INHIBIN

WITH AN OLD CONCEPT TOWARDS A NEW APPROACH IN REPRODUCTIVE PHYSIOLOGY

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J. SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 28 APRIL 1982 DES NAMIDDAGS
TE 2.00 UUR

DOOR

WILHELMINA PAULINA HERMANS
geboren te Nijmegen

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CONTENTS page

I. EFFECTS OF AN INHIBIN - LIKE FACTOR AS PRESENT IN BOVINE AND PORCINE FOLLICULAR FLUID ON GONADOTROPHIN LEVELS IN OVARIECTOMIZED RATS

R. Welschen, W. P. Hermans, J. Dullaart and F. H. de Jong (1977)
Journal of Reproduction and Fertility 50: 129-131

II. POSSIBLE INVOLVEMENT OF INHIBIN IN THE INTERRELATIONSHIP BETWEEN NUMBERS OF ANTRAL FOLLICLES AND PERIPHERAL FSH CONCENTRATIONS IN FEMALE RATS

R. Welschen, W. P. Hermans and F. H. de Jong (1980)
Journal of Reproduction and Fertility 60: 485-493

III. QUANTIFICATION OF THE CONTENT OF INHIBIN IN SPENT MEDIA FROM CULTURED RAT OVARIAN GRANULOSA CELLS

W. P. Hermans, E. C. M. van Leeuwen, M. H. M. Debets, H. J. Sander and F. H. de Jong (1982)
(submitted)

IV. INVOLVEMENT OF INHIBIN IN THE REGULATION OF FOLLICLE STIMULATING HORMONE CONCENTRATIONS IN PREPUBERTAL AND ADULT, MALE AND FEMALE RATS

W. P. Hermans, E. C. M. van Leeuwen, M. H. M. Debets and F. H. de Jong (1980)
Journal of Endocrinology 86: 79-92

V. TIME - RELATED SECRETION OF GONADOTROPHINS AFTER A SINGLE INJECTION OF STEROID - FREE BOVINE FOLLICULAR FLUID IN PREPUBERTAL AND ADULT FEMALE RATS

W. P. Hermans, M. H. M. Debets, E. C. M. van Leeuwen and F. H. de Jong (1981)
Journal of Endocrinology 90: 69-76

VI. EFFECT OF SINGLE INJECTIONS OF BOVINE FOLLICULAR FLUID ON GONADOTROPHIN CONCENTRATIONS THROUGHOUT THE OESTROUS CYCLE OF THE RAT

W. P. Hermans, M. H. M. Debets, E. C. M. van Leeuwen and F. H. de Jong (1982)
Journal of Endocrinology 92: 425-432.

LIST OF ABBREVIATIONS

bFF	: bovine follicular fluid
BP	: bovine plasma
cAMP	: cyclic adenosine monophosphate
^{o}C	: degrees centigrade
D	: dalton
DNA	: deoxyribonucleic acid
FSH	: follicle stimulating hormone (follitropin)
FSH-RH	: follicle stimulating hormone releasing hormone
g	: gram
h	: hour
^{3}H	: tritium
HCG	: human chorionic gonadotrophin
i.p.	: intraperitoneal
i.u.	: international unit(s)
LH	: luteinizing hormone (lutropin)
LH-RH	: luteinizing hormone releasing hormone
M	: moles per litre
mg	: milligram
ml	: millilitre
Mw	: molecular weight
min	: minutes
n	: number of observations
ng	: nanogram
OTX	: orchidectomy; orchidectomized
OVX	: ovariectomy; ovariectomized
P	: probability
pp	: page
r	: correlation coefficient
^{35}S	: sulpher ^{35}
S.E.M.	: standard error of the mean
µl	: microlitre
μm^{3}	: cubic micrometre
vs	: versus
wt	: weight

ADDITIONAL ABBREVIATIONS ONLY APPEARING IN APPENDIX PAPERS:

BLO	: bilateral ovariectomy or bilateral orchidectomy
BSA	: bovine serum albumin
CO_2	: carbon dioxide
Di-1	: dioestrus-1 (metoestrus)
Di-2	: dioestrus-2
Di-3	: dioestrus-3
DNAse	: deoxyribonuclease
E	: oestradiol - 17 β
EB	: oestradiolbenzoate
EGTA	: ethyleneglycol-bis-2-aminoethyl-tetraacetic acid
e/s	: enzyme/substrate ratio
FCS	: foetal calf serum
g	: relative centrifugal force
GnRH	: gonadotrophin releasing hormone
HBSS	: hank's balanced salt solution
MEM	: minimal essential medium
mM	: millimolair
NaOH	: sodiumhydroxide
O	: oestrus
P	: pro-oestrus
P	: progesterone
pFF	: porcine follicular fluid
pg	: picogram
rGCCM	: rat granulosa cell culture medium
T	: testosterone
ULO	: unilateral ovariectomy; unilaterally ovariectomized
v/v	: volume/volume ratio
w/w	: weight/weight ratio

GENERAL INTRODUCTION

Fertility in mammals has received considerable attention as far back as written reports exist. In the last centennium, the study of fertility has surpassed the level of merely descriptive and empirical science and has been applied to solve some problems of the regulation of fertility. This has contributed to the treatment of fertility disorders and to birth control in man, as well as to improvement of animal husbandry.

Our knowledge of the processes controlling fertility has increased due to clinical observations and the use of experimental techniques ranging from parabiosis experiments, castration and hypophysectomy to brain lesions and implantations of materials such as tissue fragments. Information has been obtained about the interplay between central nervous structures, pituitary gland and the gonads, and about the role of hormones secreted by these organs. In the most simple scheme, these interactions firstly involve the control and release of hypothalamic releasing factors, which reach the anterior pituitary gland by way of the portal vessels. In turn, the pituitary gland releases in both male and female animals gonadotrophic hormones: follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones enter the circulation and reach their end-organs, testes or ovaries, in which they influence both morphology and function. The testes and ovaries, apart from producing gametes, respond with the production of gonadal hormones, which do not only control the secretion of hypothalamic releasing factors and the pituitary secretion of FSH and LH, but also influence the development and maintainance of accessory sex organs, secondary sex characteristics and sexual behaviour. One of the problems in testicular and ovarian (patho)physiology is the nature and action of these gonadal hormones.

In the last decades, much attention has been given to the production and secretion of gonadal steroids: androgens, progestins and oestrogens. Until recently, these steroid hormones were thought to be to gonadal hormones regulating the secretion of gonadotrophins. However, with the availability of radioimmunoassays, methods highly sensitive and specific for the measurement of concentrations of steroid hormones, gonadotrophins and releasing factors, it became evident that feedback relationships, involving merely steroids and gonadotrophins, had to be re-evaluated. This led to a renewed interest in the existence of inhibin, a hypothetical gonadal, non-steroidal hormone, produced by both male and female gonads, which was first suggested to exist in 1923 (Mottram & Cramer).

In the earliest studies inhibin was defined as "a water-soluble, testicular substance, capable of preventing the appearance of castration cells in the adeno-

hypophysis of gonadectomized animals" (McCullagh, 1932). More recently inhibin was defined as "a water-soluble, non-steroidal gonadal substance, which exerts a specific inhibition of the release of FSH from the pituitary gland" (de Jong, 1979). Since neither in the past nor in more recent studies inhibin has been defined chemically, the hormone remains hypothetical. Therefore it would be correct if factors fulfilling the definition given above were named inhibin-activity or inhibin-like activity, but for the sake of brevity they will be named inhibin.

This thesis contains firstly a review of the history of the concept of inhibin. Secondly, experimental results are reported which support the hypothesis that in both male and female animals inhibin might be involved in the regulation of gonadotrophin secretion, and thus might play a role in the regulation of fertility. Emphasis is placed on a possible physiological role for inhibin in female animals.

CHAPTER 1

DEVELOPMENT OF THE CONCEPT OF INHIBIN

1.1. The history of the inhibin concept

New perspectives with regard to reproductive physiology became available with the discovery of gonadotrophic activity of the pituitary gland at the beginning of this century and the measurement of concentrations of gonadotrophic hormones since the late 1930's (for references see Donovan, 1966; Gemzell & Roos, 1966). This was followed by the observation that the secretion of gonadotrophic hormones is controlled by both neurohumoral factors (reviews: Harris, 1955; Fink, 1976) and by substances secreted by the gonads.

The gonads seemed involved in a negative control of gonadotrophin secretion since in rats removal of the testes or ovaries results in the appearance in the adenohypophysis of highly vacuolized, basophilic cells, the so-called castration cells, in addition to atrophy of the seminal vesicles and the prostate in males or of the uterus in female animals. In the late 1920's the discovery that factors with androgenic, progestagenic and oestrogenic activity were secreted by the gonads, led to the development of the concept that gonadotrophin secretion of the pituitary gland was negatively controlled by gonadal hormones (Moore & Price, 1932). However, already in 1923 Mottram & Cramer observed an increase in the number of castration cells in the adenohypophysis without changes in the accessory sex glands after application of radium to the testes of male rats. In these experiments only the germinal epithelium and not the interstitial cells of the testicles were severely damaged, as judged by microscopy. Therefore these authors proposed that the seminiferous tubules are involved in the production of a factor specifically controlling gonadotrophin secretion. Support for this suggestion came from a study by Martins & Rocha (1931), who used parabiotic pairs of either similar or opposite sexes, which were intact, castrated or cryptorchid. Furthermore, in groups of these animals pituitary tissue or testicular fragments were implanted. These authors concluded that castration of prepubertal or adult male rats resulted in the removal of at least two hormones, of which one seemed to exert a marked influence on the activity of the pituitary gland in male rats, but, with the doses tested, not in female rats. This latter hormone was apparently produced by implanted testicular fragments. The postulated hormone was also found in water-soluble testicular extracts and was active in preventing the appearance of castration cells in the pituitary gland. In 1932 McCullagh confirmed these studies and suggested that the testis produces two hormones: a benzene-soluble hormone, androtin, responsible for the maintainance of seminal vesicles and prostate; and a water-soluble hormone which he named inhibin,

since it inhibited the appearance of castration cells in the adenohypophysis of castrated animals. In the following years data were presented with respect to the issue of male androtin and/or inhibin, which were conflicting or difficult to interpret (Nelson, 1934; Vidgoff & Vehrs, 1940; Rubin, 1941). No search was made for the existence of inhibin in female animals, although aqueous testicular extracts disturbed the oestrous cycle of female rats (McCullagh & Schneider, 1940).

The development of bioassays for the measurement of urinary concentrations of gonadotrophins offered a new possibility for investigating the control of gonadotrophin secretion in man. A positive correlation was found between the amount of gonadotrophins, mainly FSH, in the urine and the severity of spermatogenic damage (Klinefelter et al., 1942; Heller & Nelson, 1945; del Castillo et al., 1947; McCullagh & Schaffenburg, 1952), while normal urinary concentrations of FSH were found when only the Leydig cells were damaged or absent (McCullagh & Schaffenburg, 1952). These observations supported the concept of a dual endocrine control of the pituitary gland exerted by the testis, namely by way of androgens produced in the Leydig cells and of inhibin produced in the seminiferous tubules in conjunction with spermatogenic elements, but the inhibin hypothesis did not survive. The main reasons for this were (i) the identification of an increasing number of testicular steroids, while the existence of inhibin could not be demonstrated directly, (ii) the rejection of the Sertoli cells as a source of inhibin since in men with Sertoli-cell-only syndrome elevated levels of gonadotrophins were found and (iii) the absence of a morphological indication of a secretory apparatus in spermatogenic cells (Heller et al., 1952). The high urinary concentrations of gonadotrophins found in men with different degrees of spermatogenic damage were attributed to the inability of the germinal epithelium to utilize the circulating gonadotrophins: "the utilization hypothesis". From the 1950's the inhibin hypothesis was replaced by the utilization hypothesis and by the concept that steroid hormones alone could regulate gonadotrophin secretion (see also: Donovan, 1966). No attempt was made to give an alternative explanation for the prevention of development of castration cells after administration of a water-soluble testicular extract to castrated animals. In order to elucidate the control of gonadotrophin secretion, attention was focused on the administration of different types and combinations of ovarian and testicular steroids in a number of species (reviews: Baker et al., 1976; Campbell & Schwartz, 1977; Setchell et al., 1977). The concept of inhibin seemed forgotten.

1.2. New leads to the inhibin hypothesis

1.2.1. The role of FSH and LH in gonadal function: control of FSH and LH secretion

The existence of two gonadotrophic hormones, FSH and LH, originally proposed by Fevold et al. (1931) is now generally accepted. Considerable progress has been made in the elucidation of the chemical nature and specific sites of action of FSH and LH. It was shown that both hormones contain a similar α-subunit, and a β-subunit defining the specificity of each hormone (Reichert, 1972). Progress has been made also with respect to the specific roles of FSH and LH; this was mainly due to the replacement of bioassays for FSH and LH by more sensitive radioimmunological procedures.

The role of FSH and LH in male animals has been extensively reviewed by Lee et al. (1976), Setchell et al. (1977) and by Davies (1981). In short, LH binds specifically to receptors in the membranes of the Leydig cells in the testis and stimulates androgen production by these cells. In contrast, FSH has been shown to bind to cells of the seminiferous tubules, especially to the Sertoli cells; the number of binding sites in the rat testis increases towards adulthood. FSH has been shown to stimulate cAMP production in the Sertoli cells of immature rats, and plays a role in the induction of receptors for LH. Another effect of FSH is stimulation of protein synthesis, such as the production of androgen binding protein. Especially in prepubertal animals FSH influences testis weight, diameter of the seminiferous tubules and the number of pachytene spermatocytes per tubular cross section. It also increases mitotic rate and reduces degeneration of spermatogonia. Apparently FSH is, at least in the immature animal, involved in the development of the testis and specifically in gametogenesis. However, in the adult animal androgens rather than FSH appear essential for maintainance of established spermatogenesis (see Davies, 1981).

In female animals, gonadotrophins have a variety of effects on the ovary (see reviews by Lindner et al., 1974; Fraser, 1976). LH stimulates follicular, luteal and stromal steroidogenesis; LH also causes maturation of the oocyte, induces an increase in ovarian blood flow and promotes follicular rupture and ovulation. The role of FSH is more restricted to folliculogenesis: FSH stimulates recruitment and maintainance of follicular growth, in part by stimulation of proliferation of granulosa and thecal cells. It also plays a role in granulosa cell glycolysis and maintainance of sensitivity to LH. FSH seems also necessary for optimal luteal cell function. Growing large follicles seem to need a continuous support of FSH (diZerega et al., 1981), and can be rescued from a beginning atresia by additional FSH (Hirshfield & Midgley, 1978). In analogy with the situation in male animals, FSH

is clearly involved in processes supporting gametogenesis, but whether folliculogenesis is regulated quantitatively by FSH is not known.

Since processes involved in gametogenesis and folliculogenesis are induced by both FSH and LH, it is conceivable that the release of both FSH and LH is dependent on one hypothalamic releasing hormone, luteinizing hormone releasing hormone (LHRH) (Schally et al., 1975). However, under many pathological, physiological or experimental conditions, diverging secretion patterns of FSH and LH have been observed (see reviews of Franchimont et al., 1975; Setchell et al., 1977; Fink, 1977). This dissociation may be dependent on the existence of a separate FSH releasing hormone, FSHRH (Johansson et al., 1973; Fuchs et al., 1979). This dissociation could also be due to functionally diverse subpopulations of gonadotrophs (Denef et al., 1980), to a differential action of LHRH on the gonadotrophs (Wise et al., 1979), to the involvement of prolactin (McNatty et al.,1974), or to the involvement of the pineal gland (Seegal & Goldman, 1975; Cheesman et al., 1977). Regardless of the direct cause, the control of the above mentioned parameters, which might lead to this dissociation in the release of FSH and LH, is not clear.

It is known that testicular and ovarian steroids are capable of controlling the secretion of LH and FSH in a number of circumstances (for references: Everett, 1969; Setchell et al., 1977). Therefore it could be possible that the observed diverging secretion patterns of FSH and LH are caused by a specific combination of steroids and, in experiments, also by a specific route and time of administration of steroids. On the other hand, it is possible that the gonads secrete a non-steroidal factor which specifically or preferentially influences the synthesis or release of the gonadotrophins. The following section will deal with evidence which suggests that a non-steroidal gonadal factor is involved in the regulation of FSH secretion.

1.2.2. Non-steroidal control of secretion of FSH

Clinical evidence for a role of non-steroidal gonadal factors

In the human male a number of clinical conditions exist in which a selective rise in urinary or serum levels of FSH, with normal levels of LH, can be observed. In these conditions the Leydig cells appear normal and are also regarded as functionally intact, since normal levels of testosterone are found. The germinal epithelium however is damaged or destroyed. This situation is found in the Sertoli-cell-only syndrome, in cryptorchism and in azoospermia or (sometimes) oligospermia; elevated levels of FSH with normal levels of LH are also found after certain viral infections, irradiation or treatment with cytotoxic drugs (for extensive reviews: Setchell & Main, 1974; Baker et al., 1976; Lee et al., 1976; Chari, 1977; Setchell

et al., 1977).

Recently many authors (references see below) have reported inverse relationships between damage to the germinal epithelium and a selective increase in levels of FSH. This supports the view that the germinal epithelium produces a factor which specifically inhibits the secretion of FSH, and which resembles the factor named "inhibin" by McCullagh (1932). However, the data do not indicate a specific spermatogenic cell type responsible for the production of such a factor: inverse relationships with levels of FSH were found for numbers of immature germ cells (Herruzo et al., 1978), for the number of spermatogonia (van Thiel et al., 1972; De Kretser et al., 1974; Scarselli et al., 1976), for primary and meiotic spermatocytes (Franchimont et al., 1972) and for the number of mature spermatids (Johnsen, 1970; De Kretser et al., 1974; Franchimont et al., 1975; Coutant & Comhaire, 1978). Thus, every type of cell directly involved in spermatogenesis could be related with the production of this non-steroidal factor, inhibin. However, defects in germinal maturation are generally related to Sertoli cell dysfunction (Paulsen et al., 1972). Thus the selective rise in FSH could be caused also by damage to the Sertoli cell, suggesting that also the Sertoli cell might be involved in the production of this non-steroidal factor. The observation that levels of FSH in men with Sertoli-cell-only syndrome do not rise to the level found after castration (van Thiel et al., 1972; Christiansen, 1975) supports involvement of the Sertoli cells in the regulation of secretion of FSH. Sometimes defects in the control of FSH secretion are detectable only as an exaggerated response of FSH to an LHRH test: this is often the case in oligospermia (Bramble et al., 1975; Lipshultz et al., 1977). Also these latter studies therefore suggest that in the human male factors arising in the germinal epithelium might contribute to the control of FSH secretion.

In women, only few reports have suggested the involvement of a non-steroidal factor in the control of FSH secretion, despite the fact that dissociating patterns of FSH and LH secretion have been found following castration (Ostergard et al., 1970), during the follicular phase of the menstrual cycle and during the midcycle elevations of FSH and LH (Midgley & Jaffe, 1968; Ross et al., 1970). In the early menopause, specific elevations of levels of FSH throughout the cycle accompanying normal levels of LH, progesterone and oestradiol were reported by Furuhashi et al. (1976) and Sherman (1976). Sherman (1976) suggested that these elevated levels of FSH were due to a reduction in the amount of a non-steroidal factor, resembling male inhibin, as a result of a reduced number of follicles in older women. He suggested that disorders in the secretion of inhibin could lead, by way of elevated levels of FSH, to rapid follicular maturation and, as a consequence, to shorter cycle length in early menopause. Thomas et al. (1977) suggested a role for inhibin in women in whom the ovaries were damaged after treatment with cytotoxic drugs: they observed an exaggerated FSH response to LHRH, which is suggestive of a dis-

turbed control of FSH secretion. However, their study does not make clear which ovarian compartments were damaged. Recently, Koninckx et al. (1981) compared mid-cycle elevations in levels of FSH in women with a luteinized unruptured follicle syndrome with FSH patterns in normal women; they found a more steeper fall in levels of FSH in normal women than in women with the unruptured follicle, and suggested that the slower decrease of levels of FSH was caused by the absence of resorption of follicular fluid (containing inhibin) from the peritoneal cavity after ovulation.

Experimental evidence for a role of non-steroidal gonadal factors

The experimental evidence suggestive of a role for a non-steroidal factor in control of FSH secretion is accumulating rapidly. The renewed interest in non-steroidal feedback on FSH secretion was initiated by the clinical data mentioned above and by the finding that in experimental male animals steroid administration exerts different effects on the secretion of FSH and LH. It is well documented that after castration of male rats the increased secretion of both FSH and LH can be suppressed by a number of steroids, but while LH is already suppressed by administration of amounts of androgens which cause blood levels to be in a physiological range, the effect of such a dose of steroids on FSH secretion ranges from suppression to stimulation. Often levels of FSH can only be suppressed with supra-physiological amounts of androgens and oestrogens (for references: Swerdloff et al., 1973; Franchimont et al., 1975; Lee et al., 1976; Setchell et al., 1977; Labrie et al., 1978). Even when "physiological" amounts of testosterone were maintained in castrated rats by means of silastic capsules containing testosterone and a suppression of levels of FSH could thus be obtained, levels of LH could be suppressed with lower amounts of testosterone (Decker et al., 1981).

Studies on the effects of steroid replacement in castrated rats are often difficult to compare and to interpret due to the variety of steroids and experimental approaches used. This is also true for experiments measuring the differential responses of FSH and LH after castration and the relationship with disappearance of steroids. Studies extending over several weeks cannot be compared with studies measuring hormone concentrations within the first 24 h after castration. The last type of experiments clearly show non-parallel changes in secretion patterns of FSH and LH following castration (Swerdloff et al., 1971; Gupta et al., 1975; Schwartz & Justo, 1977; Hermans et al., 1980), which cannot be attributed to decreasing amounts of testosterone (Negri & Gay, 1976; Denef & Hautekeete, 1978; Hermans et al., 1980). In conclusion, it has not been possible to positively identify a known steroid or a combination of steroids which can be held responsible for a specific suppression of concentrations of FSH, or which, if absent, will give rise to a selective in-

crease of concentrations of FSH. Therefore a renewed interest arose in the existence of a non-steroidal factor which can selectively control the secretion of FSH after the interest in the search for such a factor had largely disappeared for many years.

The experimental approach used in the search for such a non-steroidal factor consisted mainly of two types of studies. In the first type disturbances predominantly in spermatogenesis were induced in rats without affecting Leydig cell function, as measured by testosterone concentrations. Selective damage of the germinal epithelium can be obtained either by x-irradiation, heat-treatment of the testis, experimental cryptorchism, efferent duct ligation, treatment with antispermatogenic drugs, feeding of vitamin deficient diets or immunological procedures (for references: see Setchell & Main, 1974). In general, these treatments result in an increased secretion of FSH. However, in many cases peripheral levels of LH were also affected, casting some doubts whether Leydig cell function remained intact. On the other hand, the increase in the secretion of FSH and (sometimes) LH seen after treatments probably inducing selective damage of the germinal epithelium was clearly less than the response seen after castration, and in general predominantly levels of FSH were increased. This led to the view that a non-steroidal factor, inhibin, arising in the tubular compartment of the testis, contributes to the control of FSH secretion together with androgens (see for reviews: Main et al., 1978; 1979). The second type of studies involves impairment of Leydig cell function, e.g. by administration of ethylenedimethanesulphonate (Morris & Jackson, 1978) or removal of circulating testosterone by a specific antiserum against testosterone (Madwha Raj et al., 1978); in these circumstances only the secretion of LH was affected. These data also support the concept of a not testosterone mediated control of FSH secretion.

Which cell type is responsible for the production of such a non-steroidal factor, specifically or preferentially regulating FSH secretion, is not clear. Relationships between the selective rise of FSH levels and the onset of spermatogenic depletion as measured by the absence of later stages of spermatogenesis indicated the spermatids as source of the non-steroidal factor (Setchell et al., 1977; Hopkinson et al., 1978; Collins et al., 1978). In rats, like in men, spermatogenic damage may also affect Sertoli cell function (Rich & De Kretser, 1977). Indeed, Bain & Keene (1975), de Jong & Sharpe (1977 a) and Hopkinson et al. (1978) have attributed the production of a non-steroidal factor directly to the Sertoli cells, possibly in conjunction with spermatogenic cells.

Also in female animals an increasing number of experiments has been reported indicating that a non-steroidal factor is involved in the regulation of FSH secretion. Since this thesis deals mainly with a role for inhibin in female animals, and since experimental evidence suggestive of such a role became available only after

the detection of inhibin in ovarian follicular fluid (to be discussed in Chapter 2), the experimental data suggestive of the involvement of a non-steroidal gonadal factor in regulation of FSH secretion in the female mammal will be discussed in greater detail in Chapter 3.

In conclusion, clinical as well as experimental data, predominantly obtained in male animals, are in conflict with the concept of steroids as sole regulators of gonadotrophin secretion. This prompted further search for inhibin, a non-steroidal gonadal factor, specifically or preferentially suppressing the secretion of FSH by the pituitary gland.

The detection of inhibin in a variety of male and female gonadal fluids will be described in the next chapter.

CHAPTER 2

DETECTION OF INHIBIN

Conflicting data on the involvement of steroids in the regulation of peripheral concentrations of FSH, as described in the previous chapter, led to the search for a non-steroidal factor, inhibin. Unfortunately, the many experiments conducted to detect inhibin suffered from a lack of uniformity from the onset:
(i) no clear definition of inhibin was used. Depending on the tests used, preparations suppressing only levels of FSH, or the levels of both FSH and LH, were considered to contain inhibin-like activity;
(ii) a variety of bioassays, both *in vivo* and *in vitro*, has been employed in the last decade, but even laboratories using comparable in vivo bioassays have sometimes obtained different results;
(iii) most laboratories use their "house standard" as reference preparation. Occasionally different standard preparations have been exchanged, but no generally accepted international standard preparation containing inhibin is (yet) available;
(iv) purification of inhibin has yielded discouraging results over the last years. Different laboratories have reported a wide range of molecular weights and biochemical behaviour of the factor with inhibin-like activity.

In this chapter an attempt will be made to evaluate and review the many data concerned with the detection of inhibin.

2.1. Definition of inhibin

Since it was found that partially purified preparations containing inhibin also contained factors which were not identical with but behaved as inhibin under certain conditions (de Jong et al., 1979 b), we prefer the following definition of inhibin: " *inhibin is a water-soluble, non-steroidal substance which suppresses the secretion from the pituitary gland of FSH, but not of LH, in a number of different tests*".

2.2. Methods for the detection of inhibin

A large number of tests for the detection of inhibin has been employed (see also reviews by de Jong, 1979; Hudson et al., 1979; Franchimont et al., 1979; Blanc, 1980). With most methods, quantification of inhibin is not possible. In general, the methods used can be separated into two groups:
(a) "non-specific" in vivo methods, estimating changes in FSH and LH secretion on the basis of measurement of organ weights after administration of the active

principle;

(b) "specific" in vivo and in vitro methods, based on measurement of radioimmunoassayable FSH and LH concentrations: *in vivo* gonadotrophins are measured in serum or plasma after injection or infusion of the active principle into the test animals; *in vitro* gonadotrophins are measured after addition of the active substance(s) in the cells or in the media from cultured pituitary cells, or in media of incubated halved pituitaries.

2.2.1. "Non-specific" in vivo methods

A number of non-specific methods have been used. Most frequently used is the method measuring the inhibition by inhibin of the increase in ovarian weight induced by human chorionic gonadotrophin (HCG) in immature female rats (Chari et al., 1976) or the comparable inhibition of the increase in uterine weight in immature female mice (Setchell & Sirinathsinghji, 1972; Ramasharma et al., 1979). These type of tests are used since the increase in ovarian or uterine weight following administration of HCG only takes place in the presence of endogenous FSH (Lamond & Emmens, 1959). Ramasharma et al. (1979) showed that within 1 hour after the administration of HCG an endogenous surge of FSH occurs. When the ovarian or uterine weight increase is inhibited after administration of test materials, this inhibition is considered as proof that the increase in the amount of circulating endogenous FSH has been selectively suppressed.Although simple and fast to perform, these tests are very sensitive to the amount of HCG given (Setchell et al., 1977) and to the timing of the administration of inhibin. Finally, poor reproducibility of these assays within one laboratory seems to occur (Franchimont et al., 1979), while other laboratories could not obtain inhibition of ovarian weight increase with preparations known to contain inhibin (Davies et al., 1978). Also in our laboratory we could not inhibit in 25-day-old female rats the ovarian weight increase following HCG administration using bovine follicular fluid (bFF) as the source of inhibin (Table 1).

Another method for monitoring changes in the secretion of biologically active FSH uses parabiosis of an immature female rat with a castrated adult male or female partner. Protein hormones can, but steroid hormones practically cannot pass from one animal to the other. Consequently, the ovaries from the immature rat increase in weight due to the increased gonadotrophin secretion of the castrated partner. Administration of inhibin to the castrated partner should decrease the ovarian weight increase in the immature female rat (Lugaro et al., 1969; 1973). However, several objections to this test exist. With this method it is not possible to show a dose-response relationship between the amount of inhibin and the reduction of the ovarian weight increase (Lugaro et al., 1973). Moreover, the specificity of the ovarian response to changes in FSH is dubious. Inhibin seems capable of entering the

Table 1. *Mean (± S.E.M.) ovarian weights in 25-day-old female rats (n = 6 for all groups) 24 h after injection of 50 i.u. human chorionic gonadotrophin (HCG) alone or combined with 0.015 up to 1.00 ml charcoal-treated bovine follicular fluid (bFF) / animal.*
Data of de Jong et al. (1979 b).

treatment	ovarian weight (mg / 100 g body wt.)
none	28.1 ± 2.1
50 i.u. HCG	47.0 ± 2.0
50 i.u. HCG + 0.015 ml bFF	48.7 ± 2.1
50 i.u. HCG + 0.060 ml bFF	44.7 ± 1.1
50 i.u. HCG + 0.250 ml bFF	46.4 ± 1.9
50 i.u. HCG + 1.000 ml bFF	45.0 ± 1.5

circulation of the parabiotic immature female partner (Johnson, 1981), thus interfering with the rationale of the test. Finally, the enlarged ovaries probably produce large amounts of endogenous inhibin (Johnson, 1981), thus affecting FSH secretion in the castrated partner.

A last non-specific type of test *in vivo* is based on the inhibition of the ovarian weight gain in 21-day-old mice following an injection with serum from an unilaterally ovariectomized mouse, the latter previously treated or not with inhibin (Sato et al., 1980). In this way the concentration of biologically active FSH in the serum of control and inhibin treated mice might be estimated, but many factors such as the amount of inhibin still present in the serum of the unilaterally ovariectomized mouse may be of influence in this assay. Also the amount of FSH necessary to increase the ovarian weight is not known.

2.2.2. "Specific" in vivo methods

Specific in vivo methods for the detection of inhibin are based on the measurement of changes in serum or plasma concentrations of radioimmunoassayable FSH and LH following administration of material thought to contain inhibin. Both male and female animals have been used as recipient animals. The suppressed levels of FSH are never lower than 25 % of the pretreatment values (de Jong, 1979), regardless the source of inhibin used. In general blood samples are taken within 3-24 h after the last injection of inhibin, since the suppressive effect starts within a few hours after injection and is waning within 6-16 h after injection (males: Nandini et al.,

1976; females: Hermans et al., 1981 a; 1982 a).

Male animals

Castrated or cryptorchid rams, either given an infusion or two injections of the active material, have been used as test models, especially when repeated sampling of blood was necessary for studies on the time-course of the FSH suppression (Baker et al., 1976; Keogh et al., 1976; Lee et al., 1976; Blanc et al., 1978; Cahoreau et al., 1979). Although the suppression of levels of FSH was evident, variable suppressive effects on LH have been reported with higher doses of inhibin-like activity (Lee et al., 1976). Also reduced levels of LH preceding the reduction in levels of FSH in castrated rams and absence of pulsatile patterns of secretion of LH in cryptorchid rams (Cahoreau et al.,1979) have been reported.

Most commonly used in in vivo tests are immature and adult male rats of various ages, but these models are also not suited for quantification of inhibin (see Baker et al., 1981), due to the relatively large amounts of materials to be tested needed.

Adult intact rats (Hermans et al., 1980; Lorenzen et al., 1981), *rats castrated 12-48 h previously* (Nandini et al., 1976; Hermans et al., 1980; Lorenzen et al., 1981) or *castrated 2-3 weeks* before treatment (Setchell & Jacks, 1974; Lugaro et al., 1974; Franchimont et al., 1975; Becker et al., 1977; Hopkinson et al., 1977; Le Lannou & Chambon, 1977 a; Peek & Watkins, 1979; Vijayalakshmi et al., 1980) all proved suitable in models for detection of inhibin depending on the number of injections given and the timing of blood sampling after the last injection.

A simple test, used widely and yielding good results in terms of suppression of FSH is based on prevention of the *post-castration rise in FSH in adult* (Hermans et al., 1980; Lorenzen et al., 1981) or *immature male rats* (Nandini et al., 1976; Davies et al., 1976; Daume et al., 1978; Peek & Watkins, 1979; Rush & Lipner, 1979; Hermans et al., 1980) following injection of inhibin-containing material. Although 15-day-old rats show the largest post-castration rise in FSH, thus giving the widest range for observing an FSH suppressing effect (Hermans et al., 1980 and Fig. 3, pp 29), the approximately 35-day-old castrated male rat is generally accepted as the better model: these animals have high basal levels of FSH, they show a good response in terms of an FSH rise after castration and a good response to the administration of inhibin within 3-8 h after injection.

Apart from practical reasons, none of the models described above is clearly preferable. We showed that the relative response of peripheral levels of FSH to varying amounts of inhibin is comparable in *15-day-old and 75-day-old castrated male rats* (Fig. 1). FSH is maximally suppressed to concentrations between 60 and 75 % of values found in control animals. Furthermore, when *intact and 48 h castra-*

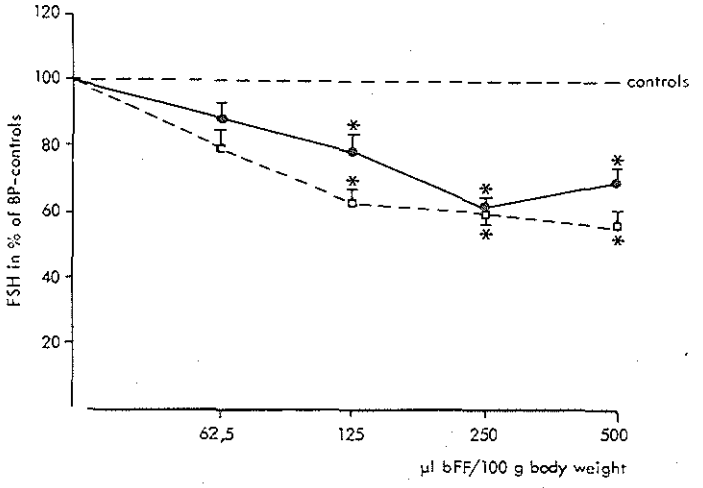


Figure 1. *Effect of a single i.p. injection at 09.00 h of various amounts of charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. on serum concentrations of FSH (mean ± S.E.M., n = 4-6) in 15-day-old (●—●) and 75-day-old (□--□) male rats, castrated 48 h earlier. Bovine plasma (BP) served as a control fluid. Rats were bled after 8 h by puncture of the ophthalmic venous plexus. Concentrations of FSH are expressed as percentages of mean values found in control animals. Control concentrations of FSH in 15-day-old rats were 2076 ± 93, and in 75-day-old rats 931 ± 96 ng NIAMDD-rat-FSH RP-1 / ml. Levels of LH were not influenced.*
Data of de Jong et al. (1978).
* *P ≤ 0.05 versus control animals (Wilcoxon, two-tailed test).*

ted male rats of 15 to 75 days of age were injected with a comparable amount of material containing inhibin (0.50 ml charcoal-treated bovine follicular fluid (bFF) / 100 g body wt.),a similar suppression in levels of FSH was obtained, both with respect to the magnitude of the suppression reached (to maximally 45-60 % of values found in control animals) as to the time at which the suppression of FSH could be detected (Fig. 2). No difference was found between the relative response in intact rats and rats castrated 48 h previously. These results indicate that the sensitivity of peripheral levels of FSH to exogenous inhibin does not change with age in male rats.

The *prevention of the immediate post-castration rise in FSH in male rats* of different ages by inhibin (to be distinguished from the just discussed suppression of (increased) levels of FSH in intact and castrated animals), is shown in Fig. 3. The largest post-castration rise in levels of FSH was found in 15-day-old rats: it

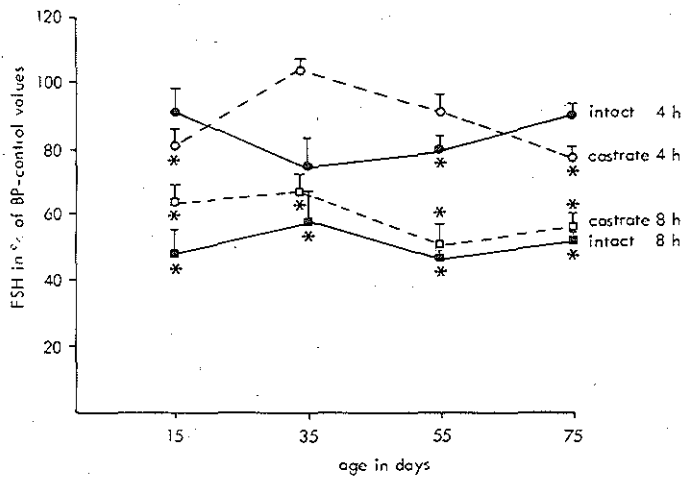


Figure 2. *Effect of age and presence or absence of the testicles in male rats of various ages on the response of FSH (mean ± S.E.M., n = 8–21) to a single i.p. injection at 09.00 h of 0.50 ml charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. Bovine plasma (BP) served as a control fluid. Rats were intact (■—■, ●—●) or castrated 48 h earlier (□---□, ○---○) and bled at 4 (●, ○) and/or at 8 h (■, □) after injection. Concentrations of FSH are expressed as percentages of mean values found in control animals. Levels of LH were not influenced. Absolute serum concentrations of FSH and LH are shown in Hermans et al. (1980), Appendix IV.*
Part of the data were presented in this form by de Jong et al. (1978).
* *P ≤ 0.05 versus control animals (Wilcoxon, two-tailed test).*

decreased steadily with age. At all ages injection of charcoal-treated bFF completely prevented the post-castration rise in levels of FSH; the influence on levels of FSH was largest in 15- and 35-day-old rats. At these ages FSH concentrations reached values between 30–40 % of values seen in otherwise untreated castrated rats. This is a stronger suppression than the amount of suppression reached 8 h after injection of bFF in intact rats or in rats which were castrated 48 h previously (Fig. 2).

Female animals

Also female animals have been used extensively in models for the detection of inhibin. In all cases, animals are bled within 3–24 h after the last injection of the preparation supposedly containing inhibin. *Oestrogenized mice, ovariectomized for*

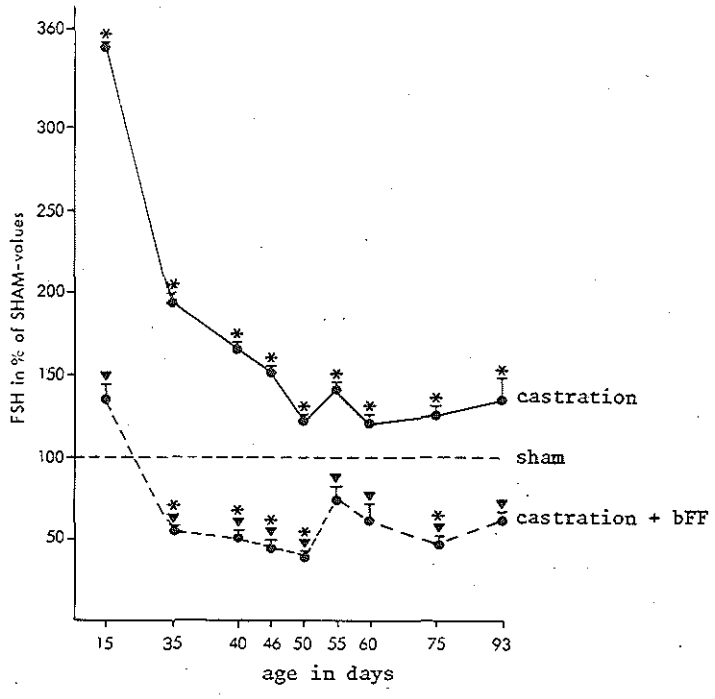


Figure 3. *Effect of age on the response of FSH (mean ± S.E.M., n = 4-24) to castration, and the influence of a single i.p. injection of 0.50 ml charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. upon this response. Male rats of various ages were either sham-operated or bilaterally castrated at 09.00 h, or castrated and given immediately afterwards an injection. Rats were bled 6 h after operation and/or injection. Concentrations of FSH are expressed as percentages of mean values found in sham-operated controls. Absolute serum concentrations of FSH and LH, on which these data are based, are shown in Hermans et al. (1980), Appendix IV.*
* *$P \leq 0.05$ versus sham-operated control rats (Wilcoxon, two-tailed test).*
▼ *$P \leq 0.05$ versus rats castrated only (Wilcoxon, two-tailed test).*

7 days (Bronson & Channing, 1978), as well as *long-term ovariectomized rhesus monkeys* (Chappel et al., 1980), *hamsters* (Chappel, 1979) and *mares* (Miller et al., 1979) have been used. The use of large animals however is impractical: large quantities of materials to be tested and manyfold injections are needed to obtain a suppression of FSH, which makes quantification of inhibin impossible (see also Baker et al., 1981). The most commonly used animal in the bioassays for inhibin is again the rat.

Adult cyclic female rats have been used at all days of the cycle (Marder et

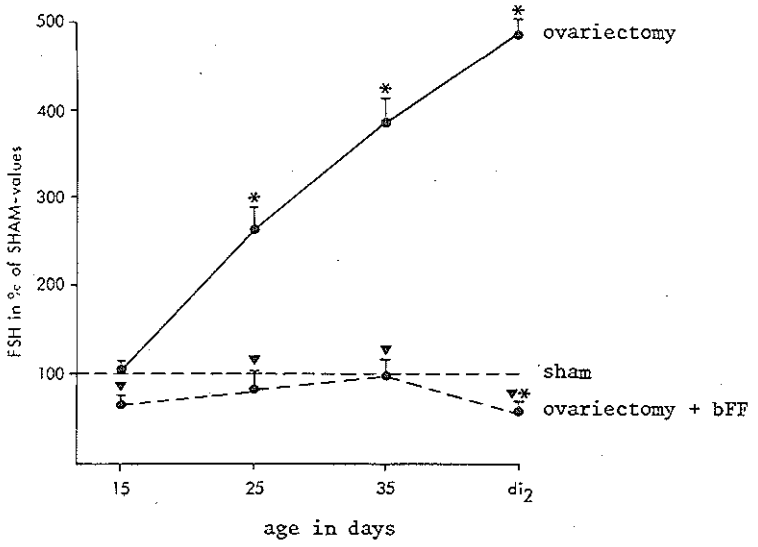


Figure 4. *Effect of age on the response of FSH (mean ± S.E.M., n = 5-18) to ovariectomy and the influence of a single i.p. injection of 0.50 ml charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. upon this response. Female rats of various ages were either sham-operated or bilaterally ovariectomized at 09.00 h, or ovariectomized and given immediately afterwards an injection. Rats were bled 8 h after operation and/or injection. Concentrations of FSH are expressed as percentages of mean values found in sham-operated control animals. Absolute serum concentrations of FSH and LH, on which these data are based, are shown in Hermans et al. (1980), Appendix IV.*

* *$P \leq 0.05$ versus sham-operated control rats (Wilcoxon, two-tailed test).*

▼ *$P \leq 0.05$ versus rats ovariectomized only (Wilcoxon, two-tailed test).*

al., 1977; Schwartz & Channing, 1977; Hoffman et al., 1979; DePaolo et al., 1979 a; Hermans et al., 1981 a; 1982 a). Experience shows that extreme care must be taken with regard to the times at which the animals are bled; the initial suppression of FSH seen after injection of inhibin decreases with time, while increased levels of FSH are found after the initial period of suppression (DePaolo et al., 1979 b; Hermans et al., 1981 a; 1982 a). This phenomenon is most pronounced at the di-oestrous days of the cycle. A suitable model, but with an even more limited time-span in which suppression of FSH can be seen, is the *acutely unilaterally ovariectomized* dioestrous rat (Welschen et al., 1977). Also *acutely bilaterally ovariectomized rats* can be used, since inhibin administered immediately after ovariectomy prevents the post-castration rise in FSH completely (Fig. 4). However, since the pituitary response to castration varies with the day of the cycle, the use of unilaterally

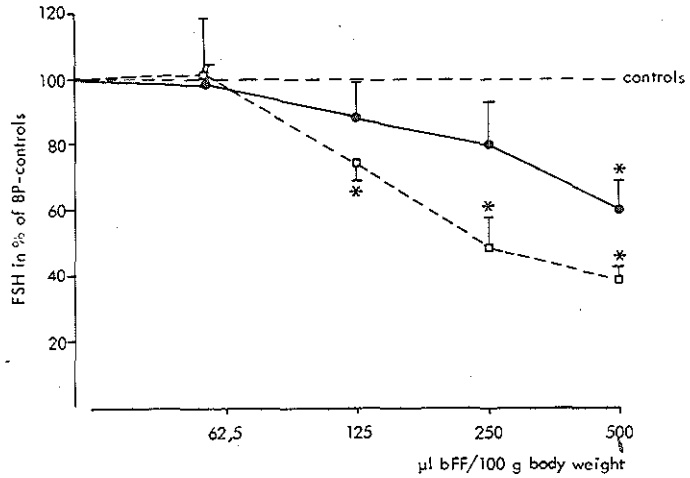


Figure 5. *Effect of a single i.p. injection at 09.00 h of various amounts of charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. on serum concentrations of FSH (mean ± S.E.M., n = 4-6) in 15-day-old (●——●) and adult (□---□) female rats, bilaterally ovariectomized 48 h earlier. Bovine plasma (BP) served as a control fluid. Rats were bled after 8 h by puncture of the ophthalmic venous plexus. Concentrations of FSH are expressed as percentages of mean values found in control animals. Control concentrations of FSH in 15-day-old rats were 2472 ± 173, and in adult rats 697 ± 67 ng NIAMDD-rat-FSH RP-1 / ml. Levels of LH were not influenced.*
Data of de Jong et al. (1978).
* *P ≤ 0.05 versus control animals (Wilcoxon, two-tailed test).*

or bilaterally castrated rats is not practical (Marder et al., 1977; Hermans et al., 1980). The models using rats *castrated for 2 days* (Hermans et al., 1980; 1981 a), *for 7 days* (Campbell & Schwartz, 1979) and *for 12 or 14 days* (Welschen et al., 1977; Hopkinson et al., 1977) are not influenced by the day of the cycle or by the occurrence of a rebound in the secretion of FSH after an initial suppression. These models have the additional advantage that initial levels of FSH are high, thus yielding a wide range for observing a decrease in levels of FSH.

Immature female rats may also be used in a model (Hermans et al., 1980), but again care must be taken in the exact timing of blood collections since the suppressive effect of inhibin is seen for an even shorter period than in adult rats and a rebound in levels of FSH may occur in intact immature rats after the initial suppression (Hermans et al., 1981 a). Furthermore, the immature rat is a less favour-

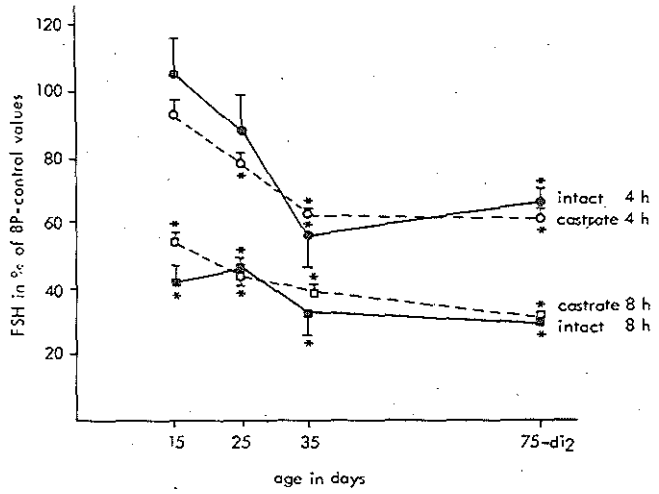


Figure 6. *Effect of age and presence or absence of the ovaries in female rats of various ages on the response of FSH (mean ± S.E.M.; n= 4-18) to a single i.p. injection at 09.00 h of 0.50 ml charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. Bovine plasma (BP) served as a control fluid. Rats were intact (●—● , ■—■) or castrated 48 h earlier (○---○ , □---□) and bled at 4 (● , ○) and/or 8 (■ , □) h after injection. Concentrations of FSH are expressed as percentages of mean values found in control animals. Adult female rats were approximately 75 days of age and in dioestrus-2 (di_2) of the cycle. Absolute serum concentrations of FSH and LH, on which these data are based, are shown in Hermans et al. (1980), Appendix IV.*

Data of de Jong et al. (1978).

* *$P \leq 0.05$ versus control animals (Wilcoxon, two-tailed test)*

able animal in a bioassay for inhibin since the pituitary response to inhibin appears to increase with age: to obtain a certain suppression of levels of FSH a higher dose of active material is needed than in adult rats (Fig. 5), while with a comparable amount of activity injected, rats of 35 days of age or older both show a faster and a larger suppression of FSH secretion than younger animals, regardless whether the animals are intact or ovariectomized two days earlier (Fig. 6).

In conclusion, in vivo methods for the detection of inhibin should preferentially use intact or ovariectomized adult female rats. For routine monitoring of different batches of follicular fluid or of purified fractions of bFF we prefer the 48 h or 12 day ovariectomized adult female rat, since in these animals, due to the high control levels of FSH, a wide range for observing a suppressive effect is pre-

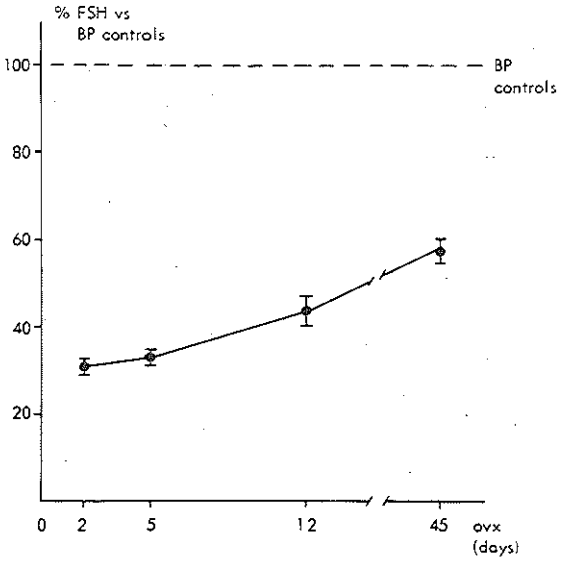


Figure 7. *Effect of time after ovariectomy on the response of FSH (mean ± S.E.M.) (n=6) to a single i.p. injection of 0.50 ml charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. Adult female rats were ovariectomized (ovx) for various hours or days before injection. Bovine plasma (BP) served as a control fluid. Rats were bled 8 h after injection by puncture of the ophthalmic venous plexus. Serum concentrations of FSH are expressed as percentages of mean values found in control animals; levels of LH were not influenced.*
Data of de Jong et al. (1979 b).

sent, since a large suppression of FSH can be reached (to concentrations between 30–45 % of control values), and since the suppression is independent of the stage of the cycle. When animals ovariectomized for a still longer period are used, the response of the pituitary gland to inhibin is decreasing with increasing time after ovariectomy (Fig. 7).

Summarizing, in vivo methods using both male and female animals, especially rats, are suitable for the detection of inhibin. However, even under the most favourable conditions large amounts of active material have to be administered, thus making these bioassays less suitable for the detection and quantification of small amounts of inhibin, and for monitoring steps in the purification of inhibin from crude preparations.

More sensitive bioassays have been developed using in vitro techniques.

2.2.3. "Specific" in vitro methods

In vitro methods used for the estimation of inhibin are based on the changes in concentrations of FSH and LH released into the culture medium by dispersed pituitary cells after addition of material containing inhibin, similar changes in intracellular concentrations of FSH and LH in the dispersed pituitary cells, or changes in the release of FSH and LH into the medium by incubated halved pituitary glands after addition of inhibin.

The assay using *incubation of halved pituitary glands from 35-day-old male rats* is fast and simple to perform (Davies et al., 1976; 1978), but is reported to be less sensitive, precise and reproducible than the assay using dispersed pituitary cells (Franchimont et al., 1979; Hudson et al., 1979). The spontaneous release of FSH from halved pituitaries during the incubation period is only slightly reduced after addition of material containing inhibin (Davies et al., 1978). A larger suppression of release of FSH could only be obtained when FSH and LH secretion was first stimulated by addition of LHRH to the halved pituitary glands (Davies et al., 1978), but in that situation a suppression of concentrations of LH also occurred when high doses of material containing inhibin-like activity were added (Davies et al., 1978). These results are in contrast with those of A. A. J. Jenner (personal communication), who obtained a selective decrease in the release of FSH after addition of charcoal-treated bFF. Also incubation with LHRH of whole pituitaries of 34-day-old mice with inhibin from bull seminal plasma showed a selective decrease of levels of FSH (Sairam, 1981), but in this study levels of FSH and LH were measured by radioreceptorassay and not by radioimmunoassay as employed in the other cited studies.

The bioassays most widely used at present are based on the suppression of the *spontaneous or LHRH-stimulated release of FSH from dispersed pituitary cells*. Pituitary cells are obtained from adult male rats (Steinberger & Steinberger, 1976; Chowdhury et al., 1978; Labrie et al., 1978; de Jong et al., 1978; 1979 a; Eddie et al., 1978; 1979; Lefevre et al., 1980; Scott et al., 1980), from adult female rats (Labrie et al., 1978; Erickson & Hsueh, 1978; Shander et al., 1980 a; Hermans et al., 1982 b), or from adult female hamsters (Chappel et al., 1980). Cells are dispersed by means of proteolytic enzymes, plated in the presence of foetal calf serum in a standardized medium, and pre-incubated during 2-3 days in order to obtain cells which are attached to the dish and which give a steady release of FSH and LH into the medium. Sometimes progesterone (1×10^{-7}M) and oestradiol (1×10^{-8}M) are added to stimulate gonadotrophin release (Shander et al., 1980 a). After the pre-incubation period the medium is renewed, and the preparations to be tested for the presence of inhibin are added for an additional 24 h (Shander et al., 1980 a) or 3 day culture period. Some authors then again renew medium, and add small amounts

(50 ng/ml) of LHRH together with the preparation to be tested for an additional 6 h culture period. Finally the media are collected and gonadotrophin concentrations in the media are estimated by radioimmunoassay.

When the spontaneous release of FSH and LH is measured, addition of inhibin causes a dose-related suppression of concentrations of FSH; concentrations of LH are not affected, except when very high doses of some preparations are added: levels of LH are reported to be increased by high doses of charcoal-treated bFF and Sertoli cell conditioned medium (de Jong et al., 1979 a) or decreased by rat granulosa cell conditioned medium (Erickson & Hsueh, 1978). When the LHRH stimulated release of FSH and LH is measured, invariably concentrations of both gonadotrophins are suppressed in a dose-related manner after addition of inhibin, but higher doses of material are needed to suppress levels of LH than to suppress levels of FSH. Furthermore, the regression lines of the suppression of FSH and LH are not parallel in that situation (Labrie et al., 1978; Baker et al., 1978; Eddie et al., 1978; 1979; de Jong et al., 1979 a; Franchimont et al., 1979). Recently, Scott et al. (1980) demonstrated the applicability of a modified form of the in vitro assay using dispersed pituitary cells: comparable results were obtained when instead of FSH and LH in the culture medium the cellular contents of FSH and LH in the pituitary cells were measured.

With the bioassay using dispersed pituitary cells very low amounts of inhibin can be detected (the assay is a 1000-fold more sensitive than the most sensitive in vivo assay: de Jong et al., 1979 b). Moreover, the amount of inhibin can be measured quantitatively. These assays are relatively simple to perform, but take quite some time before the results are known. Furthermore, the specificity of these assays needs to be regarded with caution, especially when LHRH stimulation is used. The assays might be influenced by factors present in foetal calf serum (de Jong et al., 1979 a), while addition of NaCl can cause a suppression of the secretion of FSH (Kao et al., 1977). Follicular fluid, apart from containing inhibin, contains a factor which can suppress the LHRH stimulated release of both FSH and LH, while this factor does not affect the unstimulated release of FSH and LH (de Jong et al., 1979 b). The presence in preparations containing inhibin of a "stimulin", capable of stimulating the release of LH, has also been reported (de Jong et al., 1979 a). Furthermore, material produced by cultured granulosa cells contains, besides inhibin (Erickson & Hsueh, 1978; Hermans et al., 1982 b), a low molecular weight factor (Mw < 3500 D), "gonadocrinin ", which can stimulate the unstimulated and the LHRH stimulated release of FSH and LH from dispersed pituitary cells of immature female rats (Ying et al., 1981), and may thus interfere in these assays.

On the other hand, a number of observations plead in favour of the specificity of these in vitro bioassays for the detection of inhibin. The preparations containing inhibin apparently do not exert non-specific actions affecting the viability

of the pituitary cells, as judged by the reversibility of the FSH suppression (Eddie et al., 1979; Shander et al., 1980 a). In these assays the release of prolactin (Franchimont et al., 1979), of TSH following administration of thyreotropic releasing hormone (Eddie et al., 1978) or of growth hormone (Franchimont et al., 1979; Scott et al., 1980) is not affected, thus demonstrating the specificity of the tests for the relationship between suppression of FSH secretion and addition of inhibin. After addition of inhibin to the pituitary cells the FSH or LH molecules themselves (de Jong et al., 1979 a), or LHRH (Eddie et al., 1979) are not broken down. Furthermore, the observed suppressive action of inhibin cannot be attributed to high concentrations of FSH, LH or LHRH in the crude preparations (de Jong et al., 1979 a). The suppressive action can also not be mimicked by another gonadal protein hormone, relaxin (de Jong et al., 1979 b). The effect of steroids on the secretion of FSH and/or LH in these assays are variable: oestradiol has no (Eddie et al., 1978; 1979) or a stimulatory effect (Labrie et al., 1978; de Jong et al., 1979 a; DePaolo et al., 1979 c). Progesterone and testosterone either stimulate (Labrie et al., 1978; de Jong et al., 1979 a; DePaolo et al., 1979 c) or suppress the unstimulated release of FSH and LH (Eddie et al., 1978). In the latter case, levels of LH are suppressed to a larger extent than levels of FSH, as was also found after the addition of testosterone, dihydrotestosterone and 5α-androstenediol-3α-17β diol (Eddie et al., 1979).

Thus these in vitro assays can be useful for the estimation of (semi)purified inhibin in gonadal fluids, but due to the uncertainties regarding the specificity of these assays, it appears to be better to combine an in vitro test, preferably using the unstimulated release of FSH and LH from dispersed pituitary cells in culture, occasionally with a specific (but less practical) in vivo test (de Jong, 1979; Franchimont et al., 1979). Only such a combination can offer reliable information as to whether one is really dealing with inhibin and/or with an artifact due to the bioassay chosen.

With the in vivo methods it has not been possible to detect inhibin in peripheral plasma of test animals or men; with the assay using dispersed pituitary cells, Lee et al. (1981) detected inhibin in peripheral plasma of 25-day-old rats pretreated with pregnant mare serum gonadotrophins. However, for large scale measurements of peripheral concentrations of inhibin one needs more sensitive and specific methods, such as radioimmunoassays or radioreceptor assays.

2.2.4. Radioligand assays

Radioligand assays are highly dependent on the specificity of the antisera raised against inhibin and on the purity of the inhibin preparation used for labelling. The radioligand assays developed until now for inhibin might be regarded as premature,

which is mainly due to a lack of a pure, internationally accepted standard preparation of inhibin and insufficient assay specifications (see: Franchimont et al., 1979). Also no comparisons have been made for potencies of preparations found in acceptable bioassays with the ones found in radioligand assays. Finally, it is not sufficient to test the specificity of antisera against their own antigens only or to rely on neutralisation of endogenous inhibin in rats by measuring radioimmunoassayable levels of FSH and LH, since antisera raised in rabbits can interfere with such radioimmunoassays.

In a preliminary report Sairam (1981) described a *radioreceptor assay* for inhibin from bull seminal plasma: using binding to pituitary membranes a purified fraction was obtained: this fraction was labelled with ^{125}J and used as a ligand in an assay based on binding of active principles to pituitary membranes. The purified ligand could be displaced from binding in a log-dose relationship by inhibin from bull and human seminal plasma and by bFF.

Also *radioimmunoassays* for inhibin are being developed. Franchimont et al. (1979) reported an assay using as tracer and standard purified high molecular weight inhibin obtained from ovine rete testis fluid, in combination with an antiserum (rabbit) to a semipurified fraction of inhibin from ovine rete testis fluid. Parallel inhibition curves with a much lower immunological potency were found for inhibin fractions of bovine follicular fluid, testicular extracts and human seminal plasma, but no further assay characteristics were (yet) reported. Vaze et al. (1979) reported an assay using inhibin of high molecular weight purified from human seminal plasma as tracer and standard, and an antiserum to this fraction raised in the rabbit. With this assay inhibin was shown to be absent or present in low amounts in preparations of human testes, epidydimus and in seminal vesicles, and in serum. Inhibin was found with this assay in high amounts in rat serum, while the highest levels were found in extracts of human prostates. These results cast serious doubts on the gonadal nature of the protein purified, and on the specificity for inhibin of the antiserum raised. Finally, Sairam (1981) presented a preliminary report on an assay using purified inhibin from bull seminal plasma and an antiserum from rabbits against a partially purified fraction thereof. Parallel displacement curves were found for bovine inhibin preparations, but not for porcine inhibin preparations. The specifications of the assay method have not been reported yet.

2.3. Presence of inhibin in a variety of gonadal fluids

The search for the presence of inhibin in various gonadal fluids and gonadal compartments has been hampered by the fact that the assays used are not always specific (see preceding paragraph). However, it is now generally accepted that inhibin can be found in both testicular and ovarian preparations. Since even impure prepa-

rations of ovarian as well as of testicular origin, supposedly containing inhibin, give rise to a comparable suppression of FSH in the various bioassays (Baker et al., 1978; de Jong, 1979), it is unnecessary to call ovarian inhibin-like activity "folliculostatin", the name initially used by Schwartz & Channing (1977) or inhibin-f. Other names used for inhibin have been Sertoli-cell-factor (Steinberger & Steinberger, 1976), gonadostatin (Ying & Guillemin, 1979) or, more recently, follibin (Sairam, 1981).

The various sources of material containing inhibin of male and female origin are summarized in Table 2 (male origin) and Table 3 (female origin) (pp 40-45), together with the detection methods used and, if known, the apparent molecular weights and chemical, physical and physiological characteristics of the biologically active principle.

As shown in the tables, inhibin has been found in gonadal preparations from most laboratory animals, common domestic animals and in men. The results show that the amount of inhibin can be related in female animals to the stage of the cycle (Chappel, 1979; Chappel et al., 1980), to the size of the ovarian follicles (Welschen et al., 1977; Lorenzen et al., 1978) and to the state of the follicles (cystic, atretic) (Daume et al., 1978; Channing et al., 1981 a). In male animals the amount of inhibin can be related to the number of spermatozoa present in seminal plasma (Scott & Burger, 1981). These observations seem reliable since they are based on bioassays using dispersed pituitary cells or on 2 or 3 point in vivo bioassays. Less reliable information about quantities of inhibin are found when one-point bioassays are performed, such as is the case in the studies of DePaolo et al. (1979 c) about inhibin in ovarian venous plasma of rats, of Erickson & Hsueh (1978) about inhibin in rat granulosa cell culture medium, and of Channing et al. (1981 b) about inhibin in human follicular fluid which was collected by aspiration during the cycle.

In summary, the cited studies suggest that a non-steroidal factor with a short-lasting action on FSH secretion is produced in variable quantities during gametogenesis. Indirect studies have been employed to make a physiological role for this factor plausible (see Chapter 3), but only accurate measurement of serum levels of inhibin together with depletion studies eliminating endogenous inhibin by means of antisera could give definite answers. This, however, will have to await purification of inhibin.

2.4. Purification of inhibin

Inhibin appears to be a protein: the activity can be destroyed using proteolytic enzymes such as trypsin, pepsin, papain and pronase (for references: de Jong et al., 1981). Also heat treatment in excess of 60°C destroyed the activity of inhibin

(for references: de Jong et al., 1981). Using bFF as a source for inhibin, Jansen et al. (1981) showed, by means of serial chromatography of bFF on various affinity matrices, that inhibin could be associated with a hydrophobic glycoprotein. Also the destruction of inhibin after neuraminidase treatment suggests that inhibin contains sugar residues (Setchell et al., 1977). The iso-electric point of inhibin from bovine and porcine follicular fluid is probably between pH 5.5 - 5.7 (Williams et al., 1979; Jansen et al., 1981).

The data on the molecular weight of the active principle are confusing: estimated molecular weights range from 1500 Daltons to larger than 100.000 Daltons (see Tables 2 and 3, and reviews by Franchimont et al., 1979; de Jong, 1979; Blanc, 1980; de Jong et al., 1981). Most authors, however, report molecular weights around 20.000 Daltons. The differences could be due to the use of different sources of material containing inhibin, the variety of bioassays used, and the estimation of molecular weight in only partially purified fractions containing inhibin. Also the existence of polymer forms of inhibin or a coupling of the active principle to carrier proteins could account for the observed diversity in molecular weights. Finally, Channing & Franchimont (1981) suggested that the variability could be due to proteolysis of inhibin occurring during purification with conventional methods. A better approach might be the use of purification procedures as employed by Jansen et al. (1981) and de Jong et al. (1982 a; 1982 b): i.e. serial chromatography on affinity matrices and the use of columns of immobilized antibodies against bovine plasma proteins or against a partially purified fraction of inhibin from bFF. Such procedures might lead to the - long awaited - progress in the elucidation of the physicochemical characteristics of inhibin.

TABLE 2. SOURCES OF INHIBIN IN MALE ANIMALS AND THE METHODS USED FOR DETECTION

(numbers in brackets correspond with numbers in the list of references at the bottom of tabel 3)

SOURCE AND SPECIES	METHODS USED	REMARKS AND REFERENCES
WHOLE TESTIS		
homogenate of ligated rat testis (1) medium of cultured mouse testis (2) crude testicular extracts of ram (3, 4, 5, 6) and bull (4, 7, 8, 9)	- 34, 35-day-old male rats, acute OTX (1, 3, 7) - adult male rats, 2 weeks OTX (4, 6, 8) - infusion into castrated rams (7) - HCG-induced uterine weight increase in 27-day-old female mice and rats (5)	- serum levels of FSH were lowered within 30 min (4), 3-6 h (1, 3, 6) or 12-24 h (7) after start of treatment - effects on levels of LH were variable (2, 7) or a decrease with higher doses (9) - the factor inhibited the uterine weight increase (5) - the factor was only produced when the temperature was below 37°C and when only Sertoli cells were left in culture (2) - reported molecular weights: smaller than 1500 D (6) and 10.000-70.000 D (7)
EPIDYDIMUS		
extract of rat epididymus (10, 60)	- adult male rats, 3 weeks OTX (10)	- serum levels of FSH were lowered (10) - when hypothalamic extracts of treated male rats were injected into 3 weeks OVX female rats, the increase of FSH was abolished (60)
SPERMATOZOA		
extract of bull spermatozoa (11, 12)	- parabiotic pairs of immature female - mature female rats (11) - adult male rats, 3 weeks OTX, intraventricular injection (12)	- factor inhibited ovarian weight increase of immature female rats (11) - after intraventricular injection serum FSH was lowered within 30 min. Injection of hypothalamic extracts of treated male rats to 4 weeks OVX adult female rats abolished increase of FSH (12)

spermatid enriched fraction of rat (62)	- adult male rats, 2 weeks OTX (62) - inhibin radioimmunoassay with antiserum against human seminal plasma inhibin (62)	- spermatid enriched fraction contains inhibin, both in the bioassay as well as in the immunoassay (62)
SERTOLI CELLS		
in culture, of male rats of various ages: 31-36 days old (24) 35 days old (26) 20-26 days old (27) 29-33 days old (25) 18-91 days old (61)	- pituitary cell culture (24) - pituitary cell culture with or without LHRH (26, 27) - pituitary cell culture, incorporation of ^{3}H-leucine into FSH and LH (25)	- activity decreased the unstimulated release of FSH (24, 26, 27) and in the stimulated system both the release of FSH and LH (26, 27) - it was shown that the activity decreased the synthesis of FSH in the pituitary cells (25, 27) - most activity secreted from days 5-8 in culture compared with days 2-5 (26), or most activity before day 7 in culture (24). After 21 days in culture still secretion of inhibin (61) - with higher doses of spent media the release of LH was increased (27) - reported molecular weight : larger than 10.000 D (26) - no activity with isolated germ cells (61)
SEMINIFEROUS TUBULES		
in culture, of adult rats (23)	- pituitary cell culture, with or without LHRH (23)	- the activity decreased the unstimulated release of FSH, and in the stimulated system the release of both FSH and LH (23) - reported molecular weight: 10.000 - 30. 000 D (23)
RETE TESTIS FLUID		
of rams (1, 7, 8, 13, 14, 15, 16, 17, 18, 19, 20, 21) of boars (22)	- HCG-induced uterine weight increase in 21-day-old mice (22) - adult male rats, OTX for 24 h (18) or for 3 weeks (13) or intact (13) - 35-day-old male rats, acute OTX (14) - infusion into OTX rams (7, 16) - injection into cryptorchid rams (15)	- factor inhibited uterine weight increase (22) and uptake of thymidine into DNA (17) - serum FSH was decreased (7, 13, 16) within 2-2.5 h (14, 15, 18) after injection - in vitro the factor decreased the cellular content of FSH (21), the unstimulated release of FSH (21) and the stimulated release of FSH (8, 15, 19, 20, 21) - a decrease in levels of LH has been reported (1, 8, 15, 19, 20)

(continued at page 42)

(table 2, continued)

SOURCE AND SPECIES	METHODS USED	REMARKS AND REFERENCES
(rete testis fluid)	- pituitary cell culture, with (21) or without (8, 19, 20) LHRH - incubation of demi-pituitaries (1) - uptake of ^{3}H-thymidine by fragmented ram testicles (17)	- the amount of activity could be related to the amount of sperm in the fluid (14) - the factor was not identical with androgen binding protein (8) - reported molecular weights: smaller than 5000 D (1, 17, 18, 19); larger than 10.000 D (17, 18); between 10.000-20.000 D (7, 16); 20.000 D (14); 15.000-30.000 D (1); 80.000-100.000 D (1, 14)
TESTICULAR LYMPHE		
of rams (16, 20, 21, 28)	- pituitary cell culture with (16, 20, 21) or without LHRH (16, 20, 21, 28)	- factor decreased the unstimulated release of FSH (28) and the stimulated release of FSH and LH (16, 20, 21) - the suppression was in parallel with the suppression obtained after addition of medium of cultured Sertoli cells (28)
SEMINAL PLASMA		
of bulls (1, 29, 30, 31, 32)	- adult male rats, OTX for 24 h (18), 2 weeks (29, 33), 3 weeks (31, 32) or long term (34)	- reported were a decrease in levels of FSH (30, 33, 34, 35), of serum levels of FSH within 2 h (18) or 4-25 h (31, 32), or a decrease in levels of both FSH and LH (1, 19, 31, 32)
of humans (18, 19, 33, 34, 35)	- 35, 36-day-old male rats, acute OTX (30, 31, 32) - HCG-induced ovarian weight increase in immature female rats (30) and mice (34) - pituitary cell culture, with (19, 35) or without (35) LHRH - incubation of demi-pituitaries with LHRH (1)	- the suppression was parallel with the suppression found after addition of ovine testicular lymphe (35) - the activity inhibited the ovarian weight increase (30, 34) - the activity was not identical with androgen binding protein (29) - the activity was also present in seminal plasma of vasectomized bulls (31, 32) - reported molecular weights: smaller than 5000 D (18, 19), larger than 10.000 D (18), 19.000 D (30, 34)

For references: see below table 3
For explanation of abbreviations used: see List of Abbreviations, page 10

TABLE 3. SOURCES OF INHIBIN IN FEMALE ANIMALS AND THE METHODS USED FOR DETECTION

(numbers in brackets correspond with numbers in the list of references at the bottom of this table)

SOURCE AND SPECIES	METHODS USED	REMARKS AND REFERENCES
GRANULOSA CELLS		
in culture, of female rats (36, 38) of cows (37)	- pituitary cell culture, without LHRH (36, 37, 38)	- addition of spent media caused a decrease in the release of FSH (36, 37, 38) - addition of high doses caused a decrease in the release of LH (36) - the decrease in FSH was dependent on the amount of granulosa cells in culture (36, 38) - the amount of inhibin was dependent on the number of days the cells had been in culture (38) - the decrease in levels of FSH was parallel with the decrease found after addition of standard (bovine follicular fluid) (38) - cells produced radiolabelled proteins with inhibin activity after addition of labelled methionine and fucose (37)
OVARIAN VENOUS PLASMA		
of cyclic rats (39)	- pituitary cell culture without LHRH (39)	- the activity decreased levels of FSH (39) - no activity was found in plasma collected at pro-oestrus (39)
OVARIAN EXTRACTS		
of hamsters (40)	- adult female hamsters, 4 weeks OVX (40)	- serum levels of FSH were decreased within 2-4 h (40) or within 12 h (52) after administration
of cows (52)	- adult male and female rats, 2 weeks OTX or OVX (52)	- activity was only found in extracts of ovaries collected at pro-oestrus (40)

(continued at page 44)

(table 3, continued)

SOURCE AND SPECIES	METHODS USED	REMARKS AND REFERENCES
FOLLICULAR FLUID		
of the pig (9, 20, 41, 42, 43, 44, 45, 46, 47)	- adult female rats, intact at metoestrus (41) or at pro-oestrus (42, 44) - adult female rats, acute ULO at dioestrus-2 (43, 50) or acute OVX at metoestrus (41, 44) - adult female rats (46) or mice (45), OVX for 7 days - mares, OVX for 1 year (51, 53) - adult female rats, OVX for 12 days (36, 43, 54, 57) - 34-day-old acute OTX male rats (9), or adult, 24 h OTX male rats (48) - infusion into long-term OVX rhesus monkeys (55) - cycle length in intact cows and ewes (51) - incubation of demi-pituitaries with or without LHRH (59) - pituitary cell culture, with (20, 27, 56) or without (21, 27, 37, 55, 58) LHRH - inhibition of binding of labelled FSH to granulosa cells (47)	- addition of fluid decreased concentrations of FSH (9, 27, 42, 48, 55, 56, 59) or cellular concentration of FSH (21) - concentrations of LH were also decreased (20) or the effects on levels of LH were variable (51, 53) - serum levels of FSH were decreased within 4 h (43, 45), 5.5 h (41), from 3.5-16 h (44), from 5.5-14.5 h (46), from 6-48 h (51, 53) and within 2 h (54, 57) after administration - the suppression of FSH was dose-dependent (9, 27, 42, 43, 46, 50), and in parallel with the suppression obtained after addition of spent media from cultured Sertoli cells (27) - addition of larger doses caused a longer lasting suppression of serum levels of FSH (46); administration of oestradiol-17 β and follicular fluid suppressed levels of FSH further than administration of follicular fluid alone (53) - ovarian weight increase was abolished (49, 54, 57), and cycle length was increased (51) - binding of labelled FSH to granulosa cells was reduced (47) - reported molecular weights: larger than 10.000 D (41), 23.000 D (57) and 65.000 D (58) - most activity was found in fluid collected from midcycle follicles (56), from follicles in the late follicular and luteal phase of the cycle (55), from small and medium (41), or from large follicles (50); few activity was found in cystic follicles (43, 54) - follicular fluid contains more activity than ovine testicular lymphe (20)
of the cow (27, 43, 48, 49, 50, 51, 58, 59)		
of the horse (51, 53)		
of the human (21, 54, 55, 56, 57)		

For references see next page
For explanation of abbreviations used: see List of Abbreviations, page 10

REFERENCES OF TABLES 2 AND 3

(1) Davies et al., 1978; (2) Demoulin et al., 1980; (3) Nandini et al., 1976; (4) Becker et al., 1977; (5) Ramasharma et al., 1979; (6) Vijayalakshmi et al., 1980; (7) Baker et al., 1976; (8) Lee et al., 1976; (9) Rush & Lipner, 1979; (10) Le Lannou & Chambon, 1977 a; (11) Lugaro et al., 1973; (12) Lugaro et al., 1974; (13) Setchell & Jacks, 1974; (14) Davies et al., 1976; (15) Blanc et al., 1978; (16) Baker et al., 1978; (17) Croze et al., 1980; (18) Franchimont et al., 1978; (19) Franchimont et al., 1979; (20) Eddie et al., 1979; (21) Scott et al., 1980; (22) Setchell & Sirinathsinghhii, 1972; (23) Eddie et al., 1978; (24) Steinberger & Steinberger, 1976; (25) Chowdhury et al., 1978; (26) Labrie et al., 1978; (27) de Jong et al., 1979 a; (28) Franchimont et al., 1980; (29) Franchimont et al., 1975; (30) Chari et al., 1978; (31) Peek & Watkins, 1979; (32) Peek & Watkins, 1980; (33) Franchimont et al., 1972; (34) Thakur et al., 1978; (35) Scott & Burger, 1981; (36) Erickson & Hsueh, 1978; (37) de Jong et al., 1982 b; (38) Hermans et al., 1982 b; (39) DePaolo et al., 1979 c; (40) Chappel, 1979; (41) Marder et al., 1977; (42) Schwartz & Channing, 1977; (43) Welschen et al., 1977; (44) Hoffmann et al., 1979; (45) Bronson & Channing, 1978; (46) Campbell & Schwartz, 1979; (47) Sato et al., 1980; (48) de Jong & Sharpe, 1976; (49) Chari, 1977; (50) Welschen et al., 1978; (51) Miller et al., 1979; (52) Hopkinson et al., 1977; (53) Miller et al., 1981; (54) Dawne et al., 1978; (55) Chappel et al., 1980; (56) Lefevre et al., 1980; (57) Chari et al., 1979; (58) Jansen et al., 1981; (59) Jenner, personal communication; (60) Le Lannou & Chambon, 1977 b; (61) Steinberger, 1981; (62) Sheth et al., 1981.

CHAPTER 3

A PHYSIOLOGICAL ROLE FOR INHIBIN

So far, most of the work on inhibin has dealt with questions about the existence of inhibin, about its possible gonadal source and about its chemical nature. The diversity of the results of these studies has been described in the previous chapter. Therefore, the physiological role of inhibin still remains a matter of specution. Yet, despite the limitations mentioned in the previous chapter, recent studies indicate a possible physiological role for inhibin in male and female animals. In this chapter, our own data, predominantly on female rats, which indicate a physiological role for inhibin in the regulation of FSH secretion, will be summarized and discussed, together with data from the literature.

3.1. Presence and production of inhibin in the ovary

Prerequisite for a hormonal role of a substance is the observation that this substance is produced by one organ or tissue, transported and able to affect the functioning of cells located elsewhere. Various experiments show that the ovary is producing and secreting a factor, resembling inhibin as demonstrated in male animals, and which is able to affect the pituitary secretion of FSH.

Presence of inhibin

Evidence for the presence of inhibin in the ovary came from the observations that charcoal-treated "steroid-free" follicular fluid of many species contains a factor specifically suppressing the secretion of FSH by the pituitary gland in a variety of test models (see Tables 2 and 3; Appendix I: Welschen et al., 1977; reviews of Hermans et al., 1981 b and of Channing et al., 1981 c). The observation that in the cow the highest concentration of inhibin was found in follicular fluid aspirated from medium and large follicles, and lowest concentrations in fluid aspirated from small and cystic follicles (Appendix I), led to the suggestion that inhibin might predominantly influence FSH secretion when many medium and large sized follicles are present.

Production of inhibin

The relationship between the amount of inhibin in follicular fluid with the size and state of the follicle is suggestive of the involvement of granulosa cells in the production of inhibin. More recently, both indirect and direct evidence has

supported that assumption. However, no attempts have been made to test whether corpora lutea or interstitial cells might also be involved in the production of inhibin.

Indirect evidence suggestive of the involvement of follicles, i.e. of the number of granulosa cells with the production of inhibin, is presented in Appendix II (Welschen et al., 1980). The data show that in the periovulatory period and after unilateral ovariectomy rapid changes in levels of FSH might be attributed to changing levels of inhibin and not to changing levels of steroids. Inverse relationships were found between changes in peripheral concentrations of FSH and changes in the number of follicles with a volume $\geq 200 \times 10^5$ μm^3 ($r = -0.65$, $n = 8$ in the periovulatory period; $r = -0.81$, $n = 8$ after unilateral ovariectomy). These relationships suggest that medium and large antral follicles are involved in the production of inhibin.

Direct evidence indicating that the granulosa cells are a source of inhibin was first provided by Erickson & Hsueh (1978), who showed that the medium of cultured rat granulosa cells contains inhibin. The amount of activity found in the medium was proportional to the number of granulosa cells in culture, but no comparison was made with an inhibin standard preparation, and the amount of activity was not quantified. Appendix III (Hermans et al., 1982 b) presents data showing that granulosa cells collected from antral follicles of adult female rats have and retain the capacity to secrete inhibin *in vitro* for a total of 33-37 days. In accordance with the data of Erickson & Hsueh (1978), the amount of activity produced was proportional to the number of granulosa cells in the dishes. Furthermore, the total length of the culture period influenced the amount of inhibin produced: media contained low amounts of inhibin during the period when the cells were actively growing, differentiating or aging.

In conclusion, follicles, and more precisely granulosa cells, seem to produce and contain inhibin.

The production of inhibin may be dependent on the hormonal environment or the functional state of the follicle or of the whole ovary, but little is known about this. A direct feedback between inhibin and FSH however seems to exist. Increased levels of FSH, as for instance after unilateral ovariectomy or during the periovulatory period, are involved in the initiation of growth of follicles (Welschen & Dullaart, 1976; Sheela Rani & Moudgal, 1977; Welschen et al., 1978; Hirshfield & Midgley, 1978). The increasing number of granulosa cells might produce increasing amounts of inhibin, reducing the high levels of FSH (see Appendices II and III). When a number of granulosa cells as present in about 25 medium sized antral follicles has been reached, the amount of inhibin might keep the levels of FSH at basal values. In this concept, inhibin could play a role in the determination of the number of follicles maturing during the cycle, so that follicles are regulating their

own numbers, as will be discussed below (3.4). It is not known whether FSH itself is directly necessary for inhibin production. A preliminary report of Franchimont et al. (1981) suggested that addition of FSH or LH to bovine granulosa cells in culture did not influence inhibin production, while addition of aromatisable androgens stimulated inhibin production.

3.2. Secretion of inhibin by the ovary

In the previous section it was demonstrated that inhibin is produced by follicular cells and is present in follicular fluid. However, the presence of inhibin in follicular fluid can be interpreted in at least two ways: it might represent storage without active secretion into the circulation, or, alternatively, it might represent an active production and secretion of inhibin by follicular cells, leading to both accumulation in follicular fluid and passage into the general circulation.

If the first hypothesis is correct, inhibin could be an intra-ovarian regulator of maturation processes, comparable to other factors present in follicular fluid such as the granulosa cell luteinization inhibitor (Ledwitz-Rigby et al., 1973), the luteinization stimulator (Ledwitz-Rigby & Rigby, 1981), the oocyte maturation inhibitor (Tsafriri et al., 1976), the FSH binding inhibitor (Darga & Reichert, 1978), the LH binding stimulator (Reichert et al., 1981) and an atretogenic factor (Channing et al., 1981 a). Although it cannot be ruled out that inhibin might be identical to one of these factors, the latter are generally factors with small molecular weights (Mw < 20.000 D),present only or predominantly in small or medium sized follicles (see review by Channing & Franchimont, 1981). These latter characteristics are not in line with our present knowledge about inhibin. Also a report on a direct effect of inhibin from human follicular fluid on granulosa cells, causing their degeneration (Chari et al., 1981), is no support for the hypothesis that inhibin might be an intragonadal factor exerting local effects (a cybernin), since in that study it was not excluded that changes in levels of FSH could have been responsible for the observed effects.

The assumption that inhibin might both accumulate in follicular fluid and enter the general circulation seems more likely. Secretion of inhibin-like activity by the ovary has been demonstrated now directly as well as indirectly.

Indirect evidence was obtained by Uilenbroek et al. (1978), who showed that peripheral levels of FSH were selectively suppressed in castrated male and female rats which bore intrasplenic ovarian transplants. The steroids produced by such implanted ovaries are metabolized by the liver, so that the observed effect should be ascribed to a non-steroidal, ovarian factor. This was strengthened by experiments in which the animals bore intrarenal ovarian transplants. In this situation the ovarian venous plasma, and thus ovarian steroids, immediately enter the gene-

ral circulation; as could be expected levels of both FSH and LH were suppressed now under these conditions. A similar conclusion of ovarian secretion of a non-steroidal factor with inhibin activity was reached by Johnson (1981), who connected androgenized female rats parabiotically with castrated male partners. Levels of FSH in the castrated male partner rose more slowly than levels of FSH in non-united castrated male rats, while there were no differences in the increases of the levels of LH. It should be borne in mind that steroids practically do not pass from one animal into the other in parabiosis, so that a non-steroidal factor might be involved.

The study of Johnson (1981) also showed that when the number of large, cystic follicles in the female rats was increasing concomitant with a decreasing number of healthy granulosa cells, levels of FSH in the male partner started to rise. This observation supports our finding of low concentrations of inhibin in cystic follicles of the cow (Appendix I), and again points to healthy granulosa cells as a source for inhibin production.

A direct suggestion for the secretion of inhibin by the ovary was provided by DePaolo et al. (1979 c), who detected inhibin with an in vitro assay (using dispersed pituitary cells) in steroid-free ovarian venous plasma sampled from adult cyclic female rats. The authors showed that less inhibin was present in ovarian venous plasma from late pro-oestrus and early oestrus, while more inhibin was found in venous plasma collected on the other days of the cycle. Since these results were found in a one-point assay and no comparison was made with a standard preparation known to contain inhibin, the final conclusion of the authors that the amount of inhibin in ovarian venous plasma varies inversely with plasma concentrations of FSH seems rather premature. In contrast with the results of DePaolo et al. (1979 c), Chappel (1979) found with an in vivo bioassay the highest concentration of inhibin in extracts from ovaries collected from pro-oestrous hamsters. In support of this latter finding, F. H. de Jong & D. A. M. van de Wiel (personal communication) observed larger amounts of inhibin in porcine follicular fluid collected at midcycle than at the other days of the cycle. It is possible that at pro-oestrus inhibin is no longer secreted into the circulation, but still present or accumulating in follicular fluid from medium and large sized follicles.

Irrespective of the discrepancies described above, the findings indicate that production and/or secretion of inhibin may be dependent on the day of the cycle.

3.3. Role of the pituitary gland: its response to inhibin

The regulation of the secretion of FSH takes place within the hypothalamic-pituitary unit. If an animal is to respond to varying concentrations of inhibin, as shown by changes in FSH secretion, inhibin should at least influence one component

of the hypothalamic-pituitary unit. Furthermore, the sensitivity of this component of the hypothalamic-pituitary unit to inhibin must be large enough to be able to respond to changes in the peripheral concentration of inhibin.

The fact that halved pituitary glands and pituitary cells in culture (see Chapter 2) respond to the presence of inhibin with a decreased release of FSH into the culture medium is indicative of - at least - the pituitary gland as a site of action of inhibin. In vitro studies measuring incorporation of ^{3}H-leucine into FSH (Chowdhury et al., 1978) or the cellular content of FSH (de Jong et al., 1979 a; Scott et al., 1980) after addition of inhibin showed that the synthesis of FSH in the gonadotrophic cells is suppressed by inhibin.

Thus, a direct, specific effect of inhibin on gonadotrophic cells present in the pituitary gland apparently exists. However, it has also been suggested that inhibin as present in porcine follicular fluid stimulates in hemicastrated male rats the sensitivity of the pituitary gland to LHRH *in vitro* (Wilkinson et al., 1980), but as long as sound knowledge of alterations in secretion patterns of FSH and LH following administration of porcine follicular fluid *in vivo* are unknown (see also pp 58-60), this conclusion seems premature.

Besides the pituitary gland, the hypothalamus has also been suggested as a site of action of inhibin. Demoulin et al. (1980) and Moodbidri et al. (1981) reported a decreased hypothalamic content of LHRH after addition of inhibin *in vivo* and *in vitro* respectively. Intraventricular administration of inhibin (Lugaro et al., 1974; Lumpkin et al., 1981), or injection of hypothalamic extracts of male rats treated with inhibin into ovariectomized female rats (Le Lannou & Chambon, 1977 b) suggested a central nervous site of action (Lumpkin et al., 1981), possibly by a decrease in the synthesis of FSHRH (Lugaro et al., 1974; Le Lannou & Chambon, 1977 b). Since these studies measured a selective decrease in levels of FSH after treatment and not the concentration of LHRH in pituitary stalk plasma, and since the existence of a separate FSHRH is doubtful, these conclusions might be unjustified.

Finally, a high uptake of radiolabelled inhibin, purified from human seminal plasma, was found by Vanage et al. (1980), not only in the pituitary gland, but also in the pineal gland. The binding of inhibin to the pineal gland was not discussed or explained; also doubt exists on the specific gonadal nature of the inhibin preparation used (see also pp 37). Therefore, a possible action of inhibin on the pineal gland is as yet completely speculative.

Also the gonads themselves have been suggested as a site of action for inhibin, but in these studies it could not be excluded that the observed changes were mediated by an action of FSH (for more details see pp 56).

In conclusion, results from different studies do not exclusively name one single target organ for the action of inhibin, but among several candidates the pituitary gland is an important one.

If the pituitary gland is one or the sole target organ for inhibin, it is important to specify the sensitivity of this unit to varying concentrations of inhibin. Data presented in Chapter 2, pp 32 and in Appendix IV (Hermans et al., 1980) indicate a changing sensitivity of the pituitary gland to inhibin, at least in female rats: this sensitivity does not change with age in male rats, while in female rats it seems to be increasing between 15 and 35 days of age.

In order to study to a larger extent the age-related pattern of FSH secretion after injection of inhibin, the experiments described in Appendix V (Hermans et al., 1981 a) were undertaken: adult or 25-day-old female rats, which had been ovariectomized for 48 h, were injected with bFF and the subsequent patterns of suppression of FSH secretion were followed. The results show that both the total duration of the period of suppression as well as the degree of suppression reached is less in 25-day-old female rats than in adult female animals. Since it is not known whether there is a difference in metabolic clearance rate of inhibin between prepubertal and adult rats, the effects described above do not necessarily indicate a lower pituitary sensitivity to inhibin of prepubertal compared with adult female rats. Therefore it might be better not to attribute the results to a lower pituitary sensitivity to inhibin, but to describe the end effect simply as a smaller response of the pituitary gland to inhibin in prepubertal compared to adult female rats.

3.4. The relative importance of inhibin in regulation of FSH secretion

Without knowing endogenous levels of inhibin, it is impossible to ascertain the quantitative contribution of inhibin to the regulation of peripheral concentrations of FSH. However, by using some indirect approaches we have tried to mark out the relative contribution of inhibin in a number of situations. The approaches used are based on the hypotheses that (i) inhibin is involved in the fast regulation of FSH secretion, which is likely in view of the relatively fast response of the hypothalamic-pituitary unit to inhibin, and (ii) that inhibin might play a physiological role (at least) in those situations in which diverging changes in secretion patterns of FSH and LH occur (see review of Fink, 1977). It was reasoned that interrelationships between peripheral concentrations of FSH and numbers of follicles might be interpreted as relationships between levels of endogenous inhibin and concentrations of FSH. Also, removal of endogenous inhibin by means of ovariectomy might give information about the relative contribution of inhibin in FSH regulation. Finally, a preparation containing inhibin (charcoal-treated bFF) was injected into rats in order to study the effect of increased levels of inhibin on the pituitary-ovarian axis.

3.4.1. Experiments on interrelationships between changes in blood levels of FSH and changes in ovarian follicles

Rapid changes in peripheral concentrations of FSH without concomitant changes in levels of LH could be under the control of changing levels of circulating inhibin. If this is true one might expect either changes in the number of healthy follicles, or changes in the inhibin secreting capacity of follicles, more specifically of granulosa cells. Since the latter situation cannot be investigated (yet), we chose to study in adult female rats two situations in which rapid changes in the number of ovarian follicles can be found together with diverging secretion patterns of FSH and LH (Appendix II, Welschen et al., 1980): the periovulatory period and the 32 hours following unilateral ovariectomy.

In the strain of rats used in our laboratory, levels of LH start to rise at pro-oestrus around 12.00 h, reaching peak values in the afternoon, and being low again around midnight. Levels of FSH, however, start to rise between 12.00 and 16.00 h at pro-oestrus, reach the highest values between 20.00 h at pro-oestrus and 08.00 h at oestrus, whereafter they start to decline (see Appendices II: Welschen et al., 1980 and VI: Hermans et al., 1982 a). The involvement of inhibin is likely in both the initiation and the duration of this period of high levels of FSH (see Appendix II). Summarizing the data obtained it can be said that the prolongation of the FSH rise in the periovulatory period is probably caused by a relative lack of ovarian release of inhibin, and not by a diminished inhibitory action of ovarian steroids; only administration of inhibin, but not of steroids, was effective in suppressing the high levels of FSH seen in this period. The observation of a significant negative correlation (see pp 47) between mean numbers of ovarian follicles of volume $\geq 200 \times 10^5$ μm^3 and mean levels of FSH in the period between 12.00 h pro-oestrus and 16.00 h oestrus strengthens the hypothesis that a relative lack of circulating inhibin could have occurred during that period; this may have caused levels of FSH to rise. This does not exclude the possibility that changes in the oocyte-follicular complex other than changes in the number of granulosa cells could have been the cause of a lack of inhibin: Shander et al. (1980 b) suggested that the first wave of increased levels of FSH and LH at pro-oestrus, which could be experimentally mimicked by administration of rat FSH or rat LH to phenobarbital-treated rats, is responsible for a diminished ovarian release of FSH-inhibiting activity by way of affecting ovarian components responsible for secretion of FSH-inhibiting activity.

If a relative lack of inhibin secretion accounts for - part of - the oestrous portion of the periovulatory surge of FSH, again follicles seem to regulate their own numbers by way of inhibin: Hirshfield & DePaolo (1981) made it likely that it is the second, oestrous part of the FSH surge which is responsible for the recruitment of follicles at oestrus. A decrease in the amount of circulating inhibin might

be the signal to prolong the increased secretion of FSH to such an extent that enough follicles start maturation; then, as described earlier (pp 47), the growing follicles might produce inhibin, causing a decrease in levels of FSH in later phases of oestrus.

Apart from regulation by inhibin, the second, oestrous part of the FSH surge might, possibly in a species-dependent way, be dependent on neural factors too: in the hamster intact neural connections between the pituitary gland and the medial basal hypothalamus are necessary for the full expression of the FSH surge (Chappel et al., 1979), but in the rat anterior deafferentiation had no influence on the second part of the FSH surge (Rush et al., 1980). It has also been suggested that the pro-oestrous rises of both FSH and LH are necessary for evoking the oestrous part of the FSH surge (Ashiru & Blake, 1979). Wise et al. (1979) suggested that the high pro-oestrous discharge of LHRH causes levels of FSH and LH to rise; against this hormonal background the subsequent low levels of LHRH would give rise to a selective increase in FSH secretion by way of a changed responsiveness of the gonadotrophic cells. In this way the oestrous portion of the FSH surge would be caused. This suggestion is in accordance with the observation that priming with LHRH stimulates protein synthesis of the gonadotrophs; in this way more FSH, but also LH, might be made available for discharge (Pickering & Fink, 1977). It remains to be proven whether a decrease in levels of inhibin at pro-oestrus is the direct signal for the release of FSH by the pituitary gland, or whether a decrease in levels of inhibin permits the pituitary gland to discharge that part of the FSH surge which is dependent on neural factors.

Thus, regulation of FSH secretion by way of inhibin seems to take place when the number of medium and large ovarian follicles is changing, such as is the case around ovulation. A change in the number of follicles is also, and even more striking, the case at unilateral ovariectomy (Appendix II: Welschen et al., 1980; see also Welschen et al., 1978). Summarizing the results of these studies, it was found that immediately after unilateral ovariectomy levels of FSH, but not of LH, start to rise. This rise in levels of FSH could not be prevented by administration of steroids, but only by administration of charcoal-treated bFF, containing inhibin. Furthermore, no negative correlation was found after unilateral ovariectomy between levels of oestradiol-17β or progesterone on the one hand and FSH on the other, while a negative correlation (see pp 47) could be found between mean numbers of follicles of volume $\geq 200 \times 10^5$ μm^3 and mean levels of FSH in the period after unilateral ovariectomy.

In conclusion, studies on the interrelationships between numbers of healthy, medium and large sized antral follicles and levels of FSH have shown that in situations when numbers of follicles are reduced, a rise in levels of FSH follows; eventually a decrease in the raised levels of FSH seems to be induced by increased

levels of inhibin. Physiologically this feedback mechanism seems to be operative in the periovulatory period, while it can be shown experimentally to exist at other days of the cycle as well.

3.4.2. Age related changes in the contribution of inhibin in the regulation of FSH secretion

The previous section described a possible physiological role for inhibin in adult female rats. In order to assess at what ages inhibin might play or start to play a role, we decided to study the relative contribution of inhibin feedback on FSH secretion during development in male and female rats (Appendix IV: Hermans et al., 1980). In that study bilateral ovariectomy or orchidectomy was performed at different ages followed by administration of steroids or charcoal-treated bFF.

The results show that in female rats a diverging response of the secretion of FSH and LH immediately following bilateral ovariectomy takes place from at least 25 days of age onwards. Moreover, the relative response of FSH secretion to ovariectomy is increasing with age (see pp 30). Only administration of inhibin as present in charcoal-treated bFF could abolish the selective rise in FSH levels at all ages tested, while partial effectiveness of steroids in the prevention of the rise of FSH levels was only observed at 25 days of age. Although it is not known whether 0.50 ml bFF / 100 g body wt. contains supra-physiological or physiological amounts of inhibin, it can be concluded that inhibin appears to be one, but not necessarily the only factor responsible for the fast regulation of FSH secretion. Moreover, its role apparently becomes increasingly important towards the onset of cyclicity. This latter view is strengthened by different observations: the response of the pituitary gland to inhibin increases with age (see pp 32) and the effectiveness of different combinations of steroids in inhibition of FSH secretion decreases with age from about 25-30 days of age onwards (Meijs-Roelofs et al., 1979; 1981).

Thus, a gradual decline in the contribution of ovarian steroids with regard to regulation of FSH secretion takes place towards adulthood, and simultaneously the inhibin feedback mechanism seems to develop. This development might be caused by an increasing exposure to endogenous inhibin: from about 21 days of age onwards antral follicles, the most probable source of inhibin, are appearing in the ovary (Wilenbroek et al., 1976). Further support for this view comes from the observation that no suppression of levels of FSH following administration of charcoal-treated bFF can be seen in untreated, 5-day-old rats which lack antral follicles; only when the animals have been pretreated for 3 days with bFF a response on a testdosis of bFF in terms of an FSH suppression can be seen at this age (Table 4). Some interesting but as yet unexplained observations may be mentioned here. Pretreatment of the young animals with bFF for a longer period (from day 5 till 12) did not have a

Table 4. *Effect of pretreatment with bovine plasma (BP) or charcoal-treated bovine follicular fluid (bFF) on the FSH response (mean ± S.E.M.) to a single test dose of bFF (n = 6). During pretreatment animals were injected i.p. with 0.010 ml fluid/rat/day at 09.00 h. The final injection with the test dose (0.50 ml / 100 g body wt.) was given i.p. at 09.00 h; BP served as a control fluid. Animals were decapitated at 17.00 h and trunk blood was then collected. Levels of LH were not influenced.*

* *$P \leq 0.05$ versus BP-injected control animals (Wilcoxon, two-tailed test).*

pretreatment 0.010 ml/rat/day at days	with	ovariectomy at day	final injection 0.50 ml/100 g body wt. at day	response of FSH (ng NIAMDD-rat-FSH RP-1/ml) to final injection of	
				BP	bFF
-		-	5	507 ± 12	540 ± 73
2-4	BP	-	5	504 ± 14	
2-4	bFF	-	5		352 ± 11 *
5-12	BP	-	13	1000 ± 63	
5-12	bFF	-	13		1159 ± 153
-		13	15	3020 ± 272	1614 ± 102 *
5-13	BP	13	15	2952 ± 190	1817 ± 226 *
5-13	bFF	13	15	3065 ± 88	2614 ± 360

priming effect. This paradoxical situation was confirmed by results from an experiment in which the animals were pretreated from days 5 until 13 with bovine plasma or charcoal-treated bFF, and in some groups ovariectomized at day 13. When the animals were given a single injection with a standard dose of bFF or bovine plasma at day 15, an FSH response to bFF was seen in rats which were not pretreated or pretreated with bovine plasma, while the response was absent in bFF-pretreated rats. At present we cannot explain these data, but the results suggest that either a compensation phenomenon (see pp 59) or an immunological response to inhibin in bFF might be involved.

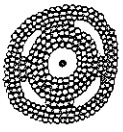
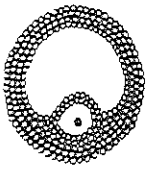
The development with age of a fast acting feedback system for the regulation of FSH secretion might serve to provide the animal with a system operating during cyclicity. It is also possible that the inhibin feedback system might already be operative from about 21 days of age onwards, regulating, through the control of secretion of FSH, the number of follicles which start growing. Thus inhibin might be involved in sexual maturation and first ovulation.

In male rats, the contribution of inhibin to regulation of FSH secretion seems to be restricted mainly to the prepubertal period. This hypothesis is supported by various lines of evidence. The relative increase in levels of FSH seen 8 h after orchidectomy is decreasing with age, while the pituitary response to inhibin remains constant with age (Appendix IV: Hermans et al., 1980 and pp 27-28). Relatively high amounts of inhibin are probably produced in the prepubertal period by the most likely source of inhibin in the male, the Sertoli cells (Steinberger & Steinberger, 1976; de Jong & Sharpe, 1977 a; Labrie et al., 1978; Demoulin et al., 1980), which stop to increase numerically around 35 days of age (de Jong & Sharpe, 1977 b). Furthermore, Main et al. (1980) and Decker et al. (1981) showed that in adult male rats the contribution of inhibin in FSH regulation is probably quite small compared with the contribution of testosterone. It is also well known that in adult male animals the role of FSH in the functioning of the testis is limited compared with its role during testicular development (review: Davies, 1981). Thus, it seems possible that inhibin plays a role during that period of life when FSH has a marked influence on the -still- developing testis, i.e. on the number of Sertoli cells, and the onset of spermatogenesis. In conclusion, a feedback system between inhibin regulating the secretion of FSH and FSH indirectly inducing the production of inhibin in prepubertal animals might exist.

It remains to be shown whether abnormalities in testis size and spermatogenesis will take place when the inhibin feedback mechanism is diminished during the prepubertal period. Local effects of inhibin on spermatogenesis have also been taken into account: inhibin has been shown to inhibit the ^{3}H-thymidine uptake into testicular DNA *in vivo* and the labelling of type B spermatogonia in prepubertal, but not adult rats, but in these studies a small molecular weight inhibin also exerted effects and it could not be excluded that the effects were mediated by changes in levels of FSH (Demoulin et al., 1981). On the other hand, de Jong et al. (1978) showed that daily treatment of 21-day-old male rats for up to 12 days with inhibin as present in charcoal-treated bFF lowered levels of FSH only during the first 6 days of treatment, together with a reduction in testicular weight and number of pachytene spermatocytes. After 12 days of treatment these parameters were no longer different from control animals 6 days younger, which suggests that the disturbances caused by exogenous material containing inhibin are only short lasting. Whether this compensation was due to hypersecretion of FSH (as found in female animals, see pp 59), either caused by changing levels of inhibin or by decreased amounts of steroids or otherwise, is not known. Also Davies et al. (1979) and Lugaro et al. (1979) could not detect long lasting changes in testicular structures after prolonged daily administration of inhibin into adult rats.

In conclusion, both in male and female rats inhibin might play a physiological role in those circumstances when FSH influences the numerical development of the

Table 5. *Mean (± S.E.M.) numbers of follicles per type class in one ovary of 25-day-old or adult rats at various hours after injection of 0.50 ml charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. Bovine plasma served as a control fluid. Adult rats were injected at dioestrus-1 (n=4). For experimental details see Hermans et al. (1981 a), Appendix V.*

follicle type	material	hours after injection: 25-day-old 8	16	24	adult, dioestrus-1 8	20	27	48
	bovine plasma	47 ± 7	46 ± 4	47 ± 7	40 ± 4	52 ± 5	46 ± 3	50 ± 6
	bovine follicular fluid	42 ± 9	56 ± 5	47 ± 13	44 ± 1	53 ± 6	49 ± 4	44 ± 4
	bovine plasma	88 ± 11	71 ± 6	52 ± 10	32 ± 7	43 ± 2	32 ± 3	33 ± 3
	bovine follicular fluid	68 ± 11	62 ± 3	54 ± 5	34 ± 5	36 ± 6	38 ± 5	32 ± 4
	bovine plasma	45 ± 1	36 ± 4	37 ± 5	24 ± 5	29 ± 2	21 ± 2	23 ± 3
	bovine follicular fluid	43 ± 3	36 ± 5	40 ± 6	29 ± 6	28 ± 2	22 ± 2	24 ± 3
	bovine plasma	25 ± 4	30 ± 1	26 ± 2	13 ± 2	18 ± 3	15 ± 1	14 ± 1
	bovine follicular fluid	28 ± 6	26 ± 3	23 ± 2	17 ± 1	17 ± 2	20 ± 4	15 ± 0

cells supporting gametogenic elements: the granulosa cells and the Sertoli cells. By producing inhibin, these cells may indirectly control their own numbers.

3.4.3. Pituitary response to exogenous inhibin during the ovarian cycle

In the previous two sections an indication for a physiological role of inhibin was sought in those situations when diverging secretion patterns of FSH and LH occur, and when levels of FSH are changing rapidly. The results suggest that during the periovulatory period, when granulosa cells increase numerically, inhibin might play a role in the control of these numbers by way of FSH. This could imply that, beside the periovulatory period, inhibin might also play a role in FSH control on the other days of the cycle, despite the fact that concentrations of FSH and LH are

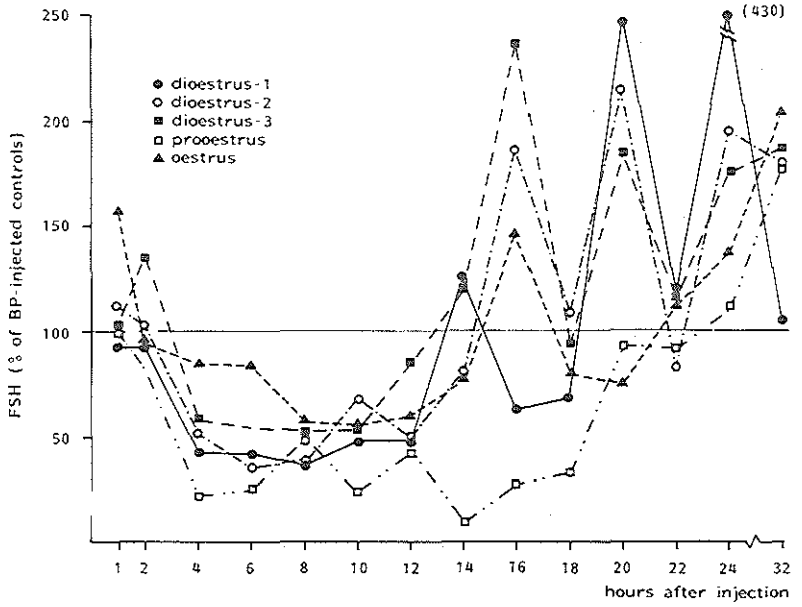


Figure 8. *Response of levels of FSH on different days of the cycle of adult female rats at various hours after a single i.p. injection at 09.00 h of 0.50 ml charcoal-treated bovine follicular fluid (bFF) / 100 g body wt. Bovine plasma (BP) served as a control fluid. Results are expressed as mean percentages of the mean values found in control animals.*
The data are calculated from data of Hermans et al. (1981 a; 1982 a), Appendices V and VI, and are to be published in this form by de Jong et al. (1982 a).

both low and stable. We decided to check firstly an assumption implicit in this view: does the hypothalamic-pituitary unit respond to inhibin with a short lasting decrease in FSH secretion on all days of the cycle? It was also investigated whether this response changes with the day of the cycle. Therefore we injected bFF into intact, adult female rats and studied the time course of its effect on levels of FSH and LH.

The first series of experiments with intact, adult rats are described in Appendix V (Hermans et al., 1981 a). Rats at dioestrus-1 of the cycle were used, and the data were compared with data obtained in ovariectomized animals. Injection of charcoal-treated bFF into intact rats yielded results quite different from the ones obtained in ovariectomized animals: in intact animals the total period of suppression was shorter than in ovariectomized rats. Furthermore, it was followed by a period of increased secretion of FSH. Apparently the hypothalamic-pituitary unit behaves

Table 6. *Effect of daily treatment (for a total of 26 days) with charcoal-treated bovine follicular fluid (bFF) on mean cycle length and number of ova shed at the fifth oestrus after start of treatment. Bovine plasma (BP) served as a control fluid. The first 17 days the rats were injected i.p. at 09.00 h with 0.25 ml, thereafter with 1.00 ml bFF / 100 g body wt. (n = 6).*
Data of de Jong et al. (1978).

treatment	mean cycle length during cycle				mean number of ova
	1	2	3	4	
BP	6.6	6.4	5.9	5.7	9.0
bFF	6.4	5.5	5.3	5.7	11.4

differently after injection of bFF in the presence of the ovaries, since it is unlikely that the metabolic clearance rate of bFF is affected by the presence of the ovaries. The period of increased secretion of FSH might serve to rescue follicles which otherwise might have become atretic after discontinued stimulation by FSH (Hirshfield & Midgley, 1978). This seems likely since no changes in the number of healthy follicles at different hours after injection of bFF were observed (Table 5). This does not exclude the possibility that secretory processes in the follicles were affected, either by a direct effect of bFF or by way of the changing levels of FSH following injection of bFF.

We then investigated whether the phenomenon of decreased followed by increased levels of FSH after injection of bFF also occurred on the other days of the cycle. The results of these experiments are described in Appendix VI (Hermans et al., 1982 a). It was found that not only at dioestrus-1 but at all other days of the cycle injection of bFF caused a selective suppression of levels of FSH. When the results are expressed in percentages of values found in control animals (Fig. 8), it can be seen that the periods of suppression and the periods with increased levels of FSH show comparable characteristics at dioestrus-1, dioestrus-2, dioestrus-3 and oestrus. At pro-oestrus the total period of suppression lasts longer, while levels of FSH start to rise later.

Thus, information was gained about the hypothalamic-pituitary response to bFF on all days of the cycle. Although the initial response to bFF is comparable at all days of the cycle, the response in the period after the decrease varies with the day of the cycle. No explanation could be given for the apparent spike-like pattern of FSH secretion in the hours following the initial decrease in levels of FSH at

oestrus and at the dioestrous days of the cycle. Yet the total periods with increased secretion of FSH were interpreted as being indicative of a safety mechanism compensating for low or decreasing levels of FSH, thus ensuring normal follicular development. This interpretation might also be used to explain undisturbed cycle length and number of ova shed following a 26 day period with daily injections of bFF into intact, cyclic female rats (de Jong et al., 1978) (Table 6). In combination these two experiments suggest that abolishment of the first, pro-oestrous portion of the FSH surge has no (lasting) influence on cycle length or initiation of follicle growth. Although the proposed existence of a safety system against disturbances in the secretion of FSH is interesting in itself, these studies did not clarify the nature of the signal(s) activating the " safety system ". In Appendices V and VI several possibilities have been suggested as an answer to this question. One among these might be a decreased synthesis and/or release of endogenous inhibin following decreased levels of circulating FSH. The results from the experiments with single injections of bFF at all days of the cycle and the results of the experiment with unilateral ovariectomy (Appendix II: Welschen et al., 1980; see also pp 53) support the view that a possible physiological role for inhibin during the cycle might be as follows: *inhibin may be involved not only in the regulation of the number of follicles starting maturation, but might also serve as the signal to protect maturational processes against disturbances in FSH secretion.*

However, it should be emphasized that as long as levels of endogenous inhibin cannot be measured directly, this hypothesis remains speculative. Moreover, since the effects seen were caused by injection of crude bFF, it is possible that other factors present in bFF and capable of influencing gonadotrophin secretion are directly responsible for the period of increased secretion of FSH. As a consequence, two conclusions should be drawn: (i) studies aimed at further elucidation of the bFF-effects described above should await its separation into factors capabable of selectively affecting gonadotrophin secretion, such as inhibin; (ii) generally results of experiments in which crude bFF has been used in order to induce changes in the pituitary-ovarian axis should be interpreted with caution as long as possible side effects of bFF are not fully known.

GENERAL DISCUSSION AND QUESTIONS FOR THE FUTURE

The previous chapters dealt with the results of studies on the question whether a non-steroidal, gonadal hormone, inhibin, exists and can be involved in regulation of gonadotrophin secretion under physiological conditions. It was shown that no definite answers can be given as long as problems concerned with purity of the hormone and reliability of the test models are not solved satisfactorily. The presence in follicular fluid of several factors which are able to influence gonadotrophin secretion makes it very difficult to interpret some of the data found in studies using charcoal-treated bFF. The use of crude preparations containing inhibin has the risk that our understanding of gonadotrophin secretion might be blurred by an abundance of theories based on side effects not attributable to inhibin. Yet, the in vivo experiments conducted until now and described in this thesis have been very useful, since (i) in vivo experiments have played an important role in the development of bioassays for measurement of inhibin and (ii) in vivo experiments have outlined the situation in which inhibin might play a physiological role. However, the theories developed so far need to be substantiated. Therefore, attention should be focused on the purification of inhibin and the development of specific radioimmunoassays for inhibin. This does not imply that in vivo investigations should be halted. On the contrary, many questions with regard to inhibin are still unsolved, but these questions can only be answered when there is no doubt about the purity of the hormone and about the method to measure it. Several possible future lines of investigation, to be briefly indicated below, have resulted from the studies conducted until now.

Unresolved fundamental questions concern the interrelationships between circulating levels of gonadotrophins and peripheral and gonadal inhibin levels. The relationship between levels of inhibin and stages of follicular and spermatogenic maturation should be investigated. Information is also lacking about the half life of inhibin and about the way of its transport into and in the general circulation in the male. In order to determine feedback relations, more information is needed about the cell type(s) capable of producing inhibin (granulosa cells, interstitial cells, luteinized cells, Sertoli cells, spermatogenic cells) and the - especially hormonal - conditions necessary for production of inhibin. More information is also needed about the action of inhibin on, and the response of, both obvious (hypophysis) and less probable (hypothalamus, testicles, ovaries, pineal) target organs. Finally, knowledge on direct and indirect interactions between gonadal steroids and inhibin might help to adjust the hypotheses concerning regulation of gonadotrophin secretion. Both in prepubertal and in adult male animals depletion studies

using antisera to inhibin could be used in order to test the hypotheses of the physiological roles for inhibin. Treatment of monoparous and multiparous animals with specific antisera to inhibin might prove the scientific and practical value of the concept of autoregulation of the number of follicles by way of inhibin and FSH.

As for practical applicability, a future for inhibin as a possible contraceptive, especially for male animals, has been suggested. On this subject we agree with B. P. Setchell, who remarked in 1980: " it is perhaps unusual to consider something as a possible contraceptive if it is not even universally accepted that the substance exists, but I am afraid that this is the situation with inhibin ". Moreover, in view of the known facts this suggestion of a possible practical use of inhibin seems rather premature: (i) it has not been possible yet to identify a single entity with inhibin activity, nor has it been attempted to synthesize inhibin; (ii) since inhibin seems to be a (glyco)protein, and possibly a large one, oral administration does not seem very effective, thus reducing the changes for inhibin as a potential contraceptive; (iii) even if it can be proven that inhibin can suppress FSH secretion permanently, it has not been shown yet to what extent levels of FSH should be suppressed in order to disturb folliculogenesis or spermatogenesis.

Nevertheless, our present knowledge on inhibin makes it tempting to speculate about possible practical applications of inhibin or of antisera against inhibin. A possibility might be regulation of the number of follicles to be ovulated in women, or the regulation of cycle length or ovulation rate in animal husbandry. Measurement of inhibin levels could possibly also be used as a tool in the diagnostic of infertility in women with a non-ruptured follicle syndrome or in men with disturbances in spermatogenesis. A problem in speculations about the future applicability of inhibin is the fact that it is not known whether disturbances in inhibin feedback represent a symptom or a cause of a pathological condition. If a disturbance in inhibin secretion is a cause, the study of inhibin might open new perspectives in treatment of fertility disorders. If a disturbance in inhibin secretion represents a symptom, measurement of levels of inhibin might be used as a parameter for the number and/or physiological condition of the cells supporting gametogenic elements.

In conclusion, inhibin might be one of the missing links in our understanding of reproductive physiology. It deserves a better fate than a repetition of history: a second fall into oblivion.

SUMMARY

Understanding of (in)fertility requires insight in the factors which regulate the morphology and function of the testes and ovaries. It is well established that in both male and female mammals the central nervous system exerts influences on the pituitary gland: its secretion of the gonadotrophic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). The ovaries and testes in turn respond with the production of gametes and gonadal hormones. Among other factors, the gonadal steroidal hormones were held responsible for the control of the secretion of FSH and LH by the pituitary gland. However, in recent years it has become evident that not only steroids are involved in the control of gonadotrophin secretion, but that also a non-steroidal factor is involved.

In Chapter 1 it is described how in the 1920's and 30's experiments were performed which led to the suggestion that the testis produces, besides steroids, a non-steroidal factor which may prevent the appearance of castration cells in the pituitary gland of castrated animals. This hypothetical factor was named "inhibin". From the 1940's on, concentrations of gonadotrophic hormones could be estimated; it was found that in men with disturbed spermatogenesis levels of FSH, but not of LH, were elevated. This led to the suggestion that the production of "inhibin" could be diminished here. This hypothesis did not gain extensive support, which was mainly due to the inability to demonstrate and purify inhibin. Therefore in the 1950's it was generally accepted that steroids alone could regulate gonadotrophin secretion. However, data on men and experimental animals accumulated indicating that steroids alone could not be held responsible for the control of especially FSH secretion. This led in the early 1970's to a revived interest in the concept of inhibin.

In Chapter 2 it is described how recently experiments were conducted to detect inhibin. Now inhibin is defined as " a water-soluble, non-steroidal substance which suppresses the secretion from the pituitary gland of FSH, but not of LH, in a number of different tests ". Inhibin could be detected in a variety of male and female gonadal fluids by using a large number of methods. The methods using changes in organ weights after administration of the test fluids are considered rather unspecific. More specific methods for detection of inhibin are the methods based on changes in concentrations of FSH and LH after administration or addition of the fluids to be tested. These methods can be either in vivo tests using predominantly rats, or in vitro tests using dispersed pituitary cells. The latter method has the advantage that small amounts of inhibin can be quantified. Also attempts were made to develop radioimmunoassays for inhibin, but - as yet - these methods are not very reliable. At present the most reliable methods for detection of inhibin appear in vitro bio-

assays using dispersed pituitary cells occasionally combined with in vivo bioassays using acutely castrated prepubertal male rats or long term ovariectomized female rats. Despite the use of all these methods and sources of fluids, supposedly containing inhibin, elucidation of the physico-chemical characteristics of inhibin, nor purification of a single entity with inhibin activity has been achieved. The experiments conducted until now indicate that the amount of inhibin varies with the size of the ovarian follicles and with the stage of the oestrous cycle. This led to the view that inhibin could have a physiological role in regulation of FSH secretion.

In Chapter 3 experimental data, predominantly obtained in female rats, are described which are suggestive of a physiological role for inhibin. Inhibin was shown to be present in ovarian follicular fluid of many species. The ovarian compartment responsible for inhibin production appears to comprise at least the medium and large antral follicles; more specifically granulosa cells. It could be made plausible that inhibin is secreted into the general circulation, so that it can reach the pituitary gland. From these observations a hormonal function for inhibin is likely. It was shown that the response of the pituitary gland to inhibin as present in charcoal-treated bovine follicular fluid (expressed as a decrease in secretion of FSH) is increasing with age in female, but not in male rats. In further experiments it was shown that regulation of FSH secretion by inhibin apparently is limited to the prepubertal period in male rats, and becomes increasingly important towards adulthood in female rats. This led to the hypothesis that especially in adult female rats inhibin plays a role in the regulation of FSH secretion. Indeed, in those physiological and experimental situations in which levels of FSH, but not of LH, start to rise in adult female rats, a relative decrease in the amount of inhibin seems to be involved. It could also be made plausible that elevated levels of FSH induce, by way of accelerated follicular growth, an increase in the production of inhibin which in turn would cause a decrease in levels of FSH. So the concept arises that inhibin plays - via control of FSH secretion - a role in regulation of the number of follicles starting maturation. Finally, results were presented which suggest that inhibin might also play a role in the initiation of a mechanism to protect follicles against dicontinued stimulation of FSH.

In the General Discussion the data presented are evaluated. Inhibin can be detected, and apparently plays a physiological role in both male and female mammals, especially when the number of granulosa cells and Sertoli cells increases. Yet, it is discussed that the impurity of the preparations containing inhibin and the limitations of the bioassays used are major factors which prevent the full substantiation of the inhibin hypothesis. Attention should be focused on the purification of inhibin. If a purified inhibin could be obtained, many lines of further investigations are open. This might lead to practical applications of inhibin in regulation of fertility and in diagnostics of disorders in fertility.

SAMENVATTING

Bestudering van vruchtbaarheid en onvruchtbaarheid bij zoogdieren vereist inzicht in de factoren die morfologie en functie van de geslachtsorganen, de testikels en de ovaria (eierstokken), reguleren. Het is bekend dat in zowel mannelijke als vrouwelijke zoogdieren het centrale zenuwstelsel invloed uitoefent op de hypofyse (hersenaanhangsel) om gonadotrope (op de geslachtsorganen inwerkende) hormonen af te geven. Deze invloed op de geslachtsorganen wordt uitgeoefend door follikel-stimulerend hormoon (FSH) en luteïniserend hormoon (LH). Op hun beurt reageren de ovaria en testikels op deze hormonen met de produktie van voortplantingscellen en geslachtshormonen. Samen met andere factoren werden steroïdale geslachtshormonen verantwoordelijk geacht voor de controle op de hoeveelheid FSH en LH afgegeven door de hypofyse. In de afgelopen jaren werd echter duidelijk dat niet alleen steroïdhormonen betrokken zijn bij de controle op de afgifte van FSH en LH, maar dat ook niet-steroïdale factoren hierbij een rol spelen.

In Hoofdstuk 1 staat beschreven hoe in de twintiger en dertiger jaren van deze eeuw proeven werden uitgevoerd die leidden tot de suggestie dat de testikel, behalve steroïdhormonen, een niet-steroïdale factor maakt die het optreden van castratiecellen in de hypofyse van gecastreerde dieren kan voorkómen. Deze hypothetische factor werd inhibine genoemd. Dankzij bepalingsmethoden voor gonadotrope hormonen ontdekte men vanaf de veertiger jaren in mannen met verstoorde spermatozoa ontwikkeling verhoogde bloedspiegels van FSH, maar niet van LH. Dit leidde tot de suggestie dat de productie van inhibine in die situaties verminderd kon zijn. Toch verkreeg de inhibine-hypothese geen uitgebreide steun, omdat het onmogelijk bleek inhibine rechtstreeks aan te tonen en in zuivere vorm te verkrijgen. Daarom werd in de vijftiger jaren algemeen aangenomen dat steroïdhormonen alléén de afgifte van FSH and LH konden reguleren. Toch bleven resultaten, verkregen bij de mens en in proefdieren, erop wijzen dat steroïdhormonen alléén niet verantwoordelijk konden zijn voor de regulatie van de afgifte van FSH. Dit leidde in de zeventiger jaren tot een hernieuwde belangstelling voor inhibine.

In Hoofdstuk 2 staan (deels eigen) experimenten van recente datum beschreven die erop gericht zijn om inhibine aan te tonen. Hierbij wordt inhibine nu gedefiniëerd als " een in water oplosbare, niet-steroïdale factor die de afgifte door de hypofyse van FSH, maar niet van LH, remt in een aantal verschillende bepalingsmethoden ". Inhibine kon aangetoond worden in een verscheidenheid van vloeistoffen afkomstig uit mannelijke en vrouwelijke voortplantingsorganen door gebruik te maken van een groot aantal methoden. De methoden waarbij veranderingen in orgaangewichten

bepaald worden na toediening van de te testen vloeistoffen worden als minder specifiek beschouwd. Als meer specifiek beschouwt men methoden om inhibine aan te tonen die gebaseerd zijn op veranderingen in concentraties van FSH en LH na toediening van de te testen vloeistoffen. Deze laatste methoden behelzen (a) in vivo proeven waarin met name ratten worden gebruikt en (b) in vitro proeven waarin gebruik gemaakt wordt van uit rattehypofyses losgemaakte cellen. De in vitro methoden hebben het voordeel dat kleine hoeveelheden inhibine kunnen worden bepaald. Ook werden er pogingen ondernomen om radioimmunologische bepalingsmethoden voor inhibine te ontwikkelen, maar deze methoden lijken nog niet erg betrouwbaar. Op dit moment lijkt de meest betrouwbare methode om de aanwezigheid van inhibine aan te tonen een combinatie van de in vitro methode waarin gebruik gemaakt wordt van uit de hypofyse losgemaakte cellen, samen met een in vivo methode waarbij gebruik gemaakt wordt van onmiddellijk tevoren gecastreerde mannelijke prepuberale of langdurig geovariectomeerde vrouwelijke volwassen ratten. Toch heeft het gebruik van veel bepalingsmethoden voor het aantonen van inhibine en het gebruik van veel bronnen die vermoedelijk inhibine bevatten niet geleid tot een opheldering van de natuurkundige en scheikundige karakteristieken van inhibine, noch tot een zuivering van één enkele stof met inhibine activiteit. Wel maakten de proeven die tot nu toe werden uitgevoerd duidelijk dat de hoeveelheid inhibine variëert met de grootte van de antrale,ovariële follikels en met het stadium van de voortplantingscyclus. Dit leidde tot de opvatting dat inhibine een fysiologische rol zou kunnen spelen in de regulatie van de secretie van FSH.

In Hoofdstuk 3 worden eigen experimenten, voornamelijk met vrouwelijke ratten, beschreven waaruit een fysiologische rol voor inhibine blijkt. Inhibine werd aangetoond in ovariëel follikelvocht van verschillende diersoorten. Tot de compartimenten van het ovarium verantwoordelijk voor de productie van inhibine lijken in ieder geval te behoren de middelgrote en grote antrale follikels, met name de granulosa cellen. Ook kon aannemelijk gemaakt worden dat inhibine wordt afgegeven aan de bloedsomloop en dus de hypofyse kan bereiken. De reactie van de hypofyse op inhibine zoals aanwezig in met actieve kool behandeld runderfollikelvocht (uitgedrukt als een daling van de afgifte van FSH) blijkt toe te nemen met de leeftijd in vrouwelijke, maar niet in mannelijke ratten. In volgende experimenten werd aangetoond dat de regulatie van de afgifte van FSH door inhibine in mannelijke ratten blijkbaar beperkt is tot de prepuberale periode, maar in vrouwelijke ratten van toenemend belang wordt naar volwassenheid toe. Dit leidde tot de veronderstelling dat vooral in volwassen vrouwelijke ratten inhibine een rol kan spelen in de regulatie van de afgifte van FSH. Inderdaad lijkt in volwassen vrouwelijke ratten inhibine betrokken te zijn bij die fysiologische en experimentele situaties waarin bloedspiegels van FSH, maar niet van LH, beginnen te stijgen. Ook kon aannemelijk gemaakt worden dat verhoogde bloedspiegels van FSH via een versnelde groei van

follikels een toename in inhibine-produktie veroorzaken, hetgeen op zijn beurt weer een verlaging in de bloedspiegels van FSH veroorzaakt. Zo ontstond de hypothese dat inhibine - via controle van de afgifte van FSH - een rol speelt in de regulatie van het aantal follikels dat begint te rijpen. Tenslotte werden resultaten gepresenteerd die suggereren dat inhibine een rol kan spelen in het op gang brengen van een mechanisme om follikels te beschermen tegen een onderbroken stimulatie door FSH.

In de Algemene Discussie worden de gepresenteerde gegevens geëvalueerd. Het bestaan van inhibine is aannemelijk en het speelt in zowel mannelijke als vrouwelijke zoogdieren blijkbaar een fysiologische rol, vooral in die situaties wanneer cellen betrokken bij de voortplanting zich in aantal vermeerderen. Echter, de onzuiverheid van de preparaten die inhibine bevatten en de beperkingen van de biologische bepalingsmethoden die gebruikt worden zijn belangrijke factoren die verhinderen dat de deugdelijkheid van de inhibine-hypothese volledig aangetoond kan worden. De aandacht dient gericht op de zuivering van inhibine. Mocht een gezuiverd inhibine verkregen worden, dan liggen veel lijnen voor onderzoek open. Dit zou kunnen leiden tot praktische toepassingen van inhibine in de regulatie van vruchtbaarheid of in de diagnostiek van (on)vruchtbaarheid.

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MET DANK AAN:

Al diegenen die direct of indirect betrokken zijn geweest bij het onderzoek hier beschreven en de uiteindelijke vormgeving van dit boekje. Dat zijn er velen.

Het legioen belastingbetalers maakte dit onderzoek financiëel mogelijk; ik ben me daar dan ook immer bewust van geweest. De faciliteiten geboden door de Erasmus Universiteit Rotterdam waren uitstekend, wat er beslist aan meegeholpen heeft mijn aversie tegen massale instellingen te doen verminderen. Maar geld en een instituut alleen zijn niet voldoende; de stimulerende invloed en inzet van velen was onontbeerlijk.

De medewerkers van de afdeling Anatomie schiepen voldoende ruimte voor een "passant". Secretariëel stonden Edith en Lous mij met raad en daad bij. Wetenschappelijke discussies met de leden van de Endo-club, vooral die met Jiet, brachten mij herhaaldelijk terug op het juiste spoor. Maar in het bijzonder de hulp, belangstelling en vriendschap van Piet, Richard, Chris, Cobie en Rob waren plezierige bestanddelen van mijn werkomgeving.

De praktische uitvoering van de proeven kon tot stand komen dankzij de continue, prima verzorgde aanvoer van talloze proefdieren, waarvoor in de loop der jaren George, Ingrid, Bas, Gerrit en Lilian tekenden. De grote hoeveelheden follikelvocht werden verkregen dankzij de welwillende medewerking van het personeel van de slachthuizen te Rotterdam en later te Bodegraven. De afdeling Biochemie 2 bood uitstekende faciliteiten voor het uitvoeren van de celkweken; evenzeer mijn dank aan de medewerkers aldaar voor de tolerantie ten opzichte van "vreemde eenden in de bijt".

Een zeer groot aandeel in het onderzoek hier beschreven werd geleverd door Liesbeth en Til. Liesbeth bleek met een nooit aflatend goed humeur ontelbare hoeveelheden monsters te kunnen verwerken en excelleerde in "lekker bezig zijn". Til moest haar voorkeur voor in vivo werkzaamheden bezuren met de nodige nachtelijke gemiste uren slaap, en was een steun en toeverlaat in statistiek, nadenk-sessies en de uitvoering van dit boekje. Maar de hoofdzaak van onze goede samenwerking zat toch wel in de bereidheid iets voor 'n ander over te hebben en in de vele, juist buiten het werk doorgebrachte, gezellige uurtjes.

Middelen en inzet zouden echter nooit dit proefschrift opgeleverd hebben indien Rein, Frank en Han zich niet over mijn wetenschappelijke opvoeding ontfermd hadden. Rein leverde, als "grote meester", de aanzet en stimulans om het inhibine-onderzoek te beginnen, en begeleidde me met de eerste wankele schreden op het wetenschappelijke pad. Frank's nooit aflatende stroom van ideeën werkte enorm stimulerend; hopenlijk heb ik wat van zijn enthousiasme, werklust en kritische houding overgenomen. Ik vond de samenwerking uiterst plezierig. Han, als direct betrokken afdelingshoofd en nu als promotor, heb ik

zeer gewaardeerd. Ik ben hem vooral dankbaar voor de snelheid van manuscripten lezen, de vele kritische kanttekeningen daarbij en zijn nooit aflatende steun en begeleiding, maar evenzeer belangrijk was de vrijheid die hij ons als team liet. De fijne puntjes op de i tenslotte werden geleverd door de coreferenten Prof. Dr. G. P. van Rees en Prof. Dr. H. J. van der Molen. Ik ben hun dankbaar voor de kostbare tijd die ze aan dit proefschrift besteed hebben en de geleverde kritiek, en Henk in het bijzonder voor de talrijke gevoerde discussies.

Rest mij te melden dat ook het thuisfront, waaraan dit proefschrift is opgedragen, zich niet onbetuigd heeft gelaten. Mijn vader, Chris, legde met zijn grote nieuwsgierigheid naar alles en nog wat bij mij de basis voor mijn latere wetenschappelijke belangstelling. Helaas heeft hij het afronden van mijn studie niet mogen meemaken. Mijn moeder, Ans, heeft mij vooral in de afgelopen maanden zodanig verwend en verzorgd dat ik mijn aandacht alleen maar op dit proefschrift hoefde te richten; bovendien zorgde zij voor de broodnodige opvang van mijn slechte buien. Bob's aandeel in dit proefschrift lijkt gering; toch was hij het die mij over de drempel heen hielp, en telkens maar weer nieuwe alternatieven verzon indien er wéér een tijdschema uit de hand liep. Dankzij zijn soepelheid konden wij beiden ons "ei" verwezenlijken. Moge dit proefschrift een - weliswaar kleine - compensatie zijn voor de vele hierdoor eenzaam doorgebrachte uren.

CURRICULUM VITAE

Elk bereikt doel
is weer het begin
van een nieuwe tocht
en zo
tot in het oneindige
Arthur Schopenhauer

Ik werd in 1952 in Nijmegen geboren, en groeide op in Bilthoven, alwaar de schooljaren probleemloos voorbij vlogen. Te Utrecht werden in 1970 het eindexamen HBS-B, en in 1973 het kandidaatsexamen Biologie behaald, waarna ik studentassistente werd voor Vergelijkende Fysiologie.Daarna brak een uiterst drukke tijd aan. In 1974 begon ik biologie les te geven aan een scholengemeenschap voor LBO en AVO te Rotterdam, naar welke stad ik alras verhuisde. De combinatie nakandidaatsstudie - lesgeven werd aanzienlijk leuker toen ik in 1976 het LBO en AVO kon inruilen voor de Rotterdamse Avondscholengemeenschap, afdelingen VWO en " Moedermavo ". Aan deze school heb ik tot medio 1981 - weliswaar voor een steeds minderend aantal lesuren per week - uiterst plezierig gewerkt. Tijdens mijn nakandidaatsstudie kwam ik in 1975 terecht op de afdeling Anatomie van de Erasmus Universiteit Rotterdam. In 1976 werd de MO-akte Biologie, en in 1979 - met een cum - het doctoraalexamen Biologie, met als hoofdvak Vergelijkende Endocrinologie en als bijvakken Pedagogiek en Didactiek van de Biologie en Algemene Plantkunde, behaald. Mijn tijdelijke aanwezigheid op de afdeling Anatomie veranderde van 1977 tot en met eind 1981 in een meer permanente, mede dankzij een poolplaats aanstelling die mij in staat stelde het onderzoek waar ik tijdens mijn studie mee was begonnen af te ronden met dit proefschrift.

APPENDIX PAPERS

J. Reprod. Fert. (1977) 50, 129–131

Effects of an inhibin-like factor present in bovine and porcine follicular fluid on gonadotrophin levels in ovariectomized rats

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De Jong & Sharpe (1976) recently observed that bovine follicular fluid from which steroids had been removed reduced peripheral levels of FSH but not of LH when injected into newly castrated male rats. The effect was ascribed to a factor resembling testicular inhibin. In the present report, observations were extended to the effects of this inhibin-like factor on FSH and LH levels in female rats. Attention has also been paid to the question of whether the factor is present in species other than the cow and whether the factor is present in antral follicles of all sizes.

Follicular fluid was aspirated from follicles of cows and pigs. Bovine follicular fluid was pooled according to the size of the follicles, i.e. <5 mm (BFF < 5), 5–10 mm (BFF 5–10), 11–20 mm (BFF 11–20) and >20 mm (BFF > 20) in diameter as measured on the ovarian surface, whereas porcine follicular fluid (PFF) was pooled without regard to the size of follicles. Histological observation of bovine follicles showed that those >20 mm in diameter were cystic. To remove steroids, follicular fluid was stirred with charcoal (50 mg/ml) at room temperature (21 ± 1°C) for 30 min and centrifuged at 12,000 *g* for 60 min. The concentrations of oestradiol-17β and progesterone after charcoal treatment were <0·5% of those before treatment (see Table 1).

Table 1. Concentrations (ng/ml) of oestradiol-17β and progesterone in follicular fluid obtained from bovine follicles of various sizes and from porcine follicles before and after charcoal treatment

Species	Follicles: Diameter (mm)	Oestradiol-17β*		Progesterone†	
		Before	After	Before	After
Bovine	<5	5	0·01	283	—
	5–10	21	0·02	115	0·52
	11–20	103	0·04	150	0·33
	>20 (cystic)	24	0·004	348	1·03
Porcine	(pooled)	14	0·02	280	1·00

* Measured by radioimmunoassay (de Jong, Hey & van der Molen, 1973).
† Measured by radioimmunoassay (de Jong, Baird & van der Molen, 1974).

The Wistar rats used (150–180 g) had shown at least two regular 5-day cycles immediately before the cycle in which they were used. They were unilaterally or bilaterally ovariectomized or sham-operated on the 2nd day of dioestrus at 09.00 h. The rats were given an intraperitoneal injection of saline (0·9% NaCl), charcoal-treated follicular fluid or bovine plasma obtained from a castrated bull which shows a protein composition similar to that of follicular fluid (Caravaglios & Cilotti, 1957). All experimental groups consisted of 6–8 rats. Blood was taken under light ether anaesthesia from the ophthalmic venous plexus 4 and 8 h after the injection. Serum FSH and LH concentrations were estimated by radioimmunoassay as described previously (Welschen *et al.*, 1975). All values are expressed in terms of the NIAMDD-rat-FSH/LH-RP1 standards. For statistical analysis of results Wilcoxon's two sample test was used.

Experiment 1. Rats were bilaterally ovariectomized or sham-operated. At 09.00 h on the 12th day after operation 250 µl saline, bovine plasma or charcoal-treated BFF 11–20 were injected. As shown in Table 2, serum FSH concentrations were increased to about 1700% and LH levels to 500–900% of control values by the ovariectomy. Bovine plasma had no effect on gonadotrophin levels, but follicular fluid injection was followed by a reduction of FSH levels but not of LH levels. The decrease of FSH concentration was modest at 4 h but reached significance at 8 h.

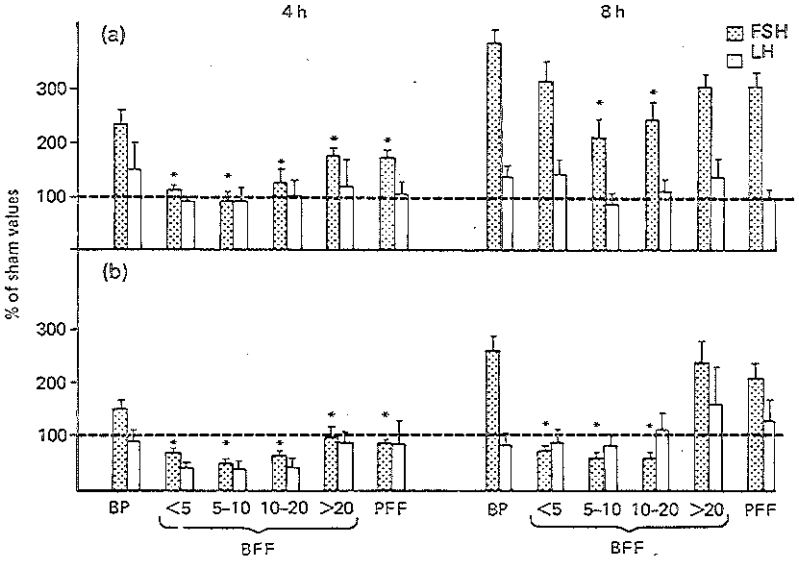
Table 2. Effect of injection of saline, bovine plasma or charcoal-treated bovine follicular fluid from follicles of 11–20 mm diameter on mean (±S.E.M.) FSH and LH levels (ng/ml) in bilaterally ovariectomized rats 4 and 8 h after injection

Ovariectomy	Material injected	FSH		LH	
		4 h	8 h	4 h	8 h
−	Saline	92 ± 7	114 ± 14	44 ± 9	87 ± 56
+	Saline	1771 ± 36	1762 ± 64	246 ± 15	287 ± 29
+	Bovine plasma	1788 ± 56	1754 ± 36	340 ± 45	361 ± 48
+	BFF 11–20	1565 ± 71	1044 ± 123*	361 ± 36	372 ± 32

* $P < 0{\cdot}01$ compared with bovine plasma value.

Experiment 2. In a first series, rats were unilaterally ovariectomized or sham-operated and received immediately afterwards an injection of 60 µl saline, bovine plasma or of one of the five preparations (see Table 1) of charcoal-treated follicular fluid. In a second series rats received 250 µl of the preparations but were otherwise treated similarly.

At 4 and 8h after injection, sham-operated saline-treated rats had mean (±S.E.M.) FSH levels of 105 ± 10 and 106 ± 8 ng/ml (first series) or of 145 ± 6 and 147 ± 12 ng/ml (second series); LH levels were 34 ± 6 and 38 ± 5 ng/ml and 31 ± 6 and 40 ± 11 ng/ml respectively. Unilaterally ovariectomized rats showed an increase of FSH levels to 183 ± 10% (at 4 h) and 344 ± 21% (at 8 h) of control values



Text-fig. 1. Effect of injection of (a) 60 µl or (b) 250 µl of bovine plasma (BP), charcoal-treated bovine follicular fluid (BFF) from follicles of <5, 5–10, 11–20 or >20 mm diameter or porcine follicular fluid (PFF) on FSH and LH levels in female rats at 4 and 8 h after unilateral ovariectomy. All values are expressed as the percentage of mean values of sham-operated rats and are given as means ± S.E.M. *$P < 0{\cdot}05$ compared with bovine plasma values.

in the first series and to 140 ± 8 and 323 ± 21% in the second series, whereas LH levels were not significantly changed. These effects of unilateral ovariectomy were similar to those described earlier (Welschen & Dullaart, 1974). Values after treatment with bovine plasma or follicular fluid are shown in Text-fig. 1. Treatment with bovine plasma did not influence gonadotrophin levels after unilateral ovariectomy. However, 60 µl of any follicular fluid preparation resulted in significantly reduced FSH levels at 4 h after injection, and at 8 h FSH levels were still reduced in rats treated with BFF 5–10 and BFF 11–20. Injection of 250 µl follicular fluid generally resulted in a more marked reduction of FSH levels, but still BFF > 20 and PFF failed to prevent the increase of FSH levels over a period of 8 h. In an additional group of rats injected with 1000 µl BFF > 20 or PFF, the FSH levels at 8 h were 108 ± 7 and 96 ± 12% of the sham values. LH levels were never significantly changed after injection of follicular fluid.

Several pieces of evidence have been reported to suggest that an inhibin-like factor might be involved in the regulation of FSH secretion in the female: a peptidic factor from bull semen prevents ovarian maturation in parabiotic pairs of immature female–mature ovariectomized female rats (Lugaro, Giannattasio, Ciaccolini, Fachini & Gianfrancesschi, 1969; Lugaro, Carrea, Casellato, Mazzola & Fachini, 1973); rete testis fluid reduces the uterine weight response to HCG in mice (Setchell & Sirinathsinghji, 1972) and reduces peripheral FSH levels in 21-day-old female rats (Setchell & Jacks, 1974). Moreover, immunization of rabbits with the inhibin fraction of bull seminal plasma has produced an antiserum which caused increased plasma FSH levels when injected into male and female rats (Franchimont, Chari, Hagelstein & Duraiswami, 1975). The present experiments demonstrate that an inhibin-like factor of ovarian origin (1) is able to suppress FSH secretion in female rats under two different experimental conditions, leaving LH secretion undisturbed; (2) is also present in porcine follicular fluid; and (3) is present in small antral follicles of cows and reaches a higher concentration in medium and large antral follicles, but shows a lower concentration in cystic follicles. Provided follicles do secrete inhibin into the blood, it seems attractive to suggest that in the female inhibin plays a role in the regulation of FSH secretion, especially during those phases of reproductive life in which medium- and large-sized follicles are present.

We gratefully acknowledge the technical assistance of Mrs A. A. de la Fosse, Miss M. Debets and Mr A. Bruynzeel. This study was supported in part by the Organization of Medical Research in the Netherlands (FUNGO).

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Received 20 October 1976

J. Reprod. Fert. (1980) 60, 485–493

II

Possible involvement of inhibin in the interrelationship between numbers of antral follicles and peripheral FSH concentrations in female rats

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Summary. The possible dependence of peripheral concentrations of FSH on a non-steroidal, ovarian factor, was studied in adult female rats. Increases in FSH levels during the periovulatory period were not correlated with decreases of steroid concentrations, and administration of steroids did not result in a reduction of FSH levels to basal values. However, a negative correlation between FSH levels and numbers of large follicles (volume $\geqslant 200 \times 10^5$ μm^3) was demonstrated, and injection of steroid-free bovine follicular fluid, which contains inhibin-like activity, suppressed FSH levels to basal values.

These results suggest that an ovarian, inhibin-like factor is involved in the fast regulation of FSH concentrations in the periovulatory period, and that this inhibin-mediated control of FSH might play a role in the regulation of the number of follicles maturing in female rats.

Introduction

Campbell & Schwartz (1977) provided evidence that in adult female rats the control of pituitary secretion of follicle-stimulating hormone (FSH) may differ from that of luteinizing hormone (LH). Secretion of FSH, but not of LH, is greatly enhanced about 5–12 h after unilateral ovariectomy (Welschen & Dullaart, 1974; Campbell, Schwartz & Gorski-Firlit, 1977; Butcher, 1977; Welschen, Dullaart & de Jong, 1978), or, in intact rats, immediately following the ovulatory surge of LH release (Gay, Midgley & Niswender, 1970). Evidence is accumulating that in such circumstances the rise of FSH secretion is not dependent on a drop in peripheral levels of ovarian steroid hormones. In studies on steroid levels after unilateral ovariectomy there was no decrease of oestradiol levels during the first 24 h (Campbell *et al.*, 1977; Butcher, 1977; Welschen *et al.*, 1978). Only Chappel & Barraclough (1977) found decreased oestradiol levels after ovariectomy at pro-oestrus in 4-day cyclic rats. However, replacement therapy with oestradiol (Chappel & Barraclough, 1977; Butcher, 1977) was ineffective in preventing the acute rise in FSH levels after unilateral ovariectomy, whereas antiserum against oestradiol-17β injected into intact rats did not provoke such a rise (Butcher, 1977). Peripheral levels of progesterone were decreased immediately after unilateral ovariectomy, but adrenalectomy, resulting in decreased oestradiol and progesterone levels (Campbell *et al.*, 1977), did not induce an acute rise in FSH secretion.

In 1932, McCullagh postulated that a non-steroidal, specifically FSH-suppressing substance, "inhibin", is secreted by the male gonad. There is considerable evidence for the existence of inhibin in male animals (see for reviews Baker *et al.*, 1976; Setchell, Davies & Main, 1977;

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Chari, 1977; de Jong, 1979), and inhibin-like activity has been detected in follicular fluid of cows (de Jong & Sharpe, 1976; Hopkinson *et al.*, 1977; Welschen, Hermans, Dullaart & de Jong, 1977), sows (Marder, Channing & Schwartz, 1977; Welschen *et al.*, 1977), women (Daume, Chari, Hopkinson, Sturm & Hirschhäuser, 1978), and mares (Miller, Wesson & Ginther, 1979), and might play an important role in the regulation of FSH secretion in the female animal (Marder *et al.*, 1977; Schwartz & Channing, 1977; Welschen *et al.*, 1978). Inhibin-like activity in bovine follicular fluid and in medium from cultured rat Sertoli cells suppresses the unstimulated release of FSH but not of LH from cultured pituitary cells in parallel ways (de Jong, Smith & van der Molen, 1979).

The present paper describes experiments investigating the involvement of inhibin-like activity in the regulation of FSH secretion by the pituitary gland during the periovulatory period of rats.

Materials and Methods

Animals. Adult female rats of a Wistar substrain (R-Amsterdam) were kept under controlled conditions of light (light period 05:00–19:00 h) and temperature (22–24°C) and received standard dry pellets and tap water *ad libitum.* Daily vaginal smears were taken and only rats with 2 consecutive 5-day cycles before the cycle of treatment were used.

Follicular fluid. Cow ovaries were obtained at a local slaughterhouse, and follicular fluid was aspirated from follicles of 10–20 mm diameter immediately after collection of the ovaries. The bovine follicular fluid was stirred with charcoal (50 mg/ml) at 21 ± 1°C for 60 min and centrifuged at 10 000 *g* for 30 min. After treatment, concentrations (ng/ml) of oestradiol-17β (0·04) and progesterone (0·33) were <1% of the original concentrations (103 and 150 respectively).

Bovine plasma was used as control fluid because it has a protein composition similar to that of follicular fluid (Caravaglios & Cilotti, 1957). Injections of bovine follicular fluid and bovine plasma were given intraperitoneally.

Experiments. All operations, injections and blood collections were performed under light ether anaesthesia. Animals were bled by puncture of the ophthalmic venous plexus. At least 6 rats were used in all groups of control and experimental animals unless otherwise stated. Details of the experiments are given in 'Results'.

Hormone determinations. Serum FSH and LH concentrations were estimated by radioimmunoassay (RIA) as described previously (Welschen *et al.*, 1975). All results are expressed in terms of NIAMDD-rat-FSH-RP1 and NIAMDD-rat-LH-RP1. Inter-assay variability (coefficients of variation) was 16% for FSH and 14% for LH.

Oestradiol, progesterone and testosterone were assayed by radioimmunoassay as described by de Jong, Hey & van der Molen (1973), de Jong, Baird & van der Molen (1974) and Verjans, Cooke, de Jong, de Jong & van der Molen (1973). Results were corrected for recovery after extraction and chromatography on LH-20 microcolumns. The latter technique was used for the assay of oestradiol and testosterone. The detection limits of the various assays, defined as the blank + twice the standard deviation at the level of the blank, were 5 (oestradiol) and 10 (progesterone and testosterone) pg/tube. Interassay variability, calculated from repeated results of pooled plasma samples, varied between 10 and 15%. The main cross-reacting steroids in these assays were dihydrotestosterone (60%, expressed as (mass of 'measured steroid' suppressing %B to 50% of B_0/mass of cross-reacting steroid causing the same suppression) × 100%) in the testosterone assay; oestrone (3%) and oestriol (5%) in the oestradiol assay and 11β-hydroxyprogesterone (17%) in the progesterone assay.

Follicle counts. Follicles were counted in ovarian sections of 5 μm after routine histological procedures (fixation in Bouin's fluid, staining with haematoxylin and eosin). Follicles were classified using the method of Boling, Blandau, Soderwall & Young (1941), as slightly modified

by Welschen (1973). The volume classes correspond with a mean follicle diameter and with the stage numbers used by Mandl & Zuckerman (1952) approximately in the following way: 200–499 × 10^5 μm³ with 350–450 μm and stages 3 and 4; ⩾500 × 10^5 μm³ with ⩾450 μm and stages 5 to 8.

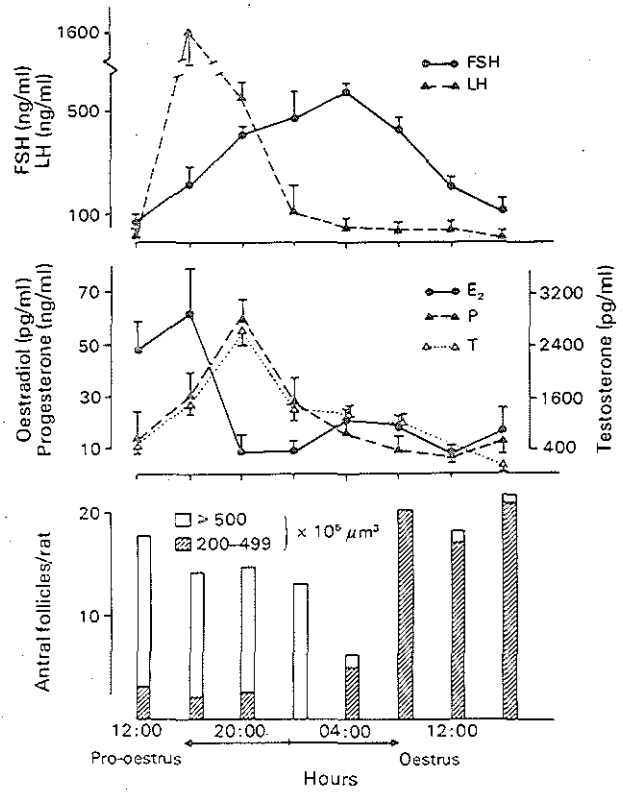
Statistics. For statistical analysis of results the Student's *t* test or the Wilcoxon two-sample test were used. A difference was considered as statistically significant when the double-tail probability was <0·05.

Results

The interrelationship between inhibin-like activity and ovarian steroid hormones on the one hand, and gonadotrophin levels and numbers of antral follicles during pro-oestrus and oestrus on the other were studied in 2 experiments to investigate the effect of bovine follicular fluid on the secondary rise of FSH at pro-oestrus.

Experiment 1

Rats were killed and bled at 4-h intervals between 12:00 h on the day of pro-oestrus and 16:00 h on the day of oestrus. Serum and plasma were used for FSH, LH, oestradiol,



Text-fig. 1. Peripheral levels (mean ± s.e.m.) of FSH, LH, oestradiol-17β (E_2), progesterone (P) and testosterone (T) and numbers of follicles ⩾200 × 10^5 μm³ in adult female rats (N = 5) between 12:00 h on the day of pro-oestrus and 16:00 h on the day of oestrus.

progesterone and testosterone determinations and ovaries were fixed in Bouin's fluid for follicle counts. The results are given in Text-fig. 1. All hormone concentrations followed the well-known patterns (see Gay & Tomacari, 1974; Nequin, Alvarez & Schwartz, 1975), although the absolute values of testosterone appeared considerably lower than those reported by Gay & Tomacari (1974). Ovulation apparently occurred in all follicles $\geqslant 500 \times 10^5$ μm³ between 00:00 and 04:00 h on the day of oestrus. A new group of follicles with a well-developed antrum (size class $\geqslant 200 \times 10^5$ μm³) was observed from 04:00 h on the day of oestrus onwards. During the period between 12:00 h on the day of pro-oestrus and 16:00 h on the day of oestrus there was a significant negative correlation between mean numbers of follicles in this size class and mean peripheral FSH levels ($r = -0{\cdot}65$, $n = 8$, $P < 0{\cdot}05$).

Experiment 2

At 14:00 h on the day of pro-oestrus the rats received 500 μl bovine follicular fluid or plasma/100 g body weight or 3 silicone-tube implants that were empty or contained oestradiol, progesterone or testosterone. The lengths and outer diameter of the tubings were 5 and 1·4 mm for oestradiol; 5 cm and 3·8 mm for progesterone; and 5 and 3·8 mm for testosterone. Oestradiol and testosterone were mixed 1:10 with cholesterol (w/w). The tubings were placed in phosphate-buffered saline, pH 7·2, overnight before implantation. The rats were bled and killed at 04:00 h and 12:00 h on the day of oestrus for hormone determinations and follicle counts. Of

Table 1. Influence of administration of steroid hormones or bovine follicular fluid on mean ± s.e.m. peripheral levels of FSH and LH and on numbers of antral follicles at early oestrus

Treatment at 14:00 h on day of pro-oestrus	Time of autopsy (h) on day of oestrus	No. of rats	Hormone conc. (ng/ml)		No. of follicles $\geqslant 200 \times 10^5$ μm³ per ovary
			FSH	LH	
Controls (3 empty tubes + bovine plasma)	04:00	8	394 ± 29	82 ± 4	2·0 ± 0·7
	12:00	8	274 ± 19	45 ± 6	11·5 ± 2·5
Steroid implants† (see text)	04:00	7	328 ± 13	41 ± 5*	4·5 ± 1·3
	12:00	8	249 ± 25	32 ± 11	8·6 ± 0·6
Bovine follicular fluid (500 μl/100 g body wt)	04:00	7	54 ± 2*	52 ± 14	1·7 ± 0·4
	12:00	7	136 ± 17*	52 ± 8	4·5 ± 0·5*

† Mean of 2 determinations in pooled plasma of control rats and implant-bearing rats at 04:00 h at oestrus: 220 and 3750 pg testosterone/ml; not determined and 831 pg oestradiol/ml; and 12 and 53 ng progesterone/ml respectively.

* $P < 0{\cdot}05$ compared with corresponding control values.

Table 2. Influence of administration of testosterone by means of an implant placed subcutaneously or on the surface of each ovary on mean ± s.e.m. plasma concentrations of FSH and numbers of antral follicles at oestrus

Treatment at 14:00 h on day of pro-oestrus	No. of rats	At autopsy at 16:00 h on day of oestrus	
		FSH (ng/ml)	No. of follicles $\geqslant 200 \times 10^5$ μm³/ovary
Control	10	188 ± 8	11·8 ± 0·6
Implant, subcutaneous	9	282 ± 18*	9·0 ± 1·4
Implant, on ovarian surface	9	559 ± 29*	5·0 ± 0·8*

Mean of 2 determinations of testosterone concentrations (pg/ml) in pooled plasma at 04:00 h on day of oestrus was 720 for controls, 3050 for s.c. implants and 3140 for implants on the ovarian surface.

* $P < 0{\cdot}05$ compared with values in both other groups.

these treatments only injection of bovine follicular fluid (Table 1) resulted in a significant suppression of FSH levels at 04:00 h and 12:00 h on the day of oestrus and in decreased numbers of follicles with a volume of 200–500 × 10^5 μm^3, but there was no effect on the occurrence of ovulation or on LH levels.

Silicone tubes containing testosterone (length 4 mm, o.d. 1·4 mm) were implanted either inside the bursa ovarica on the ovarian surface or subcutaneously. The position of the implants was confirmed at autopsy. Implants resulted in peripheral testosterone concentrations of about 4 times those found in control rats (Table 2); the implants on the ovarian surface produced significantly increased FSH levels at 16:00 h on the day of oestrus, and significantly decreased numbers of antral follicles. Implants placed s.c. induced only a marginal increase of serum FSH levels.

Discussion

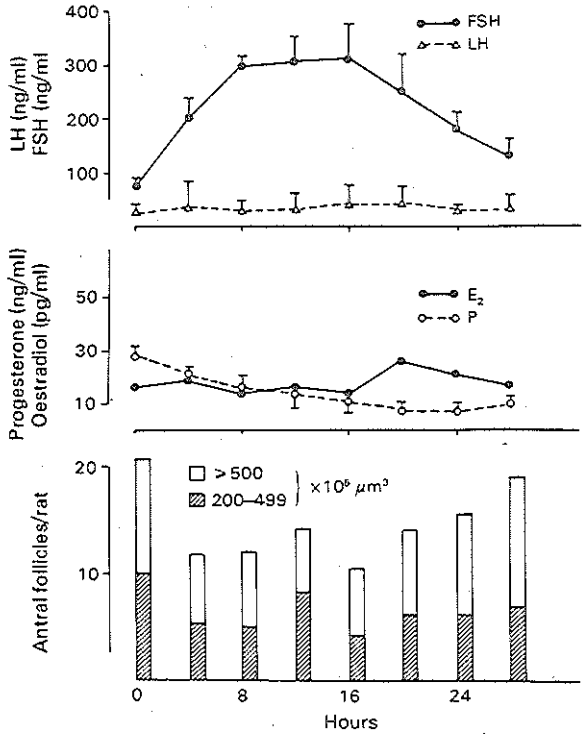
The present results support the hypothesis that in the female rat the ovaries can exert an inhibitory feed-back on the pituitary secretion of FSH via inhibin-like activity during the periovulatory period. The inverse relationship between FSH levels and numbers of follicles of volume $\geqslant 200 \times 10^5$ μm^3 suggests that these follicles secrete inhibin-like activity. Furthermore, antral follicles seem to limit their own number by the inhibin-feedback on FSH secretion.

In the adult female rat injection of inhibin-like material in a dose that causes maximal suppression of FSH levels appears to exert short-term effects on FSH secretion only (de Jong, Welschen, Hermans, Smith & van der Molen, 1978; DePaolo, Wise, Anderson, Barraclough & Channing, 1979; DePaolo, Hirshfield, Anderson, Barraclough & Channing, 1979). Probably inhibin-like activity is inactivated and removed from its target organ within a few hours. If so, changes in inhibin secretion from the ovary may be expected to be followed with a short latency by short-lived changes in FSH secretion.

Since inhibin-like activity has been defined as a factor which specifically suppresses FSH levels (de Jong, 1979) whereas steroid hormones (depending on the dose and time of treatment) influence both FSH and LH secretion, the involvement of inhibin-like activity in diverging changes of FSH and LH levels was studied in the present experiments. In the rat the second phase of FSH release about the time of ovulation occurs in the absence of a high level of LH release (Gay *et al.*, 1970). This second phase of FSH release can be prevented by injection of antiserum to testosterone (Gay & Tomacari, 1974), and was explained as reflecting a facilitatory effect of endogenous testosterone on FSH secretion. The second phase of FSH release can also be prevented by injection of steroid-free follicular fluid (Schwartz & Channing, 1977). The results of the present experiments confirm these latter data. In addition, they demonstrate that increased levels of oestradiol, progesterone and testosterone cannot prevent the second phase of the FSH surge. Thus, if this second phase is induced by diminished inhibitory feedback from the ovary on FSH secretion, it must be due to a relative lack of ovarian release of inhibin-like activity and not to a lack of steroid hormones. Schwartz & Channing (1977) suggest that after a pro-oestrus or an artificial LH stimulus, the oocyte–follicular complex is altered, resulting in secretion of decreased amounts and/or chemically altered inhibin. The second phase of FSH release, thus induced, recruits—as the present experiment shows—a new cohort of follicles of $\geqslant 200 \times 10^5$ μm^3 in size (see also Schwartz, 1969; Welschen & Dullaart, 1976). Furthermore, this cohort of antral follicles, once recruited, seems to suppress FSH secretion to baseline values.

The data from the testosterone-implant experiment seem to confirm the above observations: a subcutaneous implant did not result in decreased numbers of follicles and did not prolong the second phase of FSH release, whereas implants on the ovarian surface did. Androgens produced in the ovary in response to hCG have been shown to act locally to inhibit the effect of oestrogen on follicular growth (Louvet, Harman, Schreiber & Ross, 1975). It therefore seems likely that

testosterone implants on the ovary, instead of exerting a direct facilitatory effect on FSH release, have inhibited the growth of follicles to the antral stage and thus the secretion of sufficiently large amounts of inhibin to suppress FSH to baseline values. Assuming that this really is the case, the decreased FSH levels found by Gay & Tomacari (1974) after injection of antiserum against testosterone might reflect increased follicle growth and a greater inhibin feedback rather than a reduced direct stimulatory effect of testosterone on FSH release, although such a stimulatory effect has been shown to exist in certain circumstances (Drouin & Labrie, 1976; Juneja, Motta, Vasconi & Martini, 1977; Labrie *et al.*, 1978).

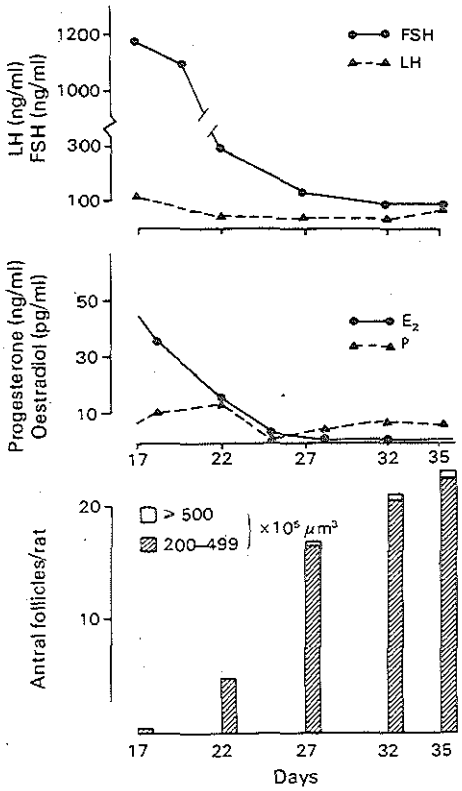


Text-fig. 2. Peripheral levels of FSH, LH, progesterone (mean ± s.e.m. for 5–6 samples) and oestradiol-17β (mean of 2–3 determinations in pooled plasma) and mean numbers of antral follicles $\geqslant 200 \times 10^5$ µm³ in adult female rats 0–28 h after unilateral ovariectomy at 09:00 h on Day 2 of dioestrus. Throughout the experimental period, FSH and LH values in control animals ranged between 50 and 100 ng FSH/ml and between 10 and 30 ng LH/ml (Welschen *et al.*, 1978).

The results of experiments with unilaterally ovariectomized rats also strongly suggest that in other circumstances regulation of FSH levels may occur via inhibin-like activity secreted by antral follicles (Welschen *et al.*, 1978). For comparison these data have been reproduced in Text-fig. 2 and the similarity of the temporal relationship between levels of FSH, LH, steroid hormones and numbers of follicles in (pro)oestrous and in unilaterally ovariectomized rats (Text-figs 1 and 2) is clear. In both cases a decrease of high concentrations of FSH to basal levels is inversely correlated with increasing numbers of healthy follicles $\geqslant 200 \times 10^5$ µm³ to about 25/animal (correlation between mean numbers of follicles and mean peripheral FSH levels in unilaterally ovariectomized rats: $r = -0{\cdot}81$, $n = 8$, $P < 0{\cdot}05$). In normal cyclic rats this number of 25 follicles/animal is never exceeded; smaller numbers (about 15/rat) are only found during the last days of the cycle. At that time, the mean diameter is greatly increased, resulting in

a total number of granulosa cells similar to that present in 25 small follicles during the first days of the cycle (for data on granulosa cell numbers see Hage, Groen-Klevant & Welschen, 1978). This suggests that inhibin is produced predominantly by granulosa cells of antral follicles *in vivo*, as has been shown by Erickson & Hsueh (1978) with in-vitro experiments, and is also supported by data of Welschen *et al.* (1977) and of Becker, Klupp, Epstein, Seidl & Lunenfeld (1977) who showed that, at least in the cow, medium-sized and large antral follicles contain the highest concentration of inhibin-like activity. The inhibin-feedback mechanism may therefore be involved in the regulation of the number of follicles $\geqslant 200 \times 10^5$ μm^3 in size which are to ovulate or to become atretic during each cycle.

A similar control might also exist in the period characterized by the first growth of antral follicles, from Day 21 of life onwards. During this period, the pituitary gland becomes sensitive to inhibin-like activity as present in bovine follicular fluid (Hermans, van Leeuwen, Debets & de Jong, 1980). Levels of FSH, LH, oestradiol and progesterone and the number of follicles $\geqslant 200 \times 10^5$ μm^3, as reported by various workers, reveal time relationships as shown in Text-fig. 3. After the dramatic fall in FSH levels around Day 20, there is a further decrease of FSH levels between 22 and 35 days of age. This decrease is not parallelled by increase of oestradiol levels and there is only a marginal increase of progesterone levels. Since injection of a steroid combination was



Text-fig. 3. Peripheral levels of FSH and LH (Ojeda & Ramirez, 1972; Döhler & Wuttke, 1975; Meijs-Roelofs, Uilenbroek, Osman, & Welschen, 1973a) and of oestradiol-17β (Meijs-Roelofs, Uilenbroek, de Jong & Welschen, 1973b; Döhler & Wuttke, 1975) and progesterone (Meijs-Roelofs, de Greef & Uilenbroek, 1975; Döhler & Wuttke, 1975) and numbers of follicles $\geqslant 200 \times 10^5$ μm^3 (Meijs-Roelofs *et al.*, 1973a; and unpublished studies) in intact female rats between 17 and 35 days of age.

only effective in suppressing FSH levels at 25 days of age and not at later ages (Hermans *et al.*, 1980), the involvement of another factor in FSH regulation seems more likely. That this factor might be inhibin-like activity is suggested by the finding that the decrease of FSH levels finds its mirror image in the increase of the number of antral follicles (Text-fig. 3) and the finding that a rapid rise of FSH levels after ovariectomy occurs from 25 days of age onwards. This increase can be prevented by injection of steroid-free bovine follicular fluid (Hermans *et al.*, 1980).

In combination, the present data seem to suggest that from the age of 25–35 days onwards the regulation of FSH secretion is (at least partly) under the control of ovarian inhibin secretion. Inhibin might be produced by the granulosa cells of antral follicles. The data suggest that in late prepubertal and adult female rats a number of granulosa cells, as present in about 25 antral follicles in the size range of 200–500 $\times$ 10^5 μm^3 or in smaller numbers of larger follicles, produce amounts of inhibin sufficient to maintain peripheral FSH concentrations at about 100 ng/ml. This level of FSH seems sufficient to keep the number of healthy granulosa cells fairly constant. Any decrease in the number of inhibin-producing granulosa cells (or follicles) results in an enhanced pituitary secretion of FSH during at least 5–20 h after the decrease. The increased FSH levels then seem to stimulate granulosa cell proliferation to such an extent that the critical number required for inhibin levels sufficient to reduce FSH concentrations to basal values is reached.

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Received 16 May 1980

QUANTIFICATION OF THE CONTENT OF INHIBIN IN SPENT MEDIA FROM CULTURED RAT OVARIAN GRANULOSA CELLS

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submitted for publication

SUMMARY

Granulosa cells were isolated from antral follicles of intact, adult female rats by treatment with EGTA and hypertonic sucrose, and kept in culture for 1, 15 or 37 days. The amount of inhibin present in the spent media was assessed using a bioassay for inhibin (measuring the unstimulated release of FSH and LH by pituitary cells in culture). After 1 day in culture detectable amounts of inhibin were present in the medium of various amounts of granulosa cells: in the bioassay maximal suppression of FSH occurred when 0.8×10^5 granulosa cells/dish were plated. Using this or a larger number of cells in long-term cultures, it was shown that spent rat granulosa cell culture media (rGCCM), collected up to 37 days after plating, contained inhibin. The suppression of FSH by addition of rGCCM in the bioassay was parallel to the suppression found after addition of charcoal-treated bovine follicular fluid, bFF, which served as a standard preparation for inhibin. The amount of inhibin in rGCCM (relative to the amount present in bFF), varied with the condition of the granulosa cells which -in turn- corresponded with the total number of days that the cells had been in culture. Inhibin release was absent or low when the cells were actively growing, differentiating or aging. Except after 1 day of culture, no substantial amounts of progesterone were detected in the media. Release of other hormones was not tested.

It is concluded that granulosa cells collected from adult, intact female rats have and retain the capacity to secrete inhibin *in vitro*.

Keywords: pituitary cell culture; FSH; LH; progesterone; bovine follicular fluid (bFF); in vitro.

INTRODUCTION

An increasing number of studies indicate that regulation of the secretion of follicle stimulating hormone (FSH) in male and female animals might be under the control of a non-steroidal factor, inhibin (reviews: Hermans et al., 1981; Franchimont et al., 1981). The ovaries seem to be the site of production of inhibin in the female. It was shown that an inhibin-like factor is secreted *in vivo* by ovaries which are implanted into the spleen of castrated male or female rats (Uilenbroek et al., 1978) and by the ovaries of immature female rats united parabiotically with castrated male rats (Johnson, 1981). Also, by means of an *in vitro* method, inhibin-like activity was demonstrated in ovarian venous plasma of cyclic rats (DePaolo et al., 1979). Healthy, antral follicles might be the ovarian compartment responsible for the production of inhibin, since in a number of situations in the rat inverse relationships were found between peripheral concentrations of FSH (and not of luteinizing hormone, LH) and numbers of medium and large antral follicles (Welschen et al., 1978; 1980). It was also shown that follicular fluid of many species contains inhibin-like activity (reviews: Hermans et al., 1981; Channing et al., 1981). These observations suggest that healthy granulosa cells in ovarian follicles produce inhibin. Indeed, Erickson & Hsueh (1978) showed that spent media of cultured rat granulosa cells, collected from

adult pro-oestrous rats or from oestrogen primed hypophysectomized 25-day-old rats, contains inhibin-like activity. More recently, Franchimont et al. (1981) and de Jong et al. (1982) presented preliminary results suggesting that bovine granulosa cells actively secrete inhibin *in vitro*.

The present report describes a study on the relationship between the amount of inhibin present in spent media from rat granulosa cells collected from intact, adult female rats and the total duration of the culture period. Inhibin was estimated by means of a bioassay based on the suppression of FSH found after addition of inhibin to pituitary cells in culture; only preparations which suppressed levels of FSH, but not of LH, in parallel with the suppression obtained after addition of charcoal-treated bovine follicular fluid (bFF), were used for calculation of the amount of inhibin.

MATERIALS AND METHODS

Animals

Adult female rats of a Wistar substrain (R-Amsterdam) were used for the collection of ovaries and pituitary glands. Animals were kept under controlled conditions of light (light period 05.00-19.00 h) and temperature (22-24°) and received standard dry pellets and tap water *ad libitum*. Rats were used when 3-6 months old, regardless of the day of the oestrous cycle. For the collection of ovaries, 6-10 rats per experiment were used: the ovaries were removed, trimmed of fat and connective tissue and placed in buffer. For the collection of pituitary glands, 12-15 rats were decapitated per experiment and, after removal of the posterior lobes, also placed in buffer.

Buffers and media

Buffers and media were prepared freshly from stock solutions which were stored in a refrigerator for not more than 2-3 weeks. All buffers and media were sterilized by passing them through 0.2 µm pore size Acrodisc filters (Gelman, Ann Arbor, Michigan, U.S.A.). The composition of the buffers and media were as follows:

For granulosa cell isolation: F-12 stock: medium F-12 (Boehringer, Mannheim, BRD), supplemented with 10 mM Hepes (Gibco, Grand Island, New York, U.S.A.), 2 mM magnesium acetate and 1 % glucose; EGTA-solution: F-12 stock supplemented with 4 mM ethyleneglycol-bis-2-aminoethyl-tetraacetic acid (EGTA) (Fluka, Buchs, Switzerland); sucrose solution: F-12 stock supplemented with 0.2 M sucrose; BSA-DNAse solution: F-12 stock supplemented with 0.25 % bovine serum albumin (BSA) (Organon, Oss, The Netherlands) and 0.001 % deoxyribonuclease (DNAse) (Sigma, St. Louis, MO, U.S.A.). All solutions were adjusted with 1 N NaOH to pH 7.2-7.4.

For pituitary cell isolation: buffer HBSS: Hank's Balanced Salt Solution (Gibco) containing 3.5 % sodiumbicarbonate (Gibco); dispase solution: HBSS containing 2.4 Units Dispase II (Boehringer); HBSS-BSA: HBSS containing 1 % BSA (Organon).

For culture of cells Eagle's Minimal Essential Medium with Earle's Salts (MEM) (Boehringer) was supplemented with 1 % non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 1 % antibiotic-antimycotic solution (Gibco, containing 100.000 Units penicillin, 100 mg streptomycin and 250 µg fungizone / L medium) and 10 % foetal calf serum (FCS, Boehringer). For culture of granulosa cells this medium was supplemented further with 1 % glucose.

Granulosa cell cultures

During all isolation procedures, siliconized (dichlorodimethylsilane, Merck, Darmstadt, BRD), heat sterilized glassware was used. The buffers and media were kept at 37° in a water bath.

Granulosa cells were obtained using a modification of the method described by Campbell (1979). After collection, 12-20 ovaries were washed 3 times with F-12 stock, placed in 5 ml EGTA-solution, incubated for 5 min. at 37° and rinsed with fresh F-12 stock. After the incubation, antral follicles were harvested *de visu* using a dissecting microscope and sterile injection needles (19 Gauge): they were collected in F-12 stock. The isolated follicles were incubated for 5 min. at 37° in 5 ml EGTA-solution; immediately afterwards the medium was replaced by 5 ml sucrose solution, in which the follicles were incubated for 2 min. After the incubation, the follicles were washed

3 times with fresh F-12 stock. Granulosa cells were collected by gently pressing the follicles in DNAse-solution with 19 Gauge needles under a dissecting microscope. The clumps of cells released were collected in a tube containing DNAse-solution and washed twice with fresh DNAse-solution, each time ended by centrifugation for 5 min. at 100 g. The final cell pellet was broken by means of a finger flip, and suspended in fresh F-12 stock solution. The number of viable cells present in the solution was counted in a hemacytometer using Trypan blue (Gibco) exclusion as criterion for viability (Campbell, 1979). On the basis of counts of viable cells the suspension was diluted to give the desired concentration of viable cells. This isolation procedure yielded 35-65 % viable granulosa cells, which were completely separated or present in clumps of 4-6 cells. Aliquots (100 µl) of the cell suspension were added to Petri dishes (Falcon plastics, 35 x 10 mm^2), containing 2 ml supplemented MEM. The dishes were placed in an incubator under 95 % air : 5 % CO_2 at 32°C. Generally cells attached 10-30 min. after plating. At various times during the culture period, spent media were collected and replaced with fresh MEM. At those times dishes were examined cytologically under a phase-contrast microscope (magnification 10 x 20). Spent rat granulosa cell culture medium (rGCCM) of dishes which had received the same treatment was pooled and stored frozen (-20°) until processed. Some batches of rGCCM were extracted with 2 volumes of ether-chloroform (1-1 v/v) and two volumes of ether; excess of ether was evaporated in air (Franchimont et al., 1975). This method was adopted since treatment with charcoal (Welschen et al., 1977) or with dextran-coated charcoal (Franchimont et al., 1981) resulted in a considerable loss of material and of inhibin activity (data not shown).

Pituitary cell cultures

A pituitary cell culture system was used as a bioassay for inhibin. Pituitary cells were prepared using an adaptation of the method described by de Jong et al. (1979): pituitary glands were placed in 5 ml HBSS-BSA solution, mechanically chopped (0.25 mm), washed twice with fresh HBSS-BSA solution and incubated for 2 h at 37°C in 5 ml dispase solution in a shaking waterbath. After the incubation, tissue fragments were washed again in fresh HBSS-BSA solution, placed in a conically shaped douncer containing MEM and dispersed. For counts of viable cells, the cell suspension was counted in a hemacytometer using Trypan blue exclusion and was diluted with supplemented MEM to obtain the desired number of viable cells (2.0-5.0 x 10^5 viable cells / 100 µl). Aliquots of 100 µl were pipetted into 64-96 Falcon Petri dishes (35 x 10 mm^2), containing 2 ml supplemented MEM, and placed in an incubator (32°C, 95 % air : 5 % CO_2). Cells were precultured for 3 days. After aspiration and renewal of the medium various doses of testsubstances (rGCCM) or standard preparations containing inhibin (charcoal-treated bovine follicular fluid, bFF, diluted 1:1000 in supplemented MEM) were each added to 4 dishes. The added volume of MEM was adjusted to maintain a total volume of 2 ml. After another 3 day culture period, the media were collected separately and stored frozen (-20°C) until assayed for concentrations of FSH and LH.

Hormone determinations

Concentrations of FSH and LH in spent media from cultured pituitary cells were estimated in duplicate by radioimmunoassay using the method described by Welschen et al. (1975). Gonadotrophin concentrations in all samples obtained from one pituitary cell culture were measured simultaneously. The coefficients of variation between assays were 15.7 % for FSH and 22.0 % for LH; within assays 3.7 % for FSH and 14.9 % for LH. Results are expressed as means ± S.D. in terms of NIAMDD-rat-FSH RP-1 or NIAMDD-rat-LH RP-1 or as percentages of mean control values found in media from dishes which contained only pituitary cells and supplemented MEM. Concentrations of inhibin in rGCCM were calculated by measuring the suppression of the release of FSH from pituitary cells after addition of various volumes of rGCCM, using a computer program kindly provided by Dr H. W. G. Baker (Melbourne, Australia). The amount of inhibin was expressed relative to the amount present in the standard preparation of bFF (for collection of bFF see Welschen et al., 1977), which was defined to have a specific activity of 1. Only preparations of rGCCM which suppressed release of FSH, but not of LH, by the pituitary cells in a dose-related manner and in parallel with the suppression obtained after addition of bFF, were used for estimation of the amount of

inhibin. Concentrations of progesterone (P) in rGCCM were estimated using a radioimmunoassay as described by de Jong et al. (1974). The detection limit of the assay was 100 pg/ml.

Statistical procedures

The significance of differences between the concentrations of FSH and LH found after addition of testsubstances and the concentrations of FSH and LH found in control dishes containing supplemented MEM only was assessed with Student's T-test. Differen-

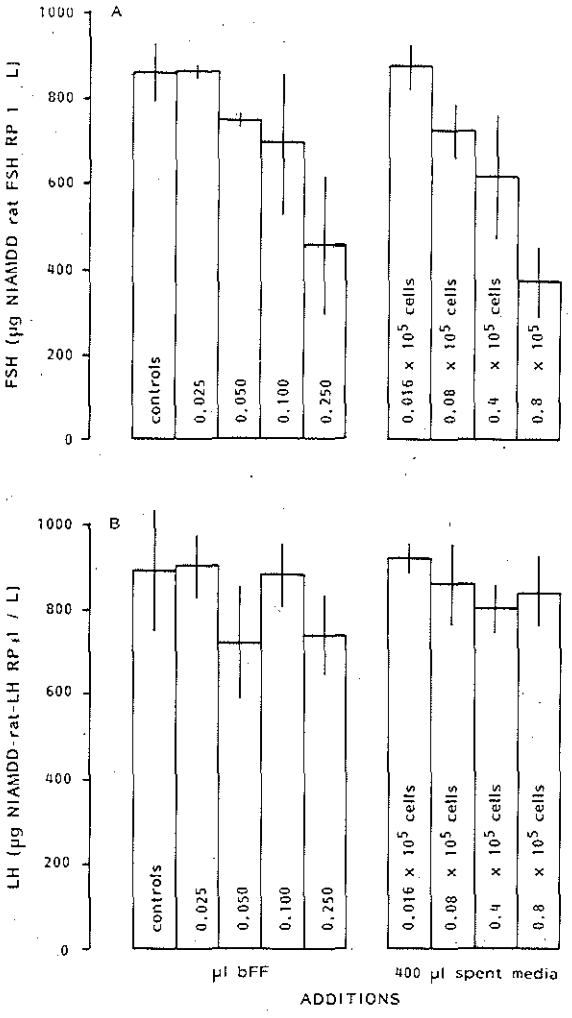

Figure 1. *Means + S.D. concentrations of (A) FSH and (B) LH in spent media from pituitary cells in culture after no additions (controls), addition of 0.025 - 0.250 µl charcoal-treated bovine follicular fluid (bFF, standard) or addition of 400 µl spent rat granulosa cell culture medium. Different numbers of cells were plated (varying from 0.016 - 0.8 x 10^5 cells / dish); spent media were collected after 1 day in culture (n=4).*

ces were considered significant when $P \leq 0.05$ (two-tailed). In the bioassay calculation of relative potencies and 95 % confidence limits and analysis of linearity and parallellism were based on the method of Finney (1964).

RESULTS

In the first experiment, addition of 400 µl rGCCM obtained after a one day incubation of different amounts of granulosa cells caused a decrease in the basal release of FSH by the pituitary cells (Fig. 1A). The amount of suppression reached was proportional to the number of granulosa cells in the dishes. Concentrations of LH were not affected after addition of rGCCM (Fig. 1B). Because the suppression could have been caused by inhibin or steroids in rat follicular fluid which was still present in the granulosa cell suspension after the isolation procedure, granulosa cells were subsequently cultured for longer periods.

In a second experiment, 1.1×10^5 viable granulosa cells/dish (total 13 dishes) were cultured for a total of 15 days. Spent media were harvested daily from day 1 to 11, and at day 13 and day 15. After 1 day in culture, the cells were well plated,

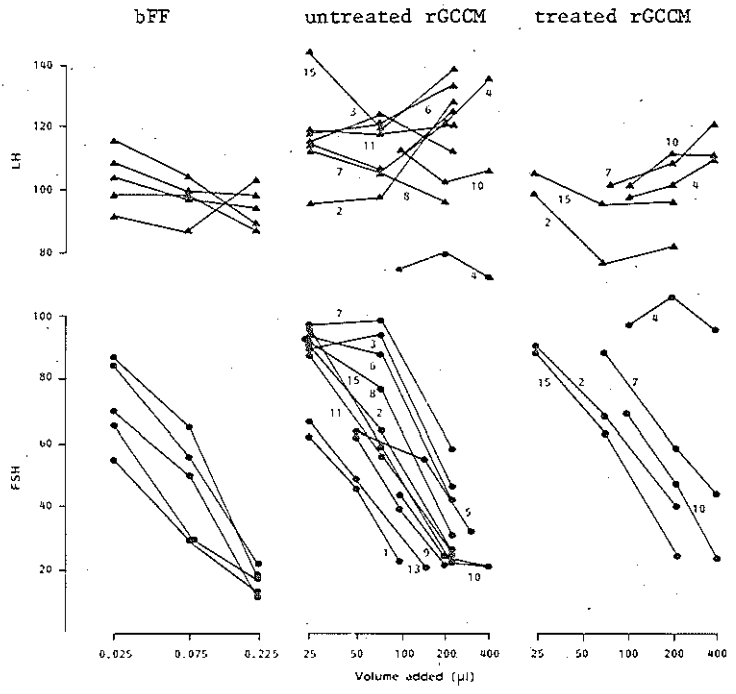

Figure 2. *Mean concentrations of FSH (●——●) and LH (▲——▲) in spent media from pituitary cells in culture after addition of 0.025 - 0.225 µl charcoal-treated bovine follicular fluid (bFF, standard) or addition of various amounts (ranging from 10 - 400 µl / dish) of spent rat granulosa cell culture media (rGCCM), either untreated or ether-chloroform treated. Rat granulosa cell media were daily obtained from 1.1 x 10^5 plated cells / dish, which were kept in culture for a total of 15 days. Numbers beside suppression lines indicate the day of collection of the media. Each point represents the mean of quadruplicate determinations. Levels of FSH and LH are expressed as percentages of mean values found in appropriate control dishes; the abcissa has a logarithmic scale. A total of 5 bioassays were used. For the sake of clarity, S.D. are omitted.*

angular and dividing. After 2 days in culture, they had an oval appearance. From day 3 onwards they were elongating until after 7 days in culture the dishes were filled with a monolayer of elongated, fibroblast-like cells. No further changes in shape were observed,but in many cells lipid-like droplets started to appear. At day 13 and 15 some cells contained 2 nucleoli. At day 16 the experiment was stopped because of contamination.

When the spent media of these cells were added to pituitary cell cultures, a dose-dependent suppression, in parallel with bFF-induced suppression, was observed. Only rGCCM obtained after 4 culture days did not suppress the concentrations of FSH in a dose dependent manner. Figure 2 gives the concentrations of FSH and LH after addition of rGCCM in 5 pituitary cell cultures, expressed as percentages of the mean concentrations found in the appropriate control dishes. The index of precision of the bioassays ranged between 0.07 and 0.16. No systematic effect on concentrations of LH after addition of rGCCM was observed, although 20 out of 54 batches raised the concentrations of LH. Since we wanted to exclude the possibility that the suppression of FSH was caused by progesterone released by the cultured granulosa cells, some batches of rGCCM were treated with ether-chloroform to remove steroids. Levels of progesterone before and after treatment are given in Table 1. Except after day 1 in culture, levels of progesterone were very low, both before and after treatment. When the ether-chloroform treated media were tested in the bioassay, a dose dependent suppression of levels of FSH, in parallel with the suppression found with bFF or untreated rGCCM, was found, while concentrations of LH were not systematically affected (Fig. 2). These results suggest that rGCCM contains inhibin. The amount of inhibin in rGCCM relative to the amount of inhibin in bFF was estimated. Results are shown in Figure 4: the amount of inhibin varied with the total duration of the culture period. In order to study the influence of a longer culture period, this experiment was repeated and extended.

Table 1. *Amount of progesterone (pg/ml) in untreated and ether-chloroform treated spent media of rat granulosa cell cultures (rGCCM) after various culture periods (days). Values are not corrected for daily production.*

rGCCM of 1.1×10^5 cells			rGCCM of 0.7×10^5 cells		
days in culture	untreated rGCCM	treated rGCCM	days in culture	untreated rGCCM	treated rGCCM
1	4566		1	548	200
2	776	<100	2-5	<100	<100
3	<100		6-9	<100	
4	<100	<100	10-13	<100	<100
5	117		14-17	<100	
6	<100		18-21	-	<100
7	<100	<100	22-25	<100	
8	<100		26-29	-	103
9	<100		30-33	<100	
10	146	<100	34-37	<100	107
11	<100				
12-13	<100	<100			
14-15	<100				

In a third experiment, 0.7×10^5 viable granulosa cells/dish (10 dishes) were cultured for a total of 37 days. Medium was renewed after 1 day in culture and subsequently every other 4 days. Cytological changes during the first 15 days were comparable with those observed in the second experiment; from day 17 onwards an increase in

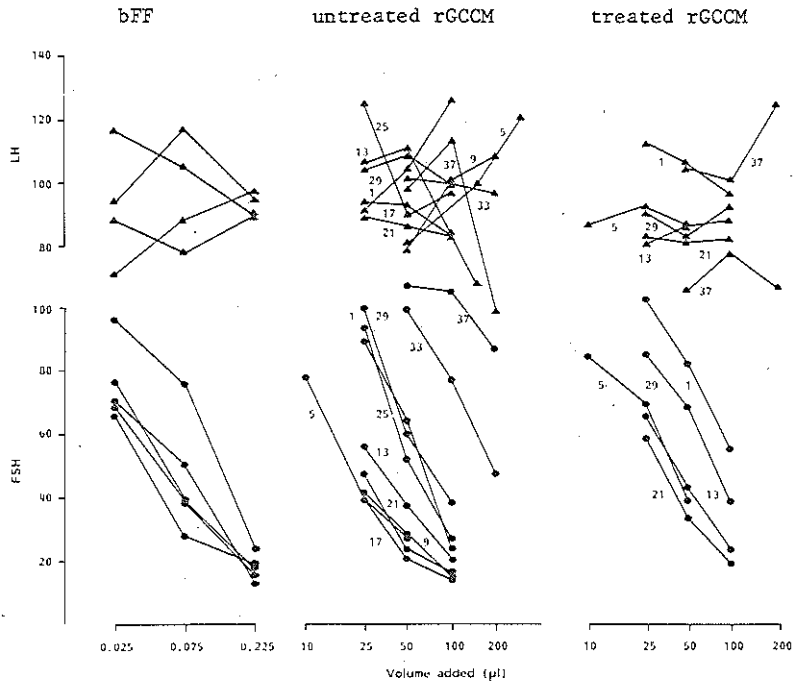

Figure 3. *For legend see Figure 2. In this experiment rat granulosa cell media were obtained from 0.7 x 10^5 plated cells / dish, renewed after 1 day in culture and subsequently every other 4 days; cells were kept in culture for a total of 37 days.*

the total number of lipid-like droplets was observed, while the cell boundaries became indistinct. At day 33 the cell boundaries were more easily visible again, and at day 37 detached cells were observed, after which the experiment was stopped. Concentrations of FSH and LH in spent media from pituitary cells after addition of rGCCM, expressed as percentages of mean values found in appropriate control dishes, are shown in Figure 3. The index of precision of the 5 bioassays used in this experiment ranged between 0.07 and 0.09. As in the second experiment, levels of LH were not systematically influenced by addition of rGCCM, but 3 out of 48 batches raised, and 6 out of 48 batches lowered levels of LH. Levels of FSH were suppressed in a dose related manner in parallel with the suppression obtained after addition of bFF. Levels of progesterone were low to undetectable except at day 1 in culture, both before and after ether-chloroform treatment (Table 1). The ether-chloroform treated spent granulosa cell media suppressed concentrations of FSH in parallel with the suppression obtained after addition of untreated rGCCM or bFF. Also in this experiment the amount of inhibin present in the media relative to the amount present in bFF was calculated (Fig. 4). Again the amount of inhibin was dependent on the total duration of the culture period, with the highest amounts present in media from culture days 2-5, 14-17 and 18-21, after which periods the concentration of inhibin started to decline. Comparison of the amount of inhibin present in untreated versus ether-chloroform treated rGCCM showed a linear relationship ($y = -0.17 + 1.51\ x$; $n = 9$; $r = 0.97$)

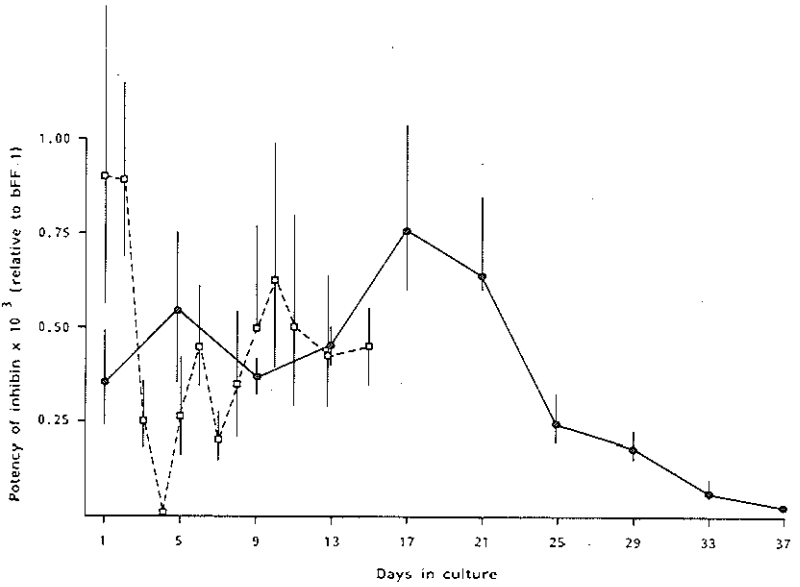

Figure 4. *Relative amount of inhibin present in untreated spent rat granulosa cell culture media (rGCCM) obtained from 1.1 x 10^5 cells (□---□) or from 0.7 x 10^5 cells (●——●) / dish and collected at various days in culture. The amount of inhibin is expressed as daily release of inhibin relative to the amount present in charcoal-treated bovine follicular fluid (bFF, standard, defined to have a potency of 1), and calculated from the individual data which fell on the regression lines shown in Figures 2 and 3. Vertical lines represent 95 % confidence limits. The numbers on the abcissa show the day that media were collected.*

DISCUSSION

When conventional methods such as application of physical pressure on the surface of the ovaries are used to isolate granulosa cells of intact, non oestrogen primed rats, cell yield (15-25 % viable cells) and survival of cells in culture is poor. Cells released after pretreatment of the ovaries with EGTA and hypertonic sucrose solutions are superior to cells collected in conventional ways with regard to viability, survival in culture and *in vitro* synthesis of proteins, RNA and DNA (Campbell, 1979). Also in our hands an adaptation of Campbell's method worked well: it was easy to dissect follicles; expressed clumps of granulosa cells could easily be dispersed, and a high percentage of viable cells (35-65 %) and a very rapid attachment (within 30 min.) of the cells to the culture dishes was observed.

By using this isolation procedure, it was possible to show that addition of rGCCM of untreated rats to pituitary cells *in vitro* selectively suppressed levels of FSH in the media of the pituitary cells (Fig. 1). The amount of suppression reached was dependent on the number of granulosa cells in culture, confirming a study of Erickson & Hsueh (1978). On basis of this experiment we chose a number of 0.8-1.0 x 10^5 viable cells per dish as a suitable number of cells for further experiments.

The two following experiments showed that rGCCM contains a factor which fulfills our criteria for inhibin: in the inhibin bioassay specific suppression of FSH was found in parallel with the standard preparation, also after ether-chloroform treatment. Although no systematic changes in levels of LH were observed, 23 out of 102 experimental groups showed elevated levels of LH. Our data do not indicate a relation-

ship between the added volume of bFF or rGCCM on the one hand and the stimulation of LH on the other hand. It is possible that the relatively high intra-assay variation for LH (14.9 %) in these experiments has been responsible for these effects.

The results of the second experiment, in which the media were renewed daily, suggest that the presence of inhibin in rGCCM is due to production of inhibin, and not to leakage of inhibin from ruptured cells or to a minute contamination with rat follicular fluid present between the plated granulosa cells. Furthermore, de Jong et al. (1982) showed that bovine granulosa cells in culture are capable of incorporation of ^{3}H-fucose and ^{35}S-methionine into protein fractions which also contain inhibin activity. Thus, rat granulosa cells in culture have and retain the capacity for a basal secretion of inhibin. Also another cell type capable of secreting inhibin, the Sertoli cell, was found to sustain *in vitro* for 21 days a basal release of inhibin (Steinberger, 1980).

The amount of inhibin present in rGCCM seemed to change together with morphological changes in the cells. The second experiment showed that during the first 5 days of the culture period, when the cells were attaching, spreading and dividing and probably differentiating, a decrease in the amount of inhibin production can be observed; thereafter a rather stable production of inhibin can be found until day 15. These results are in good agreement with those of Labrie et al. (1978), who observed a higher inhibin production of Sertoli cells in culture from days 5-8 than from days 2-5 in culture. The results from the third experiment show a similar picture when the results are expressed in terms of daily production of inhibin during the first 14 days. The production of inhibin during the first 14 days in culture might be negatively influenced by growth related processes, such as found for FSH-induced steroidogenesis in cultured rat granulosa cells (Orly et al., 1980), or as found for protein synthesis by bovine granulosa cells in culture, which only occurs after the cells have reached confluency (Savion & Gospodarowicz, 1980). The period from day 14 to 21 (third experiment) shows an increase in inhibin production, followed by a steady decrease which is probably caused by aging and eventually dying of the cells. Either aging induces a loss in the capacity of the cell organelles to produce inhibin, or a gradual loss of an intracellular precursor for inhibin production may become a limiting factor.

Whether inhibin is produced by luteinized cells derived from the granulosa cells (as was suggested by the presence of lipid-like droplets), and/or by the observed fibroblast-like cells is not known, since the composition of the *in vitro* population of cells derived from granulosa cells was not clear from this study. Furthermore, basal progesterone secretion might have been a bad parameter for demonstration of functionally luteinized cells: basal progesterone secretion was low, as was also reported by Armstrong & Dorrington (1976), Liu et al. (1981) and Koninckx et al. (1981) for rat granulosa cells in culture; this contrasts with a high basal progesterone production reported for cultured granulosa cells from the pig (Veldhuis et al., 1981; Stoklosowa et al., 1981), the rabbit (Erickson et al., 1974) and man (Moon et al., 1981). A possible explanation for the discrepancy in progesterone secretion can be found in the isolation procedure: since collection of granulosa cells from large follicles is generally performed by aspiration and scraping of the follicular wall, a minor contamination with theca cells might occur, which can account for the observed progesterone secretion (Koninckx et al., 1981).

In conclusion, this study shows that granulosa cells of antral follicles of intact rats are capable of inhibin production *in vitro*. The observation that basal release of inhibin also takes place after longer culture periods (possibly inducing changes in the cytological and biochemical characteristics of the cells), suggests that both cells from corpora lutea and interstitial cells should be investigated as potential candidates for inhibin production.

ACKNOWLEDGEMENTS

The authors wish to thank Dr J. Moll for his support and his help in preparing this manuscript. We are grateful to the NIAMDD, Bethesda, Maryland, U.S.A. for the supply of gonadotrophins used in the radioimmunoassays.

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J. Endocr. (1980), **86**, 79–92 *Printed in Great Britain*
0022–0795/80/0086–0079 $02.00/0

IV

INVOLVEMENT OF INHIBIN IN THE REGULATION OF FOLLICLE-STIMULATING HORMONE CONCENTRATIONS IN PREPUBERTAL AND ADULT, MALE AND FEMALE RATS

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(*Received* 15 *August* 1979)

SUMMARY

Administration of steroid-free bovine follicular fluid (bFF), containing inhibin-like activity, depressed levels of FSH measured 4 h after injection in intact adult and 35-day-old female rats, but not in younger females. Suppression of FSH was also observed in intact male rats, aged 55 days, but not in older and younger male rats. Eight hours after injection of bFF, FSH levels were depressed in 15-day-old and older immature and adult rats of both sexes. Male and female rats, gonadectomized 2 days earlier, responded similarly to bFF treatment as did the intact animals.

In a second experiment it was found that the rise of FSH levels, occurring within 8 h of gonadectomy, decreased with age in male and increased with age in female rats. Steroid treatment was found to prevent the rise in FSH levels partially in 15-day-old male and completely in 25-day-old female rats, whereas treatment with bFF was fully effective in blocking the FSH rise in both immature and adult rats of both sexes.

It is concluded that inhibin might be a major physiological factor in a fast-acting control of FSH concentrations from at least the age of 25 days onwards in female rats. In male rats its physiological significance might be limited to the prepubertal period, despite the fact that pituitary secretion of FSH is suppressed by exogenous inhibin-like activity at all ages studied.

INTRODUCTION

Recent evidence has indicated that the gonadal control of the pituitary secretion of follicle-stimulating hormone (FSH) may differ from that of luteinizing hormone (LH; see reviews by Baker, Bremner, Burger, de Kretser, Dulmanis, Eddie, Hudson, Keogh, Lee & Rennie, 1976; Chari, 1977; Setchell, Davies & Main, 1977; de Jong, 1979). The principle which is thought to exert a specific feedback action on the secretion of FSH was named 'inhibin' by McCullagh (1932).

Since it has been shown that in both prepubertal and adult, male and female rats peripheral levels of FSH can be selectively suppressed by preparations which contain inhibin-like material (de Jong & Sharpe, 1976; Nandini, Lipner & Moudgal, 1976; Davies, Main & Setchell, 1978; de Jong, Welschen, Hermans, Smith & van der Molen, 1978; DePaolo, Wise, Anderson, Barraclough & Channing, 1979*b*; Lorenzen & Schwartz, 1979), and since inhibin seems to be a short-term regulator of FSH concentrations (Nandini *et al.* 1976; de Jong *et al.* 1978; DePaolo *et al.* 1979*b*), a physiological role for inhibin seems possible when secretory patterns of FSH and LH diverge during a short period of time.

The aim of the present study was to investigate first whether in both prepubertal and adult, male and female rats, FSH concentrations could be selectively suppressed in different test models by injection of inhibin-like material as present in steroid-free bovine follicular fluid (bFF). Secondly, it was investigated whether in rats of various ages diverging FSH and LH responses occur after bilateral gonadectomy and whether a rise in FSH levels after gonadectomy could be prevented by injection of either a combination of steroids or of inhibin-like material.

Preliminary results from this study have appeared elsewhere (de Jong *et al.* 1978; de Jong, Welschen, Hermans, Smith & van der Molen, 1979; Welschen, Hermans & de Jong, 1980).

MATERIALS AND METHODS

Animals

Rats of a Wistar substrain (R-Amsterdam) were kept under controlled conditions of light (light period 05.00–19.00 h) and temperature (22–24 °C) and received standard dry pellets and tap water *ad libitum*. Animals were weaned at the age of 21 or 22 days. Care was taken to divide litter-mates between experimental and control groups.

The coefficient of variation between body weights of animals of any particular age ranged from 2·8 to 15·9%. Extreme care was taken in the selection of male animals of 50 and 55 days of age, since at these ages a pilot experiment had shown a negative correlation between body weight and a rise of FSH concentrations in plasma 8 h after orchidectomy ($r = 0{\cdot}775$, $n = 8$ and $r = 0{\cdot}780$, $n = 16$ at 50 and 55 days of age respectively).

Female rats of the strain used generally show first vaginal oestrus at 37–42 days of age. The day after vaginal oestrus was called dioestrus-1. Daily vaginal smears were taken from adult female rats and only rats with two consecutive 5-day cycles before the day of treatment, were used.

Follicular fluid

Bovine follicular fluid was aspirated from ovarian follicles with diameters between 10 and 20 mm and treated with charcoal to remove steroids as previously described (Welschen, Hermans, Dullaart & de Jong, 1977). After charcoal treatment, concentrations of oestradiol-17β and progesterone were 0·04 and 0·33 ng/ml respectively (Welschen *et al.* 1977). Charcoal-treated bovine plasma served as a control fluid in all experiments, since it shows a protein composition similar to that of bFF (Caravaglios & Cilotti, 1957) and no inhibin-like activity in in-vivo test systems (Welschen *et al.* 1977).

Table 1. *Effect of enzymatic digestion on the FSH-suppressing activity in bovine follicular fluid, tested in adult female rats, gonadectomized 12 days earlier. Animals were injected with 500 µl bovine follicular fluid or bovine plasma (controls)/100 g body wt. Samples were taken 8 h after injection (means ± S.E.M., n = 5–6)*

Material	Enzyme treatment	FSH (ng/ml)	LH (ng/ml)
Bovine plasma	—	1521 ± 121	265 ± 71
Bovine plasma	trypsin	1663 ± 22	209 ± 22
Bovine plasma	pronase	1659 ± 133	177 ± 12
Bovine follicular fluid	—	672 ± 42*	182 ± 31
Bovine follicular fluid	trypsin	710 ± 49*	363 ± 61
Bovine follicular fluid	pronase	1465 ± 141	242 ± 45

*$P \leq 0{\cdot}05$: compared with values in control animals injected with treated bovine plasma (two-tailed Wilcoxon test).

To show the protein nature of the inhibin-like factor, bFF and bovine plasma were incubated for 2 h in 0·1 M-phosphate buffer with trypsin (Gibco, New York; enzyme/substrate ratio (e/s) 1 : 30; 37 °C; pH 7·3) or pronase (Calbiochem., California; e/s 1 : 50; 37 °C; pH 7·3). Table 1 shows concentrations of FSH and LH in serum 8 h after injection of 500 μl test material/100 g body wt in adult female rats which had been bilaterally ovariectomized 12 days earlier.

Only incubation with pronase destroyed the inhibin-like activity present in bFF. The effect of the incubation procedure on the FSH-suppressing activity in bFF was determined by incubation of bFF and bovine plasma with denaturated enzymes (10 min heating at 90 °C). With the latter materials the amount of inhibin-like activity remained unchanged (results not shown).

Experimental design

All operations, injections and blood collections were performed under light ether anaesthesia. Bilateral orchidectomy (by the scrotal route), bilateral ovariectomy (by lateral dorsal incisions), sham-operation or anaesthesia only were performed at 09.00 h (±15 min). Either 48 h or immediately after the operation, animals were given at 09.00 h an intraperitoneal injection of 500 μl bovine plasma or bFF/100 g body wt. In these experiments this dose was always used since it causes a maximal suppression of FSH levels in both prepubertal and adult, male and female rats (de Jong *et al.* 1978; Welschen *et al.* 1980). Additional groups of rats received a subcutaneous injection of 100 μl oil, 100 μl oil containing 25 μg testosterone and 0·005 μg oestradiol benzoate (males) or 100 μl oil containing 0·012 μg oestradiol benzoate and 0·075 mg progesterone (females)/100 g body wt at 09.00 h and 13.00 h after bilateral gonadectomy performed at 09.00 h on the same day. All animals of 15 days of age were bled by decapitation, older animals were bled by puncture of the ophthalmic venous plexus. In some experiments, animals of 35 days of age or older were bled twice. Animals were bled at 13.00 and/or 17.00 h on the day of injection.

Blood was allowed to clot overnight at 4 °C before centrifugation for 15 min at 2000 *g* to obtain serum. For the collection of plasma, heparinized blood was centrifuged immediately. Serum and plasma were stored at −20 °C until used for hormone determinations.

Hormone determinations

Concentrations of FSH and LH in serum from individual rats were measured in duplicate by radioimmunoassay using the method described by Welschen, Osman, Dullaart, de Greef, Uilenbroek & de Jong (1975). Samples from both control and experimental groups were assayed at the same time.

All results are expressed as means ± S.E.M. in terms of NIAMDD-rat-LH-RP-1 or NIAMDD-rat-FSH-RP-1 from NIAMDD, Bethesda, Maryland, U.S.A. The coefficients of variation between assays were 16·6% for FSH and 14·4% for LH.

Levels of testosterone and progesterone in plasma were estimated using radioimmunological procedures (testosterone: Verjans, Cooke, de Jong, de Jong & van der Molen, 1973; progesterone: de Jong, Baird & van der Molen, 1974). The quantity of serum available did not allow measurement of oestradiol-17β.

Statistical procedures

The significance of differences between means for different groups was calculated using Wilcoxon's two-sample test. Differences were considered to be statistically significant when P was $\leqslant 0{\cdot}05$ (two-tailed test).

RESULTS

Effect of bFF on the concentrations of FSH and LH in the serum of intact and gonadectomized rats of both sexes

Figures 1 and 2 show the concentrations of FSH and LH in the serum of male rats aged 15, 35, 55 and 75 days and of female rats aged 15, 25, 35 and 70–80 days (intact female rats of the latter group were at dioestrus-2) after the injection of either bFF or bovine plasma. The rats

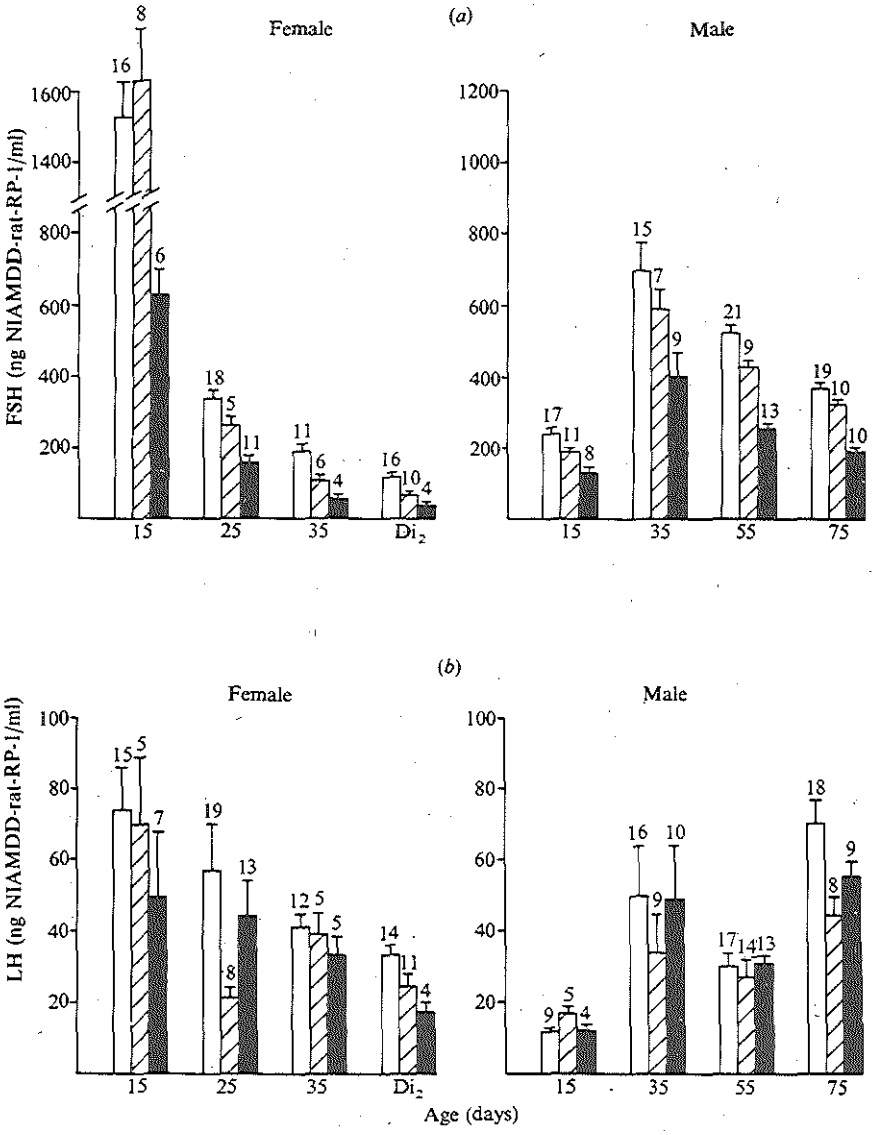

Fig. 1. Mean (±S.E.M.) concentrations of (*a*) FSH and (*b*) LH in serum 4 and 8 h after injection of 500 µl bovine plasma (open bars) or 500 µl bovine follicular fluid (hatched bars 4 h values; shaded bars 8 h values)/100 g body wt into intact male and female rats of various ages. Di_2 = dioestrus day 2. The numbers above the bars indicate the numbers of animals per group.

were either intact (Fig. 1) or bilaterally gonadectomized 2 days earlier (Fig. 2). Ovariectomy was performed on the day of oestrus in adult rats. The concentrations of FSH and LH in serum 4 and 8 h after the injection of bovine plasma were not significantly different and the data were, therefore, pooled to give a single control group for each hormone. In the case of the bFF-treated groups, however, the values of FSH and LH at both 4 and 8 h after injection are shown.

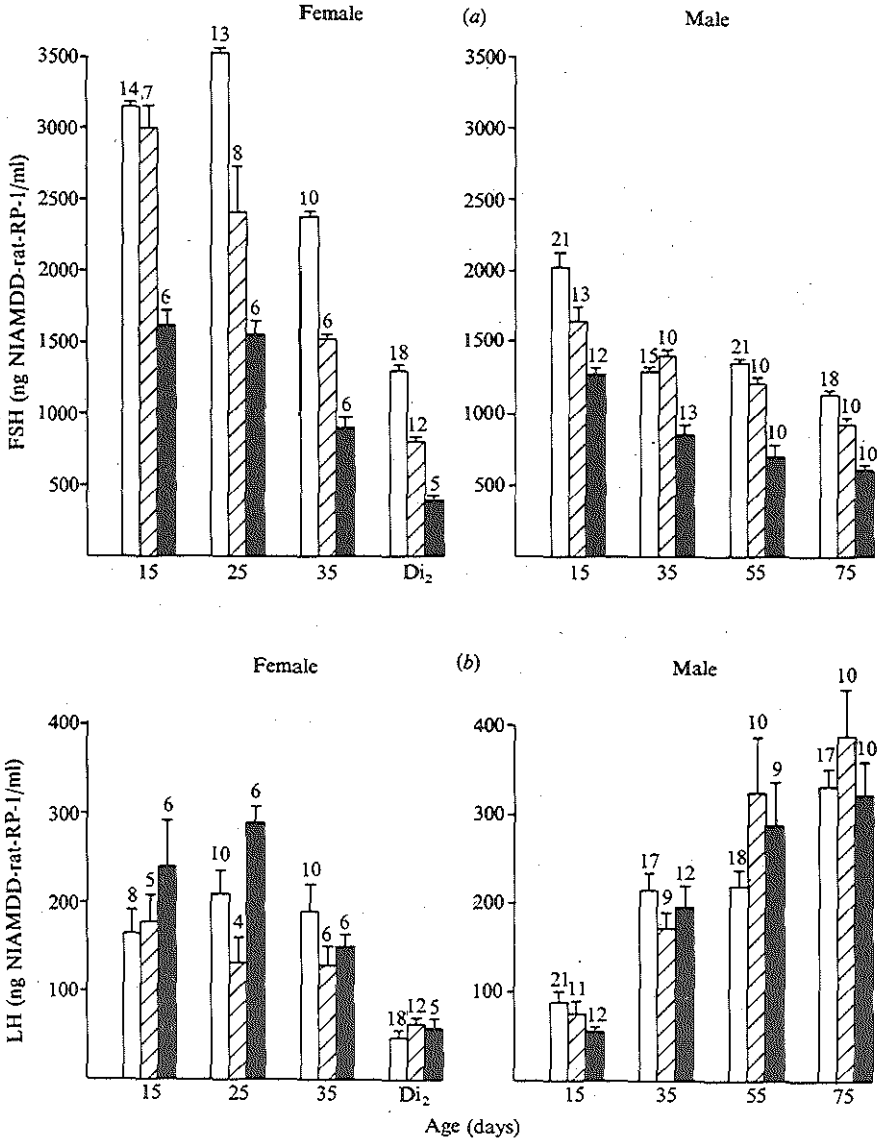

Fig. 2. Mean (±S.E.M.) concentrations of (*a*) FSH and (*b*) LH in serum 4 and 8 h after injection of 500 µl bovine plasma (open bars) or 500 µl bovine follicular fluid (hatched bars 4 h values; shaded bars 8 h values)/100 g body wt into male and female rats of various ages that were bilaterally gonadectomized 2 days before the injection. Di_2 = adult female rats gonadectomized at oestrus, aged 70–80 days. The numbers above the bars indicate the numbers of animals per group.

In female rats 4 h after the injection of bFF the concentrations of FSH were significantly ($P<0{\cdot}02$) reduced to 60–80% of those in the groups treated with bovine plasma in both intact and ovariectomized 35-day-old and adult animals and in ovariectomized 25-day-old rats. In male rats a significant suppression of FSH 4 h after treatment with bFF was observed in intact 55-day-old rats ($P<0{\cdot}02$) and in orchidectomized rats at 15 ($P<0{\cdot}05$), 55 ($P<0{\cdot}05$) and 75 ($P<0{\cdot}02$) days of age. Eight hours after the injection of bFF the concentrations of FSH in serum were significantly ($P<0{\cdot}02$) less than those of the bovine plasma-treated controls in all the groups of intact and gonadectomized rats of both sexes studied. In most groups FSH levels were suppressed to 40–60% of the values found in control groups, but the strongest suppression (to <3% of controls) was observed in intact and ovariectomized female rats at 35 days of age or older.

The concentrations of LH in serum were generally not affected by treatment with bFF with the exception that significantly decreased levels were observed at 4 h after treatment in intact 25-day-old ($P<0{\cdot}02$) and adult ($P<0{\cdot}02$) female rats and in intact 75-day-old male rats ($P<0{\cdot}05$) and 8 h after treatment in intact adult female rats ($P<0{\cdot}05$).

Acute FSH and LH response to gonadectomy

Figures 3 and 4 show the concentrations of FSH and LH in serum 8 h after bilateral gonadectomy or sham-operation in male and female rats of various ages. In this study the ovaries of adult female rats were removed at dioestrus-2. The concentrations of FSH are also expressed as percentages of the values found in sham-operated control animals (Fig. 5).

In male rats at all of the ages studied the concentrations of FSH were increased significantly ($P<0{\cdot}02$) compared to the levels observed in sham-operated control animals (Fig. 3). However, as shown in Fig. 5, the relative increase in FSH concentration after orchidectomy decreased with age from 260% in 15-day-old rats to a steady 20–30% increase from 50 days of age onwards. Concentrations of LH in serum varied considerably and significantly ($P<0{\cdot}05$) increased levels were observed after gonadectomy at 40, 55, 60, 75 and 93 days of age.

In female rats the concentrations of FSH increased significantly ($P<0{\cdot}02$) 8 h after ovariectomy at 25 and 35 days of age and in adult rats (Fig. 4). However, as shown in Fig. 5, the relative increase in levels of FSH after ovariectomy increased with age from a 160% increase at 25 days of age to a 380% increase in adult rats. The effect of ovariectomy on the levels of LH was variable: no change at 15 and 35 days of age; significantly ($P<0{\cdot}05$) decreased levels at 25 days of age and significantly ($P<0{\cdot}02$) increased levels at 70–80 days of age.

Effect of bFF and of steroids in preventing the rise in FSH after gonadectomy

To determine the nature of the signal which causes the rise in FSH immediately after gonadectomy, animals of the above mentioned ages were bilaterally gonadectomized and injected with bFF immediately after the operation. Concentrations of FSH and LH after treatment with bFF are also shown in Fig. 3 (male) and Fig. 4 (female).

At all ages studied the injection of bFF prevented the rise in FSH after gonadectomy; in general, levels of FSH were even below the values found in sham-operated rats. Levels of LH after injection of bFF varied considerably within all groups, but were only found to be significantly ($P<0{\cdot}05$) different from those in gonadectomized animals in male rats of 55 days of age.

In an additional experiment, groups of male rats of 15, 35, 55 and 75 days of age and female rats of 15, 25, 35 and 70–80 days of age were bilaterally gonadectomized (the ovaries of adult rats were removed at dioestrus-2) and injected with a total of 50 μg testosterone and 0·01 μg oestradiol benzoate (male) or 0·024 μg oestradiol benzoate and 0·15 mg progesterone (female)/100 g body wt as described in Materials and Methods. Concentrations of

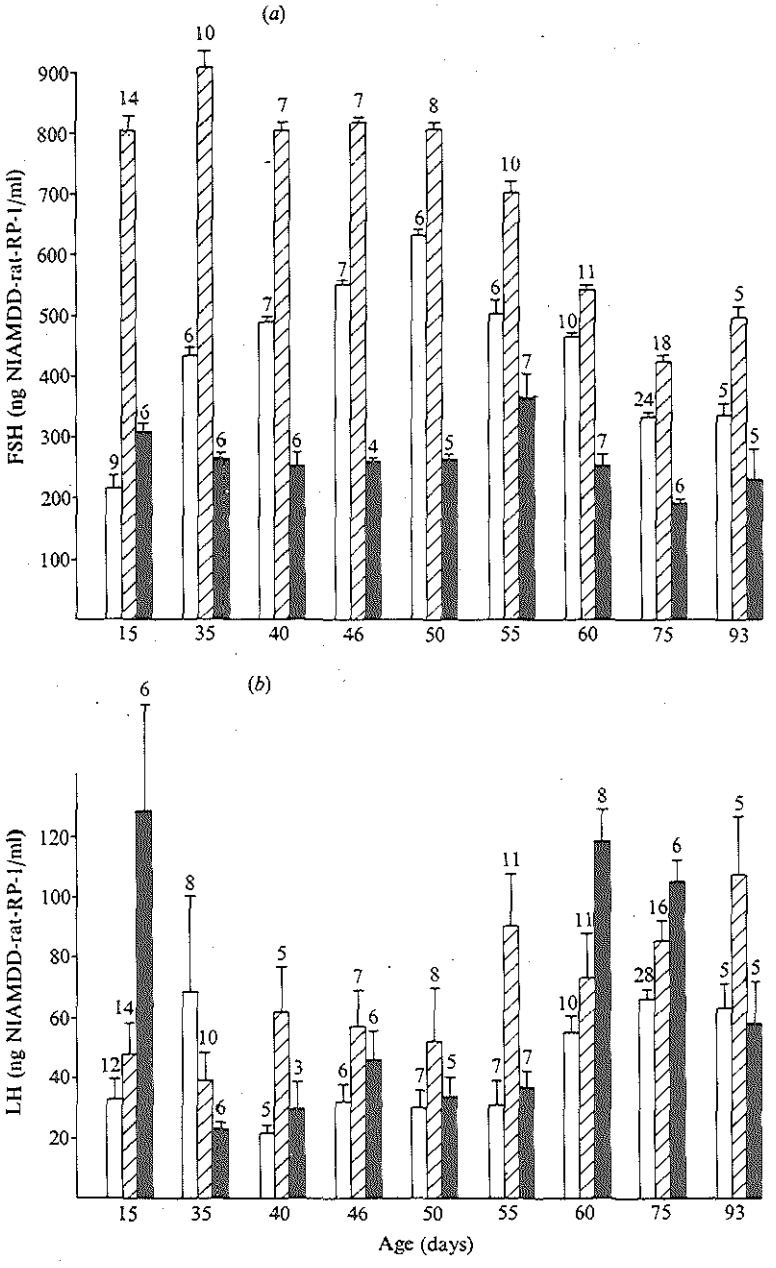

Fig. 3. Mean (±S.E.M.) concentrations of (*a*) FSH and (*b*) LH in serum 8 h after sham-operation (open bars), bilateral orchidectomy (hatched bars) or bilateral orchidectomy followed by an injection of 500 µl bovine follicular fluid/100 g body wt (shaded bars) in male rats of various ages. The numbers above the bars indicate the numbers of animals per group.

FSH and LH, and the levels of testosterone (male) or progesterone (female) in the plasma are given in Tables 2 and 3.

In male rats, orchidectomy did not cause a significant decrease in levels of testosterone. Injection of the steroid combination resulted in levels of testosterone in plasma which were significantly higher than those in oil-injected orchidectomized groups and which were , with the exception of values at day 55, also higher than those in untreated, sham-operated groups.

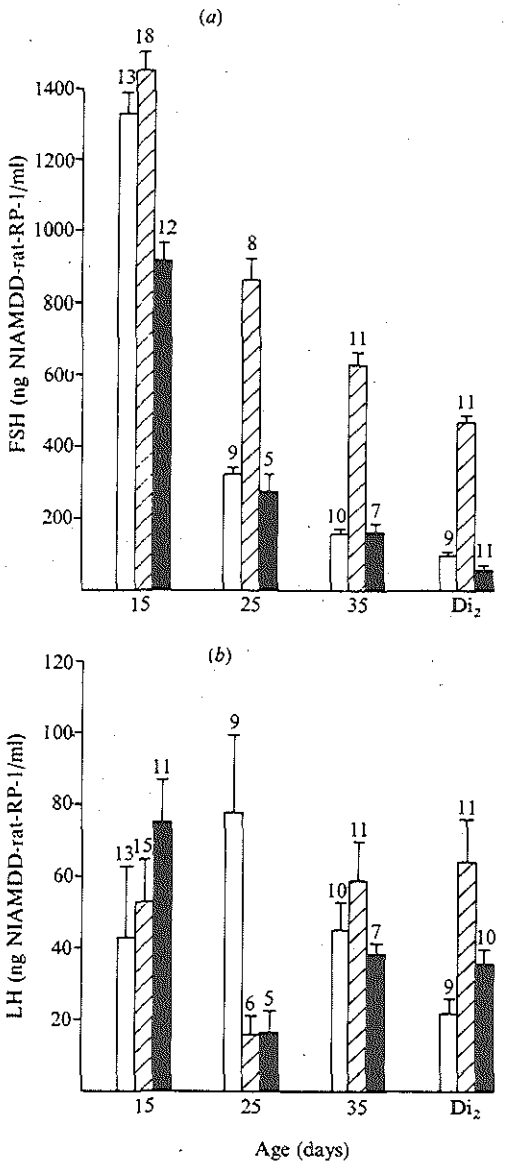

Fig. 4. Mean (±S.E.M.) concentrations of (*a*) FSH and (*b*) LH 8 h after sham-operation (open bars), bilateral ovariectomy (hatched bars) or bilateral ovariectomy followed by an injection of 500 µl bovine follicular fluid/100 g body wt in female rats of various ages. Di_2 = dioestrus day 2, aged 70–80 days. The numbers above the bars indicate the numbers of animals per group.

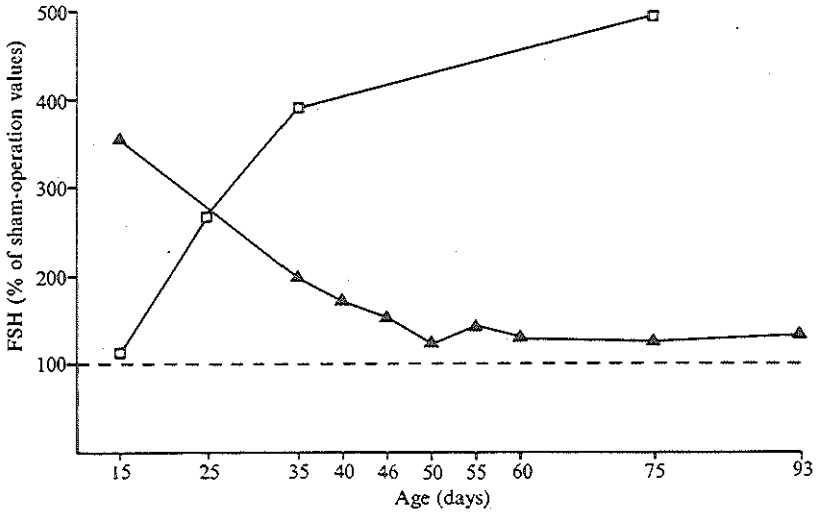

Fig. 5. Changes in concentrations of FSH 8 h after bilateral orchidectomy (▲) or ovariectomy (□) in male and female rats of various ages. Results are expressed as percentages of the values in sham-operated control animals (broken line). Numbers of animals in the various groups ranged between 4 and 24 (for exact data see Figs 3 and 4).

The levels of FSH decreased at 15 days of age only (but remained above sham-operated values, see Fig. 3). Significantly ($P < 0{\cdot}02$) suppressed levels of LH (even significantly below sham-operated values, see Fig. 3), were found in all groups with the exception of the 35-day-old male rats.

In ovariectomized rats, injection of the steroid combination resulted in approximately physiological values for plasma progesterone at 25 and 35 days of age and in adult rats. Only 25-day-old rats showed a suppression of the postovariectomy rise of FSH levels after steroid treatment, whereas levels of LH after ovariectomy were not affected after injection of the steroid combination.

DISCUSSION

Proteolytic treatment of follicular fluid

The inhibin-like activity in bFF could not be destroyed by incubation with trypsin, as was reported by Hopkinson, Daume, Sturm, Fritze, Kaiser & Hirschhäuser (1977) and Lorenzen, Channing & Schwartz (1978). Only incubation with pronase (Table 1), which is reported to be a non-substrate specific, proteolytic enzyme with a hydrolytic yield of ±90% (Nomoto, Narahashi & Murakami, 1960), destroyed the inhibin-like activity. From this result it was concluded that this activity was associated with proteinaceous material.

Comparison of three different in-vivo test models

The present results indicate that peripheral concentrations of FSH can be selectively influenced by injection of bFF in prepubertal and adult, male and female rats. This is true for three different test systems: the intact rat, the acutely gonadectomized rat and the rat gonadectomized 2 days before treatment. In-vivo effects of follicular fluid on FSH concentrations in female animals have been reported for adult mice and rats (mice: Bronson & Channing, 1978; rats: Hopkinson *et al.* 1977; Marder, Channing & Schwartz, 1977; Welschen *et al.* 1977; Lorenzen *et al.* 1978; DePaolo *et al.* 1979*b*) and recently for prepubertal rats of 18 days of age (Pomerantz, 1978) and rats of 6, 12, 21 and 26 days of age (Lorenzen & Schwartz, 1979). In-vivo effects of inhibin-like material on FSH concentrations in male

Table 2. *Concentrations of FSH and LH in serum and of testosterone in plasma 8 h after bilateral orchidectomy (BLO), sham-operation (SHAM) or BLO followed by two injections of 100 µl oil or 100 µl oil containing 25 µg testosterone (T)+0·005 µg oestradiol benzoate (E_2 B)/100 g body wt into male rats of various ages (means ± S.E.M., numbers in parentheses indicate the numbers of animals per group)*

Age (days)	Testosterone (ng/ml)			FSH (ng/ml)		LH (ng/ml)	
	SHAM	BLO + oil	BLO + E_2 B + T	BLO + oil	BLO + E_2 B + T	BLO + oil	BLO + E_2 B + T
15	2·98 ± 1·24 (5)	0·98 ± 0·56 (4)	11·6 ± 5·21* (6)	812 ± 48 (16)	590 ± 21* (7)	48 ± 10 (14)	⩽10* (7)
35	0·98 ± 0·33 (5)	0·47 ± 0·1 (4)	3·94 ± 1·58* (5)	920 ± 51 (10)	768 ± 24 (5)	39 ± 9 (10)	25 ± 5 (5)
55	3·45 ± 1·07 (5)	1·58 ± 1·49 (5)	3·05 ± 0·6* (4)	701 ± 36 (10)	649 ± 35 (5)	90 ± 17 (11)	11 ± 3* (5)
75	2·02 ± 1·32 (6)	1·12 ± 2·41 (6)	4·81 ± 1·46* (5)	448 ± 20 (18)	504 ± 21 (4)	85 ± 7 (16)	16 ± 4* (4)

*$P \leqslant 0{\cdot}05$: compared with values in orchidectomized animals injected with oil alone (two-tailed Wilcoxon test).

Table 3. *Concentrations of FSH and LH in serum and of progesterone in plasma 8 h after bilateral ovariectomy (BLO), sham-operation (SHAM) or BLO followed by two injections of 100 µl oil or 100 µl oil containing 0·012 µg oestradiol benzoate (E_2 B)+0·075 mg progesterone (P)/100 g body wt into female rats of various ages (means ± S.E.M., numbers in parentheses indicate the numbers of animals per group)*

Age (days)	Progesterone (ng/ml)			FSH (ng/ml)		LH (ng/ml)	
	SHAM	BLO + oil	BLO + E_2 B + P	BLO + oil	BLO + E_2 B + P	BLO + oil	BLO + E_2 B + P
15	2·4 ± 0·2 (4)	4·0 ± 0·8 (4)	—	1452 ± 62 (18)	1285 ± 100 (4)	53 ± 12 (15)	22 ± 6 (5)
25	2·7 ± 0·3 (4)	4·9 ± 0·8 (4)	4·8 ± 1·0 (4)	866 ± 65 (8)	482 ± 111* (5)	16 ± 5 (6)	26 ± 5 (5)
35	4·0 ± 0·4 (5)	4·2 ± 0·7 (5)	9·9 ± 4·7 (4)	630 ± 43 (11)	608 ± 47 (7)	59 ± 11 (11)	60 ± 24 (7)
Dioestrus day 2	25·8 ± 6·5 (5)	9·2 ± 0·9 (5)	20·1 ± 3·1 (5)	484 ± 26 (9)	478 ± 25 (5)	64 ± 12 (11)	52 ± 8 (5)

*$P \leqslant 0{\cdot}05$: compared with values in ovariectomized animals injected with oil alone (two-tailed Wilcoxon test).

animals have also been reported (de Jong & Sharpe, 1976; Nandini *et al.* 1976; Hopkinson *et al.* 1977; Davies *et al.* 1978; Lorenzen & Schwartz, 1979), but these authors used different test systems.

Although the relative FSH response to bFF in rats gonadectomized 2 days before treatment was comparable to the response found in intact rats, the gonadectomized rat seems to be a better test model because of a consistent lack of effect on levels of LH. However, too long an interval between gonadectomy and treatment does not seem advisable since in adult female rats a decreasing response 8 h after injection of bFF was found with increasing intervals between ovariectomy and treatment (de Jong *et al.* 1979).

It is noteworthy that in all groups and in all experiments, levels of FSH could not be suppressed below about 30% of values found in control animals, with the strongest relative suppression occurring in the acutely gonadectomized adult female rat. It is not clear whether the significant suppression of LH occurring in some groups has to be ascribed to inhibin-like activity or to other factors which might be present in the charcoal-treated bFF (de Jong *et al.* 1979).

Sex-related differences in the sensitivity of the FSH response

de Jong *et al.* (1978) showed that small amounts of bFF are only effective in suppressing FSH levels in adult female rats and not in immature female rats. The present study showed that in intact female rats from 35 days of age onwards the FSH response could by detected earlier (i.e. after 4 h) than in younger animals. These observations point to an increasing sensitivity of the hypothalamic-pituitary system to inhibin-like activity towards adulthood in female rats. In contrast, such a changing sensitivity was not found in male rats.

Indications for a physiological role of inhibin in the control of FSH secretion

The possibility that inhibin might play a role in the regulation of FSH secretion not only depends upon the sensitivity of the hypothalamic-pituitary system to inhibin-like activity, but also upon the presence and actual levels of inhibin-like activity in the animal (hereafter referred to as endogenous inhibin). The presence of inhibin-like activity in female rats has been shown by Erickson & Hsueh (1978), Uilenbroek, Tiller, de Jong & Vels (1978) and most recently by DePaolo, Shander, Wise, Barraclough & Channing (1979*a*), and in male rats by measurement of inhibin-like activity in rete testis fluid and in testicular homogenates (F. H. de Jong, unpublished observation). However, the actual levels of inhibin-like activity cannot (yet) be measured directly. The question whether, and – more specifically – at what ages inhibin may be effective, had to be approached indirectly. The criteria used here for such a possible physiological role of inhibin were, first, that removal of the gonads should result in a fast rise of FSH levels differing from the response of LH, and secondly that if such an FSH rise occurred, an injection of steroid-free bFF, but not an injection of an approximately physiological dose of testicular or ovarian steroids, could prevent this rise.

Male rats

A diverging FSH and LH response to gonadectomy was observed 8 h after the operation in male rats aged 15, 35, 46 and 50 days, but not at later ages (Fig. 3). Increased levels of FSH within 8 or 9 h of orchidectomy as well as non-parallel changes in the concentrations of FSH and LH in plasma of prepubertal and adult rats have been reported earlier (Swerdloff, Walsh, Jacobs & Odell, 1971; Gupta, Rager, Zarzycki & Eichner, 1975; Mahesh, Muldoon, Eldridge & Korach, 1975; Schwartz & Justo, 1977).

The signal causing an FSH rise after orchidectomy could be a decrease in peripheral levels of testosterone. However, in this study a significant decrease of testosterone levels in plasma could not be detected 8 h after orchidectomy (Table 2), which is in agreement with reports of Negri & Gay (1976) and Denef & Hautekeete (1978). Also, in the orchidectomized, steroid-injected rats, the observed rise in FSH occurred in the presence of plasma concentrations of

testosterone which were higher than those found in control animals. Only in the 15-day-old rats did testosterone partially suppress FSH. This might have been caused by either the higher levels of testosterone after injection at that age or by the higher sensitivity for the negative feedback action of testosterone of prepubertal rats compared with adult rats (Negro-Vilar, Ojeda & McCann, 1973; Smith, Damassa & Davidson, 1977). The observation that levels of LH were, in most cases, decreased after injection of the steroid combination is in accordance with reports that a higher dose of testosterone is needed to suppress FSH levels than to suppress LH levels (Hutchinson & Goldman, 1975; Aafjes, Vreeburg & Schenck, 1978). For the same reason it is also unlikely that the signal causing a rise of FSH after orchidectomy is a decrease in peripheral oestradiol (Hall & Gomes, 1975; de Jong, Uilenbroek & van der Molen, 1975). Since at all ages studied, injection of steroid-free bFF was capable of completely preventing the rise of FSH after orchidectomy (Fig. 3), it seems probable that the signal causing a rise of FSH is a loss of endogenous inhibin.

Female rats

A diverging FSH and LH response was observed from 25 days of age onwards within 8 h of ovariectomy (Fig. 4). The rise in FSH after ovariectomy could only be prevented at 25 days of age by an injection of oestradiol benzoate and progesterone, although the dose used results in near-physiological levels of oestradiol at 20 days of age (Meijs-Roelofs, de Greef & Uilenbroek, 1975) and in approximately physiological levels of progesterone at all ages studied (Table 3). The ineffectiveness of steroids in suppressing levels of FSH in short-term experiments was reported earlier for the adult unilaterally ovariectomized rat (Butcher, 1977; Welschen, Dullaart & de Jong, 1978) and for the pro-oestrous rat (Welschen *et al.* 1980). However, injection of follicular fluid could suppress FSH levels both in the unilateral ovariectomized rat and in the pro-oestrous rat (Marder *et al.* 1977; Welschen *et al.* 1980). Furthermore, in this study injection of steroid-free bFF was, at all ages tested, capable of preventing the rise of FSH after ovariectomy.

If the criteria chosen are correct, endogenous inhibin might play a role in the regulation of FSH levels until about 50 days of age in male rats. This conclusion concurs with the observation that the increase in Sertoli cell number (considered to be the most probable production site of male inhibin-like activity: Steinberger & Steinberger, 1976; de Jong & Sharpe, 1977*a*; Rich & de Kretser, 1977; Hopkinson, Dulisch, Gauss, Hilscher & Hirschhäuser, 1978; de Jong *et al.* 1979) is reported to stop in the period between 15 and 35 days of age (de Jong & Sharpe 1977*b*; Thanki & Steinberger, 1978). In female rats, endogenous inhibin might play a role in the regulation of FSH levels from at least day 25 onwards. This is in agreement with the observation that medium and large antral follicles (the most probable production site of female inhibin-like activity: Welschen *et al.* 1977; Erickson & Hsueh, 1978) appear for the first time around day 25 in the ovaries (Uilenbroek, Arendsen-de Wolff Exalto & Welschen, 1976). Since the short-term response of FSH to gonadectomy (Fig. 5) decreases with age in male rats, and since this response and the hypothalamic-pituitary sensitivity to inhibin-like activity increases with age in female rats, it is suggested that the biological significance of inhibin might be decreasing towards adulthood in the male rat and might become increasingly important with age in the female rat, despite the ability to respond to inhibin-like activity at all ages studied.

This study was supported in part by the Organization of Medical Research in the Netherlands (FUNGO). We appreciate the technical assistance of Mrs A. A. de la Fosse and the help of Mr A. Bruynzeel of the Rotterdam slaughterhouse in obtaining bovine ovaries. We thank Dr J. Moll and Dr H. M. A. Meijs-Roelofs for help in the preparation of the manuscript and Dr R. Welschen for stimulating discussions. We are grateful to the National Institute of Arthritis and Metabolic and Digestive Diseases (Bethesda, Maryland, U.S.A.) for the supply of gonadotrophins for use in the radioimmunoassays.

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J. Endocr. (1981), 90, 69–76 *Printed in Great Britain*
0022–0795/81/0090–0069 $02.00/0

TIME-RELATED SECRETION OF GONADOTROPHINS AFTER A SINGLE INJECTION OF STEROID-FREE BOVINE FOLLICULAR FLUID IN PREPUBERTAL AND ADULT FEMALE RATS

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(*Received* 24 *October* 1980)

SUMMARY

The time-related changes in gonadotrophin concentrations after a single injection of steroid-free bovine follicular fluid (bFF), which contains material with inhibin-like activity, were studied in 25-day-old and adult female rats which either were intact or had been ovariectomized 2 days before. In ovariectomized and intact rats administration of bFF caused a selective suppression of FSH after 4–8 h in 25-day-old rats and after 3–4 h in adult rats. No systematic changes in concentrations of LH after bFF injection were observed. Relative suppression of FSH levels in adult rats was more pronounced and of longer duration than in 25-day-old rats. Moreover, the total period of suppression lasted longer in ovariectomized than in intact rats (12 and 8 h for 25-day-old and 24 and 15 h for adult rats respectively). Hypersecretion of FSH was found in intact rats after the initial suppression; this phenomenon was more pronounced and of longer duration in adult than in 25-day-old rats. No clear change in the numbers of healthy growing follicles was observed after injection of bFF into intact rats. These results indicate that the pituitary secretion of FSH responds quickly and selectively after administration of bFF to intact and ovariectomized, 25-day-old and adult female rats. The hypersecretion of FSH in intact rats might compensate for the initial suppression of this gonadotrophin, and may thus ensure the maturation of a normal number of follicles.

INTRODUCTION

Injection of inhibin-like material as present in steroid-free follicular fluid (FF) of various origins (bovine, porcine, equine) causes a rapid, selective suppression of serum concentrations of follicle-stimulating hormone (FSH) in female animals. This is the case in adult, intact and ovariectomized rats (Marder, Channing & Schwartz, 1977; Welschen, Hermans, Dullaart & de Jong, 1977), monkeys (Channing, Anderson & Hodgen, 1979) and hamsters (Chappel & Selker, 1979) and in ovariectomized mares (Miller, Wesson & Ginther, 1979). In intact and ovariectomized immature female rats, concentrations of FSH can also be lowered selectively by injection of FF (porcine FF: Pomerantz, 1978; Lorenzen & Schwartz, 1979; bovine FF (bFF): Hermans, van Leeuwen, Debets & de Jong, 1980), but when compared with similarly treated adult female rats, the degree of suppression and its duration are less in prepubertal rats (Hermans *et al.* 1980). It was concluded from these observations that the female rat becomes increasingly sensitive to inhibin-like activity during development; apparently the animal acquires a fast (within 24 h) -acting mechanism which specifically controls concentrations of FSH in the peripheral circulation. By this mechanism, and not by increased steroid production, antral follicles (the most probable source of inhibin: Welschen

et al. 1977; Erickson & Hsueh, 1978) seem to adjust their own numbers after the rise in FSH concentrations during the periovulatory period or after unilateral ovariectomy (Welschen, Hermans & de Jong, 1980). The present study was undertaken to determine more precisely the time-related changes in FSH concentrations after a single injection of steroid-free bFF in prepubertal (25-day-old) and adult female rats. The short-term effect of a bFF-induced reduction of FSH levels on the number of growing follicles was also studied.

MATERIALS AND METHODS

Animals

Female rats, 25 days of age or approximately 3 months old, of a Wistar substrain (R-Amsterdam), were used. Rats were kept under controlled conditions of light (lights on 05.00–19.00 h) and temperature (22–25 °C), and standard dry pellets and tap water were available *ad libitum*. Animals were weaned at 21–22 days of age. Rats of this strain generally show first vaginal oestrus and ovulation at 37–42 days of age. Daily vaginal smears were taken from the adult rats and only rats with two consecutive 5 day cycles before the day of treatment were used. Adult intact rats were used on the day after vaginal oestrus, dioestrus-1 (= metoestrus). Care was taken to divide litter-mates between experimental and control groups. Only rats with body weights between 32 and 45 g at day 25 and between 180 and 210 g at 3 months of age were used.

Follicular fluid

Bovine ovaries were obtained from a local slaughterhouse. Bovine FF was aspirated from follicles of 10–20 mm diameter within 3 h after collection of the ovaries, mixed with charcoal (50 mg/ml) at 21 ± 1 °C for 60 min and centrifuged at 10 000 *g* for 30 min. Concentrations of oestradiol-17β and progesterone after treatment were less than 1% of the pretreatment levels (oestradiol-17β 103 *v.* 0·04 and progesterone 150 *v.* 0·33 ng/ml before and after treatment respectively). Bovine plasma, which was also charcoal-treated for comparability with bFF, served as a control fluid in all experiments since it has a relative protein composition similar to that of bFF (Caravaglios & Cilotti, 1957; Andersen, Krøll, Byskov & Faber, 1976) and shows no inhibin-like activity in in-vivo test systems (Welschen *et al.* 1977). Injections of bFF and bovine plasma were given intraperitoneally.

Experimental design

Operations, injections and collection of blood were always performed under ether anaesthesia. A single injection of 500 µl bovine plasma or bFF/100 g body wt was given at 09.00 h to intact or ovariectomized rats. Ovariectomy of 23-day-old or adult rats was carried out by latero-dorsal incisions, performed between 09.00 and 12.00 h. The adult rats were used regardless of the day of the oestrous cycle, since the FSH response 2 days after ovariectomy has been found to be independent of this factor (W. P. Hermans, unpublished observation). The ovariectomized animals were injected with test material 2 days after operation. Blood was sampled at various intervals after injection by puncture of the ophthalmic venous plexus; animals bled during the dark period were bled in a lighted room within 10 min. Immature rats were bled once; adult rats were bled twice: in ovariectomized rats there was at least a 6 h sampling interval between bleedings and in most groups of intact rats there was a sampling interval of 12 h. At the first bleeding not more than 1·5 ml blood was collected. Blood was allowed to clot overnight at 4 °C before centrifugation for 15 min at 2000 *g* to obtain the serum. Serum was stored at −20 °C until used for determination of hormone concentrations. In all control and experimental groups at least six rats were used unless otherwise stated.

Determination of hormone concentrations

Concentrations of FSH and luteinizing hormone (LH) in serum from individual rats were measured by radioimmunoassay as described by Welschen, Osman, Dullaart, de Greef, Uilenbroek & de Jong (1975). Concentrations of gonadotrophins in serum from comparable control and experimental groups were measured in duplicate in the same assay: the graphs were each composed from data obtained in three to four assays. All results are expressed as means (±S.E.M.) in terms of NIAMDD-rat-LH RP-1 or NIAMDD-rat-FSH RP-1. Interassay variability (coefficient of variation) was 18·6% for FSH and 13·5% for LH; mean intra-assay variability was 10·1% for FSH and 14·4% for LH.

Histology

Ovaries of 25-day-old and adult rats were collected at various times after injection. They were fixed in Bouin's fluid and after sectioning (7 μm) were stained with haematoxylin and eosin.

Since the object of this part of the study was to compare the number of follicles after administration of bFF with that after administration of bovine plasma, follicles were classified according to the method of Moore & Greenwald (1974) and not according to follicular volume, the system generally used in our laboratory (Welschen *et al.* 1975). Follicles were divided into types with three to four, five to seven, eight to 12 and more than 12 layers of granulosa cells in the largest cross-section. Every section was examined; only those follicles with the nucleolus of the oocyte nucleus visible and without obvious signs of atresia were counted.

Statistical procedures

The significance of differences between data for different groups was calculated using Wilcoxon's two-sample test. Differences were considered to be statistically significant when $P \leqslant 0{\cdot}05$ (two-tailed).

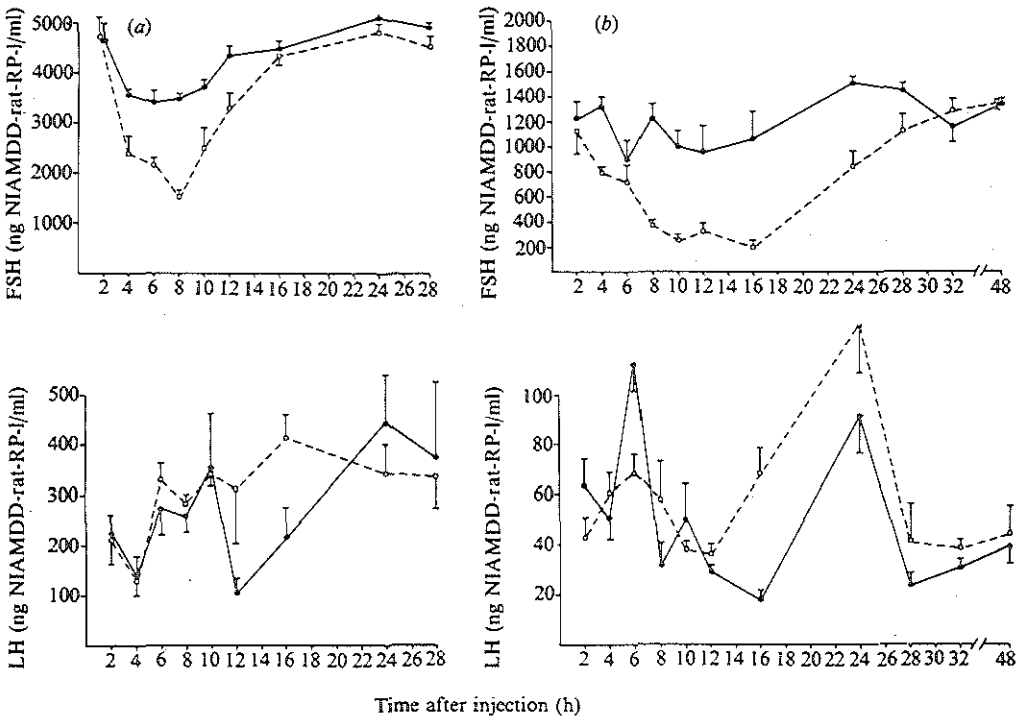
RESULTS

Effect of bFF on gonadotrophin concentrations in ovariectomized rats

Serum concentrations of FSH and LH at various times after a single injection of bovine plasma (controls) or bFF in ovariectomized rats are shown in Fig. 1*a* (25-day-old rats) and Fig. 1*b* (adult rats). Compared with values in control animals, concentrations of FSH after injection of bFF were decreased between 4 and 12 h ($P < 0{\cdot}05$) in the 25-day-old rats and, except at 6 h, between 4 and 24 h ($P < 0{\cdot}02$) in the adult rats. Maximal suppression was found at 8 h ($P < 0{\cdot}02$, reaching 44% of mean control values) in 25-day-old rats and between 8 and 16 h (suppression to 19–35% of mean control values) in adult rats. After the period of suppression, FSH levels started to rise and were never significantly different from control values from 16 h onwards in 25-day-old rats and from 28 h onwards in adult rats. Concentrations of LH were only significantly ($P < 0{\cdot}05$) different from control values at 16 h after injection in both 25-day-old and adult rats.

Effect of bFF on gonadotrophin concentrations and numbers of follicles in intact rats

The results of injection of bovine plasma or bFF on serum concentrations of FSH and LH in intact rats are shown in Fig. 2*a* (25-day-old rats) and Fig. 2*b* (adult rats). Compared with values in control animals, 25-day-old rats showed decreased concentrations of FSH at 8 h after injection of bFF ($P < 0{\cdot}02$, decrease to 48% of control values). At 12 h concentrations of FSH were not significantly different from control values, while increased concentrations were found after 16 ($P < 0{\cdot}05$) and 20 h ($P < 0{\cdot}02$) (increases to 138% of mean control values). Thereafter levels of FSH fell and reached control values at 28 h. Concentrations of



n (control)	6	7	7	6	5	8		7			8	5			6	13	5	5	4	5		4			5		6		5		5	
n (experimental)	6	8	8	6	6	7		8			9	6			6	12	4	5	4	4		4			6		6		5		6	

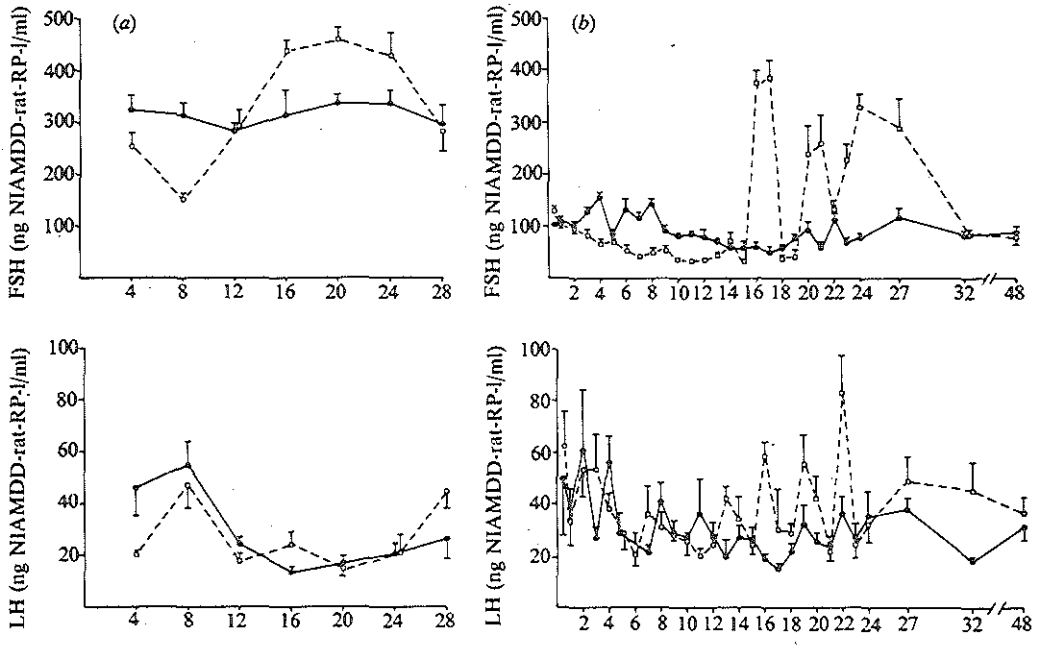
Fig. 1. Mean (±S.E.M.) concentrations of FSH (top) and LH (bottom) in the serum at various times after injection of 500 µl bovine plasma (controls, solid lines) or bovine follicular fluid (broken lines) per 100 g body wt into (*a*) 25-day-old and (*b*) adult female rats. Rats were bilaterally ovariectomized 2 days before injection. Numbers of animals per group are shown below the *x*-axes.

LH were not different from control values at any time after injection. In intact adult rats, levels of FSH were suppressed (to 36–79% of mean control values) from 3 to 13 h and at 15, 18 and 19 h after injection of bFF ($P<0{\cdot}02$; $P<0{\cdot}05$ at 5 h only). Increased levels of FSH, between 244 and 774% of mean control values, were found at 16, 17, 20, 21, 23, 24 ($P<0{\cdot}02$) and 27 h ($P<0{\cdot}05$); no significant differences were found at 14, 22, 32 and 48 h after injection. Concentrations of LH were different at 13 ($P<0{\cdot}05$), 16 and 17 ($P<0{\cdot}02$) and 22 h ($P<0{\cdot}05$) after injection (increases to 200–310% of mean control values).

The results of histological examination of the ovaries at 8, 16 and 24 h after injection in 25-day-old rats and after 8, 20, 27 and 48 h in adult rats revealed no significant differences between groups treated with bovine plasma and those treated with bFF ($n=4$, data not shown). The largest difference was observed at 8 h after injection in 25-day-old rats in the number of follicles with five to seven layers of granulosa cells (88 ± 11 (S.E.M.) in control animals *v.* 68 ± 11 in bFF-injected groups). However, after 16 and 24 h this difference became even smaller (71 ± 6 *v.* 62 ± 3 and 52 ± 10 *v.* 54 ± 5 respectively).

DISCUSSION

The aim of this study was to investigate the time-related changes in gonadotrophin secretion after a single injection of bFF in 25-day-old and adult female rats which either were intact or



n (control)	13	18	8	8	8	7	8
n (experimental)	10	17	8	8	8	7	9

Fig. 2. Mean (± S.E.M.) concentrations of FSH (top) and LH (bottom) in the serum at various times after injection of 500 µl bovine plasma (controls, solid lines) or bovine follicular fluid (broken lines) per 100 g body wt into (*a*) intact 25-day-old and (*b*) intact adult female rats (injected at dioestrus-1). Numbers of animals per group are shown below (*a*), and in (*b*) were 6–8 at 20, 21, 23 and 27 h and 4–6 at all other times.

had been ovariectomized 2 days before. Since levels of FSH were, but levels of LH were generally not, affected by injection of bFF, the observed effects can be ascribed to the presence of an inhibin-like factor in bFF (de Jong, 1979). The results of injection of bFF to intact and ovariectomized rats showed that the selective FSH response to bFF was more pronounced and of longer duration in adult rats than in 25-day-old rats. The first observation confirms and the second extends the observations of an earlier study (Hermans *et al.* 1980). These findings also provide additional support for the concept of an increasing role of inhibin in the regulation of FSH concentrations during development, which might lead to an increasing ability to adjust the number of maturing follicles (Welschen *et al.* 1980).

In ovariectomized animals the time at which a selective change in FSH levels can first be detected (4 h after injection) confirms earlier reports: latencies in the FSH response after injection of inhibin-like material in the absence of the ovaries have been reported to be 2–5 h (rats: Campbell & Schwartz, 1979; Hoffmann, Lorenzen, Weil & Schwartz, 1979; hamsters: Chappel, 1979). The apparent fall in levels of FSH in 25-day-old control animals between 2 and 12 h after injection may be caused by abnormally high levels of FSH at 2 h after injection. Since this was also found in the animals which were injected with bFF, the high concentrations of FSH might find their cause in the short recovery period after the ether anaesthesia or, less likely, in a short-lasting reaction of FSH secretion in the 25-day-old ovariectomized rat to factors present in the injected fluids. The duration of the FSH suppression was longer in adult ovariectomized rats (from 4 to 24 h after injection) than that

reported by Campbell & Schwartz (1979) (from 4 to 15 h after porcine FF injection) and Chappel (1979) (4 h after injection of crude extracts of hamster ovaries into hamsters). These differences might be caused by the differences in the dose of inhibin in the injected preparations.

In intact adult rats, preliminary results showed highly raised levels of FSH between 16 and 24 h after injection and blood was therefore sampled every hour. The selective suppression of FSH in intact rats, first detectable 3 h after injection of bFF (Fig. 2*b*), is comparable with the results of Hoffmann *et al.* (1979) and DePaolo, Wise, Anderson, Barraclough & Channing (1979) for intact rats and with the results found for the ovariectomized adult rats in the present study. However, the suppression of FSH lasted only until 13–15 h after injection (as was also reported by DePaolo, Wise *et al.* 1979), which contrasts with the suppression until 24 h found in the ovariectomized rats. After the initial suppression, a pulsating hypersecretion of FSH followed between 15 and 32 h after injection. DePaolo, Hirshfield, Anderson, Barraclough & Channing (1979) also reported increased levels of FSH following an initial suppression after injection of porcine FF into intact rats at pro-oestrus and oestrus. Decreased followed by increased secretion of FSH after bFF administration was also found in 25-day-old intact animals.

Comparison of Figs 1*b* and 2*b* (adult rats) shows that during the period when bFF still exerts a suppressive effect on FSH secretion in ovariectomized rats, the rebound phenomenon is already occurring in intact rats. Although there was a difference in sampling frequency between intact and ovariectomized rats, there was no indication that a hypersecretion of FSH could have been missed in the ovariectomized rats. Since it seems unlikely that the rebound effect can be attributed to a faster disappearance of the injected inhibin-like material from the circulation in the presence of the ovaries, the ovaries must be involved in another way in the hypersecretion of FSH.

Since changes in the numbers of growing follicles were not observed either before, during or after the hypersecretion of FSH, the ovarian involvement in the rebound phenomenon might be one of an altered synthesis or secretion of steroids and/or endogenous inhibin. Recent studies suggest that short-term increases in FSH levels (such as after unilateral ovariectomy and during the periovulatory period) cannot be attributed to changing levels of steroids (Butcher, 1977; Welschen, Dullaart & de Jong, 1978; Welschen *et al.* 1980), but must be ascribed to lowered endogenous inhibin levels (Schwartz & Channing, 1977; Hermans *et al.* 1980; Welschen *et al.* 1980). Thus, a diminished endogenous production of inhibin, possibly caused by the low levels of FSH (30 ng/ml) during the 4–15 h after injection of bFF, seems the most likely cause for the start of the enhanced secretion of FSH after 16 h (as was also suggested by DePaolo, Hirshfield *et al.* 1979).

The results of injection of bFF into prepubertal rats support this hypothesis. Since at the age of 25 days the effectiveness of oestrogens and progesterone in regulating FSH concentrations is diminishing (Meijs-Roelofs, Kramer & Gribling-Hegge, 1981), and since the role of inhibin in the regulation of FSH concentrations seems to be just developing at this age (Hermans *et al.* 1980), a less pronounced hypersecretion of FSH after the initial suppression would be expected if a loss of endogenous inhibin is the cause for the hypersecretion of FSH. This is indeed the case.

However, the above hypothesis presumes that the total amount of endogenous plus exogenous (bFF) inhibin during the period preceding the hypersecretion of FSH is lower than the amount of endogenous inhibin before injection of bFF. At present this cannot be substantiated by measurement of inhibin levels. Furthermore, although this hypothesis might serve as an explanation for the onset of the pituitary hypersecretion of FSH, it cannot fully explain the apparently oscillating pattern of FSH secretion and the slight rise in LH concentrations in some of the groups of adult rats. It remains possible that the subsequent pulsating secretion of FSH is a result of a combined effect of altered steroid and inhibin release.

Alternatively, the hypersecretion of FSH and the changes in LH concentrations found during that period might find their cause in an enhanced secretion of hypothalamic gonadotrophin releasing hormone (GnRH), resulting from the very low levels of FSH present in the intact rats during the period of suppression (Motta, Fraschini & Martini, 1969). Finally, Wilkinson, Moger & Selin (1980) suggested an increased pituitary sensitivity to GnRH after exposure to inhibin-like activity, but these authors used a long-term exposure (7 days) to inhibin-like activity.

In conclusion, the present results show that disturbances in FSH secretion by a single injection of bFF in intact rats are of short duration: basal levels of FSH are reached again within 32 h. The hypersecretion of FSH after a period with lower FSH levels seems sufficient to ensure a continuation of the maturation of a normal number of follicles during the cycle. This compensation might explain why cycles continue with little or no disturbance after a single daily injection with bFF at 09.00 h for 26 days (de Jong, Welschen, Hermans, Smith & van der Molen, 1978). The fact that ovulation in hamsters was blocked after a severe reduction of FSH levels following more frequent injections of bFF (Chappel & Selker, 1979), emphasizes the need for a compensation mechanism following disturbances in FSH secretion.

The authors wish to thank Dr J. Moll and Dr H. M. A. Meijs-Roelofs for their help in the preparation of the manuscript. We are grateful to the National Institute of Arthritis and Metabolic and Digestive Diseases (Bethesda, Maryland, U.S.A.) for the supply of gonadotrophins used in the radioimmunoassay.

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J. Endocr. (1982), 92, 425–432 *Printed in Great Britain*
0022–0795/82/0092–0425 $02.00/0

VI

EFFECTS OF SINGLE INJECTIONS OF BOVINE FOLLICULAR FLUID ON GONADOTROPHIN CONCENTRATIONS THROUGHOUT THE OESTROUS CYCLE OF THE RAT

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(*Received* 19 *June* 1981)

SUMMARY

A single injection of steroid-free bovine follicular fluid (bFF), which contains inhibin-like activity, was given to adult female rats at 09.00 h on dioestrus-2 (the day after metoestrus), dioestrus-3, pro-oestrus or oestrus. Peripheral concentrations of gonadotrophins were measured at 2-h intervals after injection. Compared with values in control animals treated with bovine plasma, injection of bFF did not influence concentrations of LH. In contrast, at all days studied injection of bFF resulted in suppressed concentrations of FSH, during a period which started between 4 and 10 h after injection and lasted 4–12 h, depending on the day of the cycle. With the exception of pro-oestrus, the period of suppression was followed by one in which fluctuating levels of FSH were found; in general, resulting levels were higher though not significantly increased. This latter effect was most pronounced on dioestrus-2 and dioestrus-3, when levels of FSH, which were already low in control animals, were first suppressed during the 6 h after injection of bFF.

These data, in conjunction with results from an earlier study in rats at dioestrus-1, showed that administration of bFF induces a fast and selective suppression of FSH secretion on all days of the cycle. This period of suppression was followed by one with fluctuating levels of FSH which showed a tendency to be higher, indicating that disturbances in FSH secretion, such as are caused by bFF, can be compensated for quickly. In this way the process of follicular maturation might be protected.

INTRODUCTION

In adult female rats the pituitary secretion of follicle-stimulating hormone (FSH), but not of luteinizing hormone (LH) seems, at least partly, to be under the inhibitory control of an inhibin-like factor (for reviews see de Jong, 1979; Welschen, Hermans & de Jong, 1980) which is secreted by the ovaries (Erickson & Hsueh, 1978; Uilenbroek, Tiller, de Jong & Vels, 1978; DePaolo, Shander, Wise, Barraclough & Channing, 1979) and is present in steroid-free follicular fluid (FF) (de Jong & Sharpe, 1976; Marder, Channing & Schwartz, 1977; Welschen, Hermans, Dullaart & de Jong, 1977). Since presumed changes in the peripheral concentration of inhibin-like activity are followed within 3–4 h by inverse changes in the pituitary secretion of FSH but not of LH (DePaolo, Shander *et al.* 1979; Hermans, van Leeuwen, Debets & de Jong, 1980; Shander, Anderson & Barraclough, 1980; Welschen *et al.* 1980), it has been postulated that the physiological role of inhibin could be a fast-acting control of FSH secretion. In turn, in the periovulatory period this regulation of FSH secretion might determine the number of follicles destined to ovulate in the next cycle

(Shander *et al.* 1980; Welschen *et al.* 1980). However, single daily injections of bovine FF (bFF) for up to 26 days did not result in disturbances of the ovarian cycles or ovulation in rats (de Jong, Welschen, Hermans, Smith & van der Molen, 1978). These results could be due to levels of FSH after bFF injection insufficiently decreased to affect follicular development on different days of the cycle. Alternatively, this observation may be explained from the results of recent studies showing that after a single injection of porcine FF (pFF) on pro-oestrus (DePaolo, Hirshfield, Anderson, Barraclough & Channing, 1979) or of bFF on dioestrus-1 (= metoestrus) (Hermans, Debets, van Leeuwen & de Jong, 1981), initial suppression of levels of FSH is followed by a rebound, resulting in increases in plasma concentrations of FSH.

The present study was undertaken to determine whether there is not only an initial suppression but also a later rebound of concentrations of FSH after a single injection of bFF in intact female rats on dioestrus-2, dioestrus-3, pro-oestrus and oestrus, thereby extending a previous study which used a similar treatment on dioestrus-1 (Hermans *et al.* 1981).

MATERIALS AND METHODS

Animals

Adult female rats of a Wistar substrain (R-Amsterdam), approximately 3 months old and weighing 180–210 g, were used. Rats were kept under controlled conditions of light (light period 05.00–19.00 h) and temperature (22–25 °C). Standard dry pellets and tap water were available *ad libitum*. Vaginal smears were taken daily and only rats which exhibited two consecutive 5-day cycles before the day of treatment were used. The day after vaginal oestrus was called dioestrus-1 as in earlier publications (Hermans *et al.* 1980, 1981).

Experimental design

Bovine follicular fluid was aspirated from ovarian follicles with diameters of between 5 and 20 mm and treated with charcoal to remove steroids; charcoal-treated bovine plasma served as a control fluid (for details see Hermans *et al.* 1981). Injections of bFF and bovine plasma were given intraperitoneally. Blood was collected by puncture of the ophthalmic venous plexus. Injections and blood collections were performed under ether anaesthesia.

Rats at various stages of the oestrous cycle received at 09.00 h a single injection of 0·5 ml bFF or bovine plasma/100 g body wt. These rats were assigned at random to form groups, ultimately of 5–7 animals, and they were bled at various times after injection (see Results). Blood (for preparation of serum) was sampled twice: the first bleeding consisted of not more than 1·5 ml blood and there was at least a 12-h interval between bleedings. Finally, groups of untreated animals were exsanguinated at 4-h intervals throughout the oestrous cycle. All animals bled during the dark period were bled within 10 min of transfer to a lighted room. Blood was allowed to clot overnight in a refrigerator before centrifugation for 15 min at 2000 ***g***. Serum was stored at −20 °C until used for determination of hormone concentrations.

Determination of hormone concentrations

Concentrations of FSH and LH in serum from individual rats were measured in duplicate by radioimmunoassay as described by Welschen, Osman, Dullaart, de Greef, Uilenbroek & de Jong (1975). Samples from rats injected with bovine plasma or bFF were measured in five different assays; every assay contained one to three serum samples from each treatment group. Gonadotrophins in samples from untreated animals were determined in separate assays. All results are expressed as means (±S.E.M.) in terms of NIAMDD-rat-LH RP-1 or NIAMDD-rat-FSH RP-1. Interassay variability (coefficient of variation) was 9·1% for FSH and 19·5% for LH; mean intra-assay variability was 8·2% for FSH and 11·1% for LH.

Statistical procedures

The significance of differences between mean hormone concentrations in groups treated with bovine plasma or bFF at various times after injection was calculated using Wilcoxon's two-sample test. The significance of differences between standard deviations of mean gonadotrophin values of groups treated with bovine plasma or bFF over longer test periods was calculated using the *F*-test, in order to show fluctuations in individual values after treatment. Differences were considered to be statistically significant when $P \leqslant 0{\cdot}05$ (two-tailed tests).

RESULTS

Concentrations of FSH and LH, measured at 2-h intervals in rats given one injection of either bovine plasma or bFF on the morning of dioestrus-2, dioestrus-3, pro-oestrus or oestrus are shown in Figs 1 and 2.

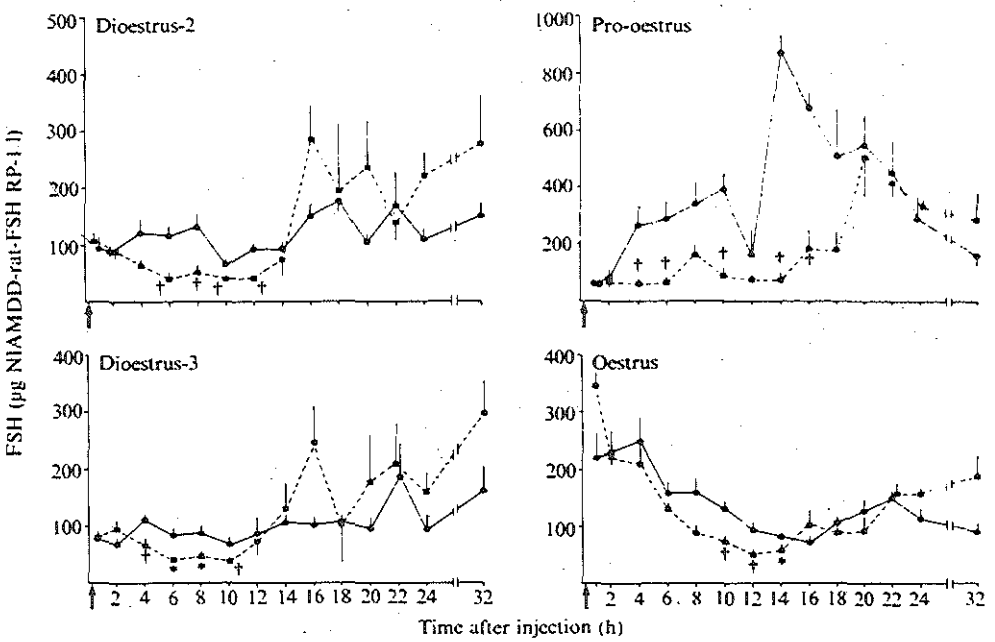

Fig. 1. Concentrations of FSH in serum at different times after a single injection of bovine plasma (controls, solid lines) or bovine follicular fluid (broken lines) in intact female rats at various days of the oestrous cycle (means ± S.E.M., $n = 5$–7). Injections (0·5 ml/100 g body wt) were given at 09.00 h shown by arrow). Note the different scale at pro-oestrus. $^{*}P \leqslant 0{\cdot}05$, $\dagger P \leqslant 0{\cdot}02$ compared with control group (Wilcoxon, two-tailed test).

Compared with values in control animals injected with bovine plasma, a significantly higher concentration of FSH was found at 1 h after injection of bFF at oestrus. Significantly lower concentrations of FSH after injection of bFF were found at dioestrus-2 between 6 and 12 h, at dioestrus-3 between 4 and 10 h, at pro-oestrus between 4 and 16 h and at oestrus between 10 and 14 h after injection. In the following periods levels of FSH were not significantly different from control values with the exception of values 32 h after treatment at oestrus, when levels were higher than in the controls. In this period mean FSH

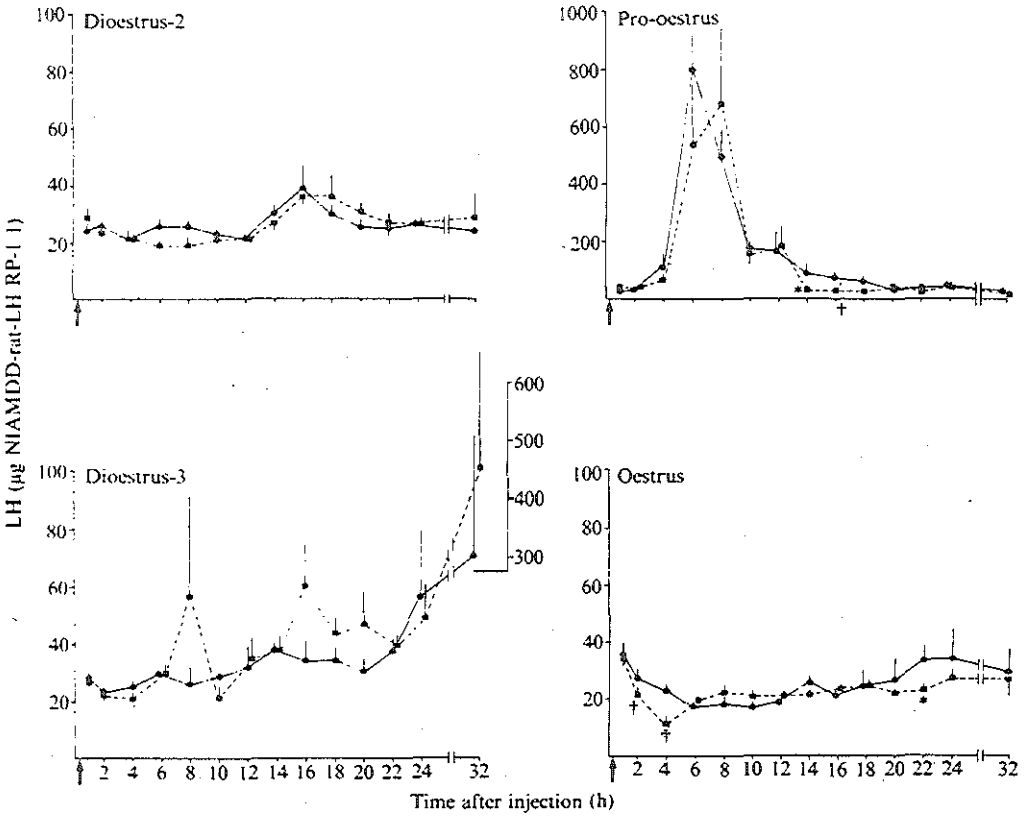

Fig. 2. Concentrations of LH in serum at different times after a single injection of bovine plasma (controls, solid lines) or bovine follicular fluid (broken lines) in intact female rats at various days of the oestrous cycle (means ± S.E.M., $n = 5$–7). Injections (0·5 ml/100 g body wt) were given at 09.00 h (shown by arrow). Note the different scale at pro-oestrus. $*P \leqslant 0{\cdot}05$, $\dagger P \leqslant 0{\cdot}02$ compared with control group (Wilcoxon, two-tailed test).

levels tended to be higher in bFF-treated rats and large variations between individual values in rats injected with bFF were found between 14 and 32 h after injection on dioestrus-2 and dioestrus-3: values within groups were generally either higher (250–600 µg/l) or lower (30–80 µg/l) than control values (90–200 µg/l). Therefore differences between standard deviations of the means of FSH values over periods of several hours were compared in animals injected with bovine plasma (n_1) with those given bFF (n_2) on all days. Significant differences in the period from 14 to 32 h after treatment were found at dioestrus-2 ($F = 5{\cdot}9$; $n_1 = 40$, $n_2 = 40$), at dioestrus-3 ($F = 4{\cdot}6$; $n_1 = 36$, $n_2 = 37$) and at oestrus ($F = 3{\cdot}2$; $n_1 = 38$, $n_2 = 41$).

Concentrations of LH after injection of bFF were only significantly different from concentrations in control animals at 14 and 16 h after injection on pro-oestrus when values were lower and at 2, 4 and 22 h after injection on oestrus when values were also lower.

Finally, the results of the measurements of FSH and LH in plasma from uninjected control rats are shown in Fig. 3*a*, together with the data from the animals which were injected with bovine plasma (Fig. 3*b*); the latter part of Fig. 3 contains the data also shown in Figs 1 and 2 and the levels found at dioestrus-1 in an earlier study (Hermans *et al.* 1981).

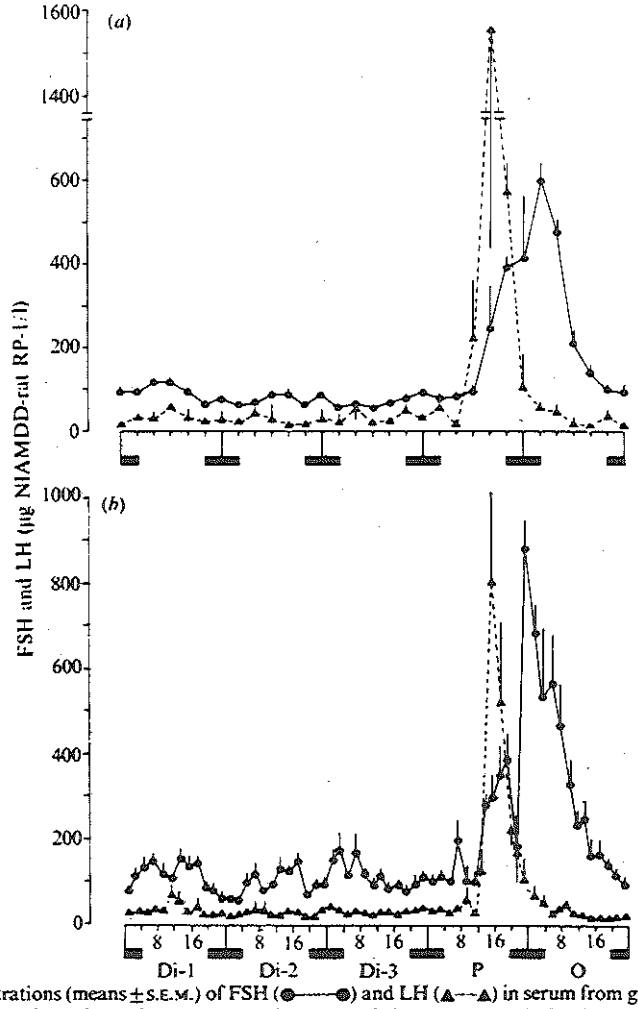

Fig. 3. Concentrations (means ± S.E.M.) of FSH (●——●) and LH (▲--▲) in serum from groups of intact adult female rats throughout the oestrous cycle at (*a*) 4-h intervals and (*b*) 2-h intervals. In (*a*) rats (n = 4–6) were intact and bled once. In (*b*) rats (n = 5–7) were injected once with bovine plasma and bled twice (data on dioestrus-1 from Hermans *et al.* (1981); data on other days of the cycle from the present study). Solid bars indicate periods of darkness and numbers indicate the hours of the day. Di-1 = dioestrus-1; Di-2 = dioestrus-2, Di-3 = dioestrus-3; P = pro-oestrus; O = oestrus.

These results indicate that the injection of bovine plasma under ether anaesthesia does not affect gonadotrophin concentrations in plasma in a systematic way.

DISCUSSION

The aim of the present study was to investigate effects of bFF on concentrations of gonadotrophins, which were measured at 2-h intervals in female rats with a 5-day cycle and compared with values found in control animals treated with bovine plasma. The possibility exists, however, that gonadotrophin levels in rats treated with bovine plasma do not reflect the normal situation because of the use of anaesthesia at injection and the repeated

bleedings. Therefore the secretion patterns of gonadotrophins after injection of bovine plasma were compared with the ones observed in a study in which concentrations of gonadotrophins were measured at 4-h intervals in intact, untreated rats of the same strain (Fig. 3). Similar patterns of concentrations of gonadotrophins were seen in untreated rats and in rats injected with bovine plasma. However, the absolute values of the pro-oestrous surges of FSH and LH were different, while levels of FSH showed larger fluctuations on the days of dioestrus in the animals injected with bovine plasma. This latter observation corresponds with the rhythmic FSH surges found in intact rats by Nequin, Alvarez & Schwartz (1979) who also measured at 2-h intervals. These differences may be ascribed to the shorter time-intervals between measurements in the rats injected with bovine plasma rather than to the more frequent anaesthesia or bleeding or to a non-specific protein effect of bovine plasma.

Compared with values in animals injected with bovine plasma, results of injection of bFF showed no major or consistent changes in levels of LH: in only five out of 56 groups of bFF-injected rats did a significant reduction of LH occur. In contrast, levels of FSH were consistently influenced. At all stages of the cycle tested, a decrease in levels of FSH was found, as was also observed previously at dioestrus-1 (Hermans *et al.* 1981). The period of FSH suppression was followed at dioestrus-2, dioestrus-3 and less conspicuously at oestrus by a period in which fluctuating, generally increased levels of FSH occurred.

At dioestrus-2 and dioestrus-3 results found after injection of bFF were comparable with those obtained at dioestrus-1 (Hermans *et al.* 1981). The time during which reduced levels of FSH were found, from 4 until 10–12 h after injection, was similar. The fluctuations in levels of FSH, observed in the period from 14–16 until 32 h after injection, might be caused by oscillating patterns of FSH secretion, since within groups values occurred which were either higher or lower than control values. Such secretion patterns of FSH had, more clearly, also been seen in the same period after injection in the dioestrous-1 rats. However, in the latter group final control values were reached earlier (at 32 h after injection) than in this study.

At pro-oestrus the results after treatment with bFF were different from those on the days of dioestrus and oestrus. Apart from the fact that, expressed as percentage of control values, a greater suppression was found, the total period of suppression also lasted longer (about 12 h compared with 6 and 4 h respectively). Moreover, this period was not followed by fluctuating or increased levels of FSH within the period tested. This resembles the situation in gonadectomized adult female rats in which high levels of FSH were present (Hermans *et al.* 1981), and disagrees with findings by others of increased levels of FSH 21 h after injection of porcine FF at 11.00 h on pro-oestrus (DePaolo, Hirshfield *et al.* 1979).

At oestrus the period of suppression differed from that at dioestrus: lowered levels of FSH occurred later, but control values were reached at about the same time, thus resulting in a shorter period of suppression than was found on the other days. The changes in secretion of FSH in the period after suppression were not as clear as on the days of dioestrus; this may be due to a delay in the increase in FSH secretion. However, DePaolo, Hirshfield *et al.* (1979) observed increased levels of FSH 24 h after the last injection of pFF of a series of two or three given at 08.00 h on oestrus. Finally, the increased FSH value found at oestrus 1 h after injection of bFF cannot be explained, but a specific effect of bFF seems unlikely.

The fluctuations in FSH secretion after the period of suppression, found on the days of dioestrus and oestrus, might reflect a rebound in FSH secretion. The presence or absence of such rebounds seems to be inversely related to normal levels of FSH and LH. When high levels of FSH and LH are present (at pro-oestrus), a reduction in levels of FSH is not followed by an increased secretion of FSH; when levels of FSH and LH are declining (at oestrus), a delayed increase in secretion of FSH after suppression seems to occur; when levels of FSH and LH are low (on the days of dioestrus), a reduction in levels of FSH is

followed by increased levels within a short period. This suggests that only on those days when a reduction of FSH results in very low (below 100 μg/l) levels and when suppression lasts for several hours does a compensation in FSH secretion after suppression occur.

Although the minimal amount of FSH required for follicular development is not known, the rebound phenomenon might protect the process of follicular development (de Jong *et al.* 1978; Hermans *et al.* 1981; Hirshfield & DePaolo, 1981). Apparently the female rat possesses a fast acting 'safety' system against disturbances in FSH secretion, which might ensure follicular development on all days of the cycle.

The question of which factors might lead to the increased release of FSH has been discussed earlier (Hermans *et al.* 1981). A role may be played by a diminished ovarian production of inhibin, caused by depressed, insufficient FSH stimulation at oestrus, more clearly on the days of dioestrus, but not at pro-oestrus. On the other hand, if an increased secretion of gonadotrophin releasing hormone (GnRH) after the period of FSH suppression is involved, the absence of increased FSH release at pro-oestrus could possibly be explained by decreased synthesis and release of GnRH following the pro-oestrous surge (Sarkar, Chiappa & Fink, 1976; Oshima, Morishita, Omura & Saito, 1978; Kalra & Kalra, 1979). Finally, a direct action on GnRH production by factors present in crude preparations of bFF (Le Lannou & Chambon, 1977; Lumpkin, Negro-Vilar, Franchimont & McCann, 1981), and the presence of factors which stimulate the pituitary release of gonadotrophins (de Jong, Welschen, Hermans, Smith & van der Molen, 1979; Ying, Ling, Böhlen & Guillemin, 1981), might also play a role. Because of this plethora of possibilities explanation of the effects found awaits further purification of active factors from bFF.

In conclusion, the results show that throughout the cycle bFF can selectively lower FSH secretion. At oestrus and dioestrus, when growing preovulatory follicles require relatively low amounts of substitutional gonadotrophins for their growth (Welschen, 1973), and when levels of FSH are low, a safety system to compensate for severe disturbances in FSH secretion seems to exist. Thus, follicle growth during the cycle might be ensured.

The authors wish to thank Drs J. Moll and H. M. A. Meijs-Roelofs for their help in the preparation of the manuscript. We are grateful to the NIAMDD, Maryland, U.S.A. for the supply of gonadotrophins used in the radioimmunoassays.

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