, de Jong *et al.* (1978) reported increased peripheral levels of LH after injection of bFF into immature male rats. It is concluded from this study that investigation of the regulation of testis size by exogenous inhibin in immature male rats requires repeated administration of inhibin, due to a number of compensating mechanisms, which make it difficult to disequilibrate the gonadotroph-Sertoli cell axis.

5.4 Effects of antibodies against inhibin on pituitary and gonadal functions in prepubertal rats

5.4.1 Introduction

Several authors have developed polyclonal antisera against inhibin and have used these antibodies for the characterization of inhibin from various sources. Antibodies raised against pFF inhibin (Channing *et al.*, 1982) or bFF inhibin (de Jong *et al.*, 1982b; van Dijk *et al.*, 1986) were shown to neutralize inhibin activity in monkeys *in vivo* (Channing *et al.*, 1982) and in porcine, human and bovine (de Jong *et al.*, 1982b) follicular fluids *in vitro*. Van Dijk et al (1986) used an ovine antiserum against bovine follicular fluid inhibin for immunoneutralization studies *in vitro*. This antiserum neutralized inhibin bioactivity from ovine and rat testicular as well as ovarian origin although the degree of immunoneutralization was higher for preparations of female than for those of male origin. Antibodies against purified inhibin from bFF (Mc Lachlan *et al.*, 1986a; Robertson *et al.*, 1986a) showed cross-reactivity with inhibin activity in hFF and human serum. This antiserum immunoneutralized the biological activity of inhibin containing preparations from gonadal fluids of bovine, human, rat and ovine origin (Mc Lachlan *et al.*, 1986a). In our study an ovine antiserum against an approximately 20 to 30 fold purified inhibin containing preparation from bovine follicular fluid (van Dijk *et al.*, 1986) was injected into prepubertal male rats, in order to investigate the influence of *in vivo* immunoneutralization of inhibin on early testicular development.

5.4.2 Results and discussion

Table 5.3 Weight of testes and hormone levels in 14-day-old male rats treated repeatedly with 30 µl inhibin antiserum/injection (s.c), starting one day after birth. Mean ± S.E.M.

TREATMENT*	No of rats	TESTES WEIGHT (mg)	PLASMA FSH (µg/l)	PLASMA LH (µg/l)	PITUITARY FSH (µg/gland)	PITUITARY LH (µg/gland)
CONTROL	5	66.7 ± 3.5	332 ± 20	30 ± 21	24.6 ± 1.4	50.1 ± 4.4
AS 35; 1x/2 days	5	66.5 ± 8.6	307 ± 39	5.8 ± 1.3	26.5 ± 3.2	48.3 ± 5.5
AS 35 3x/2 weeks	4	70.5 ± 7.2	325 ± 29	12.8 ± 3.3	26.8 ± 3.3	50.0 ± 4.2

*: AS 35, ovine antiserum against bovine ovarian inhibin injected at the frequency as indicated

Data on testicular weights and hormone levels from prepubertal rats treated with the antiserum against inhibin are summarized in Table 5.3. Passive immunization of newborn male rats with subcutaneous injections of 30 μl inhibin antiserum caused no significant effects on pituitary or testicular function at 2 weeks of age, while this ovine antiserum has been shown to block testicular inhibin activity as present in rat Sertoli cell conditioned medium or testicular homogenates *in vitro* (van Dijk *et al.*, 1986). Apparently, the application of a repeated dose of 30 μl in prepubertal male rats (body weight at birth: 6 g) was not effective in the *in vivo* immunoneutralization of peripheral inhibin activity which should have resulted in increased testicular weights. This phenomenon could reflect absence of inhibin in the circulation, or presence of too high levels of inhibin in the circulation at this age. It is unlikely that these results were due to a neutralizing activity of second antibodies produced by the treated animal against the foreign ovine material, since injections were already started in neonatal rats at an age at which immunocompetence has not yet been established (Dijkstra & Döpp, 1983).

5.5 Effects of exogenous gonadotrophins on testicular development and endogenous hormone levels

There is a close temporal relationship between the pattern of FSH and testicular growth during the prepubertal period, whereas LH is hardly detectable in peripheral plasma of rats of these ages (*appendix* paper 1). Therefore, it seems reasonable to postulate that FSH stimulates initial testis growth, while during later stages of development, increasing levels of LH may influence Leydig cell function.
Orth (1984) has shown that after decapitation *in utero* or injection of antiserum to rat-FSH into foetuses a dramatic reduction of the percentage of Sertoli cell nuclei incorporating [^{3}H]thymidine arises. Addition of exogenous FSH to organ cultures of the testes from the decapitated fetuses caused a dramatic increase in the percentage of labeled Sertoli cells. Other authors have already demonstrated that injections of gonadotrophins in prepubertal animals cause an increase of testis weight probably due to an increase of the number of Sertoli cells (bull: Meyers & Swanson, 1983; rat: Bentley *et al.*, 1978). Long term effects on testis and pituitary function after such a treatment have been reported in *appendix* paper 4. The results of these studies indicate that FSH is important for the establishment of the Sertoli cell population in the testis, while LH affects the interstitial volume and the function of the Leydig cells. When only FSH was injected pituitary FSH was suppressed to 5% of control levels concomitant with increased levels of testicular inhibin in the testes at 3 weeks of age. Plasma FSH levels exhibited significantly suppressed values until 9 weeks of age, presumably as a result of increased concentrations of inhibin in the circulation. Administration of FSH together with LH resulted in suppressed levels of both FSH and LH in the pituitary gland to 3 and 2% of control, respectively, concomitant with increased levels of testicular inhibin in the testes and increased weight of the accessory sex organs at 3 weeks of age. However, to maintain increased testicular weights at older ages, treatment for a

longer period will be needed because further development of the testis is retarded after the cessation of the injections. This is probably due to the suppression of endogenous gonadotrophins. However, an increase of the length of seminiferous tubules was still observed at 9 weeks of age and more inhibin was measured in the enlarged testes of animals which had been treated with FSH alone. From the results of these experiments it is concluded that FSH and, therefore, inhibin are important in the control of the number of Sertoli cells in the testis and therefore of testicular size. Although Sertoli cells do not possess receptors for LH (Fritz, 1978), administration of LH may have indirect effects on Sertoli cell function via paracrine regulation by Leydig cells (Benhamed *et al.*, 1986; Perrard-Sapori *et al.*, 1986; Verhoeven & Cailleau, 1987). In this respect, Chemes *et al.* (1979) showed that in rats injected from birth to 32 days of age with antibodies against LH the differentiation and growth of Sertoli cells were severely inhibited while neutralization of FSH caused specific changes in the endoplasmic reticulum of Sertoli cells. However, these results have to be interpreted cautiously, because antisera raised against FSH or LH are not fully specific as long as anti α-subunit antibodies are present. Therefore, highly specific antibodies have been developed against the hormone-specific β-subunit (Poirier *et al.*, 1984). Passive immunization of lambs with antibodies against the β-subunit of ovine FSH from 4 to 28 weeks reduced the testicular development from week 12 to week 18: the number of Sertoli cells per testis was reduced significantly as well as the daily production of spermatocytes (Courot *et al.*, 1984b). Passive immunization with the same antiserum injected in adult rams also revealed that the production of spermatocytes was decreased, while circulating levels of testosterone were not affected (Courot *et al.*, 1984a).
Passive or active immunization against FSH is a possible approach to study the role of FSH in the regulation of testicular function, but highly specific antibodies are needed to exclude side effects.

5.6 Conclusions

From the results presented in this chapter and results described in the literature it was concluded that testis size can be manipulated in prepubertal rats, where Sertoli cells are still dividing, by applying a number of experimental protocols:

1. Removal of a source of inhibin, *i.e.* one of the testes early in life results in a selective increase of peripheral concentrations of FSH. This increase is not sufficient to stimulate the total number of Sertoli cells or the inhibin production in the remaining testis to the levels found in intact rats, or to suppress pituitary FSH to control levels at the age of 21 days. While no further relative growth of the testis occurs after this age, at older ages normal gonadotrophin concentrations are found.
2. Injections of high doses of exogenous FSH during the first 3 weeks of life cause a significant increase of testicular weight. These enlarged testes contain and secrete increased amounts of inhibin resulting in suppressed endogenous FSH levels.
3. Injections of both exogenous FSH and LH cause an even larger increase of testicular

weight. In addition to an increase in tubular length, an increase of interstitial volume was observed. In this model inhibin and testosterone are likely to be responsible for the suppression of endogenous gonadotrophins.

4. A suppression of endogenous FSH by repeated injection of inhibin containing preparations such as bFF leads to a reduced testicular weight due to suppression of endogenous FSH. However, no effects on pituitary and testicular function were observed in rats injected with antibodies against bFF proteins. This may reflect a low affinity of these antibodies against substances (including inhibin) in ovarian follicular fluid for testicular inhibin.
5. The results on pituitary and testicular function obtained after early hemicastration, and prepubertal treatment with exogenous gonadotrophins or inhibin containing preparations indicate the significance of inhibin in the regulation of FSH secretion in prepubertal rats.

6

GENERAL DISCUSSION

6.1 Introduction

Regulation of the development and functions of the testis is achieved by a number of endocrine changes which take place in the compartments of the hypothalamus-pituitary-gonadal axis. In this interaction system, the feedback regulation of pituitary gonadotrophin secretion is not only dependent on the action of gonadal steroids at the hypothalamic or pituitary level, but also on the feedback action of a water soluble gonadal substance, inhibin (McCullagh, 1932), which exerts a preferential suppressive effect on pituitary FSH secretion. With the development of improved bioassay systems for the detection of inhibin activity (see chapter 2) studies on the existence and physiological significance of inhibin became possible. The studies described in this thesis were performed to investigate the importance of inhibin in the regulation of FSH secretion in the male rat. We have tried to identify which cells are involved in the production of inhibin activity and which factors can regulate inhibin production *in vitro* and *in vivo*. For the studies described in this thesis bioactive inhibin has been determined and we will discuss the application of the bioassay method of inhibin with respect to possible interferences of other active products in section 6.2.
In section 6.3 the significance of inhibin for testicular development in rats and the physiological role of inhibin with respect to male reproduction are discussed.

6.2 The bioassay of inhibin

Inhibin is a gonadal dimeric polypeptide hormone composed of dissimilar α and β subunits which are derived from separate genes (see chapter 2). Assays for inhibin activity based on specific suppression of FSH secretion or cell content of rat anterior pituitary cells in culture have been extensively used for the estimation of inhibin activity (see chapter 2). However, recent information indicates that $\beta_A\beta_A$ or $\beta_A\beta_B$ homo-or heterodimers (activins) may be present in *non* purified inhibin containing preparations.In contrast to the effects of inhibing these dimers can stimulate FSH release, when added to pituitary cells in culture (Ling *et al.*, 1986; Vale *et al.*, 1986). In this way, bioassays of inhibin may underestimate the actual level of the hormone in a sample if the β dimers are also present and counteract the effects of

inhibin. Results of Northern blot analyses of rat testicular RNA using rat ovarian cDNA probes show that mRNAs encoding the α and β_B subunits of inhibin were present, while the mRNA encoding the β_A chain was absent. This could indicate that rat Sertoli cells might not be able to synthesize or secrete activins (Esch *et al.*, 1987). This is result is in agreement with data obtained by Toebosch *et al.* (1988). The latter authors could not detect the mRNA for the β_A-subunit of inhibin in cultured Sertoli cells from 21 to 23-day-old rats in the absence or presence of FSH using a bovine β_A-subunit probe. The question if β_A-subunits are present in testicular material from younger animals has to be investigated yet. It is unlikely, however, that the presence of these substances would influence the conclusions reached in chapter 3, since in case of underestimation of inhibin activity in the medium of immature Sertoli cells, differences between immature and adult inhibin productions would only become larger.

Because of the possible interferences of activin or TGF-β (Ying *et al.*, 1986), in bioassays for inhibin, a critical reevaluation of the bioassay method for inhibin will be necessary to establish whether "inhibin" bioactivity correlates with the concentration of inhibin molecules in tissues or fluids. It would be useful to compare the results of measurements of inhibin by bioassay with those of recently developed radioimmunoassay systems (McLachlan *et al.*, 1986a), although using the latter method an overestimation of the amount of inhibin in a sample may occur because loose subunits might be detected. For this reason, final validation of methods for the estimation of inhibin can be obtained only after chromatographic separations of the various dimers and monomers, which are translation products of the mRNAs of the "inhibin family".

6.3 Physiological role and significance of inhibin in the male

Since inhibin preferentially affects the pituitary biosynthesis and secretion of FSH, much of the earlier evidence for regulation of inhibin production was obtained from *in vivo* experiments in which peripheral levels of FSH were used as a parameter for inhibin secretion. In male patients with oligospermia (Bramble *et al.*, 1975) and in surgically induced cryptorchidism in rats (Swerdloff *et al.*, 1971) normal levels of plasma LH and testosterone were reported while concentrations of FSH in blood were increased. In both studies it was concluded that this increase of FSH levels was due to a decrease of inhibin production. Because concentrations of inhibin in blood are too low to detect with the currently available *in vitro* bioassay methods, levels of inhibin in the testis have been correlated with peripheral levels of FSH by a number of authors. In this way, Au *et al.* (1984b) have shown that testicular inhibin content did not correlate directly with changes of peripheral FSH levels after experimental cryptorchidism. In further studies, these authors applied efferent duct ligation as a more sensitive technique to monitor changes in inhibin production by the testis (Au *et al.*, 1985). However, this method is not practicable in immature rats. In addition, for inhibin secretion in adult animals, it is difficult to indicate which amount of the produced inhibin is secreted into the circulation and which part is excreted with the rete testis fluid

(Gunsalus *et al.*, 1980). Evidence for inhibin production *in vivo* came from experiments in adult animals, but the regulation of the inhibin production under *in vitro* conditions has been studied mainly using immature Sertoli cells which are relatively easy to isolate and can be kept in culture for several days. In this thesis much attention has been paid to factors, which regulate inhibin production *in vitro*, because contradicting results had been published earlier by several authors (chapter 4). It appears that Sertoli cells *in vitro* secrete larger amounts of inhibin at raised temperatures, indicating that the decreased levels of inhibin after cryptorchidism are rather due to a damaged Sertoli cell function than a direct effect on Sertoli cell metabolism.

The production of inhibin by Sertoli cells probably is related also to the age of the animal and to the presence of spermatogenic cells: a fast decrease of the basal production of inhibin per 10^6 Sertoli cells in culture was observed after 2 weeks of age. The fact that around day 21 of age the blood-testis barrier becomes functional, may result in the gradually increasing levels of inhibin as measured in total testis tissue throughout development of the testis. Furthermore, the proliferative activity of Sertoli cells (Orth, 1984) and the inhibin production per cell (chapter 4) decrease after the age of 3 weeks. Hence, it is likely that the positive correlation between the amount of inhibin present in total testis and testis weight after 3 weeks of age is not a real estimation of the production rate of inhibin *in vivo*, but rather a measure of intracellular or intratubular accumulation of the hormone.

In vitro, FSH appears to stimulate the inhibin production by immature Sertoli cells (chapter 4), while testosterone shows opposite effects on the inhibin production at all ages. It has been reported earlier that Sertoli cells become refractory to FSH by 25 days of age despite an increase of the number of FSH receptors per testis (Steinberger *et al.*, 1978). To create endogenous increased FSH levels *in vivo*, the source of inhibin production was partly removed by neonatal hemicastration. This treatment resulted in elevated peripheral levels of FSH during the prepubertal period. This increase of FSH is likely to be the cause for compensatory growth of the remaining testis. Although the weight of the remaining testis never reached that of the two testes in control animals, normal levels of FSH were found in 42- and 63-day-old neonatally hemicastrated rats. This observation suggests that regulation of pituitary FSH secretion by testicular inhibin may not be as important in adult rats.

Since FSH can regulate the number of Sertoli cell divisions in immature animals, experiments were performed in which exogenous FSH or inhibin containing preparations were injected chronically into prepubertal rats in order to increase or decrease the population of Sertoli cells, respectively, and to study the development of the function of the pituitary-testicular axis in such experimental models. Administration of exogenous gonadotrophins in prepubertal rats caused enlargement of the testes concomitant with an increased inhibin production as reflected by decreased pituitary and plasma FSH levels. In addition, the larger testes contained more inhibin activity probably due to an increase of the number of Sertoli cells per testis. In contrast, injection of follicular fluid in prepubertal rats resulted in decreased pituitary FSH and lowered testis weight as compared with controls.

From these results we have concluded that the hypothalamic-pituitary-testicular axis is a rather dynamic system during the prepubertal period: changes in levels of FSH or inhibin

influence the number of Sertoli cells and therefore, spermatogenesis and the reproductive capacity at adult age. In contrast, disturbances of the FSH-inhibin feedback loop do not readily result in counterbalancing effects.
It is difficult to indicate which role inhibin plays in adult animals. However, it may be that in seasonal breeders FSH and inhibin are needed to establish a normal number of Sertoli cells per testis at the start of each breeding season.
Summarizing, FSH and inhibin are important in the regulation of the number of Sertoli cells in the testis of immature animals, thus determining quantitative aspects of spermatogenesis in adult animals. Since it has been shown that testosterone and LH are more important than FSH for spermatogenesis in adult testis, it is unlikely that inhibin may play an important role in fertility control. Further research is necessary to elucidate the possible intratesticular role of inhibin and its related peptides and to determine whether inhibin measurements are of clinical significance for the study of gonadal function in males.

7

REFERENCES

Alexander, D.C., and Miller, W.L. (1982). Regulation of ovine follicle-stimulating hormone β-chain mRNA by 17β-estradiol *in vivo* and *in vitro*. J. Biol. Chem. 257, 2282-2286.

Au, C.L., Robertson, D.M., and de Kretser, D.M. (1983). *In vitro* bioassay of inhibin in testes of normal and cryptorchid rats. Endocrinology 112, 239-244.

Au, C.L., Robertson, D.M., and de Kretser, D.M. (1984a). An *in-vivo* method for estimating inhibin production by adult rat testes. J. Reprod. Fertil. 71, 259-265.

Au, C.L., Robertson, D.M., and de Kretser, D.M. (1984b). Relationship between testicular inhibin content and serum FSH concentrations in rats after bilateral efferent duct ligation. J. Reprod. Fert. 72, 351-356.

Au, C.L. Robertson, D.M., and de Kretser, D.M. (1985). Effects of hypophysectomy and subsequent FSH and testosterone treatment on inhibin production by adult rat testes. J. Endocr. 105, 1-6.

Au, C.L., Robertson, D.M., and de Kretser, D.M. (1986). Measurement of inhibin and an index of inhibin production by rat testes during postnatal development. Biol. Reprod. 35, 37-43.

Bablok, Z., Janczewski, Z., Kwiatkowska, Z., and Fracki, S. (1978). The relationship between plasma FSH, testosterone levels and testicular histology in males with azoospermia. Andrologia 10, 502-505.

Baker, H.W.G., Bremner, W.J., Burger, H.G., de Kretser, D.M., Dulmanis, A., Eddie, L.W., Hudson, B., Keogh, E.J., Lee, V.W.K., and Rennie, G.C. (1976). Testicular control of follicle-stimulating hormone secretion. Recent Prog. Horm. Res. 32, 429-476.

Baker, H.W.G., Eddie, L.W., Higginson, R.E., Hudson, B., Keogh, E.J., and Niall, H.D. (1981). Assays of inhibin. In: Intragonadal Regulation of Reproduction (eds. P. Franchimont and C.P. Channing) pp. 193-228. Academic Press, London.

Baker, H.W.G., Eddie, L.W., Higginson, R.E., Hudson, B., and Niall, H.D. (1982). Studies on the purification of inhibin from ovine rete testis fluid. In: Non-steroidal Regulators in Reproductive Biology and Medicine (eds. T. Fujii and C.P. Channing) pp. 123-132. Pergamon Press, Oxford.

Baker, H.W.G., Eddie, L.W., Hudson, B., and Niall, H.D. (1985). Variability in the apparent molecular weight of inhibin. Austr. J. Biol. Sci. 38, 327-338.

Baker, M.E. (1985). The PDC-109 protein from bovine seminal plasma is similar to the gelatin-binding domain of bovine fibronectin and a Kringle domain of human tissue-type plasminogen activator. Biochem. Biophys. Res. Commun. 130, 1010-1014.

Bandivdekar, A.H., Vijayalakshmi, S., Jaswaney, V.L., and Sheth A.R. (1981). Inhibin in human placenta. Indian J. Exp. Biol. 19, 744-745.

Beaumont, H.M. (1960). Changes in the radiosensitivity of the testis during foetal development. Int. J. Rad. Biol. 2, 247-256.

Bellvé, A.R., and Feig, L.A. (1984). Cell proliferation in the mammalian testis: biology of the seminiferous growth factor (SGF). Rec. Progr. Horm. Res. 40, 531-567.

Benahmed, M., Morera, A.M., and Chauvin, M.A. (1986). Evidence for a Sertoli cell, FSH-suppressible inhibiting factor(s) of testicular steroidogenic activity. Biochem. Biophys. Res. Commun. 139, 169-178.

Bentley, M.J., Gass, G.H., and Leidl, W. (1978). Effects of FSH and LH administration on the testes and seminal vesicles. Andrologia 10, 357-361.

Bibro, M.C., Fourcroy J.L., and Eil, C. (1984). A unique case of Sertoli cell only syndrome with normal gonadotropins. Fert. & Steril. 42, 655-658.

Bicsak, T.A., Tucker, E.M., Cappel, S., Vaughan, J., Rivier, J., Vale, J., and Hsueh, A.J.W. (1986). Hormonal regulation of granulosa cell inhibin biosynthesis. Endocrinology 119, 2711-2719.

Bicsak, T.A., Vale, W., Vaughan, J., Tucker, E.M., Cappel, S., and Hsueh, A.J.W. (1987). Hormonal regulation of inhibin production by cultured Sertoli cells. Mol. Cell. Endocr. 49, 211-217.

Boitani, C., Mather, J.P., and Bardin, C.W. (1986). Stimulation of adenosine 3',5'-monophosprate production in rat Sertoli cells by α-melanotropin-stimulating hormone (αMSH) and desacetyl αMSH. Endocrinology 118, 1513-1518.

Borland, K., Mita, M., Oppenheimer, C.L., Blinderman, L.A., Massagué, J., Hall, P.F., and Czech, M.P. (1984). The actions of insulin-like growth factors I and II on cultured Sertoli cells. Endocrinology 114, 240-246.

Bramble, F.J., Houghton, A.L., Eccles, S., O'Shea, A., and Jacobs, H.S. (1974). Reproductive and endocrine function after surgical treatment of bilateral cryptorchidism. Lancet 2, 311-314.

Bramble, F.J., Houghton, A.L., Eccles, S., Murray, M.A.F., and Jacobs, H.S. (1975). Specific control of follicle stimulating hormone in the male: postulated site of action of inhibin. Clin. Endocrinology 4, 443-449.

Brown (1985). Immunohistochemical localization of the transferrin receptor in the eminiferous epithelium of the rat. Gamete Res. 12, 317-326.

Burger, H.G., de Kretser, D.M., and Findlay, J.K. (1988). Inhibin-*non* steroidal regulation of FSH secretion. Raven Press, New York, in press.

Cahoreau, C., Blanc, M.R., Dacheux, J.L., Pisselet, C., and Courot, M. (1979). Inhibin activity in ram rete testis fluid: depression of plasma follicle-stimulating hormone and luteinizing hormone in the castrate and cryptorchid ram. J. Reprod. Fertil. Suppl. 26, 97-116.

Carson, D.D., Rosenberg L.I., Blaner, W.S. Kato, M., and Lennarz, W.J. (1984). Synthesis and secretion of a novel binding protein for retinol by a cell line derived from Sertoli cells. J. Biol. Chem. 259, 3117-3123.

del Castillo, E.B., Trabucco, A., and de la Balze, F.A. (1947). Syndrome produced by absence of the germinal epithelium without impairment of the Sertoli or Leydig cells. J. Clin. Endocr. 7, 493-502.

Cate, R.L., Mattaliano, R.J., Hession, C., Tizard, R., Farber, N.M., Cheung, A., Ninfa, E.G., Frey, A.Z., Gash, D.J., Chow, E.P. Fisher, R.A., Bertonis, J.M., Torres, G., Wallner, B.P., Ramachandran, K.L., Ragin, R.C., Manganaro, T.F., MacLaughlin, D.T., and Donahoe, P.K. (1986). Isolation of the bovine and human genes for Müllerian inhibiting

substance and expression of the human gene in animal cells. Cell 45, 685-698.
Channing, C.P., Anderson, L.D., and Hodgen G.D. (1979). Inhibitory effect of charcoal-treated porcine follicular fluid upon serum FSH levels and follicular development in the rhesus monkey. In: Advances in experimental medicine and biology vol. 112 "Ovarian follicular and corpus luteum function" (eds. C.P. Channing, J. Marsh, W. Sadler) pp. 407-415. Plenum, New York.
Channing, C.P., Tanabe, K., Turner, C.K., and Hodgen, G.D. (1982). Antisera to porcine follicular fluid in monkeys: neutralization of human and pig inhibin activity *in vivo* and *in vitro*. J. Clin. Endocr. Metab. 55, 481-486.
Channing, C.P., Tanabe, K., Chacon, M., and Tildon, J.T. (1984). Stimulatory effects of follicle-stimulating hormone and luteinizing hormone upon secretion of progesterone and inhibin activity by cultured infant human ovarian granulosa cells. Fert. Steril. 42, 598-605.
Chappel, S.C., Acott, T., and Spies, H.G. (1979). Inhibition of *in vivo* and *in vitro* FSH secretion by steroid-free hamster ovarian extracts. In: Ovarian Follicular and Corpus Luteum Function (eds. C.P. Channing, J.M. Marsh and W.A. Saddler) pp. 361-373. Plenum Press, New York.
Chappel, S.C., Holt, J.A., and Spies, H.G. (1980). Inhibin: differences in bioactivity within human follicular fluid in the follicular and luteal stages of the menstrual cycle. Proc. Soc. Exp. Biol. Med. 163, 310-314.
Chari, S., Duraiswami, S., and Franchimont, P. (1976). A convenient and rapid bioassay for inhibin. Horm. Res. 7, 129-137.
Chari, S., Duraiswami, S., and Franchimont, P. (1978). Isolation and characterization of inhibin from bull seminal plasma. Acta Endocr. Copenh. 87, 434-448.
Chari, S., Hopkinson, C.R.N., Daume, E., and Sturm, G. (1979). Purification of "inhibin" from human ovarian follicular fluid. Acta Endocr. Copenh. 90, 157-166.
Chemes, H.E., Dym, M., and Ray, H.G.M. (1979). Hormonal regulation of Sertoli cell differentiation. Biol. Reprod. 21, 251-262.
Chen, C.L.C., Mather, J.P., Morris P.L., and Bardin, C.W. (1984). Expression of pro-opiomelanocortin-like gene in the testis and epididymis. Proc. Natl. Acad. Sci. USA 81, 5672-5672.
Cheng, C.Y., and Bardin, C.W. (1986). Rat testicular testibumin is a protein responsive to follicle stimulating hormone and testosterone that shares immunodeterminants with albumin. Biochemistry 25, 5276-5288.
Chevalier, M., and Dufaure, J-P. (1981). The effect of FSH, testosterone and Ca^{2+} on Sertoli cell microfilaments in the immature pig testis. Biol. Cell. 41, 105-112.
Childs, G.V., Hyde, C., Naor, Z., and Catt, K. (1983). Heterogeneous luteinizing hormone and follicle-stimulating hormone storage patterns in subtypes of gonadotropes separated by centrifugal elutriation. Endocrinology 113, 2120-2128.
Childs, G.V. (1985). Shifts in gonadotropin storage following GnRH stimulation *in vitro*. Peptides 6, 103-107.
Childs, G.V., Unabia, G., and Tibolt R. (1985). How the fixation-embedding protocol affects the specificity and efficiency of immunocytochemical stains for gonadotropin subunits. Am. J. Anat. 174, 409-417.
Chin, W.W. (1985). Organization and expression of glycoprotein hormone genes. In: The Pituitary Gland (ed. H. Imura) pp. 103-125. Raven Press, New York.
Chowdhury, M., and Steinberger, E. (1976). Pituitary and plasma levels of gonadotrophins

in foetal and newborn male and female rats. J. Endocr. 69, 381-384.
Christensen, A.K., Wisner, J.R., and Orth, J. (1977). Preliminary observations on the localization of androgen and FSH receptors in the rat seminiferous tubule, studied by autoradiography at the light microscope level. In: The testis in normal and infertile men. (Eds. P. Troen and R. Nankin.) pp. 153-171. Raven Press, New York.
Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol. Rev. 52, 198-236.
Clermont, Y., and Perey, B. (1957). Quantitative study of the cell population of the seminiferous tubules in immature rats. Am. J. Anat. 100, 241-260.
Colberg, C., Graesslin, D., and Bettendorf, G. (1982). Inhibin in human seminal plasma: size heterogeneity and FSH as well as LH suppression. Acta Endocrinol. Suppl. 246, 92-93.
Counis, R., Corbani, M., and Jutisz M. (1983). Estradiol regulates mRNAs encoding precursors to rat LH and FSH subunits. Biochem. Biophys. Res. Commun. 14, 65-72.
Courot, M., Hochereau-de Reviers, M.T., Pisselet, C., Kilgour, K.J., Dubois, M.P., and Sairam, M.R. (1984a). Effect of passive immunization against ovine βFSH on spermatogenesis in the ram. In: The male in farm animal reproduction (ed. M. Courot) pp. 75-79. Current topics in Vet. Med. Anim. Sci. M. Nijhoff publ.
Courot, M., Pisselet, C., Kilgour, Sairam, M.R., and Dubois, M.P. (1984b). Development testiculaire, nombre de cellules de Sertoli et spermatogenese chez des agneaux immunises passivement contre βFSH ovine. In: Recent Progress in Cellular Endocrinology of the Testis (eds. J.M. Saez, M.G. Forest, A. Dacord, and J. Bertrand) Inserm 123, pp., 163-168.
Cunningham, G.R., Tindall, D.J., Huckins, C., and Means, A.R. (1978). Mechanisms for the testicular hypertrophy which follows hemicastration. Endocrinology 102, 16-23.
Currie, B.L., Warberg, J., Folkers, K., Bowers, C.Y., Paulsen, E., Mulder, J., and Paulsen F. (1977). Presence of peptide hormones that control gonadotropin secretion in extrahypothalamic areas of the brain. Biochem. Biophys. Res. Comm. 79, 1207-1211.
David, K., Dingemanse, E., Freud, J., and Laqueur, E. (1935). Uber krystallinisches mannliches Hormon aus Hoden (Testosteron), wirksamer als aus Harn oder aus Cholesterin bereitetes Androsteron. Hoppe-Seylers Z. Physiol. Chem. 233, 281-282.
Davies, R.V., Main, S.J., Young, M.G.W.L., and Setchell, B.P. (1976). Bioassay of inhibin-like activity in rete testis fluid and its partial purification. J. Endocr. 68, 26P-27P.
Davies, R.V., Main, S.J., and Setchell, B.P. (1978). Inhibin: evidence for its existence, methods of bioassay and nature of the active material. Int. J. Androl., Suppl. 2, 102-114.
Davies, R.V., Main, S.J., and Setchell, B.P. (1979). Inhibin in ram rete testis fluid. J. Reprod. Fert., Suppl. 26, 87-95.
Davis, S.R., Dench, F., Nikolaidis, I., Clements, J.A., Forage, R.G., Krozowski, Z., and Burger, H.G. (1986). Inhibin A-subunit gene expression in the ovaries of immature female rats is stimulated by pregnant mare serum gonadotrophin. Biochem. Biophys. Res. Comm. 138, 1191-1195.
Debeljuk, L., Arimura, A., and Schally, A.V. (1973). Pituitary and serum FSH and LH levels after massive and selective depletion of the germinal epithelium in the rat testis. Endocrinology 92, 48-54.
Decker, M.H., Loriaux, D.L., and Cutler Jr. G.B. (1981). A seminiferous tubular factor is not obligatory for regulation of plasma follicle-stimulating hormone in the rat. Endocrinology 108, 1035-1039.
Delic, J.I., Hendry, J.H., Morris, I.D., and Shalet, S.M. (1986). Dose and time relationships in

the endocrine response of the irradiated adult rat testis. J. Androl. 7, 32-41.
De Philip, R.M., and Kierzenbaum, A.L. (1982). Hormonal regulation of protein synthesis, secretion, and phosphorylaton in cultured rat Sertoli cells. Proc. Natl. Acad. Sci. USA 79, 6551-6555.
Derynck, R., Jarret, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B., and Goeddel, D.V. (1985). Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature Lond. 316, 701-705.
Dobos, M., Burger, H.G., Hearn, M.T.W., and Morgan F.J. (1983). Isolation of inhibin from ovine follicular fluid using reversed-phase liquid chromatography. Mol. Cell. Endocr. 31, 187-198.
Doctor, V. M., Sheth, A.R., Simha, M.M., Arbatti, N.J., Aaveri J.P.,and Sheth N.A. (1986). Studies on immunocytochemical localization of inhibin-like material in human prostatic tissue: Comparison of its distribution in normal, benign and malignant prostates. Br. J. Cancer 53, 547-554. Dodson, W.C., and Schomberg, D.W. (1987). The effect of TGFβ on FSH-induced differentiation of cultured rat granulosa cells. Endocrinology 120, 512-516.
Dorrington, J.H., and Armstrong D.T. (1975). Follicle-stimulating hormone stimulates estradiol-17β synthesis in cultured Sertoli cells. Proc. Natl. Acad. Sci. USA 72, 2677-2681.
van Dijk, S., Steenbergen, J., Gielen, J.Th., and de Jong, F.H. (1986). Sexual dimorphism in immunoneutralization of bioactivity of rat and ovine inhibin. J. Endocr. 111, 255-261.
van Dijk, S. (1986). Inhibin. Purification and characterization. (Ph.D. Thesis). Rotterdam: Erasmus Universiteit.
Dijkstra, C.D., and Döpp, E.A. (1983). Ontogenetic development of T- and B- lymphocytes and non-lymphoid cells in the white pulp of the rat spleen. Cell Tissue Res. 229, 351-363.
Eddie, L.W., Baker, H.W.G., Higginson, R.E., and Hudson, B. (1979). A bioassay for inhibin using pituitary cell cultures. J. Endocr. 81, 49-60.
Elias, K.A., and Blake, C.A. (1981). A detailed *in vitro* characterization of the basal follicle-stimulating hormone and luteinizing hormone secretion rates during the rat four-day estrous cycle. Endocrinology 109, 708-713.
Erickson, G.F., and Hsueh, A.J.W. (1978). Secretion of "inhibin" by rat granulosa cells *in vitro*. Endocrinology 103, 1960-1963.
Esch, F.S., Ling, N.C., Bohlen, P., Ying, S.Y., and Guillemin, R. (1983). Primary structure of PDC-109, a major protein constituent of bovine seminal plasma. Biochem. Biophys. Res. Commun. 113, 861-867.
Esch, F., Shimasaki, S. Ueno, N., Ling, N., and Ying, S-Y, (1987). cDNA cloning and DNA sequence analysis of rat ovarian inhibin. The Endocrine Society, Indianapolis, Abstract, 15, p 25.
Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y., and Shibai, H. (1987). Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem. Biophys. Res. Commun. 142, 1095-1103.
Fabbri, A., Tsai-Moris, C.H., Luna, S., Fraioli, F., and Dufau, M.L. (1985). Opiate receptors are present in rat testis. Identification and localization in Sertoli cells. Endocrinology 117, 2544-2546.

Fachini, G., Toffoli, C., Gaudiano, A., Marabelli, M., Polizzi, M., and G. Mangili (1963). Nemasperm and pituitary inhibition. Nature Lond. 199, 195-196.

Fachini, G., and Ciacooini, C. (1966). Pituitary inhibition by effluent blood from the testis. Endokrinologie 50, 79-82.

Finney, D.J. (1964). Parallel line assay. In: Statistical Method in Biological Assay, pp. 100-138. Griffin, London.

Fletcher, P.W., Dias, J.A., Sanzo, M.A., and Reichert, Jr., L.E. (1982). Inhibition of FSH action on granulosa cells by low molecular weight components of follicular fluid. Mol. Cell. Endocr. 25, 303-315.

Forage, R.G., Ring, J.M., Brown, R.W., McInerney, B.V., Cobon, G.S., Gregson, R.P., Robertson, D.M., Morgan, F.J., Hearn, M.T.W., Findlay, J.K., Wettenhall, R.E.H., Burger, H.G., and de Kretser, D.M. (1986). Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc. Natl. Acad. Sci. U.S.A. 83, 3091-3095.

Franchimont, P., Millet, D., Vendrely, E., Letawe, J., Legros, J.J., and Netter, A. (1972). Relationship between spermatogenesis and serum gonadotropin levels in azoospermia and oligospermia. J. Clin. Endocr. Metab. 34, 1003-1008.

Franchimont, P., Chari, S., Hagelstein, M.T., and Duraiswami, S. (1975). Existence of a follicle-stimulating hormone inhibiting factor "inhibin" in bull seminal plasma. Nature Lond. 257, 402-404.

Franchimont, P., Chari, S., Hazee-Hagelstein, M.T., Debruche, M.L., and Durai swami, S. (1977). Evidence for the existence of inhibin. In: The Testis in Normal and Infertile Men (eds. P. Troen and H.R. Nankin) pp. 253-270. Raven Press, New York.

Franchimont, P., Demoulin, A., Verstraelen-Proyard J., Hazee-Hagelstein, M.T., Walton, J.S., and Waites, G.M.H. (1978). Nature and mechanisms of action of inhibin: perspective in regulation of male fertility. Int. J. Androl. Suppl. 2, 69-79.

Franchimont, P., Demoulin, A., Verstraelen-Proyard, J., Hazee-Hagelstein, M.T., and Tunbridge, W.M.G. (1979a). Identification in human seminal fluid of an inhibin-like factor which selectively regulates FSH secretion. J. Reprod. Fert., Suppl. 26, 123-133.

Franchimont, P., Verstraelen-Proyard, J., Hazee-Hagelstein, M.T., Renard, Ch., Demoulin, A., Bourgùignon, J.P., and Hustin, J. (1979b). Inhibin: from concept to reality. Vitams. Horm. 37, 243-302.

Fritz, I.B. (1978). Sites of action of androgens and follicle-stimulating hormone on cells of the seminiferous tubule. In: Biochemical Actions of Hormones, Vol. 5 (ed. G. Litwack) pp. 249-281. Academic Press, New York.

Fritz, I.B., Rommerts, F.F.G., Louis, B.G., and Dorrington, J.H. (1976). Regulation by FSH and dibutyryl cyclic AMP of the formation of androgen-binding protein in Sertoli cell-enriched cultures. J. Reprod. Fert. 46, 17-24.

Fuchs, E. Lundanes, J. Leban, K. Folkers, and C. Bowers (1979). On the existence and separation of the follicle stimulating hormone releasing hormone from the luteinizing hormone releasing hormone. Biochem. Biophys. Res. Commun. 88, 92-96.

Fukuda, M., Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., and Matsuo, H. (1986). Isolation of bovine follicular fluid inhibin of about 32 kDa. Mol. Cell. Endocr. 44, 55-60.

Galdieri, M., Monaco, L., and Stefanini, M. (1984). Secretion of androgen binding protein by Sertoli cells is influenced by contact with germ cells. J. Androl. 5, 409-415.

Galil, K.A.A., and Setchell, B.P. (1980). The effects of heating of the testes of rats on

testosterone production *in vivo* and *in vitro*. Programme of the Society for the Study of Fertility, Oxford, Abstract 95, p. 60.

Galil, K.A.A., and Setchell, B.P. (1981). Changes in testicular blood flow and testosterone production following a single exposure of the testis to heat. Programme of the Society for the Study of Fertility, Edinburgh, Abstract 87, p.54.

Goodman, A.L. (1984). *In vitro* evidence of a role for inhibin in female rabbits. Am. J. Physiol. 246, E243-E248.

Guillemin, R. (1972). Physiology and chemistry of the hypothalamic releasing factors for gonadotropins: a new approach to fertility control. Contraception 6, 1-19.

Gunsalus, G.L., Musto, N.A., and Bardin C.W. (1980). Bidirectional release of a Sertoli cell product, androgen binding protein, into the blood and seminiferous tubule. In: Testicular Development, Structure, and Function, edited by A. Steinberger and E. Steinberger. New York: Raven Press, 1980, p. 291-297.

Hagenäs, L., and Ritzen, E.M. (1976). Impaired Sertoli cell function in experimental cryptorchidism in the rat. Mol. Cell. Endocrinol. 4, 25-34.

Hagenäs, L., Ritzen, E.M., Svensson, J., Hansson, V., and Purvis, K. (1978). Temperature dependence of Sertoli cell function. Internat. J. Androl. Suppl. 2, 449-458.

Hall, P.F., Kew, D., and Mita, M. (1985). The influence of temperature on the functions of cultured Sertoli cells. Endocrinology 116, 1926-1932.

Heindel, J.J., Steinberger, A., and Strada, S.J. (1981). Identification and characterization of a β_1-adrenergic receptor in the rat Sertoli cell. Mol. Cell. Endocr. 22, 349-358.

Heller, C.G., and Nelson, W.O. (1945). Hyalinization of the seminiferous tubules associated with normal or failing Leydig cell function. Discussion of the relationship to eunuchoidism, gynecomastia, gonadotrophins, depressed 17- keto steroids and estrogens. J. Clin. Endocr. 5, 1-12.

Heller, C.G., and Nelson, W.O. (1948). The testis-pituitary relationship in man. Recent Prog. Horm. Res. 3, 229-255.

Heller, C.G., Paulson, C.A., Mortimore, G.E., Junck, E.C., and Nelson, W.O. (1952). Urinary gonadotrophins, spermatogenic activity, and classification of testicular morphology - their bearing on the utilization hypothesis. Ann. N.Y. Acad. Sci. 55, 685-702.

Henderson, K.M., and Franchimont, P. (1981). Regulation of inhibin production by bovine ovarian cells *in vitro*. J. Reprod. Fert. 63, 431-442.

Henderson, K.M., and Franchimont, P. (1983). Inhibin production by bovine ovarian tissues *in vitro* and its regulation by androgens. J. Reprod. Fert. 67, 291-298.

Henderson, K.M., Franchimont, P., Charlet-Renard, Ch., and McNatty, K.P. (1984). Effect of follicular atresia on inhibin production by bovine granulosa cells *in vitro* and inhibin concentrations in the follicular fluid. J. Reprod. Fert. 72, 1-8.

Hermans, W.P. (1982). Inhibin. With an old concept towards a new approach in reproductive physiology. Doctoral dissertation, Rotterdam.

Hermans, W.P., van Leeuwen, E.C.M., Debets, M.H.M., and de Jong, F.H. (1980). Involvement of inhibin in the regulation of follicle-stimulating hormone concentrations in prepubertal and adult, male and female rats. J. Endocr. 86, 79-92.

Hermans, W.P., van Leeuwen, E.C.M., Debets, M.H.M., Sander, H.J., and de Jong, F.H. (1982). Estimation of inhibin-like activity in spent medium from rat ovarian granulosa cells during long-term culture. Mol. Cell. Endocr. 27, 277-290.

Hochereau-de Reviers, M.T., and Courot, M. (1978). Sertoli cells and development of

seminiferous epithelium. Ann. Biol. anim. Bioch. Biophys. 18, 573-583.
Hopkinson, C.R.N., Daume, E., Sturm, G., Fritze, E., Kaiser, S., and Hirschhauser C. (1977). Inhibin-like activity of bovine ovarian extracts in male and female rats. J. Reprod. Fert. 50, 93-96.
Howard, R.P., Sniffen, R.C., Simmons, F.A., and Albright, F. (1950). Testicular deficiency: a clinical and pathologic study. J. Clin. Endocr. 10, 121-186.
Huang, E.S.-R., and Miller, W.L. (1984). Porcine ovarian inhibin preparations sensitize cultured ovine gonadotrophs to luteinizing hormone-releasing hormone. Endocrinology 115, 513-519.
Huang, H.F.S., Dyrenfurth, I., and Hembree, W.C. (1983). Endocrine changes associated with germ cell loss during vitamin A deficiency and vitamin A-induced recovery of spermatogenesis. Endocrinology 112, 1163-1171.
Hudson, B., Baker, H.W.G., Eddie, L.W., Higginson, R.E., Burger, H.G., de Kretser, D.M., Dobos, M., and Lee, V.W.K. (1979). Bioassays for inhibin: a critical review. J. Reprod. Fert., Suppl. 26, 17-29.
Huggenvik, J., and Griswold, M.D. (1981). Retinol binding protein in rat testicular cells. J. Reprod. Fertil. 61, 403-408.
Hutson, J.C., and Stocco, D.M. (1981). Peritubular cell influence on the efficiency of ABP secretion by Sertoli cells in culture. Endocrinology 108, 1362-1368.
Ireland, M.E., Rosenblum, B.B., and Welsh, M.J. (1986). Two-dimensional gel analysis of Sertoli cell protein phosphorylation effect of short term exposure to follicle-stimulating hormone. Endocrinology 118, 526-532.
Ireland, M.E., and Welsh, M.J. (1987). Germ cell stimulation of Sertoli cell protein phosphorylation. Endocrinology 120, 1317-1326.
Jansen, E.H.J.M., Steenbergen, J., de Jong, F.H., and van der Molen, H.J. (1981). The use of affinity matrices in the purification of inhibin from bovine follicular fluid. Mol. Cell. Endocr. 21, 109-117.
Jégou, B., Laws, A.O., and de Kretser, D.M. (1983). The effect of cryptorchidism and subsequent orchidopexy on testicular function in adult rats. J. Reprod. Fertil. 69, 137-145.
Jenner, A.A.J., de Koning, J., and van Rees, G.P. (1982). Time course of inhibition of synthesis and basal release of FSH from pituitary glands of female rats by bovine follicular fluid *in vitro*. Acta Endocrinol. (Copenhagen) 101, 501-506.
Johansson, J., Sheth, A., Cederlund, E., and Jornvall, H. (1984). Analysis of an inhibin preparation reveals apparent identity between a peptide with inhibin-like activity and a sperm-coating antigen. FEBS Lett. 176, 21-26.
de Jong, F.H., and Sharpe, R.M. (1976). Evidence for inhibin-like activity in bovine follicular fluid. Nature Lond. 263, 71-72.
de Jong, F.H., and Sharpe, R.M. (1977a). The onset and establishment of spermatogenesis in rats in relation to gonadotrophin and testosterone levels. J. Endocr. 75, 197-207.
de Jong, F.H., and Sharpe, R.M. (1977b). Gonadotrophins, testosterone and spermatogenesis in neonatally irradiated male rats: evidence for a role of the Sertoli cell in follicle-stimulating hormone feedback. J. Endocr. 75, 209-219.
de Jong, F.H., Welschen, R., Hermans, W.P., Smith, S.D., and van der Molen, H.J. (1978). Effects of testicular and ovarian inhibin-like activity, using *in vitro* and *in vivo* systems. Int. J. Androl., Suppl. 2, 125-138.
de Jong, F.H. (1979a). Inhibin - fact or artifact. Mol. Cell. Endocr. 13, 1-10.

de Jong, F.H., Smith, S.D., and van der Molen, H.J. (1979b). Bioassay of inhibin-like activity using pituitary cells *in vitro*. J. Endocr. 80, 91-102.

de Jong, F.H., Welschen, R., Hermans, W.P., Smith, S.D., and van der Molen, H.J. (1979c). Effects of factors from ovarian follicular fluid and Sertoli cell culture medium on *in vivo* and *in vitro* release of pituitary gonadotrophins in the rat: an evaluation of systems for the assay of inhibin. J. Reprod. Fert., Suppl. 26, 47-59.

de Jong, F.H., Jansen, E.H.J.M., Hermans, W.P., and van der Molen, H.J. (1982a). Purification, characterization and physiological significance of inhibin from ovarian follicular fluid. In: Non-steroidal Regulators in Reproductive Biology and Medicine (eds. T. Fujii and C.P. Channing) pp.73-82. Pergamon Press, Oxford.

de Jong, F.H., Jansen, E.H.J.M., Hermans, W.P., and van der Molen, H.J. (1982b). Purification, characterization, and *in vitro* production of inhibin. In: Intraovarian Control Mechanisms (eds. C.P. Channing and S.J. Segal) pp. 37-52. Plenum Press, New York.

de Jong, F.H., Jansen, E.H.J.M., Steenbergen, J., van Dijk, S. and van der Molen, H.J. (1983a). Assay and purification of inhibin. In: Role of Peptides and Proteins in Control of Reproduction (eds. S.M. McCann and D.S. Dhindsa) pp. 257-273. Elsevier, New York.

de Jong, F.H., Steenbergen, J., van Dijk, S., and van der Molen, H.J. (1983b). Purification and characterization of inhibin from bovine ovarian follicular fluid. In: Hormones and Cell Regulation, Vol.7 (eds. J.E. Dumont, J. Nunez and R.M. Denton) pp. 203-216. Elsevier, Amsterdam.

de Jong, F.H., van Dijk, S., and van der Molen, H.J. (1984). Inhibin from bovine ovarian follicular fluid: a 65 kD glycoprotein. In: Gonadal Proteins and Peptides and their Biological Significance (eds. M.R. Sairam and I.E. Atkinson) pp. 85-96. World Scientific Publishing Co., Singapore.

de Jong, F.H., Sander, H.J., Ultee-van Gessel, A.M., and van der Molen, H.J. (1985a). Specific regulation of the secretion of follicle-stimulating hormone from the pituitary gland: the inhibin concept. In: Frontiers of Hormone Research, Vol. 14 (ed. Tj.B. van Wimersma Greidanus) pp. 53-69. Karger, Basel.

de Jong, F.H., and Robertson, D.M. (1985b). Inhibin: 1985 update on action and purification. Mol. Cell. Endocr. 42, 95-103.

de Jong, F.H., Grootenhuis, A.J., Sander H.J., Steenbergen, J., Timmerman, M.A., and van Dijk, S. (1988). Comparison between inhibin from bovine follicular fluid and rat Sertoli cell culture medium. In: Inhibin-*non* steroidal regulation of FSH secretion. (eds. H.G. Burger, D.M. de Kretser and J.K. Findlay). Raven Press, New York, in press.

Jutisz, M., Kerdelhue, B., Berault, A., and Paloma de la Llosa, M. (1972). On the mechanism of action of the hypothalamic gonadotropin releasing factors. In: Gonadotropins. (eds. B.B. Saxena, C.G. Beling., and H.M. Gandy) pp. 64-71, New York.

Jutte, N.H.P.M., Jansen, R., Grootegoed J.A., Rommerts, F.F.G., and van der Molen, H.J. (1983). FSH stimulation of the production of pyruvate and lactate by rat Sertoli cells may be involved in hormonal regulation of spermatogenesis. J. Reprod. Fertil. 68, 219-226.

Kalla, N.R., and Zarabi, S. (1984). Studies on follicle stimulating hormone receptor binding inhibitor (FSH-RBI). Horm. Metabol. Res. 16, 212-213.

Karl, A.F., and Griswold, M.D. (1980). Actions of insulin and vitamin A on Sertoli cells. Biochem. J. 186, 1001-1003.

Keogh, E.J., Lee, V.W.K., Rennie, G.C., Burger, H.G., Hudson, B., and de Kretser, D.M. (1976). Selective suppression of FSH by testicular ex tracts. Endocrinology 98, 997-1004.

Kerr, J.B., Rich, K.A., and de Kretser, D.M. (1979). Alterations of the fine structure and androgen secretion of the interstitial cells in the experimentally cryptorchid rat testis. Biol. Reprod. 20, 409-422.

Kierszenbaum, A.L., Spruill, W.A., White, M.G., Tres, L.L., and Perkins, J.P. (1985). Rat Sertoli cells acquire a β-adrenergic response during primary culture. Proc. Natl. Acad. Sci. USA 82, 2049-2053.

Klinefelter, H.F., Reifenstein, E.C., and Albright, F. (1942). Syndrome characterized by gynecomastia, aspermatogenesis without aleydigism and increased excretion of follicle-stimulating hormone. J. Clin. Endocr. 2, 615-627.

Knecht, M., Feng, P., and Catt, K.J. (1986). TGFβ regulates the expression of LH receptors in ovarian granulosa cells. Biochem. Biophys. Res. Commun. 139, 800-807.

Kofler, R. (1982). Immunofluorescence localization of glycoprotein hormones in the rat anterior pituitary gland using monoclonal antibodies and polyclonal antisera. J. Histochem. Cytochem. 30, 645-649.

Kohan, S., Froysa, B., Cederlund, E., Fairwell, T., Lerner, R., Johansson, J., Khan, S., Ritzen, M., Jornvall, H., Cekan, S., and Diczfalusy, E. (1986). Peptides of postulated inhibin activity. Lack of *in vitro* inhibin activity of a 94-residue peptide isolated from human seminal plasma, and of a synthetic replicate of its C-terminal 28-residue segment. FEBS Lett. 199, 242-248.

Kormano, M. (1967). Development of the rectum-testis temperature difference in the post-natal rat. J. Reprod. Fert. 14, 427-437.

de Kretser, D.M., Catt, K.J., and Paulsen, C.A. (1971). Studies on the *in vitro* testicular binding of iodinated luteinizing hormone in rats. Endocrinology 88, 332-337.

de Kretser, D.M., Burger, H.G., Fortune, D., Hudson, B., Long, A.R., Paulsen, C.A., and Taft, H.P. (1972). Hormonal, histological and chromosomal studies in adult males with testicular disorders. J. Clin. Endocrinol. Metab. 35, 392-401.

de Kretser, D.M., Au, C.L., le Gac, F., and Robertson, D.M. (1983). Recent studies on inhibin. In: Recent. Adv. in male reprod.: Molec. Basis & Clin. Impl. (eds. R. D'Agata, M.B. Lipsett, P. Polosa and H.J. van der Molen) pp. 91-99.

Krishnan, K.A., Panse, G.T., and Sheth, A.R. (1982). Comparative study of inhibin from human testis, prostate and seminal plasma. Andrologia 14, 409-415.

Krishnan, K.A., Sluss, P.M., and Reichert Jr., L.E. (1986). Low molecular weight FSH binding inhibitor in bovine testis. J. Androl. 7, 42-48.

Krueger, P.M., Hodgen, G.D., and Sherins, R.J. (1974). New evidence for the role of the Sertoli cell and spermatogonia in feedback control of FSH secretion in male rats. Endocrinology 95, 955-962.

Labrie, F., Lagacé, L., Ferland, L., Kelly, P.A., Drouin, J., Massicotte, J., Bonne, C., Raynaud, J-P., and Dorrington, J.H. (1978). Interactions between LHRH, sex steroids and "inhibin" in the control of LH and FSH secretion. Int. J. Androl. Suppl. 2, 303-318.

Labrie, F., Lagacé, L., Ferland, L., Kelly, P.A., Drouin, J., Massicotte, J., Bonne, C., Raynaud, J.-P., and Dorrington, J.H. (1978). Interactions between LHRH, sex steroids and "inhibin" in the control of LH and FSH secretion. Int. J. Androl., Suppl. 2, 81-101.

Lacroix, M., and Fritz I.B. (1982).The control of the synthesis and secretion of plasminogen activator by rat Sertoli cells in culture. Mol. Cell. Endocr. 26, 247-258.

LaQuaglia, M., Shima, H., Hudson, P., Takahashi, M., and Donahoe, P.K. (1986). Sertoli production of Müllerian Inhibiting Substance *in vitro*. J. Urol. 136, 219-224.

Le Magueresse, B., and Jégou, B. (1986). Possible involvement of germ cells in the regulation of oestradiol 17β and ABP secretion by immature rat Sertoli cells (*in vitro* studies). Biochem. Biophys. Res. Commun. 141, 861-869.

Leblond, C.P., and Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N.Y. Acad. Sci. 55, 548-573.

Lee, V.W.K., Keogh, E.J., Burger, H.G., Hudson, B., and de Kretser, D.M. (1976). Studies on the relationship between FSH and germ cells: evidence for selective suppression of FSH by testicular extracts. J. Reprod. Fertil Suppl. 24, 1-15.

Lee, V.W.K., McMaster, J., Quigg, H., and Leversha, L. (1982). Ovarian and circulating inhibin levels in immature female rats treated with gonado tropin and after castration. Endocrinology 111, 1849-1854.

Lee, V.W.K., Craft, N.C., Atkins, R.C., and Burger, H.G. (1986). Monoclonal antibody to rat ovarian inhibin. J. Endocr. 109, 379-383.

Le Gac, F., and de Kretser, D.M. (1982). Inhibin production by Sertoli cell cultures. Mol. Cell. Endocr. 28, 487-498.

Le Lannou, D., and Chambon, Y., (1977). Présence dans l'epididyme d'um facteur abaissant fortement le taux sanquin de FSH chez le rat. C.R. Soc. Biol. 171, 636-638.

Levy, F.O., Eikvar, L., Piotrowska, B., and Hansson, V. (1986). Characterization and cellular localization of glucocorticoid receptors in rat seminiferous tubules. Testis Workshop Nashville, abstract p 75.

Lippschultz, A. (1922). The so-called compensatory hypertrophy of the testicle after unilateral castration. J. Physiol. 56, 451-458.

Lipschultz, L.I., Murthy, L., and Tindall, D.J. (1982). Characterization of human Sertoli cells *in vitro*. J. Clin. Endocrinol. Metab. 55, 228-237.

Lincoln, G.A. (1979). Differential control of luteinizing hormone and follicle-stimulating hormone by luteinizing hormone releasing hormone in the ram. J. Endocr. 80, 133-140.

Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L., and Guillemin, R. (1985). Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. Proc. Natl. Acad. Sci., U.S.A. 82, 7217-7221.

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. (1986). Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature Lond. 321, 779-782.

Liu, L., Booth, J., Merriam, G.R., Barnes, K.M., Sherins, R.J., Loriaux, D.L., and Cutler, Jr., G.B. (1985). Evidence that synthetic 31-amino acid inhibin-like peptide lacks inhibin activity. Endocr. Res. 11, 191-197.

Louis, B.G., and Fritz, I.B. (1979). Follicle-stimulating hormone and testosterone independently increase the production of androgen binding protein by Sertoli cells in culture. Endocrinology 104, 454-461.

Lugaro, G., Giannattasio, G., Ciaccolini, C., Fachini, G., and Gianfranceschi, G.L. (1969). Biochemical study on the pituitary inhibition of gonadal origin. Experientia 25, 147-148.

Lugaro, G., Carrea, G., Casellato, M.M., Mazzola, G., and Fachini, G. (1973). Evidence for a peptidic factor in spermatozoa inhibiting the ovarian maturation. Biochim. Biophys. Acta 304, 719-724.

Lugaro, G., Casellato, M.M., Mazzola, G., Fachini, G., and Carrea, G. (1974). Evidence for the existence in spermatozoa of a factor inhibiting the follicle stimulating hormone

releasing hormone synthesis. Neuroendocrinology 15, 62-68.
Lugaro, G., Sarvamangala, B.S., and Sheth, A.R. (1984). *In vitro* effect of a seminal inhibin-like factor on FSH and LH uptake by rat testis receptors. Cell Biol. Int. Reports 8, 538.
Main, S.J., Davies, R.V., and Setchell, B.P. (1979). The evidence that inhibin must exist. J. Reprod. Fertil. Suppl. 26, 3-14.
Main, S.J., and Setchell, B.P. (1980). Responsiveness of the pituitary gland to androgens and of the testis to gonadotrophins following damage to spermatogenesis in rats. J. Endocr. 87, 445-454.
Mallea, L.E., Machado A.J., Navaroli, F., and Rommerts F.F.G. (1986). Epidermal growth factor stimulates lactate production and inhibits aromatization in cultured Sertoli cells from immature rats. Int. J. Androl. 9, 201-208.
Martins, T., and Rocha, A. (1931). The regulation of the hypophysis by the testicle, and some problems of sexual dynamics. Endocrinology 15, 421-434.
Mason, A.J., Hayflick, J.S., Ling, N., Esch, F., Ueno, N., Ying, S.Y., Guillemin, R., Niall, H., and Seeburg, P.H. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β . Nature Lond. 318, 659-663.
Mason, A.J., Niall, H.D., and Seeburg, P.H. (1986). Structure of two human ovarian inhibins. Biochem. Biophys. Res. Commun. 135, 957-964.
Massagué, J. (1987). The TGF-β family of growth and differentiation factors. Cell 49, 437-438.
Massicotte, J., Lagacé, L., Labrie, F., and Dorrington, J.H. (1984). Modulation of gonadotropin secretion by Sertoli cell inhibin, LHRH, and sex steroids. Am. J. Physiol. 247, E495-E504.
Mather, J.P., Gunsalus, G.L., Musto, N.A, Cheng, C.Y., Parvinen, M., Wright, W., Perez-Infante, V., Margioris, A., Liotta, A., Becker, R., Krieger, D.T., and Bardin, C.W. (1983). The hormonal and cellular control of Sertoli cell secretion. J. Steroid Biochem. 19, 41-51.
Mather, J.P., Zhuang, L-Z, Perez-Infante, V., and Phillips, D.M. (1982). Culture of testicular cells in hormone-supplemented serum-free medium. Ann. NY Acad. Sci. 383, 44-68.
Mayo, K.E., Cerelli, G.M., Spiess, J., Rivier, J., Rosenfeld, M.G., Evans, R.M., and Vale, W. (1986). Inhibin A-subunit cDNAs from porcine ovary and human placenta. Proc. Nat. Acad. Sci. USA 83, 5849-5853.
McCullagh, D.R. (1932). Dual endocrine activity of the testes. Science, N.Y. 76, 19-20.
McCullagh, D.R., and Schneider, I. (1940). The effect of a non-androgenic testis extract on the estrous cycle in rats. Endocrinology 27, 899-902.
McCullagh, E.P., and Schaffenburg, C.A. (1952). The role of the seminiferous tubules in the production of hormones. Ann. N.Y. Acad. Sci. 55, 674-684.
McLachlan, R.I., Robertson, D.M., Burger, H.G., and de Kretser, D.M. (1986a). The radioimmunoassay of bovine and human follicular fluid and serum inhibin. Mol. Cell. Endocr. 46, 175-185.
McLachlan, R.I., Healy, D.L., Robertson, D.M., Burger, H.G., and de Kretser D.M. (1986b). The human placenta: a novel source of inhibin. Biochem. Biophys. Res. Comm. 140, 485-490.
Means, A.R., Fakunding, J.L., Huckins, C., Tindall, D.J., and Vitale, R. (1976). Follicle-stimulating hormone, the Sertoli cell, and spermatogenesis. Rec. Progr. Horm. Res. 32, 477-527.

Means, A.R., Dedman, J.R. Tash, J.S., Tindall, D.J., Van Sickle, M., and Welsh, M.J. (1980). Regulation of the testis Sertoli cell by follicle stimulating hormone. Am. Rev. Physiol. 42, 59-70.

Mecklenburg, R.S., Hetzel, W.D., Gulyas, R.J., and Lipsett, M.B. (1975). Regulation of FSH secretion; use of hydroxyurea to deplete germinal epitheliuim. Endocrinology 96, 564-570.

Meijers, S.A., and Swanson, L.V. (1983). The effect of exogenous follicle stimulating hormone. Treatment on anterior pituitary and testicular function in the prepubertal bull. J. Andrology 4, 371-377.

Miller, K.F., Wesson, J.A., and Ginther, O.J. (1979). Changes in of circulating gonadotropins following administration of equine follicular fluid to ovariectomized mares. Biol. Reprod. 21, 867-872.

Miyamoto, K., Hasegawa, Y., Fukuda, M., Nomura, M., Igarashi, M., Kangawa, K., and Matsuo, H. (1985). Isolation of porcine follicular fluid inhibin of 32K daltons. Biochem. Biophys. Res. Commun. 129, 396-403.

Miyamoto, K., Hasegawa, Y., Fukuda, M., and Igarashi, M. (1986). Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32 K inhibin. Biochem. Biophys. Res. Commun. 136, 1103-1109.

Mizunuma, H., Samson, W.K., Lumpkin, M.D., and McCann, S.M. (1983). Evidence for an FSH-releasing factor in the posterior portion of the rat median eminence. Life Sci. 33, 2003-2009.

Moger, W.H. (1977). Endocrine responses of the prepubertal male rat to hemiorchiectomy. Biol. Reprod. 17, 661-667.

Mohapatra, S.K., Duraiswami, S., and Chari, S. (1985). On the identity of bovine seminal plasma inhibin. Mol. Cell. Endocr. 41, 187-196.

Monaco, L., and Conti, M. (1986). Localization of adenosine receptors in rat testicular cells. Biol. Reprod. 35, 258-266.

Moore, C.R., and Price, D. (1932). Gonad hormone functions, and the reciprocal influence between gonads and hypophysis with its bearing on the problem of sex hormone antagonism. Am. J. Anat. 50, 13-71.

Moriarty, G.W. (1976). Immunocytochemistry of the pituitary glycoprotein hormones. J. Histochem. Cytochem. 24, 846-863.

Mottram, J.C., and Cramer, W. (1923). On the general effects of exposure to radium on metabolism and tumour growth in the rat and the special effects on testis and pituitary. Quart. J. Exp. Physiol. 13, 209-228.

Moudgal, N.R., Murthy, H.M.S., Murthy, G.S., and A.J. Rao (1984). Regulation of FSH secretion in the primate by inhibin: studies in the bonnet monkey (M. Radiata). In: Gonadal proteins and peptides and their biological significance, edited by M.R. Sairam and L.E. Atkinson. Singapore: World Scientific Publishing Co., p. 21-37.

Mulder, E., Peters, M.J., van Beurden, W.M.O., Galdieri, M., Rommerts, F.F.G., Janszen, F.H.A., and van der Molen, H.J. (1976). Androgen receptors in isolated cell preparations obtained from rat testicular tissue. J. Endocr. 70, 331-332.

Murphy, H.D. (1965). Sertoli cell stimulation following intratesticular injections of FSH in the hypophysectomized rat. Proc. Soc. Exp. Biol. Med. 118, 1202-1205.

Murthy, H.M., Ramasharma, K., and Moudgal, N.R. (1979). Studies in purification and characterization of sheep testicular inhibin. J. Reprod. Fert. Suppl. 26, 61-70.

Murty, G.S.R.C., Sheela Rani, C.S., Moudgal, N.R., and Prasad, M.R.N. (1979). Effect of

passive immunization with specific antiserum to FSH on the spermatogenic process and fertility of adult male bonnet monkeys (Macaca radiata). J. Reprod. Fertil. Suppl. 26, 147-163.

Nakhla, A.M., Mather, J.P., Jänne, O.A., and Bardin, C.W. (1984). Estrogen and androgen receptors in Sertoli, Leydig, myoid, and epithelial cells: effects of time in culture and cell density. Endocrinology 115, 121-128.

Nandini, S.G., Lipner, H., and Moudgal, N.R. (1976). A model system for studying inhibin. Endocrinology 98, 1460-1465.

Nelson, W.O., and Gallagher, T.F. (1935). Studies on the anterior hypophysis. 4. The effect of male hormone preparations upon the anterior hypophysis of gonadectomized male and female rats. Anat. Rec. 64, 129-145.

Nieschlag, E., Wickings, E.J., and Mauss, J. (1979). Endocrine testicular function *in vivo* and *in vitro* in infertile men. Acta Endocr. (Kbh.) 90, 544-551.

Odell, W.D., and Swerdloff, R.S. (1976). Etiologies of sexual maturation: a model system bases on the sexually maturing rat. Rec. Prog. Horm. Res. 32, 245-288.

Oonk, R.B., Grootegoed, J.A., and van der Molen, H.J. (1985). Comparison of the effects of insulin and follitropin on glucose metabolism by Sertoli cells from immature rats. Mol. Cell.Endocr. 42, 39-48.

Oonk, R.B., and Grootegoed, J.A. (1987). Indentification of insulin receptors on rat Sertoli cells. Mol. Cell. Endocr. 49, 51-62.

Orth, J.M. (1982). Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. Anat. Rec. 203, 485-492.

Orth, J.M. (1984). The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology 115, 1248-1255.

Orth, J.M. (1986). FSH-induced Sertoli cell proliferation in the developing rat is modified by β-endorphin produced in the testis. Endocrinology 119, 1876-1878.

Padgett, R.W., Johnston, R.D.St., and Gelbart, W.M. (1987). A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-β family. Nature 325, 81-84.

Papavasiliou, S.S., Zmeili S., Herbon L., Duncan-Weldon J., Marshall J.C., and Landefeld, T.D. (1986). α and luteinzing hormone β messenger ribonucleic acid (RNA) of male and female rats after castration: quantitation using an optimized RNA dot blot hybridization assay. Endocrinology, 119, 691-698.

Parvinen, M. (1982). Regulation of the seminiferous epithelium. Endocr. Reviews 3, 404-417.

Perez-Infante, V., Bardin, C.W., Gunsalus, G.L., Musto, N.A., Rich, K.A., and Mather, J.P. (1986). Differential regulation of testicular transferrin and androgen-binding protein secretion in primary cultures of rat Sertoli cells. Endocrinology, 118, 383-392.

Perrard-Sapori, M.H., Chatelain, P., Vallier, P., and Saez, J.M. (1986). *In vitro* interactions between Sertoli cells and steroidogenic cells. Biochem. Biophys. Res. Comun. 134, 957-962.

Picard, J-Y, Bernarms, R., Guerrier, D., Josso, N., and Kahn, A. (1986). Cloning and expression of cDNA for anti-Müllerian hormone. Proc. Natl. Acad. Sci. USA 83, 5464-5468.

Pierce, G.P., and Parsons, T.F. (1981). Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50, 465-495.

Plant, T.M., Hess, D.L., Hotchkiss, J., and Knobil, E. (1978). Testosterone and the control of

gonadotropin secretion in the male rhesus monkey (Macaca mulatta). Endocrinology 103, 535-541.

Poirier, J.C., Combarnous, Y., and Blanc, M.R. (1984). Highly specific antisera against native ovine follitropin (oFSH) obtained from crude antisera by affinity chromatography on solid-phase undissociable lutropin (LH) column. Acta Endocr. (Kbh.) 106, 38-42.

Putra, D.K.H., and Blackshaw, A.W. (1982). Morphometric studies of compensatory testicular hypertrophy in the rat after hemicastration. Aust. J. Biol. Sci. 35, 287-293.

Ramasharma, K., Shashidara Murthy, H.M., and Moudgal, N.R. (1979). A rapid bioassay for measuring inhibin activity. Biol. Reprod. 20, 831-835.

Ramasharma, K., Sairam, M.R., Seidah, N.G., Chretien, M., Manjunath, P., Schiller, P.W., Yamashiro, D., and Li, C.H. (1984). Isolation, structure, and synthesis of a human seminal plasma peptide with inhibin-like activity. Science, N.Y. 223, 1199-1202.

Reichert, Jr., L.E., Sanzo, M.A., Fletcher, P.W., Dias, J.A., and Lee, C.Y. (1982). Properties of follicle stimulating hormone binding inhibitors found in physiological fluids. In: Intraovarian Control Mechanisms (eds. C.P. Channing and S.J. Segal) pp. 135-144. Plenum Press, New York.

Rich, K.A., and de Kretser, D.M. (1977). Effect of differing degrees of destruction of the rat seminiferous epithelium on levels of serum follicle stimulating hormone and androgen binding protein. Endocrinology 101, 959-968.

Rich, K.A., Kerr, J.B., and de Kretser, D.M. (1979). Evidence for Leydig cell dysfunction in rats with seminiferous tubule damage. Mol. Cell. Endocr. 13, 123-135.

Rich, K.A., Bardin, C.W., Gunsalus, G.L., and Mather, J.P. (1983). Age- dependent pattern of androgen-binding protein secretion from rat Sertoli cells in primary culture. Endocrinology 113, 2284-2293.

Risbridger, G.P., Kerr, J.B., Peake, R.A., and de Kretser, D.M. (1981). An assessment of Leydig cell function after bilateral or unilateral efferent duct ligation: further evidence for local control of Leydig cell function. Endocrinology 109, 1234-1241.

Rivier, J., McClintock, R., Spiess, J., Vaughan, J., Dalton, D., Corrigan, A., Azad, R., and Vale, W. (1984). Isolation from porcine follicular fluid of a protein exhibiting potent inhibin like biological activity. In: Endocrinology (eds. F. Labrie and L. Proulx) pp. 1141-1144. Elsevier, Amsterdam.

Rivier, J., Spiess, J., McClintock, R., Vaughan, J., and Vale, W. (1985). Purification and partial characterization of inhibin from porcine follicular fluid. Biochem. Biophys. Res. Commun. 133, 120-127.

Rivier, C., Rivier, J., and Vale, W. (1986). Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. Science (N.Y.) 234, 205-208.

Robb, G.W., Amann, R., and Killian G.J., (1978). Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J. Reprod. Fertil. 54, 103-107.

Robertson, D.M., Au, C.L., and de Kretser, D.M. (1982). The use of ^{51}Cr for assessing cytotoxicity in an *in vitro* bioassay for inhibin. Mol. Cell. Endocr. 26, 119-127.

Robertson, D.M., Foulds, L.M., Leversha, L., Morgan, F.J., Hearn, M.T.W., Burger, H.G., Wettenhall, R.E.H., and de Kretser, D.M. (1985). Isolation of inhibin from bovine follicular fluid. Biochem. Biophys. Res. Commun. 126, 220-226.

Robertson, D.M., Giacometti, M.S., and de Kretser, D.M. (1986a). The effects of inhibin purified from bovine follicular fluid in several *in vitro* pituitary cell culture systems. Mol. Cell. Endocr. 46, 29-36.

Robertson, D.M., de Vos, F.L., Foulds, L.M., McLachlan, R.I., Burger, H.G., Morgan, F.J., Hearn, M.T.W., and de Kretser, D.M. (1986b). Isolation of a 31 kDa form of inhibin from bovine follicular fluid. Mol. Cell. Endocr. 44, 271-277.

Rommerts, F.F.G., Krüger-Sewnarain, B.Ch., van Woerkom-Blik, A., Grootegoed, J.A., and van der Molen, H.J. (1978). Secretion of proteins by Sertoli cell enriched cultures: effects of follicle stimulating hormone, dibutyryl cAMP and testosterone and correlation with secretion of oestradiol and androgen binding protein. Mol. Cell. Endocr. 10, 39-55.

Rommerts, F.F.G., de Jong, F.H., Grootegoed, J.A., and van der Molen, H.J. (1980). Metabolic changes in testicular cells from rats after long-term exposure to 37°C *in vivo* or *in vitro*. J. Endocr. 85, 471-479.

Rothman, C.M., Sims, C.A., and Stotts, C.L. (1982). Sertoli cell only syndrome 1982. Fert. Steril. 38, 388-390.

Rubin, D. (1941). The question of an aqueous hormone from the testicle. Endocrinology 29, 281-287.

Russell, L.D., Alger, L.E., and Nequin, L.G. (1987). Hormonal control of pubertal spermatogenesis. Endocrinology 120, 1615-1632.

Sairam, M.R., Ranganathan, M.R., Ramasharma, K., and Lamothe, P. (1981a). Isolation and characterization of a bovine seminal plasma protein inhibiting pituitary FSH secretion. Mol. Cell. Endocr. 22, 231-250

Sairam, M.R., Ranganathan, M.R., and Ramasharma K. (1981b). Binding of an inhibin-like protein from bull seminal plasma to ovine pituitary membranes. Mol. Cell. Endocr. 22, 251-264.

Sairam, M.R., Kato, K., Manjunath, P., and Ramasharma K. (1984). Isolation and characterization of a protein with inhibin-like activity from pig follicular fluid. In: Gonadal Proteins and Peptides and their Biological Significance (eds. M.R. Sairam and L.E. Atkinson) pp. 65-83. World Scientific Publishing Co., Singapore.

Sanborn, B.M., Steinberger, A., Meistrich, M.L., and Steinberger, E. (1975). Androgen binding sites in testis cell fractions as measured by a nuclear exchange assay. J. Steroid Biochem. 6, 1459-1465.

Sander, H.J., van Leeuwen, E.C.M., and de Jong, F.H. (1984). Inhibin-like activity in media from cultured rat granulosa cells collected throughout the oestrous cycle. J. Endocr. 103, 77-84.

Sander, H.J., Meijs-Roelofs, H.M.A., Kramer, P., and van Leeuwen, E.C.M. (1985). Inhibin-like activity in ovarian homogenates of prepubertal female rats and its physiological significance. J. Endocr. 107, 251-257.

Sandow, J., Enzmann, F., Arimura, A., Redding, T.W., and Schally, A.V. (1975). Purification and characterization of two porcine hypothalamic fractions with LH-releasing activity: evidence for a single LH and FSH-releasing hormone. Acta Endocr. (Kbh.) 80, 209-219.

Sarvamangala, B., and Sheth, A.R. (1984). Effect of inhibin on hypothalamo-pituitary-gonadal axis in prepubertal rats. Indian J. Exp. Biol. 22, 292-294.

Saucier, J.S., Tremblay, R.R., and Dubé, J.Y. (1983). Changes in insulin binding in rat testis following testosterone and gonadotrophins administration. J. Receptor. Res. 3, 439-450.

Savoy-Moore, R.T., and Schwartz, N.B. (1980). Differential control of FSH and LH secretion. In: Reproductive Physiology III (ed. R.O. Greep) pp. 203-248. Internat. Rev. of Physiology vol. 22.

Schally, A.V., Kastin, A.J., and Arimura, A. (1972). FSH- releasing hormone and LH-releasing hormone. Vitams. Horm. 30, 83-164.

Scott, R.S., Burger, H.G., and Quigg, H. (1980). A simple and rapid *in vitro* bioassay for inhibin. Endocrinology 107, 1536-1542.

Scott, R.S., and Burger, H.G. (1981). Mechanism of action of inhibin. Biol. Reprod. 24, 541-550.

Scott, R.S., Burger, H.G., Quigg, H., Dobos, M., Robertson, D.M., and de Kretser, D.M. (1982). The specificity of inhibin bioassay using cultured pituitary cells. Mol. Cell. Endocr. 27, 307-316.

Seethalakshmi, L., Steinberger, A., and Steinberger E. (1984). Pituitary binding of ^{3}H-labelled Sertoli cell factor *in vitro*: a potential radioreceptor assay for inhibin. Endocrinology 115, 1289-1294.

Seidah, N.G., Arbatti, N.J., Rochemont, J., Sheth, A.R., and Chretien, M. (1984a). Complete amino acid sequence of human seminal plasma β-inhibin. Prediction of post Gln-Arg cleavage as a maturation site. FEBS Lett. 175, 349-355.

Seidah, N.G., Ramasharma, K., Sairam, M.R., and Chrétien, M, (1984b). Partial amino acid sequence of a human seminal plasma peptide with inhibin-like activity. FEBS Lett. 167, 98-102.

Sertoli, E. (1865). Dell' esistenza di particulari cellule ramificate nei canalicoli seminiferi del testicolo umano. Il Morgagni 7, 31-39.

Setchell, B.P. (1967). The blood-testicular fluid barrier in sheep. J. Physiol. London 189, 63P-65P.

Setchell, B.P., and Sirinathsinghji, D.J. (1972). Antigonadotrophic activity in rete testis fluid, a possible 'inhibin'. J. Endocr. 53, lx-lxi.

Setchell, B.P., and Wallace, A.L.C. (1972). The penetration of iodine-labelled follicle-stimulating hormone and albumin into the seminiferous tubules of sheep and rats. J.Endocr. 54, 67-77.

Setchell, B.P., and Jacks, F. (1974). Inhibin-like activity in rete testis fluid. J. Endocr. 62, 675-676.

Setchell, B.P., Davies, R.V., and Main, S.J. (1977). Inhibin. In: The Testis, Vol. 4 (eds. A.D. Johnson and W.R. Gomes) pp. 189-238. Academic Press, New York.

Sherins, R.J., Patterson, A.P., Brightwell, D., Udelsman, R., and Sartor, J. (1982). Alteration in the plasma testostrone:estradiol ratio: an alternative to the inhibin hypothesis. Ann. N.Y. Acad. Sci. 383, 295-306.

Sheth, A.R., Vaze, A.Y., and Thakur, A.N. (1978). Development of a radioimmunoassay for inhibin. Ind. J. Exp. Biol. 16, 1025-1026.

Sheth, A.R., Joshi, L.R., Moodbidri, S.B., and Rao, S.S. (1979). Characterization of a gonadal factor involved in the control of FSH secretion. J. Reprod. Fert., Suppl. 26, 71-85.

Sheth, A.R., Vanage, G.R., Vaze, A.Y., and Thakur, A.N. (1980). Negative feedback of secretion of follicle-stimulating hormone by the pituitary gland of developing male rats. J. Endocr. 87, 401-407.

Sheth, N.A., Vaze, A.Y., and Sheth, A.R. (1982). A peptide in gastric secretion with inhibin-like properties. Clin. Endocrinol. 17, 157-163.

Sheth, A.R., Arabatti, N., Carlquist, M., and Jornvall, H. (1984). Characterization of a polypeptide from human seminal plasma with inhibin (inhibition of FSH secretion)-like activity. FEBS Lett. 165, 11-15.

Shivaji, S., Bhargava, P.M., and Scheit, K.H. (1984). Immunological identification of seminalplasmin in tissue extracts of sexglands of bull. Biol. Reprod. 30, 1237-1241.

Skinner, M.K., and Griswold, M.D. (1980). Sertoli cells synthesize and secrete transferrin-like protein. J. Biol. Chem. 255, 9523-9525.

Skinner, M.K., and Griswold, M.D. (1982). Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. Biol. Reprod. 27, 211-221.

Skinner, M.K., and Fritz, I.B. (1985). Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions. Proc. Natl. Acad. Sci. USA 82, 114-118.

Sluss, P.M., and Reichert, Jr., L.E. (1983). Presence of bacteria in porcine follicular fluid and their ability to generate an inhibitor of follicle-stimulating hormone binding to receptor. Biol. Reprod. 29, 335-341.

Smith, B.C., and Griswold, M.D. (1981). Primary culture of supporting cells from bovine testes. In Vitro 17, 612-618.

Smith, P.E. (1930). Hypophysectomy and a replacement therapy in the rat. Am. J. Anat. 45, 205-256.

Steelman, S.L., and Pohley, F.M. (1953). Assay of the follicle stimulating hormone based on augmentation with human chorionic gonadotropin. Endocrinology 53, 604-616.

Steinberger, A., and Steinberger, E. (1976). Secretion of an FSH-inhibiting factor by cultured Sertoli cells. Endocrinology 99, 918-921.

Steinberger, A., Hintz, M., and Heindel, J.J. (1978). Changes in cyclic AMP responses to FSH in isolated rat Sertoli cells during sexual maturation. Biol. Reprod. 19, 566-572.

Steinberger, A. (1979). Inhibin production by Sertoli cells in culture. J. Reprod. Fert. Suppl. 26, 31-45.

Steinberger, A. (1980). Factors affecting *in vitro* secretion of inhibin by isolated Sertoli cells. In: Endocrinology (eds. I.A. Cumming, J.W. Funder, and F.A.O. Mendelsohn) pp. 259-262. The Australian Academy of Science, Canberra City, Australia.

Steinberger, A. (1981). Regulation of inhibin secretion in the testis. In: Intragonadal Regulation of Reproduction (eds. P. Franchimont and C.P. Channing) pp. 283-298. Academic Press, London.

Steinberger, A., Seethalakshmi, L., Kessler M., and Steinberger, E. (1982). Binding of ^{3}H-Sertoli cell factor to rat anterior pituitary *in vitro*. Endocrinology 111, 696-698.

Swerdloff, R.S., Walsh, P.C., Jacobs, H.S., and Odell, W.D. (1971). Serum LH and FSH during sexual maturation in the male rat: effect of castration and cryptorchidism. Endocrinology 88, 120-128.

Tash, J.S., Welsh, M.J., and Means A.R. (1981). Regulation of protein kinase inhibitor by follicle-stimulating hormone in Sertoli cells *in vitro*. Endocrinology 108, 427-434.

Thakur, A.M., Vaze, A.Y., Dattatreyamurthy, B., and Sheth, A.R. (1981). Isolation and characterization of inhibin from human seminal plasma. Indian J. Exp. Biol. 19, 307-313.

Tindall, D.J., and Means, A.R. (1980). Properties and hormonal regulation of androgen binding proteins. In: Advances in Sex Hormone Research vol. 4 (eds. J.A. Thomas and R.L. Singhal) pp. 295-328. Urban & Schwarzenberg, Baltimore, Maryland.

Tindall, D.J., Tash, J.S., and Means, A.R. (1981). Factors affecting Sertoli cell function in the testis. Environmental Health Perspectives 38, 5-10.

Toebosch, A.M.W., Robertson, D.M., Trapman, J., Klaassen, P., de Paus, R.A., de Jong, F.H., and Grootegoed, J.A. (1988). Effects of FSH and IGF-I on immature rat Sertoli

cells: inhibin α- and β-subunit mRNA levels and inhibin secretion. Mol. Cell. Endocr., in press.

Tsonis, C.G., McNeilly, A.S., and Baird, D.T.(1986). Measurement of exogenous and endogenous inhibin in sheep serum using a new and extremely sensitive bioassay for inhibin based on inhibition of ovine pituitary FSH secretion *in vitro*. J. Endocr. 110, 341-352.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature Lond. 321, 776-779.

Van Thiel, D.H., Sherins, R.J., Myers, G.H., and De Vita, V.T. (1972). Evidence for a specific seminiferous tubular factor affecting follicle-stimulating hormone secretion in man. J. Clin. Invest. 51, 1009-1019.

Vaze, A.Y., Thakur, A.N., and Sheth, A.R. (1979). Development of a radioimmunoassay for human seminal plasma inhibin. J. Reprod. Fert., Suppl. 26, 135-146.

Verhoeven, G., and Cailleau J. (1987). A Leydig cell stimulatory factor produced by human testicular tubules. Mol. Cell. Endocr. 49, 137-147.

Verhoeven, G., and Franchimont, P. (1983). Regulation of inhibin secretion by Sertoli cell-enriched cultures. Acta Endocr. Copenh. 102, 136-143.

Verjans, H.L., Eik-Nes, K.B., Aafjes, J.H., Vels, F.J.M., and van der Molen, H.J. (1974). Effects of testosterone propionate, 5α-dihydrostestosterone propionate and oestradiol benzoate on serum levels of LH and FSH in the castrated adult male rat. Acta Endocr. Copenh. 77, 643-654.

Vigier, B., Picard, J.Y., Tran, D., Legeai, L., and Josso, N. (1984). Production of anti-Müllerian Hormone: another homology between Sertoli and Granulosa cells. Endocrinology 114, 1315-1320.

Vijayalakshmi, S., Bandivdekar, A.H., Joshi, L.R., Moodbidri, S.B., and Sheth, A.R. (1980). Isolation and characterization of ovine testicular and ovarian inhibin. Arch. Androl 5, 179-188.

Vitale, R.D., Fawcett, D.W., and Dym, M. (1973). The normal development of the blood-testis barrier and the effect of clomiphene and estrogen treatment. Anat. Rec. 176, 333-344.

Waites, G.M.H., and Gladwell, R.T. (1982). Physiological significance of fluid secretion in the testis and blood-testis barrier. Physiol. Rev. 62, 624-671.

Waites, G.M.H., Wenstrom, J.C., Crabo, B.G., and Hamilton, D.W. (1983). Rapid compensatory hypertrophy of the lamb testis after neonatal hemiorchidectomy: endocrine and light microscopial morphometric analyses. Endocrinology 112, 2159-2167.

Walton, J.S., Evins, J.D., and Waites, G.M.H. (1978). Feedback control of follicle-stimulating hormone in pre- and postpubertal rams as revealed by hemicastration. J. Endocr. 77, 75-84.

Wang, J., Galil, K.A.A., and Setchell, B.P. (1983). Changes in testicular blood flow and testosterone production during aspermatogenesis after irradiation. J. Endocr. 98, 35-46.

Ward, D.N., Glenn, S.D., Liu, W.K., and Gordon, W.L. (1983). Chemistry and physiology of gonadal peptides. In: Factors Regulating Ovarian Functions (eds. E.G. Greenwald and P.F. Terranova.) pp. 141-156. Raven Press, New York.

Wilkinson, M., Moger, W.H., and Selin, L.K. (1980). An unexpected increase in pituitary sensitivity to gonadotrophin releasing hormone after treatment with porcine follicular

fluid (inhibin) in immature hemicastrated rats. Can. J. Physiol. Pharmacol. 58, 220-222.

Williams, A.T., Rush, M.E., and Lipner, H. (1979). Isolation and preliminary characterization of inhibin-f. In: Ovarian Follicular and Corpus Luteum Function (eds. C.P. Channing, J. Marsh and W.S. Sadler) pp. 429-435. Plenum Press, New York.

Wise, P.M., Rance, N., Barr, G.D., and Barraclough, C.A. (1979). Further evidence that luteinizing hormone-releasing hormone also is follicle-stimulating hormone-releasing hormone. Endocrinology, 104, 940-947.

Wright, W.W., Musto, N.A., Mather, J.P., and Bardin, C.W. (1981). Sertoli cells secrete both testis-specific and serum proteins. Proc. Natl. Acad. Sci. USA 78, 7565-7569.

Yamashiro, D., Li, C.H., Ramasharma, K., and Sairam, M.R. (1984). Synthesis and biological activity of human inhibin-like peptide-(1-31). Proc. Natl. Acad. Sci. USA 81, 5399-5402.

Ying, S.-Y., Ling, N., Bohlen, P., and Guillemin, R. (1981). Gonadostatins and gonadocrinin: peptides from the gonads regulating the secretion of gonadotropins. In: Bioregulators of Reproduction (eds. G. Jagiello and H.J. Vogel) pp. 389-401. Academic Press, New York.

Ying, S.-Y., Becker, A., Baird, A., Ling, N., Ueno, N., Esch, F., and Guillemin, R. (1986a). Type beta transforming growth factor (TGF-β) is a potent stimulator of the basal secretion of follicle stimulating hormone (FSH) in a pituitary monolayer system. Biochem. Biophys. Res. Commun. 135, 950-956.

Ying, S.-Y., Becker, A., Ling, N., Ueno, N., and Guillemin, R. (1986b). Inhibin and beta type transforming growth factor (TGFβ) have opposite modulating effects on the follicle stimulating hormone (FSH)-induced aromatase activity of cultured rat granulosa cells. Biochem. Biophys. Res. Commun. 136, 969-975.

PART TWO

Inhibin-like activity in Sertoli cell culture media and testicular homogenates from rats of various ages

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ABSTRACT

The influence of age on testicular inhibin in untreated, neonatally hemicastrated and prenatally irradiated rats was studied using in-vivo and in-vitro experiments.

In testicular cytosols prepared from 1-, 7-, 14-, 21-, 42- and 63-day-old rats concentrations of testicular inhibin could be measured with an in-vitro bioassay method using dispersed pituitary cells. Preparations of testicular cytosols caused a dose-dependent suppression of pituitary FSH secretion, whereas no effects were found on LH secretion. Testicular content of inhibin increased gradually with age, while after 14 days of age a relatively large increase of peripheral FSH concentrations occurred in all experimental groups. Neonatal hemicastration or prenatal irradiation resulted in decreased inhibin content of the testis and increased plasma FSH levels.

The production of inhibin activity by Sertoli cells obtained from 7-, 14-, 21-, 42- and 63-day-old normal rats was measured during a 24-h incubation period on the third day of culture. The inhibin production per 10^6 plated Sertoli cells decreased rapidly after 14 days of age and the lowest production of inhibin was found in Sertoli cells from rats of 63 days of age. After preincubation with ovine FSH significantly larger amounts of inhibin activity were detected in spent media from 21-day-old rat testes. In contrast, suppression of inhibin production was found after preculture in the presence of testosterone at most of the ages studied.

These data from in-vivo and in-vitro experiments indicate that a reciprocal relationship exists between pituitary FSH secretion and inhibin production before the age of 21 days. This relationship supports the concept that inhibin is a physiologically important modulator of FSH secretion before puberty, while the role of the large amount of testicular inhibin present at the older ages remains to be determined.

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INTRODUCTION

The concept of a water soluble testicular hormone which affects the secretion of gonadotrophins from the pituitary gland was originally proposed by McCullagh (1932), who coined the name 'inhibin' for this principle. Later studies indicated that testicular inhibin is a protein hormone, which acts on the pituitary gland and selectively inhibits the secretion of follicle-stimulating hormone (FSH), i.e. without affecting the secretion of luteinizing hormone (LH) (Setchell & Jacks, 1974; Franchimont, Chari, Hagelstein & Duraiswami, 1975; de Jong, 1979). Inhibin activity has been detected using in-vivo and in-vitro bioassay methods in testicular extracts (Keogh, Lee, Rennie *et al.* 1976; Au, Robertson & de Kretser, 1983), in fluids from the male reproductive tract (Setchell & Jacks, 1974; Franchimont, Chari & Demoulin, 1975) and in media from primary cultures of Sertoli cells (Steinberger & Steinberger, 1976; Le Gac & de Kretser, 1982; Verhoeven & Franchimont, 1983; Ultee-van Gessel, Leemborg, de Jong & van der Molen, 1986). These studies have provided experimental evidence that testicular inhibin is a specific Sertoli cell product and that production of inhibin by Sertoli cells isolated from immature rat testes is affected by FSH and/or testosterone.

Most of the work on the in-vivo regulation of inhibin production has been performed in adult rats (Au, Robertson & de Kretser 1984, 1985; Au, Irby, Robertson & de Krester, 1986), while most of the in-vitro studies used Sertoli cells from immature animals (Le Gac & de Kretser, 1982; Verhoeven & Franchimont, 1983; Ultee-van Gessel *et al.* 1986). So

far, relatively little attention has been paid to the effect of age on inhibin production. Steinberger (1979) reported no change in the secretion of inhibin by cultured Sertoli cells from rats with ages ranging from 18 to 90 days; her results indicated an increase in the sensitivity of pituitary cells to inhibin from about 35 days of age onwards in the rat. Massicotte, Lagacé, Labrie & Dorrington (1984) suggested that Sertoli cells from younger rats secrete more inhibin than those from older animals. Finally, Hermans, van Leeuwen, Debets & de Jong (1980) have suggested that the role of inhibin in the regulation of peripheral FSH concentrations is more important in prepubertal than in adult male rats.

The current study was designed to examine the ability of Sertoli cells from rats of various ages to secrete inhibin-like activity and to study the factors regulating inhibin secretion by these cells. In addition, these data on inhibin secretion were compared with the concentration of inhibin in the testes of animals of the same ages. Finally, the relationships between testicular inhibin concentration and peripheral concentrations of FSH, LH and testosterone were investigated in normal, prenatally irradiated and neonatally hemicastrated, prepubertal and adult rats.

MATERIALS AND METHODS

Animals

Male Wistar rats with ages between 1 and 63 days were used. The size of the litters was reduced to eight pups on the first day after birth. The animals were weaned at 21 days of age, and kept in an animal room under controlled conditions of temperature (22–24 °C) and light (lights on 04.45–19.00 h). Standard dry pellets and tap water were available *ad libitum*. Prenatal irradiation (150 rad) was performed on day 20 of gestation and resulted in testes without spermatogenic cells (Beaumont, 1960). Hemicastration was carried out 1 day after birth under ether anaesthesia. After recording of body weight, animals of various ages were killed with CO_2 and decapitated. Trunk blood was collected, clotted at room temperature for 1 h and overnight at 4 °C, and centrifuged before collection of serum. Serum was stored at −20 °C until assayed for FSH, LH and testosterone.

Preparation of testicular cytosols

After weighing, the testes from normal, neonatally hemicastrated or in-utero irradiated rats were decapsulated, combined and put into a 5 ml Teflon–glass homogenizer (Braun, Melsungen, F.R.G.; four up and down strokes at 1100 rev./min at 4 °C) together with Eagle's Minimum Essential Medium (MEM; Gibco, Grand Island, NY, U.S.A.), which contained non-essential amino acids (Gibco), fungizone (600 ng/ml), streptomycin (100 µg/ml) and penicillin (100 i.u./ml). Weight/volume ratios varied between 1:60 (7-day-old rat testes) and 1:1 (63-day-old rat testes). Homogenates were centrifuged at 100 000 *g* for 1 h at 4 °C in a Beckmann L5-65 ultracentrifuge using a SW 60 rotor. Subsequently, high speed supernatants were collected and incubated with an equal volume of Dextran-coated charcoal suspension (1% Norit + 0·1% Dextran T300 in phosphate-buffered saline (0·01 mol/l) pH 7·0) at 4 °C for 30 min in order to remove steroids. Thereafter, charcoal was removed by centrifugation at 1500 *g* for 10 min and rat testicular cytosols were collected, sterilized by passing through a disposable membrane filter (0·2 µm; Schleicher & Schuell, Dassel, F.R.G.) and stored at −20 °C until estimation of inhibin activity. To prepare a testicular cytosol from 1-day-old normal rat testes one batch of ten testes was homogenized in 1 ml using the same method as described above.

Sertoli cell culture

Sertoli cell cultures were initiated from testes of normal rats. The method used for the isolation of Sertoli cells from testes of 7-, 14- and 21-day-old rats for short-term culture experiments has been described in detail previously (Ultee-van Gessel *et al.* 1986). Briefly, the Sertoli cells were isolated after two incubations of total testis tissue with collagenase in a shaking water bath (shaking velocity 120 cycles/min), followed by mechanical dispersion and cultured for 3 days in chemically defined medium with 1% fetal calf serum (Gibco) during the first 24 h. The cultures were kept at 32 °C in a humidified atmosphere of 5% CO_2 in air. To prepare tubular explants from testes of 42- or 63-day-old rats, these testes were incubated with collagenase (0·5 mg/ml) for 30 and subsequently for 60 min in a more gently shaking water bath at 32 °C (shaking velocity 80 cycles/min) in order to maintain the tubular structure while most peritubular cells were stripped away. This treatment was followed by an incubation with 10 mg trypsin/20 ml (24 units/mg; Worthington, Freehold, NY, U.S.A.) for 20 min in the presence of 100 µg ribonuclease A/20 ml (3950 units/mg; Worthington) in order to obtain tubular fragments and to remove most spermatogenic cells yielding aggregates of mainly Sertoli cells. Subsequently, an appropriate amount of trypsin inhibitor (type I; Sigma, St Louis, MO, U.S.A.) was added to the suspension and Sertoli cell preparations were washed three times by sedimentation at unit gravity for 3–5 min before they were cultured for 3 days as described above.

Culture medium was renewed 24 and 32 h after the start of the culture, and cells were cultured for another 24 h period. Inhibin activity was estimated in the spent culture media collected at the end of the latter period. Sertoli cells from rat testes at different ages were cultured also in the presence of ovine FSH (NIH-FSH S13, 500 ng/ml medium; a gift from NIH, Bethesda, MD, U.S.A.) and/or testosterone (700 nmol/l; Steraloids, Wilton, NH. U.S.A.) for an 8-h preincubation period before this 24-h period in order to avoid interference of the added hormones in the inhibin bioassay (see Ultee-van Gessel *et al.* 1986). After the culture period the cells were lysed in 1mol NaOH/l and protein was estimated (Lowry, Rosebrough, Farr & Randall, 1951). From parallel culture wells cells were detached with a rubber scraper for flow cytometric DNA analysis on a FACS II Cell Sorter (Vindeløv, Christensen & Nissen, 1983). DNA content was measured with a fluorimetric assay according to Kapuscinski & Skoczylas (1977) as modified by Lee, Thornthwaite & Rasch (1984).

Morphology of the Sertoli cell cultures was examined at regular intervals using a microscope with phase contrast optics (Nikon, Tokyo, Japan).

Hormone determination

In-vitro bioassay of inhibin activity

Inhibin activity was measured using dispersed pituitary cells from adult normal male rats cultured in 1 ml MEM with the additions described above (de Jong, Smith & van der Molen, 1979*a*; Hermans, van Leeuwen, Debets *et al.* 1982). The suppression of FSH release caused by the addition of aliquots of charcoal-treated testicular cytosols (added volume ranged from 10 to 200 µl) or Sertoli cell culture medium (SCCM; added volume 25–450 µl) was compared with the suppression of FSH release in pituitary cell cultures incubated under similar conditions with a standard preparation of charcoal-treated bovine follicular fluid (bFF) with the arbitrary potency of 1 inhibin unit/µg protein. This standard is 312 times more potent than the ovine testicular lymph protein preparation used as a standard by Eddie, Baker, Higginson & Hudson (1979) as described earlier by de Jong, Jansen & van der Molen (1981). The median effective value for the bFF standard in the rat pituitary cell bioassay amounted to $2{\cdot}65 \pm 0{\cdot}07$ µg protein/ml (mean ± S.E.M., $n = 15$). Inhibin potencies were calculated using statistics for parallel-line assay (Finney, 1964). No significant deviations from parallelism were observed between the dose–response curves obtained after addition of bFF, testicular cytosols or SCCM. No significant effects on pituitary LH release were detected. The index of precision (λ) amounted to $0{\cdot}10 \pm 0{\cdot}01$ (means ± S.E.M., $n = 15$ range 0·06–0·15). Data on reproducibility of the inhibin bioassay have been reported by de Jong, Jansen, Steenbergen *et al.* (1983). Testicular cytosols from rats of different ages or various experimental groups were measured in the same bioassay for inhibin.

Estimation of gonadotrophins and steroids

Concentrations of FSH and LH in culture media of pituitary cells and in sera were estimated by radioimmunoassay as described by Welschen, Osman, Dullaart *et al.* (1975). All serum samples were measured in the same radioimmunoassay. Results have been expressed in terms of NIADDK-rat-FSH RP1 and NIADDK-rat-LH RP1. Intra-assay coefficients of variation were 12·0% for FSH and 10·8% for LH respectively.

Testosterone concentrations in SCCM, serum and charcoal-treated testicular cytosols were assayed by radioimmunoassay (RIA) as described by Verjans, Cooke, de Jong *et al.* (1973). The testosterone concentrations in SCCM (after hormone-supplemented preincubation) and charcoal-treated testicular cytosols were < 3 nmol/l as measured by RIA.

Statistical procedures

The significance of differences between means of results of various treatment groups for different ages was assessed using Student's *t*-test. Differences were considered significant when $P < 0{\cdot}05$ (two-tailed).

RESULTS

In-vivo experiments

Testis weights in non-treated control rats on the one hand and in hemicastrated and irradiated animals on the other were significantly different from 7 and 14 days of age onwards (Table 1). In rats exposed to prenatal irradiation, testis weights were significantly lower than in normal animals, while in neonatally hemicastrated rats the remaining testis showed hypertrophic growth. No significant effects of hemicastration or irradiation on body weight were observed (data not shown).

The data in Fig. 1 show amounts of inhibin/total testicular mass per rat (Fig. 1*a*) and peripheral concentrations of FSH (Fig. 1*b*) and testosterone (Fig. 1*c*) in all groups of animals. The total amount of testicular inhibin increased gradually with age in all groups (Fig. 1*a*). This increase was significantly ($P < 0{\cdot}01$) smaller in the experimental groups when compared with that in normal rats of the same age. The smallest amounts of inhibin were found in testes from irradiated rats. Testicular concentrations of inhibin, expressed per gram testis tissue, declined with age

TABLE 1. Testis weights (g) from normal, neonatally hemicastrated and prenatally irradiated rats at different ages. Values are means ± S.E.M., numbers of rats are shown in parentheses

Age (days)	Total testis weight (g)‡		
	Normal	Hemicastrated	Irradiated
1	0·0055 ± 0·0001 (9)	—	—
7	0·0171 ± 0·0004 (4)	0·0127 ± 0·0008 (4)*	0·0166 ± 0·0005 (5)
14	0·068 ± 0·0018 (6)	0·040 ± 0·0053 (3)*	0·047 ± 0·0011 (4)†
21	0·268 ± 0·009 (10)	0·186 ± 0·012 (4)*	0·123 ± 0·0057 (4)†
42	1·70 ± 0·06 (9)	1·19 ± 0·03 (8)*	0·50 ± 0·011 (5)†
63	2·84 ± 0·06 (7)	1·80 ± 0·05 (6)*	0·62 ± 0·050 (4)†

*Significantly ($P < 0·05$) lower than the combined weight of two testes, but significantly higher than the weight of a single testis in normal rats; †significantly lower than the combined weight of two testes in normal rats (two-tailed Student's *t*-test).
‡In normal and irradiated rats the 'total testis weight' reflects the combined weight of the two testes. In the hemicastrated rats it reflects the weight of the single remaining testis.

in normal and hemicastrated rats to a comparable, low level at 42 and 63 days of age (Fig. 1*a* and Table 1). Prenatally irradiated animals showed a low testicular concentration of inhibin activity at all ages studied. The peripheral concentration of FSH increased with age in all groups, reached its highest level on day 42 of age and decreased thereafter (Fig. 1*b*). In contrast, concentrations of testosterone in serum increased between the ages of 42 and 63 days (Fig. 1*c*). Peripheral levels of FSH in neonatally hemicastrated animals with hypertrophic testicular growth were significantly ($P < 0·01$) increased when compared with levels in untreated rats at 7 and 14 days of age, while in irradiated rats levels of FSH in serum were significantly ($P < 0·01$) decreased at the age of 14 days and significantly ($P < 0·05$ and $P < 0·01$) increased at the ages of 42 and 63 days (Fig. 1*b*). No significant influences of treatment on the levels of LH in serum were detected (data not shown).

Until the age of 42 days an inverse correlation between the amount of inhibin expressed per gram testicular tissue and peripheral FSH concentrations was found in non-treated animals ($r = -0·75$, $n = 22$, $y = 1317·24 - 0·72x$).

In-vitro experiments

Phase-contrast microscopic examination of the Sertoli cell cultures indicated that the cultures from testes of older animals still contained a number of spermatogenic cells at the end of the incubation period. The cell preparations were therefore studied in more detail by flow cytometric DNA analysis. Cultures from 7-, 14-, and 21-day-old rat testes contained 92, 87 and 82% diploid cells respectively, while cultures derived from older rat testes contained only about 30% diploid cells. It is not possible to discriminate between spermatogonia and Sertoli cells, which contain the same amount of DNA, but the number of spermatogonia was assumed to be 10% of the number of Sertoli cells from 21 days of age onwards, as calculated by de Jong & Sharpe (1977). Taking these corrections into account, inhibin activity was expressed per 10^6 plated Sertoli cells per 24 h (Fig. 2). Sertoli cells from 7- and 14-day-old testes secreted significantly ($P < 0·01$) more inhibin than cells from the older testes. From 21 days of age onwards, the amount of inhibin secreted into the media decreased further at 42 ($P < 0·01$) and 63 ($P < 0·05$) days of age.

Addition of FSH caused a significantly increased production of inhibin by Sertoli cells from 21-day-old rat testes, while no significant effects were found at either the younger ages where inhibin production was relatively high or at the older ages where inhibin production was relatively low (Fig. 3). In contrast, preincubation with testosterone caused a significantly ($P < 0·01$) lower release of inhibin from Sertoli cells at all ages studied, except from those from 42-day-old rats (Fig. 3). After preincubation with testosterone in the presence of FSH, inhibin production returned to values as found under control conditions until the age of 63 days, at which age no inhibin activity was detectable in spent media.

DISCUSSION

The relationship between testicular inhibin levels and resulting circulating inhibin concentrations may be influenced by a number of factors, which change during the development of the animal. First, the number of testicular cells which produce inhibin (the Sertoli cells) (Steinberger & Steinberger, 1976; de Jong, Welschen, Hermans *et al.* 1979*b*; Verhoeven & Franchimont, 1983; Ultee-van Gessel *et al.* 1986) increases up to the age of 15 (Clermont & Perey,

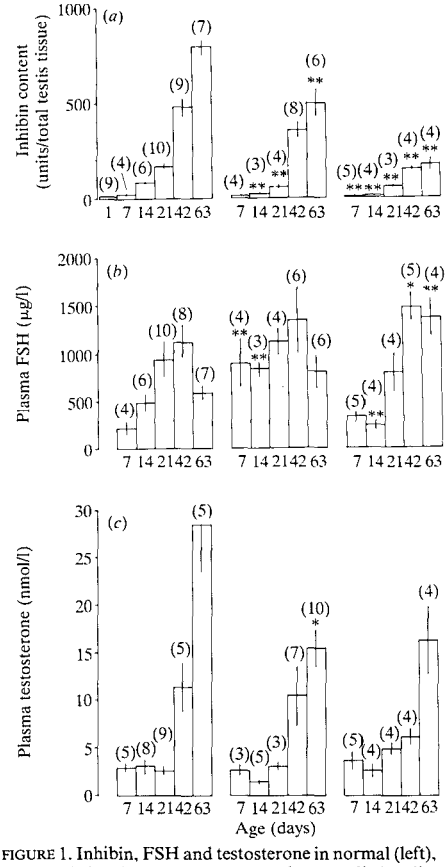

FIGURE 1. Inhibin, FSH and testosterone in normal (left), neonatally hemicastrated (centre) and prenatally irradiated (right) male Wistar rats at different ages. Values are means ±S.E.M., numbers are shown in parentheses. (*a*) Total amount of inhibin in paired testes, (*b*) peripheral plasma FSH and (*c*) peripheral plasma testosterone. $*P<0.05$, $**P<0.01$ compared with appropriate values in control animals of the same age (Student's *t*-test).

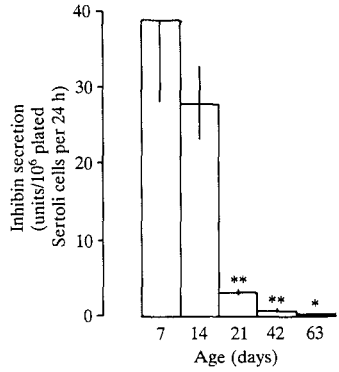

FIGURE 2. Amounts of inhibin in culture media of Sertoli cells isolated from normal rat testes at different ages. Data are expressed as units inhibin activity/10^6 plated Sertoli cells measured in the culture medium collected after a 24-h incubation period on the third day of culture (means ± S.E.M., $n=3$). $*P<0.05$, $**P<0.01$ compared with the value of the age before (Student's *t*-test).

1957), 18 (Steinberger & Steinberger, 1971), 21 (Orth, 1982) or 32 (Nagy, 1972; de Jong & Sharpe, 1977) days. After this period of increasing numbers of Sertoli cells, the weight of the testis increases considerably through the further development of the spermatogenic cells. Secondly, the amount of inhibin produced per Sertoli cell is clearly influenced by the age of the animal (Massicotte *et al.* 1984, this study); this development counteracts the influence of the increasing number of Sertoli cells. Thirdly, the architecture of the seminiferous tubules changes during development: around the age of 18 days the blood–testis barrier is closed (Dym & Fawcett, 1970; Vitale, 1975), making it less likely that inhibin from the luminal compartment of the tubule will reach the interstitial compartment and therefore the peripheral circulation easily; both the rete testis fluid and testicular lymph contain inhibin activity (see Setchell & Jacks, 1974; Hudson, Baker, Eddie *et al.* 1979). Hormones might influence the distribution of inhibin secretion between the luminal and basal side of the Sertoli cell, as is the case with androgen-binding protein (Gunsalus, Musto & Bardin, 1980). Finally, once a certain amount of inhibin has been secreted from the testis, it will be distributed through the general circulation, the volume of which changes with the size of the animal. Nothing is known about possible changes in metabolic clearance of inhibin with age, and reports on changes in pituitary sensitivity for inhibin have yielded conflicting results (Steinberger, 1979; Hermans *et al.* 1980). All of these factors should be considered when evaluating the relationships between the results of the present experiments.

In-vivo experiments

Inhibin activity measured with the in-vitro bioassay method could be detected in homogenates of testicular tissue of rats from birth to adulthood. The validity of this method has been evaluated by Au, Robertson & de Kretser (1983), who found that charcoal-treated

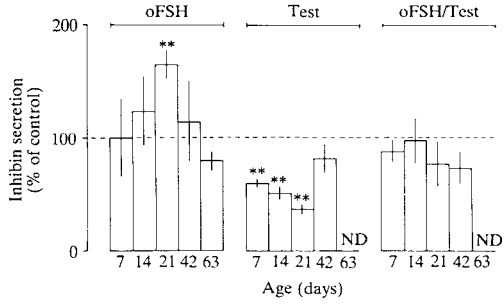

FIGURE 3. Relative amounts of inhibin in cell culture media of Sertoli cells after a 8-h preincubation with the addition of ovine FSH (oFSH) (500 ng/ml), testosterone (Test) (700 nmol/l) or testosterone in the presence of ovine FSH. Sertoli cells were isolated from rats of different ages. Data are expressed as percentage of appropriate controls (means ± S.E.M., $n = 3$). ND, not detectable. $^{**}P < 0{\cdot}01$ compared with the value of controls of the same age without added hormones (dotted line) (Student's *t*-test).

cytosols from rat testes caused a dose-dependent suppression of FSH without effect on the LH content of cultured pituitary cells. The concentration of inhibin activity in testes from 63-day-old normal rats as found in this study corresponds with the concentration reported by de Kretser, Au, Le Gac & Robertson (1983) in normal adult rats after correction for the difference in standard preparation used in the bioassay. Although both testicular inhibin content and peripheral FSH levels increased gradually with age, up to day 42 an inverse correlation between the amount of inhibin expressed per gram testicular tissue and peripheral FSH concentrations was found in untreated animals up to this age. At the age of 63 days the further decrease of testicular inhibin concentration was not accompanied by a further rise in serum FSH. The drop in the level of FSH at this age might be caused by increased concentrations of testosterone in the circulation.

After neonatal hemicastration a significant increase of plasma FSH was found in animals of 7 and 14 days of age. This increase was associated with a lower testicular inhibin content of the remaining testis in the 14-day-old animals. Previous studies have shown that a diminished pituitary feedback of FSH secretion by testicular inhibin from the remaining testis occurred in 21-day-old hemicastrated rats despite compensatory growth, while in older animals pituitary FSH secretion reached control levels (Ultee-van Gessel, Leemborg, de Jong & van der Molen, 1985) as was found here.

The testes in prenatally irradiated animals contained lower amounts of inhibin at all ages studied. Comparable results were obtained for levels of testicular plasminogen activator (PA) (Fritz, 1982). This may be associated with impairment of the secretory function of Sertoli cells in aspermatogenic rats as described by Rich & de Kretser (1977). Wang, Galil & Setchell (1983) reported that after destruction of seminiferous epithelium a decrease in testicular blood flow was responsible for the limited capacity of the testes to secrete testosterone into the circulation. However, in this study normal concentrations of testosterone in serum were found, while circulating levels of FSH were found to be higher than those in control groups at the ages of 42 and 63 days but lower at 14 days. In addition, it has been reported that absence of spermatogenic cells as a result of in-utero irradiation delays the appearance of Sertoli cell-occluding junctions in rats (Vitale, 1975). Apparently, after prenatal irradiation the architecture of the testis is important for the amount of inhibin secreted into the circulation.

In-vitro experiments

Cultured Sertoli cells from 21-day-old rat testes secreted significantly less inhibin activity when compared with Sertoli cells from 7- and 14-day old rats, while at 42 and 63 days even lower amounts of inhibin activity were secreted. This confirms the data of Massicotte *et al.* (1984), who used 10- to 40-day-old animals and collected Sertoli cell culture media for inhibin measurements during the last 5 days of culture of a 7-day culture period. In contrast, Steinberger (1979) found no change in inhibin secretion from Sertoli cells obtained from rats between 18 and 90 days of age, as

measured in media collected on day 5 of culture. The decreasing in-vitro secretion of inhibin with age apparently reflects changes in the secretory function of Sertoli cells with age, as did the decreasing secretion of oestradiol with age (Rommerts, de Jong, Brinkmann & van der Molen, 1982) and the increasing secretion of androgen-binding protein (ABP) (Rich, Bardin, Gunsalus & Mather, 1983) and of transferrin (Skinner & Griswold, 1982; Perez-Infante, Bardin, Gunsalus *et al.* 1986).

The observation that stimulation of inhibin production *in vitro* by FSH is possible only around day 21 of age suggests that feedback regulation of pituitary FSH is important at this age. The amount of ABP and the activity of PA were also increased by the addition of FSH to the medium during culture of Sertoli cells from 20-day-old rats (Rich *et al.* 1983; Lacroix, Smith & Fritz, 1977 respectively). In contrast, inhibin production by Sertoli cell cultures from 7- and 14-day-old animals is relatively high and was not affected by the presence of FSH. This situation may be comparable to that in Sertoli cells of 21-day-old rats cultured at 37 °C, where again a relatively high amount of inhibin in the culture medium was not increased after addition of FSH to the culture medium (Ultee-van Gessel *et al.* 1986). The lack of stimulation of inhibin production by FSH in Sertoli cell cultures from older animals may be explained by the age-related decline in cyclic AMP response to FSH as reported by Means (1975).

The addition of relatively high amounts of testosterone to the culture medium suppressed the in-vitro secretion of inhibin significantly in Sertoli cells of 7-, 14-, 21- and 63-day-old rats. This suggests that increasing testicular levels of testosterone could be a factor causing the low secretion rate of inhibin at the older ages.

The results of the present study indicate that the in-vitro secretion of inhibin by Sertoli cell-enriched primary cultures is related to the age of the animals. The production of inhibin per Sertoli cell decreases with the age of the animal, but the population of Sertoli cells continues to increase until 21–35 days of age. The levels of testicular inhibin increase during development; this might be partly due to accumulation of inhibin activity within compartments of the growing testis which become less penetrable for relatively large molecules. Finally, the amount of inhibin which is secreted into the circulation, and which is needed for pituitary feedback regulation, might be deduced from peripheral FSH levels. Earlier data have shown that the postcastration rise of plasma FSH decreases with age in male rats, while at all ages injections of exogenous inhibin suppress this rise of FSH (Hermans *et al.* 1980). This makes it likely that the inhibin content of the testis does not reflect the amount of inhibin secreted into the circulation which would be important for feedback regulation of pituitary FSH secretion.

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REFERENCES

Au, C. L., Irby, D. C., Robertson, D. M. & de Kretser, D. M. (1986). Effects of testosterone on testicular inhibin and fluid production in intact and hypophysectomized adult rats. *Journal of Reproduction and Fertility* **76**, 257–266.

Au, C. L., Robertson, D. M. & de Kretser, D. M. (1983). *In-vitro* bioassay of inhibin in testes of normal and cryptorchid rats. *Endocrinology* **112**, 239–244.

Au, C. L., Robertson, D. M. & de Kretser, D. M. (1984). Relationship between testicular inhibin content and serum FSH concentrations in rats after bilateral efferent duct ligation. *Journal of Reproduction and Fertility* **72**, 351–356.

Au, C. L., Robertson, D. M. & de Kretser, D. M. (1985). Effects of hypophysectomy and subsequent FSH and testosterone treatment on inhibin production by adult rat testes. *Journal of Endocrinology* **105**, 1–6.

Beaumont, H. M. (1960). Changes in the radiosensitivity of the testis during foetal development. *International Journal of Radiation Biology* **2**, 247–256.

Clermont, Y. & Perey, B. (1957). Quantitative study of the cell population of the seminiferous tubules in immature rats. *American Journal of Anatomy* **100**, 241–267.

Dym, M. & Fawcett, D. W. (1970). The blood–testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biology of Reproduction* **3**, 308–326.

Eddie, L. W., Baker, H. W. G., Higginson, R. E. & Hudson, B. (1979). A bioassay for inhibin using pituitary cell cultures. *Journal of Endocrinology* **81**, 49–60.

Finney, D. J. (1964). Parallel line assays. In *Statistical Method in Biological Assay*, edn 2, pp. 101–138. London: Griffin.

Franchimont, P., Chari, S. & Demoulin, A. (1975). Hypothalamus-pituitary-testis interaction. *Journal of Reproduction and Fertility* **44**, 335–350.

Franchimont, P., Chari, S., Hagelstein, M. T. & Duraiswami, S. (1975). Existence of a follicle-stimulating hormone inhibiting factor 'inhibin' in bull seminal plasma. *Nature* **257**, 402–404.

Fritz, I. B. (1982). Comparison of granulosa and Sertoli cells at various stages of maturation: similarities and differences. In *Intraovarian Control Mechanisms*, pp. 357–384. Eds C. P. Channing, & S. J. Segal. New York: Plenum Press.

Gunsalus, G. L., Musto, N. A. & Bardin, C. W. (1980). Bidirectional release of a Sertoli cell product, androgen binding protein, into the blood and seminiferous tubule. In *Testicular Development, Structure, and Function*, pp. 291–297. Eds A. Steinberger & E. Steinberger. New York: Raven Press.

Hermans, W. P., van Leeuwen, E. C. M., Debets, M. H. M. & de Jong, F. H. (1980). Involvement of inhibin in the regulation of follicle-stimulating hormone concentrations in prepubertal and adult, male and female rats. *Journal of Endocrinology* **86,** 79–92.

Hermans, W. P., van Leeuwen, E. C. M., Debets, M. H. M., Sander, H. J. & de Jong, F. H. (1982). Estimation of inhibin-like activity in spent medium from rat ovarian granulosa cells during long-term culture. *Molecular and Cellular Endocrinology* **27,** 277–290.

Hudson, B., Baker, H. W. G., Eddie, L. W., Higginson, R. E., Burger, H. G., de Kretser, D. M., Dobos, M. & Lee, V. W. K. (1979). Bioassays for inhibin: a critical review. *Journal of Reproduction and Fertility* Suppl. **26,** 17–29.

de Jong, F. H. (1979). Inhibin, fact or artifact. *Molecular and Cellular Endocrinology* **13,** 1–10.

de Jong, F. H., Jansen, E. H. J. M. & van der Molen, H. J. (1981). Purification and characterization of inhibin. In *Intragonadal Regulation of Reproduction*, pp. 229–250. Eds P. Franchimont & C. P. Channing. New York: Academic Press.

de Jong, F. H., Jansen, E. H. J. M., Steenbergen, J., van Dijk, S. & van der Molen, H. J. (1983). Assay and purification of inhibin. In *Role of Peptides and Proteins in Control of Reproduction*. Eds S. M. McCann & D. S. Dhindsa. New York: Elsevier Science Publishing Co.

de Jong, F. H. & Sharpe, R. M. (1977). The onset and establishment of spermatogenesis in rats in relation to gonadotrophin and testosterone levels. *Journal of Endocrinology* **75,** 197–207.

de Jong, F. H., Smith, S. D. & van der Molen, H. J. (1979*a*). Bioassay of inhibin-like activity using pituitary cells *in vitro*. *Journal of Endocrinology* **80,** 91–102.

de Jong, F. H., Welschen, R., Hermans, W. P., Smith, S. D. & van der Molen, H. J. (1979*b*). Effects of factors from ovarian follicular fluid and Sertoli cell culture medium on in-vivo and in-vitro release of pituitary gonadotrophins in the rat: an evaluation of systems for the assay of inhibin. *Journal of Reproduction and Fertility* Suppl. **26,** 47–59.

Kapuscinski, J. & Skoczylas, B. (1977). Simple and rapid fluorimetric method for DNA microassay. *Analytical Biochemistry* **83,** 252–257.

Keogh, E. J., Lee, V. W. K., Rennie, G. C., Burger, H. G., Hudson, B. & de Kretser, D. M. (1976). Selective suppression of FSH by testicular extracts. *Endocrinology* **98,** 997–1004.

de Kretser, D. M., Au, C. L., Le Gac, F. & Robertson, D. M. (1983). Recent studies on inhibin. In *Recent Advances in Male Reproduction: Molecular Basis and Clinical Implications*, pp. 91–99. Eds R. D'Agata, M. B. Lipsett, P. Polosa & H. J. van der Molen. New York: Raven Press.

Lacroix, M., Smith, F. E. & Fritz, I. B. (1977). Secretion of plasminogen activator by Sertoli cell enriched cultures. *Molecular and Cellular Endocrinology* **9,** 227–236.

Lee, G. M., Thornthwaite, J. T. & Rasch, E. M. (1984). Picogram per cell determination of DNA by flow cytofluorometry. *Analytical Biochemistry* **137,** 221–226.

Le Gac, F. & de Kretser, D. M. (1982). Inhibin production by Sertoli cell cultures. *Molecular and Cellular Endocrinology* **28,** 487–498.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193,** 265–275.

McCullagh, D. R. (1932). Dual endocrine activity of the testes. *Science* **76,** 19–20.

Massicotte, J., Lagacé, L., Labrie, F. & Dorrington, J. H. (1984). Modulation of gonadotropin secretion by Sertoli cell inhibin, LHRH, and sex steroids. *American Journal of Physiology* **247,** E495–E504.

Means, A. R. (1975). Biochemical effects of follicle-stimulating hormone on the testis. In *Handbook of Physiology*, section 7, vol. 5, pp. 203–218. Eds D. W. Hamilton & R. O. Greep.

Nagy, F. (1972). Cell division kinetics and DNA synthesis in the immature Sertoli cells of the rat testis. *Journal of Reproduction and Fertility* **28,** 389–395.

Perez-Infante, V., Bardin, C. W., Gunsalus, G. L., Musto, N. A., Rich, K. A. & Mather, J. P. (1986). Differential regulation of testicular transferrin and androgen-binding protein secretion in primary cultures of rat Sertoli cells. *Endocrinology* **118,** 383–392.

Orth, J. (1982). Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anatomical Record* **203,** 485–492.

Rich, K. A., Bardin, C. W., Gunsalus, G. L. & Mather, J. P. (1983). Age-dependent pattern of androgen-binding protein secretion from rat Sertoli cells in primary culture. *Endocrinology* **113,** 2284–2293.

Rich, K. A. & de Kretser, D. M. (1977). Effect of differing degrees of destruction of the rat seminiferous epithelium on levels of serum follicle stimulating hormone and androgen binding protein. *Endocrinology* **101,** 959–968.

Rommerts, F. F. G., de Jong, F. H., Brinkmann, A. O. & van der Molen, H. J. (1982). Development and cellular localization of rat testicular aromatase activity. *Journal of Reproduction and Fertility* **65,** 281–288.

Setchell, B. P. & Jacks, F. (1974). Inhibin-like activity in rete testis fluid. *Journal of Endocrinology* **62,** 675–676.

Skinner, M. K. & Griswold, M. D. (1982). Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. *Biology of Reproduction* **27,** 211–221.

Steinberger, A. (1979). Inhibin production by Sertoli cells in culture. *Journal of Reproduction and Fertility* Suppl. **26,** 31–45.

Steinberger, A. & Steinberger, E. (1971). Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. *Biology of Reproduction* **4,** 84–87.

Steinberger, A. & Steinberger, E. (1976). Secretion of an FSH-inhibiting factor by cultured Sertoli cells. *Endocrinology* **99,** 918–921.

Ultee-van Gessel, A. M., Leemborg, F. G., de Jong, F. H. & van der Molen, H. J. (1985). Influence of neonatal hemicastration on in-vitro secretion of inhibin, gonadotrophins and testicular steroids in male rats. *Journal of Endocrinology* **106,** 259–265.

Ultee-van Gessel, A. M., Leemborg, F. G., de Jong, F. H. & van der Molen, H. J. (1986). In-vitro secretion of inhibin-like activity by Sertoli cells from normal and prenatally irradiated immature rats. *Journal of Endocrinology* **109,** 411–418.

Verhoeven, G. & Franchimont, P. (1983). Regulation of inhibin secretion by Sertoli cell-enriched cultures. *Acta Endocrinologica* **102,** 136–143.

Verjans, H. L., Cooke, B. A., de Jong, F. H., de Jong, C. M. M. & van der Molen, H. J. (1973). Evaluation of a radioimmunoassay for testosterone estimation. *Journal of Steroid Biochemistry* **4,** 665–676.

Vindeløv, L. L., Christensen, I. J. & Nissen, N. I. (1983). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* **5,** 323–327.

Vitale, R. (1975). The development of the blood testis barrier in Sertoli cells of rats. *Anatomical Record* **181,** Abstract 501.

Wang, J., Galil, K. A. A. & Setchell, B. P. (1983). Changes in testicular blood flow and testosterone production during aspermatogenesis after irradiation. *Journal of Endocrinology* **98,** 35–46.

Welschen, R., Osman, P., Dullaart, J., de Greef, W. J., Uilenbroek, J. Th. J. & de Jong, F. H. (1975). Levels of follicle-stimulating hormone, luteinizing hormone, oestradiol-17β and progesterone, and follicular growth in the pseudopregnant rat. *Journal of Endocrinology* **64,** 34–47.

In-vitro secretion of inhibin-like activity by Sertoli cells from normal and prenatally irradiated immature rats

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ABSTRACT

The influence of in-vitro conditions on the production of inhibin by Sertoli cells from 21-day-old normal and prenatally irradiated rat testes was studied by measuring inhibin activity in culture media, using the suppression of the release of FSH from cultured rat pituitary cells. Sertoli cells secreted inhibin-like activity during at least 21 days of culture, and cells cultured at 37 °C produced significantly more inhibin than those cultured at 32 °C. The presence of fetal calf serum had no significant effect on inhibin production at 32 °C, while at 37 °C the production was decreased. The presence of ovine FSH stimulated inhibin secretion, while inhibin concentrations in Sertoli cell culture media were decreased after the addition of testosterone. Testosterone, added together with ovine FSH, suppressed inhibin secretion when compared with the levels found in the presence of FSH alone. The presence of spermatogenic cells decreased the release of inhibin.

From these results it was concluded that both Sertoli cells isolated from normal immature rat testes and those from testes without spermatogenic cells can secrete inhibin-like activity in culture. A number of discrepancies with in-vivo observations was observed. Therefore, it is likely that the in-vivo situation is too complicated for direct study of the regulation of inhibin production, because of mutual interactions between the testicular compartments.

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INTRODUCTION

It is generally accepted that Sertoli cells produce and secrete inhibin (Steinberger & Steinberger, 1976; de Jong, Welschen, Hermans *et al.* 1978; Le Gac & de Kretser, 1982; Verhoeven & Franchimont, 1983), a protein hormone which exerts a specific suppressing activity on the secretion of follicle-stimulating hormone (FSH) from the pituitary gland (Setchell, Davies & Main, 1977; de Jong, 1979). During the last few years several authors have investigated the production and secretion of inhibin by Sertoli cells *in vivo* (Steinberger, 1981; Au, Robertson & de Kretser, 1983, 1984) and *in vitro* (Steinberger, 1981; Le Gac & de Kretser, 1982; Verhoeven & Franchimont, 1983). The results of these investigations show a number of discrepancies. Verhoeven & Fanchimont (1983) reported that basal secretion of inhibin was undetectable when Sertoli cells were incubated in the absence of fetal calf serum (FCS), while Le Gac & de Kretser (1982) described a toxic effect of FCS-containing Sertoli cell culture media on the pituitary cell culture when FCS was present for more than 48 h. Steinberger (1981) and Le Gac & de Kretser (1982) found a stimulatory effect of FSH on inhibin secretion, while Verhoeven & Franchimont (1983) could not demonstrate any effect of FSH. In contrast, the latter authors observed increased inhibin release after the addition of high concentrations of testosterone. The described differences between the results from different laboratories might be related to differences in experimental protocol.

The aim of the present study was to compare, under various in-vitro conditions, the inhibin production by Sertoli cells isolated from normal immature rat testes and from those without spermatogenic cells. In addition, the influence of spermatogenic cells on inhibin production was examined in recombination experiments and by comparing the inhibin secretion by Sertoli cells from normal and prenatally irradiated 21-day-old animals, using monolayers of dispersed cells or intact seminiferous tubule cultures.

MATERIALS AND METHODS

Animals

Throughout this study, 21-day-old male Wistar rats were used. They were kept with their mothers in an animal room under controlled conditions of temperature (21–22 °C) and lighting (lights on 04.45–19.00 h). One group of animals was irradiated *in utero* on day 19 *post coitum*, to obtain spermatogenic-cell-depleted testes (Beaumont, 1960). The animals were killed with CO_2 before excision of the testes.

Preparation of Sertoli cell cultures and influence of hormones

Long-term culture experiments

Testes from six–eight immature rats were quickly removed, decapsulated, placed in a 100 ml siliconized Erlenmeyer flask in 20 ml Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY, U.S.A.) containing 1 mg collagenase/ml (type I; Worthington Biochemical Corporation, Freehold, NJ, U.S.A.) and incubated for 40 min at 32 °C in a shaking water bath (frequency of 120 cycles/min). The tubular fragments, obtained after this procedure, were washed three times with HBSS and subsequently resuspended in 10 ml Eagle's Minimum Essential Medium (MEM; Gibco) which contained non-essential amino acids (Gibco), fungizone (Squibb bv, Rijswijk, The Netherlands; 600 ng/ml), streptomycin (Specia, Paris; 100 µg/ml) and penicillin (Gist-Brocades nv, Delft, The Netherlands; 100 i.u./ml). After mechanical dispersion using a plastic 10 ml pipette, small aggregates of Sertoli cells (1–1·5 mg protein/flask, as estimated at the end of the culture period using the method of Lowry, Rosebrough, Farr & Randall (1951)) were placed in 25 cm^2 plastic culture flasks (Falcon; Becton Dickinson Labware, Oxnard, CA, U.S.A.) in 4 ml supplemented MEM. The isolated Sertoli cells, still containing spermatogenic elements when derived from normal animals, were cultured at 32 or 37 °C with or without 5% FCS (Gibco) for 1 or 3 weeks in a humidified incubator in an atmosphere of 5% CO_2 in air. Every 2 or 3 days, Sertoli cell culture media (SCCM) were removed and replaced by 4 ml supplemented MEM. The collected media were centrifuged at 1500 *g* for 10 min to remove cellular debris which might exert toxic effects in pituitary cell cultures (Le Gac & de Kretser, 1982). The removed SCCM were stored at −20 °C until estimation of inhibin-like activity.

Ovine FSH (NIH-FSH S13; 500 ng/ml medium; a gift from NIH, Bethesda, MD, U.S.A.) was added to some cultures of Sertoli cells in the absence of FCS, at the start of the culture period. Medium was collected after 48 h of culture and stored at −20 °C until assayed for inhibin activity.

Short-term culture experiments

In these experiments a slightly different method was used for the preparation of Sertoli cell cultures in order to compare the inhibin production by Sertoli cells from normal rat testes with those from testes without spermatogenic cells. To remove peritubular cells more extensively, decapsulated testes were incubated twice for 30 min at 32 °C in a thermostatic shaking water bath (frequency of 120 cycles/min) in HBSS containing 0·5 mg collagenase type I/ml. In between these incubations, tubular fragments were allowed to sediment for 5 min at unit gravity and washed once with HBSS. Subsequently, tubular fragments were washed three times with MEM supplemented as described above. These tubular fragments stripped from peritubular cells were either used directly for culture experiments or dispersed using 15 strokes of a Dounce homogenizer (de Jong, Smith & van der Molen, 1979) to prepare small aggregates of Sertoli cells. Both for culture of seminiferous tubules or small aggregates of Sertoli cells, samples of the cell preparations were placed into the wells of 12-well tissue culture plates (Costar, Cambridge, MA. U.S.A.; about 400 µg protein/well, as estimated at the end of the culture period (Lowry *et al.* 1951)). The cells were initially cultured in 2 ml supplemented MEM in a humidified incubator in an atmosphere of 5% CO_2 in air. After this initial 24-h period in the presence of 1% FCS in order to stimulate attachment of the cells, culture media were replaced by 1 ml fresh medium without FCS, with or without ovine FSH (500 ng/ml) and/or testosterone (Steraloids, Wilton, NH, U.S.A.; 200 µg/l). Cells were cultured in the absence or presence of these hormones for the next 8 h. Finally, the cells were washed three times with fresh medium and subsequently incubated for an additional 24-h period in 1 ml medium without hormones. The latter media were collected and stored at −20 °C until bioassay for inhibin-like activity.

Effects of spermatogenic cells

Recombination experiments

Pachytene spermatocytes and round spermatids from testes of normal Wistar rats (30–40 days old) were isolated by the 'Staput' method (Grootegoed, Grollé-Hey, Rommerts & van der Molen, 1977). Fractions containing 80–90% spermatocytes or early spermatids, as judged by light microscopic investigation (10^6 cells/ml), were used for recombination with Sertoli cell monolayers isolated from the testes of prenatally irradiated rats. These recombination

experiments were carried out on day 7 of long-term cultures at 37 °C in the absence or presence of ovine FSH.

As a control, thymocytes were isolated from immature rats by mechanical dispersion of the thymus. The cells were washed three times before addition to Sertoli cell monolayers.

Short-term culture experiments

The influence of the presence of spermatogenic cells was also studied by comparing inhibin production by cultures of Sertoli cells from normal and prenatally irradiated rat testes under similar in-vitro conditions.

Hormone determinations

Bioassay of inhibin activity

Inhibin-like activity was measured using cultures of dispersed rat pituitary cells (de Jong *et al.* 1979; Hermans, van Leeuwen, Debets *et al.* 1982). Samples of SCCM were assayed in quadruplicate at two dose levels. The suppression of FSH was compared with that caused by the addition of three quadruplicate doses of a standard preparation of charcoal-treated bovine follicular fluid with the arbitrary potency of 1 unit/μg protein; concentrations of luteinizing hormone (LH) in the media were not affected. Inhibin potencies of the media were calculated using statistics for parallel line assay (Finney, 1964). Significant non-parallelism was never observed. The precision index (λ) amounted to $0{\cdot}13 \pm 0{\cdot}01$ (mean ± S.E.M., $n = 18$; range 0·07–0·25). Data on the reproducibility of the assay have been summarized by de Jong, Jansen, Steenbergen *et al.* (1983). Inhibin-like activities are expressed in units/day per culture flask or units/day per mg protein. Whenever inhibin activity was measured in media from Sertoli cells cultured in the presence of ovine FSH, FSH concentrations in the medium of the pituitary cells were corrected for the amount of ovine FSH added, as measured in control pituitary cell cultures which contained the same amount of ovine FSH. These corrections amounted maximally to 22% of the FSH estimated.

Estimation of gonadotrophins and steroids

Concentrations of FSH and LH in pituitary cell culture media were estimated by radioimmunoassay as described previously (Welschen, Osman, Dullaart *et al.* 1975). All results are expressed in terms of NIADDK-rat FSH-RP1 and NIADDK-rat LH-RP1. Intra- and interassay coefficients of variation were 11·0 and 5·1% for FSH and 16·3 and 14·2% for LH respectively.

Oestradiol and testosterone concentrations in SCCM were assayed by radioimmunoassay as described by de Jong, Hey & van der Molen (1973) and Verjans, Cooke, de Jong *et al.* (1973) respectively.

Statistical analysis

The significance of differences between inhibin secretion by Sertoli cells in different culture conditions was assessed using paired or non-paired Student's *t*-test. Differences were considered significant when $P < 0{\cdot}05$ (two-tailed).

RESULTS

Effects of FCS and temperature on the production of inhibin activity by cultured Sertoli cells

When Sertoli cells from normal rat testes were cultured in the presence or absence of FCS at 32 or 37 °C for a period of 21 days, inhibin-like activity could be detected in media collected over 2- to 3-day periods. Data on the secretion of inhibin by Sertoli cells cultured at 32 °C in one representative experiment are shown in Fig. 1; similar results were obtained in other experiments. The amount of inhibin-like activity in spent media varied with the time of culture: most inhibin was secreted between days 5 and 12 of the culture period. The effect of temperature on the amount of inhibin in the medium of these long-term cultures became clear only after a longer culture period: there was no significant difference between the amounts of inhibin released from Sertoli cells cultured at 32 °C (145 ± 49 units/day per culture flask; mean ± S.E.M., $n = 4$) or 37 °C (133 ± 72 units/day per culture flask; $n = 4$) up to day 10 of culture; thereafter Sertoli cells at 37 °C produced significantly ($P < 0{\cdot}01$) more inhibin

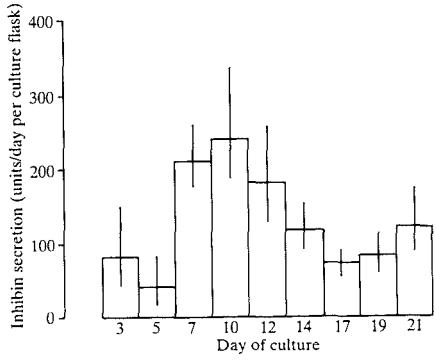

FIGURE 1. Inhibin secretion by Sertoli cells from normal rat testes cultured at 32 °C without addition of hormones and serum to the culture medium. Media were changed every 2–3 days. Results are given as units inhibin/day per culture flask; 95% confidence limits are shown (1 μl bovine follicular fluid standard preparation contains 65 units inhibin activity).

TABLE 1. Inhibin activity in spent media of Sertoli cells from normal and prenatally irradiated 21-day-old rats, cultured at 32 or 37 °C. Values are means ± S.E.M.; the number of cultures is shown in parentheses

		Inhibin activity (units/day per mg protein)	
	Culture	32 °C	37 °C
Treatment			
None	Monolayer	50·6 ± 5·1 (3)	185·5 ± 12·7 (3)†
	Tubules	36·8 ± 13·9 (2)	95·8 ± 3·3 (2)*†
Prenatally irradiated	Monolayer	89·7 ± 2·0 (6)*	232·0 ± 25·0 (6)†
	Tubules	64·4 ± 15·7 (6)	252·6 ± 88·2 (5)

*$P < 0.01$ compared with results obtained from Sertoli cells from normal rats, cultured as a monolayer at the appropriate temperature.
†$P < 0.01$ compared with results obtained at 32 °C using the same Sertoli cell preparation (Student's *t*-test).

than at 32 °C (263 ± 34 and 115 ± 19 units/day per culture flask respectively; $n = 5$).

The presence of FCS had no significant effect on the release of inhibin from Sertoli cells at 32 °C (without, 128 ± 23; with, 94 ± 18 units/day per culture flask; $n = 9$), while at 37 °C the presence of FCS significantly ($P < 0.05$) suppressed the production of inhibin from 205 ± 41 to 101 ± 19 units/day per culture flask ($n = 9$). The total inhibin production after 21 days of culture without FCS was 2704 units/culture flask at 32 °C and 4100 units/culture flask at 37 °C, while in the presence of 5% FCS, production was decreased to 1942 units/culture flask at 32 °C and 2098 units/culture flask at 37 °C.

During short-term cultures of Sertoli cell monolayers or tubular fragments from normal or prenatally irradiated rats, a similar effect of temperature on the amount of inhibin in the culture medium was observed. Inhibin release at 37 °C amounted to 312 ± 25% ($n = 14$) of that at 32 °C, indicating a highly significant ($P < 0.01$) increase of inhibin production at 37 °C (Table 1). Mean values of inhibin activity in spent media of Sertoli cell cultures from normal and prenatally irradiated testes cultured in monolayer or in tubular fragments at different temperatures are shown in Table 1. The concentration of inhibin in spent media of tubules derived from prenatally irradiated rat testes did not differ significantly from that in media of cultured tubules from normal rat testes.

Effect of temperature on protein concentrations of short-term Sertoli cell cultures

Table 2 shows the amount of protein/culture well at 37 °C, expressed as a percentage of the amount of protein at 32 °C under the same culture conditions. The amount of protein in cultures of testicular cells from normal and prenatally irradiated rats, estimated at the end of the culture period (after 56 h), was decreased significantly ($P < 0.01$) at the higher temperature. In addition, in cultures with Sertoli cells from prenatally irradiated rat testes this effect was larger than in cultures from normal rat testes. Cultures of seminiferous tubules from irradiated rats showed a significantly ($P < 0.01$) lower protein content than monolayer cultures of Sertoli cells derived from the same testes (data not shown). There was no significant effect of hormones on the amount of protein after 8-h preincubation with various hormones (data not shown).

TABLE 2. Relative amounts of protein/culture flask in which Sertoli cells or tubular fragments from normal and prenatally irradiated 21-day-old rats were cultured at 37 or 32 °C. The amount of protein in cultures at 32 °C = 100%. Values are means ± S.E.M.; the number of cultures is shown in parentheses

		Protein (%)	
	Culture	32 °C	37 °C
Treatment			
None	Monolayer	100 ± 3 (12)	86 ± 1 (12)*
	Tubules	100 ± 10 (12)	84 ± 5 (12)
Prenatally irradiated	Monolayer	100 ± 2 (24)	76 ± 5 (24)*
	Tubules	100 ± 12 (24)	56 ± 2 (24)*

*$P < 0.01$ compared with 32 °C (Student's *t* test).

Effect of hormones on in-vitro production of inhibin activity by Sertoli cells

In long-term incubation experiments, addition of ovine FSH stimulated inhibin production by Sertoli cells from normal rats at both 32 °C (basal, 4 ± 2; stimulated, 125 ± 23 units/day per culture; $P < 0.01$, $n = 2$) and 37 °C (basal, 25 ± 3; stimulated, 154 ± 22 units/day per culture; $P < 0.01$, $n = 2$). The relative

stimulation of inhibin production was lower at 37 than at 32 °C. This difference probably reflects the increased basal production at higher temperatures, since the absolute values of FSH-stimulated production of inhibin were not different.

The effects of preincubation with ovine FSH and/or testosterone on inhibin secretion by short-term cultures of monolayers of Sertoli cells or seminiferous tubules were comparable. Therefore, data from both types of experiments have been combined in Fig. 2. Addition of ovine FSH to cultures of Sertoli cells from prenatally irradiated rats caused a significant ($P<0{\cdot}01$) stimulation of inhibin production at both temperatures ($2{\cdot}7\pm0{\cdot}2$- and $2{\cdot}4\pm0{\cdot}3$-fold at 32 and 37 °C respectively; $n=12$), while inhibin production by Sertoli cells from normal rats was significantly ($P<0{\cdot}05$) stimulated by the presence of ovine FSH only at 32 °C ($1{\cdot}6\pm0{\cdot}2$-fold; $n=5$). Addition of testosterone during the preincubation period of 8 h caused a significant suppression of inhibin secretion in all cultures, to $39{\cdot}5\pm0{\cdot}6\%$ of control secretion ($n=4$). Addition of testosterone together with ovine FSH to cultured Sertoli cells had a similar suppressive effect on the secretion of inhibin, when compared with results of cultures to which only ovine FSH was added. Inhibin production by Sertoli cells from prenatally irradiated rats was significantly ($P<0{\cdot}01$ and $P<0{\cdot}05$) higher after hormone additions at 32 and 37 °C respectively, except when testosterone alone was added during preincubation (Fig. 2). Concentrations of testosterone and oestradiol in these samples of SCCM were below the detection limits of the assays (0·5 nmol/l and 20 pmol/l respectively; data not shown).

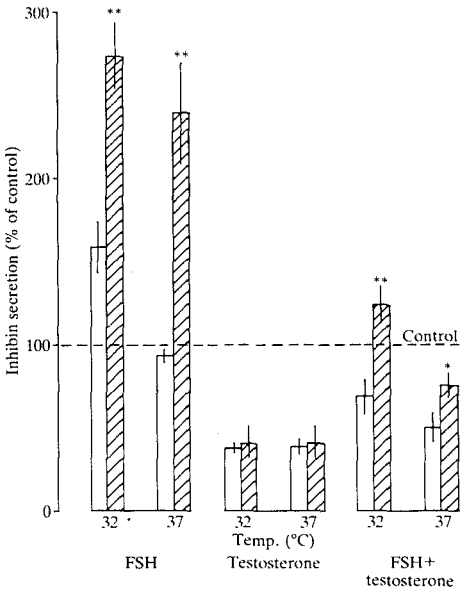

FIGURE 2. Inhibin secretion by Sertoli cells from normal (open bars) or prenatally irradiated (hatched bars) rat testes in the presence of various hormones at different temperatures during 24-h culture periods. Data are expressed as percentage of inhibin secretion in absence of hormones at the same temperature (means ± S.E.M., $n=3$–12). $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ compared with corresponding value for Sertoli cells from normal rats (Student's t-test).

Influence of spermatogenic cells on in-vitro production of inhibin activity by Sertoli cells

Results from recombination of spermatogenic and Sertoli cells during long-term experiments indicate that the presence of spermatocytes or spermatids significantly ($P<0{\cdot}01$) suppressed inhibin secretion from ovine FSH-stimulated immature Sertoli cells in culture at 37 °C (Fig. 3). The addition of non-testicular cells (thymocytes derived from normal immature male rats) had no effect on inhibin production under the same conditions.

During short-term cultures about 90% of the spermatogenic cells were washed away during changes of the media. Under these conditions, monolayers of Sertoli cells from prenatally irradiated testes secreted significantly more inhibin activity *in vitro* than Sertoli cells from normal testes, when results were expressed

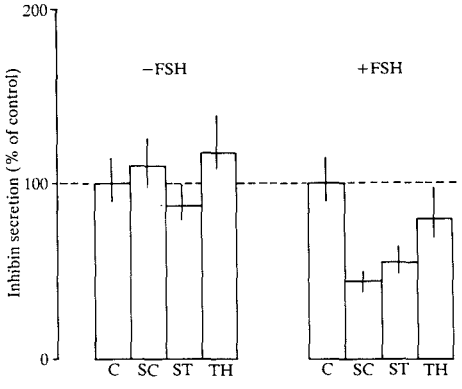

FIGURE 3. Inhibin secretion by Sertoli cells from rat testes without spermatogenic cells which were cultured for 24 h at 37 °C with spermatocytes (SC), spermatids (ST) or thymocytes (TH) in the absence or presence of 500 ng ovine FSH/ml (means and range for duplicate experiments). C, control.

on the basis of the amount of protein (Table 1). In contrast, spent media from cultures of seminiferous tubules without spermatogenic elements, cultured at 32 or 37 °C, did not contain significantly more inhibin than those with spermatogenic elements.

DISCUSSION

The results of the present study show that cultured Sertoli cells isolated from normal 21-day-old rat testes and those from testes containing only Sertoli cells secrete inhibin-like activity. The inhibin-like activity was secreted by cultured Sertoli cells for at least 21 days (Fig. 1) at 32 and 37 °C, but not by cultured testicular fibroblast-like cells or isolated spermatogenic cells (data not shown). These results confirm previous observations that inhibin-like activity in testes is a specific product of Sertoli cells.

The continued production of inhibin by Sertoli cells in culture is of interest, since under the same conditions in the absence of hormonal stimulation the secretion of androgen-binding protein decreased greatly (to approximately 10% on day 5) (Rommerts, Kruger-Sewnarain, van Woerkom-Blik *et al.* 1978). On the other hand, the same authors also showed that the production of oestradiol by Sertoli cells cultured in the presence of ovine FSH and testosterone remained constant or even increased with increasing length of culture. In long-term cultures at 32 °C, inhibin secretion between days 5 and 10 was higher than during the period before day 5. This might be caused by the loss of spermatogenic elements during changes of the medium (see below). Steinberger (1981), however, reported that spermatogenic cells had no effect on inhibin secretion by Sertoli cells in culture, but these cultures were derived from adult rat testes.

Results from long-term cultures showed that inhibin production at 37 °C was higher than at 32 °C from day 10 of culture onwards, when medium was renewed every 2 or 3 days; before that time no difference between inhibin production at 32 and 37 °C was observed. During short-term culture experiments the effect of temperature on inhibin secretion was already detectable after 56 h of culture (Table 1). These results are again in contrast with those of Steinberger (1981), who showed that inhibin production *in vitro* was significantly lower at 38 °C when compared with that at 32 °C. The difference between the present study and that of Steinberger (1981) is that, in the latter, cultures of Sertoli cells were derived from adult rats. Apparently, adult Sertoli cells cannot produce more inhibin at higher temperatures *in vitro*. This is in agreement with observations *in vivo* by Au, Robertson & de Kretser (1983), who found that testicular inhibin concentrations were decreased in experimentally cryptorchid adult rats. Increased inhibin production by Sertoli cells of immature rats at increased temperatures may be important in view of the observation that the temperature of the testes in 21-day-old rats is still higher than 32 °C, because the testes have not yet reached a scrotal position (Kormano, 1967).

The presence of FCS in the medium of the Sertoli cell cultures affected the amount of secreted inhibin adversely. Le Gac & de Kretser (1982) reported toxic effects of SCCM on cultured pituitary cells when Sertoli cells were cultured in the presence of FCS for periods longer than 48 h. Such effects were not observed in the present study, but due to these reported effects of FCS we have concluded that culture of Sertoli cells without FCS might represent the better model for the study of regulation of inhibin production. In addition, FCS might interact with added substances and thus influence their effect on inhibin production.

Stimulating effects of FSH on inhibin production by adult (Steinberger, 1981) and immature (Le Gac & de Kretser, 1982) Sertoli cells *in vitro* have already been shown. On the other hand, Verhoeven & Franchimont (1983) suggested that addition of FSH (100 ng ovine FSH S5/ml) to cultures did not influence inhibin production when added on day 7 of culture. Our results from long-term experiments indicate that FSH stimulated inhibin-like activity at the beginning of the culture period (media collected after the first 48 h of culture), which might partly explain the different results found by the authors mentioned above. In addition FCS might have inhibited hormone action in the experiments of Verhoeven & Franchimont (1983). Follicle-stimulating hormone stimulated inhibin secretion to the same levels at 32 and 37 °C, although basal secretion was increased at the higher temperature. This may reflect the use of supraphysiological amounts of FSH at both temperatures; maximal secretion rates of inhibin are apparently not different at 32 and 37 °C. Recently it has been reported that FSH stimulated the production of lactate and cyclic AMP in immature Sertoli cells *in vitro* also to the same level at 34 and 38 °C (Hall, Kew & Mita, 1985).

Testosterone inhibited inhibin secretion significantly in all cultures. This observation is in contrast with results from Steinberger (1981) and Verhoeven & Franchimont (1983), who found that inhibin secretion was stimulated by testosterone *in vivo* and *in vitro*. So far, no explanation for these differences can be found.

For the experiments in which Sertoli cells were recombined with spermatogenic cells (Fig. 3), a temperature of 37 °C was chosen because of the increased basal inhibin production at this temperature; this might cause larger differences in inhibin production after the attachment of spermatogenic cells. When a

fraction of spermatids or spermatocytes was combined with Sertoli cell monolayers from prenatally irradiated rats in the presence of FSH, the inhibin concentration in the medium collected after renewal of the medium was significantly lower, while the addition of thymocytes did not affect the production of inhibin (Fig. 3). This might indicate that specific spermatogenic cells are a signal for the Sertoli cell to produce less inhibin, as is the case *in vivo* when testicular development proceeds from an immature to the adult stage (Hermans, van Leeuwen, Debets & de Jong, 1980). This decrease of inhibin production after addition of spermatogenic cells to Sertoli cells contrasts with reports on increased FSH levels after destruction of the seminiferous epithelium (Krueger, Hodgen & Sherins, 1974; Mecklenburg, Hetzel, Gulyas & Lipsett, 1975). The latter studies, however, generally used adult rats, and it is not clear whether the seminiferous epithelium was selectively damaged; Sertoli and/or Leydig cells might also have been damaged. Therefore, the effect of spermatogenic cells on inhibin secretion was also examined by comparing inhibin production by Sertoli cells from both normal and prenatally irradiated immature testes in monolayer and tubular-fragment cultures.

Non-stimulated cultures of Sertoli cells from prenatally irradiated rat testes secreted about twice as much inhibin activity when compared with those from normal rat testes (Table 1); however, these data were expressed on the basis of protein. At the end of short-term culture experiments some spermatogenic cells (10% or less) were still attached when a monolayer culture was started from a Sertoli cell preparation of normal rat testes. In contrast, most of the spermatogenic cells were still present when seminiferous tubules were cultured from normal rat testes, while in tubular preparations from irradiated animals no spermatogenic cells were found. Therefore, it is not clear whether spermatogenic cells or specific populations of spermatogenic cells had an effect on inhibin production and/or secretion by Sertoli cells *in vitro*. Contacts between Sertoli cells and spermatogenic cells were preserved when seminiferous tubules were cultured, but it cannot be excluded that inhibin activity measured in these culture media did not reflect the inhibin secretion during these culture conditions, and it has been suggested that selective release of inhibin from the Sertoli cells is possible into the tubular lumen or interstitial compartment (Au *et al.* 1984).

In summary, immature Sertoli cells in culture appear to be a good model for the study of inhibin production *in vitro* under various conditions. There is, however, a number of discrepancies with in-vivo observations. Inhibin production is low *in vivo*, both at 37 °C (cryptorchidism) and in the absence of spermatogenesis in adult rats. This suggests that the age of the animal may influence the factors which regulate inhibin production and/or secretion or, alternatively, that the in-vivo situation is too complicated for direct study of the regulation of inhibin production, because of mutual interactions between the testicular compartments.

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REFERENCES

Au, C. L., Robertson, D. M. & de Kretser, D. M. (1983). *In vitro* bioassay of inhibin in testes of normal and cryptorchid rats. *Endocrinology* **112**, 239–244.

Au, C. L., Robertson, D. M. & de Kretser, D. M. (1984). An in-vivo method for estimating inhibin production by adult rat testes. *Journal of Reproduction and Fertility* **71**, 259–265.

Beaumont, H. M. (1960). Changes in the radiosensitivity of the testis during foetal development. *International Journal of Radiation Biology* **2**, 247–256.

Finney, D. J. (1964). Parallel line assays. In *Statistical Method in Biological Assay*, edn 2, pp. 101–138. London: Griffin.

Grootegoed, J. A., Grollé-Hey, A. H., Rommerts, F. F. G. & van der Molen, H. J. (1977). Ribonucleic acid synthesis *in vitro* in primary spermatocytes isolated from rat testis. *Biochemical Journal* **168**, 23–31.

Hall, P. F., Kew, D. & Mita, M. (1985). The influence of temperature on the functions of cultured Sertoli cells. *Endocrinology* **116**, 1926 1932.

Hermans, W. P., van Leeuwen, E. C. M., Debets, M. H. M. & de Jong, F. H. (1980). Involvement of inhibin in the regulation of follicle-stimulating hormone concentrations in prepubertal and adult, male and female rats. *Journal of Endocrinology* **86**, 79–92.

Hermans, W. P., van Leeuwen, E. C. M., Debets, M. H. M., Sander, H. J. & de Jong, F. H. (1982). Estimation of inhibin-like activity in spent medium from rat ovarian granulosa cells during long-term culture. *Molecular and Cellular Endocrinology* **27**, 277–290.

de Jong, F. H. (1979). Inhibin, fact or artifact. *Molecular and Cellular Endocrinology* **13**, 1–10.

de Jong, F. H., Hey, A. H. & van der Molen, H. J. (1973). Effect of gonadotrophins on the secretion of oestradiol-17β and testosterone by the rat testis. *Journal of Endocrinology* **57**, 277–284.

de Jong, F. H., Jansen, E. H. J. M., Steenbergen, J., van Dijk, S. & van der Molen, H. J. (1983). Assay and purification of inhibin. In *Role of Peptides and Proteins in Control of Reproduction*, pp. 257–274. Eds S. M. McCann & D. S. Dhindsa. New York: Elsevier Science Publishing Co.

de Jong, F. H., Smith, S. D. & van der Molen, H. J. (1979). Bioassay of inhibin like activity using pituitary cells *in vitro*. *Journal of Endocrinology* **80**, 91–102.

de Jong, F. H., Welschen, R., Hermans, W. P., Smith, S. D. & van der Molen, H. J. (1978). Effects of testicular and ovarian inhibin-like activity, using *in vitro* and *in vivo* systems. *International Journal of Andrology* Suppl. **2**, 125–138.

Kormano, M. (1967). Development of the rectum-testis temperature difference in the post-natal rat. *Journal of Reproduction and Fertility* **14**, 427–437.

Krueger, P. M., Hodgen, G. D. & Sherins, R. J. (1974). New evidence for the role of the Sertoli cell and spermatogonia in feedback control of FSH secretion in male rats. *Endocrinology* **95**, 955–962.

Le Gac, F. & de Kretser, D. M. (1982). Inhibin production by Sertoli cell cultures. *Molecular and Cellular Endocrinology* **28**, 487–498.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

Mecklenburg, R. S., Hetzel, W. D., Gulyas, B. J. & Lipsett, M. B. (1975). Regulation of FSH secretion: use of hydroxyurea to deplete germinal epithelium. *Endocrinology* **96**, 564–570.

Rommerts, F. F. G., Kruger-Sewnarain, B. Ch., van Woerkom-Blik, A., Grootegoed, J. A., & van der Molen, H. J. (1978). Secretion of proteins by Sertoli cell enriched cultures: effects of follicle stimulating hormone, dibutyryl cAMP and testosterone and correlation with secretion of oestradiol and androgen binding protein. *Molecular and Cellular Endocrinology* **10**, 39–55.

Setchell, B. P., Davies, R. V. & Main, S. J. (1977). Inhibin. In *The Testis*, vol. 4, pp. 189–238. Eds. A. D. Johnson & W. R. Gomes. New York: Academic Press.

Steinberger, A. (1981). Regulation of inhibin secretion in the testis. In *Intragonadal Regulation of Reproduction*, pp. 283–298. Eds P. Franchimont & C. P. Channing. London: Academic Press.

Steinberger, A. & Steinberger, E. (1976). Secretion of an FSH-inhibiting factor by cultured Sertoli cells. *Endocrinology* **99**, 918–921.

Verhoeven, G. & Franchimont, P. (1983). Regulation of inhibin secretion by Sertoli cell-enriched cultures. *Acta Endocrinologica* **102**, 136–143.

Verjans, H. L., Cooke, B. A., de Jong, F. H., de Jong, C. M. M. & van der Molen, H. J. (1973). Evaluation of a radioimmunoassay for testosterone estimation. *Journal of Steroid Biochemistry* **4**, 665–676.

Welschen, R., Osman, P., Dullaart, J., de Greef, W. J., Uilenbroek, J. Th. J. & de Jong, F. H. (1975). Levels of follicle-stimulating hormone, luteinizing hormone, oestradiol-17β and progesterone, and follicular growth in the pseudopregnant rat. *Journal of Endocrinology* **64**, 37–47.

Influence of neonatal hemicastration on in-vitro secretion of inhibin, gonadotrophins and testicular steroids in male rats

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ABSTRACT

Pituitary secretion of FSH in male animals is regulated, at least partly, by a protein hormone, inhibin, which is produced by Sertoli cells in the testes. To establish at which age the role of testicular inhibin in the regulation of FSH secretion becomes apparent, groups of male rats were hemicastrated or sham-operated on day 1 of life and pituitary and testicular function were investigated *in vitro* at 21, 42 or 63 days of age.

Testis weights were increased in hemicastrated rats at all ages studied. Peripheral concentrations of gonadotrophins generally showed a good correlation with the concentrations of FSH and LH measured in the medium of hemipituitary glands which were incubated *in vitro* at 37 °C in the absence or presence of LH-releasing hormone. Peripheral testosterone concentrations in hemicastrated animals were not significantly different from those in sham-operated rats at all ages studied. Steroid production by Leydig cells *in vitro* was not significantly influenced by hemicastration. The secretion of inhibin by Sertoli cells from 21-day-old hemicastrated rats was decreased while Sertoli cells from 42- and 63-day-old hemicastrated animals secreted slightly but not significantly more inhibin than Sertoli cells from sham-operated rats.

It is concluded that although compensatory increases of testosterone and inhibin production at later ages make it difficult to draw conclusions about the relative importance of inhibin in the feedback regulation of FSH secretion at different ages, it is likely that inhibin plays a role in the feedback of FSH in immature, rather than in mature male rats.
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INTRODUCTION

The mechanisms which regulate the synthesis and secretion of follicle-stimulating hormone (FSH) in male animals are still not completely understood. McCullagh (1932) observed that the 'castration cells' which are found in the pituitary gland of castrated male rats disappeared after administration of an aqueous extract from bovine testes. He ascribed this phenomenon to the presence of 'inhibin' in the testicular extracts. Since that time, inhibin has been redefined as a gonadal principle, which specifically suppresses the secretion of FSH from the pituitary gland (Setchell & Jacks, 1974; Franchimont, Chari, Hagelstein & Duraiswami, 1975; de Jong, 1979; Scott & Burger, 1981; Au, Robertson & de Kretser, 1983), and many investigators have shown that testicular fluids or charcoal-treated testicular extracts contain such FSH-suppressing activity (Setchell & Jacks, 1974; Franchimont *et al.* 1975; Au *et al.* 1983). The origin of testicular inhibin has been debated for a long time (see Setchell, Davies & Main, 1977), but recently several in-vitro studies have shown that isolated Sertoli cells secrete inhibin-like activity (Steinberger & Steinberger, 1976; de Jong, Smith & van der Molen, 1979; Le Gac & de Kretser, 1982; Verhoeven & Franchimont, 1983).

Removal of one testis early in life causes compensatory hypertrophy of the remaining testis in several mammalian species (rat: Hochereau-de Reviers, 1971; Cunningham, Tindal, Huckins & Means, 1978; Putra & Blackshaw, 1982; sheep: Walton, Evins & Waites, 1978) and results in an imbalance of gonadotrophin secretion through a specific increase in FSH in the circulation (Walton, Evins, Hillard & Waites, 1980). This increased secretion of FSH might influence testicular development through an increased production of inhibin by Sertoli cells (Le Gac & de Kretser, 1982) and through changes in the development of Leydig cells

(van Beurden, Mulder, de Jong & van der Molen, 1977).

The present study was undertaken to investigate the influence of neonatal hemicastration on the pituitary-testicular axis. In order to avoid interactions between the components of the axis, the hormone production by the pituitary gland, the Leydig cells and the Sertoli cells were studied separately *in vitro*. It was expected that we might obtain information on the age at which inhibin may become important for the regulation of pituitary FSH secretion by studying prepubertal, pubertal and young adult animals.

MATERIALS AND METHODS

Animals

Male Wistar rats were used in this study. Litter-mates were distributed between experimental and control groups; the size of the litters was adjusted to six or seven pups. The animals were kept under controlled conditions of light (lights on 04.45–19.00 h) and temperature (22–24 °C) and standard dry pellets and tap water were available *ad libitum*.

Hemicastration in the experimental group and sham-operation in the corresponding controls were performed 1 day after birth under ether anaesthesia. Groups of animals were killed by decapitation at 21, 42 or 63 days of age after CO_2 anaesthesia. After the animals were weighed and decapitated, blood, pituitary glands, testes, seminal vesicles and prostate glands were collected and weights recorded. The blood was allowed to clot for 1 h at room temperature and overnight at 4 °C. Serum was collected after centrifugation and stored at −20 °C until assayed for FSH, luteinizing hormone (LH) and testosterone.

Hemipituitary glands

After opening the skull, the brain and posterior lobe of the pituitary gland were removed. The anterior pituitary gland was cut into two equal parts and the hemipituitaries were placed in separate counting vials (Packard Instrument Company, Zurich, Switzerland) which contained 1 ml Medium TC 199 (Gibco Europe BV, Breda, The Netherlands). After a preincubation of 1–1·5 h at room temperature the medium was replaced with 2 ml fresh Medium TC 199 with or without synthetic LH-releasing hormone (LHRH; 1 mg/l; Relefact-Hoechst AG, Frankfurt am Main, F.R.G.) and the hemipituitary glands were incubated for 8 h at 37 °C in a thermostatic shaking water bath (frequency of 140 cycles/min) in an atmosphere of 5% CO_2 in air. At hourly intervals 200 µl aliquots were taken from the flasks and stored at −20 °C until assayed for FSH and LH. After the incubations the hemipituitary glands were placed in 2 ml phosphate-buffered saline (0·01 mol/l, pH 7·0), homogenized and stored at −20 °C until assayed for FSH and LH content.

Testicular cells

For isolation of Leydig and Sertoli cells, decapsulated testes from control or experimental animals were pooled in Krebs–Ringer bicarbonate buffer containing 0·2% glucose at pH 7·4 and 1 mg collagenase type I/ml (Sigma Chemical Company, St Louis, MO, U.S.A.) as described by Molenaar, Rommerts & van der Molen (1983). After incubation at 37 °C for 20 min, Leydig cells were obtained from the supernatant fraction. These cells were cultured in plastic Petri dishes (Lux Scientific Corporation, Newbury Park, CA, U.S.A.) for measurements of production of basal and LH-stimulated (NIH-LH-S18; 100 ng/ml) pregnenolone (21-day-old rats) and testosterone (42- and 63-day-old rats) as described by Rommerts, van Roemburg, Lindh *et al.* (1982). The tubular fragments obtained after the collagenase treatment were washed three times with Eagle's Minimum Essential Medium (MEM; Gibco Europe BV) supplemented with non-essential amino acids (Gibco Europe BV), fungizone (600 ng/ml), streptomycin (100 µg/ml) and penicillin (100 i.u./ml). Small Sertoli cell aggregates, which still contained some germinal cells, were obtained by mechanical dispersion of the tubules with a 10 ml plastic pipette. These Sertoli cell preparations were cultured in 25 cm^2 plastic culture flasks (Falcon, Becton Dickinson Labware, Oxnard, CA, U.S.A.) in 4 ml MEM without serum at 32 or 37 °C in a humidified atmosphere with 5% CO_2 in air. Sertoli cell culture media were collected after 2- or 3-day- culture periods and stored at −20 °C until assayed for inhibin activity. At the end of the culture period, cells were dissolved in 2 ml NaOH (1 mol/l) and cellular protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Hormone estimations

The concentrations of FSH and LH in serum, pituitary culture medium and pituitary homogenates after incubation were measured using the radioimmunoassay technique as described by Welschen, Osman, Dullaart *et al.* (1975). Intra- and interassay variations were 9·3 and 10·2% for FSH and 9·2 and 22·6% for LH. All results are expressed in terms of NIADDK-rat-FSH RP-1 and NIADDK-rat-LH RP-1 per litre plasma or per hemipituitary gland. Testosterone in serum was measured by the radioimmunoassay technique described by Verjans, Cooke, de Jong *et al.* (1973), while the amounts of testosterone and pregnenolone in the culture medium of Leydig cells were measured by radioimmunoassay as described by Rommerts, van

Roemburg, Lindh *et al.* (1982). Inhibin activity was measured with the in-vitro bioassay using dispersed cell preparations of anterior pituitary glands collected from adult male rats according to the method of de Jong *et al.* (1979), as modified by Hermans, van Leeuwen, Debets *et al.* (1982). The concentration of inhibin was expressed relative to the concentration present in a standard preparation of bovine follicular fluid (bFF), which was defined as having an activity of 1 unit/μg protein.

Statistical procedures

The significance of differences between the results for different treatment groups was assessed using Student's *t*-test (unpaired) or two-way analysis of variance followed by Duncan's multiple range tests. Differences were considered to be statistically significant when P was $<0{\cdot}05$ (two-tailed). In the bioassay of inhibin, calculation of relative potencies and 95% confidence limits and analysis of linearity and parallelism were based on the method of Finney (1964).

RESULTS

Organ weights and peripheral hormone levels

Testis weights and hormone concentrations in the serum of hemicastrated and sham-operated animals are given in Table 1. At all ages the weight of the remaining testis after neonatal hemicastration was significantly ($P<0{\cdot}01$) increased when compared with mean testis weight of sham-operated animals of the same age. No significant effects of hemicastration on either body weight or the weights of prostate and seminal vesicles were detected (data not shown). Concentrations of FSH in serum of hemicastrated 21- and 63-day-old animals were significantly ($P<0{\cdot}01$ and $P<0{\cdot}05$ respectively) increased when compared with appropriate controls while in 42-day-old rats this increase was not significant (Table 1). The concentration of LH was raised ($P<0{\cdot}05$) in 21-day-old hemicastrated rats, but not at the other ages. The levels of testosterone in serum were not significantly different in animals from the hemicastrated and sham-operated groups at all ages studied.

In-vitro release of gonadotrophins from pituitary glands

Patterns of release of gonadotrophic hormones from hemipituitary glands during in-vitro incubations are shown in Fig. 1. In the 21-day-old rats basal and LHRH-stimulated release of FSH in the hemicastrated animals were significantly ($P<0{\cdot}01$) higher than in the control group. The pituitary glands from hemicastrated and control rats of 42 and 63 days showed no significant differences in the release of FSH under both basal and LHRH-stimulated conditions. The amounts of FSH, which were retained in the incubated pituitary tissue, are shown in Table 2. LHRH-stimulated and non-stimulated hemipituitary glands from 21-day-old hemicastrated rats and LHRH-stimulated hemipituitary glands from 42-day-old hemicastrated rats contained significantly more FSH when compared with values in control animals of the same age. In 42-day-old rats the hemipituitary content of FSH was significantly stimulated after incubation with LHRH. In 63-day-old rats no significant changes in FSH content were detected.

In 21-day-old rats the basal and LHRH-induced LH release of hemipituitary glands from hemicastrated rats was significantly increased (Fig. 1*b*), while in 42- and 63-day-old animals this release was not different when compared with that in controls.

Furthermore, LH content of hemipituitary glands from hemicastrated 63-day-old rats was significantly ($P<0{\cdot}01$) decreased in the presence of LHRH (Table 2), while at day 42 LHRH-stimulated hemipituitaries from sham-operated animals contained significantly more LH than those from non-stimulated controls.

Testosterone production by isolated Leydig cells

No significant differences were detected between basal and LH-stimulated steroid production in cultured

TABLE 1. Testis weights and concentrations of FSH, LH and testosterone in the serum of neonatally hemicastrated (HC) and control animals (SH) at various ages. Values are means ± S.E.M.

	Number		Testis weight (mg)		FSH (μg/l)		LH (μg/l)		Testosterone (nmol/l)	
	SH	HC	SH	HC	SH	HC	SH	HC	SH	HC
Age (days)										
21	14	16	121 ± 7	151 ± 5**	662 ± 68	1336 ± 117**	35 ± 8	94 ± 23*	5·5 ± 0·7	5·9 ± 0·8
42	8	7	800 ± 12	1050 ± 38**	1283 ± 76	1501 ± 77	108 ± 43	99 ± 31	10·4 ± 2·5	10·5 ± 3·2
63	9	10	1472 ± 37	1867 ± 50**	676 ± 37	853 ± 68*	80 ± 25	80 ± 38	11·4 ± 1·7	15·5 ± 2·0

*$P<0{\cdot}05$, **$P<0{\cdot}01$ compared with appropriate controls (Students's unpaired *t*-test).

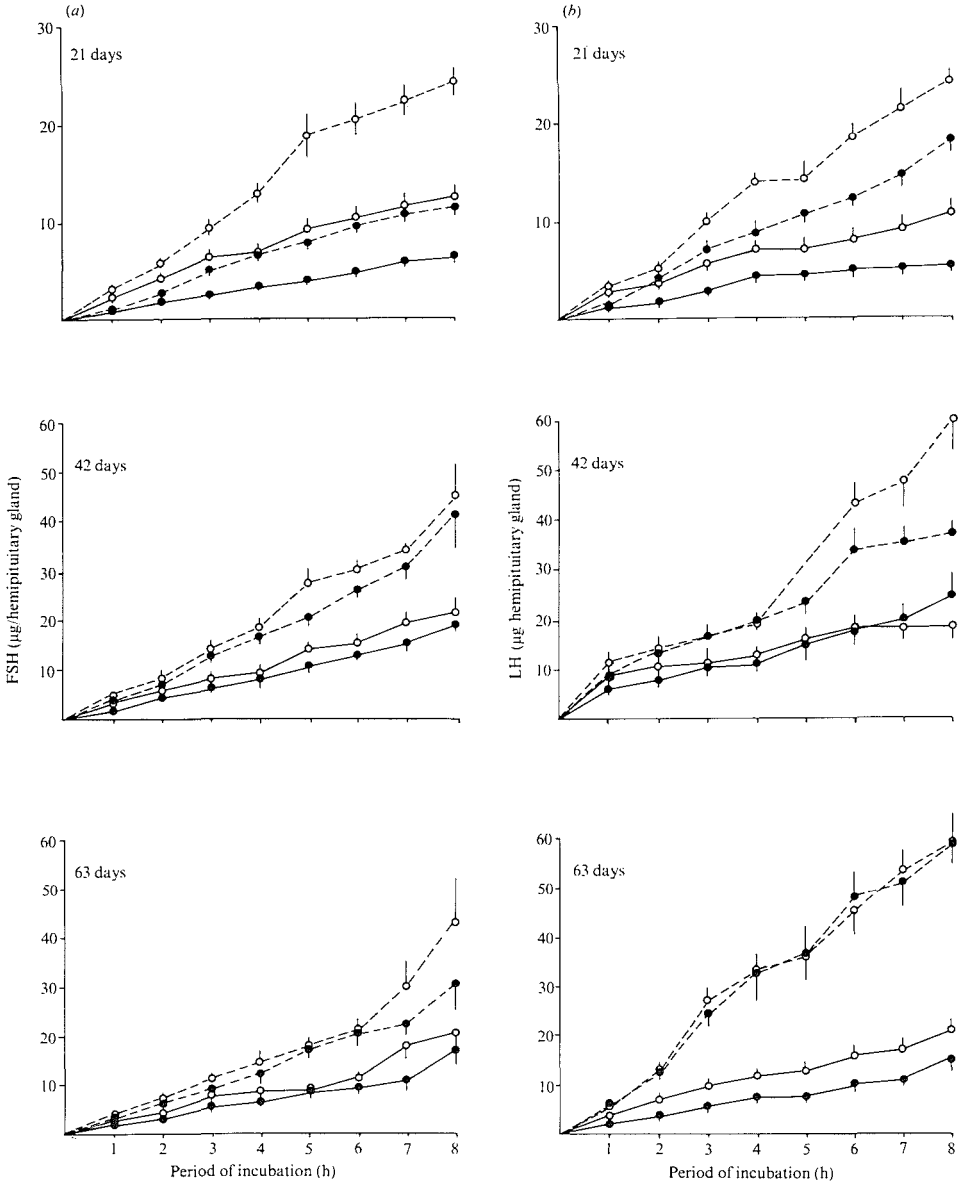

FIGURE 1. Release of (*a*) FSH and (*b*) LH by hemipituitary glands from sham-operated (●) and neonatally hemicastrated (○) rats of 21, 42 and 63 days of age during 8 h of incubation in medium without (solid lines) or with (broken lines) LH-releasing hormone (1 mg/l). Values are means ± S.E.M. There were 7–16 rats per group.

TABLE 2. Amounts of FSH and LH in hemipituitary glands from neonatally hemicastrated (HC) and sham-operated (SH) rats at various ages after 8-h incubation at 37 °C with or without the addition of 1 mg LH-releasing hormone (LHRH)/l. Values are means ± S.E.M.; numbers of rats are shown in parentheses

Age (days)	Treatment	LHRH	FSH (μg NIADDK-rat-FSH-RP-1/ hemipituitary)	LH (μg NIADDK-rat-LH-RP-1/ hemipituitary)
21	SH	–	19·17 ± 1·92 (13)	31·77 ± 5·62 (10)
	HC	–	33·21 ± 4·55 (15)	46·23 ± 8·28 (10)
	SH	+	12·64 ± 1·66 (13)	24·11 ± 4·78 (9)
	HC	+	24·97 ± 3·65 (15)	32·73 ± 4·97 (10)
42	SH	–	4·30 ± 1·37 (6)	16·60 ± 3·33 (5)
	HC	–	12·51 ± 6·19 (6)	15·75 ± 3·16 (3)
	SH	+	21·82 ± 4·14 (6)	36·81 ± 4·77 (5)
	HC	+	42·44 ± 8·88 (6)	26·55 ± 4·49 (4)
63	SH	–	39·48 ± 5·45 (5)	93·02 ± 9·72 (7)
	HC	–	44·51 ± 11·16 (7)	126·15 ± 17·20 (7)
	SH	+	35·01 ± 4·35 (5)	96·78 ± 10·51 (7)
	HC	+	34·04 ± 1·93 (6)	54·26 ± 8·63 (6)

TABLE 3. In-vitro steroid production by Leydig cells from neonatally hemicastrated (HC) and sham-operated (SH) rats measured under basal conditions and after LH stimulation. Pregnenolone was measured in media from 21-day-old rat testes, testosterone in media from the other age groups. Data are expressed as ng/h per 3×10^5 cells. Values are means ± S.E.M.; numbers of cultures are shown in parentheses

Age (days)	Treatment	Basal	LH stimulated	Stimulation factor
21	SH	1·20 ± 0·12 (4)	27·8 ± 3·9 (4)	23·5 ± 3·2 (4)
	HC	1·30 ± 0·18 (5)	31·5 ± 5·2 (5)	23·8 ± 2·7 (5)
42	SH	2·60 ± 0·17 (3)	32·5 ± 10·6 (3)	12·3 ± 3·4 (3)
	HC	4·40 ± 1·14 (3)	44·9 ± 5·7 (3)	11·6 ± 2·9 (3)
63	SH	1·80 ± 1·00 (3)	24·5 ± 4·9 (3)	19·8 ± 5·9 (3)
	HC	3·03 ± 1·09 (3)	46·6 ± 4·3* (3)	18·2 ± 4·1 (3)

*$P < 0.05$ compared with appropriate control (Student's unpaired *t*-test).

TABLE 4. Production of inhibin-like activity *in vitro* by Sertoli cells isolated from testes of sham-operated (SH) or neonatally hemicastrated (HC) rats at different ages. Data are expressed as percentage of control. Values are means ± S.E.M.; numbers of cultures are shown in parentheses

Age (days)	Treatment	Inhibin production (%)
21	SH	100 ± 1 (4)
	HC	67 ± 5 (4)**
42	SH	100 ± 10 (3)
	HC	152 ± 25 (4)
63	SH	100 ± 10 (4)
	HC	131 ± 39 (3)

**$P < 0.01$ compared with appropriate control (Student's unpaired *t*-test).

Leydig cells from hemicastrated and control rats, except at 63 days, where LH-stimulated steroid production was significantly ($P < 0.05$) raised in the experimental group (Table 3). Stimulation factors (LH-stimulated steroid production divided by non-stimulated steroid production) were similar for Leydig cells from control and hemicastrated animals of the same age.

Secretion of inhibin into the medium of cultured Sertoli cells

Table 4 shows that the secretion of inhibin by Sertoli cells *in vitro* was significantly ($P < 0.01$) lower in hemicastrated 21-day-old animals compared with sham-operated controls, while at the other ages inhibin secre-

tion was not significantly higher in the experimental group. Absolute values for inhibin production in the control groups were $47{\cdot}4 \pm 4{\cdot}2$, $62{\cdot}4 \pm 6{\cdot}0$ and $87{\cdot}1 \pm 31{\cdot}5$ units/day per mg protein on days 21, 42 and 63 respectively (means $\pm$ S.E.M., 1 μl bFF = 65 μg protein = 65 units).

DISCUSSION

The present study confirms that neonatal hemicastration causes compensatory testicular hypertrophy (rat: Hochereau-de Reviers, 1971; sheep: Jenkins & Waites, 1983; Waites, Wenstrom, Crabo & Hamilton, 1983). The compensatory hypertrophy was already observed at 21 days of age and the weight of the remaining testis in hemicastrated rats of 42 and 63 days of age was also significantly increased when compared with that in controls. This increase in testicular weight can be attributed mainly to an increased length of the seminiferous tubules (Hochereau-de Reviers, 1971) and an increased number of Sertoli cells (Orth, Higginbotham & Salisbury, 1984) and spermatogenic cells (Putra & Blackshaw, 1982).

The concentrations of pituitary hormones, as found in control animals of 21, 42 and 63 days of age, agree well with those found by de Jong & Sharpe (1977). After removal of one testis on day 1 of life the negative feedback of the gonads on the pituitary secretion is disturbed, as reflected in the significantly increased FSH levels in 21- and 63-day-old rats. Plasma LH levels were raised only in 21-day-old rats, while at the other ages normal LH levels were found. Hochereau-de Reviers (1975) also found no significant differences between plasma LH levels in normal adult rats and adult rats which were hemicastrated before puberty. A possible explanation for this finding at day 21 of age might be that more LH is needed to stimulate the smaller Leydig cell population in the hemicastrated animals in order to obtain normal peripheral testosterone concentrations.

The spontaneous and LHRH-induced FSH release by hemipituitary glands *in vitro* were significantly ($P < 0{\cdot}01$) increased in the 21-day-old experimental group, while in older animals basal and LHRH-induced FSH secretions were similar in the experimental and control groups (Fig. 1*a*). This indicates a diminished secretion of testicular inhibin from the remaining testis in 21-day-old hemicastrated rats, assuming that estimations *in vitro* can still detect differences in inhibin levels *in vivo*. This suggestion is confirmed by the data in Table 4 which, in combination with the testicular weights in Table 1, indicate that the total amount of inhibin produced per rat is much lower in hemicastrated than in control animals at this age. Furthermore, the amount of pituitary FSH is increased significantly only at 21 days of age, while in older hemicastrated animals this difference in in-vitro FSH secretion and total amount of pituitary FSH between hemicastrated and sham-operated animals was not observed. This might be caused by a larger number of Sertoli cells in the remaining testis in older animals (Hochereau-de Reviers, 1971), by a larger production of inhibin per Sertoli cell in hemicastrated animals, or by a smaller influence of inhibin on FSH secretion in older male rats. The first possibility is unlikely, since Sertoli cell divisions stop around day 15–18 of age (Steinberger & Steinberger, 1971). Hence, a possible compensation of Sertoli cell numbers should have occurred before day 21. The data in Table 4 indicate that Sertoli cells from older neonatally hemicastrated rats did not secrete significantly more inhibin than those from control animals, while in immature neonatally hemicastrated animals inhibin secretion *in vitro* is much lower than in controls. This suggests that compensatory actions do not play an important role at older ages. With respect to the last possibility, the increase of peripheral levels of FSH secretion (8 h) after castration of male rats is more marked in immature rats than in adult animals (Hermans, van Leeuwen, Debets & de Jong, 1980). This is confirmed by the present results, where the increase of peripheral levels, in-vitro secretion and pituitary content of FSH after neonatal hemicastration is much larger at day 21 of age than at the other ages studied. This might indicate that inhibin plays a relatively minor role in the feedback regulation of FSH secretion in older animals.

Testosterone might be a more important factor in the regulation of FSH secretion by the pituitary gland in adult animals as suggested earlier (de Jong & Sharpe, 1977). The observation of normal peripheral testosterone levels and normal weights of prostate glands and seminal vesicles in hemicastrated rats is not reflected in increased androgen production by 3×10^5 isolated Leydig cells *in vitro* (Table 3). This discrepancy between in-vivo and in-vitro results suggests that the number of Leydig cells per testis in hemicastrated rats may be larger than in the control animals as indicated by Hochereau-de Reviers (1975).

In conclusion, the present results provide evidence that neonatal hemicastration causes a selective increase of plasma FSH in immature and adult rats, but it is not possible to draw conclusions on the importance of inhibin in the regulation of pituitary FSH secretion in older animals because of the occurrence of hypertrophic growth of the testis and increased hormone secretion by testicular cells. The observation that castration in young animals causes a relatively larger rise in peripheral FSH combined with the observation that both the in-vitro secretion and pituitary content of FSH were influenced by hemicastration in 21-day-old rats but not in older animals, support the idea that

inhibin plays a more important role in the feedback regulation of FSH secretion in immature than in adult animals.

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REFERENCES

Au, C. L., Robertson, D. M. & de Kretser, D. M. (1983). In vitro bioassay of inhibin in testes of normal and cryptorchid rats. *Endocrinology* **112**, 239–244.

van Beurden, W. M. O., Mulder, E., de Jong, F. H. & van der Molen, H. J. (1977). The effect of estrogens on luteinizing hormone plasma levels and on testosterone production in intact and hypophysectomized rats. *Endocrinology* **101**, 342–349.

Cunningham, G. R., Tindall, D. J., Huckins, C. & Means, A. R. (1978). Mechanisms for the testicular hypertrophy which follows hemicastration. *Endocrinology* **102**, 16–23.

Finney, D. J. (1964). Parallel line assays. In *Statistical method in biological assay*, pp. 101–138. Edn 2. London: Griffin.

Franchimont, P., Chari, S., Hagelstein, M. T. & Duraiswami, S. (1975). Existence of a follicle-stimulating hormone inhibiting factor "inhibin" in bull seminal plasma. *Nature* **257**, 402–404.

Hermans, W. P., van Leeuwen, E. C. M., Debets, M. H. M. & de Jong, F. H. (1980). Involvement of inhibin in the regulation of follicle stimulating hormone concentrations in prepubertal and adult, male and female rats. *Journal of Endocrinology* **86**, 79–92.

Hermans, W. P., van Leeuwen, E. C. M., Debets, M. H. M., Sander, H. J. & de Jong, F. H. (1982). Estimation of inhibin-like activity in spent medium from rat ovarian granulosa cells during long-term culture. *Molecular and Cellular Endocrinology* **27**, 277–290.

Hochereau-de Reviers, M. T. (1971). Action de l'hemicastration sur l'évolution du testicule restant chez le rat. *Bulletin de l'Association des Anatomistes* **151**, 364–371.

Hochereau-de Reviers, M. T. (1975). Augmentation de l'efficacité de la spermatogénèse par l'hemicastration chez le rat et le bélier. *Annales de Biologie Animale, Biochimie et Biophysique* **15**, 621–631.

Jenkins, N. & Waites, G. M. H. (1983). Effects of hemicastration at various ages and of oestradiol-17β on plasma concentrations of gonadotrophins and androgens, testicular growth and interstitial cell responses in prepubertal lambs. *Journal of Reproduction and Fertility* **67**, 325–334.

de Jong, F. H. (1979). Inhibin, fact or artifact. *Molecular and Cellular Endocrinology* **13**, 1–10.

de Jong, F. H. & Sharpe, R. M. (1977). The onset and establishment of spermatogenesis in rats in relation to gonadotrophin and testosterone levels. *Journal of Endocrinology* **75**, 197–207.

de Jong, F. H., Smith, S. D. & van der Molen, H. J. (1979). Bioassay of inhibin like activity using pituitary cells *in vitro*. *Journal of Endocrinology* **80**, 91–102.

Le Gac, F. & de Kretser, D. M. (1982). Inhibin production by Sertoli cell culture. *Molecular and Cellular Endocrinology* **28**, 487–498.

Lowry, O. H., Rosebrough, N. H., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

McCullagh, D. R. (1932). Dual endocrine activity of the testes. *Science* **76**, 19–20.

Molenaar, R., Rommerts, F. F. G. & van der Molen, H. J. (1983). The steroidogenic activity of isolated Leydig cells from mature rats depends on the isolation procedure. *International Journal of Andrology* **6**, 261–274.

Orth, J. M., Higginbotham, C. A. & Salisbury, R. L. (1984). Hemicastration causes and testosterone prevents enhanced uptake of [^{3}H]thymidine by Sertoli cells in testes of immature rats. *Biology of Reproduction* **30**, 263–270.

Putra, D. K. H. & Blackshaw, A. W. (1982). Morphometric studies on compensatory testicular hypertrophy in the rat after hemicastration. *Australian Journal of Biological Sciences* **35**, 287–293.

Rommerts, F. F. G., van Roemburg, M. J. A., Lindh, L. M., Hegge, J. A. J. & van der Molen, H. J. (1982). The effects of short-term culture and perifusion on LH-dependent steroid production in isolated rat Leydig cells. *Journal of Reproduction and Fertility* **65**, 289–297.

Scott, R. S. & Burger, H. G. (1981). Mechanism of action of inhibin. *Biology of Reproduction* **24**, 541–550.

Setchell, B. P., Davies, R. V. & Main, S. J. (1977). Inhibin. In *The testis*, vol. 4, pp. 189–238. Eds A. D. Johnson & W. R. Gomes. New York: Academic Press.

Setchell, B. P. & Jacks, F. (1974). Inhibin-like activity in rete testis fluid. *Journal of Endocrinology* **62**, 675–676.

Steinberger, A. & Steinberger, E. (1971). Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. *Biology of Reproduction* **4**, 84–87.

Steinberger, A. & Steinberger, E. (1976). Secretion of an FSH-inhibiting factor by cultured Sertoli cells. *Endocrinology* **99**, 918–921.

Verhoeven, G. & Franchimont, P. (1983). Regulation of inhibin secretion by Sertoli cell-enriched cultures. *Acta Endocrinologica* **102**, 136–143.

Verjans, H. L., Cooke, B. A., de Jong, F. H., de Jong, C. M. M. & van der Molen, H. J. (1973). Evaluation of a radioimmunoassay for testosterone estimation. *Journal of Steroid Biochemistry* **4**, 665–676.

Waites, G. M. H., Wenstrom, J. C., Crabo, B. G. & Hamilton, D. W. (1983). Rapid compensatory hypertrophy of the lamb testis after neonatal hemiorchidectomy: endocrine and light microscopical morphometric analyses. *Endocrinology* **112**, 2159–2167.

Walton, J. S., Evins, J. D., Hillard, M. A. & Waites, G. M. H. (1980). Follicle stimulating hormone release in hemicastrated prepubertal rams and its relationship to testicular development. *Journal of Endocrinology* **84**, 141–152.

Walton, J. S., Evins, J. D. & Waites, G. M. H. (1978). Feedback control of follicle-stimulating hormone in pre- and postpubertal rams as revealed by hemicastration. *Journal of Endocrinology* **77**, 75–84.

Welschen, R., Osman, P., Dullaart, J., de Greef, W. J., Uilenbroek, J. Th. J. & de Jong, F. H. (1975). Levels of follicle-stimulating hormone, luteinizing hormone, oestradiol-17β and progesterone, and follicular growth in the pseudopregnant rat. *Journal of Endocrinology* **64**, 37–47.

EFFECTS OF TREATMENT OF NEONATAL RATS WITH HIGHLY PURIFIED FSH ALONE AND IN COMBINATION WITH LH ON TESTICULAR FUNCTION AND ENDOGENOUS HORMONE LEVELS AT VARIOUS AGES

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Abstract

Factors which play a role in the regulation of testicular size in rats were investigated using neonatal animals treated with exogenous gonadotrophins for 2 or 3 weeks, starting on the day after birth. Effects on testis weight and various aspects of the pituitary-testicular axis were evaluated up to the age of 9 weeks.

Daily treatment with human FSH (Metrodin, 0.15 U/g b.w.) for two or three weeks, starting on the first day or one week after birth, resulted in enlargement of the testes, increased testicular content of inhibin and a suppression of pituitary and plasma FSH. The relative increase of testis weight decreased after cessation of treatment. Injections of human FSH combined with administration of human LH (Pergonal) for three weeks, starting on the first day after birth resulted in larger testes immediately after treatment. In addition, an increased amount of interstitial tissue was observed in these animals. Pituitary and plasma FSH and LH were suppressed after this treatment, while the growth of the accessory sex organs was significantly stimulated.

In animals treated with human FSH during the first two or three weeks of life, levels of rat FSH in blood samples collected with weekly intervals, were +ignificantly suppressed until the animals were killed at the age of 9 weeks. In the animals treated with human FSH and human LH, both FSH and testosterone concentrations were significantly lower than those in control animals between the ages of 4 and 9 weeks. At the age of 9 weeks testicular weights were still higher than those in control animals after these treatments. In the treated animals, no histological abnormalities of spermatogenesis were observed.

We have concluded, that the first three weeks after birth are important for the establishment of testis size in the rat because during this period it is possible to stimulate mitoses of Sertoli cells with FSH. To obtain a permanent increase in testis size a longer period of treatment with exogenous gonadotrophins is needed. A possible feedback suppression of endogenous gonadotrophins by inhibin in FSH-treated rats and by inhibin and testosterone in the FSH and LH-treated 3-weeks-old animals could be the reason for the relative delay of testicular development after cessation of the treatments. The observed low endogenous level of LH caused impaired Leydig cell function in animals which were treated with FSH and LH.

Introduction

Development and function of the hypothalamo-pituitary-testicular axis is dependent on a balance between stimulatory and inhibiting actions of products secreted by the hypothalamus, pituitary gland and gonads.
Stimulating actions can be exerted by follicle-stimulating hormone (FSH) acting on Sertoli cells (Means, Dedman, Tash *et al.* 1980), and by luteinizing hormone (LH), which stimulates Leydig cells to produce androgens. These androgenic hormones in turn can suppress the secretion of LH and partly of FSH from the pituitary gland by a negative feedback mechanism while it has been shown that inhibin, produced by Sertoli cells, has specific inhibitory effects on pituitary FSH secretion (de Jong, 1979). The production of inhibin activity can be stimulated by adding FSH to Sertoli cells from 21-day-old rats in culture (Steinberger, 1981; Le Gac & de Kretser, 1982; Ultee-van Gessel, Leemborg, de Jong & van der Molen, 1986). The production of inhibin by immature Sertoli cells *in vitro* shows a marked decrease (Ultee-van Gessel & de Jong, 1987) during a period of increasing peripheral levels of FSH as observed *in vivo* (Swerdloff, Walsh, Jacobs & Odell, 1971; Lee, de Kretser, Hudson & Wang, 1975; Ultee-van Gessel & de Jong, 1987). It has been suggested that a diminished feedback regulation of pituitary FSH secretion by testicular inhibin could be an explanation for the testicular hypertrophy which follows after early postnatal hemicastration (Hochereau-de Reviers & Courot, 1978; Cunningham, Tindall, Huckins & Means, 1978; Ultee-van Gessel, Leemborg, de Jong & van der Molen, 1985), but not after operation at older ages (Putra & Blackshaw, 1982). This hypothesis is supported by the observation of increased release of FSH *in vitro* by hemipituitary glands from neonatally hemicastrated rats of 21 days of age, which was not observed with pituitaries from older animals (Ultee-van Gessel *et al.*, 1985).
Removal of the pituitary gland in prepubertal rats resulted in a decrease in Sertoli cell number per testis, while administration of FSH to these animals resulted in normal testis weights at the end of the treatment period (Hochereau-de Reviers & Courot, 1978). It is still not clear, whether both FSH and LH are essential for the development of all testis functions. Studies using antibodies against FSH or LH indicated that both gonadotrophins are needed for growth and differentiation of Sertoli cells (Chemes, Dym & Raj, 1979). Daily administration of FSH to normal immature animals for 3-30 days may result in increased testicular weights primarily due to lengthening of the seminiferous tubules (mice: Davies, 1971, 1976; rats: Leidl & Hansel, 1972, Bentley, Gass & Leidl, 1978; bulls: Meyers & Swanson, 1983). The interpretation of findings in some of these studies is complicated because of contamination of FSH preparations with LH (Davies, 1971). The aim of the present study was to investigate the effects of a preparation of FSH, which is devoid of LH activity, on neonatal testicular development in rats. To study possible interactions of the two hormones in the regulation of Sertoli cells, the same preparation of pure FSH was given also concomitantly with LH. We have also studied the development of pituitary gonadotrophic and testicular function after cessation of treatment.

Materials and methods

Animals and experimental procedures

Male Wistar rats were used throughout this study. Litter mates were distributed between experimental and control groups. The size of the litters was adjusted to eight pups on the first day after birth when the treatments were started. All animals were kept under controlled conditions of light (lights on: 04.45 - 19.00 h) and temperature (22-24°C). After weaning on day 21 they received standard dry pellets and tap water ad libitum.
Groups of animals were injected daily subcutaneously with ovine pituitary FSH (NIH FSH-S13 containing less than 5% of LH, expressed as NIH LH-S1) or human urinary FSH with specific activity of more than 120 IU/mg (Metrodin, Serono, Geneva, Switzerland) containing less than 0.1 IU LH per 75 IU FSH (Harlin, Khan & Diczfalusy, 1986) or a combination of human urinary FSH and LH, which contains equal amounts of both gonadotrophins (Pergonal, Serono), beginning one day or 1 week after birth for periods of two or three weeks. Control animals were injected with saline. The animals were weighed, anesthesized with carbondioxide and killed by decapitation at the ages of 2 or 3 weeks twenty-four hours after the last injection. Trunk blood was collected in heparinized plastic tubes immediately after killing. Other groups of animals, which had been treated during the first two or three weeks of life, were killed at the age of 9 weeks. In these groups of animals blood was collected weekly between the ages of 3 and 9 weeks by puncture of the ophthalmic plexus under light ether anesthesia.
Blood samples were centrifuged and the plasma was separated and stored at -20°C until assayed for FSH, LH and testosterone. At autopsy, testes, prostate and seminal vesicles were removed and weighed. From each pair of testes one testis was decapsulated and placed in a tube with chilled Eagle's Minimum Essential Medium (MEM; Gibco, Grand Island, NY, USA), homogenized and treated with a charcoal suspension as described previously (Ultee-van Gessel & de Jong, 1987). These steroid-free testicular homogenates were stored at -20°C until assayed for inhibin content. The other testis was fixed in Bouin's fluid for histological examination.
The anterior pituitary glands were removed and placed in 1 ml phosphate buffered saline (0.01 M; pH 7.0). The glands were homogenized and stored at -20°C until assay of FSH and LH. To investigate possible direct effects of exogenous FSH on the peripheral and pituitary levels of FSH, human FSH was also injected into groups of animals which had been castrated under ether anesthesia one day or one week after birth.

Histology

Using standard histological techniques for dehydrating and clearing, testes were embedded in paraffin and cut into 5 μ slices perpendicular to the long axis of the organ. The sections were stained by the periodic acid-Schiff's-haematoxylin technique.
The relative volume of total interstitial tissue was estimated with the help of an ocular

containing a 100 points grid. The number of points falling on interstitial tissue and space per frame were counted in ten different fields of randomly selected slices from each testis.

Hormone estimations

a) *In vitro* inhibin bioassay

Inhibin activity was measured using cultures of dispersed pituitary cells from adult normal male rats (de Jong, Smith & van der Molen,1979; Hermans, van Leeuwen, Debets *et al.*, 1982). This pituitaryculture system was validated for the measurement of inhibinactivity in rat testis homogenates (Ultee-van Gessel & de Jong, 1987). Testicular cytosols were assayed in quadruplicate at two or three dose levels. In each bioassay the inhibin activity in testicular cytosols from rats of various treatment groups were measured and compared. The suppression of FSH release caused by the addition of aliquots of charcoal-treated testicular cytosols was compared with the suppression of FSH release in pituitary cells incubated under similar conditions with a standard preparation of charcoal-treated bovine follicular fluid (bFF) with the arbitrary potency of 1 inhibin unit/μg bFF protein. Inhibin potencies were calculated using statistics for parallel line assay (Finney, 1964). No significant deviations from parallelism were observed between the dose-response curves obtained after addition of bFF or testicular cytosol. No significant effects on pituitary LH release were detected. The average assay index of precision (λ) in 11 assays was 0,15 (range: 0,08-0,25).

b) Radioimmunoassays

Concentrations of FSH and LH in culture media of pituitary cells, in plasma and in pituitary homogenates were estimated by radioimmunoassay as described by Welschen, Osman, Dullaart *et al.* (1975). Results have been expressed in terms of NIADDK-rat-FSH RP1 and NIADDK-rat-LH RP1. Intra- en interassay coefficients of variation were 8.7 and 10.1% for FSH and 9.1 and 15.6% for LH, respectively. Hormone concentrations in all plasma samples of one experiment were measured in the same assay. Testosterone concentrations in plasma were assayed using the radioimmunoassay method as described by Verjans, Cooke, de Jong *et al.* (1973). Intra- and interassay coefficients of variation were 8.1 and 11.8%, respectively.

Statistical analyses

The significance of differences between mean values of different groups were estimated using Student's t-test. Differences were considered significant when $P<0.05$ (two-tailed).

Results

Preliminary experiments

Daily treatment with ovine FSH (1 μg/g body weight) for the first 2 weeks of life caused a significantly increased testicular weight : from 86 ± 3.6 mg (mean weight of two testes ± S.E.M., n=18) in control animals to 192 ± 5.3 mg (mean weight of two testes ± S.E.M., n=8) in FSH-injected rats. Data on endogenous FSH could not be interpreted because ovine FSH crossreacts in the radioimmunoassay for rat FSH (data not shown). Concentrations of FSH and LH, estimated in pituitary glands after this treatment period were suppressed to 6 ± 1% and 43 ± 5%, respectively (means ± S.E.M.; n=5) of values found in control animals. In addition, the amount of inhibin activity measured in charcoal-treated testicular cytosols from FSH-treated animals was increased to 196 ± 12% of that in control rats.
To investigate which dose of human FSH had a clearcut effect on testicular weight, groups of animals received daily injections with 0.05 or 0.15 U of human FSH. Only the highest dose caused a significant increase of testis weight (96 ± 8 vs 188 ± 8 mg, n=4), while pituitary FSH content was suppressed to 6.4% of control values. This dose was used in all further experiments.
The effect of 2 weeks of daily treatment with human FSH, beginning on the first day or one week after birth, on pituitary and plasma FSH and LH levels was also examined in castrated rats. Results have been summarized in Table 1. Significantly lower ($P<0.01$) concentrations of both gonadotrophins were measured in the pituitary glands of castrated animals, which were injected with hFSH during the first two weeks of life. Normal values were found when these injections were performed during week 2 and 3 of life. Peripheral plasma levels of FSH were not significantly affected.

Table 1 Effects of daily treatment with hFSH (0.15 U/g b.w.) on endogenous gonadotrophins in rats castrated one day (a) or one week (b) after birth. Treatment for two weeks started on the day after castration. Animals were killed 24h after the last injection. (Means ± S.E.M., numbers of animals in parentheses)

TREATMENT		PLASMA (μg/l)		PITUITARY (μg/gland)	
period	group	FSH	LH	FSH	LH
a) wk 1+2	castr.	5481±1479 (3)	-	22.6±1.0 (5)	158± 8 (5)
	castr.+hFSH	4267±1479 (5)	-	15/4±1.9 (6)**	107±10 (6)**
b) wk 2+3	castr.	2816±160 (7)	339±31 (7)	36.6±4.2 (7)	260±27 (7)
	castr.+hFSH	2685±168 (6)	462±89 (6)	41.3±7.7 (6)	284±28 (6)

**p< 0.01, compared with appropriate control (Student's t-test).

Effects on organ weights

Effects of 2 or 3 weeks of treatment with FSH or FSH and LH on organ weights have been summarized in Table 2. Significantly enlarged testes were found when human FSH was injected daily for two or three weeks within the first 3 weeks of life. After 2 or 3 weeks of treatment, starting one day after birth, similar relative increases in testicular weight were found (69 ± 7 vs 73 ± 5%). A lower relative increase (47 ± 7%) was found in rats injected only during weeks 2 and 3 of life. After treatment during the first 3 weeks of life a relative increase of testis weight was still observed at 9 weeks of age (43 ± 7%, n=7). However, the relative increase found after 2 weeks of FSH injections starting one day after birth declined within one week after treatment to 16 ± 4%, and was 26 ± 4% at the age of 9 weeks. The weights of the accessory sex organs were not affected by treatment with FSH alone (Table 2). The largest relative increase in testicular weight was observed at 3 weeks of age when FSH was daily injected together with LH (115 ± 8%). In addition, the weights of the accessory sex organs were significantly increased. Six weeks after cessation of the treatment with both gonadotrophins, the relative increase of testis weight was 12 ± 4%, while weights of prostate and seminal vesicles were significantly reduced (Table 2).

Table 2 Organ weights from rats after various periods of daily administration (s.c.) of gonadotrophins (0.15 U/g body weight) or saline. Mean ± S.E.M.

AGE at autopsy (weeks)	TREATMENT hormone	weeks	No. of rats	TESTES WEIGHT (mg)	VESICLES WEIGHT (mg)	PROSTATE WEIGHT (mg)
2	saline	1+2	8	101± 5	-	-
	hFSH	1+2	9	171± 8**	-	-
3	saline		20	264± 7	7.1± 0.4	21± 3
	hFSH	1+2	7	306± 7**	-	-
	hFSH	2+3	7	387± 23**	-	-
	hFSH	1+2+3	7	456± 20**	7.5± 0.6	19± 1
	hFSH+hLH	1+2+3	7	567± 40**	49.6± 5.8**	56± 5**
9	saline		13	3024± 93	263 ± 11	298±20
	hFSH	1+2	6	3805±112**	280 ± 16	312±19
	hFSH	1+2+3	7	4311±288**	257 ± 17	289±15
	hFSH+hLH	1+2+3	7	3394± 57**	125 ± 14**	205±37*

*: $P < 0.05$ and **: $P < 0.01$, compared with control values at the same age (Student's t-test).

Histology

Treatment with FSH alone had no effect on the relative volume of the interstitial compartment immediately after treatment (15.3 ± 1.2% vs 15.8 + 1.7% in control vs FSH treated resp. n=3-4) Treatment with both gonadotrophins, however, resulted in an significant increase of the Leydig cell compartment immediately after the treatment period of 3 weeks (control 15.2 ± 1.2% vs 21.3 ± 1.5% in FSH+LH treated animals n=3-4), but a significantly reduced volume was found at the age of 9 weeks (control: 16.8 ± 2.1% FSH treated: 21.4 ± 3.9% and FSH+LH treated 6.0 ± 1.8% n=3-4)
Further histological examinations revealed tubular cross-sections with normal spermatogenesis in testes of the hormone-injected groups at 3 and 9 weeks of age.

Hormonal changes after treatment

Endogenous concentrations of plasma and pituitary FSH were significantly lowered at 2 and 3 weeks of age in all FSH-treated groups of animals (Figure 1a and Table 3), while plasma LH values were found to be not different from those in control animals (data not shown) despite lower concentrations of LH in the pituitary glands (Table 3). The enlarged prepubertal testes contained significantly increased amounts of inhibin activity after treatment with FSH for different periods (Table 3). Injection of FSH in prepubertal rats during 2 or 3 weeks resulted in a sustained suppression of endogenous plasma FSH after the cessation of the injections (Figure 1a). These lowered peripheral FSH levels, however, did not affect the onset or sequence of pubertal changes in plasma testosterone (Fig. 1b). Endogenous concentrations of plasma FSH were also significantly lowered at all ages when both FSH and LH were injected (Fig. 1a), while plasma LH values were significantly suppressed immediately after cessation of the 3 weeks treatment period (3 ± 1 μg/l vs 18 ± 3 μg/l in control animals) and reached normal levels at the age of 9 weeks (data not shown). Plasma LH values were not measured in samples collected between the ages of 3 and 9 weeks, because not enough blood was available. Significantly diminished concentrations of plasma testosterone were observed during the period between 4 and 9 weeks of age in the animals treated with the combination of FSH and LH (Fig. 1b). Significant reductions of pituitary FSH contents were measured in both treatment groups at the age of 3 weeks, while at 9 weeks of age the pituitary content of FSH was still lower than levels in control animals. In contrast to the group of animals treated with FSH alone, the group treated with FSH and LH showed very low levels of LH in the pituitary gland at 3 weeks of age and significantly ($P<0.01$) decreased levels of pituitary LH were still observed at 9 weeks of age (Table 3). Finally, at 3 weeks of age the enlarged testes contained significantly more inhibin activity after treatment with FSH alone, or FSH combined with LH. However, at 9 weeks of age increased inhibin activity ($P<0.01$) was only found in the testes of the FSH-injected group (Table 3).

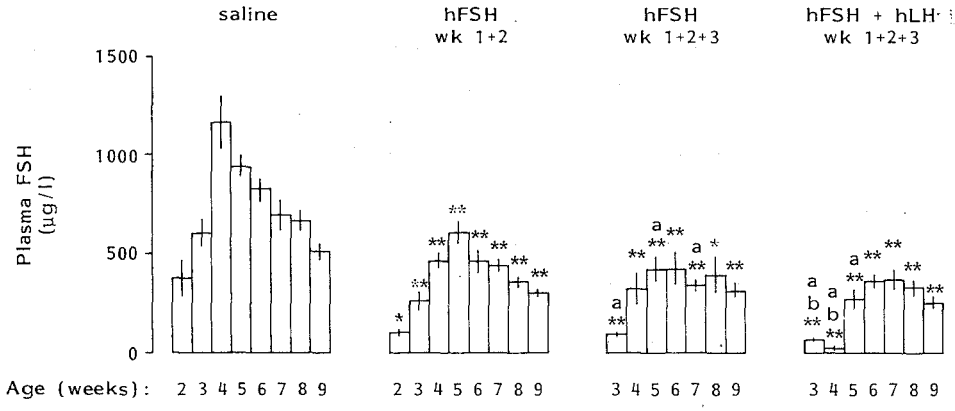

Fig. 1a

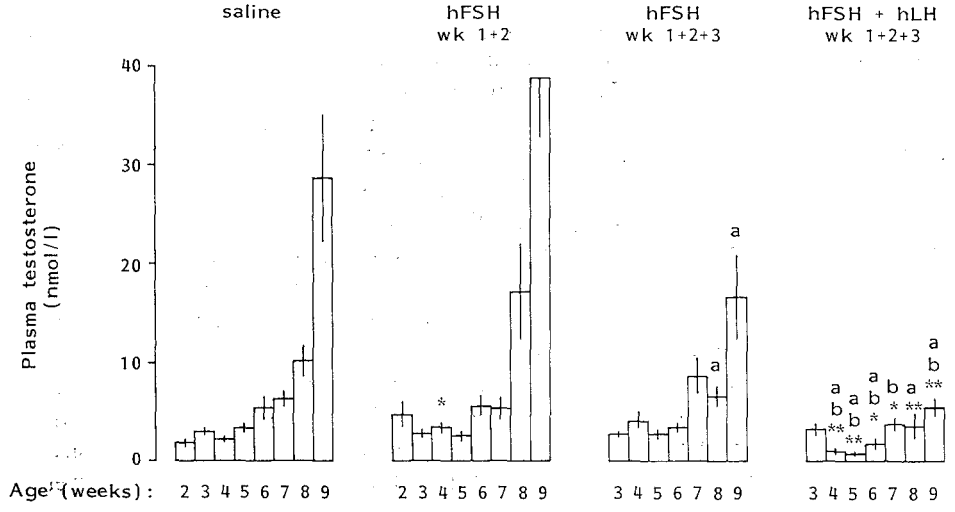

Fig. 1b

Fig. 1 Concentrations of FSH (a) and testosterone (b) in the plasma of male rats of various ages after daily treatment with gonadotrophins or saline.
Means ± S.E.M., n= 6-7.
**P<0.05 and **P<0.01, significantly different from appropriate control.*
a: significantly different from values in rats treated with hFSH for 2 weeks
b: significantly different from values in rats treated with hFSH for 3 weeks

Table 3 Levels of pituitary FSH and LH, and testicular inhibin in rats injected daily (s.c.) for various periods with gonadotrophins (0.15 U/g body weight). Means ± S.E.M.

AGE at autopsy (weeks)	TREATMENT hormone	weeks	No. of rats	PITUITARY FSH (μg/gland)	PITUITARY LH (μg/gland)	TESTICULAR INHIBIN (IU/paired testes)
2	saline	1+2	8	34± 7	44± 7	103± 7
	hFSH	1+2	9	2± 1**	19± 2**	180± 32**
3	saline		13	59± 6	81±11	162± 8
	hFSH	1+2	7	13± 3**	64±10	-
	hFSH	2+3	7	9± 3**	27± 7**	349± 9**
	hFSH	1+2+3	7	3± 1**	14± 1**	461±100**
	hFSH+hLH	1+2+3	7	2± 1**	2± 0.2**	466± 64**
9	saline		13	173±31	453±48	868± 71
	hFSH	1+2	6	178±18	557±44	1033±118
	hFSH	1+2+3	7	74±10**	222± 0**	2779±239**
	hFSH+hLH	1+2+3	7	77±16**	159±13**	1174±170

**: $P<0.01$, compared with values in control of the same age group (Student's t-test).

Discussion

The administration of FSH during the first 2 or 3 weeks of life stimulated prepubertal testis growth in rats, probably reflecting the increase of the number of Sertoli cells and of the length of the seminiferous tubules. This observation confirms data of Leidl & Hansel (1972) and Bentley *et al.* (1978) in immature rats, of Davies (1971, 1976) in immature mice and of Meyers & Swanson (1983) in prepubertal bulls. Davies (1971, 1976) also reported an increase of the amount of cytoplasm per Sertoli cell and a significant increase of the mass of interstitial tissue per pair of testes after treatment with FSH. In the studies reported by Davies, testicular weight was not affected significantly by treatment with LH alone. Injection of LH together with FSH caused augmentation of the effect of FSH on the total amount of Sertoli cell cytoplasm (Davies, 1976). In 4-months-old bulls, FSH treatment resulted in significantly heavier testes in the summer, but no effects were observed in late winter (Meyers & Swanson, 1983). In none of these studies on FSH and LH treatment during the prepubertal period long term effects on testis and pituitary function have been investigated. The above mentioned observations indicate that testicular growth is dependent on peripheral concentrations of gonadotrophins, especially FSH, during the prepubertal period. The results from the present study demonstrate that in rats at least the whole period between birth and 3 weeks of age is important for the determination of testis size (Table 2):

when treatment started on the day after birth, similar relative testis weights were observed after 2 and 3 weeks of treatment. When, however, animals were only treated during the first two weeks or second and third week, at the age of 3 weeks relative testicular weights were lower than those in animals which were treated for the whole 3 weeks period.
Treatment with FSH combined with LH caused the largest increase of testis growth at 3 weeks of age, which confirms earlier observations by Davies (1976). Relative testicular weights in the adult animals which were treated with gonadotrophins during the prepubertal period were lower than immediately after treatment. These observations are likely to be due to the increased number of Sertoli cells and increased tubular length. Testicular growth in control animals apparently went on to catch up with that in treated animals, in which a relative retardation of testicular growth occurred after cessation of treatment. An explanation for this phenomenon may be found in the endocrine data, obtained after treatment (Table 3, Figure 1).
Administration of FSH alone caused suppressed endogenous levels of plasma and pituitary FSH at the ages of 2 and 3 weeks. This suggests that more inhibin has been produced by the enlarged testes, which is supported by the increased testicular concentrations of this hormone. This increase may reflect either a larger production of inhibin activity per Sertoli cell, the increased number of Sertoli cells per animal or both. Since the weight of the accessory sex organs was not affected by the injections with FSH, the amount of androgens, secreted by the Leydig cells was probably not affected by this treatment. Hence, it appears unlikely that the suppression of FSH reflects a feedback by increased concentrations of steroids in the FSH-treated animals.
Treatment with the combination of exogenous FSH and LH stimulated the production of both inhibin and androgens as appears from the increased testicular inhibin content and reduced levels of endogenous FSH, and from the increased weight of accessory sex organs and suppressed endogenous concentrations of LH. This combination of suppressed FSH and LH contents is likely to be the reason for the strong decrease of the relative stimulation of testicular weight in this experimental model 6 weeks after the cessation of treatment, and for the suppression of circulating testosterone levels and weights of accessory sex organs. The absence of a significant rise of peripheral testosterone at 3 weeks of age, 24 hours after the last injection of FSH and LH may be due to the relatively long interval between injection and sampling. Moger and Armstrong (1974) demonstrated that in response to one LH injection, testosterone levels in immature rats were elevated after 30 min and returned to control values after 180 min.
Finally, it is unlikely that the drastic suppression of pituitary and plasma gonadotrophins after treatment with FSH is due to a direct effect of exogenous gonadotrophin on the hypothalamic-pituitary system, since the gonadotrophin production in the pituitary gland from castrated hormone-injected rats was not, or only slightly lower when compared with those in appropriate control animals (Table 1).
In summary, the present results demonstrate an increase of testicular weight and inhibin production after early postnatal gonadotrophin injections. Injections of highly pure preparations of human FSH apparently resulted in specific effects on testicular inhibin

production by Sertoli cells, the primary target cells for FSH. Subsequently, inhibin released from the testes suppressed the pituitary secretion of FSH. The advantage of using human FSH was that peripheral concentrations of rat FSH could be measured without interference of human FSH in the radioimmunoassay for rat FSH. Endogenous levels of peripheral FSH remained decreased in these animals until 9 weeks of age due to the initially increased population of Sertoli cells, which had been induced during the hormonal stimulation in the prepubertal period. Thereafter, the mitotic activity of the Sertoli cell population was apparently no longer maintained by the low endogenous FSH levels. This indicates that treatment for a longer period could be essential to increase Sertoli cell number per testis permanently. A similar "functional hypophysectomy" for both LH and FSH was observed after treatment with the combination of the two gonadotrophins; in this situation both FSH and peripheral testosterone levels remained low until at least 6 weeks after the end of treatment.

The results obtained after treatment with FSH suggest that the interaction between inhibin and FSH is an important determinant for testicular growth and development in the prepubertal male rat and, therefore, for the size of the testis and the numbers of spermatozoa produced in the adult animal. A possible physiological role of testicular inhibin in the adult male rat remains to be elucidated. The relatively large amounts of inhibin activity found in adult testes (Au *et al.*, 1986; Ultee-van Gessel & de Jong, 1987) may be attributed to a low secretion rate from the testes and possible local effects of inhibin inside the testes have to be considered in further studies.

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References

Au, C.L., Robertson, D.M. & de Kretser, D.M. (1986). Measurement of inhibin and an index of inhibin production by rat testes during postnatal development. Biology of Reproduction 35, 37-43.

Bentley, M.J., Gass, G.H. & Leidl, W. (1978). Effects of FSH and LH administration on the testes and seminal vesicles. Andrologia 10, 357-361.

Chemes, H.E., Dym, M. & Raj, H.G.M. (1979). Hormonal regulation of Sertoli cell differentiation. Biology of Reproduction 21, 251-262.

Cunningham, G.R., Tindall, D.J., Huckins, C. & Means, A.R. (1978). Mechanisms for the

testicular hypertrophy which follows hemicastration. Endocrinology 102, 16-23.
Davies, A.G. (1971). Histological changes in the seminiferous tubules of immature mice following administration of gonadotrophins. Journal of Reproduction and Fertility 25, 21-28.
Davies, A.G. (1976). Gonadotrophin-induced changes in the Sertoli cells of the immature mouse testis. Journal of Reproduction and Fertility 47, 83-85.
Finney, D.J. (1964). Parallel line assays. In Statistical Method in Biological Assay, edn 2, pp. 101-128. London: Griffin.
Harlin, J. Khan, S.A. & Diczfalusy, E. (1986). Molecular compositionof luteinizing hormone and follicle-stimulating hormone in commercial gonadotropin preparations. Fertility and Sterility 46, 1055-1061.
Hermans, W.P., van Leeuwen, E.C.M., Debets, M.H.M., Sander, H.J. & de Jong, F.H. (1982). Estimation of inhibin-like activity in spent medium from rat ovarian granulosa cells during long term culture. Molecular and Cellular Endocrinology 27, 277-290.
Hochereau-de Reviers, M.T. & Courot, M. (1978). Sertoli cells and development of seminiferous epithelium. Annales de Biologie Animale, Biochimie et Biophysique 18, 573-583.
de Jong, F.H. (1979). Inhibin, fact or artifact. Molecular and Cellular Endocrinology 13, 1-10.
de Jong, F.H., Smith, S.D. & van der Molen, H.J. (1979). Bioassay of inhibin like activity using pituitary cells *in vitro*. Journal of Endocrinology 80, 91-102.
Lee, V.W.K., de Kretser, D.M., Hudson, B & Wang, C. (1975). Variations in serum FSH, LH and testosterone levels in male rats from birth to sexual maturity. Journal of Reproduction and Fertility 42, 121-126.
Le Gac, F. & de Kretser, D.M. (1982). Inhibin production by Sertoli cell culture. Molecular and Cellular Endocrinology 28, 487-498.
Leidl, W. & Hansel, W. (1972). Longitudinal growth of the seminiferous tubules in rats and its hormonal control. Advances in the biosciences 10, 117-125.
Means, A.R., Dedman, J.R., Tash, J.S., Tindall, D.J., van Sickle, M. & Welsh, M.J. (1980). Regulation of the testis Sertoli cell by follicle stimulating hormone. Annual Review of Physiology, 42,59-70.
Meyers, S.A. & Swanson, L.W. (1983). The effect of exogenous follicle stimulating hormone treatment on anterior pituitary and testicular function in the prepubertal bull. Journal of Andrology 6, 371-377.
Moger, W.H. & Armstrong, D.T. (1974). Changes in serum testosterone levels following acute LH treatment in immature and mature rats. Biology of Reproduction 11, 1-6.
Putra, D.K.H. & Blackshaw, A.W. (1982). Morphometric studies on compensatory testicular hypertrophy in the rat after hemicastration. Australian Journal of Biological Sciences 35, 287-293.
Steinberger, A. (1981). Regulation of inhibin secretion in the testis. In Intragonadal Regulation of Reproduction, pp. 283-298. Eds P. Franchimont & C.P. Channing. London: Academic Press.
Swerdloff, R.W., Walsh, P.C., Jacobs, H.S. & Odell W.D. (1971). Serum LH and FSH during sexual maturation in the male rat: effect of castration and cryptorchidism. Endocrinology 88, 120-128.
Ultee-van Gessel, A.M. & de Jong, F.H. (1987). Inhibin-like activity in Sertoli cell culture media and testicular homogenates from rats of various ages. Journal of Endocrinology 113, 103-110.

Ultee-van Gessel, A.M., Leemborg, F.G., de Jong, F.H. & van der Molen, H.J. (1985). Influence of neonatal hemicastration on in-vitro secretion of inhibin, gonadotrophins and testicular steroids in male rats. Journal of Endocrinology 106, 259-265.

Ultee-van Gessel, A.M., Leemborg, F.G., de Jong, F.H. & van der Molen, H.J. (1986). In-vitro secretion of inhibin-like activity by Sertoli cells from normal and prenatally irradiated immature rats. Journal of Endocrinology 109, 411-418.

Verjans, H.L., Cooke, B.A., de Jong, F.H., de Jong, C.M.M. & van der Molen, H.J. (1973). Evaluation of a radioimmunoassay for testosterone estimation. Journal of Steroid Biochemistry 4, 665-676.

Welschen, R., Osman, P., Dullaart, J., de Greef, W.J., Uilenbroek, J.Th.J. & de Jong, F.H. (1975). Levels of follicle-stimulating hormone, luteinizing hormone, oestradiol-17β and progesterone, and follicular growth in the pseudopregnant rat. Journal of Endocrinology 64, 37-47.

PAPERS RELATED TO THIS THESIS

de Jong, F.H., Sander, H.J., Ultee-van Gessel, A.M. and van der Molen, H.J. (1985). Specific regulation of the secretion of follicle-stimulating hormone from the pituitary gland: the inhibin concept. In: Frontiers of Hormone Research, vol. 14, (eds. Tj.B. van Wimersma Greidanus and S.W.J. Lamberts.), pp. 53-69. Karger, Basel.

van der Donk, J.A., de Ruiter-Bootsma, A.L., Ultee-van Gessel, A.M. and Wauben-Penris, P.J.J. (1986). Cell-cell interaction between rat Sertoli cells and mouse germ cells *in vitro*. Experimental Cell Research 164, 191-198.

van Steenbrugge, G.J., Ultee-van Gessel, A.M., Groen, M., de Jong, F.H. and Schroeder, F.H. (1987). Administration of an LHRH-antagonist to male mice: effects on *in vivo* secretion of hormones and on the growth of a transplantable human prostatic carcinoma. Life Sciences 40, 1335-1343.

Ultee-van Gessel, A.M. and de Jong, E.H. (1987). Effects of neonatal gonadotrophin treatment on testicular development and endoenous gonadotrophins in the rat. Annals of the New York Academy of Sciences, in press.

Ultee-van Gessel, A.M., Timmerman, M.A. and de Jong, F.H. (1988). Function of the pituitary-gonadal axis after administration of gonadotrophins or steroid-free bovine follicular fluid to prepubertal male rats. In: Neuro-Endocrinology of Reproduction. International Congress Series 751. (Ed. R. Rolland) Excerpta Medica, Amsterdam, in press.

Ultee-van Gessel, A.M., van Steenbrugge, G.J., Leemborg, F.G., de Jong, F.H., and Schroeder, F.H. (1988). *In vivo* administration of an LHRH-antagonist to male mice: effects on pituitary gonadotrophin secretion *in vitro*. Acta Endocrinologica (Copenhagen), in press.

PART THREE

SUMMARY

Growth and function of the reproductive organs in male mammals are influenced by a large number of factors, produced in the components of the hypothalamo-pituitary-gonadal axis. The pituitary gland secretes two gonadotrophic hormones, follicle-stimulating hormone (FSH), which can stimulate Sertoli cell function and is thought to be important for the initiation of spermatogenesis, and luteinizing hormone (LH) acting directly on the Leydig cells, which produce and secrete steroid hormones. The secretion of FSH and LH by the pituitary gland is influenced by a stimulating factor, luteinizing hormone-releasing hormone (LH-RH), produced by the hypothalamus, and by inhibiting factors produced by the testes such as steroid hormones. In addition, FSH secretion is suppressed also by a *non* steroidal, gonadal factor, the protein hormone "inhibin" (McCullagh, 1932).
The aim of the present studies was to investigate the factors, that can affect testicular inhibin production *in vivo* and inhibin secretion by Sertoli cells *in vitro* in order to get insight in the role of inhibin in the regulation of male reproductive function.
Relevant aspects of the components of the hypothalamic-pituitary-testicular axis have been described in *chapter 1.*

In *chapter 2* background information on the inhibin concept has been provided. The first observations which suggested the existence of a hormone, produced by the seminiferous tubules affecting pituitary function, have been reported in 1923 by Mottram and Cramer. This hormone was called "inhibin" by McCullagh (1932). Thereafter, much controversy arose about the existence of inhibin, resulting in the temporary abandonment of the inhibin concept. New interest in inhibin started in the early 1970's with the development of more reliable and sensitive bioassay systems for the detection and estimation of inhibin activity. This led to the isolation and characterization of ovarian inhibin. The structure of testicular inhibin has not been fully elucidated yet. Inhibin is a dimer with two different subunits, α and β, and is a member of a much larger group of protein hormones and growth factors such as transforming growth factor-β, activin and Müllerian inhibiting substance.

Chapter 3 and *appendix paper 1* describe results on the regulation of inhibin production by the testis *in vivo*. Concentrations of inhibin in blood are too low to detect with the currently available *in vitro* bioassay methods. Therefore, levels of inhibin in the testis were compared with peripheral levels of FSH at various ages and in different experimental models. The testicular inhibin content increased with age, but was not correlated inversely with levels of

plasma FSH. This discrepancy may be explained by differences in the secretion rate of inhibin into the circulation at various ages.

To evaluate this further, production and secretion of inhibin by Sertoli cells from rats of various ages, was studied *in vitro*, as described in *chapter 4* and *appendix papers 1* and *2*. Sertoli cells from immature rats secrete larger amounts of inhibin than those from adult animals. Furthermore, it became apparent that the production of inhibin is temperature dependent: at 37°C more inhibin is secreted *in vitro* than at 32°C. FSH can stimulate the secretion of inhibin from immature Sertoli cells, whereas testosterone inhibits inhibin secretion under these conditions. Spermatogenic cells appear to have a negative effect on inhibin production.

In *chapter 5* and *appendix papers 3* and *4* deal with manipulation of peripheral levels of FSH and inhibin in prepubertal animals. These experiments were performed in order to examine the consequences of changes of inhibin or FSH secretion on pituitary and testicular function, both immediately after treatment and in adult animals.
The source of inhibin was partly removed by hemicastration at an early age, to decrease endogenous inhibin and thus FSH concentrations *in vivo*. This resulted in compensatory growth of the remaining testis. The maximum relative increase of testicular weight was already observed in 21-day-old animals. Furthermore the role of FSH in the regulation of testis size was investigated in a more direct way by injecting exogenous FSH into prepubertal rats. This treatment stimulated testis growth and inhibin production. In the FSH-treated animals, pituitary FSH levels were reduced to below 10% of control values, presumably because of increased endogenous inhibin secretion.
It was not possible to increase endogenous levels of FSH by injecting an antiserum against inhibin, probably because of a relative low activity of the antiserum or a too high production of inhibin by the testes. Injection of inhibin containing preparations suppressed peripheral FSH levels and caused impaired testicular growth.
Apparently, manipulation of the pituitary-Sertoli cell axis, can result in effects on testicular growth. The balance between FSH secretion and the production of inhibin is an important factor in the determination of final testicular size.

In *chapter 6* the specificity of the bioassay used to detect inhibin in the experiments described in this thesis has been discussed. Since dimers of the β-subunit of inhibin, activin, may counteract the FSH-suppressive effect of inhibin in pituitary cell cultures, it is possible that these bioassay results give too low values for the amounts of inhibin present in the various samples.
It is concluded that FSH can activate prepubertal Sertoli cells to divide and to produce and secrete inhibin. Combined actions of testosterone and the appearance of spermatogenic cells at older ages may result in a decrease of inhibin production by Sertoli cells. The possibility that inhibin acts also as an intratesticular factor, requires further investigations. FSH is not an important factor in the regulation of testicular function in adult rats.

Summary

Regulation of FSH by inhibin may only play an important role during the period of development when FSH is involved in testis growth and initiation of spermatogenesis, regulating the number of Sertoli cells. These Sertoli cells in turn support a fixed number of developing spermatogenic cells at the adult age. It is not likely that inhibin can be used as a male contraceptive agent.

SAMENVATTING

Hormonen die worden gesecerneerd door de organen van het hypothalamus-hypofyse-gonade systeem, reguleren groei en functie van de mannelijke geslachtsklier (testis). De gonadotrope cellen van de hypofyse-voorkwab synthetiseren twee hormonen: het follikel-stimulerend hormoon (FSH) en het luteiniserend hormoon (LH). Beide kunnen een "trope" (stimulerende) invloed uitoefenen op de functie van de testis. De regulatie van de hormoonafgifte door de hypofyse-voorkwab staat onder directe, stimulerende, invloed van een uit de hypothalamus afkomstig product, het "LH-releasing" hormoon (LH-RH).
In het algemeen wordt verondersteld dat FSH belangrijk is bij het op gang komen van de spermatogenese via een stimulerende werking op Sertoli cellen in de testis. Deze cellen bezitten specifieke receptoren voor FSH en voeden en ondersteunen de zaadcellen-inwording via een nauw cel-cel contact; bovendien produceren zij het eiwithormoon inhibine. LH stimuleert in de testis de Leydig cellen, waarin de productie van steroidhormonen, zoals testosteron, plaats heeft. Testosteron kan de afgifte van beide gonadotrope hormonen door de hypofyse-voorkwab remmen. Daarnaast blijkt dat de afgifte van FSH specifiek onderdrukt kan worden door inhibine.

Doel van het onderzoek, beschreven in dit proefschrift, was opheldering te verkrijgen omtrent de regulatie van de inhibineproductie in de mannelijke rat, teneinde het belang van inhibine voor de regulatie van de FSH-productie bij de man te kunnen vaststellen.
De onderdelen van het hypothalamus-hypofyse-gonade systeem zijn in *hoofdstuk 1* kort beschreven.

Hoofdstuk 2 geeft historische informatie over de discussies rondom het zgn. "inhibine-concept". In het begin van de twintiger jaren suggereerden enkele onderzoekers het bestaan van een hormoon dat geproduceerd zou worden in de zaadbuisjes van de testis en dat van invloed zou zijn op de morfologie van cellen in de hypofyse-voorkwab (Mottram en Cramer, 1923). McCullagh noemde dit hormoon in 1932 "inhibine", omdat het "castratie-cellen" in de hypofyse deed verdwijnen wanneer na verwijdering van de testes (castratie) een testis-extract ingespoten werd. In later onderzoek werden de resultaten van deze klassieke experimenten evenwel niet bevestigd. Mede door de ontdekking van vele nieuwe steroid-hormonen en de onmogelijkheid inhibine te zuiveren, achtte men nader onderzoek naar de inhibine-hypothese overbodig.

Samenvatting

Hernieuwde belangstelling voor het bestaan van inhibine ontstond in de zeventiger jaren. Meer specifieke, betrouwbare bioassays (biologische bepalingen) voor inhibine werden ontwikkeld. Het heeft echter meer dan vijftien jaar geduurd voordat onderzoekers erin slaagden het eiwit te zuiveren. Thans is inhibine gezuiverd uit ovariëel follikelvocht, afkomstig van verschillende zoogdieren. Voorts is inhibine aangetroffen in placentaweefsel. Inhibine is een eiwithormoon en bestaat uit een dimeer met twee verschillende subeenheden, α en β, die behoren tot een grotere groep eiwithormonen en groeifactoren, zoals transforming growth factor-β, activine en Müllerian inhibiting substance. De structuur van testiculair inhibine is nog niet geheel opgehelderd; er lijken echter veel overeenkomsten met het ovariëel inhibine te zijn.

In de experimenten beschreven in *hoofdstuk 3* en *appendix publicatie 1*, is getracht middels verschillende proefdiermodellen de regulatie van de inhibineproductie door de ratte-testis *in vivo* te bestuderen. In tegenstelling tot de zeer lage concentraties van inhibine in het bloed, bevat de testis steeds een meetbare hoeveelheid bioactief inhibine. Uit ons onderzoek bleek dat de concentratie van inhibine in de testis afneemt met de leeftijd, terwijl de totale hoeveelheid testiculair inhibine toeneemt. Dit fenomeen kan mogelijk verklaard worden uit een hoge inhibine secretie door de nog delende populatie Sertoli cellen in jonge dieren, terwijl op oudere leeftijd bij een afnemende secretie-snelheid van inhibine, toch ophoping ervan in de testis kan optreden door het functioneel worden van de zgn. bloed-testis barriere.

In *hoofdstuk 4* en de *appendix publicaties 1 en 2* zijn de resultaten geëvalueerd van *in vitro* experimenten, die betrekking hebben op de regulatie van inhibineproductie door Sertoli cellen. Gekweekte Sertoli cellen van jonge ratten kunnen meer inhibine produceren dan de cellen van volwassen ratten. De productie blijkt afhankelijk te zijn van de temperatuur (recht evenredig), van de incubatie tijd (variabele productie) en van hormonen (stimulatie door FSH, remming door testosteron), alsmede van de aan- of afwezigheid van spermatogene cellen (remmend effect) en foetaal kalfsserum (toxisch).

Omdat uit bovengenoemde experimenten bleek dat FSH een belangrijke rol speelt in de inhibineproductie door Sertoli cellen, geïsoleerd uit testes van jonge ratten, is vervolgens getracht FSH- en inhibineconcentraties in prepuberale ratten te variëren, zoals beschreven in *hoofdstuk 5* en *appendix publicaties 3 en 4.*
Verhoogde FSH-spiegels kunnen worden verkregen door direct na de geboorte één testikel - een potentiële bron van inhibine - te verwijderen (neonatale hemicastratie). Uit de resultaten van deze proeven bleek, dat verhoogde FSH-spiegels in bloed verantwoordelijk kunnen zijn voor een extra toename in testisgewicht bij jonge ratten. Bij ratten ouder dan 21 dagen werd geen extra gewichtstoename van de testis gevonden en werden normale FSH-waarden gemeten. Dit laatste kan betekenen dat de overgebleven testikel normale hoeveelheden inhibine produceert, of dat inhibine geen rol meer speelt bij de regulatie van FSH in volwassen mannelijke dieren. Het inspuiten van FSH-preparaten in prepuberale ratten

resulteerde in een versnelde testisgroei. De concentratie van FSH in de hypofyse bleek sterk verlaagd. Dit vindt waarschijnlijk zijn oorzaak in een verhoogde inhibineproductie door de groter geworden testis. Het lukte niet met een antilichaam tegen inhibine, de endogene FSH-spiegels te verhogen. Wellicht was het gebruikte antiserum niet voldoende potent of was de inhibineproductie door de testis te hoog. Injectie van inhibine-bevattende preparaten verlaagde de perifere FSH-spiegels en veroorzaakte een vertraging van de testisgroei.
Manipulaties met de hypofyse-Sertoli cel as, kunnen derhalve resulteren in effecten op de testisgroei. De balans tussen FSH-afgifte en inhibineproductie is kennelijk bepalend voor de testis-grootte.

In *hoofdstuk 6* is de gebruikte *in vitro* bioassay voor de meting van inhibine-aktiviteit besproken. Nu recentelijk dimeren van de β-subeenheid van inhibine ontdekt zijn die een aan inhibine tegensteld effect kunnen hebben op hypofysecellen in kweek, is het niet uitgesloten dat de werkelijke inhibine-aktiviteit in sommige monsters onderschat is. Om in de toekomst de reële inhibine activiteit te meten, moet inhibine van de activinen gescheiden worden, voordat inhibinepreparaten aan hypofysecellen worden toegevoegd.
Voorts is gebleken dat FSH de delingsactiviteit van Sertoli cellen kan stimuleren en daardoor mede de productie van inhibine kan vergroten. Op latere leeftijd zijn Sertoli cellen niet in staat te delen en maken zij aanzienlijk minder inhibine. Mogelijk is een samenspel van factoren, zoals de concentratie van testosteron en het verschijnen van bepaalde stadia van spermatogenetische cellen, een signaal voor de vermindering van de inhibineproductie door Sertoli cellen in oudere testes. Of inhibine in de testis ook een regulerende rol vervult, dient nader onderzocht te worden met het thans zuivere materiaal.
Inhibine speelt waarschijnlijk geen belangrijke rol in de regulatie van de afgifte van FSH door de hypofyse-voorkwab bij volwassen ratten. De verhouding van FSH en inhibine is echter wel van belang tijdens de groei en ontwikkeling van de mannelijke geslachtsklier. Een juiste balans tussen FSH-afgifte en inhibineproductie is een voorwaarde voor het bereiken van een normaal aantal Sertoli cellen aan het eind van de puberteit. Deze Sertoli cellen, waarvan het aantal na de puberteit niet meer verandert, staat uiteindelijk borg voor een kwalitatief en kwantitatief normale spermatogenese in het volwassen mannelijke dier. Toepassing van inhibine als anticonceptivum voor de man lijkt uitgesloten.

CURRICULUM VITAE

Annemarie Ultee-van Gessel behaalde in 1972 aan het Sint Ludgercollege te Doetinchem het HBS B-diploma. In dat jaar begon zij met de studie Biologie aan de Katholieke Universiteit Nijmegen en legde in 1975 het kandidaatsexamen (B1g) af. Het doctoraal-examen (1979) omvatte de hoofdvakken Geobotanie (Prof.Dr. V. Westhoff) en Biochemische Antropogenetica (Prof.Dr. S.J. Geerts), alsmede het bijvak Aquatische Oecologie (Prof.Dr. C. den Hartog). Tijdens de doctoraalfase werd de eerste graads onderwijsbevoegdheid voor Biologie/Scheikunde behaald en - in het kader van het tweede hoofdvak - enige tijd onderzoek verricht aan het Institute Pasteur te Parijs (Prof.Dr. P. Hösli).
Vanaf 1979 was de schrijfster van dit proefschrift werkzaam als wetenschappelijk assistent te Utrecht aan de Rijks Universiteit, Medische Faculteit, afdeling Histologie en Celbiologie (Prof.Dr. M.F. Kramer). Aansluitend volgde in 1982 een aanstelling als wetenschappelijk medewerker aan de Medische Faculteit van de Erasmus Universiteit Rotterdam, afdeling Biochemie II (Chemische Endocrinologie), alwaar het onderzoek, beschreven in deze dissertatie, werd voltooid.
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