# I N H I B I N

Role of inhibin in the endocrinology of reproduction
of the female laboratory rat.

# I N H I B I N E

Betekenis van inhibine voor de endocrinologie van de voortplanting
van de vrouwelijke laboratorium rat.

## PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.Dr. A.H.G. Rinnooy-Kan
en volgens besluit van het college van dekanen.
De openbare verdediging zal plaatsvinden
op woensdag 7 december om 15.45 uur.

door

HENDRIK JAN SANDER

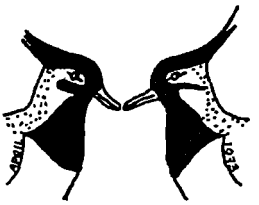
geboren te Jelsum.

Gedrukt bij
Offsetdrukkerij Kanters B.V.
Alblasserdam

## PROMOTIECOMMISSIE

| | | |
|---|---|---|
| Promotor | : | Prof.Dr. J. Moll |
| Overige leden | : | Prof.Dr. S.W.J. Lamberts |
| | | Prof.Dr. J.J. van der Werff ten Bosch |
| | | Dr. F.H. de Jong |
| Co-promotor | : | Dr. H.M.A. Meijs-Roelofs |

Dit proefschrift werd bewerkt bij de Afdeling Anatomie,
Faculteit der Geneeskunde en Gezondheidswetenschappen,
Erasmus Universiteit Rotterdam.



voor mijn ouders,
voor Ariëtta en de kinderen.

De illustratie op de voorkant werd getekend
door Willem van Leeuwen.

Contents.

## Appendix Papers - Titles.

I Compensatory ovulatory mechanisms operative after first ovulation in rats unilaterally ovariectomized prepubertally.
H.M.A.Meijs-Roelofs, P.Kramer, P.Osman & H.J.Sander (1984). Biology of Reproduction 31, 44-51.

II Changes in serum concentration of luteinizing hormone in the female rat approaching puberty.
H.M.A.Meijs-Roelofs, P.Kramer & H.J.Sander (1983). Journal of Endocrinology 98, 241-249.

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

IV Inhibin increases in the ovaries of female rats approaching first ovulation: relationships with follicle growth and serum FSH concentrations.
H.J.Sander, H.M.A.Meijs-Roelofs, E.C.M.van Leeuwen, P.Kramer & W.A.van Cappellen (1986). Journal of Endocrinology 111, 159-166.

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

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

---

The papers are reprinted with permission from :
The Journal of Endocrinology (II, III, IV & VI),
Biology of Reproduction (I) and,
Molecular and Cellular Endocrinology (V).

## Abbreviations & Symbols.

| | |
|---|---|
| AFP | alpha-foetoprotein |
| bFF(-I) | bovine follicular fluid (-derived inhibin) |
| bTE | bovine testicular extract |
| CNS | central nervous system |
| D1,D2,D3 | di-oestrus day-1, -2 and -3 |
| E2 | oestradiol-17$\beta$ |
| eCG | equine chorionic gonadotroin (formerly PMSG) |
| EOP | endogenous opioid peptide |
| FSH | follicle-stimulating hormone, follitropin |
| GH | growth hormone |
| hCG | human chorionic gonadotrophin |
| HPO axis | hypothalamus-pituitary-ovary axis |
| HPX | hypophysectomy (-ized) |
| ILA | inhibin-like activity |
| IU | international unit |
| LH | luteinizing hormone, lutropin |
| LHRH | LH-releasing hormone, GnRH |
| OVP | ovarian vein plasma |
| OE | day of oestrus |
| P4 | progesterone |
| P | day of pro-oestrus |
| pFF(-I) | porcine follicular fluid (-derived inhibin) |
| PMS(G) | pregnant mare's serum (gonadotrophin); obsolete, see eCG |
| PRL | prolactin |
| pS | porcine serum |
| RIA | radio immuno assay |
| S-D | Sprague-Dawley strain rats |
| W | Wistar strain rats |
| WRA | Wistar R-Amsterdam strain rats |

---

| | |
|---|---|
| $\phi$ | diameter |

# PART ONE

## 1. General Introduction.

Regulation of reproduction is of great interest, both because of its economical value in animal husbandry and its social and personal importance for man. For that reason the mechanisms underlying normal and abnormal reproduction have always drawn much scientific attention, and, as far as mankind is concerned, the study of abnormal reproductive ability (i.e. the inability to achieve natural fertilization and pregnancy) and its subsequent remedy have received much attention. At the same time however, over the years a great deal of research has been devoted to the possibility of voluntary restriction of fecundity.

Historically, research on normal as well as abnormal reproduction has mainly been focussed on the contribution of the female. This is partly because in the female the state of sexual maturity is marked by clear, repetitive events: ovulations occurring at given intervals or after given stimuli and accompanied by abrupt changes in blood hormone levels, while in the male sexual maturity is characterized by a single condition: presence of viable spermatozoa in the ejaculate with constant blood levels of sex hormones. These facts made the female contribution to reproduction more open for investigation and manipulation than the male part. Also, contrary to males, females deliver offspring (necessitating an endocrinological more sophisticated system) and (female domestic animals) are therefore more valuable to society.

Ovulation is a real landmark in the growth process of an ovarian follicle, whereby the ovary sheds an oocyte that has been primed for fusion with a spermatocyte. The growth of such a (primordial) follicle towards the preovulatory stage is stimulated when gonadotrophic hormones (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) reach a primary oocyte and its adjacent stromal cells (Dorrington & Armstrong,1979; Richards,1980). Both LH and FSH are peptide hormones secreted by the anterior pituitary (syn. adenohypophysis) and their secretion is regulated by a factor of hypothalamic origin, luteinizing hormone-releasing hormone (LHRH) (Harris,1955; Schally, Kastin & Arimura,1972; Chappel,1985), as well as by gonadal hormones (Labrie, Drouin, Ferland et al.,1978; Plant,1986). When the gonadotrophic hormones challenge the cells of the ovarian follicle (their main target), the reaction of these cells depends on the stage of development of the follicle and on the environment provided by other hormones (Richards,1980).

Vice-versa, the cells involved in follicle growth (oocyte and cells of the follicle wall, i.e. granulosa cells and theca cells) also produce and secrete hormones (gonadal hormones). These modulate the activity of the hypothalamus as well as that of the pituitary, influencing synthesis and release of (a.o.) LHRH, LH and FSH. One category of gonadal hormones, the steroidal or sex hormones, is also very important as regulator of general, sex-specific somatic growth and of behavioural patterns (e.g. courtship, mating and nursing) (Parkes,1962; Callard,1983).

Ovulation per-se is a result of specific external and/or internal cues. Often, external cues (e.g. changes in day-length, ambient temperature or the copulation stimulus) induce reactions in the sexually mature female leading to internal, physiological cues for ovulation. These are of neural and humoral origin. In the human and in some other species (e.g. the laboratory rat) ovulation almost completely depends on internal cues. These are formed by the interplay of signals and feedback-signals along the

hypothalamo-pituitary-ovarian axis. This interplay finally leads to the expulsion of a mature (primary) oocyte from a pre-ovulatory (or Graaffian) follicle (Asdell,1962; Espey,1980). This event also marks the endpoint of the growth of the follicle in its present form. At this stage the oocyte starts its second meiotic division to become a secondary oocyte. After ovulation the follicle is transformed into a reproductive organ of a different nature, the corpus luteum which may sustain (part of) pregnancy or becomes atretic.

All this demonstrates that regulation of female reproductive activity is a dynamic process governed mainly by the hypothalamo-pituitary-ovarian triangle and that its hormonal and neuronal signals form a stimulating and inhibiting (in part short-loop) feedback system. Other data indicate that gradual development of these feedback mechanisms is involved in the development towards reproductiveness (during sexual maturation) (Meijs-Roelofs & Kramer,1979; Meijs-Roelofs, Kramer & Gribling-Hegge,1981; Meijs-Roelofs, Kramer & Gribling-Hegge,1982a; Meijs-Roelofs, Uilenbroek et al., 1975; Ojeda, Andrews et al.,1980; Donham, Creyaufmiller et al.,1985; Waldhauser & Dietzel,1986), whereas loss of these interactions results in the loss of regular, sexual reproductive function with aging (post maturitas) (Meites & Huang,1976; Wise,1983; Felicio, Nelson & Finch,1984; Matt, Lee et al.,1986). Regulation of serum levels of both LH and FSH around a given tonic, basal value has long been thought to be mainly dependent on the action of LHRH as well as ovarian steroidal hormones (oestradiol-17$\beta$ (E2), progesterone (P4), testosterone, androstanediol (adiol)). However, diverging patterns of secretion of FSH and LH were seen around the time of ovulation (pro-oestrus/oestrus), when there is a combined LH/FSH surge on the afternoon of pro-oestrus, but only in the case of FSH there is a second surge on the subsequent day of oestrus, suggestive of differential regulation of the gonadotrophic hormones (Schwartz,1974; Schwartz & Channing,1977). Differential regulation was also evident after (unilateral-) ovariectomy (ULO,OVX), when serum FSH levels increase well ahead (within 5 hours) of LH levels (after 12 hours) (Howland & Skinner,1973; Welschen, Dullaart & De Jong,1978; De Jong & Robertson,1985a). During the late 1970-s and early '80-s it became clear that physiological amounts of ovarian steroids alone were insufficient to prevent the (short-term) rise in serum FSH levels after ovariectomy (ULO or OVX) of adult and prepubertal female rats (Meijs-Roelofs, Uilenbroek et al.,1973a,b; Welschen, Hermans et al.,1977; Chappel,1980; Williams & Lipner,1982). Also, it was found that the gradual decline of serum FSH levels seen in immature, intact female rats approaching first ovulation could not solely be accounted for by action of ovarian steroids (Meijs-Roelofs & Kramer,1979; Meijs-Roelofs et al.,1981,1982a). In a different approach, it was found that injection of steroid-free bovine follicular fluid (bFF) caused a rapid (within 4 hours) and short lasting (<16h) decrease in serum FSH levels in male and female rats (Welschen et al.,1977; De Jong, Welschen et al.,1978) of different ages, with or without gonadectomy (Hermans, Van Leeuwen et al.,1980; Hermans, Debets et al.,1981a,1982a; Hermans,1982). Welschen, Hermans & De Jong (1980) suggested a relationship between the number of antral follicles and serum FSH levels on the days of pro-oestrus and oestrus. All these data pointed to the existence of a non-steroidal factor of gonadal origin, which is involved in the regulation of FSH. Such a factor had already been postulated in 1923 by Mottram and Cramer (1923). And thus, after half a century, the quest about "inhibin" - the name originally coined for this

factor by McCullagh (1932) - experienced a revival.
Now, it is generally accepted that

> "inhibin is a glycoprotein hormone consisting of two dissimilar, disulfide-linked subunits, which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of FSH" (Burger & Igarashi,1988).

The relevance of inhibin in the regulation of reproduction was amply indicated by results from experiments with preparations (not yet pure in the biochemical sense) thought to contain inhibin-like activity (ILA) (De Jong & Sharpe,1976; Schwartz & Channing,1977; De Jong, Sander et al., 1985b). Mostly fluid of ovarian follicles (depleted of steroids by adsorption to activated charcoal) was used as a source for ILA in in-vivo or in-vitro experiments, or for purification of inhibin. In view of the quantities of follicular fluid (FF) needed for these experiments, usually material of porcine (pFF), bovine (bFF), ovine (oFF) or human (hFF) origin was used. Also, the effects on reproductive endocrinology of immunoneutralization of endogenous inhibin were studied (Schwartz & Channing,1977; Channing, Tanabe et al.,1982; Henderson et al.,1984).

Attempts at isolation and characterization of inhibin from different sources have recently resulted in a number of publications describing various amino-acid sequences, isoelectric points (pI) and molecular weights (see: De Jong & Robertson,1985a; De Jong,1988). Availability of purified inhibin could contribute to refinement, facilitation and acceleration of investigation of the physiological role of inhibin in the regulation of sexual maturation and reproduction, and disorders thereof.

The work described and discussed in this thesis deals with investigation of the possible role of ILA in the regulation of sexual maturation and reproduction, as studied in the immature, adult and senescent female rat. A concise review of the most recent data on characterization of inhibin will be given first.

## 2. Characteristics of inhibin.

### 2.1. Discovery and Recognition.

In 1923, Mottram & Cramer first mentioned the possible existence of a non-steroidal, anti-gonadotrophic factor of gonadal origin when they found that X-irradiation of the testis of juvenile rats did not affect the interstitial tissue but caused atrophy of the seminiferous tubules in conjunction with hypertrophy of basophilic cells of the anterior pituitary (giving "large cells" or "castration cells")(Mottram & Cramer,1923). This effect was not seen after efferent duct ligation and they concluded that an endocrine factor had to be involved. In addition, Martins & Rocha (1931) performed parabiosis experiments on castrated and intact, juvenile, male and female rats, and demonstrated the existence of more than one testicular hormone : one - water soluble - for regulation of gonadotrophin secretion from the pituitary, and a second - not water soluble - for activation of the accessory genital glands. They also noticed different reactions of male and female littermates after treatment with similar, inhibin containing preparations of testicular origin (Martins & Rocha,1931). In 1932 the name inhibin was coined by McCullagh (1932) , who considered the material to be complementary to androtin, a benzene soluble testicular hormone (now known as testosterone (David, Dingemanse et al.,1935)).

The putative role of inhibin as a regulator of reproduction was indicated by several observations on anomalies of mainly the male reproductive system (spermatogenic compartment) and accompanying disturbances of the hypothalamo-pituitary-testis axis (Mottram & Cramer,1923; McCullagh,1932; McCullagh & Schneider,1940); a number of other investigators, however, could not corroborate these data and conclusions (Nelson,1934; Nelson & Gallagher,1935; Vidgoff & Vehrs,1940; Rubin,1941) and so, until the late '70-s, gonadal steroids remained the vehicles of choice in concepts of the regulation of gonadotrophin secretion (see Donovan,1966).

The information and conclusions brought forward in reproductive endocrinology up to the fifties were derived from detection of gross, spontaneous changes in macroscopical- or microscopical anatomy of (endocrine) organs involved in regulation of reproduction, together with observations on the effects of natural or experimental, primary or secondary aberrations of the reproductive hormone system. More direct estimation of levels of gonadotrophic hormones was greatly facilitated after the introduction of bioassays (for LH: ovarian interstitial tissue augmentation: Evans, Simpson et al.,1939; ventral prostate weight: Greep, Van Dyke & Chow,1941; ovarian ascorbic acid depletion test: Parlow,1958 and, for FSH : ovarian and uterine weight gain: Steelman & Pohley,1953, testis weight gain: Paesi, De Jongh et al.,1955) and even more so when radioligand immunoassays (RIA) took over (Midgley, 1969; Welschen, Osman et al.,1975). The RIA improved (1) the distinction between FSH and LH (review Schwartz,1974) and (2) detection of changes in their levels in the nanogram per millilitre (ng/ml) range in virtually every (biological) fluid, which is a more than 1000-fold improvement compared with the sensitivity of bioassays. Obviously, these developments facilitated investigation of the role of inhibin in regulation of reproduction via control of gonadotrophin levels. Recently, in vitro bioassays have regained importance for studying the relationship between, on the one hand, heterogeneity of FSH- as well as LH molecules and, on the other hand, differences in biological activity as a result thereof (for FSH: Jia & Hsueh,1985 and, for LH: Harlow, Hearn & Hodges,1984).

In 1977, Setchell, Davies & Main reinvestigated the association of

impaired spermatogenesis and elevated serum FSH levels, and tentatively explained this elevation through an accompanying impairment of inhibin-mediated suppression of FSH secretion (Setchell, Davies & Main, 1977). In 1948, the aforementioned rise in serum FSH levels had been explained by Heller & Nelson (1948) with reduced FSH utilization (uptake) caused by impaired activity of the spermatogenic compartment. This explanation was, in part, refuted when Howard, Sniffen et al.(1950) demonstrated that in these cases pituitary FSH content was increased as well, an observation not explained by the "utilization theory".

The possible role of inhibin in reproductive processes was first investigated via in-vivo bioassays, focussing on changes in serum FSH levels or biological effects caused by these changes (see: Hudson, Baker et al.,1979). Soon, in-vitro bioassays were developed that focussed on inhibin-mediated suppression of basal or LHRH-stimulated secretion of FSH from pituitary halves (Jenner, De Koning & Van Rees,1982) or dispersed pituitary cells (De Jong, Smith & Van der Molen,1979; Eddie, Baker et al.,1979) or on lowering of in-vitro pituitary cell content of FSH (Scott, Burger & Quigg,1980). The effects of inhibin on the basal secretion of LH was small in comparison to the effect on FSH secretion, but in the LHRH-stimulated bioassays, secretion of LH was suppressed as well.

Using in-vivo bioassays, several authors described selective suppression of serum FSH levels after injection of steroid-free preparations of ovine rete testis fluid (oRTF: Setchell & Jacks,1974), bovine seminal plasma (bSP: Franchimont, Chari et al.,1975) or bovine ovarian follicular fluid (bFF: De Jong & Sharpe,1976). A few years later the cellular origin of the active principle, a "non-steroidal, selectively FSH secretion suppressing factor of gonadal origin", was, in rats, pinpointed to the Sertoli-cell of the testis (Steinberger & Steinberger,1976; De Jong & Sharpe,1977) and the granulosa cell of the ovarian follicles (Erickson & Hsueh,1978; Hermans, Van Leeuwen et al.,1982b). In their 1985 review, De Jong & Robertson (1985a) redefined inhibin as "presumably a protein produced by the gonads under FSH control to, putatively, act at the pituitary level to control FSH secretion by a receptor mechanism", and thus left room for a possible stimulatory action, besides the already accepted suppressive action of inhibin on FSH secretion; once more the need for a generally accepted standard preparation of inhibin for further research was stressed.

## 2.2. Purification.

After the above mentioned confirmation and extension of the original view of Mottram & Cramer (1923) that the gonads release a FSH secretion suppressing principle, now generally known as inhibin, its purification was attempted by several groups in the past decade.

Purification efforts were at first focussed on possible sources of male inhibin: homogenates of testis and epididymis, preparations of spermatozoa, rete testis fluid (RTF), seminal plasma (SP) and testicular lymph (TL), and estimates for molecular weight ranging from 1000 to 90,000 Dalton emerged (see: De Jong, Jansen & Van der Molen,1981 and De Jong & Robertson,1985a for references). Mostly, these (putatively) inhibin containing preparations were validated by their ability to suppress pituitary cell FSH secretion in vivo and/or in vitro. Soon afterwards the purification of female inhibin from pools of follicular fluid was judged a more economical approach, and so the field of interest shifted that way;

molecular weight of female inhibin was reportedly between 10,000 and 194,000 Dalton (review De Jong et al.,1981, 1985a and Channing, Gordon et al.,1985). However, all preparations used for estimation of molecular weight until then were, in biochemical sense, not pure.

Recently, a number of papers described (tentatively) the definite purification of inhibin from bovine follicular fluid (Robertson, Foulds et al.,1985; Fukuda, Miyamoto et al.,1986) and porcine follicular fluid (Miyamoto, Hasegawa et al.,1985, Rivier, Spiess et al.,1985). From these reports it was concluded that inhibin is a dimeric, glycosylated proteinaceous molecule with disulfide bridges linking the $\alpha$ and $\beta$ chain (see Burger & Igarashi,1988). Molecular weight data (estimated after sodiumdodecylsulphate polyacrylamide gel electrophoresis, SDS-PAGE) of the $\alpha$ and $\beta$ subunits and the intact molecule are summarized in table 2.1. With the exception of the approach of Forage, Ring et al. (1986) all preparations were tested in vitro for inhibin-like potency via reduction of FSH secretion of dispersed rat anterior pituitary cells in culture. The inhibin molecule (ovine or bovine) thus appears to be composed of an $\alpha$-chain of 18-20, 43-44 or 57 kDa linked via disulfide bridges of cysteine residues to a $\beta$-chain of 13-15 kDa giving a total apparent molecular weight of approximately 32, 58 or 65 kDa. The different molecular weights reported for the $\alpha$-chain seem to result from proteolysis either during intracellular processing of inhibin(-precursors) in vivo (Fukuda et al.,1986) or, during acidic purification steps that activate acidic proteases present in follicular fluid (Beers,1975; Channing et al,1985; Van Dijk,1986).

With the use of monoclonal antibodies to 32 kDa inhibin Miyamoto, Hasegawa et al. (1986) detected biologically active forms of inhibin in bFF, with a molecular weight of 88, 108 and 120 kDa, and suggested the existence of a third, disulfide bridge linked subunit of inhibin with a molecular weight of 62 kDa. This subunit would be immunologically related to the shorter ($\beta$-) subunit of 32 kDa inhibin (Miyamoto et al.,1986). About simultaneously Ling, Ying et al. (1986) and Vale, Rivier et al.(1986) reported the isolation of a heterodimer of the $\beta$-subunit of inhibin

Table 2-1. Molecular weight of inhibin and its subunits (1000 x Dalton; estimated after SDS-PAGE, except Forage e.a.,1986 who used cDNA).

| $\alpha$ | $\beta$ | $\alpha$-$\beta$ | source | reference |
|---|---|---|---|---|
| 20 | 13 | 32 | pFF | Miyamoto e.a.,1985 |
| 44 | 14 | 56 | bFF | Robertson e.a.,1985 |
| 18 | 14 | 32 | pFF | Rivier e.a.,1985 |
| 20 | 13 | 32 | bFF | Fukuda e.a.,1986 |
| 20,44,57 | 13 | 32,55,65 (88,108,120) | bFF | Miyamoto e.a.,1986, (+"62 kDa subunit") |
| 20,43 | 15 | 31,58 | bFF | Forage e.a.,1986 |

($\beta_a$-$\beta_b$) from pFF and termed it activin to emphasize its capacity to stimulate FSH secretion from dispersed rat anterior pituitary cells in vitro.

Mason, Hayflick et al. (1985) reported structural homology between a part of the $\alpha$-chain (denoted $A_c$) and the $\beta$-chain of inhibin, and suggested a common ancestral gene for both subunits. Another point of interest is the reported partial structural homology of inhibin with products of the gene family that produces growth regulating proteins such as transforming growth factor-$\beta$ (TGF-$\beta$; Mason et al.,1985; Sporn, Roberts et al.,1986) and Müllerian inhibitory substance (MIS; Sporn et al.,1986). Ying, Becker et al. (1986) reported TGF-$\beta$ induced stimulation of FSH release in vitro. For a review see De Jong (1988).

The meaning of differences in potency of inhibin preparations obtained from male or female animals as well as from different species, as reported in several reviews (De Jong et al.,1981a, 1985a; Channing et al,1985), remains uncertain as long as a generally available and accepted standard preparation (necessarily an arbitrary one) is not in use.

## 2.3. Summary.

Inhibin, as purified from bovine or porcine ovarian follicular fluid, appears to be a glycosylated proteinaceous hormone consisting of an $\alpha$ and a $\beta$ chain linked by disulfide bridges, with a molecular mass between 32,000 and 65,000 Daltons. Variation in the molecular mass seems to be caused by variations in the reported composition of the $\alpha$-chain.

The physiological role of inhibin is the suppression of synthesis and secretion of FSH by the anterior pituitary gland, although further research may reveal other endocrine as well as paracrine functions. It is produced by a member of a gene family that produces other, growth regulating, factors.

## 3. Inhibin in the life of the female rat.

With sexual reproduction as a central theme, the life of the female rat may be divided in three stages :

3.1) prepubertal, immature : the sexually immature stage, in which reproduction is not possible,

3.2) adult, mature : the sexually mature stage, in which reproduction may take place,

3.3) adult, aged : the sexually senescent stage, in which the capacity for reproduction is (gradually) lost.

The transition period from the immature stage to the mature stage is called puberty. In the rat this encompasses vaginal opening accompanied by first ovulation and the ensuing first oestrous cycle on the one hand, and, on the other hand, a specific shift of hormone levels over the days immediately preceding the day of first ovulation.

Transition from the mature to the senescent stage takes place in absence of a clear, physiological event. On the contrary, oestrous cyclicity is gradually losing its regular character together with changes in the regulation of reproductive hormone levels and of follicular growth and maturation.

### 3.1. The prepubertal female rat.

The prepubertal or immature episode in the life of the female rat spans the time between the day of birth and the day of first ovulation. At the time of birth, the neonate is abruptly deprived of the hormones supplied via the maternal circulation and it will start to build a new "milieu interieur" from its own resources, a milieu that is continuously changing as the animal grows and maturation of the reproductive organs progresses.

#### 3.1.1. Staging of the prepubertal period.

Subdivision of the prepubertal period of the rat, based on major physiologically and/or histologically recognizable features of the reproductive apparatus, was proposed by Critchlow & Bar-Sela (1967), Schwartz (1974), Ramaley (1979) and Ojeda, Urbanski & Ahmed (1986), who subdivided this period of maturation and growth of the reproductive system of the female rat, from birth to first ovulation, as given in Table 3.1-1.

At the time of birth the infantile (Critchlow & Bar-Sela,1967) or neonatal (Ramaley,1979; Ojeda et al.,1986) period (1) starts, in which follicle growth and follicle maturation seem to be more or less autonomous and rather insensitive to gonadotrophic hormone stimulation, a situation that gradually starts to change after day 5, when the brain begins to get grip over ovarian function.

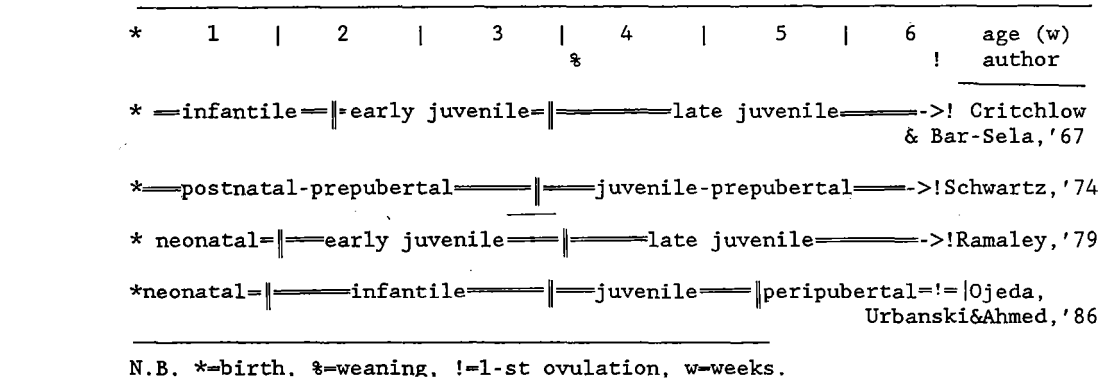
(2) Around day 7-10, the early juvenile (Critchlow & Bar-Sela,1967; Ramaley,1979) or infantile (Ojeda et al.,1986) period sets in, in which follicle growth (i.e. mainly growth rate (Richards,1980)) and follicle maturation start to be clearly under the influence of gonadotrophic hormones. Also, in this period, due to growth and development of the central nervous system (CNS)-pituitary axis the levels of gonadotrophic hormones show peak values around day 12-15. Furthermore, ovarian production of steroids is becoming manifest at day 5 and is positively controlled by gonadotrophic hormones, sporadic formation of bioactive inhibin starts (details in paragraph 3.1.4.) and follicles containing an antrum may be

sparsely found. These follicles can not yet ovulate, a quality that is only attained around day 17-25 when the first follicles may have completed their growth (which takes about 15-19 days (Richards,1980).

(3) Around day 17-25, the juvenile (Ojeda et al.,1986), late juvenile (Critchlow & Bar-Sela,1967; Ramaley,1979) or juvenile-prepubertal (Schwartz,1974) period starts, characterized by a gradual increase in numbers of growing follicles reaching the antral stage. These follicles may develop as far as the Graaffian (i.e. ovulable) stage and, consequently, may be induced to ovulate precociously or will spontaneously ovulate at the first day of oestrus, around day 35-40. Oestradiol, which had already established a clear negative feedback on tonic gonadotrophic hormone secretion, now also develops a positive feedback resulting in a surge-like gonadotrophin release. From day 18 to 33 ovarian production of inhibin gradually increases (details in paragraph 3.1.4.). Evidently, the hypothalamus-pituitary-ovary (HPO) axis is fully functioning. Also, prolactin (PRL) and growth hormone (GH) secretion increase during this period and facilitate the effects caused by the gonadotrophic hormones, mainly via initiation and/or promotion of LH-receptor development (Ojeda et al.,1986). A separate, peri-pubertal period from day 30-32 to the day after the day of first ovulation (i.e. first day of first dioestrus), and further subdivided in anoestrus, early pro-oestrus and late pro-oestrus, based on the amount of uterine fluid present, was proposed by Ojeda et al. (1986). The peri-pubertal period is characterized by increased average levels of serum LH resulting from increased release of presumably a pulsatile nature, during the afternoon (Meijs-Roelofs, Kramer & Sander,1983, Appendix Paper II). The change towards a basal, diurnal mode of LH release is most likely autonomously generated in the CNS, since it is not ovary dependent and not induced via oestradiol (Ojeda et al.,1986; Urbanski & Ojeda, 1987). However, development of afternoon mini-surges in prepubertal rats, finally leading to the pre-ovulatory LH peak, was reported to be ovary dependent (Urbanski & Ojeda, 1986).

With regard to first ovulation accompanied by vaginal opening and ensuing oestrous cycles, display of a mature-type of behaviour and,

Table 3.1-1. Staging of the period between birth and the day of first ovulation of the female rat.




N.B. *=birth, %=weaning, !=1-st ovulation, w=weeks.

thereby, attainment of the capacity to reproduce, it is generally accepted that "there is not one single event (...) that triggers puberty onset" (Ojeda, Smith et al.,1984). From table 3.1-1 it may be seen that there is general agreement regarding the subdivision of the immature period of the female rat, while differences may be explained by the criteria used, i.e. mainly ovarian parameters or taking into account developmental steps of the whole HPO axis (Ojeda et al.,1986). Moreover, some of the differences in timing may result from the use of different strains of rats (mainly Sprague-Dawley (S-D) or Wistar (W) derived).

Since we want to take into account as many developmental features of the HPO axis as possible and are the first to include developmental data on inhibin levels, we feel the need to define the staging of the immature period of females of our rat strain. Generally this staging scheme agrees with the one proposed by Ojeda et al. (1986). The immature stages of our strain are summarized below and in Table 3.1-2. The immature or prepubertal period starts with :

(1) the neonatal period, from birth to approx. day 7 (first week of life), followed by
(2) the infantile period, from day 7 to approx. day 20 (week 2 and 3), leading to
(3) the juvenile period from day 21 to day 32 (week 4 and 5), which is a prelude to
(4) the peripubertal period (day 32 to the day after the day of first ovulation; week 6 and 7).

In contrast to other authors, we have studied the peripubertal period with, as a guideline, the number of days preceding the day of first ovulation : days -10 to -1 (or +1, if needed), using an experimental set-up that involves unilateral ovariectomy in order to study ovarian development in a well-timed fashion (Meijs-Roelofs, Osman & Kramer,1982b; Meijs-Roelofs et al.,1983; Sander, Meijs-Roelofs et al.,1986).

### 3.1.2. Ovarian follicle growth.

A large pool of non-growing follicles is present in the ovary of the neonatal female rat. Out of this pool of follicles a number is transformed each day into growing follicles (Peters, 1969; Lintern-Moore & Moore,1979; review: Richards,1980). The quantity of transformed follicles is positively correlated with the size of the pool of non-growing follicles, being maximal in neonatal rats and declining steadily during the normal course of life until ovarian oestrous cyclicity is terminated, a point of transition that, in women, would occur at menopause (Richards,1980).

After the initiation of growth of a follicle, it requires 15-19 days of growth to reach the mature, antral stage (Peters,1969; Richards,1980), and it is not until approximately day 18, during the infantile period, that antral follicles may be found in the ovary of our rats (Meijs-Roelofs Uilenbroek et al.,1973c). During the following days growth of the largest follicles stops and they become atretic.

Table 3.1-2. Staging of the immature period of life of Wistar R-Amsterdam-strain, female rats.

| AFTER DAY | IN MOST ANIMALS...STARTS | DAY | STAGE |
|---|---|---|---|
| 5 | ovarian steroidogenesis | 0 | |
| 7 | FSH sensitive follicle growth, injected E2 may lower FSH secretion | | NEONATAL |
| | | 7 | |
| 12 | E2 and FSH reach peak levels | | |
| 18 | appearance of antral follicles | | INFANTILE |
| 18-23 | ovary releases bioactive inhibin | | |
| 20 | reduced inflow class 3B follicles, large scale atresia of follicles | | |
| | | 21 | |
| 20-22 | E2 and FSH (and LH) levels lowered | | |
| 23 | short-term control of FSH by inhibin | | |
| 25 | E2-positive feedback on LH secretion | | |
| 28 | a few Graaffian follicles present, hCG induced ovulation possible, reduced E2 suppression of FSH | | JUVENILE |
| 28-33 | increased ovarian inhibin levels | | |
| 30-1$^{st}$P (-10/-1) | ovarian inhibin content parallels growth of class III-V follicles, follicle growth becomes cycle-like | | |
| -8 (day 32) | increased basal LH levels, pulsatile mode of LH secretion, FSH further reduced to first pro-oestrus | 32 | |
| -5/-1 | cycle-like ovarian inhibin content, inhibin tightly controls short-term FSH secretion | | |
| -3/-1 | FSH reaches adult, basal levels | | PERI-PUBERTAL |
| -1 | inhibin, E2, LH and FSH reach (preovulatory) peak levels | | |
| 0 | first ovulation & vaginal opening followed by oestrous cycles. | 40 | |

Legends on opposite page.

It is only after day 28, in the juvenile period, that small numbers of follicles complete their maturational growth and become (ovulable or) Graaffian follicles. However, spontaneous ovulation of these follicles does not yet take place, although ovulation may be induced after appropriate injection with hCG (Meijs-Roelofs, Kramer & Osman,1985). During the last 10 days preceding the day of first ovulation (day -10 to -1; the last part of the juvenile period plus part of the peri-pubertal period), maturational growth and subsequent atresia of follicles occur in a more cycle-like fashion. This results in a cohort of approximately 10 Graaffian follicles being expelled from the ovary at the time of first ovulation (Meijs-Roelofs et al.,1982b).

### 3.1.3. Gonadotrophin levels.

Generally, the gonadotrophin levels decline during day 1-5 and, thereafter start to increase again from day 5-6 onward to reach a maximum around day 12-15 (Meijs-Roelofs et al.,1973b). The maximum serum level of FSH around day 12-15 (800-1500 $\mu$g/l) equals that found in ovariectomized adult animals (800-1400 $\mu$g/l) and appears to be somewhat higher than the peri-ovulatory level in pubertal and adult animals (900 $\mu$g/l), while the highly variable mean level of LH (generally low but with episodic bursts of release: 50-275 $\mu$g/l) remains well below that of castrated (>2000 $\mu$g/l) as well as that of pubertal and adult pro-oestrous values (1200$\mu$g/l; Meijs-Roelofs et al.,1973b). From day 17 to day 22 the level of FSH declines rapidly to approx. 500 $\mu$g/l, subsequently it gradually decreases further and attains adult di-oestrous levels (<200 $\mu$g/l) at about three days before the day of first ovulation (Meijs-Roelofs et al.,1982b; Sander et al.,1986, Appendix Paper IV). The level of LH around day 25 reaches basal values of 10-20 $\mu$g/l and remains generally low until approximately 12-7 days before the day of first ovulation, when the average (daily) serum level starts to increase, mainly as a result of raised afternoon serum levels, and reaches about 50 $\mu$g/l on the morning of the day preceding the day of first ovulation: first pro-oestrus (Meijs- Roelofs et al.,1983). Figure 3.1-4 gives a graphical overview of these data.

#### 3.1.3.1. Regulation of FSH and LH levels.

Factors influencing the release of FSH and LH stem mainly from the hypothalamus (LHRH) and the ovaries (oestrogens, progestins, androgens, inhibins), and the effectiveness of their interactions depends on the maturational age of the animal.

The initial decrease (on day 1-5) in the levels of FSH and LH may represent the resultant of, on the one hand, (metabolic) clearance of

Table 3.1-2 (opposite page). The data in this table were derived from: Meijs-Roelofs (1972); Meijs-Roelofs, Uilenbroek et al.,(1973a,b,c); Uilenbroek, Arendsen De Wolff-Exalto & Welschen (1976); Hage, Groen-Klevant & Welschen (1978); Meijs-Roelofs & Kramer (1979); Meijs-Roelofs, Osman & Kramer,(1982); Meijs-Roelofs, Kramer & Osman,(1985), Sander, Meijs-Roelofs et al.,(1985,1986) and Ojeda, Urbanski & Ahmed (1986).

residual maternal factors involved in the regulation of levels of endogenous gonadotrophic hormones and, on the other hand, the start of autonomous production and secretion of gonadotrophic hormones. It is generally accepted that the peak values of FSH (and LH) reached around day 12-15 result from LHRH-induced secretion (Ojeda et al.,1984). Meijs-Roelofs et al. (1973c), studying follicle growth-characteristics in immature, intact or hypophysectomized, and in part pregnant mare serum (PMS) treated, female rats from day 18 to the day of first ovulation, suggested that until day 18 neither the extremely high endogenous serum concentration of FSH nor a high PMS dose (8 IU + 4 IU/100 g body weight within 24 h) can induce the development of follicles with a volume >100 x $10^5$ $\mu m^3$ (i.e. follicles that have a small antrum and show a cyclic variation in number in the oestrous cycle of the adult rat). The biological significance of the endogenous serum levels of FSH may be that ovarian follicles need approximately 3 weeks of uninterrupted (i.e. FSH stimulated) growth to reach this volume (Hage, Groen-Klevant & Welschen,1978). Other studies on the regulation of FSH secretion in immature rats of various ages have shown that up to 20 days of age but not thereafter, physiological, age-related amounts of oestradiol (Meijs-Roelofs et al.,1973a,b; Meijs-Roelofs & Kramer,1979), in combination with progesterone (Meijs-Roelofs et al.,1981) and perhaps 5$\alpha$-androstane-3$\beta$-diol (Meijs-Roelofs et al.,1982a), are sufficient to maintain an FSH concentration in ovariectomized rats not different from that in intact rats. Although at this age (day 12-20) the serum level of oestradiol is rather high (Meijs-Roelofs et al.,1973a,b), and although oestradiol is a potent suppressor of gonadotrophin secretion, its actual physiological action is limited by the presence in serum of a binding protein, $\alpha$-foeto-protein (AFP), of which the level gradually decreases from a maximum around the time of birth to undetectable levels at day 25 (Meijs-Roelofs & Kramer,1979; Greenstein & Adcock,1985). From the total body of data the conclusion was reached (Meijs-Roelofs et al., 1979, 1981, 1982a) that after 20 days of age an ovarian factor, different from the steroids mentioned, perhaps inhibin (McCullagh,1932; De Jong & Sharpe,1976), must be involved in the regulation of FSH secretion.

### 3.1.4. Inhibin in the immature female rat.

The first indication of a possible action of inhibin in the regulation of (levels of) FSH in the immature female rat was obtained by Franchimont et al.(1975). With an antiserum against a partially purified fraction of bull seminal plasma, they neutralized endogenous inhibin in 21-24 day-old female rats which resulted in elevated levels of FSH and to a lesser extent of LH, and they pointed to the possibility that inhibin might be important for the transition of females from sexual immaturity to adulthood (Franchimont et al.,1975). Pomerantz (1978) reported that bilateral ovariectomy (24h earlier) of 17 day-old (S-D) rats caused increased levels of serum FSH that were suppressed again at 8h after injection of inhibin as present in porcine follicular fluid (pFF); in intact rats no comparable suppression was seen. Lorenzen & Schwartz (1979) demonstrated that pFF caused suppression of serum levels of FSH when it was injected in 21- or 26-day-old (S-D) rats, but not in 6 or 12 day-old rats, when compared with porcine serum (pS) injected controls. In contrast with the data of Pomerantz (1978), experiments by Hermans et al.(1980), using 15 day-old (WRA) rats, showed that in intact rats as well as in rats bilaterally

ovariectomized 48h earlier, injection of bovine FF (bFF) causes a 50-60% suppression of serum levels of FSH. In the same paper, Hermans et al. (1980) hypothesized that indeed inhibin may have a physiological role at about day 20, since on the one hand, bilateral ovariectomy of 25-day-old (WRA) rats resulted, within 8 hours, in a significant increase in FSH secretion, while, on the other hand, 8h after injection of bFF an effective suppression of FSH secretion in both intact and bilaterally ovariectomized rats was seen. Sander, Meijs-Roelofs et al., (1985, Appendix Paper III) narrowed the age at which inhibin might begin to play a major role in the regulation of serum levels of FSH, to between day 18 and day 23 (see below). Rivier & Vale (1987) noted that injection of an inhibin antiserum caused increased serum levels of FSH only in (S-D) rats of 20 days or older. The combined available data thus show that inhibin may exert a specific, short-term effect on the regulation of FSH secretion in immature female rats after approx. 20 days of age.

Lee, McMaster et al.(1982) reported that, after PMSG treatment of 25 day-old (S-D) rats, inhibin bio-activity could be detected in ovarian vein plasma. A comparable result was obtained by Rivier, Rivier & Vale (1986) who detected (by RIA) increased levels of immunoreactive inhibin in ovarian homogenates and in ovarian vein plasma of 25 day old, PMSG injected (S-D) rats and loss of immunoreactivity in plasma 24h after bilateral ovariectomy. More recently, Rivier & Vale (1987) detected immunologically reactive inhibin in pooled ovarian homogenates of 5- to 30-day-old (S-D) rats. In the reports cited above, changes in the serum levels of FSH after manipulation of serum levels of inhibin occurred on the short-term, within 8h. The short-term character of effect of inhibin on serum levels of FSH can presumably be attributed to its reported relatively short half-life time (30 min-3h; Lee et al,1982). Changes in the levels of endogenous gonadal steroids do not cause significant changes of FSH levels within 8h (Campbell & Schwartz,1977).

Involvement of inhibin in the process of sexual maturation in the female rat was already suggested by Franchimont et al. (1975), Hermans, De Jong & Welschen (1981b) and Lorenzen (1981) and was thereafter confirmed by Sander et al. (1985), who, using an inhibin bioassay, reported rising ovarian content of inhibin (Figure 3.1-1). Sander et al. (1985) also found in 18 to 33 day-old female rats, after both unilateral and bilateral ovariectomy, characteristic changes in blood levels of FSH measured 5, 8 and 24h post-operatively (figure 3.1-2). Furthermore, at 33 days of age it was found that the ovarian bioactive inhibin-content amounted to

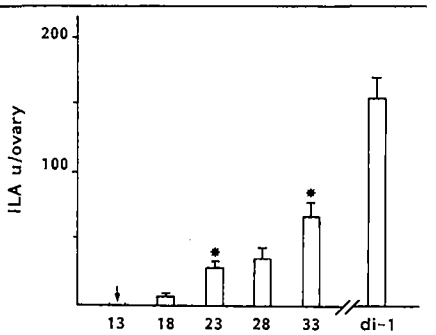



Figure 3.1-1. Ovarian inhibin content (units per ovary; means ±s.e.m.) of immature Wistar-R-Amsterdam rats, at 13, 18, 23, 28 and 33 days of age. For comparison the content of adult di-oestrus day 1 ovaries (D-1) has been depicted as well. * , $p<0.05$ compared with previous age. † , undetectable. Data, with permission from Sander, Meijs-Roelofs et al.(1985).

approximately 40% of that in cyclic di-oestrous rats (Sander et al.,1985). Rivier & Vale (1987), using a RIA based on an antibody raised against a synthetic pFF-inhibin (pFF-I) analog, reported a comparable rise of serum level of inhibin for rats up to 30 days of age. Hermans et al. (1980) found, in 15-day-old female rats, no reaction of serum levels of FSH 8h after bilateral ovariectomy but did notice suppression of FSH levels 8h after injection of 0.5 ml bFF/100g body weight. Taken together, these data would suggest that, in 13- to 18- day-old rats, the pituitary FSH release is already sensitized for interaction with inhibin, while, at the same time, the ovary does not generally release sufficient amounts of bioactive inhibin to induce significant suppression of FSH. From, at least, 23 days of age onward the short term inhibin feedback loop from ovary to pituitary

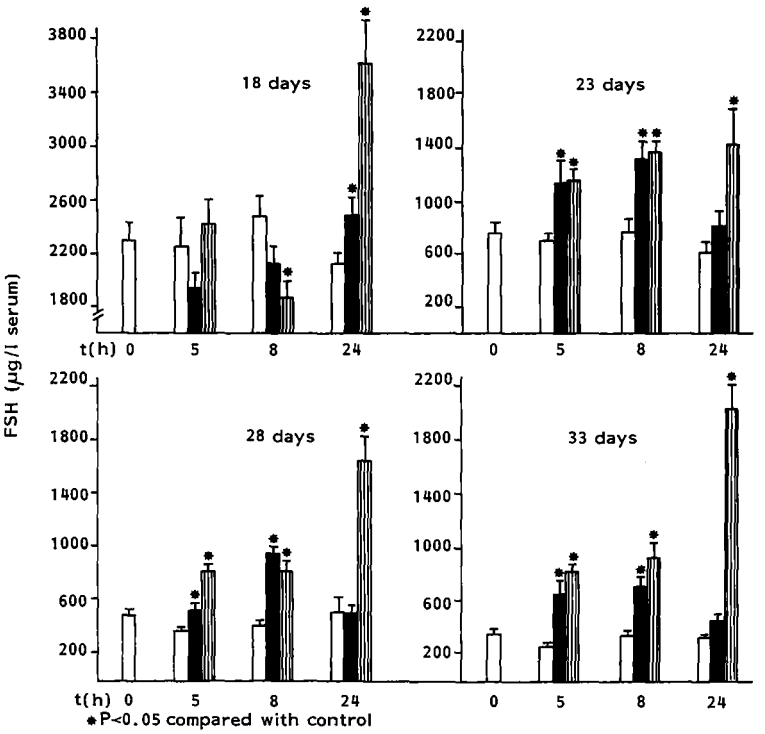



Figure 3.1-2. Concentrations of FSH in the serum of rats which were sham operated (open bars), unilaterally ovariectomized (filled bars) or bilaterally ovariectomized (hatched bars), at 5, 8 and 24h after operation performed at 18, 23, 28 and 33 days of age. Open bars at time 0 represent concentrations of FSH in intact control rats. * P<0.05, compared with sham operated controls (Wilcoxon-test). Data, with permission, from Sander, Meijs-Roelofs et al.,(1985).

may be functionally active and its physiological significance may be increasing. This is illustrated by a consistent increase of FSH levels 5 and 8h after unilateral ovariectomy (ULO) at 23, 28 and 33 days of age and a return to pre-treatment levels after 24h (Figure 3.1-2) due to compensatory follicle growth and compensatory inhibin release by the remaining ovary, stimulated by increased FSH levels acutely after ovariectomy (Sander et al.,1985).

These results provide a nice explanation for the decrease in concentration of serum levels of FSH seen in intact rats after 18-20 days of age, which cannot solely be explained by suppression of FSH secretion by circulating steroids (Meijs-Roelofs & Kramer, 1979; Meijs-Roelofs et al., 1981, 1982a).

During the juvenile period (day 21-32) and up to the time of first ovulation, exogenous ovarian steroids become less and less effective in suppressing FSH secretion (Meijs-Roelofs et al.,1982a). Thus it seems likely that this change is accompanied by an increasing role of inhibin-mediated, more subtle, short-term regulation of FSH secretion, enabling fine regulation of follicle growth (Hermans et al.,1981a; Lorenzen, 1981; Sander et al.,1985, 1986).

Sander et al. (1986) studied changes in rat ovarian bioactive-inhibin content and in the follicle population during the last 10 days before the

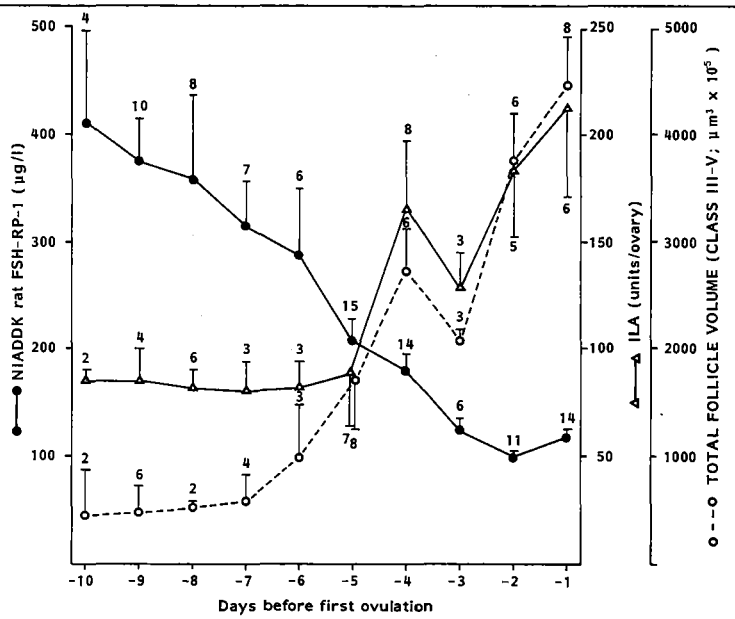



Figure 3.1-3. Concentrations of FSH in peripheral plasma (filled symbols), content of inhibin-like activity (ILA) in charcoal treated ovarian homogenates (triangular symbols) and total volume of all healthy follicles (classes III-V) with a volume > 350 * $10^5$ $\mu m^3$ per ovary (open symbols) on the 10 days before the day of first ovulation in rats. Values are means ± S.E.M.; numbers of rats are given for each point. Data, with permission, from Sander, Meijs-Roelofs et al.(1986).

day of first ovulation (sometimes referred to as the late-prepubertal period). They reported a significant, positive correlation between mean ovarian inhibin content and mean total volume of (antral) follicles of classes III-V (i.e. volume > $350*10^5$ $\mu m^3$ or $\phi$ > 410 $\mu m$; Figure 3.1-3), but not between inhibin content and mean total volume of follicles of lower classes. These larger antral follicles (classes III-V) contain the cohort of follicles that is going to ovulate on the oncoming day of oestrus. In this period, serum concentrations of FSH show a clear negative correlation with age -as related to the day of first ovulation- and follicular growth follows a pattern comparable to that seen during the adult cycle, although more slowly (Meijs-Roelofs et al.,1982b). These data indicate that in peri-pubertal female rats the amount of ovarian inhibin increases towards the day of first pro-oestrus. At that day a maximum is reached (comparable (55%) to that at adult pro-oestrus) and the increase in content with time is significantly related with the development of follicles of classes III-V. Also between days -5 and -1 (in contrast to days -10 to -5) the increase in inhibin apparently causes a more precise adjustment of serum FSH to levels below 200 $\mu g/l$, the level also found during adult dioestrus. This fine regulation is presumably due to inhibin released by follicles of classes III-V, maintaining serum FSH levels within narrow limits, and might serve to usher just the normal number of large preovulatory follicles contained in these classes to the point of ovulation.

Although we mainly emphasized here the role of inhibin, it should be noted that at least two other hormones of the pituitary-ovarian axis are of major importance in the process of sexual maturation, notably oestradiol-17$\beta$ (E2) and LH. The physiological role of E2 probably resides mainly in its more long-term inhibitory action on the secretion of FSH and LH, in its positive feedback action i.e. induction of the preovulatory gonadotrophin surge, as well as in the attainment of mature, secondary sex characteristics (e.g. behaviour). An important role may be attributed to LH during the 10 days preceding the day of first ovulation, since it was found that in the peripubertal period the mode of secretion of LH changes (around day -8), causing elevated average daily levels (Meijs-Roelofs et al.,1983) and the occurrence of a pulsatile secretion pattern (Urbanski & Ojeda, 1985). Complementary to this, it was shown that changes in endogenous LH may play a significant role in stimulating follicle growth from day -8 onward until the ensuing first ovulation (Meijs-Roelofs et al.,1985).

### 3.1.5. Summary.

The presence (Figure 3.1-4) and physiological role of inhibin during the immature period of the female rat may be summarized as follows. During the neonatal period (days 0-7) and part of the infantile period until approx. day 15-18, no physiologically active inhibin is demonstrable in the ovary and in the peripheral circulation. During this period (day 0-18) modulation of serum levels of FSH seems to result mainly from stimulation of pituitary secretion of FSH by hypothalamic factors (notably LHRH) and, after day 8, from suppression of FSH secretion by oestradiol.

Around day 15-18, pituitary FSH secretion gradually becomes sensitive to suppression by inhibin, which now may add to the already existing, more long-term steroidal feedback control of FSH secretion, although the ovarian compartment (i.e. follicles) does not yet secrete sufficient amounts of bioactive inhibin to fully "exploit" this option. In the period from day

17-21, a characteristic, sharp decline of serum levels of FSH (and less clearly of LH) takes place that coincides with increasing levels of immunoreactive serum (OVP)- and bioactive ovarian inhibin from day 18 to day 23 and with declining levels of AFP. From there on, serum FSH levels gradually decrease and reach adult dioestrous levels at about three days before the day of first ovulation. The content of bioactive inhibin in the ovary increases gradually between day 18 and 28. From day 28 to day 30-33 there is an abrupt increase, whereafter, during the 10-day-period preceding the day of first ovulation, the ovarian content of inhibin is rather constant until about day -5. Then, from day -4 on (approaching first ovulation), a rapid increase starts and finally a time related pattern of ovarian inhibin content is achieved that is comparable to the one found during adult oestrous cycles, with a maximum on the day of first pro-oestrus, day -1. Over this period of days -10 to -1, the average serum level of FSH declines continuously, and no acceleration in this decline is seen after day -5. However, the fluctuations of serum levels of FSH around their average value (fig. 3.1-3) are much greater before than after day -5, indicating that large fluctuations in secretion of FSH are prevented and that, thereby, undisturbed growth of the prevailing crop of preovulatory follicles towards the day of first ovulation is guaranteed.

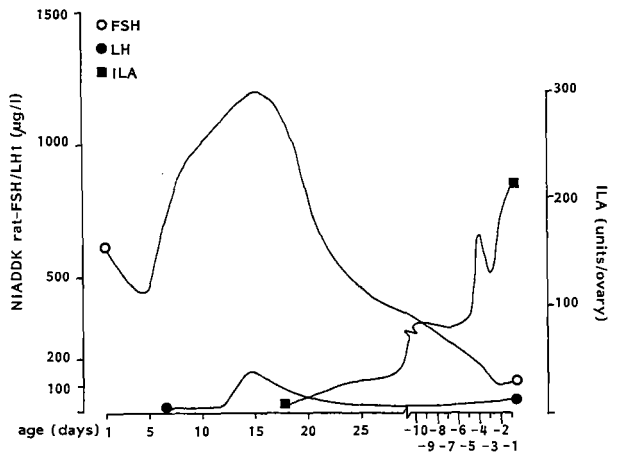



Figure 3.1-4. Levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both expressed as µg NIADDK rat-FSH or -LH RP-1/1 in serum during the immature period of life of the female Wistar R-Amsterdam rat and, of inhibin-like activity (ILA; units per ovary; means ± S.E.M.) in the ovaries of immature female rats up to the day before the day of first (spontaneous) ovulation (day -1). Inhibin-like activity was estimated at 13, 18, 23 and at 28 days of age (n=6-13; at 13 days of age no ILA was detected in individual ovaries) and, during the last 10 days before the day of first ovulation. -10 to -1 : the 10 days preceding the day of first ovulation. n for inhibin is as indicated in fig. 3). Data for ILA, with permission, from Sander, Meijs-Roelofs et al.(1985,1986). Data for FSH and LH redrawn with permission, from Meijs-Roelofs, Uilenbroek et al.(1973a,b) and Sander et al.(1986).

Thus, ovarian follicular inhibin may be essential during the final phases of sexual maturation as a specific and independent, short-term and subtle regulator of FSH synthesis and secretion as opposed to the more long-term and coarse regulation exerted via steroids.

The data presented here make it likely that the main physiological roles of inhibin in the immature WRA-rat are:

(1) to add to steroid suppression of the average serum levels of FSH between days 17-21;
(2) to suppress the occurrence of great fluctuations in secretion of FSH from day -5 to -1, thereby
(3) safeguarding preovulatory growth of a specific (normal) number of follicles up to the point of first ovulation and sexual maturity.

## 3.2. The adult female rat.

Female rats are usually considered adult from the time of concomitant occurrence of vaginal opening and first ovulation, and subsequent happening of more or less regular oestrous cycles (of 5 days in our rats). During heat, when ovulation occurs, the female rat may be fertilized. After a period of cyclicity (which may be interrupted by pregnancies), fecundity diminishes and the oestrous cycles gradually become irregular (5-8 days). During senescence the female ultimately becomes truly a-cyclic and has reached the end of her reproductive life.

### 3.2.1. Staging of the adult or postpubertal period.

In our rat strain the animals generally display regular oestrous cycles for 4-6 months following first ovulation. The cycles predominantly have a duration of 5 days (in this thesis designated di-oestrus day-1,-2 and -3 (D1, D2, D3), pro-oestrus (P) and oestrus (OE)) and are characterized by a recurrent pattern of maturation of antral follicles and by changes in levels of pituitary and ovarian hormones in blood and tissues that are controlled by the hypothalamus-pituitary-ovary (HPO) axis. Between 4 and 12 months after first ovulation the animals display the already mentioned gradual transition from regular 5-day cyclicity to irregular cyclicity. Cycle length increases and day-to-day changes begin to falter. The main cause for the breakdown of oestrous cyclicity may be aging of the hypothalamus (Finch, Felicio et al.,1984; see also section 3.3) which gradually loses its potential to be stimulated by oestradiol to release a surge of LHRH, and, by this loss of oestradiol positive feedback the ovulatory surge of FSH and LH deteriorates and ovulation fails. Generally, the female laboratory rat is reproductively senescent when 12-15 months old. The aging of the hypothalamus results, as already stated, in faltering of the ovulation stimulus itself, but also, via absence of the oestrous part of the FSH surge, in inadequate stimulation of follicle growth and maturation and of steroid production which renders the ovary gradually non-functional. However, especially during early senescence sporadic follicle growth and ovulation may still occur.

### 3.2.2. The cyclic female rat.

After completion of the process of ovarian maturation, culminating in the occurrence of first ovulation around day 39-40 (Meijs-Roelofs et al., 1982b), the subsequent (first) oestrous cycle usually takes 7-8 days (Osman & Meijs-Roelofs, 1976; Appendix Paper I, Meijs-Roelofs, Kramer et al.,1984) followed by 5-day oestrous cycles. At this first ovulation 7-14 ova are shed (10.7 ± 1.2; mean ± s.e.m.; Meijs-Roelofs et al.,1982b; Meijs-Roelofs, Kramer et al.,1987), a number the same as the average number of ova shed during the subsequent cycles (Osman & Dullaart,1976; Meijs-Roelofs et al.,1984) and later cycles (Welschen,1972; this Thesis). In our rat-strain ovulation generally takes place during the early morning hours (after midnight) of the day of oestrus (Osman,1975,1977).

Oestrous cyclicity in the laboratory rat is easily detected by studying the accompanying cyclic changes in vaginal cytology as detected in vaginal smears (Long & Evans,1922). These changes are related to a recurrent pattern of follicle maturation and ovulation (Everett, Sawyer & Markee,1949) which, in turn, cause cyclic changes in the blood levels of

oestradiol-17$\beta$, the hormone that induces vaginal cornification (Schwartz,1969).

It is generally known that the ovary contains a very large number of follicles of various stages of maturation. This population of follicles may be divided into two main pools: one pool that is formed by a gradually diminishing reservoir of non-growing (primordial) follicles, from which there is continuous outflow to the second pool, which contains the growing follicles (see section 3.1.2.). Follicles in this second pool may be further subdivided in a first group containing non-antral follicles (volume $< 100*10^5$ $\mu m^3$ or $\phi < 260$ $\mu m$) which grow at a relatively slow rate and a second group consisting of follicles with an antrum filled with fluid (follicular fluid, FF)(follicle volume $> 100*10^5$ $\mu m^3$, $\phi > 260$ $\mu m$) and with a relatively fast growth rate (Pedersen,1972; Welschen,1972; Hirshfield,1981). Maturation of follicles from the pre-antral to the antral stage requires a basal level of FSH since hypophysectomy (HPX) prevents maturational growth beyond the largest pre-antral stage, which may be remedied by exogenous FSH (review: Richards,1980; Hirshfield,1985). During the oestrous cycle the number of antral follicles shows significant day-to-day changes in contrast to the number of small, pre-antral follicles (Mandl & Zuckerman,1952; Welschen & Rutte,1971).

It has been suggested that a cohort of pre-antral follicles starts maturational growth during each cycle after appropriate stimulation by the peri-ovulatory FSH surge (Schwartz,1969; Welschen & Rutte,1971; Welschen,1972), i.e. the oestrous part of this surge (Hirshfield,1981). This growth ultimately leads to either ovulation or atresia of the follicles (Welschen,1972; Hirshfield & Midgley,1978).

Follicle growth and maturation in our strain of rats have been documented by Welschen & Rutte(1971), Welschen (1972,1973), Meijs-Roelofs et al. (1982b,1985) and Osman (1975,1985) and, may be summarized as follows :

On the morning of oestrus in each single ovary approximately the number of follicles that will finally ovulate per 2 ovaries is recruited from the pre-antral group. These follicles have developed an antrum and start to grow at a fast rate. Subsequently this number is reduced via two waves of atresia to the number (7-14) of follicles that normally ovulates from the two ovaries during the period of oestrus. The first wave of atresia occurs shortly after recruitment, yielding reduced numbers of follicles on di-oestrus day-1. The second wave starts on di-oestrus day-3 and causes further reduction to a number within the normal range of pre-ovulatory follicles on the afternoon of pro-oestrus. These follicles will ovulate during the early morning of the following day (oestrus).

Reduced recruitment of antral follicles may be achieved by various means causing suppression of the above mentioned surge of FSH on the morning of oestrus : suppression by anti-FSH serum (Welschen & Dullaart, 1976), by barbiturates (Hirshfield & Midgley,1978), or by porcine follicular fluid (Hoak & Schwarz,1980; Hirshfield,1981). However, administration of FSH on the morning of oestrus to rats in which the peri-ovulatory surges of endogenous FSH (and LH) have been blocked by barbiturates (which block LHRH secretion), reinstates recruitment of follicles (Hirshfield & Midgley,1978). On the other hand, augmenting the effect of the oestrous-surge of FSH via injection of equine chorion gonadotrophin (eCG, formerly PMSG) at 08.00h on the day of oestrus, results in a supranormal number of large antral follicles and reduced numbers of

atretic follicles at 12.00h on di-oestrus day-1 (Hirshfield,1986).

While the first wave of atresia may possibly be reduced by increased levels of FSH on the morning of oestrus (Hirshfield,1986), the mechanism underlying the second wave of atresia (on di-oestrus day-3) is not yet fully understood. However, gradual changes in levels of circulating hormones (oestradiol-17$\beta$, in conjunction with FSH and LH) may possibly cause atresia of specific follicles, mainly dependent on their stage of maturity as indicated by the rate of oestradiol-17$\beta$ secretion (Lacker, Beers et al.,1987), a self propagating process (Adashi & Hsueh,1982).

The pituitary releases gonadotrophic hormones into the blood of adult female rats in a cyclic pattern (figure 3.2-1; Hermans et al., 1982a). Throughout the oestrous cycle, except on the morning of oestrus, levels of FSH and LH in blood follow a nearly parallel course at differing average concentrations. Average FSH levels during the di-oestrous period (based on 2-hour sampling intervals) fluctuate between 50 and 200 $\mu$g/l, while average levels of LH are below 50 $\mu$g/l with only minor deviations (Hermans et al.,1982a).

On the day of pro-oestrus, levels of both hormones begin to increase from 12.00h onwards and reach a maximum around 16.00h for LH and around 20.00h for FSH. This rapid increase constitutes the LHRH-induced, pro-oestrous surge of gonadotrophins that is necessary for induction of ovulation (Welschen & Dullaart,1976). Luteinizing hormone plays a major role and FSH seems to be of minor importance (DePaolo, Wise et al.,1979c). After this surge, the level of LH rapidly decreases to dioestrous values before 08.00h on oestrus. The level of FSH, however, shows only a transient decrease on pro-oestrus with a nadir around 22.00h, whereafter it increases

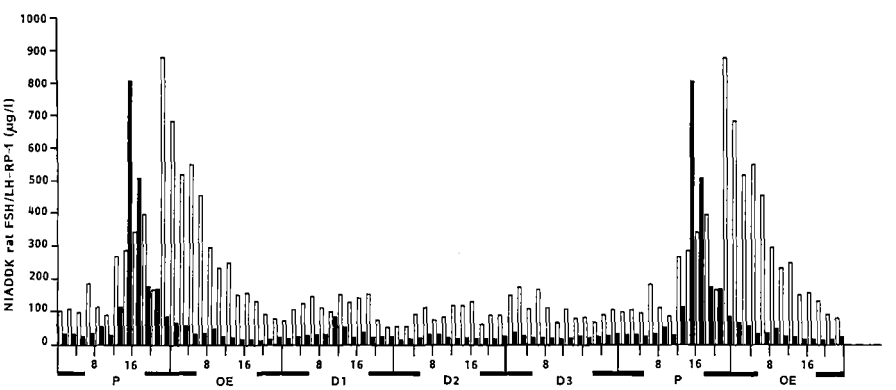



Figure 3.2-1. Pattern of serum levels of FSH (open bars) and LH (filled bars) throughout the oestrous cycle, of females from our strain of rats. The light period lasted from 05.00h-19.00h. FSH and LH are expressed as $\mu$g NIADDK rat FSH or LH RP-1/L. D1, D2 and D3, di-oestrus day-1,-2 and-3; P, pro-oestrus; OE, oestrus; black lines at bottom, dark period; 8, 8.00h a.m.; 16, 16.00h (redrawn after Hermans, Debets et al.,1982a, fig. 3b). Number of rats per group is 4-7.

again and reaches a maximum around 24.00h. Then it gradually declines and attains di-oestrous levels again around 12.00h on oestrus (Hermans et al.,1982a). This second surge of FSH encompasses the time-span in which ovulation takes place (Nequin, Alvarez & Schwartz,1979; Hashimoto, Isomoto et al.,1987).

Ovarian follicles release steroidal hormones to the blood in a cyclic fashion. These steroids are synthesized by theca cells (androgens, progestins) or granulosa cells (progestins) or by the 'two cell' complex (Peters & McNatty,1980; Hillier,1981) of theca and granulosa cells (oestrogens); in this complex, theca cells release androgens that serve as substrate for oestradiol-17$\beta$ synthesis by the granulosa cells (Short,1962; Moon & Duleba,1982; review: Erickson, Magoffin et al.,1985). Recently some doubt has been raised about the validity of the 'two cell' theory of follicular oestradiol-17$\beta$ synthesis (Krishna & Terranova,1987).

Gonadal steroids influence gonadotrophin secretion. However, the mechanism involved in the regulation of gonadotrophin secretion is complicated : the same steroid may stimulate or suppress serum levels of gonadotrophins, depending on the reproductive status of the female (Campbell & Schwartz,1977; Schuiling, Moes et al.,1987). Also, the ratio of the concentration of oestradiol and progesterone plays a role in the suppression of pulse amplitude and/or frequency of LH release (Leipheimer, Bona-Gallo & Gallo,1984,1985,1986). While feedback regulation of the secretion of LH by gonadal steroids is well documented, this is less clear for FSH (Campbell & Schwartz,1977; McCann, Mizunuma et al.,1983; Chappel,1985). Negative feedback regulation of the secretion of FSH during the oestrous cycle seems to be mediated not only to a great extent by steroids but also by proteinaceous gonadal factors called inhibins, activins or follistatins (De Jong et al.,1985b; Findlay,1986; Tsonis & Sharpe,1986; Li & Ramasharma,1987; Sairam, Ramasharma & Li,1987; Ying, Czvik et al.,1987). Some of these factors supposedly play an intragonadal paracrine role as well, modulating LH-stimulated androgen release (Hsueh, Dahl et al.,1987). Also, in concert with oestradiol they are thought to suppress LH secretion in ovariectomized rats (Lumpkin, DePaolo & Negro-Vilar,1984; Babu, Bona-Gallo & Gallo,1986).

The gonadotrophin-regulating effect of steroids occurs in part at the level of the hypothalamus and is exerted there in combination with endogenous opioid peptides (EOP's) via neurotransmitters influencing LHRH release (Blank, Panerai & Friesen,1979; Bhanot & Wilkinson,1984; Plant,1986; Piva, Limonta et al.,1986; Babu, Marco et al.,1987). At the level of the pituitary, oestradiol causes a reduction in LHRH sensitivity early in the cycle (Apfelbaum,1981) but afterwards, on pro-oestrus, it sensitizes the pituitary for interaction with LHRH (Apfelbaum,1981; Leipheimer et al.,1985; Karsch,1987). The sensitivity of the pituitary for LHRH inducing secretion of gonadotrophins, thus shows cyclic variations and reaches a peak around 17.00h on pro-oestrus, when the pre-ovulatory surge of LH reaches a maximum. However, interpretation of the observations is hampered by the finding that the blood level of FSH displays another peak approximately 7h later, at a time when the pituitary has lost its greatest sensitivity for LHRH (Aiyer, Fink & Greig,1974; Apfelbaum,1981).

The role of inhibin in the regulation of blood and tissue levels of FSH and of follicle recruitment is discussed in detail in the next paragraph.

3.2.2.1. Inhibin in the cyclic female rat.

The putative role of inhibin in the physiology of reproduction of the adult, female rat has been studied by many authors. Their results, conclusions and remarks have been summarized in the Tables 3.2-1 to -5.

Mostly, intact or (unilaterally) ovariectomized rats have been used to study the effect of injection with inhibin-containing preparations on parameters of reproductive physiology (table 3.2-1). The effects of administration of such preparations during the periovulatory period are summarized in Table 3.2-2. The first data on the physiological role of endogenous inhibin were obtained via various treatments aimed at a (transient) change of blood levels of inhibin, and are summarized in Table 3.2-3. Data on qualitative and quantitative estimation of levels of inhibin present in various biological fluids or tissues in the rat is summarized in Table 3.2-5.

The time-related pattern of suppression of FSH after a bolus injection with inhibin may be described as follows (references in table 3.2-1).

Generally, the first reduction of blood levels is seen after 2-5h and minimum values occur after 8-10h, while the suppressive effect disappears after 16-30h (Hermans et al,1981a). The period of sub-normal levels of FSH is followed by a period where levels of FSH show a series of surge-like increases lasting 2-3h each, the "rebound" phenomenon (Hermans et al., 1982a). These surges occur superimposed on apparently normal, basal levels of (immunoassayable) FSH.

When inhibin activity is administered as single, daily injections to adult female rats, oestrous cyclicity generally is not affected, even after prolonged treatment (De Jong et al.,1978). This possibly may be the result of the rebound of FSH (DePaolo, Hirshfield et al.,1979a; Hermans et al.,1981a,b, 1982a), that functionally compensates the previous deprivation of FSH-bioactivity and, thereby, causes the regulatory mechanism subserving oestrous cyclicity to keep its normal pace. Interruption of regular oestrous cyclicity occurs only when inhibin injections are given more frequently (at 8-12h intervals)(McCullagh & Schneider,1940) This causes prolonged suppression of levels of FSH (Lumpkin et al.,1984). However, when inhibin treatment is stopped, a rebound of levels of FSH is seen and cyclicity is re-instated. A side-effect of repeated injection with exogenous inhibin may be the formation of antibodies and the subsequent neutralization of endogenous inhibin activity (in monkeys: Channing et al., 1982; in ewes: Henderson, Franchimont et al.,1984).

After a bolus injection with bFF in intact rats, the ensuing period of suppressed levels of FSH is followed by the rebound phenomenon, but this is not the case in ovariectomized rats, where suppression of FSH is more profound than in intact rats and levels of FSH afterwards return directly to pretreatment levels (Hermans et al.,1981a).

In adult rats the HPO axis appeared to be more sensitive to inhibin-like activity (ILA) than in immature rats (Hermans et al.,1980). Although initially the effects of ILA on levels of FSH were judged from changes in the average, basal level, recent evidence supports the view that the effect of ILA (in vivo) resides at least in part in reduction of episodic FSH secretion (Lumpkin et al.,1984). This may possibly result from a blunted reaction of the pituitary to stimulation by LHRH. Also, enhanced clearance of FSH from the circulation as a result of changed molecular heterogeneity

Table 3.2-1. Effect of administration of various preparations containing inhibin on cyclicity and serum level of FSH in adult female rats pretreated in various ways.

| IN from | Treatment | Effects observed and remarks. | Authors |
|---|---|---|---|
| bTE | OVX | -pituitary castration cells diminished; no effect on vaginal cornification | Nelson & Gallagher, 1935 |
| bTE | 3-9 days multiple daily | -oestrous cyclicity disrupted | McCullagh & Schneider, 1940 |
| bFF, pFF | D,OVX | -FSH suppressed at 4 and 8h post-OVX; follicle size and FF inhibin potency are related | Welschen et al., 1977 |
| bFF | 26 days single daily | -FSH initially suppressed, after day 3 elevated; oestrous cyclicity normal; ovulation rate elevated | De Jong et al., 1978 |
| bFF | D,ULO | -FSH suppressed in intact rat; no postovariectomy rise of FSH after ULO + bFF | Welschen et al., 1978 |
| rat ovary | OVX | -ovarian homotranspl. intrasplenic: FSH suppressed, under kidneycapsule: FSH unaltered; E2b and P4 no effect | Uilenbroek et al., 1978 |
| bFF | D,OVX | -FSH suppression more pronounced after OVX; E2b and P4 ineffective; HPO axis more sensitive for IN in adult than in immature | Hermans et al., 1980 |
| bFF | D,OVX | -serum FSH suppression more profound after OVX; rebound in intact, not in OVX, after 16-30h. | Hermans et al., 1981a |
| bFF | D, P, OE single | -time related FSH suppression for 4-12h + rebound | Hermans et al., 1982a |
| pFF | OVX+ steroids | -Suppression of FSH secretion by inhibin and steroids | Williams & Lipner, 1982 |
| pFF | D,OVX | -episodic FSH secretion reduced; FSH response to LHRH blunted | Lumpkin et al., 1984 |
| pFF | 14d.OVX | -FSH secretion 60% suppressed; IN acts distal to GnRH receptor and lowers LHRH induced LH secretion | Charlesworth et al., 1984 |

| | | | |
|---|---|---|---|
| bFF | OVX | -reduced half-life and changed molecular heterogeneity of FSH | Van der Schaaf-Verdonk et al., 1984 |
| pFF, inf. | OVX, 90d.OVX | -FSH maximally suppressed for 20 days, but no suppression after day 30. | Thomas & Nikitovitch -Winer, 1984 |
| pFF | MSG,ULO | -FSH suppression unaltered after MSG, but no COH | Rush, 1986 |
| pFF | D,OVX | -FSH suppressed; bioassay model | Savoy-Moore et al., 1986 |
| bFF | 14d.OVX | -pulsatile FSH secretion suppressed; LH and PRL unaffected | De Greef et al., 1987 |
| rgcm IN-A | OVX | -suppression of FSH | Ying et al., 1987 |

Abbreviations: bFF/pFF, follicular fluid of bovine/porcine origin; bTE, aqueous bull testis extract; COH, compensatory (contralateral) ovarian hypertrophy (after ULO); D, di-oestrus; E2b, oestradiol benzoate; FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone, probably identical to LHRH, luteinizing hormone-releasing hormone; IN(-A), (type A or $\alpha\beta_A$) inhibin; inf., infusion; LH, luteinizing hormone; MSG, monosodium-glutamate; OVX, ovariectomy; P4, progesterone; PRL, prolactin; rgcm, rat granulosa cell-conditioned medium containing IN-A; ULO, unilateral OVX; 14d.OVX, 14 days after OVX.

of FSH and reduced half-life after injection with inhibin, supposedly causes lowered FSH levels (Van der Schaaf-Verdonk, Harryvan et al.,1984).

When bFF is administered immediately following ovariectomy the short-term increase in serum levels of FSH that follows ovariectomy is prevented (Welschen et al.,1978), which supports the idea that the ovary normally releases a factor with inhibin-like activity.

One of the observations that originally provoked speculations about a role for inhibin in the divergent regulation of secretion of FSH and LH was the combined surge of FSH and LH in the afternoon of pro-oestrus, as opposed to the solitary surge of FSH on the morning of oestrus. Much attention has therefore been given to the possible dissimilar role of inhibin in the regulatory mechanisms that cause the first (LH and FSH) and the second (FSH only) surge of gonadotrophin during the periovulatory period (i.e. generally between pro-oestrus 12.00h and the afternoon of oestrus). Data and remarks are summarized in Table 3.2-2.

Injected preparations containing ILA may dissimilarly suppress the separate FSH surges of pro-oestrus and oestrus, provided that the lag-time of two to three hours for inhibin to become operative is taken into account. The surge of LH on the afternoon of pro-oestrus is not affected by this treatment (Schwartz & Channing,1977; DePaolo et al.,1979c; Welschen et al.,1980). Recruitment of a new cohort of follicles, which normally occurs during the periovulatory period, was shown to be solely dependent on the FSH surge on the morning of oestrus (Hirshfield,1981).

Table 3.2-2. Effect of administration of various preparations containing inhibin on serum level of FSH in adult female rats during the peri-ovulatory period.

| IN from | Treatment | Effects observed and remarks | Authors |
|---|---|---|---|
| pFF | P | -oestrous surge of FSH suppressed; no effect on steroid levels or follicle rupture | Schwartz & Channing, 1977 |
| pFF | P&OE | -suppression of FSH surge -> rebound; normal peri-ovulatory parameters at next oestrus | DePaolo et al., 1979a |
| pFF | P/OE, PB & LHRH | -suppression of FSH surges, not of LH lag-time 2-5h, duration 10-14h; oocyte maturation normal; pituitary is focus of action of IN. | DePaolo et al., 1979c |
| pFF | P 16.00h | -oestrous surge of FSH suppressed and no follicle recruitment on D1 | Hoak & Schwartz, 1980 |
| bFF | P | -FSH reduced on OE; number of antral follicles and level of serum FSH are correlated | Welschen et al., 1980 |
| pFF | P/OE | -suppression of periovulatory surges of FSH; rebound next day; fewer large antral follicles. | DePaolo et al., 1981 |
| pFF | P&OE | -FSH surge on P and/or OE suppressed; follicle recruitment depends on FSH-surge on OE | Hirshfield, 1981 |
| bFF | hCG-ovulation | -premature ovulation not affected; FSH surge next day delayed; retardation of follicle recruitment. | Sasamoto et al., 1981 |

Abbreviations: bFF/pFF, follicular fluid of bovine/porcine origin; D, di-oestrous; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotrophin; IN, inhibin; LH, luteinizing hormone; LHRH, luteinizing hormone-releasing hormone; OE, oestrus; P, pro-oestrus; PB, pentobarbitone.

Suppression of the surges of FSH on pro-oestrus and oestrus resulted in normal ovulation but in reduced numbers of antral follicles on di-oestrus day-1 (Hoak & Schwartz,1980). At the next oestrus, however, the peri-ovulatory parameters were normal again (DePaolo et al.,1979a). This suggests that the adverse effects of reduced levels of FSH on the process of follicle recruitment and maturation on oestrus following injection with ILA, had been compensated for by a rebound of levels of FSH. Comparable results with regard to compensation of follicle maturation were obtained when hCG-induced ovulation on di-oestrus day-2 was studied instead of

spontaneous ovulation, which suggests that the compensatory feedback mechanism operates also beyond the naturally occurring periovulatory period (Hasegawa, Miyamoto et al.,1981; Sasamoto, Otani & Shirota,1981). Furthermore, a good inverse correlation was found between serum levels of FSH and the number of antral follicles in the ovary, the main source of circulating ILA (Welschen et al.,1980; 1-st pre-ovulatory period: Sander et al.,1986). From these data it may be concluded that during the oestrous cycle large antral follicles are responsible for inhibin-mediated suppression of FSH and that the process of ovulation, with loss of large antral follicles, and occurrence of the second or oestrous surge of FSH are closely related phenomena.

In agreement with this conclusion were results obtained by Wang & Greenwald (1987), who injected hamsters on pro-oestrus with cycloheximide (which prevents protein synthesis and thus formation of microtubules in eukaryotic cells), and found that this effectively blocked follicle growth and ovulation but not the LHRH-induced surges of FSH and LH on pro-oestrus. Most interestingly in this context, the surge of FSH on the morning of oestrus was absent in this experiment. From these results they concluded that on oestrus morning there possibly is a causative, inverse relation between the presence in the ovary of preovulatory follicles and, serum levels of FSH (Wang & Greenwald,1987).

In conclusion, it is evident that during the periovulatory period also, inhibin may be held responsible for the (short-term) regulation of FSH, including part of the FSH surge on the morning of oestrus. Inhibin is released predominantly by the large antral- and pre-ovulatory follicles (as is oestradiol), and, transformation of these follicles and loss of follicular fluid and part of the granulosa cells at ovulation also causes disappearance of the bulk of inhibin from the ovary and reduced levels of inhibin in ovarian venous plasma (OVP). This decreased inhibin level may induce the second or oestrous surge of FSH. Thereby it is these follicles that control the level of FSH. From these conclusions it follows that (lowering of) the serum level of inhibin is, indirectly, responsible for the magnitude of the oestrous surge of FSH and thus also for recruitment of a new cohort of antral follicles for the next oestrous cycle.

Evidence for the presence of bioactive inhibin in the female rat was initially rather circumstantial, as may be seen in Table 3.2-3. After unilateral ovariectomy (ULO), the amount of ovarian inhibin and steroids released into the circulation is suddenly halved and within 5-8h a rise in serum levels of FSH to peri-ovulatory values was seen, followed by a return to pretreatment levels between 16 and 24h (Welschen & Dullaart,1974). Throughout the first oestrous cycle after ULO, FSH bioactivity is greater than in controls (Peppler,1972), but immunoactivity (RIA) during that period was not different from control values (Welschen & Dullaart,1974).

Table 3.2-3. Indirect evidence for the presence of physiologically active inhibin in female rats, as apparent from various treatments that supposedly result in altered levels of endogenous inhibin.

| Treatment | Effects observed and conclusions. | Authors. |
|---|---|---|
| ULO | -FSH elevation causes COH; FSH, not LH, elevated through 1-st cycle; bio-assays for FSH and LH. | Peppler 1972 |
| ULO | -prolonged FSH surge at next OE; FSH and LH elevated at 24h post-ULO | Howland & Skinner, 1973 |
| ULO | -FSH elevated between 5 and 16-24h post-ULO; LH incidentally elevated after 24h | Welschen & Dullaart, 1974 |
| OVX | -acute regulation of FSH in part LHRH-independent, long-term is LHRH dependent | Berardo & DePaolo, 1986 |
| unilateral X-irradiat. ovary | -fewer large antral follicles; CL smaller post-irradiat.; presumed granulosa cell damage; superovulation contralaterally | Mandl, 1964 |
| X-irradiat. ovaries | -fewer antral follicles, more atresia; increased FSH, not LH | Jarrell et al., 1986 |
| pituitary transplant, D1 | -increased number of antral follicles; E2 level normal, FSH surge on P and OE blocked; LH suppressed in part | Tsukamoto et al., 1986 |
| anti-IN ser. (bTE) | -elevated FSH (15x) and LH (8x); E2 unchanged; effect more pronounced in immatures | Franchimont et al., 1975 |
| anti-IN ser. (pFF-IN) | -FSH rise 3-5h after injection on all days of cycle ; FSH surges on P and on OE elevated | Rivier et al., 1986 |
| anti-IN ser. (pFF-IN) | -elevated FSH, not LH | Ying et al., 1987 |
| anti-IN ser. (bFF-IN) D1 | -FSH elevated at 8h, not LH; increased ovulation rate; more atretic follicles at next oestrus | Sander, 1988 (this Thesis) |

Abbreviations : bFF or pFF, follicular fluid of bovine or porcine origin; bTE, aqueous extract of bovine testis; CL, corpus luteum; COH, compensatory ovarian hypertrophy after ULO; D1, di-oestrus day-1; E2, oestradiol-17$\beta$; IN, inhibin; LHRH, luteinizing hormone-releasing hormone; OE, oestrus; OVX or ULO, bi- or unilateral ovariectomy; P, pro-oestrus; ser., serum; transpl., transplant; X-irradiat., X-irradiated.

This may be explained, as mentioned before, by postulating an effect of inhibin on the heterogeneity of FSH molecules (Van der Schaaf- Verdonk et al.,1984), thereby changing the ratio of bioactive to immunoactive molecules

Since the effect of ULO on levels of FSH, could not be prevented by administration of physiological amounts of steroids, it was concluded that possibly elimination of inhibin-mediated suppression of pituitary secretion of FSH was responsible for the observed effects (Welschen et al.,1978). After ULO, compensatory growth of antral follicles occurs in the remaining ovary (COH), which is initiated by the surge of FSH 8h after ULO and almost completely compensates the loss of antral follicles present in the removed ovary (Peppler,1972; Welschen,1972). This results in doubled numbers of antral follicles after 24h (Welschen,1972) and thus in doubling the inhibin releasing potential of the remaining ovary, which would explain why levels of FSH have again reached control levels after 24h (Welschen et al.,1978).

Using a different approach, Mandl (1964) and Jarrell, Younglai et al. (1986) found that ovaries treated with a cell-damaging dose of X-rays showed decreased development of antral follicles, increased rates of atresia and smaller corpora lutea; observations pointing to possibly considerable damage of the granulosa cell compartment of the follicles. This was accompanied by elevated levels of serum FSH, but not of LH (Jarrell et al.,1986) which is in accord with the presumed origin (granulosa cells from ovarian antral follicles) and function of inhibin (preferential suppression of synthesis and secretion of FSH); in unilaterally irradiated rats this FSH-rise may have caused the observed superovulation in the contralateral, not-irradiated ovary (Mandl,1964).

Another approach, passive immunoneutralization, reduces rat endogenous inhibin in a more elegant way. The anti-inhibin sera employed were all raised against inhibin preparations of non-rat origin, but cross-reacted sufficiently with rat-inhibin (as proven in vitro) to take effect after injection in rats. Rats passively immunized against inhibin showed within 3-8h a surge-like elevation of levels of FSH, not LH, on each individual day of the cycle (Rivier et al.,1986).

Next, our experiments with anti-inhibin sera will be described in more detail. In cyclic female rats a bolus injection of antiserum against bFF-inhibin was given on di-oestrus day-1 (D1). Ovulation rate, follicle dynamics and blood levels of gonadotrophic hormones and oestradiol were then studied. One series of rats was followed until the day of first ovulation (1-OE) following treatment, the second series was followed until the day of second ovulation (2-OE).

Cyclic female rats from our inbred strain were used and littermates were equally divided between three treatment groups. Vaginal smears were taken every morning and after at least two successive 5-day oestrous cycles rats were used in the experiment. Bodyweight was 182 ± 2.1 g (range 162-207), age was 84 ± 0.8 days (range 75-88). Antiserum to partially purified bovine follicular fluid-inhibin (bFF-I) raised in ovariectomized ewes (Van Dijk, Steenbergen et al.,1986) was used in the antiserum group (AS). This antiserum inhibited, as shown in a previous experiment, the FSH secretion suppressing-potential (i.e. inhibin activity) present in culture media from rat granulosa cells and in homogenates of rat ovaries (Van Dijk et al.,1986).Pre-immune serum from ovariectomized ewes was used for one control group (CS). The second control group was injected with saline (SAL) in order to detect possible effects of ovine serum. Intraperitoneal

injections and, blood sampling via the ophthalmic venous plexus were performed under light ether anaesthesia between 09.00h and 10.00h. At the start of treatment, on the morning of a di-oestrous day-1 (designated 1-D1), rats in each treatment-group were injected with 0.5 ml of serum or saline /100 g bodyweight and a blood sample was taken (time 0h, t=0).

In the first series, animals were followed until the first ovulation following 1-D1, i.e. 1-OE (n = 8 per group) and blood samples were obtained at 0h, after 24h and on the day of oestrus (1-OE). In the second series, animals were followed until the second ovulation following 1-D1 (2-OE; n = 6-7 per group) and blood was obtained at 0h and after 8h and 48h and on the day of second oestrus (2-OE). At sacrifice the weight of total body, pituitary and of ovaries was recorded. Pituitary tissue was stored at -20°C until estimation of content of FSH and LH followed. The ovaries were fixed in Bouin's fluid, embedded in paraffin wax after routine histological procedures and then ovulation rate was determined by counting the number of fresh corpora lutea (CL) in haematoxylin and eosin stained serial sections.

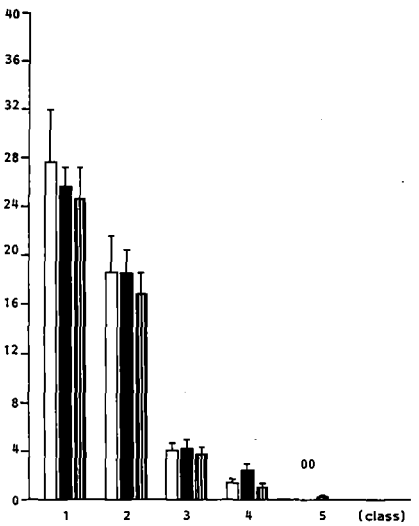



Figure 3.2-2a. Average numbers (± s.e.m.) of healthy antral follicles with a volume >100*$10^5$ $\mu m^3$ (or $\phi$ > 267 $\mu$m) in the ovaries of cyclic rats on oestrus (09.00h-10.00h), that were injected with either anti-inhibin serum (AS, striped bars n=8), control serum (CS, solid bars, n=8) or saline (SAL, open bars, n=8) (all three 500 $\mu$l/100 g BW) on the previous di-oestrus day-1 (09.00h-10.00h). Follicle volumes were classified as follows: class 1, 100-200*$10^5$ $\mu m^3$; 2, 200-350*$10^5$ $\mu m^3$; 3, 350-500*$10^5$ $\mu m^3$; 4, 500-1000*$10^5$ $\mu m^3$; 5, ≥ 1000*$10^5$ $\mu m^3$. 0, no follicles present. All groups n=8.

Table 3.2-4. Number of corpora lutea after anti-inhibin treatment.

| | CS | SAL | AS |
|---|---|---|---|
| 1-OE: (I) | 11.8±0.7 (9-15) | 11.8±0.4 (10-13) | 13.9±0.4 (13-16) |
| 2-OE: (II) | 12.6±0.6 (10-15) | 10.6±0.6 (9-13) | 14.5±0.7 (13-16) |

Average number (± s.e.m.) and range (in brackets) of fresh corpora lutea per animal per treatment group at first (1-OE; n=8-10) or second (2-OE; n=6-7) ovulation following injection on di-oestrus day-1. AS, antiserum; CS, control serum; SAL, saline injected. For statistics see text.

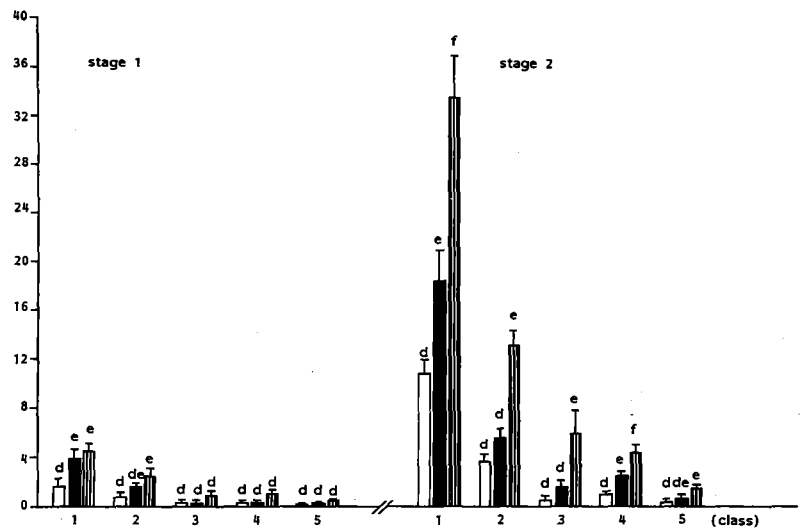



Figure 3.2-2. Average numbers (± s.e.m.) of atretic follicles with a volume >100*$10^5$ $\mu m^3$ (or $\phi$ > 267 $\mu$m) in the ovaries of cyclic rats on oestrus, that were injected with either anti-inhibin serum (AS), control serum (CS) or saline (SAL) on the previous di-oestrus day-1. For further explanation see legends to figure 3.2-2a. Stage 1, early atretic follicles have granulosa cells with pyknosis and their oocyte with nucleus; stage 2, late atretic follicles have granulosa cells with pyknosis and their oocyte without nucleus. Within blocks, columns with different superscripts differ significantly ($p<0.05$).

In the first series, differential follicles counts were also made, as described by Meijs-Roelofs et al.,(1984). Concentrations of FSH and LH in serum and in supernatants of aqueous pituitary homogenates were estimated by radioimmunoassay as described by Welschen et al. (1975) and Sander et al. (1986). Oestradiol-17$\beta$ was estimated in serum as described by De Jong, Hey & Van der Molen (1973). Statistical verification of results was performed via analysis of variance to detect overall differences and, the Student-t test to detect day-to-day differences within and between groups (significant when $p<0.05$).

Counting the number of corpora lutea in the ovaries collected on 1-OE and 2-OE (series I and II), revealed greater numbers in the AS-group as compared to the CS and SAL group (Table 3.2-4). The difference between AS and SAL was significant in series I and II ($p<0.05$), in series I the difference between AS and CS was significant as well, but it just failed to reach significance ($p=0.057$) in series II.

Differential follicle counts performed on ovaries collected during the morning of 1-OE revealed that injection on 1-D1 had not affected follicle recruitment, since the numbers of healthy, antral follicles (volume > 100 * $10^5$ $\mu m^3$, $\phi$>260 $\mu$m) were not different between the groups (fig. 3.2-2a). Analysis of variance of the data on atretic follicles (i.e. follicles with local or general pyknosis in the granulosa cell layer) of all five volume classes taken together, revealed a significantly greater number of atretic follicles in the AS group as compared to both control groups. When the atretic follicles were subdivided according to their degree of atresia and to volume class, the AS-group, as compared to both control groups, contained significantly more late atretic follicles (fig.3.2-2, stage 2: oocyte with lysed nucleus), but not more early atretic follicles (fig.3.2-2, stage 1: oocyte with intact nucleus) follicles. When the late-atretic follicles were further divided in stage 2a (oocyte-cumulus complex present) and stage 2b (oocyte-cumulus complex dispersed) and grouped per volume class (no figure), subsequent analysis of variance of these groups revealed that only the number of follicles in stage-2a of all volume-classes taken together was significantly greater in the AS-group as compared to both control groups. In the AS-group a total average of 96 antral follicles (healthy and atretic) and corpora lutea was counted per rat, as compared to 86 in both control groups; from these data we concluded that AS treatment on di-oestrus day-1 caused addition of 10 extra follicles to the fast growing group of antral follicles, from which ultimately the Graafian follicles are derived. Whether the follicle addition following AS-treatment on di-oestrus day-1 was a result of extra recruitment of follicles from the slow-growing or pre-antral group, or resulted from rescue of early atretic follicles, or from a combination of these phenomena, can not yet be concluded from these data.

Levels of FSH, LH and oestradiol in the animals in series I, at 24h and 1-OE, were not different between the groups (data not shown). In series II, the level of FSH (figure 3.2-3) of the AS-group at 8h and at 48h was significantly elevated as compared to SAL and CS (SAL and CS were not different). In fact, at 8h the level of FSH was elevated from 160 $\mu$g/l (t=0h) to 530 $\mu$g/l, a value comparable to the one found at 8h after unilateral or bilateral ovariectomy. At 2-OE, the levels of FSH were not different between the groups. Serum levels of LH showed no significant differences, except that in series II in the CS-group the reduction from 0h to 8h was significant (60±13 to 27±4 $\mu$g/L, s.e.m.,n=7)

In figure 3.2-4 serum levels of oestradiol (E2) from series II are plotted as averaged values (fig.3.2-4a) and as values for individual animals (fig.3.2-4b,c,d), since in our opinion this best illustrates the effect of injection with AS as opposed to CS or SAL. Analysis of variance of the data for E2 from series II revealed significantly lower overall levels for the AS-group, as compared to both control groups. At individual timepoints there were no significant differences between groups. At 8h the AS-animals did not yet show lowered oestradiol values. The AS-treated animals (fig.3.2-4d) displayed little variation in their serum oestradiol levels, while the individual levels of CS or SAL treated animals (fig.3.2-4b and c) were highly variable.

The length of the oestrous cycle in the saline, CS and AS group, containing 1-D1 and 1-OE in series I was 6-7 days, except for 2 animals with a cycle length of 13-14 days (1 in SAL and 1 in CS). In series II, the first oestrous cycle was either a 6 to 7-day cycle (n = 11) or, a 12-15 day period of anoestrus (n = 10), both terminated by a normal oestrous smear and, followed by a 5-day cycle that was terminated at 2-OE. Within the setting of the experiment we could not detect a relation between an extended time-span from injection to (first or second) oestrus and ovulation rate (nor any of the other parameters studied).

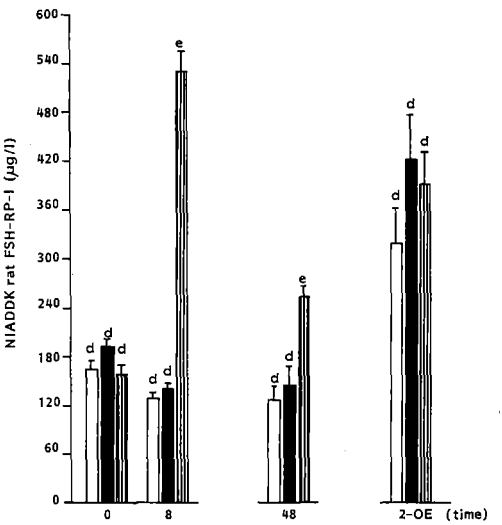



Figure 3.2-3. Mean serum level of FSH (µg NIADDK rat FSH-RP-I/L) per group at the indicated times (h) after injection with inhibin neutralizing antiserum (AS, hatched bars), control serum (CS, open bars), or saline (SAL, solid bars) at 09.00h a.m. on a 1-st di-oestrus day-1 (0h-). Within blocks, columns with different superscripts differ significantly ($p<0.05$). Abbreviations : 2-OE, second oestrus and day of sacrifice. All groups,n=7.

In summary, from this experiment we conclude that :

1) injection of cyclic female rats with anti-inhibin serum on di-oestrus day-1 (D1) is followed 8h later (i.e. short-term) by a surge in the serum level of FSH. It is unlikely that oestradiol caused this surge, since serum levels at 8h were not different between the groups.
2) ovulation rate in the AS-treated animals was greater than in the controls, but with the AS-average still within the normal range.
3) the ovaries of the AS-group on the day of first oestrus following injection (1-D1) contained 10 atretic follicles more than found in the control group. The evoked surge of FSH on D1 may have caused either ingrowth of additional follicles into the group of fast growing antral follicles, salvage of early-atretic antral follicles or, a combination of both phenomena. However, most of these follicles eventually must have become atretic. The evoked surge of FSH on 1-D1 did not affect recruitment on the following day of oestrus.
4) Long-term (48h) effects of antiserum injection on serum levels of FSH and oestradiol seem present but more data are needed.

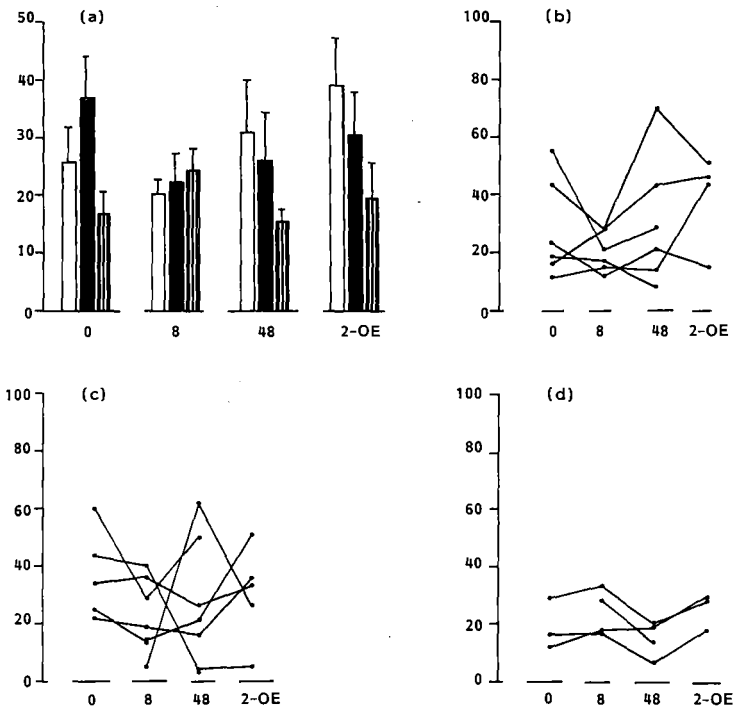



Figure 3.2-4a,b,c,d. Serum levels of oestradiol-17$\beta$ (pmol/L) plotted as average per group (a; n=3-5) and, as values for individual animals (b,c,d), as a function of time. In b-d at some timepoints values are missing due to insufficient serum sample volume. b, saline; c, control serum; d, antiserum treated. For further explanation see legends to figure 3.2-3.

In general, the data are in line with the suggestion that the interaction between inhibin and FSH constitutes a fast-acting component of the feedback loop that can compensate for short-term disruptions the FSH levels of the mechanism that regulates follicle maturation and ovulation rate (Hermans et al.,1981a; Schwarz,1981).

In previous studies, possible neutralization of endogenous inhibin (like-activity) via active immunization after repeated, single daily injections with preparations containing exogenous inhibin has been reported for rhesus monkeys (Channing et al.,1982) and ewes (Cummins, O'Shea et al.,1986; Henderson et al.,1984), but without measurable effect on ovulation rate. In other experiments, ovulation rate in the ewe increased after injections with bFF twice daily for at least three days during the follicular phase (Henderson, Prisk et al.,1986) or two injections per day for 10 days during the luteal phase (Wallace, McNeilly & Baird,1985). Taking together the above mentioned results in monkeys and ewes and our present results in rats, it is apparent that active or passive immunization against inhibin may be a tool for manipulating ovulation rate. Passive immunization seems to be the method of choice in view of its simple treatment protocol.

Increased serum levels of FSH after neutralization of endogenous inhibin through passive immunization of intact cyclic female rats were already reported before we carried out these experiments (Franchimont et al.,1975; Rivier et al.,1986; Ying et al.,1987). In our experiments, the level of FSH at 8h after the start of AS treatment (short-term reaction) increased to a level comparable to the one seen at 8h after bilateral ovariectomy. Therefore, it appears that neutralization of inhibin mimics the short-term effect of bilateral ovariectomy.

At 48h after treatment, serum FSH levels were generally found to remain elevated above controls, which suggests continued inhibin-neutralizing activity of the antiserum and thereby reduced suppression of FSH secretion at the level of the pituitary. This hypothesis is supported by data on disappearance rates of immunoglobulin activity from serum (6-10 days; Talwar, Gupta et al.(1985); Hurkadli & Sheth (1985)). The observation that levels of FSH in our AS group at second oestrus were not elevated as compared to controls, might be explained by assuming a very low actual level of inhibin on oestrus morning in serum and in the ovary, which renders the antiserum ineffective at this time.

Other investigators also found a 4-fold increase in serum levels of FSH 8h after injection with antiserum. However, the further course of FSH remains to be clarified. From Table 3.2-3 it appears that after interruption of administration of antiserum, levels of FSH reach control values after 24h. However, in series II levels of FSH were elevated at 48h after treatment.

The increase in average ovulation rate in the AS group as compared to the control groups, may, as already stated, be explained by postulating a) addition of follicles from the slow-growing pool to the fast-growing-pool as a result of increased levels of FSH following injection with anti-inhibin (Schwartz,1974; Welschen & Dullaart,1976) or, b) decreased atresia of preovulatory follicles, also due to elevated serum levels of FSH (Hirshfield,1986). The ovulation rate in series II in the CS-group was significantly elevated when compared to that in saline controls. It is unlikely that an (ovine) ovarian factor is involved since the Control Serum (CS) was obtained from pre-immune, ovariectomized ewes. The possibility

that oFSH present in CS (and AS) caused the increased ovulation rate in the CS-group seems unlikely, because with our ovine-rat RIA for FSH (Welschen et al.,1975) we did not detect different levels of serum FSH between the CS and the SAL group. However, this evidence is not conclusive since we did not check the FSH-biopotency of the ovine sera. From their studies with immunoneutralization of rat inhibin in vivo, Rivier et al. (1986) could not decide for a physiological role for inhibin, while we have demonstrated a relation between levels of inhibin and of FSH, as well as an indirect dependence of growth and atresia of follicles and of ovulation rate on inhibin. In Chapter 3.1 we already proposed such relations to be operative in prepubertal female rats during the last five days preceding the day of first ovulation (Appendix Paper IV, Sander et al.,1986).

Another point of interest are the blood levels of oestradiol following antiserum treatment. At 8h, as compared to 0h, the levels were low and equal in all three groups. This is in accordance with reduced afternoon levels in the rat (Kalra & Kalra,1974) and does not favour oestradiol as a candidate for the short-term control of levels of FSH. However,the great variance of the values found for oestradiol levels, as illustrated by the differences between the treatment groups at 0h, makes firm statements on the short-term effects of oestradiol impossible. Probably, as indicated by series II, the antiserum suppresses all levels of oestradiol to a common, low level. This may, on the longer term, cause levels of FSH to increase.

Between the three treatment groups we did not find significantly different (average) levels of LH at the times investigated that could possibly have explained the suppressed levels of oestradiol; this strongly points to a local action of the antiserum at the level of ovarian granulosa cell steroidogenesis, via an as yet obscure mechanism.

In conclusion, the results from these experiments demonstrate, for the first time, that in the female rat ovulation rate can be increased via an intra-peritoneal bolus injection with an inhibin neutralizing antiserum.

A further issue of great interest are the actual blood levels of inhibin. Direct estimation of levels of rat inhibin (Table 3.2-5) has mainly been performed by studying its bioactivity in various biological fluids and tissues. To this end, generally the suppression of FSH secretion from primary monolayer cultures of dispersed anterior pituitary cells was used as endpoint.

It was found that dispersed rat granulosa cells in monolayer culture secreted inhibin into the culture-medium (rgcm)(Erickson & Hsueh,1978) for up to 37 days (Appendix Paper V, Hermans et al.,1982b). When granulosa cells of antral ovarian follicles from adult 5-day cyclic rats were cultured on each day of the cycle (Appendix Paper VI, Sander, Van Leeuwen & De Jong,1984), it was found that on day 1 of culture the inhibin activity in the medium was higher than on the following 2-4 days, when low and constant levels were found. Also, media collected on days 1 and 2 from pro-oestrous cells contained larger amounts of inhibin than media collected on other days of the cycle (Appendix Paper VI, fig.1). From these data it was concluded that material with inhibin activity is secreted at a higher rate by granulosa cells collected on pro-oestrus than on any other day of the cycle.

Table 3.2-5. Estimation of levels of endogenous rat inhibin in various biological fluids or tissues of female rats as estimated in the in vivo bioassay using the inhibin-suppressed FSH secretion by a monolayer culture of dispersed, adult female rat anterior pituitary cells as an endpoint.

| Medium tested | Effects observed and remarks. | AUTHORS |
|---|---|---|
| rgcm | -IN present in medium from 3-day culture of granulosa cells collected on P | Erickson & Hsueh,1978 |
| OVP, cycle | -steady level of IN throughout oestrous cycle from OE to P, but lowered periovulatory from P 14.00h to OE 16.00h; inverse relation with plasma FSH levels | DePaolo et al. 1979b |
| OVP, ULO | -IN concentration contralaterally elevated 4-32h post-ULO; FSH elevated 4-24h post-ULO | DePaolo et al. 1981 |
| rgcm | -IN present in medium from 37-day culture; biphasic pattern of secretion | Hermans et al. 1982b |
| OVP | -IN increased at 6, 12 & 18h, FSH at 12 & 18h after hCG injection on D-1; levels of IN and FSH inversely related; hCG may reduce ovarian IN release | Kimura et al. 1983 |
| rat FF, cycle | -IN level steady throughout oestrous cycle but surge from P 9.00h to P 16.00h | Fujii et al. 1983 |
| rgcm, cycle | -steady IN release throughout oestrous cycle from OE to P, but increased on P | Sander et al. 1984 |
| ovarian homog., cycle | -IN increase during oestrous cycle from OE to P; maximum on P; relation with number of follicles with $\phi > 400$ $\mu$m | Sander et al. 1986 |
| OVP, cycle | -gradual rise in IN during oestrous cycle from OE 11.00h to P 11.00h, periovulatory decline from P 11.00h to OE 11.00h | Tsukamoto cycle et al. 1986 |

Abbreviations: FF, follicular fluid; homog., homogenate; hCG, human chorionic gonadotrophin; IN, inhibin; OE, oestrus; OVP, ovarian vein plasma; P, pro-oestrus; rgcm, rat granulosa cell-conditioned medium; ULO, unilateral ovariectomy.

However, on the day of pro-oestrus, transport of inhibin activity from the follicle to the circulation is apparently impeded since this increased inhibin production (Sander et al.,1984, 1986) is not reflected in a reduction in plasma FSH concentration (Hermans et al.,1982a).

Estimation of the inhibin content of ovarian homogenates of 5-day cyclic rats (Appendix Paper IV, Sander et al.,1986) revealed a gradual increase from oestrus to pro-oestrus (3-fold) and a sharp decline from pro-oestrus to oestrus (Appendix Paper IV, fig.3). This was, again, not reflected in a proportional decrease in serum FSH concentrations from oestrus to pro-oestrus. This may be explained, in part, by non-parallel increase of ovarian content and of ovarian release of inhibin, since the amount of inhibin in ovarian vein plasma appears to be rising at a much slower rate from oestrus to pro-oestrus (Tsukamoto, Taya et al.,1986) than ovarian inhibin content, but here too, a conspicuous, periovulatory decline was seen in ovarian venous plasma (DePaolo et al.,1979a; Tsukamoto et al., 1986) (Table 3.2-5).

When follicular fluid from all antral follicles on the surface of the ovaries of individual 4-day cyclic rats was aspirated and pooled, the level of inhibin was found to be constant throughout the oestrous cycle, except that between 9.00h and 16.00h on pro-oestrus a surge was found (Fujii, Hoover & Channing, 1983).

Inhibin estimations have also been carried out in experimental conditions. In unilaterally ovariectomized (ULO) rats, levels of inhibin in OVP of the remaining ovary were elevated for 4-32h following ULO, while levels of FSH in serum were elevated for 4-12h (DePaolo, Anderson & Hirschfield,1981). The temporal changes in the levels of inhibin and of FSH after ULO may be explained as follows :
Removal of one ovary reduces the total ovarian release of inhibin by 50% and, after a lag-time of 2-5h (Hermans et al.,1982a; Welschen et al.,1978, 1980), the pituitary secretion of FSH is gradually freed from part of the inhibin-mediated suppression and increases. Increased levels of FSH act both to increase the number of antral follicles present in the remaining ovary, and, to increase inhibin production by granulosa cells present in these follicles (Welschen et al.,1978). This causes increased serum levels of inhibin and thus reduced secretion of FSH by the pituitary and, finally, this leads to a new equilibrium of the short-term part of the mechanism that regulates the release of FSH and inhibin.

Concluding the remarks on the results of inhibin estimations, it can be said that we are still far from fully understanding the interrelations between the levels of inhibin in the ovary (and its constituents), in culture media, in ovarian venous plasma and in peripheral blood and - of course - the relation between these parameters and the physiological role of inhibin. Also, the relation between inhibin production and its release into the circulation remains to be established.

### 3.2.3. Summary.

The temporal changes in inhibin levels and the physiological role of inhibin during the oestrous cycle of the female rat may tentatively be described as follows (see also figure 3.2-5).

After ovulation has taken place on the day of oestrus, the ovarian content and blood level of inhibin are relatively low. This results in

relatively little suppression of pituitary secretion of FSH, causing high serum levels of FSH on the morning of oestrus. This stimulates growth of a cohort of small antral follicles and thus the magnitude of the follicular granulosa cell compartment increases. Since granulose cells synthesize and secrete inhibin, ovarian content and serum level of inhibin start to increase, and so does, in consequence, its FSH-suppressing activity at the level of the pituitary gland (in concert with more long-term effects of oestradiol). This causes a gradual decrease in levels of FSH to the basal values found on di-oestrus at the afternoon of oestrus. During the di-oestrous period, ovarian content of inhibin and presumably levels of inhibin in the peripheral circulation both increase further as a result of two phenomena : the compartment of granulosa cells contained in the growing follicles expands and, during the di-oestrus period the individual granulosa cells secrete inhibin at a rather constant rate, as shown by in vitro experiments. Taken together, this is suggestive of a cycle-dependent control of both the synthesis and the secretion of inhibin.

Growth of antral follicles during the di-oestrus period does not seem to be subjected to sudden stimuli. Still, on di-oestrus day-3 a wave of atresia takes place which is, however, not reflected in the data known until now on inhibin or oestradiol, or FSH levels. Because inhibin levels measured in ovarian homogenates and in serum increase during di-oestrus,

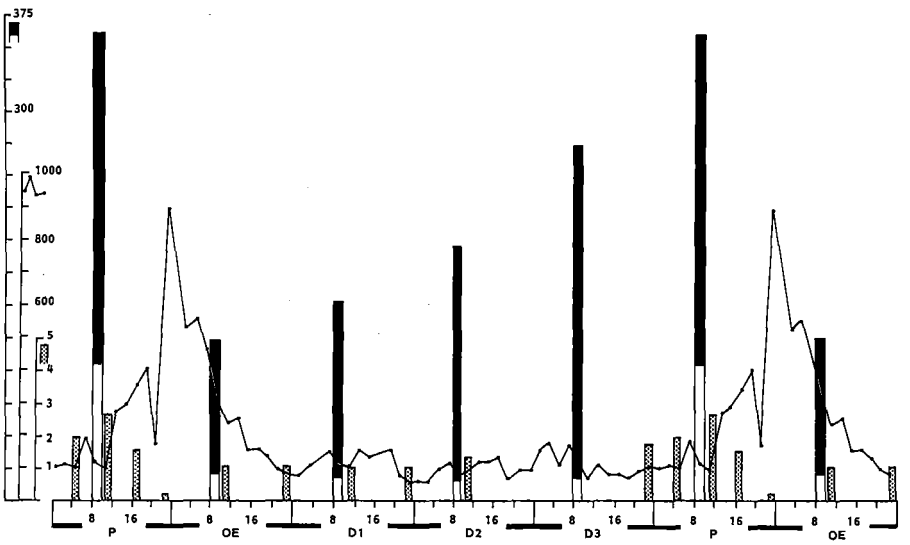



Figure 3.2-5. Graphical overview of FSH and inhibin data throughout the oestrous cycle referred to in this chapter. Total length of bars, content of inhibin in the ovary (units/ovary; data from Sander et al.,1986); open bars, release of inhibin by granulosa cells in vitro (units/$10^5$ cells per 24h (x2); data from Sander et al.,1984); solid line, levels of FSH in peripheral serum ($\mu$g/L; data from Hermans et al.,1982a); stippled bars, levels of inhibin in ovarian venous plasma (units/ml; data from Tsukamoto et al.,1986). For further explanation, see legends to figure 3.2-1.

whereas plasma levels of FSH are uniformly low during this period, this suggests that suppression by endogenous inhibin is at its physiological maximum, and also, that a synergistic effect of inhibin and other ovarian hormones, such as oestradiol, may exist.

Ovarian output of inhibin reaches a climax on pro-oestrus, as estimated from the release by granulosa cells in vitro, ovarian content and, levels in ovarian venous plasma. Plasma levels of FSH do not show a plain minimum on pro-oestrus, although lowest levels of FSH have sometimes been reported for the morning of pro-oestrus. Then, in the afternoon of pro-oestrus the CNS induces a massive release of LHRH into the portal circulation that provokes the preovulatory surge of both FSH and LH, followed shortly by a rapid decline of levels of inhibin in OVP. This decline occurs 8-12h before the second surge of FSH on the morning of oestrus, so that (the magnitude of) the pre-ovulatory surge of gonadotrophins on pro-oestrus may possibly activate, with a lag-time of 3-4h, an as yet fully hypothetical mechanism that reduces ovarian output of physiologically active inhibin and thus causes increased secretion of FSH. There are two more processes that may possibly be causally related to this second maximum of the FSH level via alteration of ovarian inhibin output : Firstly, at the time of ovulation, the oocyte and antral fluid (and thus inhibin) of large antral follicles are expelled from the ovary into the space between the ovary and the bursa ovarica (not directly into the peritoneal cavity). In this way the ovarian content of bioactive inhibin as well as its level in OVP, are reduced as compared to levels found on the morning of pro-oestrus. It should be stressed, however, that levels of inhibin in OVP start to decrease on the afternoon of pro-oestrus, well ahead of the time of ovulation.
Secondly, following ovulation the remnants of the preovulatory follicles are transformed into corpora lutea that still contain granulosa cells, and thereby may possibly synthesize and secrete inhibin for some time (Davis, Dench et al.,1986).

In the late afternoon and early evening of pro-oestrus, LHRH stimulation of the pituitary subsides and LH levels rapidly return to the basal values found during dioestrus. Levels of FSH, however, remain - after a relative dip - elevated, possibly as a result of the above mentioned reduction of serum levels of inhibin, and FSH reaches a second maximum during the early hours of the morning of oestrus. This second surge of FSH of the normal oestrous cycle is held responsible for recruitment of a new cohort of antral follicles into the group of rapidly growing follicles, that finally provides the preovulatory follicles of the next cycle.

Research into the role of FSH and/or LH in the mechanism that controls the release of inhibin from the ovary during the oestrous cycle -with emphasis on the periovulatory period- , as well as the future availability of a RIA for direct measurement of inhibin in the peripheral circulation (Robertson, Hayward et al.,1988), will further clarify the interactions between gonadotrophins, inhibin and follicle growth kinetics.

In conclusion, during the cycle, inhibin (together with oestradiol) and FSH play a pivotal role in regulating the kinetics of follicle growth , and atresia and in keeping the ovulation rate at what may be called the appropriate quota (9 to 15) of the adult, cyclic female rat of our strain.

## 3.3. The aged female rat.

In chapter 3.1 the role of inhibin in the process of sexual maturation of the female rat was discussed. Inhibin proved to be an important factor in the regulation of secretion of FSH and thus in the regulation of follicle growth during the period immediately preceding the day of first ovulation, i.e. in the timing of puberty.

This section deals with the possible physiological role of inhibin in the adult, senescent female rat. Its inhibin secretion is of interest since during the period of senescence, oestrous cycles and hormonal levels that are different from those in the cyclic animal have been reported.

A generally accepted, universal definition of aging in biological terms still seems a question of debate, although characterizations of aging organisms usually include a decrease in the ability to survive (Masoro,1987). Decreased survival is caused by diseases and disorders that are either, as defined by Brody & Schneider (1986), age-related (mainly occurring at a specific age of the host) or age-dependent (as a result of aging of the host).

Biological aging manifests itself as changes over time affecting living systems from the level of the molecule up to the level of the entire organism and, consequently, for each level theories of aging may be constructed (review: Hayflick,1985; Holehan & Merry,1986; Meites, Goya & Takahashi,1987). Bortz (1986) proposed that aging is a consequence of the Second Law of Thermodynamics, which states that an isolated system left to itself will, in the course of time, go toward greater disorder (entropy), thus focussing on age-related use of energy.

### 3.3.1. Staging of the period of aging.

The reproductive system of the female rat also shows changes that are generally referred to as signs of aging. As already stated in paragraph 3.2, following the pre-cyclic and the cyclic period of life, the female rat passes through a semi-cyclic stage to finally reach the post-cyclic or aged (senescent) stage.

The time course for the aging process of reproductive functions in the female rat is dependent on the strain that is studied, but generally the following scheme is accepted and will be used in this chapter : young or cyclic rats are 3-5 months old, middle-aged or semi-cyclic rats are 7-12 months old and, old or post-cyclic rats are more than 18 months old.

Oestrous cycles gradually lengthen during the semi-cyclic period (see diagram 3.3-1), then lose their regular occurrence and, subsequently, are replaced by a state of either prolonged di-oestrus (PD; also called repetitive pseudopregnancy, RPP) or of persistent vaginal cornification (PVC; also named constant oestrus, CE) followed by PD (Aschheim,1976,1983; Lu et al.,1979; Finch et al.,1984); a small fraction of the irregularly cyclic females becomes directly anoestrous.

Also, with aging of the female a reduction in fecundity is seen with reduced littersize, due to factors such as a high rate of embryo resorption and stillbirths (reviews by Aschheim, 1976; Wise, 1983; Finch et al., 1984; Meites et al.,1987). This reduction in fecundity does not appear to be related to alterations of ovarian steroidogenesis (Albrecht, 1984). Finally, the female rat becomes a-cyclic or an-oestrous, is no longer fertile and the post-cyclic or senescent stage sets in.

### 3.3.2. The aging female rat.

In the aging, semi- or post-cyclic rat, quantitative studies of follicle growth and development have been performed, but a precision comparable to that achieved in the adult, cyclic rat has not been attained (and cannot be attained) due to the increasingly irregular character of the oestrous cycles with increasing age of the rat and the prominent variability between individuals (see: Aschheim,1976,1983; Butcher & Page,1981).

In semi-cyclic, middle-aged rats, follicles of pre-ovulatory size (volume > $500*10^5$ $\mu m^3$ or $\phi$ > 450 $\mu m$) are present on each day of the (prolonged) cycle and a normal number of follicles reaches ovulation, but many of the ovulated oocytes are defective and thus account in part for the reduced fecundity seen in these animals (Peluso, Hutz & England-Charlesworth,1982; Matt, Sarver & Lu,1987). The number of antral follicles per ovary on all days of the cycle is smaller in cyclic, middle-aged rats than in young rats and, also, the percentage of such follicles with a viable oocyte is lower, especially on pro-oestrus when it is less than 50% of that of young rats (Peluso et al.,1982).

Furthermore, it appears that, approaching the end of the middle-age period, normal numbers of preovulatory follicles develop which are not all ovulated. This results in more large follicles (Lu, LaPolt et al.,1985) that continue to secrete oestradiol and testosterone. These follicles may subsequently develop into ovarian cysts that in turn go on secreting oestradiol (Wise,1983). This leads to persistent vaginal cornification (PVC) (Wise, 1983). No corpora lutea were detected in the ovaries of rats with PVC (Lu et al., 1979).

In some rats cyclicity is replaced by a state of PD, in which the ovary contains numerous corpora lutea (CL) that release progesterone (review: Steger & Peluso,1987) and are possibly maintained by prolactin (Lu et al.,1979), that, together with progesterone blocks the ovulatory LH surge. The PD-state is comparable to the one found during pregnancy, post-partum lactation, pseudopregnancy, or following implantation of progesterone-containing capsules (review: Lu,1983; Lu et al.,1985).

Lacker et al.(1987) proposed a theory of follicle selection, which states that the physiological mechanism controlling ovulation rate is relatively insensitive to the number of interacting follicles in the developing cohort and that ovulation rate will increase with decrease

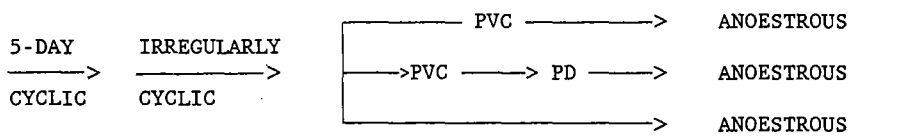



Diagram 3.3-1. Diagram of the subsequent, different reproductive states that an adult female rat may go through. PVC, persistent vaginal cornification; PD, prolonged vaginal dioestrous.

the follicle reserve pool. At the same time, timing of ovulation (i.e. cycle length) is, according to these authors, much more sensitive to the size of the pool of developing follicles and, with increasing age of the animal shows increased variation around a slightly lower, mean cycle length (Lacker et al.,1987). Since in the rat the actual, age-dependent alterations both in ovulation rate and cycle length are, to a large extent, opposite to the theoretical alterations predicted by the mathematical model (Lacker et al.,1987) this model does not adequately describe the kinetics of follicle growth in the aging rat.

Finally, the female rat is reproductively senescent when the PVC- or PD-state shifts to a state of anoestrus when follicular cysts or CL have disappeared.

Summarizing these data on ovarian follicle growth, it appears that age- dependent changes at the level of the ovary become manifest first as qualitative alterations in the developmental pattern of preovulatory follicles. Gradually, the development of follicles also changes quantitatively and finally comes to an end, though an incidental ovulation is still possible. Inevitably, these changes result in altered feedback signals from the ovary (follicles) involved in regulation of the function of the HPO axis.

The changes in levels of hormones involved in the regulation of reproduction that occur concomitantly with the above mentioned, age dependent changes in follicular growth, may be summarized as follows.

The first sign of aging of the HPO axis is that the pro-oestrous surge of LH is delayed and its steepness reduced, resulting in a reduction of the pro-oestrus surge of LH in middle-aged (8-10 mo) rats that are still cyclic or are about to cease cyclicity (Van der Schoot,1976).

The basal serum level of LH throughout the cycle is reduced in rats with ages increasing from 3 to 24 months, while that of FSH increases with increasing age; in these rats the rise of serum level of FSH and LH at 4 days after ovariectomy was progressively reduced (Costoff,1985). When the rats were 24 months old, serum levels of FSH of OVX and intact animals were almost equal, while values for serum LH were still much higher in OVX than in control animals. Both phenomena are possibly caused by oestradiol induced damage of certain parts of the hypothalamic neurons that are part of the mechanism regulating the secretion of LHRH (Brawer, Schipper & Naftolin,1980). Since impairment of the regulation of levels of LH is gradual, it was concluded that this process is not closely correlated to chronologic age (Finch & Mobbs,1983) as it is the case with FSH (DePaolo & Rowlands, 1986), but depends on other factors (see next page). Also, Costoff (1985) reported that in aged OVX rats progesterone was unable to reduce serum levels of either FSH or LH, whereas with oestradiol gonadotrophin levels could be reduced but with progressively less effectiveness with increasing age. In rats aged 9 months or more, no significant effect was found; FSH could not be suppressed to control level. When oestradiol and progesterone were injected in tandem, serum levels of LH (not of FSH) were suppressed more effectively at all ages studied, except for the observation that in animals of 90-270 days increased levels were found.

When comparing the dioestrous basal, and periovulatory surge values of FSH in rats aged 3, 5, 7 and 9 months, it was found that, apart from increased basal dioestrous levels after 5 months, the surge levels were progressively elevated by 7 months and, in part due to a delayed preovulatory surge, the characteristic biphasic elevation in levels of FSH on pro-oestrus and oestrus was no longer present when the rats were 9 months old (DePaolo & Chappel,1986). Also, these authors found that the preovulatory surge of LH was diminished only in 9-months old rats and that within the setting of the experiment levels of prolactin were not related to age. Further, DePaolo & Chappel (1986) reported that in the aging female rat the (LHRH independent) mechanism controlling the (secondary) surge of FSH on oestrus changes prior to that of the (LHRH dependent) surge on pro-oestrus and later than that controlling the basal level of blood FSH. These authors postulated that this is due to age-dependent alterations in as yet unidentified - possibly inhibin - feedback signals from the ovary. When comparing young and middle-aged and, regularly and irregularly cycling rats, Nass, LaPolt et al. (1983) also found the already mentioned change in preovulatory surge level of LH, but not that in FSH. Results from both reports point to differential aging rates of the mechanisms regulating secretion of FSH and of LH in middle-aged rats, as has been reported following comparison of LH and prolactin secretion (Akema, Mitsugi et al.,1985).

The possible role of oestradiol (E2) and progesterone (P4) in the control of regular oestrous cyclicity in aging rats was discussed by many authors. It was found that both repeated pregnancies and the use of progesterone-containing implants contributed to the maintenance of regular cyclicity in aging female rats, possibly as a result of high levels of progesterone in combination with low levels of oestradiol (high P4/E2 ratio) as compared to those in untreated controls (Lu,1983; Lu et al.,1985; DePaolo & Rowlands,1986; LaPolt, Matt et al.,1986). Increased levels of E2 and testosterone (T) were found in middle-aged rats with irregular cycles, as compared to middle-aged and young rats with regular cycles. These increased E2 and T levels apparently progressively altered the pro-oestrous surge of LH and finally may render it inadequate for normal ovulatory function (Nass et al.,1983). This phenomenon may be attributed to a gradual erosion of the CNS-response to stimulatory steroidal feedback on LHRH-induced LH release (Brawer et al.,1980; Nass et al.,1983; Blake, Elias & Huffman,1983). It should be noted that impairment of neuroendocrine function by altered oestradiol-to-progesterone ratio is rather independent of age but depends to a great extent on how often positive E2-feedback has occurred, so that pregnancy and lactation may possibly retard the aging process (see: Finch & Mobbs,1983).

The role of the age-dependent decline of follicle numbers and change in hormonal output of the ovary in the aging of the mechanism regulating reproduction (HPO axis) has not been fully elucidated.

As the pre-cyclic and cyclic stages of life proceed, a continuous loss of follicles occurs, which makes reduction of the ovarian pool of follicles a natural, age-dependent phenomenon. Artificial reduction of the mass of ovarian tissue via ULO of middle-aged, cyclic rats was followed by partial compensatory ovarian hypertrophy (COH; also in rats with PVC) accompanied by significantly higher serum levels of FSH up to 90 days post-surgery and increased incidence of irregular oestrous cycles and PVC, as compared to intact controls (Sopelak & Butcher,1982; Butcher,1985). This suggests that

the mechanism that regulates serum levels of FSH and inhibin, and follicular growth kinetics is functioning at a different level in middle-aged as compared to young, cyclic females.

A different approach was followed by using orthotopic, heterochronic ovarian grafts. It was found that regular cyclicity could be supported for a shorter time by ovaries from middle-aged rats than by ovaries from young rats (Sopelak & Butcher,1982). When both middle-aged and young rats were grafted with either 2 months old, or 12 months old ovaries, most of the middle-aged recipients ended cyclicity in a state of PVC, whereas most of the young rats ended in PD, with no difference as regards the age of the ovaries. Interestingly, the numbers of growing follicles (healthy as well as atretic) in the ovaries of PVC and of PD rats were not different, an observation in favour of a hypothalamic/pituitary site of aging of the HPO axis; however, in that case one has to assume that the follicles are functionally equivalent. It was also concluded that the amount of functional ovarian tissue, as determined by the reduction in number of follicles, plays a major role in the maintenance of cyclicity.

These results would suggest that the waning of reproductive life is, apart from faltering hypothalamus-pituitary function (see above), associated with (a decline in) the number of remaining oocytes in the ovary (Butcher,1985) and of the reduction in the amount of ovarian tissue (Sopelak & Butcher,1982). Since ULO of young, cyclic rats was not associated with long-term elevations of serum levels of FSH (Welschen,1972; Butcher,1977) and did not (for a certain period) influence oestrous cyclicity (Welschen,1972; Sopelak & Butcher,1982). This indicates that the reaction of the HPO axis to ULO is age-dependent in causing increased incidence of deviant COH and oestrous cyclicity with increasing age, both of which are possibly associated with long-term elevated levels of serum FSH (Butcher,1985).

Pituitary responsiveness to LHRH, or potential secretion rate of FSH and LH, does not change in middle-aged rats with PVC, as compared to young, cyclic rats and, therefore, does not seem to be a very important factor in the transition to the (acyclic) PVC-state (Wise & Ratner,1980; Wise,1983). In contrast, transition from the cyclic to the (acyclic) PD-state is accompanied by a reduction in LHRH sensitivity of the pituitary (Howland,1976; Wise & Ratner,1980). However, it cannot be excluded that subtle, as yet unnoticed alterations in blood levels of ovarian hormones are involved in the above mentioned shift in LHRH sensitivity of the pituitary. Howland (1976) concluded that the pituitary of old rats is possibly less sensitive to stimulation by LHRH than that of young rats.

### 3.3.3. Inhibin in the aging female rat.

Involvement of inhibin in the reproductive aging process of female rats supposes the occurrence of alterations with age of the number and rate of growth of antral follicles and/or their production rate of inhibin and, of alterations in the secretion rate of FSH and LH (see previous chapters).

As mentioned earlier, in middle-aged and old female rats the basal level of FSH is elevated and that of LH is reduced. Furthermore, the levels of inhibin and FSH have been shown to be closely and inversely related in late prepuberal and mature rats (chapter 3.1 and 3.2). These two observations, taken together, suggest that in the middle-aged and the old

rat relatively little bioactive inhibin reaches the cells of the anterior pituitary gland, thereby facilitating the already mentioned, increased levels of blood FSH (Butcher & Page,1981; Sopelak & Butcher,1982; Butcher,1985; DePaolo & Rowlands,1986; Steger & Peluso,1987). Recently, DePaolo (1987) reported that at 16.00h on oestrus reduced amounts of inhibin activity were found in OVP of 7 months old rats as compared to 3-months old rats. This was accompanied by increased serum levels of FSH.

We estimated inhibin activity in the ovaries of old (30 months) and young (3-4 months) rats of the Wistar R-Amsterdam strain. Further, the reactivity of primary cultures of anterior pituitary cells of young and old rats to inhibin activity present in the above mentioned ovarian homogenates was compared (unpublished data).

The old rats displayed repeated periods of prolonged di-oestrus and the young rats had shown at least 3 consecutive, 5-day oestrous cycles. From both groups, ovaries, uteri and, pituitaries were obtained during the di-oestrous period and weighed. Subsequently, ovarian homogenates were prepared as described by Sander et al. (1985,1986). Pituitaries were cut in halves, one half was used for estimation of pituitary content of FSH and LH in individual rats, and the other halves were pooled per group and used to establish reactivity of primary cultures of these pituitary cells to

Table 3.3-1. Physical and hormonal data for old (30-32 mo) and young (3-4 mo) female rats.

| | OLD (n=15) | YOUNG (n=12) | RATIO O/Y |
|---|---|---|---|
| Weight: | | | |
| body (g) | 279±5* | 214±5 | 1.3 |
| uterus (mg) | 575±34* | 385±30 | 1.5 |
| ovary (mg) | 75±4 | 68±2 | 1.1 |
| pituitary (mg) | 11.2±1.3* | 5.8±0.3 | 2.0 |
| Serum level : | | | |
| FSH (μg/L) | 382±44* | 116±8 | 3.3 |
| LH (μg/L) | 64±8 | 66±6 | 1.0 |
| Pituitary cont. (x0.5): | | | |
| FSH (mg/pit.) | 18.5±1.7* | 9.9±0.7 | 1.9 |
| LH (mg/pit.) | 346.6±28.1 | 383.2±16.9 | 0.9 |
| Ovarian inhibin cont.: | | | |
| 'old'assay (u/ov. | 250* | 405 | 0.6 |
| + 95% conf.lim.) | (253-337) | (401-530) | |
| 'young'assay (u/ov. | 293 | 461 | 0.6 |
| + 95% conf.lim.) | (163-389) | (272-626) | |
| ratio O/Y assay | 0.85 | 0.88 | |

Summary of the data obtained from experiments comparing old, prolonged di-oestrous rats and young, regularly 5-day cyclic rats. Inhibin content (cont.) of ovarian (ov.) homogenates of either age-group was estimated using two bioassays: one prepared with anterior pituitary cells from the old rats ('old'assay), and the other with cells from the young rats ('young'assay); conf.lim., 95% confidence limits. *, $p<0.05$ as compared to young rats (Student-T test); u, units.

inhibin activity in ovarian homogenates from young and old rats (see: Sander et al.,1984,1985,1986). The results have been summarized in Table 3.3-1. Weights of body, uterus and, pituitary were significantly greater in old than in young rats, while ovarian weights were not different. Levels of FSH in serum and pituitary content of FSH were both significantly higher in old as compared to young rats, while LH was not different. Ovarian content of ILA was significantly lower in old rats as compared to young rats.Also, anterior pituitary cells of old rats appeared to be less sensitive (in vitro) to ILA present in ovaries from old as well as from young rats, than were pituitary cells of young rats.

Because reduced peripheral levels of inhibin will result in increased synthesis and secretion of FSH at the pituitary level, these data on levels of inhibin activity in ovaries from old versus young rats may explain, at least in part, the high values found for pituitary and serum levels of FSH of old rats, as compared to young rats. Interestingly, in the old rats a smaller amount of inhibin was present in ovaries whose weight was not different from that of young rats. This is in concert with the earlier mentioned, reduced number of antral follicles present in the ovaries of old rats (Butcher & Page,1981; Butcher,1985). As mentioned before, DePaolo (1987) reported reduced levels of bioassayable inhibin in OVP of 7-months old rats as compared to 3-4-months old rats.

### 3.3.4. Summary.

In brief, in our opinion the data on the presence of reduced ovarian levels of bioactive inhibin in middle-aged and old rats, available so far, point to an important role for inhibin in (the ovary-resident part of) the process of reproductive aging by way of decreased inhibitory feedback on FSH synthesis and secretion.

This may be achieved via the gradual exhaustion of follicle reserves, leading to reduced numbers of growing, antral follicles, which are the main source of inhibin in the female rat. Consequently, the ovarian content of inhibin is reduced, resulting in reduced levels of inhibin in ovarian venous plasma.

Additionally, the pituitary of aging rats may become less sensitive to inhibin-mediated suppression of FSH synthesis and release, which, together with reduced levels of inhibin in the circulation and reduced oestradiol positive feedback on hypothalamus-mediated FSH secretion, would fully explain the rising serum levels of FSH found in aging rats.

# 4. Overview.

In this chapter an overview will be presented of the role of inhibin in the regulation of (the timing of) sexual maturation, in cyclic reproductive function, and in age-related waning of reproductive capacities in the life of the female laboratory rat.

Existence and a possible function of a non-steroidal, anti-gonadotrophic factor of gonadal origin -now called inhibin- were first suggested and explored in the twenties and thirties but were not generally accepted : feedback on gonadotrophin secretion was thought to be a matter of steroids only. However, research in this field was resumed in the late seventies and in the eighties and it led to findings demonstrating a role of importance of inhibin in reproductive endocrinology, as will be discussed here on the basis of the experiments presented in this thesis, and findings in the literature.

## 4.1. Inhibin assays.

Until now, estimation of levels of inhibin in biological fluids has mainly been performed via in vitro bioassays, using the amount of FSH secreted by anterior pituitary cells in culture as endpoint. Only recently reliable radio-immunoassays (RIA) have been developed (Bicsak, Tucker et al.,1986; Hasegawa, Miyamoto et al.,1986; McLachlan, Robertson et al., 1986; Robertson et al.,1988; see review De Jong,1988). This will facilitate research on inhibin. At present the inhibin-RIA is not yet generally available.

So far, it has been reported that levels of biological- and of immunological inhibin activity in serum show parallel changes, which suggests a rather constant bio-to-immuno (B/I) inhibin ratio in the circulation.

Use of RIA for inhibin may help to further clarify the time dependent relation that exists between inhibin and FSH, especially in those situations where (abrupt) changes in the level of the one are reported without (apparent) complementary temporal changes in the level of the other, as is the case in the late-prepubertal period, in the peri-ovulatory period, and during the post-cyclic or aging phase of reproductive life.

Inhibin from the gonads of all mammalian species studied until now (bovine, human, murine, ovine, porcine), reportedly is a dimeric glycoprotein consisting of an alpha ($\alpha$) and either, a beta-A ($\beta$-A) or, a beta-B ($\beta$-B) subunit (see: De Jong,1988). The $\alpha$ and $\beta$ subunits combine to form two classes of molecules: inhibins (inhibin-A ($\alpha$-$\beta_A$) and -B ($\alpha$-$\beta_B$)) and activins (activin-A ($\beta_A$-$\beta_A$) and -AB ($\beta_A$-$\beta_B$); nomenclature: Burger & Igarashi,1988).

When, in inhibin-RIAs, specific antibodies, raised against (part of) the inhibin-$\alpha$ chain, are used, it is possible to estimate selectively the amount of inhibin that is present, without interference from activin. This is an advantage over bioassays, where the endpoint is the net result of factors suppressing (inhibin) as well as of factors stimulating (e.g. activin) the secretion of FSH by cultured pituitary cells (De Jong,1988).

## 4.2. Inhibin in the female laboratory rat.

### 4.2.1. The female rat during sexual maturation.

After birth, the period of sexual maturation may be divided in four phases.

1). The first phase lasts from birth until the end of the characteristic, prepubertal surge-like increase of gonadotrophins and oestradiol (E2) that reaches peak levels around day 15.

The earliest age that inhibin immunoactivity has been detected in peripheral serum is from day 5 onward, (RIA, Rivier & Vale,1987), whereas no inhibin bioactivity could be detected in ovarian homogenates of 13-day-old rats (Sander et al.,1985). At 17 days of age the blood level of inhibin (RIA) is approximately three-fold higher than at day 5, while the blood level of FSH rises two-fold from day 5 to day 17 (Rivier & Vale,1987). This suggests that, until this age, inhibin may be present in the circulation as a physiologically inactive molecule as far as suppression of blood levels of FSH is concerned.

However, the pituitary of the 15-day-old female rat is moderately susceptible to exogenous inhibin causing suppression of FSH secretion but ovariectomy at this age does not result within 8h in increased blood levels of FSH (Hermans et al.,1980).

From day 5 to day 18 oestradiol-17$\beta$ (E2) may, and does, suppress serum levels of FSH, but, while increasing amounts of E2 are measured in serum, most of it is bound to alpha foeto protein (AFP) and thus physiologically inactive as far as suppression of blood levels of FSH is concerned, explaining why high levels of E2 and of FSH may coexist in rats up to day 18-20 (Meijs-Roelofs & Kramer,1979).

In summary, in immature female rats there seems to be, before the age of 18 days, no clear physiological role of inhibin in regulation of serum levels of FSH.

2). The second prepubertal phase starts at approximately day 18, when the prepubertal peak of FSH (LH and, E2) has been passed and antral follicles (the main source of inhibin in the female rat) occur for the first time. This phase lasts until approximately day 28 (see later). From day 18 on, exogenous inhibin suppresses serum levels of FSH with increasing effectiveness. Also, there is a step-wise increase in the amounts of inhibin in serum around days 18-23 (Rivier & Vale,1987) and in the ovary around days 18-23 and days 28-33, with a rather steady ovarian content from day 23 to 28 (Sander et al.,1985).

Unilateral (ULO) and bilateral ovariectomy after day 18 cause a progressively more pronounced, short-term (at 5h and 8h) increase in the serum level of FSH which, in the case of ULO, has disappeared again at 24h presumably due to the effect of compensatory inhibin production by the remaining ovary, suggesting a physiological role for inhibin at this age (Sander et al.,1985). From day 25 on the effectiveness of inhibin in suppression of pituitary secretion of FSH increases, possibly via increased pituitary sensitivity for inhibin (Hermans et al.,1980). In parallel, after day 20, amounts of E2 and P4 previously capable of maintaining uterine weights in ovariectomized rats, at levels not different from those in intact rats, will no longer maintain physiological concentrations of FSH (Meijs-Roelofs et al.,1981). Thus, again, inhibin as an additional FSH secretion inhibiting factor appears to be physiologically significant.

The presence of inhibin could be demonstrated for the first time in ovarian homogenates of 18-day-old rats. Follicle maturation in the rat is initiated in the neonatal period (Peters & McNatty,1980); growth up to the largest pre-antral stage takes 15-19 days and is rather independent of gonadotrophins (mouse: Peters,1969; rat: Hage, Groen-Klevant & Welschen,1978; Richards,1980). This explains, why, in rats, antral follicles are not found before day 18 (Meijs-Roelofs et al.,1973c) and is in line with the above mentioned absence of follicular, inhibin-mediated regulation of blood levels of FSH before day 18.

Thus, in contrast to the situation during the first post-natal phase, during this second phase inhibin is increasingly present and is involved (together with E2 and P4) in the regulation of FSH secretion.

3). A third phase starts around day 28 and lasts until approximately day -6, i.e. 6 days before the day of first ovulation which takes place at about day 40.

From day 28 to day 33 there is a step-wise increase in ovarian content of inhibin, which is easily explained, since from day 28 on, antral follicles (from which inhibin originates) reach progressively more mature, larger stages and, occasionally, attain the pre-ovulatory stage (Meijs-Roelofs et al.,1973c). The increase in ovarian inhibin content does not seem to be reflected in serum levels of inhibin, as reported by Rivier & Vale (1987). During the remaining days of this phase, after day 33, ovarian content of inhibin remains constant (Sander et al.,1986).

From day 28 to day -6, levels of FSH are still declining steadily, presumably as a result of the combined action of E2 and inhibin.

In summary, the available evidence indicates that during the third phase, the role of inhibin in the regulation of blood levels of FSH is more important than in the previous phase: inhibin is present in larger amounts and the effectiveness of steroids in suppression of FSH declines.

4). Phase 4 starts at day -6, when ovarian inhibin content begins to rise abruptly (see 3.1.4) and lasts until the day of first ovulation.

From day -8 to day -1 (i.e. the day of first pro-oestrus), the follicle growth pattern becomes comparable to that seen in adult rats (Meijs-Roelofs et al.,1982b). This results in an increasing ovarian content of inhibin after day -6, highest on the day of first pro-oestrus (Sander et al.,1986). Changes in inhibin content are significantly correlated with the total volume of follicles of classes III, IV and V, or $\phi > 405$ $\mu$m, but not with smaller follicles, suggesting it is only the larger antral follicles that contribute to cycle-dependent alterations of the total inhibin content. During this period, basal levels of FSH decline and after day -6 increasing levels of inhibin secreted by growing antral follicles ensure a stricter (short-term) regulation of serum FSH concentrations, as is the case during adult cycles, thereby keeping the number of (pre-) ovulatory follicles within the normal range (Sander et al.,1986).

Summarizing the role of inhibin in the immature female rat in the period between birth and the occurrence of first ovulation, it is evident that after day 18 inhibin adds to the action of ovarian steroids, which are no longer sufficient on their own to keep blood levels of FSH in check. Then, from day 28 on, increasing amounts of ovarian inhibin are found as a result of the occurrence of pre-ovulatory follicles, and this contributes progressively to the regulation of blood levels of FSH. Thus, inhibin is

essential in producing the gradual decrease of blood levels of FSH from day 18 until day -6. Additionally, after day -6 until the day of first ovulation, inhibin secreted by growing (pre-)antral follicles is responsible for the tight and precise regulation of levels of FSH and thus for the regulation of a normal number of oocytes to be ovulated.

From the above it is clear that inhibin contributes decisively to normal sexual maturation and a normal (first) ovulation rate.

4.2.2. The adult, cyclic female rat.

The oestrous cycle may be divided into two phases. The first or peri-ovulatory phase is characterized by abrupt changes, both hormonally (fig.3.2-5) and at the ovarian follicular level (ovulation!), and lasts approximately from 12.00h on the day of pro-oestrus until 12.00h on the day of oestrus. The second phase is characterized by more gradual changes, hormonally as well as in follicle growth, and covers the rest of the cycle.

1). During the peri-ovulatory phase, a short, downward plunge of ovarian and blood levels of inhibin takes place between, approximately, 12.00h on the day of pro-oestrus and 12.00h on the day of oestrus (see 3.2.2.1 and fig. 3.2-5).

It is generally known, that the process of ovulation is initiated on the afternoon of pro-oestrus by the LHRH-induced surge of LH (and FSH). The hypothalamus and pituitary have been primed for the occurrence of this surge by gradually rising levels of E2 during the preceding days of the cycle, resulting in a peak on di-oestrous day-3 and the morning of pro-oestrus. Involvement of inhibin in the peri-ovulatory events seems to be as follows. The surge of FSH and LH is followed by a decrease of levels of inhibin in ovarian venous plasma (OVP) from 16.00h on the day of pro-oestrus until 11.00h on the day of oestrus (DePaolo et al.,1979a; Tsukamoto et al.,1986) and, by reduced expression of inhibin messenger RNA in pre-ovulatory follicles at pro-oestrus 24.00h (Woodruff, D'Agostino et al.,1988). Further, ovarian inhibin content decreases abruptly from the morning of pro-oestrus to the morning of oestrus (Sander et al.,1986). Additionally, high doses of LH may suppress inhibin secretion by cultured rat granulosa cells (Zhang, Lee et al.,1988). Thus, the (primary) surge of LH (and FSH) on the day of pro-oestrus may cause reduced synthesis and secretion of inhibin during the afternoon and evening of that day. Subsequently, this results in increasing blood levels of FSH, leading to the second- or oestrous surge of FSH, which may thus be an inhibin-dependent event (for more detailed discussion see 3.2.2.1).

The second (or recruitment-) surge of FSH reaches its maximum around 24.00h on the day of pro-oestrus, whereafter it starts to decline. This decline may possibly result in part from increased suppression of FSH secretion due to rising blood levels of inhibin, derived from at least two sources.

First, during the final phase of ovulation the content of the antra of the ovulating follicles (and thus inhibin) is deposited in the space between the ovary and the bursa ovarica (peri-ovarian space). It is conceivable that lymphatic drainage of the ovarian region (described in the ewe: Morris and Sass,1966) and of the tuba uterina may possibly carry inhibin to the circulation. This suggestion is supported by the finding

that lymphatic drainage of the testis is an important factor for transport of inhibin to the circulation (Eddie et al.,1979). Secondly, secretion of increasing amounts of inhibin to the circulation by the cohort of newly recruited, growing follicles on the day of oestrus, may also contribute to the termination of the surge levels of FSH (DePaolo, Shander et al.,1979b; Tsukamoto et al.,1986).

Large, early atretic follicles may possibly also contribute to ovarian inhibin content and secretion (Kaneko, Taya & Sasamoto,1987), while for newly formed corpora lutea this may (Cuevas, Ying et al.,1987; Merchenthaler, Culler et al.,1987) or may not be the case (Woodruff et al.,1988). Synthesis and secretion of inhibin by other parts of the ovary seem highly unlikely (review: De Jong,1988). Thus, the normal course of the FSH levels during this part of the cycle, seems to be inhibin-dependent.

That the process of ovulation is needed for a normal course of the second surge of FSH, was demonstrated in the hamster by Wang & Greenwald (1987), who retarded this process with cycloheximide at 14.00h on the day of pro-oestrus and did not find a secondary surge of FSH the following day. Since this second surge of FSH is inhibin and not LHRH dependent, the required decrease in serum levels of inhibin apparently did not take place in the absence of the ovulatory process. Inversely, when ovulation is induced in di-oestrous rats via injection of hCG, this results in a peak of blood levels of FSH on the following day (Sasamoto et al.,1981).

Future radio-immuno assay systems enabling precise estimation of inhibin levels in the peripheral circulation should facilitate the interpretation of experimental results such as obtained by Wang & Greenwald (1987).

Taken together, the results indicate an interdependency between, on the one hand, the process of ovulation of the present cycle and, on the other hand, recruitment by the inhibin-dependent, second surge FSH of a new cohort of follicles for the next cycle.

2). The second phase of the oestrous cycle lasts from 12.00h on the day of oestrus until 12.00h on the day of pro-oestrus.Looking for a possible role of inhibin during this phase it should be noted that no dramatic changes in the blood level of FSH are seen here (Butcher, Collins & Fugo,1974; Hermans et al.,1982a), while the cohort of fast growing antral follicles is reduced via two waves of atresia (Osman,1985).

The first wave of atresia occurs between oestrus and di-oestrus day-1, after the oestrous surge of FSH has ended. The second wave occurs between di-oestrus day-3 and pro-oestrus, but no precise data are available yet to support the idea of a direct, cause-effect relation between atresia, inhibin and FSH.

During the second phase, inhibin is synthesized and secreted at a rather constant rate (figure 3.2-5), but the pattern of inhibin concentrations in the circulation is, as yet, unknown. At the same time, the increasing volume of the cohort of growing, antral follicles may be held responsible for increasing amounts of inhibin stored inside the ovary (c.f. sections 3.1.4 and 3.2.2). On pro-oestrus morning, inhibin synthesis and secretion are accelerated, but no correlation with decreasing blood levels of FSH has been reported yet, due to the absence of reliable methods for precise estimation of levels of inhibin in the peripheral circulation.

Summarizing the role of inhibin in the adult rat during the cyclic period, it is clear that inhibin is a key factor for initiation of FSH-

induced follicle recruitment at the start of each oestrous cycle and, that, during the ensuing period of follicle growth, until the primary surge of FSH and LH, it contributes to maintaining blood levels of FSH between narrow limits at a low, basal level.

In this way, during each oestrous cycle inhibin ensures the undisturbed, continuous growth of a normal number of antral follicles up to the point of ovulation once they have been recruited by a surge of FSH.

4.2.3. The aged female rat.

The period of aging, that follows after the cyclic period, is characterized by gradual deterioration of the hormonal mechanism that regulates oestrous cyclicity and ovulation. In aging rats, cyclicity gradually becomes irregular and ends in a state of anoestrus, either via a period of persistent vaginal cornification (PVC) or via PVC followed by prolonged dioestrus (PD), or without intermediate stages (see figure 3.3-1). Thus, aging rats constitute a group with a great variety of "reproductive" processes. Comparable to the cyclic female, a peri-ovulatory (first) phase may be present, but the following (second) phase may be of a very different nature.

During the peri-ovulatory period, the surge level of LH was found to be decreased in 7-9-months-old rats (Van der Schoot,1976; DePaolo & Chappel,1986). At 7 months, the secondary surge of FSH occurred approximately 4h later, while at 9 months the primary surge also was retarded by 4h, and merged with the secondary surge (DePaolo & Chappel,1986). It seems likely therefore, that the ovary-dependent, inhibin regulated part of the secretion of FSH (the second surge) changes prior to the hypothalamus-dependent, LHRH regulated first FSH surge.

Continuing this line of reasoning, it could be that reproductive aging is associated with, either, lower amounts of bioactive inhibin reaching the pituitary (DePaolo,1987), or a diminishing pituitary sensitivity for inhibin-mediated suppression of FSH secretion (section 3.3.3), or a combination of the two.

During the growth-phase (second phase), the reduced number of ovarian follicles (Peluso & Downey,1982; Peluso et al.,1982) causes a smaller ovarian inhibin content (section 3.3.3) and output (DePaolo,1987). The presence of reduced, basal blood levels of LH may possibly also reduce the basal granulosa cell production of inhibin (Zhang et al.,1988). Taken together, these factors may be responsible for the elevated blood levels of FSH found in aging rats (Butcher & Page,1981; DePaolo & Chappel,1986; section 3.3.3). The elevated blood level of FSH is, apparently, unable to induce increased, compensatory ovarian synthesis and release of inhibin. This may be the result of reduced numbers of primary follicles, of relatively inadequate gonadotrophin stimulation of the ovary, or of altered levels of local, ovarian factors which may cause reduced ovarian reactivity following stimulation by gonadotrophins. This is in agreement with reported, deficient compensatory ovarian hypertrophy (COH) following unilateral ovariectomy (ULO) of middle- aged rats (Sopelak & Butcher,1982).

Possibly, reduced sensitivity of the pituitary for inhibin mediated suppression of FSH may also contribute to the rising blood levels of FSH (section 3.3.3).

Some assumptions may be made about the possible role of inhibin in animals that are either in a state of PD or of PVC, based on data from ovarian histology (follicle counts) and blood levels of FSH, but no pertinent inhibin-data have been reported.

During the state of PD, corpora lutea are maintained, while follicle recruitment is halted and follicle growth retarded. Supposedly, this would lead to reduced ovarian secretion of inhibin and, thus, allow for blood levels of FSH to be elevated, which was actually reported by Wise & Ratner (1980). Interestingly, elevated levels of FSH are also found during PVC, a state that is characterized by presence in the ovary of large, persistent pre-ovulatory follicles that continuously secrete E2 (Wise & Ratner,1980) but, apparently, not bioactive inhibin. The idea of reduced ovarian production of inhibin and possibly also of E2 in rats with PVC, as compared to young rats, is substantiated by a smaller rise in plasma FSH (and LH) after ovariectomy (Wise & Ratner,1980), and by incomplete COH at 16 days after ULO, reported by Sopelak & Butcher (1982).

In summary, aging of the reproductive endocrine system of the female rat is accompanied, during the early phase, by significantly decreased levels of inhibin in blood and in the ovary. This points to a role for inhibin in the increase in basal blood levels of FSH as seen during the process of reproductive aging.

No pertinent data are available (yet) that point to a causative role for inhibin in the initiation of reproductive aging. Nonetheless, alterations in inhibin-mediated regulation of periovulatory blood levels of FSH appear to occur earlier than alterations of LHRH-mediated secretion of FSH.

## 5. Summary.

Study of the mechanisms regulating reproduction of female mammals has always been of great interest because of possible quantitative and qualitative control of fecundity (chapter 1). While pituitary gonadotrophins (FSH and LH) and gonadal steroids are the classical hormones regulating reproductive functions, already in 1923 it was suspected, and reinvestigated recently, that another, proteinaceous, hormone of gonadal origin - inhibin (chapter 2) - may play a role as well.

Chapter 3 and Appendix Papers I-VI, report in detail on the role of inhibin in reproductive physiology of the female rat between birth and the end of the reproductive phase of life.

During the period of sexual maturation (Section 3.1), first occurrence of antral follicles around day 18 and of pre-ovulatory follicles after day 28, are closely related to abrupt increases of the ovarian content of inhibin (Appendix Paper III). After day 18, inhibin becomes gradually more effective in suppression of pituitary secretion of FSH, which suggests increasing pituitary sensitivity for inhibin. Thus, after day 18 increased ovarian production of inhibin is associated with increased pituitary sensitivity for the action of inhibin.

Use of accurate timing of developmental age with respect to the day of first ovulation (Appendix Paper I) revealed a significant correlation between ovarian inhibin content and the total volume of antral follicles of class III-V ($\phi$ > 400 $\mu$m) during the last 10 days preceding the day of first ovulation (Appendix Paper IV). During this period, inhibin, in addition to its, above mentioned, general suppressive effect on FSH release after day 18, apparently causes blood levels of FSH to remain within very narrow limits, consequently ensuring an undisturbed, final phase of growth of preovulatory follicles and regulating their number. This suggests that specifically follicles with a diameter > 400 $\mu$m are responsible for (cyclic) alterations of the level of inhibin above a basal value. During this period the mechanism that regulates blood levels of LH also changes, which results in elevated levels in the afternoon from day -5 on (Appendix Paper II).

In summary, during the neonatal period (day 0-7) there seems to be no role for inhibin in the regulation of blood levels of FSH. During the infantile period (day 7-21) the pituitary attains sensitivity for inhibin before the ovaries contain biologically effective inhibin, and gradually the production and role of inhibin in the regulation of blood levels of FSH is established. During the juvenile (day 21-32) period steroids are gradually becoming inadequate in the regulation of blood levels of FSH, while inhibin is gaining significance through increased production and raised effectiveness at the level of the pituitary. In the peripubertal period (day 32 until first ovulation, i.e. approximately day 40) inhibin is becoming notably important in the regulation of specific numbers of ovulatory follicles via a more strict control of blood levels of FSH.

Section 3.2 delineates the role of inhibin in the adult female rat. Ovarian content (Appendix Paper IV), serum levels of inhibin in ovarian vein plasma and synthesis of inhibin in the follicular granulosa cells all change in parallel with the oestrous cycle (although no direct, accurate

measurement is available yet)(Appendix Papers V & VI). This seems to be related to reproductive physiology in the following way.

During the oestrous cycle, inhibin serves two functions. Firstly, during the period of gradual follicle growth (approximately from 12.00h oestrus to 12.00h pro-oestrus), blood levels of FSH are kept at a low, basal level and within narrow limits, via specific short-term negative feedback of inhibin. Thus, inhibin may prevent undue recruitment of additional follicles. A personal experiment is reported which supports the presented concept of the physiological role of inhibin : injection on di-oestrous day-1 of antiserum against inhibin caused an endogenous peak of FSH after 8h and, a significantly increased ovulation rate at the next day of oestrus (section 3.2.1).

Secondly, a rapid decrease of blood (OVP) levels of inhibin is seen after the (primary) surge of LH (and FSH) on pro-oestrous. Consequently, reduced suppression by inhibin of pituitary secretion of FSH takes place, which initiates the event of the second- or oestrous surge of FSH (not LH). Thus, during the peri-ovulatory phase the absence of inhibin induces the secondary surge of FSH and, therefore, inhibin is involved in recruitment of the follicles destined to ovulate at the next oestrus.

In conclusion, during the oestrous cycle, inhibin is an important factor for follicle recruitment, for control of the balance between growth and atresia within a cohort of growing antral follicles and, thus, for regulation of ovulation rate.

The delicate balance between stimulation and suppression of reproductive processes via LHRH, gonadotrophins, steroids and inhibin(s), needed for cyclic reproductive functioning, gradually regresses during the aging process, as described in Section 3.3. Depreciation of the oestradiol-mediated mechanism that induces ovulatory LHRH release appears to be the first sign of reproductive aging. This is soon followed by altered periovulatory surges of LH and FSH, impaired follicle recruitment and follicle growth and irregularly occurring and deficient ovulation. Thus, reduced numbers of large, antral follicles will be present in the ovaries of aged rats, leading to reduced ovarian synthesis and secretion of inhibin, as indicated by smaller ovarian content and blood (OVP) levels of inhibin. As a result, basal blood levels of FSH rise and the secondary surge of FSH appears to be reduced.

Taken together, a clear, but on the whole, secondary role for inhibin in the ovary-resident part of reproductive aging seems present : negative feedback on the pituitary secretion of FSH diminishes. This is mainly due to age-related, progressive reduction of ovarian follicle reserves and inadequate hypothalamic stimulation, leading to decreased numbers of large antral follicles, the main source of ovarian inhibin.

In conclusion, the data presented in this Thesis combined with data from the literature (Chapter 4), indicate that inhibin has an important role to play in the endocrine mechanism that regulates reproduction in the female rat. During the period of sexual maturation, the physiological role of inhibin gradually emerges around day 18 and, is obvious from day 23 onwards. However, its most important function is in the "cycle" that precedes first ovulation (peri-pubertal period) and, during ensuing, adult, cyclic reproductive functioning. Finally, in the aging female rat the capability to reproduce is gradually lost, and, in parallel, the role of inhibin wanes.

Therefore, during each reproductive stage of life, inhibin levels in the ovary are closely related to the ovarian follicle population, and, the role it plays may be deducted from blood levels of FSH. More accurate data on levels of inhibin will further clarify its role, but must await the general availability of an inhibin RIA.

This warrants emphasis on inhibin in the further study of reproductive physiology and its disorders.

## References.

Adashi,E.Y. & Hsueh,A.J.W. (1982). Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells. Journal of Biological Chemistry 257, 6077-6083

Aiyer,M.S., Fink,G. & Greig,F. (1974). Changes in the sensitivity of the pituitary gland to luteinizing hormone-releasing factor during the oestrous cycle of the rat. Journal of Endocrinology 60, 47-64

Akema,T., Mitsugi,N., Tadokoro,Y. & Kimura,F. (1985). Differential changes in the mechanisms controlling luteinizing hormone and prolactin secretion in middle-aged female rats. Endocrinologica Japonica 32, 153-161

Albrecht,E.D. (1984). Pregnancy in young and aged rats. I. Ovarian progesterone and 20α-hydroxypregn-4-en-3-one formation. Endocrinology 115, 1418-1425

Apfelbaum,M.E. (1981). Basal and GnRH-induced release and synthesis of LH and FSH from incubated pituitary glands throughout the 4-day estrous cycle of the rat. Hormone Research 15, 109-121

Aschheim,P. (1976). Aging in the hypothalamic-hypophyseal ovarian axis in the rat. In: Hypothalamus, pituitary and aging. Editors: A.V.Everitt & J.A.Burgess. Springfield, Illinois, U.S.A.:Charles C.Thomas. pp.376-418.

Aschheim,P. (1983). Relation of the neuroendocrine system to reproductive decline in female rats. In: Neuroendocrinology and aging. Editor:J.Meites. New York:Plenum Press. pp.73-101

Asdell,S.A. (1962). Mechanism of ovulation. In: The ovary. Editor: S.Zuckerman. London:Academic Press. p.436.

Babu,G.N., Bona-Gallo,A. & Gallo,R.V. (1986). Interaction between estradiol and a nonsteroidal factor in porcine follicular fluid in regulating LH pulse amplitude between the mornings of diestrus-2 and proestrus in the rat. Neuroendocrinology 44, 8-14

Babu,G.N., Marco,J., Bona-Gallo,A. & Gallo,R.V. (1987). Steroid-independent endogenous opioid peptide suppression of pulsatile luteinizing hormone release between estrus and diestrus-1 in the rat estrous cycle. Brain Research 416, 235-242

Beers,W.H. (1975). Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell 6, 379-386

Berardo,P.V. & DePaolo,L.V. (1986). Different neuroendocrine mechanisms regulate the acute pituitary follicle-stimulating hormone response to orchidectomy and ovariectomy. Neuroendocrinology 43, 511-518

Bhanot,R. & Wilkinson,M. (1984). The inhibitory effect of opiates on gonadotrophin secretion is dependent upon gonadal steroids. Journal of Endocrinology 102, 133-141

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

Blake,C.A., Elias,K.A. & Huffman,L.J. (1983). Ovariectomy of young adult rats has a sparing effect on the ability of aged rats to release luteinizing hormone. Biology of Reproduction 28, 575-585

Blank,M.S., Panerai,A.E. & Friesen,H.G. (1979). Opioid peptides modulate luteinizing hormone secretion during sexual maturation. Science 203, 1129-1131

Bortz,W.M. (1986). Aging as entropy. Experimental Gerontology 31, 321-328

Brawer,J.R., Schipper,H. & Naftolin,F. (1980). Ovary dependent degeneration in the hypothalamic arcuate nucleus. Endocrinology 107, 274-279

Brody,J.A. & Schneider,E.L. (1986). Diseases and disorders of aging: an hypothesis. Journal of Chronic Diseases 39, 871-876

Burger,H.G. & Igarashi,M. (1988). Inhibin: definition and nomenclature, including related substances. Endocrinology 122, 1701-1702

Butcher,R.L. (1977). Changes in gonadotropins and steroids associated with unilateral ovariectomy of the rat. Endocrinology 101, 830-840

Butcher,R.L. (1985). Effect of reduced ovarian tissue on cyclicity, basal hormonal levels and follicular development in old rats. Biology of Reproduction 32, 315-321

Butcher,R.L. & Page,R.D. (1981). Role of the aging ovary in cessation of reproduction. In: Dynamics of ovarian function. Editors: N.B.Schwartz and M.Hunzicker-Dunn. New York:Raven Press. pp.253-271

Butcher,R.L., Collins,W.L. & Fugo,N.W. (1974). Plasma concentrations of LH, FSH, prolactin, progesterone and estradiol-17-$\beta$ throughout the 4-day estrous cycle of the rat. Endocrinology 94, 1704-1708

Callard,G.V. (1983). Androgen and estrogen actions in the vertebrate brain. American Zoologist 23, 607-620

Campbell,C.S. & Schwartz,N.B. (1977). Steroid feedback regulation of luteinizing hormone and follicle-stimulating hormone secretion rates in male and female rats. Journal of Toxicology and Environmental Health 3, 61-95

Channing,C.P., Gordon,W.L., Liu,W.K. & Ward,D.N. (1985). Physiology and biochemistry of ovarian inhibin. Proceedings of the Society for Experimental Biology and Medicine 178, 339-361

Channing,C.P., Tanabe,K., Turner,C.K. & Hodgen,G.D. (1982). Antisera to porcine follicular fluid in monkeys : neutralization of human and pig inhibin activity in vivo and in vitro. Journal of Clinical Endocrinology and Metabolism 55, 481-486

Chappel,S.C. (1980). Restoration of pro-estrous and estrous release of gonadotropins in ovariectomized hamsters: a possible role for inhibin in the female. Biology of Reproduction 22, 798-804

Chappel,S.C. (1985). Neuroendocrine regulation of luteinizing hormone and follicle stimulating hormone: a review. Life Sciences 36, 97-103

Charlesworth,M.C., Grady,R.R., Shin,L., Vale,W.W., Rivier,C., Rivier,J. & Schwartz,N.B. (1984).Differential suppression of FSH and LH secretion by follicular fluid in the presence or absence of GnRH. Neuroendocrinology 38, 199-205

Costoff,A. (1985). Effects of estradiol and estradiol-progesterone treatment on gonadotropin secretion in castrate aging female rats. Maturitas 7, 351-365

Critchlow,V. & Bar-Sela,M.E. (1967). Control of the onset of puberty. In: Neuroendocrinology, volume 2. Editors: L.Martini & W.F.Ganong. New York, London:Academic Press. pp.101-162

Cuevas,P., Ying,S.-Y., Ling,N., Ueno,N., Esch,F. & Guillemin,R. (1987). Immunohistochemical detection of inhibin in the gonad. Biochemical and Biophysical Research Communications 142, 23-30

Cummins,L.J., O'Shea,T., Al-Obaidi,S.A.R., Bindon,B.M. & Findlay,J.K. (1986). Increase in ovulation rate after immunization of merino ewes with a fraction of bovine follicular fluid containing inhibin activity. Journal of Reproduction and Fertility 77, 365-372

David,K., Dingemanse,E., Freud,J. & Laqueur,E. (1935). Über krystallinisches männliches hormon aus hoden (testosteron), wirksamer als aus harn oder aus cholesterin bereitetes androsteron. Biological Chemistry Hoppe-Seyler 233, 281-282

Davis,S.R., Dench,F., Nikolaidis,I., Clements,J.A., Forage,R.G., Krozowsky,Z. & Burger,H.G. (1986). Inhibin-A subunit gene expression in the ovaries of immature female rats is stimulated by pregnant mare serum gonadotrophin. Biochemical and Biophysical Research Communications 138, 1191-1195

De Greef,W.J., Eilers,G.A.M., De Koning,J., Karels,B. & De Jong,F.H. (1987). Effects of ovarian inhibin on pulsatile release of gonadotrophins and secretion of LHRH in ovariectomized rats : evidence against a central role of action of inhibin. Journal of Endocrinology 113, 449-455.

De Jong,F,H. (1988). Inhibin. Physiological Reviews 68, 555-607

De Jong,F.H. & Robertson,D.M. (1985a). Inhibin: 1985 update on action and purification. Molecular and Cellular Endocrinology 42, 95-103

De Jong,F.H. & Sharpe,R.M. (1976). Evidence for inhibin-like activity in bovine follicular fluid. Nature 263, 71-71

De Jong,F.H. & Sharpe,R.M. (1977). Gonadotrophins, testosterone and spermatogenesis in neonatally irradiated male rats: evidence for a role for the sertoli cell in follicle-stimulating hormone feedback. Journal of Endocrinology 75, 209-219

De Jong,F.H., Hey,A.H. & Van der Molen,H.J. (1975). Effect of gonadotrophins on the secretion of oestradiol-17 beta and testosterone by the rat testis. Journal of Endocrinology 57, 277- 284

De Jong,F.H., Jansen,E.H.J.M. & Van der Molen,H.J. (1981). Purification and characterization of inhibin. In: Intragonadal regulation of reproduction. Editors: P.Franchimont & S.J.Segal. London, Academic Press. pp.229-250

De Jong,F.H., Sander,H.J., Ultee-Van Gessel,A.M. & Van der Molen,H.J. (1985b). Specific regulation of the secretion of follicle-stimulating hormone from the pituitary gland: the inhibin concept. In: Regulation of pituitary function. Editors: T.B. Van Wimersma-Greidanus & S.W.J.Lamberts. Basel, Karger. pp.54-69

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

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

DePaolo,L.V. (1987). Age-associated increases in serum follicle-stimulating hormone levels on estrus are accompanied by a reduction in the ovarian secretion of inhibin. Experimental Aging Research 13, 3-7

DePaolo,L.V. & Chappel,S.C. (1986). Alterations in the secretion and production of follicle-stimulating hormone precede age- related lengthening of estrous cycles in the rat. Endocrinology 118, 1127-1133

DePaolo,L.V. & Rowlands,K.L. (1986). Deceleration of age-associated changes in the preovulatory but not secondary follicle-stimulating hormone surge by progesterone. Biology of Reproduction 35, 320-326

DePaolo,L.V., Anderson,L.D. & Hirshfield,A.N. (1981). Possible existence of a long-loop feedback system between FSH and inhibin in female rats. American Journal of Physiology 240, E544-549

DePaolo,L.V., Hirschfield,A.N., Anderson,L.D., Barraclough,C.A. & Channing,C.P. (1979a). Suppression of pituitary secretion of follicle-stimulating hormone by porcine follicular fluid during pro-oestrus and oestrus in the rat: effects on gonadotrophin and steroid secretion, follicular development and ovulation during the following cycle. Journal of Endocrinology 83, 355-368

DePaolo,L.V., Shander,D.S., Wise,P.M., Barraclough,C.A. & Channing,C.P. (1979b). Identification of inhibin-like activity in ovarian venous plasma of rats during the estrous cycle. Endocrinology 105, 647-654

DePaolo,L.V., Wise,P.M., Anderson,L.D., Barraclough,C.A. & Channing,C.P. (1979c). Suppression of the pituitary FSH secretion during pro-estrus and estrus in rats by porcine follicular fluid: possible site of action. Endocrinology 104, 402-408

Donham,R.S., Creyaufmiller,N., Lyons,T.J. & Stetson,M.H. (1985). Temporal relationships between the onset of daily gonadotrophin surges and of puberty in the female golden hamster. Journal of Endocrinology 108, 219-224

Donovan,B.T. (1966). The regulation of the secretion of follicle-stimulating hormone. In: The pituitary gland. Editors: G.W.Harris & B.T.Donovan. London:Butterworths. pp.49-98.

Dorrington,J.H. & Armstrong,D.T. (1979). Effects of fsh on gonadal functions. Recent Progress in Hormone Research 35, 301-341

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

Erickson,G.F. & Hsueh,A.J.W. (1978). Secretion of "inhibin" by rat granulosa cells in vitro. Endocrinology 103, 1960-1963

Erickson,G.F., Magoffin,D.A., Dyer,C.A. & Hofeditz,C. (1985). The ovarian androgen producing cells: a review of structure/function relationships. Endocrine Reviews 6, 371-399

Espey,L.L. (1980). Ovulation as an inflammatory reaction - a hypothesis. Biology of Reproduction 22, 73-106

Evans,H.M., Simpson,M.E., Tolksdorf,S. & Jensen,H. (1939). Biological studies of the gonadotropic principles in sheep pituitary substances. Endocrinology 25, 529-546

Everett,J.W., Sawyer,C.H. & Markee,J.E. (1949). A neurogenic timing factor in control of the ovulatory discharge of luteinizing hormone in the cycling rat. Endocrinology 44, 234-250

Felicio,L.S., Nelson,J.F. & Finch,C.E. (1984). Longitudinal studies of estrous cyclicity in aging c57bl/6j mice: ii cessation of cyclicity and the duration of persistent vaginal cornification. Biology of Reproduction. 31, 446-453

Finch,C.E. & Mobbs,C.V. (1983). Hormonal influences on hypothalamic sensitivity during aging in female rodents. In:Neuroendocrinology and aging. Editor: J.Meites. New York:Plenum Press. pp.143-171

Finch,C.E., Felicio,L.S., Mobbs,C.V. & Nelson,J.F. (1984). Ovarian and steroidal influences on neuroendocrine aging processes in female rodents. Endocrine Reviews 5, 467-497

Findlay,J.K. (1986). The nature of inhibin and its use in the regulation of fertility and diagnosis of infertility. Fertility and Sterility 46, 770-783

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

Franchimont ,P., Chari,S., Hagelstein,M.T. & Duraiswami,S. (1975). Existence of a follicle-stimulating hormone inhibiting factor "inhibin" in bull seminal plasma. Nature 275, 402-404

Fujii,T., Hoover,D.J. & Channing,C.P. (1983). Changes in inhibin activity, and progesterone, oestrogen and androstenedione concentrations, in rat follicular fluid throughout the oestrous cycle. Journal of Reproduction and Fertility 69, 307-314

Fukuda,M., Miyamoto,K., Hasegawa,Y., Nomura,M., Igarashi,M., Kangawa,K. & Matsuo,H. (1986). Isolation of bovine follicular fluid inhibin of about 32 kda. Molecular and Cellular Endocrinology 44, 55-60

Greenstein,B.D. & Adcock,I.M. (1985). Role of steroid hormone receptors in development and puberty. In:The receptors, Vol.II. New York, Academic Press. pp.341-366

Greep,R.O., Van Dyke,H.B. & Chow,B.F. (1941). Use of the anterior lobe of the prostate gland in the assay of metakentrin. Proceedings of the Society for Experimental Biology, New York. 46, 644-649

Hage,A.J., Groen-Klevant,A.C. & Welschen,R. (1978). Follicle growth in the immature rat ovary. Acta Endocrinologica 88, 375-382

Harlow,C.R., Hearn,J.P. & Hodges,J.K. (1984). Ovulation in the marmoset monkey - endocrinology, prediction and detection. Journal of Endocrinology 103, 17-24

Harris,G.W. (1955). Neural control of the pituitary gland. London:Edward Arnold.

Hasegawa,Y., Miyamoto,K., Fukuda,Y., Takahashi,Y. & Igarashi,M. (1986). Immunological study of ovarian inhibin. Endocrinologica Japonica 33, 645-654

Hasegawa,Y., Miyamoto,K., Yazaki,C. & Igarashi,M. (1981). Regulation of the second surge of follicle-stimulating hormone; effects of anti-luteinizing hormone-releasing hormone serum and pentobarbital. Endocrinology 109, 130-135

Hashimoto,I., Isomoto,N., Eto,M., Kawaminami,M., Sunazaka,C. & Ueki,N. (1987). Preovulatory secretion of progesterone, luteinizing hormone, and prolactin in 4-day and 5-day cycling rats. Biology of Reproduction 36, 599-605

Hayflick,L. (1985). Theories of biological aging. Experimental Gerontology 20, 145-159

Heller,C.G. & Nelson,W.O. (1948). The testis pituitary relationship in man. Recent Progress in Hormone Research 3, 229-255

Henderson,K.M., Franchimont,P., Lecomte-Yerna,P., Hudson,N. & Ball,K. (1984). Increase in ovulation rate after active immunization of sheep with inhibin partially purified from bovine follicular fluid. Journal of Endocrinology 102, 305-309

Henderson,K.M., Prisk,M.D., Hudson,N., Ball,K., McNatty,K.P., Lun,S., Heath,D., Kieboom,L.E. & McDiarmid,J. (1986). Use of bovine follicular fluid to increase ovulation rate or prevent ovulation in sheep. Journal of Reproduction and Fertility 76, 623-635

Hermans,W.P. (1982). Inhibin, with an old concept towards a new approach in reproductive physiology. Ph.D.Thesis. Rotterdam, Erasmus University.

Hermans,W.P., Debets,M.H.M., Van Leeuwen,E.C.M. & Jong,F.H.de. (1981). Time-related secretion of gonadotrophins after a single injection of steroid-free bovine follicular fluid in prepubertal and adult female rats. Journal of Endocrinology 90, 69-76

Hermans,W.P., Debets,M.H.M., Van Leeuwen,E.C.M. & De Jong,F.H. (1982a). Effects of single injections of bovine follicular fluid on gonadotropin concentrations throughout the oestrous cycle of the rat. Journal of Endocrinology 92, 425-432

Hermans,W.P., De Jong,F.H. & Welschen,R. (1981). The possible role of ovarian inhibin-like activity in the regulation of peripheral concentrations of follicle-stimulating hormone. In: Functional morphology of the human ovary, Editor: J.R.T.Coutts. Lancaster, England:MTP-Press Ltd. pp.85-93

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

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

Hillier,S.G. (1981). Regulation of follicular oestrogen synthesis: a survey of current concepts. Journal of Endocrinology 89, 3P-18P

Hirshfield,A.N. (1981). Role of FSH in the selection of ovulatory follicles. In: Dynamics of ovarian function. Editors: N.B.Schwarz & M.Hunzicker-Dunn. New York:Raven Press. pp.79-82.

Hirshfield,A.N. (1985). Comparison of granulosa cell proliferation in small follicles of hypophysectomized, prepubertal, and mature rats. Biology of Reproduction 32, 979-987

Hirshfield,A.N. (1986). Effect of a low dose of pregnant mare's serum gonadotropin on follicular recruitment and atresia in cycling rats. Biology of Reproduction 35, 113-118

Hirshfield,A.N. & Midgley,A.R. (1978). Morphometric analysis of follicular development in the rat. Biology of Reproduction. 19, 597-605

Hoak,D.C. & Schwartz,N.B. (1980). Blockade of recruitment of ovarian follicles by suppression of the secondary surge of follicle-stimulating hormone with porcine follicular fluid. Proceedings of the National Academy of Sciences, U.S.A. 77, 4953-4956

Holehan,A.M. & Merry,B.J. (1986). The experimental manipulation of ageing by diet. Biological Reviews 61, 329-368

Howard,R,P.,Sniffen,R,C.,Simmons,F,A.,Albright,F. (1950). Testicular deficiency: a clinical and pathologic study. The Journal of Clinical Endocrinology 10, 151-186

Howland,B.E. (1976). Reduced gonadotropin release in response to progesterone or gonadotropin releasing hormone (GnRH) in old female rats. Life Sciences 19, 219-224

Howland,B.E. & Skinner,K.R. (1973). Effect of hemiovariectomy on serum FSH and LH levels during the oestrous cycle in the rat. Journal of Reproduction and Fertility 32, 501-503

Hsueh,A.J.W., Dahl,K.D., Vaughan,J., Tucker,E., Rivier,J., Bardin,C.W. & Vale,W. (1987). Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. Proceedings of the National Academy of Sciences, U.S.A. 84, 5082-5086

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

Hurkadli,K.S. & Sheth,A.R. (1985). Studies on immunoneutralization of inhibin : a time-course study. Indian Journal of Experimental Biology 23, 561-565

Jarrell,J., Younglai,E.V., Barr,R., O'Connell,G., Belbeck,L. & McMahon,A. (1986). An analysis of the effects of increasing doses of ionizing radiation to the exteriorized rat ovary on follicular development, atresia, and serum gonadotropin levels. American Journal of Obstetrics and Gynecology 154, 306-309

Jenner,A.A.J., De Koning,J. & Rees,G.P.van. (1982). Time course of inhibition of synthesis and basal release of fsh from pituitary glands of female rats by bovine follicular fluid in vitro. Acta Endocrinologica 101, 501-506

Jia,X.-C. & Hsueh.A.J.W. (1985). Sensitive in vitro bioassay for the measurement of follicle-stimulating hormone. Neuroendocrinology 41, 445-448

Kalra,S.P. & Kalra,P.S. (1974). Temporal interrelationships among circulating levels of estradiol, progesterone and LH during the rat estrous cycle: effects of exogenous progesterone. Endocrinology 95, 1711-1717

Kaneko,H.,Taya,K. & Sasamoto,S. (1987). Changes in the secretion of inhibin and steroid hormones during induced follicular atresia after hypophysectomy in the rat. Life Sciences 41, 1823-1830

Karsch,F.J. (1987). Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. Annual Reviews of Physiology 49, 365-382

Kimura,J. Katch,K., Taya,K. & Sasamoto,S. (1983). An inverse relationship between inhibin and follicle stimulating hormone during the period of ovulation induced by human chorionic gonadotrophin in dioestrous rats. Journal of Endocrinology 79, 313-318

Krishna,E. & Terranova,P.F. (1987). Effect of the gonadotrophin surge at pro-oestrus on steroidogenesis and gonadotrophin binding of preantral follicles in the cyclic hamster. Journal of Endocrinology 114, 49-54

Labrie,F., Drouin,J., Ferland,L., Lagac,L., Beaulieu,M., De Lean,A., Kelly,P.A., Caron,M.G. & Raymond,V. (1978). Mechanism of action of hypothalamic hormones in the anterior pituitary gland and specific modulation of their activity by sex steroids and thyroid hormones. Recent Progress in Hormone Research 34, 25-93

Lacker,H.M., Beers,W.H., Meuli,L,E. & Akin,E. (1987). A theory of follicle selection: II. Computer simulation of estradiol administration in the primate. Biology of Reproduction 37, 581-588

LaPolt,P.S., Matt,D.W., Judd,H.L. & Lu,J.K.H. (1986). The relation of ovarian steroid levels in young female rats to subsequent estrous cyclicity and reproductive function during aging. Biology of Reproduction. 35, 1131-1139

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

Leipheimer,R.E., Bona-Gallo,A. & Gallo,R.V. (1984). The influence of progesterone and estradiol on the acute changes in pulsatile luteinizing hormone release induced by ovariectomy on diestrous day 1 in the rat. Endocrinology 114, 1605-1612

Leipheimer,R,E.,Bona-Gallo,A. & Gallo,R,V. (1985). Ovarian steroid regulation of pulsatile luteinizing hormone release during the interval between the mornings of diestrus-2 and proestrus in the rat. Neuroendocrinology 41, 252-257

Leipheimer,R.E., Bona-Gallo,A. & Gallo,R.V. (1986). Ovarian steroid regulation of basal pulsatile luteinizing hormone release between the mornings of proestrus and estrus in the rat. Endocrinology 118, 2083-2090

Li,C.H. & Ramasharma,K. (1987). Inhibin. Annual Reviews of Pharmacology and Toxicology 27, 155-217

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

Lintern-Moore,S. & Moore,G.P.M. (1979). The initiation of follicle and oocyte growth in the mouse ovary. Biology of Reproduction. 20, 773-778

Long,J.A. & Evans,H.M. (1922). The estrous cycle of the rat and its associated phenomena. Memoirs of the University of California 5, 5-148

Lorenzen,J.R. (1981). The development of sexual maturity in the rat: an alternate hypothesis. In: Dynamics of Ovarian Function. Editors: N.B.Schwartz and M.Hunzicker-Dunn. New York:Raven Press. pp.273-278

Lorenzen,J.R. & Schwartz,N.B. (1979). The differential ability of porcine follicular fluid to suppress FSH in female rats from 6 days of age to adulthood. In: Advances of Experimental Biology and Medicine, vol.112: Ovarian follicular and corpus luteum function. Editors: C.P.Channing, J.Marsh & W.A.Sadler. London:Plenum Press. pp.375-380

Lu,J.K.H. (1983). Changes in ovarian function and gonadotropin and prolactin secretion in aging female rats. In: Neuroendocrinology and aging, Editor: J.Meites. New York:Plenum Press. pp.103-122

Lu,J.K.H., Lapolt,P.S., Nass,T.E., Matt,D.W. & Judd,H.L. (1985). Relation of circulating estradiol and progesterone to gonadotropin secretion and estrous cyclicity in aging female rats. Endocrinology 116, 1953-1959

Lu,K.H., Hopper,B.R., Vargo,T.M. & Yen,S.S.C. (1979). Chronological changes in sex steroid, gonadotropin and prolactin secretion in aging female rats displaying different reproductive states. Biology of Reproduction 21, 193-203

Lumpkin,M.D., DePaolo,L.V. & Negro-Vilar,A. (1984). Pulsatile release of follicle-stimulating hormone in ovariectomized rats is inhibited by porcine follicular fluid (inhibin). Endocrinology 114, 201-206

Mandl,A,M. (1964). Superovulation following ovarian X-irradiation. Journal of Reproduction and Fertility 8, 375-396

Mandl,A.M. & Zuckerman,S. (1952). Cyclical changes in the number of medium and large follicles in the adult rat ovary. Journal of Endocrinology 8, 341-346

Martins,T. & Rocha,A. (1931). The regulation of the hypophysis by the testicle, and some problems of sexual dynamics. Endocrinology 15, 421-434

Mason,A.J., Hayflick,J.S., Ling,N., Esch,F., Ueno,N., Ying,S-Y., Guillemin,R., Niall,H. & Seeburg,P.H. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and sequence homology with transforming growth factor-$\beta$. Nature 318, 659-663

Masoro,E.J. (1987). Biology of aging. Archives of Internal Medicine 147, 166-169

Matt,D.W., Lee,J., Sarver,P.L., Judd,H.L. & Lu,J.K.H. (1986). Chronological changes in fertility, fecundity and steroid hormone secretion during consecutive pregnancies in aging rats. Biology of Reproduction. 34, 478-487

Matt,D.W., Sarver,P.L. & Lu,J,K,H. (1987). Relation of parity and estrous cyclicity to the biology of pregnancy in aging female rats. Biology of Reproduction. 37, 421-430

McCann,S.M., Mizunuma,H., Samson,W.K. & Lumpkin,M.D. (1983). Differential hypothalamic control of fsh secretion: a review. Psychoneuroendocrinology 8, 299-308

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

McCullagh,R. & Schneider,I. (1940). The effect of a non-androgenic testis extract on the estrous cycle in rats. Endocrinology 27, 899-902

McLachlan,R.I., Robertson,D.M., Burger,H.G. & De Kretser,D.M. (1986). The radioimmunoassay of bovine and human follicular fluid and serum inhibin. Molecular and Cellular Endocrinology 46, 175-185

Meijs-Roelofs,H.M.A. (1972). Effect of electrical stimulation of the hypothalamus on gonadotrophin release and the onset of puberty. Journal of Endocrinology 54, 277-284

Meijs-Roelofs,H.M.A. & Kramer,P. (1979). Maturation of the inhibitory feedback action of estrogen on follicle-stimulating hormone secretion in the immature female rat: a role for alpha-foetoprotein. Journal of Endocrinology 81, 199-208

Meijs-Roelofs,H.M.A. Kramer,P. & Gribling-Hegge,L. (1981). Role of progesterone in the control of secretion of follicle-stimulating hormone in the immature female rat. Journal of Endocrinology 89, 99-106

Meijs-Roelofs,H.M.A., Kramer,P. & Gribling-Hegge,L. (1982a). Possible role of 5$\alpha$-androstane-3$\alpha$,17$\beta$-diol in the control of follicle-stimulating hormone secretion in the immature female rat. Journal of Endocrinology 92, 37-42

Meijs-Roelofs,H.M.A., Kramer,P. & Osman,P. (1985). Precocious ovulation induced by human chorionic gonadotrophin in the immature female rat: comparison of follicle growth induced by treatment with human chorionic gonadotrophin and by electrical stimulation of the hypothalamus. Journal of Endocrinology 106, 61-66

Meijs-Roelofs,H.M.A., Kramer,P. & Sander,H.J. (1983). Changes in serum concentration of luteinizing hormone in the female rat approaching puberty. Journal of Endocrinology 98, 241-249

Meijs-Roelofs,H.M.A., Kramer,P., Osman,P & Sander,H.J. (1984). Compensatory ovulatory mechanisms operative after first ovulation in rats unilaterally ovariectomized prepubertally. Biology of Reproduction 31, 44-51

Meijs-Roelofs,H.M.A., Kramer,P., Van Cappellen,W.A. & Schuiling,G.A. (1987). Inhibition of first ovulation: administration of an LHRH antagonist to immature female rats. Journal of Endocrinology 112, 407-415

Meijs-Roelofs,H.M.A., Osman,P. & Kramer,P. (1982b). Ovarian follicular development leading to first ovulation and accompanying gonadotrophin levels as studied in the unilaterally ovariectomized rat. Journal of Endocrinology 92, 341-349

Meijs-Roelofs,H.M.A., Uilenbroek,J.Th.J., De Greef,W.J., De Jong,F.H. & Kramer,P. (1975). Gonadotrophin and steroid levels around the time of first ovulation in the rat. Journal of Endocrinology 67, 275-282

Meijs-Roelofs,H.M.A., Uilenbroek,J.Th.J., De Jong,F.H. & Welschen,R.W. (1973b). Plasma concentrations of oestradiol-17α and serum levels of follicle stimulating hormone in the immature female rat. Annales de Biologie animale, Biochimie, Biophysique 13, 197-198

Meijs-Roelofs,H.M.A., Uilenbroek.J.Th.J., De Jong,F.H. & Welschen,R. (1973a). Plasma oestradiol-17α and its relationship to serum follicle-stimulating hormone in immature female rats. Journal of Endocrinology 59, 295-304

Meijs-Roelofs,H.M.A., Uilenbroek,J.Th.J., Osman,P. & Welschen,R. (1973c). Serum levels of gonadotrophins and follicular growth in prepubertal rats. In: The development and maturation of the ovary and its functions; Editor: H.Peters. Amsterdam:Excerpta Medica. pp.3-11

Meites,J., Goya,R. & Takahashi,S. (1987). Why the neuroendocrine system is important in aging processes. Experimental Gerontology 22, 1-15

Meites.J. & Huang,H.H. (1976). Relation of neuroendocrine system to loss of reproductive functions in aging rats. In: Neuroendocrine regulation of fertility. Editor: A.Kumar. Basel:Karger. pp.246-258

Merchenthaler,I., Culler,M.D., Petrusz,P. & Negro-Vilar,A. (1987). Immunocytochemical localization of inhibin in rat and human reproductive tissues. Molecular and Cellular Endocrinology 54, 239-243

Midgley,A.R. (1969). Radioimmunoassay employing double antibody techniques. In: Karolinska symposium on immunoassay of gonadotropins, Editor: E.Diczfalusy. Copenhagen, Bogtrykkeriet Forum. pp.247-256

Miyamoto,K., Hasegawa,Y., Fukuda,M. & Igarashi,M. (1986). Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) using monoclonal antibodies to bFF 32 k inhibin. Biochemical and Biophysical Research Communications 136, 1103-1109

Miyamoto,K., Hasegawa,Y., Fukuda,M., Nomura,M., Igarashi,M., Kangawa,K. & Matsuo,H. (1985). Isolation of porcine follicular fluid inhibin of 32k daltons. Biochemical and Biophysical Research Communications 129, 396-403

Moon,Y.S. & Duleba,A.J. (1982). Comparative studies of androgen metabolism in theca and granulosa cells of human follicles in vitro. Steroids 39, 419-430

Morris,B. & Sass,M.B. (1966). The formation of lymph in the ovary. Proceedings of the Royal Society for Biology 164, 577-591

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

Nass,T.E., LaPolt,P.S., Judd,H.L. & Lu,J.K.H. (1983). Alterations in ovarian steroid and gonadotrophin secretion preceding the cessation of regular oestrous cycles in aging female rats. Journal of Endocrinology 100, 43-50

Nelson,W.O. (1934). Effect of oestrin and gonadotropic hormone injections upon hypophysis of the adult rat. Proceedings of the Society for Experimental Biology and Medicine 32, 452-454

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

Nequin,L.G., Alvarez,J. & Schwartz,N.B. (1979). Measurement of serum steroid and gonadotropin levels and uterine and ovarian variables throughout 4-day and 5-day estrous cycles in the rat. Biology of Reproduction. 20, 659-670

Ojeda,S.R., Andrews,W.W., Advis,J.P. & Smith-White,S. (1980). Recent advances in the endocrinology of puberty. Endocrine Reviews 1, 228-257

Ojeda,S.R., Smith,S.S., Urbanski,H.F. & Aguado,L.I. (1984). The onset of female puberty: underlying neuroendocrine mechanisms. In: Neuroendocrine perspectives, vol.3. Editors: E.E.Müller & R.M.Mc Leod. Amsterdam:Elsevier Science Publishers. pp.225-278

Ojeda,S.R., Urbanski,H.F. & Ahmed,C.E. (1986). The onset of female puberty: studies in the rat. Recent Progress in Hormone Research 42, 385-442

Osman,P. (1975). Preovulatory changes in the ovaries during the first spontaneous pro-oestrus in the rat. Journal of Endocrinology 67, 259-265

Osman,P. (1977). Ovarian changes during the first spontaneous ovulation in intact indomethacin treated rats. Endokrinologie 69, 351-354

Osman,P. (1985). Rate and course of atresia during follicular development in the adult cyclic rat. Journal of Reproduction and Fertility 73, 261-270

Osman,P. & Dullaart,J. (1976). Intraovarian release of eggs in the rat after indomethacin treatment at pro-oestrus. Journal of Reproduction and Fertility 47, 101-103

Osman,P. & Meijs-Roelofs,H.M.A. (1976). Effects of sodium pentbarbitone administration on gonadotrophin release, first ovulation, and ovarian morphology in pubertal rats. Journal of Endocrinology 68, 431-437

Paesi,F.J.A., De Jongh,S.E., Hoogstra,M.J. & Engelbregt,A. (1955). The follicle-stimulating hormone content of the hypophysis of the rat as influenced by gonadectomy and oestrogen treatment. Acta Endocrinologica 19, 49-60

Parkes,A.S. (1962). The rise of reproductive endocrinology, 1926-1940. Journal of Endocrinology 34, 20-32

Parlow,A.F. (1958). A rapid bioassay method for LH and factors stimulating LH secretion. Federation Proceedings 13, 402

Pedersen,T. (1972). Follicle growth in the mouse ovary. In: Oogenesis. Editors: J.D.Biggers & A.W.Schutz. Baltimore:University Park Press. London:Butterworth, pp.261-276

Peluso,J.J., Hutz,R. & England-Charlesworth,C. (1982). Development of preovulatory follicles and oocytes during the oestrous cycle of mature and aged rats. Acta Endocrinologica 100, 434-443

Peppler,R.D. (1972). FSH and LH levels in the intact and unilaterally ovariectomized cycling rat. Acta Endocrinologica 69, 267-280

Peters,H. (1969). The development of the mouse ovary from birth to maturity. Acta Endocrinologica 62, 98-116

Peters,H. & McNatty,K.P. (1980). The ovary. Frogmore, England:Granada Publishing Ltd.

Piva,F., Limonta,P., Maggi,R. & Martini,L. (1986). Stimulatory and inhibitory effects of the opioids on gonadotropin secretion. Neuroendocrinology 42, 504-512

Plant,T.M. (1986). Gonadal regulation of hypothalamic gonadotropin-releasing hormone release in primates. Endocrine Reviews 7, 75-88

Pomerantz ,D.K. (1978). Effect of porcine follicular fluid injection on FSH secretion in the immature female rat. Canadian Journal of Physiology and Pharmacology 56, 1076-1078

Ramaley,J.A. (1979). Development of gonadotropin regulation in the prepubertal mammal. Biology of Reproduction. 20, 1-31

Richards,J.S. (1980). Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiological Reviews 60, 51-89

Rivier,C. & Vale,W. (1987). Inhibin: measurement and role in the immature female rat. Endocrinology 120, 1688-1690

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

Rivier,J., Spiess,J., McClintock,R., Vaughan,J. & Vale,W. (1985). Purification and partial characterization of inhibin from porcine follicular fluid. Biochemical and Biophysical Research Communications 133, 120-127

Robertson,D.M., Foulds,L.M., Leversha,L., Morgan,F.J., Hearn,M.T.W., Burger,H.G., Wettenhall,R.E.H. & De Kretser,D.M. (1985). Isolation of inhibin from bovine follicular fluid. Biophysical and Biochemical Research Communications 126, 260-265

Robertson,D.M., Hayward,S., Jacobsen,I.J., Clarke,L., McLachlan,R.I. & De Kretser,D.M. (1988). Radioimmunoassay of rat serum inhibin: changes after PMSG stimulation and gonadectomy. Molecular and Cellular Endocrinology 58, 1-8

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

Rush,M.E. (1986). Follicle-stimulating hormone secretion in monosodium glutamate-lesioned rats: response to unilateral gonadectomy or porcine follicular fluid (inhibin). Life Sciences 38, 1941-1949

Sairam,M.R., Ramasharma,K. & Li,C.H. (1987). Synthetic peptide with inhibin-like activity preferentially inhibits follitropin secretion in comparison with lutropin-releasing hormone antagonists. Proceedings of the National Academy of Sciences, U.S.A. 84, 2043-2046

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

Sander,H.J., Meijs-Roelofs,H.M.A., Van Leeuwen,E.C.M., Kramer,P. & Van Cappellen,W.A. (1986). Inhibin increases in the ovaries of female rats approaching first ovulation: relationships with follicle growth and serum fsh concentrations. Journal of Endocrinology 111, 159-166

Sander,H.J., Van Leeuwen,E.C.M. & De Jong,F.H. (1984). Inhibin-like activity in media from cultured rat granulosa cells collected throughout the oestrous cycle. Journal of Endocrinology 103, 77-84

Sasamoto,S., Otani,T. & Shirota,M. (1981). Delay of the selective surge of follicle-stimulating hormone by bovine follicular fluid during the period of ovulation induced by human chorionic gonadotropin in dioestrous rats. Journal of Endocrinology 89, 205-212

Savoy-Moore,R.T., Grady,R.R. & Schwartz,N.B. (1986). Detection of folliculostatin: a sensitive and specific bioassay using the female rat. Life Sciences 38, 1281-1288

Schally,A.V., Kastin,A.J. & Arimura,A. (1972). The hypothalamus and reproduction. American Journal of Obstetrics and Gynecology 114, 423-441

Schuiling,G.A., Moes,H., De Koning,J. & Koiter,T.R. (1987). Suppression by LHRH of the stimulatory effect of oestradiol on the secretion of LH by the rat pituitary gland. Acta Endocrinologica 114, 488-496

Schwartz,N.B. (1969). A model for the regulation of ovulation in the rat. Recent Progress in Hormone Research 25, 1-55

Schwartz,N.B. (1974). The role of FSH and LH and of their antibodies on follicle growth and on ovulation. Biology of Reproduction. 10, 236-272

Schwartz,N.B. & Channing,C.P. (1977). Evidence for ovarian "inhibin": suppression of the secondary rise in serum follicle-stimulating hormone levels in pro-oestrous rats by injection of porcine follicular fluid. Proceedings of the National Academy of Sciences,U.S.A. 74, 5721-5724

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

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

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

Short,R.V. (1962). Steroids in the follicular fluid and the corpus luteum of the mare. A 'two cell' theory of ovarian steroid synthesis. Journal of Endocrinology 24, 59-63

Sopelak,V.M. & Butcher,R.L. (1982). Contribution of the ovary versus hypothalamus-pituitary to termination of estrous cycles in aging rats using ovarian transplants. Biology of Reproduction. 27, 29-37

Sporn,M.B., Roberts,A.B., Wakefield,L.M. & Assolan,R.K. (1986). Transforming growth factor-$\alpha$: biological function and chemical structure. Science 233, 532-534

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

Steger,R.W. & Peluso,J.J. (1987). Sex hormones in the aging female. Endocrinology and Metabolism Clinics 16, 1027-1043

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

Talwar,G.P., Gupta,S.K., Singh,V., Sahal,D. Iyer,K.S.N.& Singh,O. (1985). Bioeffective monoclonal antibody against the decapeptide gonadotropin-releasing hormone: reacting determinant and action on ovulation and estrus suppression. Proceedings of the National Academy of Sciences,U.S.A. 82, 1228-1231

Thomas,C.L. & Nikitovitch-Winer,M.B. (1984). Complete suppression of follicle-stimulating hormone in castrated male and female rats during continuous administration of porcine follicular fluid. Biology of Reproduction 30, 427-433

Tsonis,C.G. & Sharpe,R.M. (1986). Dual gonadal control of follicle-stimulating hormone. Nature 321, 724-725

Tsukamoto,I., Taya,K., Watanabe,G. & Sasamoto,S. (1986). Inhibin activity and secretion of gonadotropin during the period of follicular maturation. Life Sciences 39, 119-125

Uilenbroek,J.Th.J., Arendsen De Wolff-Exalto,E. & Welschen,R. (1976). Studies on the significance of the high levels of follicle stimulating hormone for follicular development in immature rats. Annales de Biologie animale, Biochemie, Biophysique 16, 297-305

Uilenbroek,J.Th.J., Tiller,R., De Jong,F.H. & Vels,F. (1978). Specific suppression of follicle-stimulating hormone secretion in gonadectomized male and female rats with intrasplenic ovarian transplants. Journal of Endocrinology 78, 399-406

Urbanski,H.F. & Ojeda,S.R. (1985). The juvenile-peripubertal transition period in the female rat: establishment of a diurnal pattern of pulsatile luteinizing hormone secretion. Endocrinology 117, 644-649

Urbanski,H.F. & Ojeda,S.R. (1986). The development of afternoon mini-surges of luteinizing hormone secretion in prepubertal female rats is ovary dependent. Endocrinology 118, 1187-1193

Urbanski,H.F.,Ojeda,S.R. (1987). Activation of luteinizing hormone-releasing hormone release advances the onset of female puberty. Neuroendocrinology 46, 273-276

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

Van Dijk,S. (1986). Inhibin, purification and characterization. Ph.D.Thesis. Rotterdam:Erasmus University.

Van Dyk,S., Steenbergen,J., Gielen,J.Th. & De Jong,F.H. (1986). Sexual dimorphism in immunoneutralization of bioactivity of rat and ovine inhibin. Journal of Endocrinology 111, 255-261

Van der Schaaf-Verdonk,G.C.J., Harryvan,J., Schuiling,G.A. & Koiter,T.R. (1984). Steroid-free bovine follicular fluid affects the nature and degradation of FSH in the ovariectomized rat. IRCS Medical Sciences 12, 673-673

Van der Schoot, P. (1976). Changing pro-oestrous surges of luteinizing hormone in aging 5-day cycling rats. Journal of Endocrinology 69, 287-288

Vidgoff,B. & Vehrs,H. (1940). Studies of the inhibitory hormone of the testes. IV. effect on the pituitary, thyroid and adrenal glands of the adult male rat. Endocrinology 26, 656-661

Waldhauser,F. & Dietzel,M. (1986). Daily and annual rhythms in human melatonin secretion : role in puberty control. Annals of the New York Academy of Sciences 453, 205-214

Wallace,J.M., McNeilly,A.S. & Baird,D.T. (1985). Ovulation rate and embryo survival in damline ewes after treatment with bovine follicular fluid in the luteal phase of the oestrous cycle. Journal of Reproduction and Fertility 75, 101-109

Wang,S.-C. & Greenwald,G.S. (1987). Effects of cycloheximide during the periovulatory period on ovarian follicular FSH, hCG, and prolactin receptors and on follicular maturation in the hamster.(42516) Proceedings of the Society for Experimental Biology and Medicine 185, 55-61

Welschen,R. (1972). Effect of unilateral ovariectomy on follicular growth in hypophysectomized rats treated with pregnant mare serum gonadotrophin. Journal of Endocrinology 55, 227-228

Welschen,R. (1973). Amounts of gonadotropins required for normal follicular growth in hypophysectomized adult rats. Acta Endocrinologica 72, 137-155

Welschen,R. & Dullaart,J. (1974). Serum concentrations of follicle--stimulating hormone and luteinizing hormone after unilateral ovariectomy in the adult rat. Journal of Endocrinology 63, 421-422

Welschen,R. & Dullaart,J. (1976). Administration of antiserum against ovine follicle-stimulating hormone or ovine luteinizing hormone at pro-oestrus in the rat: effects on follicular development during the oncoming cycle. Journal of Endocrinology 70, 301-306

Welschen,R. & Rutte,M. (1971). Ovulation in adult rats after treatment with pregnant mare serum gonadotrophin during oestrus. Acta Endocrinologica 68, 41-49

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

Welschen,R., Dullaart,J. & De Jong,F.H. (1978). Interrelationships between circulating levels of oestradiol-17$\alpha$, progesterone, FSH and LH immediately after unilateral ovariectomy in the cyclic rat. Biology of Reproduction 18, 421-427

Welschen,R., Hermans,W.P. & De Jong,F.H. (1980). Possible involvement of inhibin in the interrelationship between numbers of antral follicles and peripheral FSH concentrations in female rats. Journal of Reproduction and Fertility 60, 485-493

Welschen,R., Hermans,W.P., Dullaart,J. & De Jong,F.H. (1977). Effects of an inhibin-like factor present in bovine and porcine follicular fluid on gonadotropin levels in ovariectomized rats. Journal of Reproduction and Fertility 50, 129-131

Williams,A.T. & Lipner,H. (1982). The contribution of gonadostatin (inhibin-f) to the control of gonadotropin secretion in a simulated estrous cycle in steroid-treated ovariectomized rats. Endocrinology 111, 231-237

Wise,P.M. (1983). Aging of the female reproductive system. Review of Biological Research in Aging 1, 195-222

Wise,P.M. & Ratner,A. (1980). LHRH-induced LH and FSH responses in the aged female rat. Journal of Gerontology 35, 506-511

Woodruff,T.K., D'Agostino,J., Schwartz,N.B. & Mayo,K.E. (1988). Dynamic changes in inhibin messenger RNAs in rat ovarian follicles during the reproductive cycle. Science 239, 1296-1299

Ying,S-Y., Becker,A., Ling,N., Ueno,N. & Guillemin,R. (1986). Inhibin and beta-type transforming growth factor (TGF$\beta$) have opposite modulating effects on the follicle-stimulating hormone (FSH)-induced aromatase activity of cultured rat granulosa cells. Biochemical and Biophysical Research Communications 136, 969-975

Ying,S-Y., Czvik,J., Becker,A., Ling,N., Ueno,N. & Guillemin,R. (1987). Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related. Proceedings of the National Academy of Sciences, U.S.A. 84, 4631-4635

Zhang,Z., Lee,V.W.K., Carson,R.S. & Burger,H.G. (1988). Selective control of rat granulosa cell inhibin production by FSH and LH in vitro. Molecular and Cellular Endocrinology 56, 35-40

# PART TWO

APPENDIX PAPER I

# Compensatory Ovulatory Mechanisms Operative after First Ovulation in Rats Unilaterally Ovariectomized Prepubertally

H.M.A. MEIJS-ROELOFS,[1] P. KRAMER, P. OSMAN and H. J. SANDER

*Department of Anatomy, Medical Faculty*
*Erasmus University*
*Rotterdam, The Netherlands*

## ABSTRACT

Ovarian steroid contents and serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin were measured during the days after first ovulation in rats unilaterally ovariectomized in late prepuberty. In addition, follicle counts were made at second estrus and second metestrus.

During the cycle following first ovulation, ovarian estradiol contents in unilaterally ovariectomized (ULO) rats were significantly increased as compared to intact rats on the day of metestrus, on diestrus 1 and on second estrus. Ovarian progesterone was significantly increased on the days of metestrus, on diestrus 1, second proestrus and second estrus, but no differences were seen in ovarian androgen contents. After ULO there was an indication of an augmented FSH surge at the first and the second ovulation.

Follicle counts revealed that the total number of healthy as well as of atretic antral follicles on the day of second estrus was significantly increased after ULO, due to increased numbers of the smallest antral follicles. At second metestrus the number of larger antral follicles (350–500 $\mu m^3$) and the total number of healthy antral follicles was higher after ULO.

It is concluded that the compensatory process after ULO involved increased recruitment of small antral follicles. Activities in the remaining ovary were not simply doubled but a new hormonal balance was established.

## INTRODUCTION

Unilateral ovariectomy (ULO) in the adult cyclic rat may lead to functional compensation of follicle growth and ovulation in the remaining ovary during the ongoing cycle (Peppler and Greenwald, 1970a); the compensatory mechanism involves increased secretion of follicle-stimulating hormone (FSH) 6–18 h after ULO (Butcher, 1977; Welschen et al., 1978).

In a recent study we found also that after late prepubertal ULO, rats were capable of immediate compensation of follicle growth and of the number of eggs shed from the remaining ovary at first ovulation. These events were induced, as in adult rats, by an increase in FSH secretion (Meijs-Roelofs et al., 1982; Osman et al., 1982).

Butcher has reported (1977) that in the adult rat subjected to ULO, continued compensatory function of the remaining ovary during the subsequent cycles is due to a prolongation of the periovulatory FSH surge. In the long-term ULO adult rat, according to Hirshfield (1982), compensatory ovulation is brought about by diminished follicular atresia rather than by recruitment of an increased number of small antral follicles (both being FSH-dependent phenomena).

It now seemed of interest to study a later phase of the hormonal and follicular compensatory mechanisms in rats that had been unilaterally ovariectomized in late prepuberty, i.e., before cyclic ovarian function had started. Since in our intact adult rats characteristic cyclic changes in content of ovarian steroids have been found to occur (Toorop and Gribling-Hegge, 1982; Toorop et al., 1982), and since ovarian steroids may play an intraovarian role in regulating follicle growth and atresia (see Richards, 1980), the patterns of their ovarian content were compared in ULO and in intact rats. In addition, serum concentrations of prolactin, luteinizing hormone (LH) and FSH as well as follicular recruitment were studied.

Accepted March 15, 1984.
Received January 24, 1984.

[1] Reprint requests: Dr. H.M.A. Meijs-Roelofs, Dept. of Anatomy, Medical Faculty, P. O. Box 1738, Erasmus University, Rotterdam, The Netherlands.

## MATERIALS AND METHODS

Female rats of a Wistar substrain, R-Amsterdam, were used. The rats were weaned at 22 days of age and kept in a controlled temperature of 22–25°C with a light period from 0500–1900 h daily. Standard dry pellets and tap water were available ad libitum. Prepubertal rats of equal age and body weight were paired and from one rat of each pair the right ovary was removed between 1000 and 1100 h; the time interval between unilateral ovariectomy (ULO) and first ovulation was later calculated to be 4.3 ± 0.2 days (mean ± SEM; n=97; see Toorop et al., 1984). From the day of vaginal opening, generally coincident with the day of first ovulation at about 40 days of age, vaginal smears were taken daily until sacrifice.

Experimental and intact rats were killed at 1100 h on the day of first estrus, the 2 subsequent days (metestrus and diestrus 1), the day of second proestrus or the day of second estrus. Rats in which the day of second proestrus had not occurred 7 days after first ovulation were discarded, based on results obtained in a separate group of rats. Blood was taken under ether anesthesia by puncture of the ophthalmic venous plexus. Rats were then killed and the remaining ovary (experimental rats, n=84) or both ovaries (control rats, n=52) were removed, weighed on a torsion balance and then homogenized in 0.5 ml absolute ethanol for extraction of steroids. The efficiency of extraction was ⩾90% for all steroids (Toorop et al., 1984).

Additional groups of experimental (n=97) and control (n=93) rats were killed for blood sampling at 1100, 1500 and 1700 h on the day of second proestrus and at 1100 and 1500 on the day of second estrus. For the two groups of rats killed at 1100 h on the day of second estrus and metestrus, ovaries were fixed in Bouin's fluid for histological examination. In a separate group of ULO rats (n=10), the length of the cycle following first ovulation was recorded and the number of ova shed at the second ovulation was estimated by counting tubal ova.

### *Histology and Follicle Counts*

Serial sections (10 μm) of the ovaries, embedded in paraffin wax, were stained with haematoxylin and eosin. Follicle counts were made using the method of Boling et al. (1941) as modified by Welschen (1973). All follicles with a volume ⩾100 × $10^5$ $\mu m^3$ (mean diameter ⩾275 μm; generally comparable to Types 6, 7 and 8 of Pedersen and Peters, 1968) were counted and classified into five volume classes (I: 100–199; II: 200–349; III: 350–499; IV: 500–999; V: ⩾1000 × $10^5$ $\mu m^3$).[2] Atretic follicles were counted and classified similarly. Criteria for atresia were changes in granulosa cells, and changes or loss of the oocyte nucleus as reported by Osman (1975). Follicles in advanced stages of atresia (i.e., with nude or fragmented oocytes) were also taken into consideration.

[2] These volume classes correspond approximately to the following mean follicle diameters: I: 275–350 μm; II: 351–400 μm; III: 401–450 μm; IV: 451–575 μm; V: ⩾576 μm.

### *Hormone Determinations*

In serum, concentrations of LH, FSH and prolactin were measured. Blood was allowed to clot in a refrigerator before centrifugation. The serum was separated and stored at −20°C until assay. The concentration of LH and/or FSH was estimated by radioimmunoassay (RIA) with anti-ovine LH and FSH as antisera and rat LH and FSH as tracer (Welschen et al., 1975). Serum LH and FSH concentrations are expressed as μg reference preparation/l (NIAMDD-rat-LH or -FSH-RP-1). Serum prolactin was estimated by double-antibody RIA (de Greef and Zeilmaker, 1978) using NIAMDD-RP-1 as standard. Intraassay variations were below 10% for all three assays; for FSH estimations two assays were performed with an interval of several months, interassay variation was 22%. Data from these two assays have been presented in separate figures (Figs. 2C and D). Data in Fig. 2C involved 48 experimental and 44 control rats, and in Fig. 2D, 49 rats for each group.

In ovarian homogenates, estradiol, androgen and progesterone were estimated as described before (Toorop et al., 1982). The concentrations of estradiol and androgen were determined by RIA (androgen: Verjans et al., 1973; estradiol: de Jong et al., 1973). Since the antiserum raised against testosterone cross-reacts with 5α-dihydrotestosterone (31%) and 5α-androstane-3α-17β-diol (25%; Verjans et al., 1973) steroids measured in this assay are referred to as androgen.

Progesterone was estimated by the RIA method of de Jong et al. (1974). Intra- and interassay variations for steroid assays were between 8% and 15%, respectively.

### *Statistical Analysis*

All data are presented as means ± SEM. The Wilcoxon two-sample test was used for statistical analysis of the results. Moreover, two-way analysis of variance was performed for overall differences in LH concentrations in intact versus unilaterally ovariectomized rats. Differences of $P \leqslant 0.05$ were considered to be statistically significant.

## RESULTS

The number of ova released at second ovulation in the separate group of rats that were prepubertally unilaterally ovariectomized amounted to 10.6 ± 0.3 (mean ± SEM; n=10), a number not different from that found in intact control rats, 10.3 ± 0.3 (n=9). In this group no differences were found in either age or body weight at first ovulation, being 41.3 ± 1.0 days and 95.2 ± 3.7 g in ULO rats and 40.4 ± 0.6 days and 100.2 ± 0.8 g in intact rats. The length of the cycle subsequent to first ovulation was 6.8 ± 0.7 days in ULO rats and 7.1 ± 0.5 days in intact rats.

### *Ovarian Steroids*

Ovarian contents of estradiol, progesterone and androgen as well as ovarian weights during

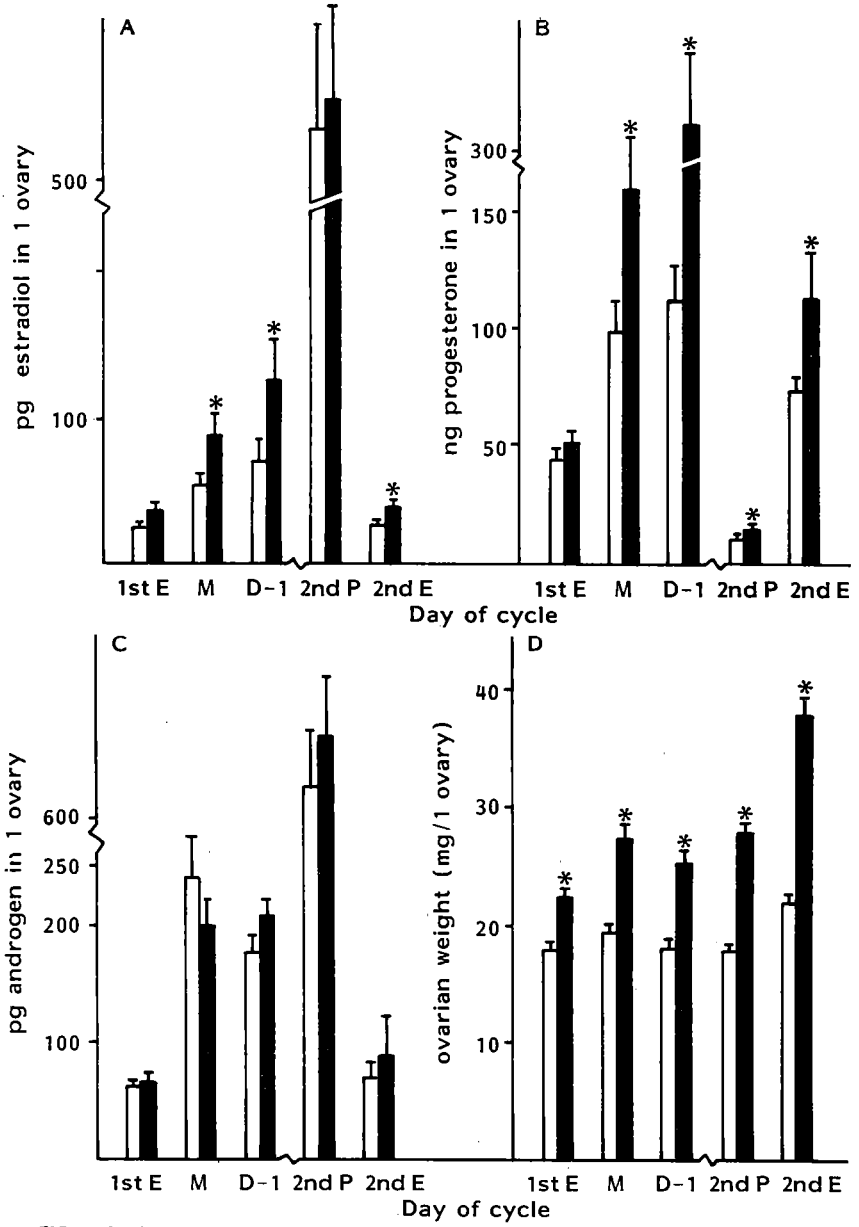



FIG. 1. Ovarian estrogen (*A*), progesterone (*B*) and androgen (*C*) content and ovarian weight (*D*) of intact (*open bars*) or ULO (*black bars*) rats measured at 1100 h on the day of first estrus and subsequent days. Each bar represents the mean ± SEM of estimations of 6–16 pairs of ovaries expressed per one ovary. *P≤0.05 ULO vs. control; Wilcoxon test.

the days between first and second ovulation in ULO rats[3] are shown in Fig. 1. Ovarian estradiol contents, expressed as pg per 1 ovary, were significantly higher ($P<0.05$) in ULO rats as compared to intact rats on the days of metestrus and diestrus 1; no difference was seen on the day of second proestrus, but at second estrus the ovarian estradiol content was again higher in ULO rats.

Ovarian progesterone contents (ng per 1 ovary) were higher ($P\leq0.05$) in ULO rats than in intact rats on the day of metestrus, diestrus 1, second proestrus and second estrus.

Ovarian androgen contents (pg per 1 ovary) in ULO and intact rats did not differ on any of the days studied; the weight of the remaining ovary of the ULO rats was always higher ($P<0.05$) than half the weight of the two ovaries in intact rats.

*Serum Concentrations of Prolactin, LH and FSH*

Concentrations in the serum of prolactin and LH (Fig. 2A and B), as measured at 1100 on the day of first estrus and subsequent days, did not differ between intact and ULO rats on any day. However, two-way analysis of variance revealed that over the period from first estrus

[3] Data in these rats on ovarian steroid contents in the prepubertal ovaries, removed at ULO, were incorporated in another study (Toorop et al., 1984).

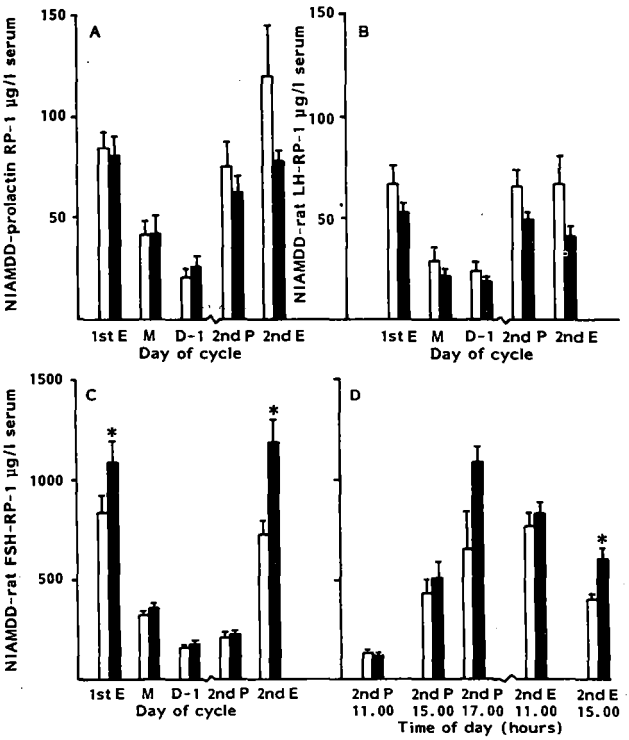



FIG. 2. Serum concentrations of prolactin (*A*) and LH (*B*) in intact rats (*open bars*) or in ULO rats (*black bars*) measured at 1100 h on the day of first estrus and subsequent days; serum FSH concentrations at 1100 h on the day of first estrus and subsequent days (*C*) and at various times on the days of second proestrus and estrus (*D*). Each bar represents the mean ± SEM; numbers of rats were 6–16. *$P\leq0.05$ ULO vs. control; Wilcoxon test.

to second estrus LH concentrations were lower ($P<0.001$) in the ULO group than in the intact group.

Serum FSH concentrations (Fig. 2C) were significantly higher in ULO rats at 1100 h on the day of first estrus ($P<0.05$, Wilcoxon) as well as at 1100 h on the day of second estrus ($P<0.01$). FSH concentrations, measured in a separate series at various times on the days of second proestrus and second estrus (Fig. 2D) differed only at 1500 h on the day of second estrus ($P<0.01$).

*Follicle Counts*

Numbers of healthy and atretic follicles present on the days of second estrus and metestrus in one ovary of control rats, or in the remaining ovary of ULO rats, have been compared and are shown in Fig. 3. On the day of estrus the number of healthy smallest antral follicles (Class I) was significantly increased ($P<0.05$) in ULO rats as compared to control rats; the same is true for atretic antral follicles in Classes I and II on this day. Moreover, the total number of healthy antral follicles in ULO rats (Classes I-V: 29.3 ± 4.2) was higher ($P<0.05$) than in control rats (17.3 ± 2.4); the same is true for total numbers of atretic antral follicles ($P<0.02$), being 23.8 ± 1.7 in ULO and 13.8 ± 0.7 in control rats.

On the day of metestrus the number of larger antral follicles (Class III) was higher in ULO rats ($P<0.05$). No differences in numbers of atretic follicles were seen in any size class on this day. With regard to the total number of healthy antral follicles, a higher number was

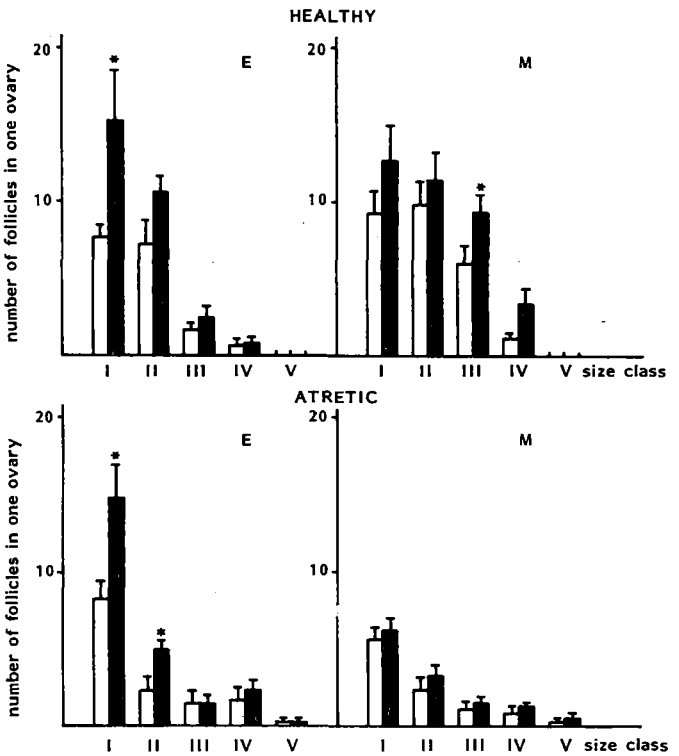



FIG. 3. Number of healthy (*upper panels*) or atretic (*lower panels*) follicles in one ovary in the various size classes (*I*: 100–199, *II*: 200–349, *III*: 350–499; *IV*: 500–999, *V*: ≥1000 × $10^5$ $\mu m^3$) on the day of second estrus (*E*) and second metestrus (*M*) in ULO (*black bars*) and control (*open bars*) rats. Each bar represents the mean ± SEM of 6–7 rats. *$P \leq 0.05$ ULO vs. control; Wilcoxon test.

present in ULO rats (36.8 ± 3.4) as compared to control rats (26.3 ± 2.0; $P<0.02$). Total numbers of atretic follicles (12.8 ± 1.1 vs. 10.0 ± 0.5) were not different.

## DISCUSSION

Prepubertal ULO was found to result in a normal number of ovulations at second ovulation, as was found previously for the first ovulation (Meijs-Roelofs et al., 1982); the length of the cycle after first ovulation did not differ between ULO and intact rats. At first ovulation, compensation of number of ova shed was achieved by decreased atresia of the growing follicles, presumably caused by the (acute) rise in serum FSH concentration (8 h) after ULO (Osman et al., 1982). Which regulatory mechanism(s) may be held responsible for ovulatory compensation at the subsequent ovulations?

It is generally accepted that the periovulatory FSH surge, and presumably the estrous portion of this surge (Hirshfield and de Paolo, 1981), is responsible for recruitment of a new cohort of small antral follicles, of which a selected number will ovulate during the subsequent cycle (Welschen, 1973; Welschen and Dullaart, 1976; Schwartz, 1974; Hirshfield and Midgley, 1978; Hirshfield, 1981). Butcher (1977) reported a prolongation of the FSH surge on the day of estrus after ULO in the adult rat. Our present data in the pubertal rat, showing significantly increased FSH concentrations at 1100 h, both at first and at second estrus after ULO in one group of rats and at 1500 h on the day of second estrus after ULO in a second group of rats, seem in line with these results. Butcher (1977) proposed that the prolonged FSH surge maintains a continued compensatory ovarian hyperfunction after ULO. According to Hirshfield (1982, 1983) the total number of follicles recruited at the start of a new cycle in adult long-term ULO rats amounts to only half that in intact rats, the compensation in ova shed then being achieved by diminished follicular atresia. Our present data clearly show that after prepubertal ULO, at second estrus an increased number of small antral follicles was recruited: not only the number of healthy but also the number of atretic smallest antral follicles was significantly increased as compared to the intact situation, negating the idea that follicles normally becoming atretic are "rescued" in this case. Also, Peppler and Greenwald (1970b) reported an increase in proliferation of smaller follicles rather than a decrease in follicular atresia in short-term ULO, adult rats.

Taken together the results from previous studies and the present one would seem to indicate that after ULO compensatory ovulation in the ongoing cycle is achieved by rescuing already growing follicles from atresia. During the immediately following cycle(s) a prolonged estrous FSH surge may recruit an increased number of preantral follicles, whereas at later cycles the (oocyte-saving) mechanism of decreased atresia of normal numbers of follicles initially recruited may take over again. However, alternative explanations based on differences in rat strain used or the age at which ULO was performed cannot be excluded. It might also be of interest to know how long after ULO the prolonged FSH surges are being maintained. The occurrence or the role of the overall decrease in serum LH concentration in the period from first to second estrus in ULO rats as compared to intact rats cannot be readily explained as yet.

Apart from rescuing small antral follicles from atresia or recruiting high numbers of them for further growth, FSH may also stimulate aromatase activity in these follicles (Moon et al.,1975; Erickson and Hsueh, 1978). This, in conjunction with the presence of a larger number of healthy antral follicles in the remaining ovary, may account for the significantly increased ovarian estradiol content as early as on the day of metestrus; because the increase seen on the day of first estrus just failed to reach significance ($P<0.09$). It should be noted that the estradiol content in the remaining ovary never reached twice the content found in one ovary of intact rats. In contrast to estradiol contents, no significant increases in ovarian androgens were seen. Both in intact and in ULO rats the pattern characteristic for the estrous cycle, with peak values on the day of proestrus, were seen for estradiol and androgen contents (Toorop and Gribling-Hegge, 1982).

A role for intraovarian androgens and estrogens in, respectively, promoting and preventing follicular atresia has often been demonstrated (Payne et al., 1956; Payne and Runser, 1958; Harman et al., 1975; Louvet et al., 1975; Zeleznik et al., 1979; Hillier and Ross, 1979; Hillier et al., 1980; Bagnell et al., 1982). Moreover, intraovarian inhibition of androgen production by estradiol has been reported (Leung et al., 1978; Leung and Armstrong, 1979; Magoffin and Erickson, 1981). It

thus seems that, in the present study, the absence of an increase in ovarian androgens after ULO may be the result of the aromatase activity of the approximately doubled number of healthy small antral follicles, producing increased amounts of ovarian estrogens which, in turn, inhibit accumulation of ovarian androgens. This then, as argued in the literature quoted above, seems like a favorable condition for maintenance of the doubled number of healthy antral follicles in the remaining ovary during the days of the cycle where FSH concentrations are no longer increased: their atresia may thus be prevented and a doubled number of antral follicles may reach the preovulatory stage with ensuing compensatory ovulation. Alternatively, it may be argued that the steroid levels found in the remaining ovary merely reflect the presence of a relatively high number of healthy antral follicles, resulting from changes in gonadotropic stimulation.

Although we cannot readily explain the finding that on the day of proestrus ovarian contents of estradiol are not significantly increased in ULO rats as compared to intact rats, the following teleological reasoning comes to mind. Ovarian estradiol secretion into the blood, in order to reach adequate concentrations for induction of the ovulatory gonadotropin surge, has to be accomplished by the one remaining ovary which (see above) shows an estradiol content of less than that of the two ovaries of the intact rat; thus depletion of estradiol stores may occur. The increases in progesterone content in the remaining ovary on the day of metestrus and subsequent days may be readily explained by the presence of a doubled number of corpora lutea in this ovary.

In conclusion, prepubertal ULO resulted in short-term compensation of numbers of ova shed by a mechanism probably involving prolongation of the FSH surge and involving recruitment of an increased number of small antral follicles at the start of the new cycle. Further maturation of these follicles may have been ensured by a change in the ratio of intraovarian concentrations of estradiol and androgens. Results indicate that compensatory ovulation after ULO is not simply a "doubling" of activities in the remaining ovary, but involves establishment of a new balance between gonadotropic and ovarian functions.

#### ACKNOWLEDGMENTS

The authors wish to thank Mrs. E.C.M. van Leeuwen and Mrs. L. Gribling-Hegge for their technical assistance, Dr. W. J. de Greef for estimation of prolactin and Dr. J. Moll for his criticism of the manuscript.

#### REFERENCES

Bagnell, C. A., Mills, T. M., Costoff, A. and Mahesh, V. B. (1982). A model for the study of androgen effects on follicular atresia and ovulation. Biol. Reprod. 27:903–914.

Boling, J. L., Blandau, R. J., Soderwall, A. L. and Young, W. C. (1941). Growth of Graafian follicle and time of ovulation in the albino rat. Anat. Rec. 79:313–331.

Butcher, R. L. (1977). Changes in gonadotropins and steroids associated with unilateral ovariectomy of the rat. Endocrinology 101:830–840.

Erickson, G. F. and Hsueh, A.J.W. (1978). Stimulation of aromatase activity by follicle stimulating hormone in rat granulosa cells in vivo and in vitro. Endocrinology 102:1275–1282.

de Greef, W. J. and Zeilmaker, G. H. (1978). Regulation of prolactin secretion during the luteal phase in the rat. Endocrinology 102: 1190–1198.

Harman, S. M., Louvet, J.-P. and Ross, G. T. (1975). Interaction of estrogen and gonadotrophins on follicular atresia. Endocrinology 96:1145–1152.

Hillier, S. G. and Ross, G. T. (1979). Effects of exogenous testosterone on ovarian weight, follicular morphology and intraovarian progesterone concentration in estrogen-primed hypophysectomized immature female rats. Biol. Reprod. 20:261–268.

Hillier, S. G., Zeleznik, A. J., Knazek, R. A. and Ross, G. T. (1980). Hormonal regulation of preovulatory follicle maturation in the rat. J. Reprod. Fertil. 60:219–229.

Hirshfield, A. N. (1981). Role of FSH in the selection of ovulatory follicles. In: Dynamics of Ovarian Function (N. B. Schwartz and M. Hunzicker-Dunn, eds). Raven Press, New York, pp. 79–82.

Hirshfield, A. N. (1982). Follicular recruitment in long-term hemicastrate rats. Biol. Reprod. 27:48–53.

Hirshfield, A. N. (1983). Compensatory ovarian hypertrophy in the long-term hemicastrate rat: size distribution of growing and atretic follicles. Biol. Reprod. 28:271–278.

Hirshfield, A. N. and De Paolo, L. V. (1981). Effect of suppression of the surge of follicle-stimulating hormone with porcine follicular fluid on follicular development in the rat. J. Endocrinol. 88:67–71.

Hirshfield, A. N. and Midgley, R. A., Jr. (1978). The role of FSH in the selection of large ovarian follicles in the rat. Biol. Reprod. 19:606–611.

de Jong, F. H., Hey, A. H. and van der Molen, H. J. (1973). Effect of gonadotrophins on the secretion of oestradiol-17$\beta$ and testosterone by the rat testis. J. Endocrinol. 57:277–284.

de Jong, F. H., Baird, D. P. and van der Molen, H. J. (1974). Ovarian secretion rates of oestrogens, androgens and progesterone in normal women and in women with persistent ovarian follicles. Acta Endocrinol. 77:575–587.

Leung, P.C.K. and Armstrong, D. T. (1979). A mechanism for the intraovarian inhibitory action of estrogen on androgen production. Biol. Reprod. 21:1035–1042.

Leung, P.C.K., Goff, A. K., Kennedy, T. G. and Armstrong, D. T. (1978). An intraovarian inhibitory action of estrogen on androgen production in vivo. Biol. Reprod. 19:641–647.

Louvet, J.-P., Harman, S. M., Schreiber, J. R. and Ross, G. T. (1975). Evidence for a role of androgens in follicular maturation. Endocrinology 97:366–372.

Magoffin, D. A. and Erickson, G. F. (1981). Mechanism by which 17-$\beta$ estradiol inhibits ovarian androgen production in the rat. Endocrinology 108:962–969.

Meijs-Roelofs, H.M.A., Osman, P. and Kramer, P. (1982). Ovarian follicular development leading to first ovulation and accompanying gonadotrophin levels as studied in the unilaterally ovariectomized rat. J. Endocrinol. 92:341–349.

Moon, Y. S., Dorrington, J. H. and Armstrong, D. T. (1975). Stimulating action of follicle stimulating hormone on estradiol-17$\beta$ secretion by hypophysectomized rat ovaries in organ culture. Endocrinology 97:244–247.

Osman, P. (1975). Preovulatory changes in the ovaries during the first spontaneous pro-oestrus in the rat. J. Endocrinol. 67:259–265.

Osman, P., Meijs-Roelofs, H.M.A. and Kramer, P. (1982). Compensatory follicle growth and ovulation after unilateral ovariectomy during the late prepubertal period in the rat. In: Development and Function of Reproductive Organs (A. G. Byskov and H. Peters, eds.). Excerpta Medica, Amsterdam, ICS no. 599, pp. 299–306.

Payne, R. W. and Runser, R. H. (1958). The influence of estrogen and androgen on the ovarian response of hypophysectomized immature rats to gonadotropins. Endocrinology 62:313–321.

Payne, R. W., Hellbaum, A. A. and Owens, J. N., Jr. (1956). The effect of androgens on the ovaries and uterus of the estrogen treated hypophysectomized immature rat. Endocrinology 59: 306–316.

Pedersen, T. and Peters, H. (1968). Proposal for a classification of oocytes and follicles in the mouse ovary. J. Reprod. Fertil. 17:555–557.

Peppler, R. D. and Greenwald, G. S. (1970a). Effects of unilateral ovariectomy on ovulation and cycle length in 4- and 5-day cycling rats. Am. J. Anat. 127:1–8.

Peppler, R. D. and Greenwald, G. S. (1970b). Influence of unilateral ovariectomy on follicular development in cycling rats. Am. J. Anat. 127: 9–14.

Richards, J. S. (1980). Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol. Rev. 60:51–89.

Scwartz, N. B. (1974). The role of FSH and LH and of their antibodies on follicle growth and ovulation. Biol. Reprod. 10:236–272.

Toorop, A. I. and Gribling-Hegge, L. (1982). Androgen and oestrogen concentrations in the ovary of the cyclic rat. J. Endocrinol. 93:25–30.

Toorop, A. I., Gribling-Hegge, L. and Meijs-Roelofs, H.M.A. (1982). Ovarian steroid concentrations in rats with spontaneous and with delayed or advanced ovulation. J. Endocrinol. 95:287–292.

Toorop, A. I., Meijs-Reolofs, H.M.A., Kramer, P. and de Greef, W. J. (1984). Ovarian steroid concentrations in rats approaching first ovulation. J. Endocrinol. 100:281–286.

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

Welschen, R. (1973). Amounts of gonadotrophins required for normal follicular growth in hypophysectomized adult rats. Acta Endocrinol. 72: 137–155.

Welschen, R. and Dullaart, J. (1976). Administration of antiserum against ovine follicle-stimulating hormone or ovine luteinizing hormone at pro-oestrus in the rat: effects on follicular development during the oncoming cycle. J. Endocrinol. 70:301–306.

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

Welschen, R., Dullaart, J. and de Jong, F. H. (1978). Interrelationships between circulating levels of oestradiol-17$\beta$ progesterone, FSH and LH immediately after unilateral ovariectomy in the cyclic rat. Biol. Reprod. 18:421–427.

Zeleznik, A. J., Hillier, S. G. and Ross, G. T. (1979). Follicle stimulating hormone-induced follicular development: an examination of the role of androgens. Biol. Reprod. 21:673–681.

APPENDIX PAPER II

# Changes in serum concentration of luteinizing hormone in the female rat approaching puberty

H. M. A. Meijs-Roelofs, P. Kramer and H. J. Sander
*Department of Anatomy, Medical Faculty, Erasmus University, Rotterdam, The Netherlands*

(*Received* 31 *December* 1982)

SUMMARY

Concentrations of LH in the serum were estimated in rats bled either once or twice during a 15-day period preceding first ovulation. In rats bled once (between 09.00 and 17.00 h) serum concentrations showed little change between 15 and 9 days before first ovulation and averaged 16 μg/l (days −15 to −9). A shift in LH level, to a mean of 31 μg/l, was seen on day −8, whereafter LH concentrations increased gradually. Basal LH values of $<10$ μg/l were only found until day −4.

The finding that LH values increased with age was confirmed by data from rats bled twice with an interval of $\geqslant 3$ days between bleedings. Furthermore, both in rats bled twice at 11.00 h and in rats bled twice at 15.00 h LH concentrations were significantly higher in the second sample.

Both morning (11.00 h) and afternoon (15.00 h) LH concentrations in rats bled once also indicated a rise in LH concentrations with age but it became apparent that only morning values showed a shift in LH concentration (from ~15 to ~42 μg/l) from day −9 to day −8. In contrast, mean afternoon values showed a gradual increase from day −15 on. From day −8 on the number of rats with LH values $\geqslant 50$ μg/l increased, and from day −5 on they were more frequent at 15.00 than at 11.00 h.

Thus a clear increase in LH secretion, most likely of a pulsatile nature, was found in the female rat approaching puberty. The correlation in time and possible functional relationship with late-prepubertal follicular growth is discussed.

## INTRODUCTION

The importance of possibly subtle changes in the mode and quantity of luteinizing hormone (LH) secretion as a triggering factor during the final phases of female sexual maturation has often been stressed. In man, during early puberty, an increase in LH secretion, associated with sleep, is a well-recognized phenomenon (Boyar, Finkelstein, Roffwarg, Kapen, Weitzman & Hellman, 1972; Swerdloff, 1978; Chipman, 1980). Evidence of prepubertal changes in LH secretion has also been presented in various animal species: in the lamb the occurrence of pulsatile LH secretion with increasing pulse frequency and a concomitant increase in basal LH level have been described (Ryan & Foster, 1980). In the hamster it has been suggested that development of a clock-timed daily surge of gonadotrophin release is a key event in the pubertal process (Smith & Stetson, 1980).

Evidence of episodic or pulsatile release has also been obtained in prepubertal rats (MacKinnon, Mattock & ter Haar, 1976; MacKinnon, Puig-Duran & Laynes, 1978; Andrews & Ojeda, 1981; Kimura & Kawakami, 1981, 1982; Meijs-Roelofs, Osman & Kramer, 1982), but no clear indication of any substantial increase in late prepubertal LH secretion has so far been obtained. Various factors may account for the lack of more precise information on the rat. For example, its small size allows only limited amounts of blood to

be obtained, normal LH secretion is easily disturbed by mild stress (MacKinnon *et al.* 1976) and repeated blood sampling changes basal LH concentrations in a way that depends upon the steroidal state of the animal (Seyler & Reichlin, 1973; Lee & de Kretser, 1981). Moreover, large biological variations in LH levels are to be expected because of possible pulsatile release, the quick disappearance rate of LH in rats (Weick, 1977) and because of individual differences in the final stages of sexual maturation at a fixed age.

In the present study a new attempt was made to study LH secretion, at various times of the day, during the late prepubertal period in the female rat. Each rat was bled only once or twice and blood samples were dated in relation to the day of first ovulation as described before (Meijs-Roelofs, Uilenbroek, de Greef, de Jong & Kramer, 1975) in order to eliminate biological variation due to differences in the stage of maturation.

## MATERIALS AND METHODS

Immature female rats of a Wistar substrain, R-Amsterdam, were used. The rats were weaned at 22 days of age and kept in a controlled temperature of 22–25 °C with a light period from 05.00 to 19.00 h daily. Standard dry pellets and tap water were available *ad libitum*. To establish LH concentrations from 15 days before (day −15) to 2 days before the day of first ovulation, one or two blood samples (0·5–1·0 ml) were collected from individual rats ranging in age from 28 to 42 days; after bleeding rats were checked daily for body weight and vaginal opening. In order to establish the day of bleeding in relation to the day of first ovulation (= day 0) animals were allowed to live and daily vaginal smears were taken until the day following the day of first oestrus, as judged by a metoestrous smear following the first oestrous smear. The day of vaginal opening was found to coincide with the day of first ovulation in 312 out of 333 rats, the remaining 21 rats showed vaginal opening on the day of first pro-oestrus.

Four independent experiments were performed in which rats were bled at various times of the day, either once or twice, with different intervals between bleedings; further details are described in the Results.

Blood was obtained under light ether anaesthesia by puncture of the ophthalmic venous plexus and allowed to clot overnight in a refrigerator before centrifugation. The serum was separated and stored at −20 °C until assay.

The concentration of LH in serum was estimated by radioimmunoassay with anti-ovine LH as antiserum and rat LH as tracer (Welschen, Osman, Dullaart, de Greef, Uilenbroek & de Jong, 1975). Serum LH concentration is expressed as μg reference preparation/l (NIAMDD rat LH-RP-1). All serum samples were assayed at least in duplicate and care was taken to spread samples from any experiment over at least two assays. Four assays were performed in total; the interassay variability (coefficient of variation) was 12·5% and the intra-assay variability was 9·4%.

### *Statistical analysis*

Statistical analysis of results was performed by regression analysis followed by the *F*-test. For these tests the original data were transformed logarithmically to normalize distribution and to reduce differences in variances. Furthermore the sign test, Student's *t*-test, Wilcoxon's two-sample test and Wilcoxon's matched-pairs signed rank test were applied. A difference was considered to be significant if the double-tail probability was $<0{\cdot}05$.

## RESULTS

### *Experiment I*

Data on LH concentrations measured in rats bled once between 09.00 and 17.00 h in the period from 15 days (day −15) until 2 days before the day of first ovulation are presented in

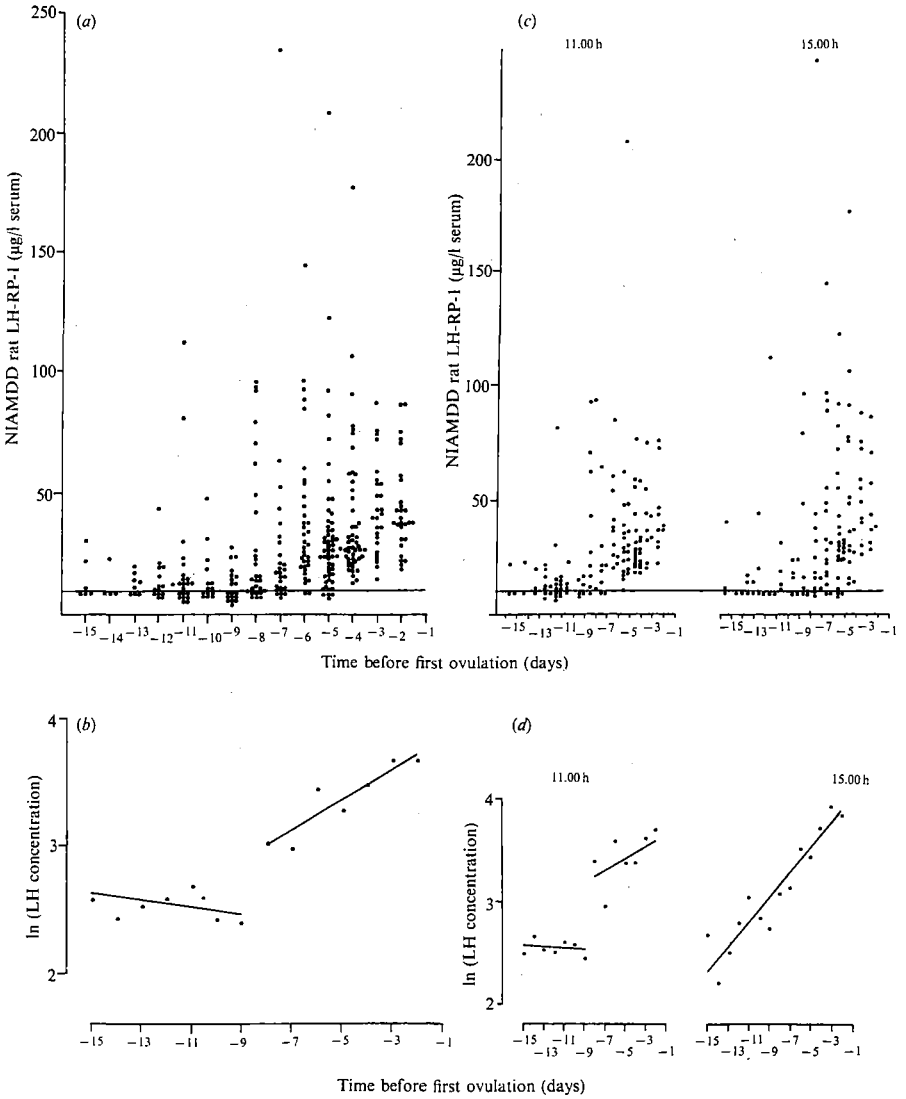



Fig. 1. Concentrations of LH in the serum of intact rats (*a*) bled once between 09.00 and 17.00 h ($n = 333$) and (*c*) bled once at 11.00 h ($n = 136$) or at 15.00 h ($n = 123$) on various days before the day of first ovulation. The horizontal line shows the limit of detection. Figure 1*a* also includes data from experiments II, III and IV (see text). Figures 1*b* and *d* show lines of best fit of (*a*) $n = 333$ and (*c*) 11.00 h $n = 136$ and 15.00 h $n = 123$ respectively after logarithmic transformation of the data. The points represent mean ln (LH concentration) per age group.

Fig. 1*a*. From day −15 until day −9, low serum LH concentrations were generally seen: only in two rats were concentrations higher than 50 μg/l observed (an arbitrary limit for 'high' values). From day −8 on, more rats showed values >50 μg/l, whereas the low values of ≤10 μg/l (limit of detection) gradually disappeared with increasing age. Figure 1*b* shows the lines of best fit after logarithmic transformation. This model of two separate lines resulted in a significant further reduction of the variance ($P<0{\cdot}005$, *F*-test) as compared to the model of one single line. For the period from day −15 until day −9 the regression equation was $y = 2{\cdot}22\ (\pm 0{\cdot}44) - 0{\cdot}0275\ (\pm 0{\cdot}039)x$ (S.E.M.), showing that in this period no significant changes in LH concentration were found. The mean LH concentration over this period was 16 μg/l (Fig. 1*a*). For the period from day −8 until day −2 the regression equation was $y = 3{\cdot}96\ (\pm 0{\cdot}46) + 0{\cdot}118\ (\pm 0{\cdot}045)x$, showing a significant rise in LH concentration from day −8 until day −2 ($P<0{\cdot}005$, Student's *t*-test). Thus there was a shift in mean LH concentration from 16 to 31 μg/l from days −9 to −8, followed by a gradual increase towards a value of 43 μg/l on day −2. Furthermore, day-to-day changes in LH concentration were significant only between days −9 and −8 ($P<0{\cdot}05$, Wilcoxon's two-sample test).

With regard to the LH values in relation to the time of day at bleeding relatively low concentrations were found more often around 11.00 h and relatively high concentrations around 15.00 h (data not shown); for this reason in the subsequent experiment LH values in rats bled at either 11.00 or 15.00 h or at both 11.00 and 15.00 h were compared.

Data based on (first) samples in experiments II, III and IV were combined with those of experiment I into Fig. 1*a–b*.

*Experiment II*

Data on LH concentrations measured in rats bled once at either 11.00 or 15.00 h, in the period from day −15 until day −2, are presented in Fig. 1*c*. Both morning and afternoon values increased while approaching first ovulation.

Values ≤10 μg/l were not seen from day −4 on; whereas values >50 μg/l became more frequent after day −9. The percentage of rats with an LH concentration of >50 μg/l in morning versus afternoon samples on days −5, −4, −3 and −2 were 9 vs 19, 17 vs 35, 17 vs 43 and 14 vs 36% respectively. Thus from day −5 onwards, but not before (see Fig. 1*c*), LH concentrations of >50 μg/l were always more frequent in the afternoon than in the morning. Figure 1*d* shows the lines fitting the data, after logarithmic transformation, for values at either 11.00 or 15.00 h. Two periods can be discerned for morning values, the model of two separate lines resulting in a significant further reduction of the variance ($P<0{\cdot}01$, *F*-test) as compared with the model of one single line. The regression equation for the period from day −15 until day −9 was $y = 2{\cdot}46\ (\pm 0{\cdot}59) - 0{\cdot}007\ (\pm 0{\cdot}051)x$, indicating a constant (mean) LH value over this period (~15 μg/l). The regression equation for the period from day −8 until day −2 was $y = 3{\cdot}71\ (\pm 0{\cdot}61) + 0{\cdot}058\ (\pm 0{\cdot}060)x$, showing a shift in mean LH level (to ~42 μg/l) as compared with the previous period; the seeming rise in mean LH concentration from day −8 until day −2 was not significant (Student's *t*-test). Day-to-day changes in LH concentration at 11.00 h were significant only from days −9 to −8 ($P<0{\cdot}05$, Wilcoxon's two-sample test).

A different pattern emerged from the data for afternoon values. The line of best fit was a single regression equation for the total period from day −15 until day −2, of $y = 4{\cdot}09\ (\pm 0{\cdot}45) + 0{\cdot}118\ (\pm 0{\cdot}037)x$, demonstrating a continuous and significant ($P<0{\cdot}001$, Student's *t*-test) rise between days −15 and −2, whereas no significant day-to-day changes were present.

*Experiment III*

Data from rats bled twice, either at 11.00 h and then at 15.00 h on the same day (III*a*) or first at 15.00 h and then at 11.00 h on the subsequent day (III*b*) are presented in Fig. 2. It

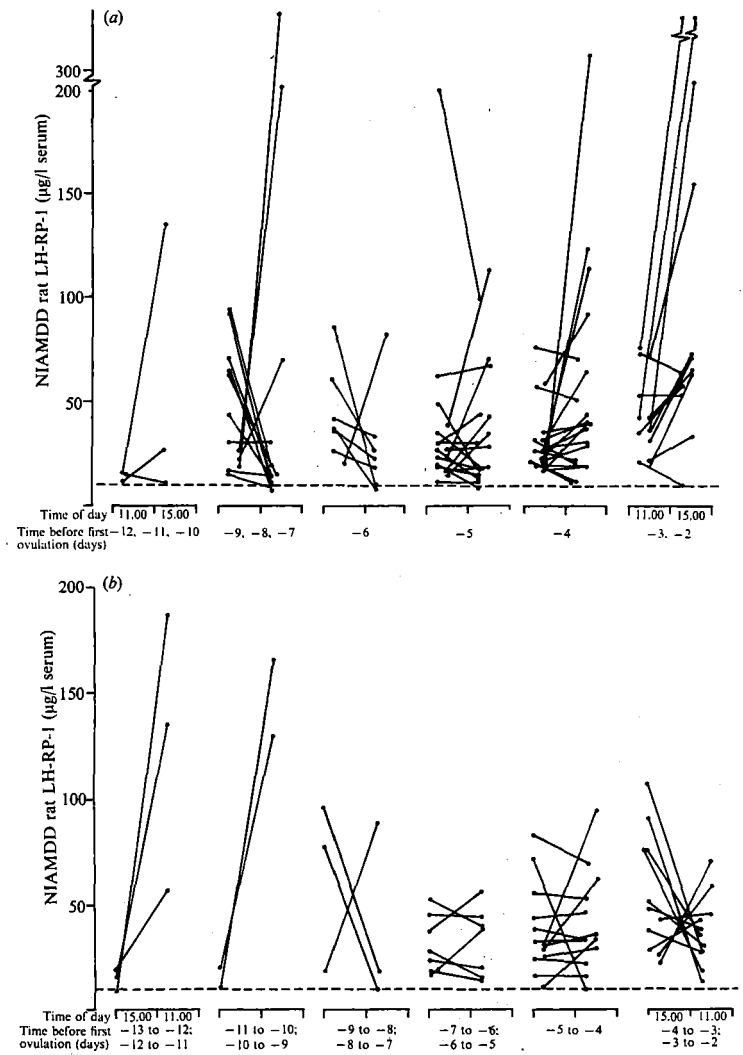



Fig. 2. Concentrations of LH in the serum of rats bled twice (*a*) at 11.00 and 15.00 h on the same day and (*b*) first at 15.00 h and then at 11.00 h on the subsequent day on various days before the day of first ovulation. The dotted line shows the limit of detection.

can be seen that in the first group of rats (III*a*), from day −5 onwards, values at 15.00 h were generally higher than values at 11.00 h, which in fact was the case in 32 out of the total number of 49 rats in this age group. On day −4 LH values measured at 15.00 h were

significantly higher than those measured at 11.00 h ($P<0{\cdot}05$, Wilcoxon's matched-pairs test), the same was true for days $-3$ and $-2$ ($P<0{\cdot}01$). This was in agreement with the previous data (Fig. 1*c*, *d*). In the second group of rats (III*b*) values at 15.00 h were higher than those at 11.00 h in only 11 out of the total number of 24 rats in the period from day $-5$ to $-2$. Moreover, in contrast to results from experiment III*a*, no significant difference between morning and afternoon values was found on any day. Taken together, results of experiments III*a* and III*b* (Fig. 2) did not show an overall influence of bleeding twice on the LH concentration measured in the second sample ($P = 1{\cdot}0$, sign test).

*Experiment IV*

Rats were bled twice but with an interval of $\geqslant 3$ days and bleeding was performed both times at either 11.00 or 15.00 h (Fig. 3).

Rats bled twice at 11.00 h on different days showed a higher LH concentration in the second sample in 39 out of 43 cases (Fig. 3*a*), thus showing an increase in morning LH concentration in individual rats approaching first ovulation ($P<0{\cdot}001$, sign test). Rats bled twice on different days at 15.00 h showed higher LH concentrations in the second sample in 28 out of 37 cases (Fig. 3*b*), thus demonstrating a significant increase of afternoon LH values as well in individual rats approaching first ovulation ($P<0{\cdot}01$, sign test).

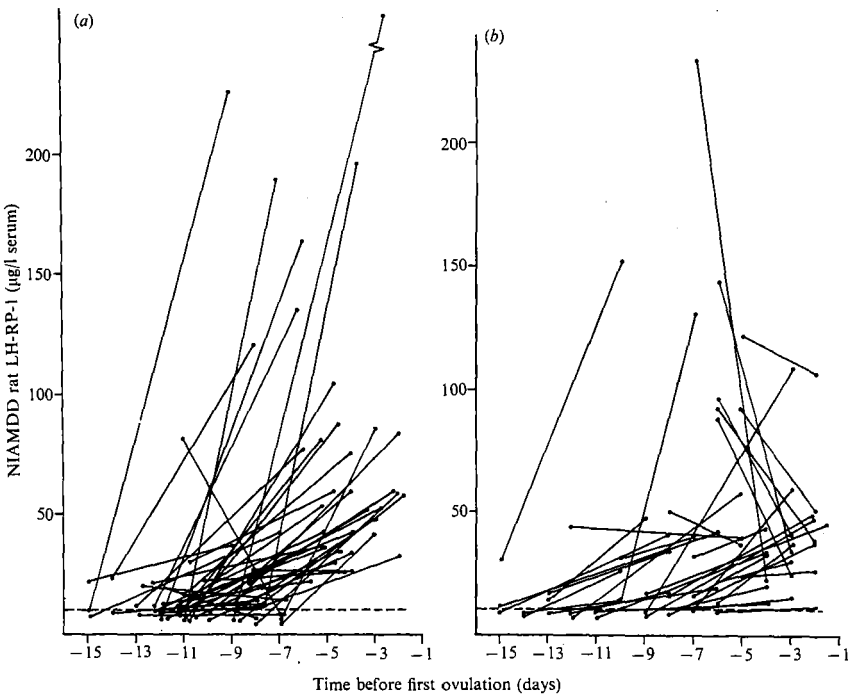



Fig. 3. Concentrations of LH in the serum of rats bled twice, both times at (*a*) 11.00 h or (*b*) 15.00 h, with an interval of $\geqslant 3$ days. The dotted lines shows the limit of detection.

## APPENDIX PAPER II

### DISCUSSION

In the present study we have been able to show for the first time a clear increase in LH secretion in the female rat approaching puberty; this is presumably due to our particular method of timing samples and to the large number of rats involved. In rats bled once, little change in LH secretion was seen from 15 until 9 days before first ovulation, both with regard to morning (11.00 h) values and with regard to values measured irrespective of the time of day (between 09.00 and 17.00 h). In both cases, most clearly for values at 11.00 h, an abrupt shift towards a higher LH level was found between 9 and 8 days before the first ovulation. This shift was followed by a more gradual increase in LH concentration closer to the first ovulation. Afternoon (15.00 h) values of LH concentration were found to increase gradually over the whole (14-day) period studied. In agreement with the foregoing, individual rats bled twice, with an interval between bleedings of several days, showed a significant increase in LH concentration with age, in both morning and afternoon values. The increase in mean LH concentration seemed to be accompanied by a gradual disappearance of very low LH values, with (from day −4 until first ovulation) no more values below the range of detection ($<10$ μg/l). Similar findings have been reported for the lamb (Ryan & Foster, 1980). Moreover, high LH values ($>50$ μg/l) not only became more frequent but, from 5 days before first ovulation onwards, usually occurred in the afternoon. This may reflect a development of the mechanism responsible for the clock-timed gonadotrophin surge; a development known to occur in the hamster (Smith & Stetson, 1980). The finding of a daily increase in pituitary responsiveness to gonadotrophin releasing hormone during the afternoon in prepubertal female rats (Wilkinson & Moger, 1981) supports this view.

If measured on the same day in individual rats, afternoon values were found to be significantly higher than morning values 4, 3 and 2 days before first ovulation (experiment III*a*). The seemingly discrepant observation that, in the same period, no such difference was seen after bleeding first at 15.00 h and then at 11.00 h on the next day (experiment III*b*) may indicate that collecting a morning sample first, stimulates LH secretion in the afternoon. Since values at 11.00 h were similar in samples being collected first or after collection at 15.00 h, no effect of repeated bleeding seems to be present at this time.

Of particular interest is the shift in LH level occurring 8 days before first ovulation, though the mechanism responsible for this shift remains unknown. However, it is noteworthy that follicular growth leading to first ovulation starts at ~8 days before this ovulation (Meijs-Roelofs *et al.* 1982). The shift in LH level found in the present study may well provide the requisite signal, thereafter followed by the necessary gonadotrophic regimen, to start and accomplish adequate follicular growth and steroidogenesis. Studies by Richards, Jonassen & Kersey (1980) and by Bogovich, Richards & Reichert (1981), showing that subtle increases in serum LH concentration may initiate preovulatory follicular growth in the immature rat (Richards & Bogovich, 1982), strongly support this view. Thus the first peak level of oestradiol, eliciting the first gonadotrophin surge (Meijs-Roelofs *et al.* 1975) may occur at a moment when follicles have reached the mature, preovulatory stage. First ovulation will then ensue.

In agreement with the findings of others (MacKinnon *et al.* 1978) and with those of a previous study (Meijs-Roelofs *et al.* 1982) LH concentrations in the prepubertal rat showed high individual variations, fluctuating from below 10 μg/l to nearly 400 μg/l. That in these earlier studies the present maturational changes in LH secretion were not detected may be explained by the facts that (1) comparatively small numbers of rats were used and (2) rats were grouped according to calendar age only (MacKinnon *et al.* 1976). In our previous study, involving rats dated in relation to first ovulation, tendencies similar to the LH changes found in the present work were present, but the number of rats was only one-third of the number of rats used in the present study. The pattern of LH secretion found is fully comparable to the pattern described for the lamb, where a pubertal LH increase, consisting

of pulsatile LH release with increasing frequency of pulses, occurs (Ryan & Foster, 1980). In the lamb marked reduction in oestradiol negative feedback was also noted during the pubertal period. Since it is not clear whether a change in sensitivity towards gonadal steroid feedback is responsible for the shift in LH secretion observed here, it seems premature to regard this shift as evidence for the so-called 'gonadostat reset' hypothesis of puberty (see Ryan & Foster, 1980).

Although no individual secretory profiles have been measured in the present study, pulsatile LH release seems to be the most likely explanation for our results. In the adult cyclic rat pulsatile LH release was reported to take place both during the ovulatory LH surge (Gallo, 1981*a*) and during periods of low-level LH secretion (Gallo, 1981*b*). In the immature rat pulsatile LH secretion at 24–27 days of age was described by Kimura & Kawakami (1982). Occurrence of LH pulses (more frequent and relatively low as compared to the previous study) in 28- to 37-day-old rats was described by Andrews & Ojeda (1981). Differences between the results of the present study and those in the study by Andrews & Ojeda (1981) seem to be first, the height of the 'peak' levels reached (never being higher than four times the basic level in the latter study whereas 'high' values may reach 40 times the basic level in our study) and secondly, the findings of decreasing basal LH levels with age (Andrews & Ojeda, 1981) versus increasing basal values (present study). These differences may be due to the use of a different strain of rat, sequential sampling as opposed to taking single blood samples, the use of different radioimmunoassay systems (both using anti-ovine LH antiserum but using ovine LH versus rat LH as tracer) and timing the rats in relation to first ovulation in our study compared with using uterine weight stages, as in the study of Andrews & Ojeda (1981).

We conclude from our data that an increase in LH secretion takes place in the immature rat approaching first ovulation, showing a shift in LH level 8 days before this ovulation. During the last 5 days high LH levels occur preferentially during the afternoon. The results support the assumption of a pulsatile nature of LH secretion, similar to the situation in the lamb.

The authors wish to thank Mrs E. C. M. van Leeuwen for her technical assistance and Mr G. Eilers for help and advice in statistical procedures. The criticism of the manuscript by Drs J. Moll and K. B. Ruf is highly appreciated.

## REFERENCES

Andrews, W. W. & Ojeda, S. R. (1981). A detailed analysis of the serum luteinizing hormone secretory profile in conscious, free-moving female rats during the time of puberty. *Endocrinology* **109**, 2023–2039.

Bogovich, K., Richards, J. S. & Reichert, J. L., Jr. (1981). Obligatory role of luteinizing hormone (LH) in the initiation of pre-ovulatory follicular growth in the pregnant rat: specific effects of human chorionic gonadotropin and follicle stimulating hormone on LH receptors and steroidogenesis in theca, granulosa and luteal cells. *Endocrinology* **109**, 860–867.

Boyar, R. M., Finkelstein, J. W., Roffwarg, H., Kapen, S., Weitzman, E. D. & Hellman, L. (1972). Synchronization of augmented luteinizing hormone secretion with sleep during puberty. *New England Journal of Medicine* **287**, 582–586.

Chipman, J. J. (1980). Pubertal control mechanisms as revealed from human studies. *Federation Proceedings*. **39**, 2391–2394.

Gallo, R. V. (1981*a*). Pulsatile LH release during the ovulatory LH surge on proestrus in the rat. *Biology of Reproduction* **24**, 100–104.

Gallo, R. V. (1981*b*). Pulsatile LH release during periods of low level LH secretion in the rat estrous cycle. *Biology of Reproduction* **24**, 771–777.

Kimura, F. & Kawakami, M. (1981). Serum hormone levels during sleep and wakefulness in the immature female rat. *Neuroendocrinology* **33**, 276–283.

Kimura, F. & Kawakami, M. (1982). Episodic LH secretion in the immature male and female rat as assessed by sequential blood sampling. *Neuroendocrinology* **35**, 128–132.

Lee, V. W. K. & de Kretser, D. M. (1981). Changes in secretion of luteinizing hormone and follicle-stimulating hormone induced by hypovolaemia in rats. *Journal of Endocrinology* **88**, 97–101.

## APPENDIX PAPER II


MacKinnon, P. C. B., Mattock, J. M. & ter Haar, M. B. (1976). Serum gonadotrophin levels during development in male, female and androgenized female rats and the effect of general disturbance on high luteinizing hormone levels. *Journal of Endocrinology* **70**, 361–371.

MacKinnon, P. C. B., Puig-Duran, E. & Laynes, R. (1978). Reflections on the attainment of puberty in the rat: have circadian signals a role to play in its onset? *Journal of Reproduction and Fertility* **52**, 401–412.

Meijs-Roelofs, H. M. A., Osman, P. & Kramer, P. (1982). Ovarian follicular development leading to first ovulation and accompanying gonadotrophin levels as studied in the unilaterally ovariectomized rat. *Journal of Endocrinology* **92**, 341–349.

Meijs-Roelofs, H. M. A., Uilenbroek, J. Th. J., de Greef, W. J., de Jong, F. H. & Kramer, P. (1975). Gonadotrophin and steroid levels around the time of first ovulation in the rat. *Journal of Endocrinology* **67**, 275–282.

Richards, J. S. & Bogovich, K. (1982). Effects of human chorionic gonadotropin and progesterone on follicular development in the immature rat. *Endocrinology* **111**, 1429–1438.

Richards, J. S., Jonassen, J. A. & Kersey, K. A. (1980). Evidence that changes in tonic luteinizing hormone secretion determine the growth of preovulatory follicles in the rat. *Endocrinology* **107**, 641–648.

Ryan, K. D. & Foster, D. (1980). Neuroendocrine mechanisms involved in onset of puberty in the female: concepts derived from the lamb. *Federation Proceedings*. **39**, 2372–2377.

Seyler, L. E. & Reichlin, S. (1973). Luteinizing hormone release in the rat induced by blood volume depletion. *Endocrinology* **92**, 295–302.

Smith, S. G., III & Stetson, M. H. (1980). Maturation of the clock-timed gonadotropin release mechanism in hamsters: a key event in the pubertal process? *Endocrinology* **107**, 1334–1337.

Swerdloff, R. S. (1978). Physiological control of puberty. *Medical Clinics of North America* **62**, 351–366.

Weick, R. F. (1977). A comparison of the disappearance rates of luteinizing hormone from intact and ovariectomized rats. *Endocrinology* **101**, 157–161.

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

Wilkinson, M. & Moger, W. H. (1981). Development of a quotidian increase in pituitary responsiveness to GnRH in prepubertal female rats. *Journal of Reproduction and Fertility* **62**, 393–398.

# Inhibin-like activity in ovarian homogenates of prepubertal female rats and its physiological significance

**H. J. Sander, H. M. A. Meijs-Roelofs, P. Kramer** and **E. C. M. van Leeuwen**

Department of Anatomy, Medical Faculty, Erasmus University, P.O. Box 1738,3000 DR Rotterdam, The Netherlands

RECEIVED 28 March 1985

## ABSTRACT

The presence and physiological significance of ovarian inhibin-like activity (ILA) in immature rats was studied by measuring ILA in ovarian homogenates and by studying the short-term changes in serum concentrations of FSH and LH after unilateral or bilateral ovariectomy. Inhibin-like activity in ovarian homogenates was estimated using an in-vitro bioassay system with dispersed rat anterior pituitary cells and subsequent measurement of FSH and LH levels in the spent pituitary cell medium. Inhibin-like activity, expressed in units/ovary, was undetectable in 13-day-old ovaries, was present in 18-day-old ovaries (5·8 units/ovary) and rose significantly to a value of 29·1 units/ovary on day 23. Another significant rise was seen between 28 and 33 days of age, with ILA reaching a value of 66·4 units/ovary, a value still well below that found in adult dioestrous ovaries (155·6 units/ovary). In the bioassay no systematic dose-dependent influence on LH secretion was found. Serum concentrations of FSH after unilateral or bilateral ovariectomy at 18 days of age showed no change at 5 h and a significant ($P<0·05$) increase compared with sham-operated controls at 24 h after operation. At 23, 28 and 33 days of age significant increases in FSH concentration were seen at 5 and 8 h after both unilateral and bilateral ovariectomy. At 24 h after unilateral ovariectomy, FSH had returned to the control values in these rats, whereas after bilateral ovariectomy a further increase in FSH was seen. Concentrations of LH generally varied widely and, compared with the situation after sham-operation, did not show a systematic trend of change during the 24-h period after unilateral or bilateral ovariectomy.

It was concluded that ILA is present in immature rat ovaries from the age of 18 days and increases, to a still sub-adult value, until at least 33 days of age. A physiological role for ILA, as a short-term regulator of FSH secretion seems to be present from at least 23 days of age and may account for the decreasing FSH concentrations seen during the late-prepubertal period, when ovarian steroids alone cannot explain the low FSH levels measured.

*J. Endocr.* (1985) **107**, 251–257

## INTRODUCTION

It has been shown by various authors that, similar to its well-established action in adults (for review see Hermans, de Jong & Welschen, 1981), porcine or bovine ovarian follicular fluid may selectively inhibit the secretion of follicle-stimulating hormone (FSH) if administered to immature female rats (Pomerantz, 1978; Lorenzen & Schwartz, 1979; Hermans, van Leeuwen, Debets & de Jong, 1980). The active principle in follicular fluid is a non-steroidal factor produced by granulosa cells of ovarian antral follicles (Erickson & Hsueh, 1978). It appears to be a short-term regulator of FSH secretion (Nandini, Lipner & Moudgal, 1976; de Jong, Welschen, Hermans *et al.* 1979) which has been termed 'inhibin', but has not yet been characterized as a pure and homogenous entity. In immature, 22- to 24-day-old rats it has been demonstrated that the ovaries contain inhibin and that the content increases after treatment with pregnant mare serum gonadotrophin or FSH (Lee, McMaster, Quigg *et al.* 1981; Lee, McMaster, Quigg & Leversha, 1982; Lee, 1983).

Studies on the regulation of FSH secretion in immature rats of various ages have shown that up to 20 days of age, but not thereafter, physiological amounts of oestradiol (Meijs-Roelofs, Uilenbroek, de Jong & Welschen, 1973; Meijs-Roelofs & Kramer, 1979), in

combination with progesterone (Meijs-Roelofs, Kramer & Gribling-Hegge, 1981*a*) and perhaps 5α-androstane-3β-diol (Meijs-Roelofs, Kramer & Gribling-Hegge, 1982), are sufficient to maintain an FSH concentration in ovariectomized rats not different from that in intact rats. Thus the conclusion was reached that after 20 days of age an ovarian factor, different from the steroids mentioned, perhaps inhibin, must be involved in the regulation of FSH secretion. Also, it was suggested by Lorenzen (1981) that, in order to establish cyclic ovarian function, ovarian inhibin may well be required during the final phases of sexual maturation as a specific independent regulator of FSH secretion. Findings by Hermans *et al.* (1980) indicate that indeed inhibin does have a physiological role to play here since bilateral ovariectomy of 25-day-old rats resulted, within 8 h, in a significant increase in FSH secretion. This short-term effect must presumably be attributed to the disappearance of endogenous inhibin in view of its reported short half-life (18 min–3 h; Lee 1983), whereas serum concentrations of gonadal steroids do not change significantly within the 8 h period (Campbell & Schwartz, 1977). Moreover, in rats of this age the administration of exogenous inhibin was effective in suppressing FSH secretion in both intact and bilaterally ovariectomized rats (Hermans *et al.* 1980).

The aim of the present study was to investigate the role of inhibin in immature female rats at various ages by (1) measuring the presence of inhibin-like activity in ovarian homogenates and (2) demonstrating its physiological significance by studying short-term changes in FSH and luteinizing hormone (LH) secretion after unilateral and bilateral ovariectomy. Part of this work was presented at the IXth Anglo–Dutch Endocrine Meeting (Sander, van Leeuwen, de Jong & Meijs-Roelofs, 1984*b*).

## MATERIALS AND METHODS

Immature female rats of a Wistar substrain (R-Amsterdam) were used. The rats were weaned at 22 days of age and kept in controlled conditions of temperature (22–25 °C) and light (lights on 05.00–19.00 h). Standard dry pellets and tap water were available *ad libitum*. Rats operated upon before weaning were kept with their mothers. Animals were used at 13, 18, 23, 28 or 33 days of age, when their body weights were $20{\cdot}0 \pm 0{\cdot}0$ (S.E.M.; $n = 4$), $28{\cdot}9 \pm 0{\cdot}2$ ($n = 100$), $38{\cdot}5 \pm 0{\cdot}3$ ($n = 94$), $55{\cdot}3 \pm 0{\cdot}3$ ($n = 95$) and $74{\cdot}0 \pm 0{\cdot}4$ g ($n = 164$) respectively. The rats were subjected to unilateral ovariectomy or bilateral ovariectomy, to sham-operation or were left intact. Operations were performed between 08.30 and 11.00 h under ether anaesthesia and litter-mates were divided among the various groups.

The ovaries, destined for estimation of inhibin-like activity (ILA; first experiment), were weighed on a torsion balance and homogenized in culture medium (1 ml/ovary; see below) without fetal calf serum and centrifuged at 100 000 *g* for 60 min at 4 °C. The supernatant was pipetted off and stored at −20 °C until bioassay.

Blood was taken from the intact control rats at the time of operation ($t = 0$) and from the sham-operated and the unilaterally or bilaterally ovariectomized rats 5, 8 or 24 h after operation to measure FSH and LH (second experiment). Each rat was bled once only, except in the 33-day-old group where some were bled two or three times after it had been found that this procedure did not affect concentrations of FSH and LH ($P$ range 0·33–0·81). Moreover, there were similar ($P < 0{\cdot}01$) differences between experimental and control groups when rats were bled once, twice or three times. The blood was allowed to clot overnight in a refrigerator before centrifugation. The serum was separated and stored at −20 °C until assay.

### Assay of ILA using dispersed pituitary cells in culture

Anterior pituitary cells were obtained as desribed by Hermans, van Leeuwen, Debets *et al.*(1982), and cultured in 24-well tissue culture plates (Falcon Multiwell; Becton-Dickenson, Oxnard, CA, U.S.A.) in 1 ml Minimal Essential Medium with Earle's Salts (Boehringer, Mannheim, F.R.G.) containing $NaHCO_3$ (25·8 mmol/l), 2% antibiotic/antimycotic (Gibco, Grand Island, NY, U.S.A.), 10% fetal calf serum (Gibco) and sodium pyruvate (Gibco; 1 mmol/l). Briefly, anterior pituitary glands were minced, treated with Dispase II and finally dispersed in a Dounce homogenizer. The cell number and viability, determined with trypan blue exclusion, were $1{\cdot}3 \pm 0{\cdot}03 \times 10^6$ cells/ml and $71 \pm 1{\cdot}1\%$ ($n = 11$) respectively. Cells were cultured (100 µl cell suspension/well) in a moist chamber at 37 °C in a 5% $CO_2$/95% air atmosphere. For further details see Sander, van Leeuwen & de Jong (1984*a*).

After 3 days of culture the spent culture medium was discarded and replaced with 1 ml fresh culture medium containing either the supernatants to be tested at two or three dose levels, each in triplicate or quadruplicate, or charcoal-treated bovine follicular fluid (bFF) diluted 1 : 1000 with culture medium (internal standard) at three dose levels in quadruplicate. After a further 3 days of culture the spent media were collected and stored at −20 °C until assayed for FSH and LH.

In the bioassay, calculation of potencies relative to the internal standard and 95% confidence limits and also analysis of linearity and parallelism were based on

the method of Finney (1964) as elaborated by Borth (1976), using a computer program kindly provided by Dr H. W. G. Baker, Howard Florey Institute of Experimental Physiology and Medicine, Parkville, Victoria, Australia. The relative potency calculated is derived from our standard sample, diluted 1:1000. One ovary was homogenized in 1000 µl medium and thus equally diluted. Inhibin like activity, in units, was obtained by multiplying the relative potency by a factor 65 since 65 µg protein is present in 1 µl bFF and 1 unit inhibin was defined as the ILA present in 1 µg bFF protein. The index of precision ($\lambda$) of the bioassay ranged from 0·066 to 0·1235 (0·0934 ± 0·006; mean ± S.E.M., $n = 11$). No significant deviations of parallelism or linearity were seen. Sensitivity of the assay was 0·78 units/ml or 12 µl bFF/l.

### Removal of steroids

Before bioassay, all homogenates were treated with dextran-coated charcoal, as described by Sander *et al.* (1984*a*) and then sterilized by filtration.

### Hormone determinations

Concentrations of FSH and LH in serum or in spent pituitary cell medium were estimated in duplicate by radioimmunoassay with anti-ovine FSH or LH as antiserum and rat FSH or LH as tracer, and expressed as µg NIADDK-rat FSH or LH RP-1/1, as described by Welschen, Osman, Dullaart *et al.* (1975). Within and between assay variations and indexes of precision were 21·8%, 17·8% and 0·0968 ± 0·0066 (S.E.M., $n = 9$) for FSH and 10·3%, 11·1% and 0·0896 ± 0·0219 ($n = 8$) for LH. Detection limits were 8 and 16 µg/l for FSH and LH respectively.

### Statistical analysis

Statistical analysis of results from radioimmunoassays was performed by Wilcoxon's two-sample test. Analysis of variance was performed for overall differences in ILA, followed by trend analysis. Differences in ILA were evaluated by Student's *t*-test. A difference was considered significant if the double-tail probability was < 0·05.

## RESULTS

### ILA of ovarian homogenates

Inhibin-like activity and weights of the ovaries of rats of various ages are shown in Fig. 1. Analysis of variance revealed overall changes ($P < 0·001$) for ILA, trend analysis confirmed the rise in ILA with age ($P < 0·002$). Thus ILA, undetectable at 13 days, increased with age and was highest at 33 days of age. For comparison, the ILA of adult ovaries weighing 35·9 ± 2·2 mg estimated on the first day of dioestrus was 155·6 ± 15·8 units/ovary ($n = 5$). Thus ILA at 33 days of age (66·4 units/ovary) was still significantly ($P < 0·001$) lower than in adult dioestrous rats.

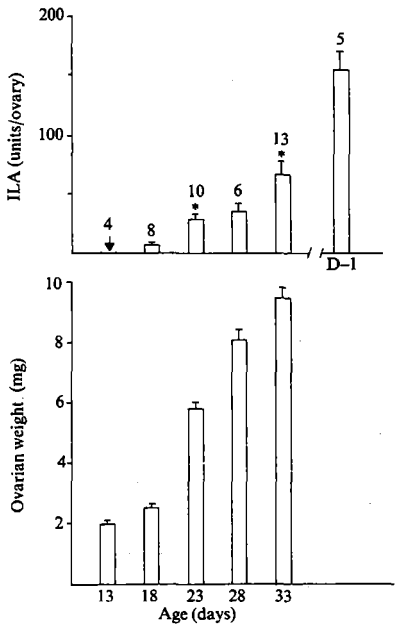



FIGURE 1. Inhibin-like activity (ILA) in ovaries from immature rats and ovarian weight at various ages. Values are means ± S.E.M. The numbers of rats per group are shown above bars. At 13 days of age no ILA was detected. D–1, first day of dioestrus (adult cycle). *$P < 0·01$ compared with previous age group (two-tailed Student's *t*-test).

### Short-term effects of unilateral ovariectomy or bilateral ovariectomy on FSH and LH secretion

Data on the age-dependent effects of unilateral or bilateral ovariectomy on the serum concentrations of FSH and LH are presented in Figs 2 and 3. At 18 days of age no changes in FSH concentration were seen 5 h after operation but 24 h after operation FSH concentrations had significantly increased, in both unilaterally and bilaterally ovariectomized rats compared with sham-operated control rats. Values after bilateral ovariectomy were significantly ($P < 0·05$) higher than after unilateral ovariectomy. At 8 h after operation bilaterally ovariectomized rats unexpectedly showed decreased FSH concentrations. At 23, 28 and 33 days

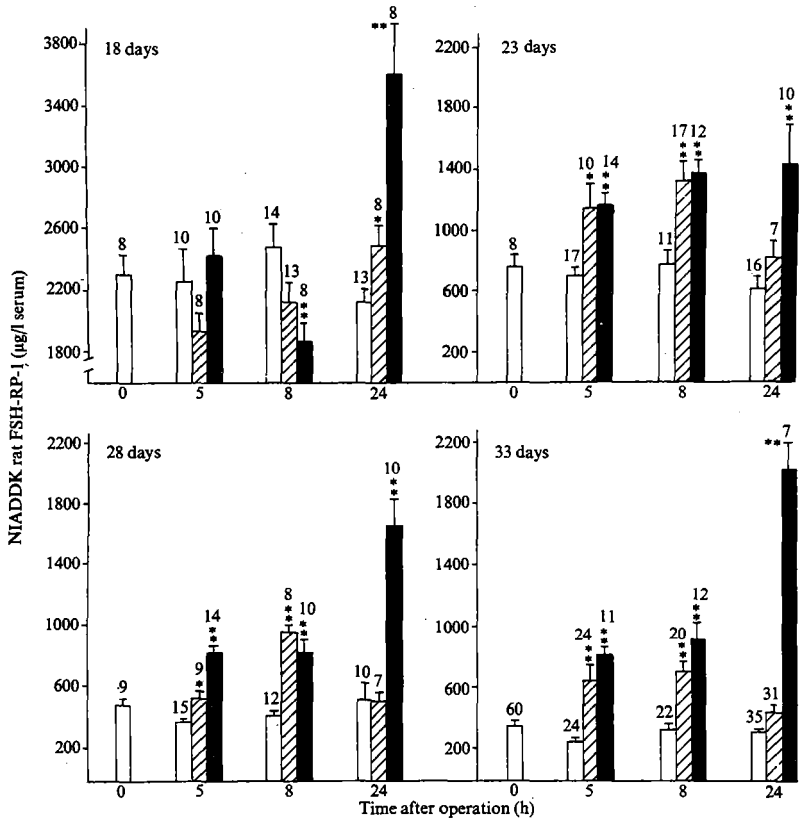



FIGURE 2. Concentrations of FSH (means ± S.E.M.) in the serum of rats which were sham-operated (open bars), unilaterally ovariectomized (hatched bars) or bilaterally ovariectomized (solid bars) at 5, 8 and 24 h after operation performed at 18, 23, 28 and 33 days of age. Open bars at time 0 represent concentrations of FSH in intact control rats. The numbers above bars show the numbers of rats per group. * $P<0.05$, ** $P<0.01$ compared with sham-operated controls (Wilcoxon-test).

of age FSH concentrations measured 5 or 8 h after operation were always significantly increased compared with the sham-operated controls, in both unilaterally and bilaterally ovariectomized rats. At 24 h after operation the effects of unilateral and bilateral overiectomy diverged: FSH concentrations in unilaterally ovariectomized rats had decreased again and were no longer different from those in the sham-operated control rats, whereas FSH concentrations in bilaterally ovariectomized rats had increased and were significantly ($P<0.01$) higher than levels in both sham-operated and unilaterally ovariectomized rats.

Concentrations of LH varied widely, even when comparing the intact and sham-operated control groups at the same age (Fig. 3). At 18 days of age, after both unilateral and bilateral ovariectomy, significantly increased LH concentrations were found 24 h after operation; at 28 days of age, 5 h after operation, bilaterally ovariectomized rats showed increased LH values compared with their sham-operated controls, whereas 8 h after operation LH concentrations were undetectable in unilaterally ovariectomized rats and thus significantly lower than in sham-operated controls. At 33 days of age only bilaterally ovariectomized rats showed, 24 h after operation, significantly increased LH concentrations compared with those in controls. In both the 28- and the 33-day-old group, 24 h after operation, LH concentrations after bilateral ovariectomy were significantly ($P<0.01$) higher than after unilateral ovariectomy.

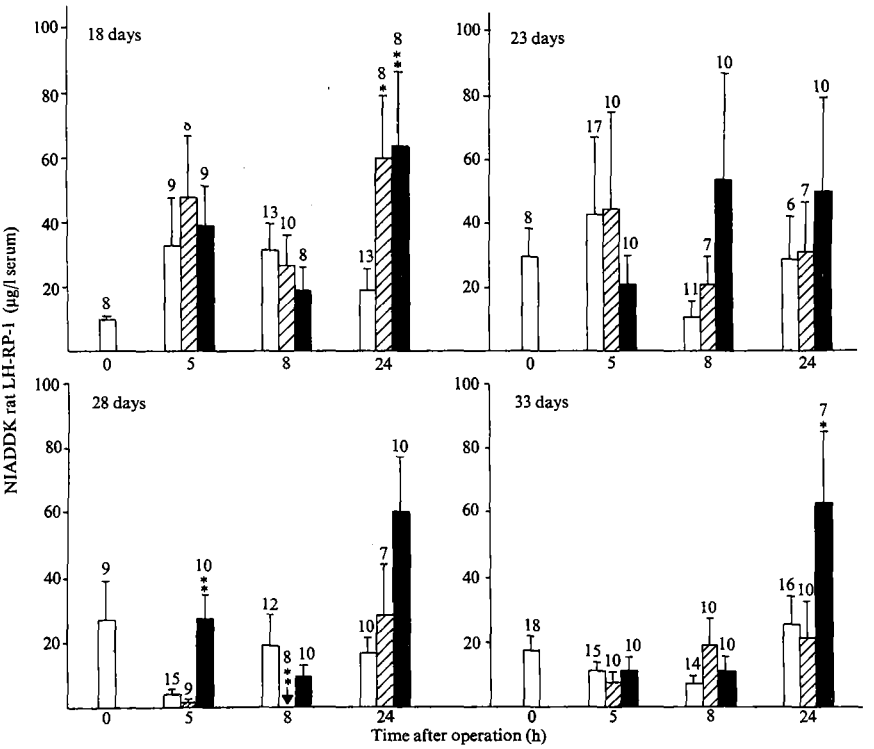



FIGURE 3. Concentrations of LH (means ± S.E.M.) in the serum of rats which were sham-operated (open bars), unilaterally ovariectomized (hatched bars) or bilaterally ovariectomized (solid bars) at 5, 8 and 24 h after operation performed at 18, 23, 28 and 33 days of age. Open bars at time 0 represent concentrations of LH in intact control rats, The numbers above bars show the numbers of rats per group. No LH was detected in unilaterally ovariectomized rats at 28 days of age and 8 h after the operation. * $P<0.05$, ** $P<0.01$ compared with sham-operated controls (Wilcoxon-test).

## DISCUSSION

At least two conditions need to be fulfilled in order to prove the existence in immature female rats of a physiologically significant role of an ovarian inhibin-like factor in the short-term regulation of FSH secretion. First, this factor should be shown to be produced under normal physiological conditions and secondly, it should be demonstrated that changes in the amount of inhibin produced result in the short-term in changes in serum FSH concentration. The present results clearly show that an inhibin-like factor is present in ovarian homogenates of immature rats aged 18 days and older. Furthermore, the ILA was relatively low at 18 days of age and increased to a value at 33 days of age of about 40% of that found during adult dioestrus.

Changes in FSH concentrations during the early period after removal of the ovaries may be expected to provide information on the effect of disappearance of endogenous ILA: in adult rats there is ample evidence that changes in FSH concentration 5–12 h after unilateral ovariectomy are not due to changes in serum concentrations of ovarian steroids, but must be attributed to loss of a different, non-steroidal ovarian factor: inhibin (Butcher, 1977; Campbell & Schwartz, 1977; Welschen, Dullaart & de Jong, 1978). Moreover, Hermans *et al.* (1980) showed that after bilateral ovariectomy in immature rats of 15 days of age and older, injections of inhibin (bovine follicular fluid) could prevent the rise in FSH concentration 8 h after gonadectomy, whereas steroid injections failed to do so. Thus studying the changes in FSH concentration during the period of 5–24 h after operation, in rats unilaterally or bilaterally ovariectomized at various ages, may pro-

vide information on the effect of the disappearance of endogenous ILA and thus on the physiological significance of ILA. In the case of unilateral ovariectomy, compensatory gonadotrophic stimulation of the remaining ovary and a return to normal, physiological hormone levels might be expected (Osman, Meijs-Roelofs & Kramer, 1982) whereas, in the case of bilateral ovariectomy, gradual disappearance of ovarian steroids may be expected to add to the acute effect of loss of inhibin and thereby cause further rises in gonadotrophins.

At 18 days of age unilateral or bilateral ovariectomy did not result in an increase of FSH secretion within 8 h, but significant increases in FSH concentration at both 5 and 8 h after operation, compared with sham-operated controls, were seen at all later ages studied. The finding of a decrease in the level of FSH 8 h after bilateral ovariectomy at 18 days of age must presumably be explained by the relatively high individual variation in FSH concentration in rats of this age in which a transition from high FSH levels (as found around 15 days of age) towards the much lower levels (as present after 22 days of age) takes place (Meijs-Roelofs *et al.* 1973). Again with the exception of the situation at 18 days of age, when an increase in FSH was seen 24 h after unilateral ovariectomy, at all other ages at 24 h after operation FSH levels had decreased again and were no longer different from those in control rats, indicative of compensatory production of inhibin by the remaining ovary. In contrast, 24 h after bilateral ovariectomy, a further rise in FSH concentration was found. In total these findings demonstrate that ILA may be present in the ovaries of immature rats from 18 days of age and that it plays a physiological role in the regulation of FSH secretion from at least 23 days of age. Judging from the LH concentrations found at 5 and 8 h after unilateral or bilateral ovariectomy performed at various ages, which, with the exception of results at 28 days of age, showed no changes compared with control rats, it seems justified to conclude that ILA plays a specific role in the regulation of FSH secretion only, in agreement with the results of others (e.g. Charlesworth, Grady, Shin *et al.* 1984). The differences in LH concentrations seen at 28 days of age between some experimental and control groups are most easily explained by the high variability in LH levels generally found, as clearly illustrated by the LH concentrations in control groups at this age.

The level of ILA in immature 33-day-old rats is still clearly below that found during the adult cycle and this raises the question of whether the 'adult' level will only be reached after the first ovulation has taken place (at about 40 days of age) or, perhaps, during the last few days preceding this first ovulation when ovarian follicular development has been reported to be comparable with that during the adult cycle (Meijs-Roelofs, Osman & Kramer, 1982*b*). This is currently under study.

Taking together the results of previous studies (e.g. Meijs-Roelofs *et al.* 1981) and the present study, it seems that up to 20 days of age the peripheral level of FSH is regulated by ovarian steroids (oestrogen and progesterone). However, after 20 days of age, endogenous steroids can no longer explain the further decrease in FSH which then occurs. In view of the increasing presence of ILA in the ovary (from 18 days of age) and taking into account its physiological role as demonstrated from at least 23 days of age, inhibin may, via a short-term regulatory mechanism, adjust the FSH concentrations present, and thus regulate final follicular growth leading to first ovulation.

## ACKNOWLEDGEMENTS

The authors wish to thank Dr J. Moll for his criticism of the manuscript and Ms E. Klink for expert secretarial assistance. They are grateful to the NIADDK, Bethesda, MD, U.S.A. for the gonadotrophins used in the radioimmunoassays.

## REFERENCES

Borth, R. (1976). Statistics of parallel line assay. In *Methods of hormone analysis*, pp. 500–513. Eds H. Breuer, D. Hamel & H. L. Krüskemper. Stuttgart: Georg Thieme Verlag.

Butcher, R. L. (1977). Changes in gonadotropins and steroids associated with unilateral ovariectomy of the rat. *Endocrinology* **101**, 830–840.

Campbell, C. S. & Schwartz, N. B. (1977). Steroid feedback regulation of luteinizing hormone and follicle-stimulating hormone secretion rates in male and female rats. *Journal of Toxicology and Environmental Health* **3**, 61–95.

Charlesworth, M. C., Grady, R. R., Shin, L., Vale, W. W., Rivier, C., Rivier, J. & Schwartz, N. B. (1984). Differential suppression of FSH and LH secretion by follicular fluid in the presence or absence of GnRH. *Neuroendocrinology* **38**, 199–205.

Erickson, G. F. & Hsueh, A. J. W. (1978). Secretion of 'inhibin' by rat granulosa cells *in vitro*. *Endocrinology* **103**, 1960–1963.

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

Hermans, W. P., de Jong, F. H. & Welschen, R. (1981). The possible role of ovarian inhibin-like activity in the regulation of peripheral concentrations of follicle-stimulating hormone. In *Functional morphology of the human ovary*, pp. 85–93. Ed. J. R. T. Coutts. Lancaster: M. T. P. Press Limited.

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

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

de Jong, F. H., Welschen, R., Hermans, W. P., Smith, S. D. & van der Molen, H. J. (1979). Effects of factors from ovarian follicular fluid and Sertoli cell culture medium on in-vivo and in-vitro

release of pituitary gonadotrophins in the rat: an evaluation of systems for the assay of inhibin. *Journal of Reproduction and Fertility* Suppl. **26**, 47–59.

Lee, V. W. K. (1983). PMSG treated immature female rat – A model system for studying control of inhibin secretion. In *Factors regulating ovarian function*, pp. 157–161. Eds G. S. Greenwald & P. F. Terranova, New York: Raven Press.

Lee, V. W. K., McMaster, J., Quigg, H., Findlay, J. & Leversha, L. (1981). Ovarian and peripheral blood inhibin concentrations increase with gonadotropin treatment in immature rats. *Endocrinology* **108**, 2403–2405.

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

Lorenzen, J. R. (1981). The development of sexual maturity in the rat: an alternate hypothesis. In *Dynamics of ovarian function*, pp. 273–278. Eds N. B. Schwartz & M. Hunzicker-Dunn. New York: Raven Press.

Lorenzen, J. R. & Schwartz, N. B. (1979). The differential ability of porcine follicular fluid to suppress FSH in female rats from 6 days of age to adulthood. In *Advances in experimental biology and medicine, Ovarian and corpus luteum function*, vol. 112, pp. 375–380. Eds C. P. Channing, J. Marsh & W. A. Sadler. New York and London: Plenum Press.

Meijs-Roelofs, H. M. A. & Kramer, P. (1979). Maturation of the inhibitory feedback action of oestrogen on follicle-stimulating hormone secretion in the immature female rat: a role for alpha-foetoprotein. *Journal of Endocrinology* **81**, 199–208.

Meijs-Roelofs, H. M. A., Kramer, P. & Gribling-Hegge, L. (1981). Role of progesterone in the control of secretion of follicle-stimulating hormone in the immature female rat. *Journal of Endocrinology* **89**, 99–106.

Meijs-Roelofs, H. M. A., Kramer, P. & Gribling-Hegge, L. (1982*a*). Possible role of 5α-androstane-3α,17β-diol in the control of follicle-stimulating hormone secretion in the immature female rat. *Journal of Endocrinology* **92**, 37–42.

Meijs-Roelofs, H. M. A., Osman, P. & Kramer, P. (1982*b*). Ovarian follicular development leading to first ovulation and accompanying gonadotrophin levels as studied in the unilaterally ovariectomized rat. *Journal of Endocrinology* **92**, 341–349.

Meijs-Roelofs, H. M. A., Uilenbroek, J. Th. J., de Jong, F. H. & Welschen, R. (1973). Plasma oestradiol-17β and its relationship to serum follicle-stimulating hormone in immature female rats. *Journal of Endocrinology* **59**, 295–304.

Nandini, S. G., Lipner, H. & Moudgal, N. R. (1976). A model for studying inhibin. *Endocrinology* **98**, 1460–1465.

Osman, P., Meijs-Roelofs, H. M. A. & Kramer, P. (1982). Compensatory follicle growth and ovulation after unilateral ovariectomy during the late prepubertal period in the rat. In *Development and function of reproductive organs*, ICS no. 599, pp. 299–306. Eds A. G. Byskov & H. Peters. Amsterdam: Excerpta Medica.

Pomerantz, D. K. (1978). Effect of porcine follicular fluid injection on FSH secretion in the immature female rat. *Canadian Journal of Physiology and Pharmacology* **56**, 1076–1078.

Sander, H. J., van Leeuwen, E. C. M. & de Jong, F. H. (1984*a*). Inhibin-like activity in media from cultured rat granulosa cells collected throughout the oestrous cycle. *Journal of Endocrinology* **103**, 77–84.

Sander, H. J., van Leeuwen, E. C. M., de Jong, F. H. & Meijs-Roelofs, H. M. A. (1984*b*). Ovarian inhibin-like activity: Presence in granulosa cell conditioned medium of cyclic rats and evidence of secretion in pre-pubertal female rats. *Journal of Endocrinology* **102**, (Suppl.) Abstract No. 92.

Welschen, R., Dullaart, J. & de Jong, F. H. (1978). Interrelationships between circulating levels of estradiol-17β, progesterone, FSH and LH immediately after unilateral ovariectomy in the cyclic rat. *Biology of Reproduction* **18**, 421–427.

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

APPENDIX PAPER IV

# Inhibin increases in the ovaries of female rats approaching first ovulation: relationships with follicle growth and serum FSH concentrations

**H. J. Sander, H. M. A. Meijs-Roelofs, E. C. M. van Leeuwen, P. Kramer and W. A. van Cappellen**

Department of Anatomy, Medical Faculty, Erasmus University, P. O. Box 1738, NL-3000 DR Rotterdam, The Netherlands

RECEIVED 21 January 1986

## ABSTRACT

In order to relate various prepubertal events in a group of 95 late prepubertal female rats, the following data were obtained during the last 10 days before the day of first ovulation: (1) amounts of ovarian inhibin-like activity (ILA) in some animals ($n=47$); (2) size and numbers of healthy (antral) follicles with a volume $\geqslant 100 \times 10^5\ \mu m^3$ (or diameter $\geqslant 260\ \mu m$) present per ovary in their litter-mates ($n=48$); (3) serum FSH concentrations in both groups.

Rats were unilaterally ovariectomized to obtain an ovary for either estimation of ILA content or for histological procedures and counting of follicles. At the time of unilateral ovariectomy they were bled to obtain serum for estimation of FSH concentrations. Rats were kept until the day after the day of first ovulation to determine the time-interval between the day of unilateral ovariectomy and first ovulation. They were studied between 10 and 1 days (days $-10$ to $-1$, maturational age) before first ovulation. In addition, adult cyclic rats were bilaterally ovariectomized on different days of the oestrous cycle for estimation of ovarian ILA content.

The amount of ovarian ILA was estimated in steroid-free ovarian cytosols using an in-vitro bioassay system with dispersed anterior pituitary cells and subsequent measurement of FSH and LH in the spent medium.

The amount of ovarian ILA was about 83 units/ovary from days $-10$ to $-5$, and subsequently increased ($P<0.005$) to reach a maximum on day $-1$, the day of pro-oestrus (213 units/ovary). Inhibin-like activity in adult rat ovaries at pro-oestrus amounted to 374 units/ovary. A significant relationship was found between ovarian ILA content and total volume of follicles of classes III–V ($\geqslant 350 \times 10^5\ \mu m^3$) ($r=0.9683$, $P<0.005$) except for the period between days $-7$ and $-5$ when this volume increased earlier than did the ILA content.

The total volume of all follicles $\geqslant 100 \times 10^5\ \mu m^3$ was steady from days $-10$ to $-7$. On day $-6$ this volume increased, mainly as a result of an increase of total volume of class II follicles. Thereafter, the total volume of follicles in classes III–V started to increase and was maximal on day $-1$, while the total volume of follicles in classes I plus II decreased and reached a minimum on day $-1$.

The serum FSH concentration declined between days $-10$ and $-1$ from 400 to 100 μg/l ($P<0.001$); the presence of follicles of classes III–V was always associated with FSH concentrations $\leqslant 200$ μg/l ($P<0.005$). The presence of class I and II follicles was not related to FSH concentrations. This suggested that mainly follicles of classes III–V contribute to ovarian ILA.

The present data show that in immature rats ovarian ILA content increases towards the day of first pro-oestrus, as it does later during pro-oestrus in adult cyclic rats. Inhibin-like activity seems to be produced mainly by follicles of classes III–V which are present in the ovaries during the last 5 days preceding first ovulation. In this same period FSH concentrations are kept within narrow limits ($<200$ μg/l) as is the case during the adult cycle. Thus, ILA probably plays a role in the fine regulation of FSH secretion.

*J. Endocr.* (1986) **111**, 159–166

## INTRODUCTION

The gonadal hormone inhibin, produced in the ovaries by follicular granulosa cells, may be defined as a substance which specifically suppresses pituitary secretion of follicle-stimulating hormone (FSH) (de Jong, 1979; de Jong & Robertson, 1985). Its involvement in the process of sexual maturation in the female rat has

already been suggested by Hermans, de Jong & Welschen (1981) and Lorenzen (1981). Recently, Sander, Meijs-Roelofs, Kramer & van Leeuwen (1985) reported rising ovarian amounts of inhibin and an increase in its physiological significance (i.e. short-term suppression of FSH secretion) in 18- to 33-day-old female rats. These results offer an explanation for the decrease in the concentration of FSH in serum seen in intact rats after 23 days of age; the decrease cannot be explained by suppression of FSH secretion by circulating steroids only (Meijs-Roelofs & Kramer, 1979; Meijs-Roelofs, Kramer & Gribling-Hegge, 1981, 1982*a*).

The level of ovarian inhibin in 33-day-old rats was found to be about 40 per cent of that seen in adult, cyclic dioestrous rats (Sander *et al.* 1985). It was therefore of interest to study changes of ovarian inhibin content during the subsequent late prepubertal period, the period during which serum concentrations of FSH show a clear negative correlation with age, as related to the day of first ovulation, and during which follicular growth follows a pattern comparable to that seen during the adult cycle (Meijs-Roelofs, Osman & Kramer, 1982*b*).

During the late prepubertal period, exogenous ovarian steriods become less effective in suppressing FSH secretion (Meijs-Roelofs *et al.* 1982*a*). It is possible that this change is accompanied by an increasing role of inhibin-mediated, more subtle, short-term regulation of FSH secretion, enabling fine regulation of follicle growth (Hermans *et al.* 1981; Lorenzen, 1981).

The present study set out to detect changes in ovarian follicular growth, ovarian inhibin content and serum FSH concentrations and their possible interrelationships during the 10 days preceding the day of first ovulation. The experimental approach was based on the findings that unilateral ovariectomy does not disturb sexual maturation and that, in consequence, ovaries removed at unilateral ovariectomy can be allocated to specific days preceding first ovulation (Meijs-Roelofs *et al.* 1982*a*). Part of this work has been reported previously (Meijs-Roelofs & Sander, 1985; Sander & Meijs-Roelofs, 1985).

## MATERIALS AND METHODS

Immature and adult cyclic female rats of a Wistar substrain (R-Amsterdam) were used. They were weaned at 22 days of age and kept in conditions of controlled temperature (23 ± 2 °C) and light (lights on from 05.00 to 19.00 h) and allowed free access to standard dry pellets and tap water. Unilateral ovariectomy was performed on groups of immature rats of 31, 32, 33, 35, 36, 38 or 39 days of age, when their body weights (g) were 64 ± 2·1 (S.E.M.; $n=4$), 68 ± 1·1 ($n=18$), 73 ± 1·0 ($n=16$), 84 ± 1·3 ($n=13$), 80 ± 1·3 ($n=8$), 93 ± 1·3 ($n=19$) and 93 ± 1·7 ($n=17$) respectively. Operations were performed between 08.30 and 11.00 h under ether anaesthesia and ovaries from litter-mates were used at random for estimation of inhibin-like activity (ILA) or histological evaluation. At the time of unilateral ovariectomy blood was collected from all animals by puncture of the ophthalmic venous plexus and allowed to clot. After centrifugation the serum was separated and stored at −20 °C until radioimmunoassay of FSH and luteinizing hormone (LH).

The animals were checked daily for vaginal opening and occurrence of first ovulation (Meijs-Roelofs *et al.* 1982*b*). Rats were then grouped according to age as related to the number of days that had passed between the day of operation and the day at which first ovulation was detected (days −10 to −1, maturational age), aiming at 6–15 rats per group. For comparison, the ovarian content of ILA was estimated in adult female rats aged 60—90 days after at least two consecutive 5-day oestrous cycles. Five rats were bilaterally ovariectomized on dioestrus days 1, 2 and 3, prooestrus and oestrus. Their body weight was 196 ± 2·1 g (S.E.M., $n=25$).

The ovaries (left or right, at random) were treated as follows. Those destined for histology were dissected out, fixed in Bouin's fluid and embedded in paraffin wax after routine histological procedures. Morphometric estimation of follicle volumes and numbers was performed as described by Meijs-Roelofs *et al.* (1982*b*). Follicle volumes were classified as follows: class I, 100–200 × $10^5$ μm$^3$; class II, 200–350 × $10^5$ μm$^3$; class III, 350–500 × $10^5$ μm$^3$; class IV, 500–1000 × $10^5$ μm$^3$; class V, ≥ 1000 × $10^5$ μm$^3$. No data on atretic follicles have been included in the present paper. Ovaries destined for estimation of inhibin content were homogenized in 1 ml culture medium (see below) without fetal calf serum and centrifuged at 100 000 *g* for 60 min at 4 °C. The supernatant was pipetted off and stored at −20 °C until bioassay.

### Assay of ILA using dispersed pituitary cells in culture

Anterior pituitary cells were obtained as described by Hermans, van Leeuwen, Debets *et al.* (1982*b*), and modified by Sander *et al.* (1985). In short, anterior pituitary glands were minced, treated with Dispase II (Boehringer-Mannheim, Mannheim, F.R.G.) and dispersed in a Dounce homogenizer. Cell number and viability, determined with trypan blue exclusion, were 1·2 ± 1 × $10^6$ cells/ml and 73 ± 4% (S.E.M., $n=8$) respectively and 0·1 ml cell suspension was added to each well containing 0·9 ml culture medium. Cells were cultured in a moist chamber at 37 °C in an atmosphere

of 5% $CO_2$/95% air. For further details see Sander, van Leeuwen & de Jong (1984).

After 3 days of culture the spent medium was discarded and replaced by 1 ml fresh medium containing either the supernatants to be tested at two or three dose levels, each in triplicate or quadruplicate, or standard (charcoal-treated bovine follicular fluid (bFF) diluted 1:1000 with culture medium) at three doses in quadruplicate. After a further 3 days of culture the spent media were collected and stored at −20 °C until assayed for FSH and LH.

In the bioassay, calculation of potencies relative to the internal standard, 95% confidence limits and analysis of linearity and parallelism were based on the method of Finney (1964) as elaborated by Borth (1976), using a computer program kindly provided by Dr H. W. G. Baker, Howard Florey Institute of Experimental Physiology and Medicine, Victoria, Australia. Conversion to units/ml was performed as described by Sander *et al.* (1985). The index of precision of the bioassay ranged from 0·0789 to 0·1846 (mean 0·1284±0·0126, $n=8$). No significant deviations of parallelism or linearity were seen. The sensitivity of the bioassay was 0·78 units/ml or 12 µl bFF/l; 1 unit of ILA equals 1 µg bFF-protein or 0·016 µl bFF.

### Removal of steroids

Before bioassay all homogenates were treated with dextran-coated charcoal (0·18%, w/v) with the same efficiency as described by Sander *et al.* (1984), and then sterilized by filtration.

### Hormone determinations

Concentrations of FSH and LH in serum or in spent pituitary cell culture medium were estimated in duplicate by radioimmunoassay with anti-ovine FSH or LH as antiserum and rat FSH or LH as tracer, and expressed as µg NIADDK rat FSH or LH-RP-1/l, as described by Welschen, Osman, Dullaart *et al.* (1975), with the following modification: tracer FSH and LH were labelled by the addition of 10 mg Protag-125/ml (J. T. Baker, Deventer, The Netherlands), following the supplier's protocol. Within and between assay variations and precision index were 15%, 15% and 0·0868±0·0082 ($n=8$) for FSH and 12%, 26% and 0·0870±0·0068 ($n=8$) for LH. Detection limits of the assay were 8 and 16 µg/l for FSH and LH respectively.

### Statistical analysis

Analysis of variance was performed for overall differences in ILA, total follicle volumes and FSH data. Next, for all three the line(s) of best fit were calculated with the sum of squares method followed by the *F*-test; regression analysis followed by the *F*-test was also used. In two cases, Wilcoxon's two-sample test was performed. A difference was considered to be significant if the double-tailed probability was 0·05 or less.

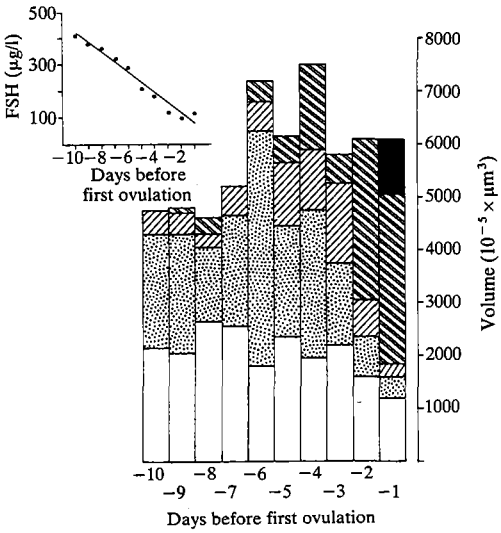



FIGURE 1. Total volume of healthy follicles in classes I (open bars; 100–200 × $10^5$ µm³), II (stippled bars; 200–350 × $10^5$ µm³), III (hatched bars; 350–500 × $10^5$ µm³), IV (double-hatched bars; 500–1000 × $10^5$ µm³) and V (solid bars; ⩾1000 × $10^5$ µm³) on the 10 days before the day of first ovulation in rats and correlation between concentrations of FSH and age expressed as days before the day of first ovulation. The points represent mean FSH values per group. For details see text.

## RESULTS

### Ovarian follicle population and serum FSH levels

After measuring and classifying all healthy antral follicles individually in each ovary, the total follicular volume (a possible measure of capacity for inhibin production) per volume class was calculated for each day of the 10-day period preceding the day of first ovulation. Results are shown in Fig. 1. From days −10 to −7 the overall volume of follicles of classes I–V was relatively constant (approx. 4800 × $10^5$ µm³. On day −6 a conspicuous increase in volume of follicles in class II (and in overall follicular volume) was seen. Over the period from days −6 to −1 there was, with

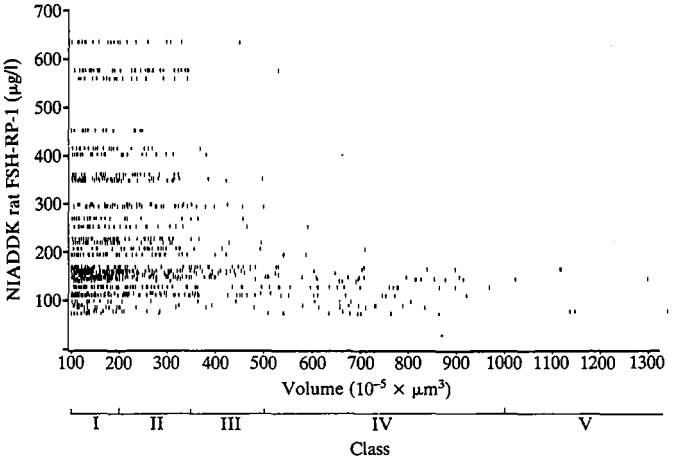



FIGURE 2. Individual concentrations of FSH plotted against volumes of all healthy follicles with a volume $\geqslant 100 \times 10^5$ μm³ present in the same rat. Rats of 10 to 1 days before the day of first ovulation were studied ($n=48$).

an interruption on day −3, a steady increase in the total volume of follicles in classes III–V (from 1000 to $4400 \times 10^5$ μm³). On a day-to-day basis this increase was significant only from days −5 to −4 (Wilcoxon's two-sample test). Analysis of variance ($P<0{\cdot}005$) and regression analysis also showed that the total volume of follicles in classes III–V increased significantly over the period between days −10 and −1 (from 400 to $4400 \times 10^5$ μm³: $r=0{\cdot}7586$, $P<0{\cdot}001$; $n=48$). Although the sum of squares (SS) was smaller when two lines were fitted (days −10 to −7 and −6 to −1) than when one line was fitted (days −10 to −1) the difference failed to reach significance.

The follicle measurements also show a clear decrease in the total volume of classes I and II from days −6 to −1 (from 4300 to $1600 \times 10^5$ μm³; $P<0{\cdot}005$).

Figure 1 also shows the mean FSH concentrations plotted against age as related to first ovulation (and the regression line of these values). A significant negative correlation was found ($P<0{\cdot}001$; regression equation: $y=-38x+39$). Note that after day −5 mean serum FSH concentration fell below 200 μg/l.

In Fig. 2 the volumes of all individual healthy follicles with a volume $\geqslant 100 \times 10^5$ μm³ (classes I–V), found in one ovary from each of 48 individual rats with ages between days −10 and −1, are related to the serum FSH concentration in the same animal. Follicles of classes III–V (volume $\geqslant 350 \times 10^5$ μm³) were found in the presence of relatively low serum FSH concentrations and their total volume was inversely correlated with serum FSH levels ($r=-0{\cdot}6036$, $P<0{\cdot}005$, $n=48$). There was no correlation between the total volume of follicles of class I plus II and serum levels of FSH. This suggests that, via production of ILA, it may be the larger (classes III–V) follicles that contribute to the suppression of FSH secretion. For this reason attention was focused on follicle classes III, IV and V. Thus, from here on, follicle data pertain to these classes only. The full results of FSH estimations are presented later.

**ILA of ovarian homogenates**

In immature rats the ovarian content of ILA did not change significantly between days −10 and −6 and averaged 83 units/ovary (Fig. 3). However, ILA increased from day −5 on (day −5 vs day −4, $P<0{\cdot}05$, Wilcoxon's two-sample test) with an interruption on day −3. Calculation of the SS indicated a breaking point between days −5 and −4 and although the SS for two lines of best fit (days −10 to −5 and days −4 to −1) was smaller than that for one line (days −10 to −1), again this difference was not significant. A maximum was reached on day −1 (213 units/ovary), the day of pro-oestrus. Regression analysis of ILA data over the period between days −10 and −1 showed a positive correlation between ILA and maturational age ($r=0{\cdot}5675$ and $P<0{\cdot}001$; $n=47$).

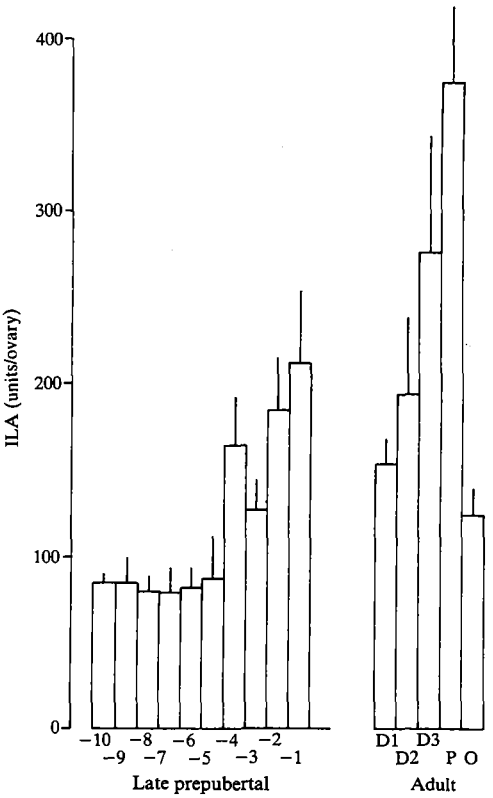



FIGURE 3. Inhibin-like activity (ILA) in the ovaries from immature female rats during the last 10 days before the day of first ovulation (late prepubertal) and from mature rats during the various days of the oestrous cycle. D1, dioestrus day 1; D2, dioestrus day 2; D3, dioestrus day 3; P, pro-oestrus; O, oestrus; −10 to −1, days before the day of first ovulation. Values are means ± S.E.M.; $n = 5$ per day for adults; $n$ for late-prepubertal is as indicated in Fig. 4.

In adult rats there was a clear increase in ovarian ILA content from 156 units/ovary on dioestrus day 1 to 374 units/ovary on pro-oestrus, followed by a sharp decrease to 125 units/ovary on oestrus (Fig. 3). Thus, in the prepubertal rat on the day before the day of first ovulation, the ILA content per ovary is approximately 55% of that found in the adult cyclic female rat on the day of pro-oestrus. Regression analysis showed a positive correlation between mean ovarian ILA levels and the days of the cycle from oestrus to pro-oestrus ($r = 0{\cdot}7950$ and $P < 0{\cdot}001$; $n = 25$).

**Serum concentration of FSH, ovarian ILA content and total volumes of follicles in classes III–V**

The serum FSH concentration gradually declined between days −10 and −2 (415–100 μg/l) (Fig. 4). The apparently steeper decline between days −6 and −5 failed to reach significance. The FSH value on day −3 (123 μg/l) was significantly higher than that on day −2 (100 μg/l), whereas on day −1 a small but significant increase in concentration of FSH (117 μg/l) was again seen. Regression analysis of FSH data over the period between days −10 and −1 showed a significant decrease of FSH concentration with time ($r = -0{\cdot}7160$, $P < 0{\cdot}001$; $n = 95$). The coefficient of variation of the mean FSH values in the period between days −10 and −6 was larger ($P < 0{\cdot}05$, Z-test) than in the period between days −5 and −1.

A clear parallel ($r = 0{\cdot}9683$, $P < 0{\cdot}005$; $n = 10$) rise of mean ILA values and of the mean total volume of follicles of classes III–V can be seen in Fig. 4 for the period between days −10 and −1. There were, however, two exceptions: the increase in follicular volume from days −7 to −6 and, to a lesser extent, from days −6 to −5, which apparently preceded the increase in ILA content.

The serum concentration of FSH was negatively correlated with increasing maturational age, the total volume of follicle classes III–V and ovarian content of ILA ($r = -0{\cdot}3993$, $P < 0{\cdot}005$; $n = 47$).

## DISCUSSION

This paper describes changes in the amount of ILA measured in ovaries of late prepubertal rats during the 10 days preceding the day of first ovulation. In addition, relationships of ILA values with follicle development and with changes in serum FSH concentrations were found in immature rats where ovarian ILA content, after reaching a level of about 155 units/ovary at 33 days of age (Sander *et al.* 1985), increased towards the day of first pro-oestrus, when a level of 213 units/ovary was reached. This level remained below that seen during pro-oestrus in adult cyclic rats (374 units/ovary). The approximately 1·8-fold increase in ILA in the ovaries of adult compared with pubertal pro-oestrous rats may be explained by the observation that the total volume of follicles (number × average class volume) of classes III–V in ovaries of adult pro-oestrous rats is about 1·8 times greater than that found in the ovaries of pubertal pro-oestrous rats (Meijs-Roelofs *et al.* 1982*b*).

The decline of serum FSH concentration found here during the last 10 days before the day of first ovulation confirms earlier findings by Meijs-Roelofs *et al.* (1982*b*). Taken together these results suggest that in late pubertal female rats the decline of serum

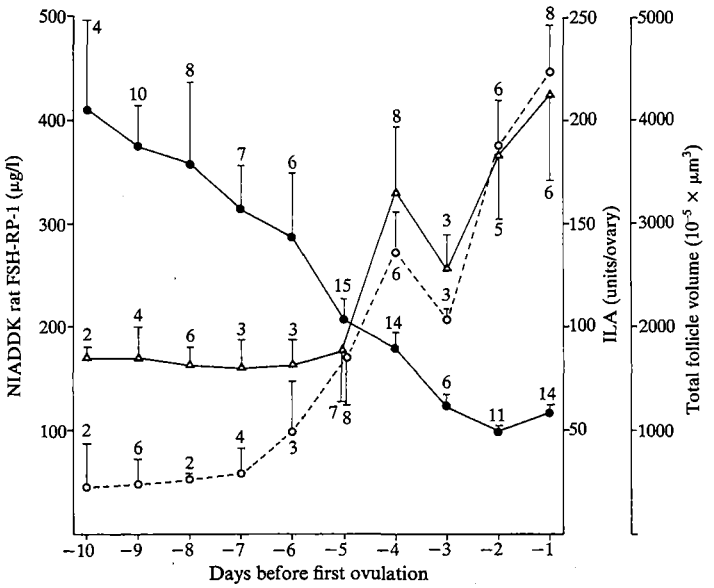



FIGURE 4. Concentrations of FSH in peripheral plasma (●), content of inhibin-like activity (ILA) in charcoal-treated ovarian homogenates (△) and total volume of all healthy follicles (classes III–V) with a volume $\geqslant 350 \times 10^5$ μm³ per ovary (○) on the 10 days before the day of first ovulation in rats. Values are means ± S.E.M.; numbers of rats are given for each point.

FSH concentration, measured daily between 10.00 and 11.00 h from day −10 onwards, plateaus 2 days before the day of first ovulation and may reach its minimum at day −2. These results contradict earlier findings by Ojeda, Wheaton, Jameson & McCann (1976) that FSH remains uniformly low in prepubertal rats until the preovulatory surge on the afternoon of first pro-oestrus. This difference is most likely to be due to the more precise estimation of maturational age (i.e. related to the day of first ovulation) in our studies.

Levels of ILA correlate significantly with the total volume of follicles with a volume $\geqslant 350 \times 10^5$ μm³ (classes III–V) which increases after day −6. It is remarkable that around the same time (after day −6) serum FSH reaches levels equal to or below 200 μg/l with much less ($P < 0{\cdot}05$) fluctuation than during the preceding period. Thus, a situation comparable to that during the adult cycle, with relatively low FSH concentrations ($\leqslant 200$ μg/l) varying within narrow limits (Hermans, Debets, van Leeuwen & de Jong, 1982*a*), then develops for the first time at this stage in pubertal rats. This strongly suggests that ovarian ILA plays a role in the fine regulation of serum FSH during these last 5 days preceding first ovulation. As Hermans *et al.* (1981) and Lorenzen (1981) have already suggested, this strict regulation of FSH concentration may be a prerequisite to achieve the appropriate number of ovulating follicles.

In adult rats a relationship between ILA and antral follicles has been suggested previously (Welschen, Hermans & de Jong, 1980), i.e. between numbers of follicles of classes II–V and ILA. However, the present study, in which follicle growth, ILA and FSH were measured in the same group of rats, strongly suggests correlation between total volume of follicles of classes III–V and ILA. The coefficient of correlation for the relationship between ILA and follicles of classes II–V was 0·6237, while that for the relationship between ILA and follicles of classes III–V was 0·9683.

The increase in adult rat ovarian ILA content (approximately 2·9-fold) from oestrus to the following pro-oestrus is not reflected in proportional decreases in serum FSH concentrations (Hermans *et al.* 1982*a*). This may be explained by a divergence between increase of ovarian content and ovarian release of ILA, since the amount of ILA in ovarian venous plasma appears to be steady, except for a decline in the periovulatory

period (14.00 h on pro-oestrus–16.00 h on oestrus; DePaolo, Shander, Wise *et al.* 1979); Tsukamoto, Taya, Watanabe & Sasamoto (1986) report a very small, gradual increase of ILA in ovarian venous plasma from dioestrus day 1 to pro-oestrus (11.00 h) followed by a decline in the periovulatory period. In pubertal rats a similar divergence between content and release might account for the discordance between the steady level of ovarian ILA content and the decline in serum FSH concentration in the period between days −10 and −6, although this may also be explained in part by assuming increasing responsiveness of the pituitary to ILA-mediated suppression of FSH secretion (Hermans, van Leeuwen, Debets & de Jong, 1980). Various studies suggest that the decline of serum FSH concentration over this period cannot be attributed to increased suppression through ovarian steroids (Meijs-Roelofs *et al.* 1981, 1982*a*; Uilenbroek, Woutersen & van der Linden, 1983). In the present study oestradiol levels showed little change over the period from day −10 to day −3 ($\leqslant$40 ng/l) and a significant increase to levels $\geqslant$50 ng/l was only found on days −2 and −1 (unpublished results from our laboratory). Other steroids, such as progesterone and androstanediol, have little, if any, effect on the regulation of serum FSH at this age (Meijs-Roelofs *et al.* 1981, 1982*a*).

Another topic that warrants discussion is the increasing volume of follicles in classes III–V over the period between days −7 and −5, as opposed to the steady level of ovarian ILA content. A possible explanation is that during the initial growth phase of the first follicles in classes III–V, the production of structural proteins prevails over the production of secretory proteins in the granulosa cells (in-vitro follicles: Hillier, Zeleznik & Ross, 1978; in-vitro granulosa cells: Orly, Sato & Erickson, 1980).

Taken together, the present data show that in pubertal female rats the amount of ovarian ILA increases towards the day of first pro-oestrus, when a maximum is reached (comparable to that at adult pro-oestrus) and that this increase of ovarian ILA with time is closely related with the development of follicles with a volume $\geqslant 350 \times 10^5$ μm$^3$. Furthermore, between days −5 and −1 (c.f. days −10 to −6), the increase in ILA apparently causes a more precise regulation of serum FSH concentrations, which reached levels (approximately 100 μg/l) which were also found during adult dioestrus. This fine regulation at levels below 200 μg/l is possibly due to inhibin released by follicles of classes III–V and might serve to usher the large preovulatory follicles, which are destined to ovulate, to the point of ovulation while maintaining FSH within narrow limits, as during the adult cycle, and keeping the number of ovulatory follicles within the normal range.

## ACKNOWLEDGEMENTS

The authors wish to thank Mrs L. Gribling-Hegge for measuring oestradiol levels and Drs F. H. de Jong and J. Moll for their criticism of the manuscript. They are grateful to the NIADDK, Bethesda, MD, U.S.A. for the gonadotrophins used in the radioimmunoassay.

## REFERENCES

Borth, R. (1976). Statistics of parallel line assay. In *Methods of Hormone Analysis*, pp. 500–513. Eds H. Breuer, D. Hamel & H. L. Krüskemper. Stuttgart: Georg Thieme Verlag.

DePaolo, L. V., Shander, D., Wise, P. M., Barraclough, C. A. & Channing, C. P. (1979). Identification of inhibin-like activity in ovarian venous plasma of rats during the estrous cycle. *Endocrinology* **105**, 647–654.

Finney, D. J. (1964). Parallel line assays. In *Statistical Methods in Biological Assays*, pp. 101–138. Ed. D. J. Finney. London: Griffin.

Hermans, W. P., Debets, M. H. M., van Leeuwen, E. C. M. & de Jong, F. H. (1982*a*). Effects of single injections of bovine follicular fluid on gonadotrophin concentrations throughout the oestrous cycle of the rat. *Journal of Endocrinology* **92**, 425–432.

Hermans, W. P., de Jong, F. H. & Welschen, R. (1981). The possible role of ovarian inhibin-like activity in the regulation of peripheral concentrations of follicle-stimulating hormone. In *Functional Morphology of the Human Ovary*, pp. 85–93. Ed. J. R. T. Coutts. Lancaster: MTP Press Ltd.

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

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

Hillier, S. G., Zeleznik, A. J. & Ross, G. T. (1978). Independence of steroidogenic capacity and luteinizing hormone receptor induction in developing granulosa cells. *Endocrinology* **102**, 937–946.

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

de Jong, F. H. & Robertson, D. M. (1985). Inhibin: 1985 update on action and purification. *Molecular and Cellular Endocrinology* **42**, 95–103.

Lorenzen, J. R. (1981). The development of sexual maturity in the rat: an alternate hypothesis. In *Dynamics of Ovarian Function*, pp. 273–278. Eds N. B. Schwarz & M. Hunzicker-Dunn. New York: Raven Press.

Meijs-Roelofs, H. M. A. & Kramer, P. (1979). Maturation of the inhibitory feedback action of oestrogen on follicle-stimulating hormone secretion in the immature female rat: a role for alpha-foetoprotein. *Journal of Endocrinology* **81**, 199–208.

Meijs-Roelofs, H. M. A., Kramer, P. & Gribling-Hegge, L. (1981). Role of progesterone in the secretion of follicle-stimulating hormone in the immature female rat. *Journal of Endocrinology* **89**, 99–106.

Meijs-Roelofs, H. M. A., Kramer, P. & Gribling-Hegge, L. (1982*a*). Possible role of 5α-androstane 3α,17β-diol in the control of follicle stimulating hormone secretion in the immature female rat. *Journal of Endocrinology* **92**, 37–42.

Meijs-Roelofs, H. M. A., Osman, P. & Kramer, P. (1982*b*). Ovarian follicular development leading to first ovulation and accompanying gonadotrophin levels as studied in the unilaterally ovariectomized rat. *Journal of Endocrinology* **92**, 341–349.

Meijs-Roelofs, H. M. A. & Sander, H. J. (1985). Ovarian follicle growth, FSH and ILA approaching puberty. *Meeting of the Society for the Study of Fertility*, December 1985. Abstract No. 31.

Ojeda, S. R., Wheaton, J. E., Jameson, H. E. & McCann, S. M. (1976). The onset of puberty in the female rat: changes in plasma prolactin, gonadotropin, luteinizing hormone-releasing hormone (LHRH), and hypothalamic LHRH content. *Endocrinology* **98,** 630–638.

Orly, J., Sato, G. & Erickson, G. F. (1980). Serum suppresses the expression of hormonally induced functions in cultured granulosa cells. *Cell* **20,** 817–827.

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

Sander, H. J. & Meijs-Roelofs, H. M. A. (1985). Ovarian inhibin-like activity (ILA) in prepubertal female rats. *Meeting of the Society for the Study of Fertility*, December 1985. Abstract No. 30.

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

Tsukamoto, I., Taya, K., Watanabe, G. & Sasamoto, S. (1986). Inhibin activity and secretion of gonadotropin during the period of follicular maturation. *Life Sciences*. (In Press).

Uilenbroek, J. Th. J., Woutersen, P. J. A. & van der Linden, R. (1983). Steroid production *in vitro* by rat ovaries during sexual maturation. *Journal of Endocrinology* **99,** 469–475.

Welschen, R., Hermans, W. P. & de Jong, F. H. (1980). Possible involvement of inhibin in the interrelationship between numbers of antral follicles and peripheral FSH concentrations in female rats. *Journal of Reproduction and Fertility* **60,** 485–492.

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

APPENDIX PAPER V

# ESTIMATION OF INHIBIN-LIKE ACTIVITY IN SPENT MEDIUM FROM RAT OVARIAN GRANULOSA CELLS DURING LONG-TERM CULTURE

W.P. HERMANS, E.C.M. van LEEUWEN, M.H.M. DEBETS, H.J. SANDER and [a] F.H. de JONG

*Departments of Anatomy and of [a] Biochemistry (Division of Chemical Endocrinology), Medical Faculty, Erasmus University, Rotterdam (The Netherlands)*

Received 1 February 1982; revision received 26 April 1982; accepted 26 April 1982

Granulosa cells were isolated from antral follicles of intact, adult female rats by treatment with EGTA and hypertonic sucrose, and were kept in culture for 1, 15 or 37 days. The amount of inhibin-like activity in spent rat granulosa-cell media (rGCCM) was assessed by using a bioassay for inhibin (measurement of the unstimulated release of FSH and LH by pituitary cells in culture).

After 1 day in culture, inhibin-like activity was detected in rGCCM of $0.016–0.8 \times 10^5$ cultured granulosa cells per dish. In the bioassay, maximal suppression of FSH occurred when $0.8 \times 10^5$ granulosa cells were plated. With this or a larger number of cells in long-term cultures, pooled rGCCM, collected up to 37 days after plating, suppressed levels of FSH (and not of LH) in the inhibin bioassay in parallel with the suppression found after addition of charcoal-treated bovine follicular fluid (bFF), which served as a standard. The amount of inhibin-like activity in rGCCM (relative to the amount present in bFF, which was given a potency of 1) ranged between 0.05 and $0.90 \times 10^{-3}$. Release of inhibin-like activity could not be estimated on day 4, and was low from days 29 to 37 in culture. Except after 1 day of culture, no substantial amounts of progesterone were detected in the media.

It is concluded that granulosa cells collected from adult, intact female rats have and retain the capacity to secrete inhibin-like activity in vitro, under basal conditions in long-term cultures.

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

An increasing number of studies indicates 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. An inhibin-like factor is secreted in vivo by ovaries that 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, in vitro, inhibin-like activity was observed in ovarian venous plasma of cyclic rats (DePaolo et al., 1979). Healthy, antral follicles might be the ovarian compartments responsible for the production of inhibin, because in a number of situations in the rat, inverse relationships were found between peripheral concentrations of FSH (but not of luteinizing hormone, LH), and numbers of medium and large antral follicles (Welschen et al., 1978, 1980). Further, follicular fluid of many species contains inhibin-like activity. (Reviews: Hermans et al., 1981; Channing et al., 1981.) Moreover, less inhibin-like activity was detected in follicular fluid of cystic or atretic follicles (Welschen et al., 1977; Channing et al., 1981). Finally, Erickson and Hsueh (1978) found that spent media of cultured rat granulosa cells, collected from adult pro-oestrus rats or from oestrogen-primed hypophysectomized 25-day-old rats, contain 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-like activity in vitro.

The present report describes a study on the relationship between the amount of inhibin-like activity present in spent media from rat granulosa cells collected from intact, adult female rats and the total duration of the culture period. Inhibin-like activity was measured, in vitro, in a bioassay system (de Jong et al., 1979).

## MATERIALS AND METHODS

### *Animals*

Adult female rats of a Wistar substrain (R-Amsterdam) were used for the collection of ovaries and pituitary glands. The rats were kept under controlled conditions of light (light period 0500–1900 h) and temperature (22–24°C) 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 were used per experiment: the ovaries were removed, trimmed free of fat and connective tissue and placed in buffer. For the collection of pituitary glands, 12–15 rats were decapitated per experiment. After removal of the posterior lobes, the hypophyses were also placed in buffer.

### *Buffers and media*

Buffers and media were prepared freshly from stock solutions that were stored in a refrigerator for not more than 2–3 weeks. All buffers

and media were sterilized by passing them through 0.2 $\mu$m pore size Acrodisc filters (Gelman, Ann Arbor, MI, U.S.A.). The composition of the buffers and media were as follows.

*Granulosa-cell isolation.* F-12 stock: medium F-12 (Boehringer, Mannheim, BRD), supplemented with 10 mM Hepes (Gibco, Grand Island, NY, 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–DNAase solution: F-12 stock supplemented with 0.25% bovine serum albumin (BSA) (Organon, Oss, The Netherlands) and 0.001% deoxyribonuclease (DNAase) (Sigma, St. Louis, MO, U.S.A.). All solutions were adjusted with 1 N NaOH to pH 7.2–7.4.

*Pituitary cell isolation.* Buffered HBSS: Hank's Balanced Salt Solution (Gibco) containing 3.5% sodium bicarbonate (Gibco). Dispase solution: HBSS containing 2.4 units Dispase II (Boehringer). HBSS-BSA: HBSS containing 1% BSA (Organon).

*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 100000 units of penicillin, 100 mg streptomycin and 250 $\mu$g 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°C in a water-bath.

Granulosa cells were obtained by 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°C and rinsed with fresh F-12 stock. After the incubation, antral follicles were harvested de visu under a dissecting microscope with sterile injection needles (19 gauge). The follicles were collected in F-12 stock. The isolated follicles were incubated for 5 min at 37°C 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 DNAase solution with 19 gauge needles under a dissecting microscope. The clumps of cells released were collected in a tube containing DNAase solution and washed twice with fresh DNAase solution, each time ending by centrifugation for 5 min at 100 *g*. The final cell pellet was broken by a finger flip, and suspended in fresh F-12 stock.

The number of viable cells present in the solution was counted in a haemacytometer with 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 single or in clumps of up to 6 cells. Aliquots (100 $\mu$l) of the cell suspension were added to petri dishes (Falcon plastics, Oxnard, CA, U.S.A., 35 mm × 10 mm), containing 2 ml supplemented MEM. The dishes were placed in an incubator under 95% air : 5% $CO_2$ at 32°C. Cells usually 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 × 20). Spent rat granulosa cell culture medium (rGCCM) from dishes that had been cultured for the same period was pooled and stored frozen (−20°C) until processed. Some batches of rGCCM were extracted with 2 vol. of ether–chloroform (1 : 1 v/v) and 2 vol. of ether; excess of ether was evaporated in air (Franchimont et al., 1975). This method was adopted because 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 by 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 water-bath. After the incubation, tissue fragments were washed again in fresh HBSS–BSA, placed in a conically shaped douncer and dispersed.

For counts of viable cells, the cell suspension was counted in a haemocytometer, with trypan blue exclusion, and was diluted with supplemented MEM to provide the desired number of viable cells (2.0–5.0 × $10^5$ viable cells/100 $\mu$l). Aliquots of 100 $\mu$l were pipetted into 64–96 Falcon petri dishes (35 mm × 10 mm), 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 test substances (rGCCM) or standard preparations containing inhibin-like activity (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 FSH and LH.

*Hormone determinations*

Concentrations of FSH and LH in spent media from cultured pituitary cells were estimated in duplicate by radioimmunoassay as described by Welschen et al. (1975). Gonadotrophin concentrations in all samples obtained from one pituitary cell culture were measured in the same assay. 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 that contained only pituitary cells and supplemented MEM.

Concentrations of inhibin-like activity in rGCCM were calculated by mesauring the suppression of the release of FSH from pituitary cells after addition of various volumes of rGCCM, by 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 those preparations of rGCCM that suppressed the 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-like activity.

Concentrations of progesterone (P) in rGCCM were estimated by the radioimmunoassay described by de Jong et al. (1974). The detection limit of the assay was 0.10 ng/ml.

*Statistical procedures*

The significance of differences between the concentrations of FSH and LH found after addition of test substances, and the concentrations of FSH and LH found in control dishes containing supplemented MEM only was assessed with Student's *t*-test. Differences were considered significant when $P \leq 0.05$ (2-tailed). In the bioassay, calculation of rela-

tive potencies and 95% confidence limits and analysis of linearity and parallelism were based on the method of Finney (1964).

## RESULTS

In the first experiment, addition of 400 μl rGCCM obtained after a 1-day incubation of various amounts of granulosa cells caused a decrease in the basal release of FSH by the pituitary cells (Fig. 1A). The suppression 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-like activity or steroids in rat follicular fluid that was still present in the granulosa-cell suspension after the isolation procedure, granulosa cells were subsequently cultured for longer periods.

In a second experiment, $1.1 \times 10^5$ viable granulosa cells per dish (total 13 dishes) were cultured for a total of 15 days. Spent media from the 13 dishes were collected and pooled daily from days 1 to 11, and at days 13 and 15. After 1 day in culture, the cells were well plated, angular and dividing. After 2 days in culture, they had an oval appearance. From days 3 till 6 they were elongating until after 7 days in culture the dishes were filled with a monolayer of elongated, fibroblast-like cells. No further change in shape was observed, but in many cells lipid-like droplets started to appear. At days 13 and 15 some cells contained two nucleoli. At day 16 the experiment was stopped because of contamination.

When the spent media of the cells were added to pituitary cell cultures, a dose-dependent suppression of the release of FSH, which was parallel with bFF-induced suppression, was observed. Only rGCCM obtained on the 4th culture day did not suppress concentrations of FSH. Fig. 2 shows 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 dose-dependent effect on concentrations of LH after addition of rGCCM was observed, although the concentrations of LH were raised by 20 out of 54 additions.

To exclude the possibility that the suppression of FSH was caused by steroids released by the cultured granulosa cells, some batches of rGCCM were extracted with ether–chloroform before addition to the pituitary cells. As a measure for the efficacy of the extraction, progesterone levels were measured. Before the extraction, levels of progesterone were 4.6,

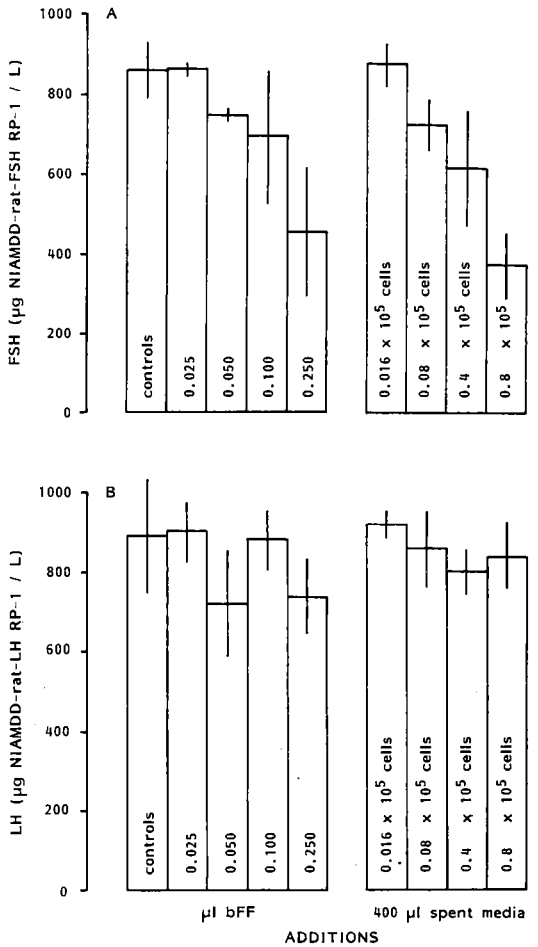



Fig. 1. Mean (±S.D., $n=4$) concentrations of (A) FSH and (B) LH in spent media from pituitary cells in culture without additions (controls), after addition of 0.025–0.250 µl bFF (standard) or after addition of 400 µl spent rat granulosa-cell culture medium. Various numbers of cells were plated (ranging from 0.016 to $0.8\times10^5$ viable cells/dish); spent media were collected after 1 day in culture.

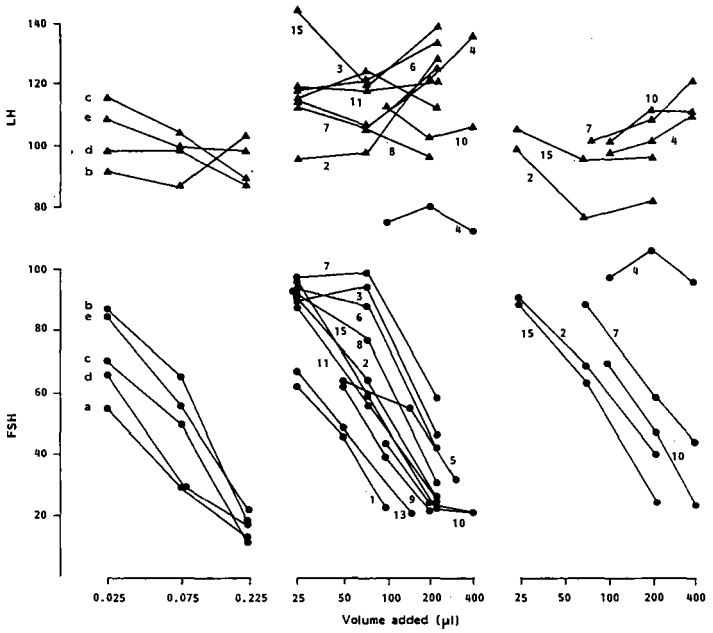



Fig. 2. Mean concentrations of FSH (●) and LH (▲) in spent media from pituitary cells in culture after addition of 0.025–0.225 µl bFF (standard) or addition of various amounts (ranging from 10 to 400 µl/dish) of spent rat granulosa-cell culture media (rGCCM), either untreated or ether–chloroform treated. Rat granulosa-cell media were obtained daily from $1.1 \times 10^5$ 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; for the sake of clarity, S.D. are omitted. The abscissa has a logarithmic scale. The bFF suppression lines correspond with the following preparations: a, days 1, 5, 9, 13; b, days 2, 6, 15; c, days 3, 4, 7; d, days 8, 10, 11; e, days 2, 4, 7, 10, 15, ether–chloroform-treated media.

0.78, 0.12 and 0.15 ng/ml at days 1, 2, 5 and 10, respectively. At all other days and after extraction, progesterone could not be detected in the media. 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

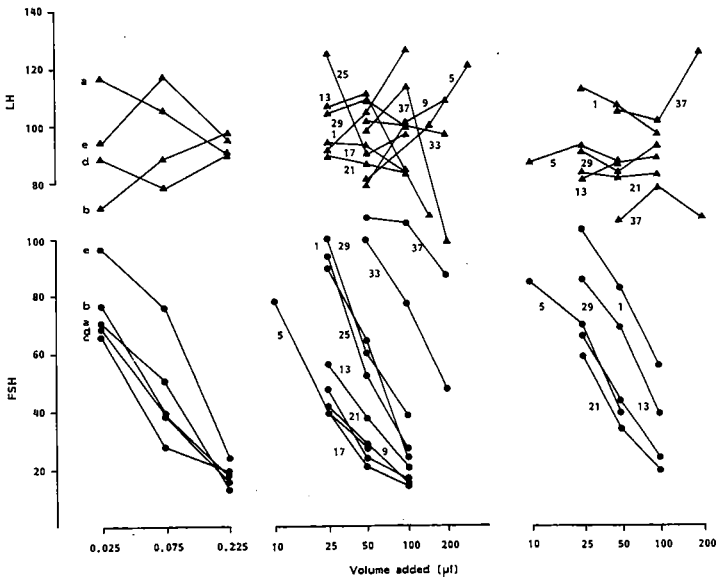



Fig. 3. For details see Fig. 2. In this experiment, rat granulosa-cell media were obtained from $0.7\times10^5$ plated cells/dish, renewed after 1 day in culture and subsequently every other 4 days; cells were kept in culture for a total of 37 days. The bFF suppression lines correspond with the following preparations: a, day 1, both untreated and ether–chloroform treated; b, days 5 (LH), 9, 13; c, day 5 (FSH); d, days 17, 21, 25, 29, 33, 37; e, days 5, 13, 21, 29, 37, ether–chloroform-treated media.

concentrations of LH were not systematically affected (Fig. 2). These results indicate that rGCCM contains inhibin-like activity. Results of the calculations of the amount of inhibin-like activity in rGCCM, relative to that in bFF, are shown in Fig. 4. To study the influence of a longer culture period, this experiment was repeated and extended in a 3rd experiment.

In this 3rd experiment, $0.7\times10^5$ viable granulosa cells per dish (total 10 dishes) were cultured for a total of 37 days. Media of the 10 dishes were renewed and pooled after 1 day in culture and subsequently every other 4 days. Cytological changes during the first 15 days were comparable to those observed in the second experiment; from day 17 onwards an

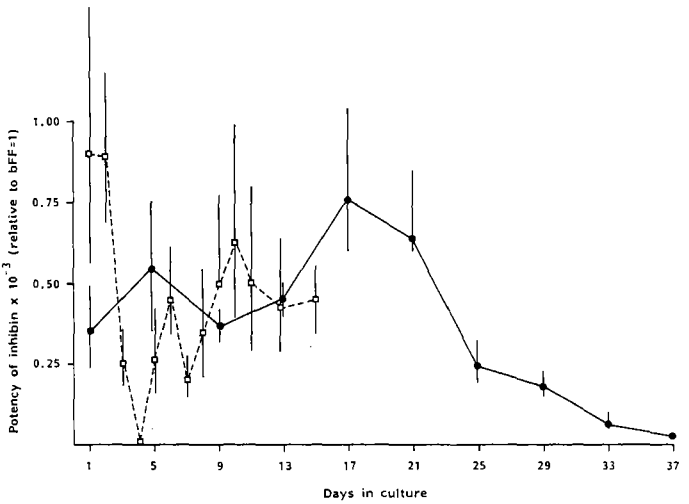



Fig. 4. Relative amount of inhibin-like activity in untreated spent rat-granulosa cell culture media (rGCCM) obtained from $1.1\times10^5$ cells (□) or from $0.7\times10^5$ cells (●)/dish and collected after various days in culture. The amount of inhibin-like activity is expressed as daily release of inhibin-like activity relative to the amount present in bFF (standard, defined to have a potency of 1) and calculated from the data shown in Figs. 2 and 3. Vertical lines represent 95% confidence limits. The numbers on the abscissa indicate the days on which media were collected.

increase in 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. At this stage, the experiment was stopped.

Concentrations of FSH and LH in the spent media from pituitary cells after addition of rGCCM, expressed as percentages of mean values found in appropriate control dishes, are shown in Fig. 3. The index of precision of the 5 bioassays used in this experiment ranged between 0.07 and 0.09. As in the 2nd 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, the 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 at day 1 in culture were 0.55 and 0.20 ng/ml before and after ether–chloroform treatment,

respectively. At the other days, levels of progesterone were below the detection limit of the assay. The ether–chloroform-treated spent granulosa cell media also suppressed concentrations of FSH in parallel with the suppression obtained after addition of untreated rGCCM or bFF. The amount of inhibin-like activity (Fig. 4) was dependent on the total duration of the experimental period, with the highest amounts present in media from culture days 2–5, 14–17 and 18–21, after which period the amount of inhibin-like activity started to decline.

Comparison of the amount of inhibin present in untreated versus ether–chloroform-treated rGCCM showed a linear relationship ($y = -0.17 + 1.51\ x$; $n = 9$; $r = 0.97$); the values for biopotencies of inhibin-like activity before and after ether–chloroform treatment did not differ significantly (paired $t$ test). Therefore it was assumed that the ether–chloroform treatment did not affect the content of inhibin-like activity in rGCCM, so that the values thus obtained were used for calculation of the coefficient of variation of the inhibin bioassays (34%; $n = 9$).

## DISCUSSION

When conventional methods, such as application of physical pressure on the surface of the ovary, are used to isolate granulosa cells from the ovaries of intact, non-oestrogen primed rats, cell yield (15–25% viable cells), as well as 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 synthesis, in vitro, of proteins, RNA and DNA (Campbell, 1979). Also, in our hands an adaptation of this 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. Foetal calf serum (FCS) was used to facilitate attachment and optimal growth of the cells (Barnes and Sato, 1980; Orly et al., 1980); a concentration of 10% FCS was chosen to maintain a final concentration of 10% FCS in the dishes with pituitary cells after addition of rGCCM.

By using this isolation procedure, we could see 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 the results of Erickson and Hsueh (1978). On the basis of this experiment, we chose $0.8–1.0 \times 10^5$ viable cells per dish as a suitable number for further experiments.

The two following experiments showed that rGCCM contains a factor that fulfills our criteria for inhibin: in the inhibin bioassay, specific suppression of FSH was found in parallel with the standard preparation, even after ether–chloroform treatment. It is not likely that the suppressive effect on levels of FSH was caused by a non-specific factor, because rGCCM collected on day 4 in the 2nd experiment did not exhibit a suppressive action on levels of FSH. Although no systematic changes in levels of LH were observed, 23 out of 102 experimental groups showed elevated levels of LH, and 6 out of 102 groups showed decreased levels of LH. Our data do not indicate a relationship 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 2nd experiment, in which the media were renewed daily, suggest that the presence of inhibin-like activity in rGCCM is due to production of inhibin-like activity, and not to leakage of inhibin-like activity 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-like activity. Thus, rat granulosa cells in culture have and retain the capacity for basal secretion of inhibin-like activity. Also, another cell type capable of secreting inhibin-like activity, the Sertoli cell, sustains release of inhibin-like activity in vitro for 21 days under basal conditions (Steinberger, 1980).

The amount of inhibin-like activity present in rGCCM seemed to change together with morphological changes in the cells. In the 2nd experiment, during the first 5 days of the culture period, when the cells are attaching, spreading and dividing and probably differentiating, a decrease in the amount of inhibin-like activity in rGCCM was observed; thereafter a rather stable production of inhibin-like activity was found until day 15. These results are in good agreement with those of Labrie et al. (1978), who observed a higher production of inhibin-like activity by Sertoli cells in culture from days 5 to 8 than from days 2 to 5 in culture.

The results from the 3rd experiment show a comparable picture when the results are expressed in terms of daily production of inhibin-like activity during the first 14 days. The production of inhibin-like activity 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 predominantly takes place after the cells have reached confluency (Savion and Gospodaro-

wicz, 1980). The period from days 14 to 21 (3rd experiment) shows an increase in the production of inhibin-like activity, followed by a steady decrease which might be caused by ageing and eventual dying of the cells. However, without quantification of the number of viable cells present at the various culture days, such explanations for the differences in the amount of inhibin-like activity found in rGCCM remain speculative.

Whether inhibin-like activity 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, because the composition, in vitro, of the 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 and 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 this discrepancy in progesterone secretion can be found in the isolation procedure: because collection of granulosa cells from large follicles is usually performed by aspiration and scraping of the follicular wall, a minor contamination with theca cells might occur, which may account for the observed progesterone secretion (Koninckx et al., 1981). Thus, the absence of progesterone secretion in this study might reflect the absence of theca cells from the isolated population of granulosa cells, which is in line with the data on the isolation procedure presented by Campbell (1979).

In conclusion, this study shows that granulosa cells of antral follicles of intact rats are capable of production of inhibin-like activity in vitro. The observation that basal release of inhibin-like activity 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 production of inhibin-like activity.

ACKNOWLEDGEMENTS

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

## REFERENCES


Armstrong, D.T., and Dorrington, J.H. (1976) Endocrinology 99, 1411–1414.

Barnes, D., and Sato, G. (1980) Cell 22, 649–655.

Campbell, K.L. (1979) Biol. Reprod. 21, 773–786.

Channing, C.P., Tanabe, K., Guthrie, H.D., Batta, S.K., and Hoover, D.J. (1981) in: Intragonadal regulation of Reproduction, Eds.: P. Franchimont and C.P. Channing (Academic Press, New York) pp. 365–376.

DePaolo, L.V., Shander, D., Wise, P.M., Barraclough, C.A., and Channing, C.P. (1979) Endocrinology 105, 647–654.

Erickson, G.F., and Hsueh, A.J.W. (1978) Endocrinology 103, 1960–1963.

Erickson, G.F., Challis, J.R.G., and Ryan, K.J. (1974) Develop. Biol. 40, 208–224.

Finney, D.J. (1964) Statistical Method in Biological Assay (Griffin, London) pp. 101–138.

Franchimont, P., Chari, S., and Demoulin, A. (1975) J. Reprod. Fert. 4, 335–350.

Franchimont, P., Henderson, K., Verhoeven, G., Hazee-Hagelstein, M.T., Charlet-Renard, Ch., Demoulin, A., Bourguignon, J.P., and Lecomte-Yerna, M.J. (1981) in: Intragonadal Regulation of Reproduction. Eds.: P. Franchimont and C.P. Channing (Academic Press, New York) pp. 167–191.

Hermans, W.P., Welschen, R., and de Jong, F.H. (1981) in: Functional Morphology of the Human Ovary. Ed.: J.R.T. Coutts (M.T.P. Press, Lancaster) pp. 85–93.

Johnson, D.C. (1981) J. Endocrinol. 88, 321–328.

de Jong, F.H., Baird, D.T., and van der Molen, H.J. (1974) Acta Endocrinol. (Copenhagen) 77, 575–587.

de Jong, F.H., Smith, S.D., and van der Molen, H.J. (1979) J. Endocrinol. 80, 91–102.

de Jong, F.H., Jansen, E.H.J.M., Hermans, W.P., and van der Molen, H.J. (1982) in: Intra-Ovarian Control Mechanisms. Eds.: C.P. Channing and S.J. Segal (Plenum, New York).

Koninckx, P.R., and de Moor, P. (1981) in: New Aspects of Ovarian Function in Man and in Rat, P.R. Koninckx, Ph.D. Thesis, Leuven.

Labrie, F., Lagace, L., Ferland, L., Kelly, P.A., Drouin, J., Massicotte, J., Bonne, C., Raynaud, J.P., and Dorrington, J. (1978) Int. J. Androl., Suppl. 2, 81–99.

Liu, W.K., Burleigh, B.D., and Ward, D.N. (1981) Mol. Cell. Endocrinol. 21, 63–73.

Moon, Y.S. (1981) Mol. Cell. Endocrinol. 23, 115–122.

Orly, J., Sato, G., and Erickson, G.F. (1980) Cell 20, 817–827.

Savion, N., and Gospodarowicz, D. (1980) Endocrinology 107, 1798–1807.

Steinberger, A. (1980) in: Endocrinology 1980. Eds.: I.A. Cumming, J.W. Funder and F.A. Mendelssohn (The Australian Academy of Science, Canberra City, Australia) pp. 259–262.

Stoklosowa, S., Gregoraszczuk, E., Holeckova, E., and Michl, J. (1981) Exp. Cell Res. 131, 430–435.

Uilenbroek, J.Th.J., Tiller, R., de Jong, F.H., and Vels, F. (1978) J. Endocrinol. 78, 399–406.

Veldhuis, J.D., Klase, P.A., and Hammond, J.M. (1981) Endocrinology 108, 1463–1468.

Welschen, R., Osman, P., Dullaart, J., de Greef, W.J., Uilenbroek, J.Th.J., and de Jong, F.H. (1975) J. Endocrinol. 64, 37–47.

Welschen, R., Hermans, W.P., Dullaart, J., and de Jong, F.H. (1977) J. Reprod. Fertil. 50, 129–131.

Welschen, R., Dullaart, J., and de Jong, F.H. (1978) Biol. Reprod. 18, 421–427.

Welschen, R., Hermans, W.P., and de Jong, F.H. (1980) J. Reprod. Fertil. 60, 485–493.

# Inhibin-like activity in media from cultured rat granulosa cells collected throughout the oestrous cycle

**H. J. Sander, E. C. M. van Leeuwen** and **F. H. de Jong***

Department of Anatomy, Medical Faculty, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

* Department of Biochemistry (Chemical Endocrinology), Medical Faculty, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

RECEIVED 22 February 1984

## ABSTRACT

Granulosa cells of antral ovarian follicles from adult 5-day cyclic rats were cultured on each day of the cycle. The rat granulosa cell conditioned medium (rGCCM) was harvested and renewed on each day of a 4-day culture period. Inhibin-like activity and progesterone were estimated in rGCCM using an in-vitro bioassay system with dispersed rat anterior pituitary cells and radioimmunoassay respectively. Removal of steroids from rGCCM with dextran-coated charcoal was effective and did not significantly change the inhibin-like activity of the treated samples.

On day 1 of culture the inhibin-like activity of rGCCM for each day of the oestrous cycle was 20–90% higher than on days 2, 3 and 4 of culture when low and constant levels were observed. Media collected after culture on days 1 and 2 from pro-oestrous cells contained larger amounts of inhibin-like activity than media collected on the other days of the cycle.

On day 1 of culture, rGCCM from pro-oestrous cells contained higher concentrations of progesterone than that from cells collected on the other days of the cycle. On days 2, 3 and 4 progesterone levels in rGCCM were undetectable ( < 320 pmol/l) except in media from pro-oestrous cultures on day 2. Addition of FSH (62 μg/l) to granulosa cell cultures in medium with or without 10% fetal calf serum (FCS) did not alter the inhibin-like activity of rGCCM from pro-oestrous cells. The presence of FCS maintained the production of inhibin-like activity since rGCCM from cells cultured without FCS was devoid of FSH-suppressing activity after 3 days of culture.

It is concluded that material with inhibin-like activity is secreted at a higher rate by granulosa cells collected on the day of pro-oestrus than on any other day of the cycle. However, on the day of pro-oestrus, transport of inhibin-like activity from the follicle to the circulation is apparently impeded since this increased inhibin production is not reflected in a reduction in plasma FSH concentration.

*J. Endocr.* (1984) **103**, 77–84

## INTRODUCTION

Inhibin, a gonadal glycoprotein which specifically suppresses the secretion of follicle-stimulating hormone (FSH) from the pituitary gland, is present in the follicular fluid of antral ovarian follicles of several mammalian species (de Jong & Sharpe, 1976; Schwartz & Channing, 1977; Welschen, Hermans, Dullaart & de Jong, 1977; Channing, Tanabe, Guthrie *et al.* 1981). Erickson & Hsueh (1978) demonstrated that granulosa cells from adult pro-oestrous rats secreted inhibin-like activity *in vitro* and Hermans, van Leeuwen, Debets *et al.* (1982*b*) showed that rat granulosa cells *in vitro* can secrete inhibin-like activity for extended periods.

Moreover, in the adult cyclic rat a relationship exists between the phase of the oestrous cycle and peripheral levels of FSH (see Nequin, Alvarez & Schwartz, 1979, for references), between the phase of the oestrous cycle and steroid and inhibin levels in ovarian venous plasma (OVP) (DePaolo, Shander, Wise *et al.* 1979) and in follicular fluid (Fujii, Hoover & Channing, 1983) and, more directly, between fluctuations in the levels of FSH and inhibin (DePaolo, Anderson & Hirshfield, 1981; Hermans, Debets, van Leeuwen & de Jong, 1981, 1982*a*). It is therefore tempting to postulate that there is a closed feedback system between FSH and inhibin which plays a role in the regulation of the oestrous cycle and follicular growth.

Reports on inhibin-like activity in OVP (DePaolo *et al.* 1979), peripheral plasma (Lee, McMaster, Quigg *et al.* 1981) and follicular fluid (Fujii *et al.* 1983) in rats are not consistent with each other. This may be caused by the kinetics governing the release of inhibin from the follicular compartment to the circulation (Anderson & Hoover, 1982). In addition, the assay methods used to estimate inhibin-like activity have not always utilized a standard reference preparation (DePaolo *et al.* 1979, 1981; Fujii *et al.* 1983) and this has made quantitative comparison difficult.

In the present study we therefore investigated whether the rate of release of inhibin-like material from granulosa cells *in vitro* on subsequent days of the oestrous cycle showed clear cyclic variations and could perhaps provide a basis for explaining time-related changes in the content of inhibin-like activity found during the cycle in OVP and follicular fluid.

## MATERIALS AND METHODS

### Animals

Adult female rats of a Wistar substrain (R-Amsterdam) aged 60–100 days were used for the collection of ovaries and anterior pituitary glands. Rats were kept under controlled conditions of light (lights on 05.00–19.00 h) and temperature (22–25 °C). Standard dry pellets and tap water were available *ad libitum*. Vaginal smears were taken every morning and five to eight rats with at least two consecutive synchronous 5-day cycles were used for collection of the ovaries. The day after oestrus was called dioestrus-1, as in previous publications (Hermans *et al.* 1981). Anterior pituitary glands were collected from rats at random stages of the cycle.

### Buffers and media

Buffers and media were prepared from stock solutions that were stored for no longer than 4 weeks in a refrigerator. All solutions were adjusted to pH 7·2 and filter-sterilized (Acrodisc 0·2 μm; Gelman, Ann Arbor, Michigan, U.S.A.) before use. For the collection of granulosa cells the following media were used: F-12 stock (F-12 (Boehringer, Mannheim, F. R. G.) containing 10 mM-Hepes (Gibco, Grand Island, New York, U.S.A.), 2 mM-magnesium acetate and 0·055 M-glucose); F-12 stock with 4 mM-EGTA; F-12 stock with 0·2 M-sucrose and F-12 stock with 0·25% (w/v) bovine serum albumin (BSA; Organon, Oss, The Netherlands) and 0·001% (w/v) deoxyribonuclease (DNAse; Sigma, St Louis, Missouri, U.S.A.). For the harvest of anterior pituitary cells the following media were used: Hanks' balanced salt solution (HBSS; Gibco) stock with 4 mM-$NaHCO_3$; HBSS stock with 1% (w/v) BSA; HBSS with 0·5% (w/v) Dispase II (Boehringer). Both granulosa and pituitary cells were cultured in 35 mm Petri dishes (Falcon, Grenoble, France) in Minimal Essential Medium with Earle's Salts (Boehringer) containing 100 ml fetal calf serum/l (FCS; Gibco) and 1 mM-sodium pyruvate (Gibco) and, for granulosa cells only, with 10 g D(+)glucose/l (Merck, Darmstadt, F. R. G.)

### Granulosa cell isolation and culture

These were performed essentially as described by Hermans *et al.* (1982*b*). Ovaries were removed between 10.00 and 10.30 h, placed in F-12 stock and, after incubation in F-12 stock with EGTA, antral follicles were dissected out. The follicles were incubated in F-12 stock with EGTA (5 min) and in F-12 stock with sucrose (3 min). Granulosa cells were harvested by gently expressing the follicles in F-12 stock containing BSA and DNAse.

The number of viable cells was assessed with the trypan blue exclusion test and adjusted to approximately $1 \times 10^6$ cells/ml. Viability was $51 \pm 2{\cdot}5\%$ (S.E.M., $n = 9$). Granulosa cells were cultured ($1 \times 10^5$ cells/dish per 2 ml medium) in a moist chamber at 32 °C in a 5% $CO_2$/95% air atmosphere. The day on which the cultures were started was designated day 0. Spent rat granulosa cell culture medium (rGCCM) was harvested and renewed every day. Medium from similarly treated dishes was pooled and stored at −20 °C until assayed for inhibin-like activity using the dispersed pituitary cell culture bioassay. In one experiment, granulosa cells were cultured with or without 10% FCS, and in the presence or absence of FSH (NIADDK-rat-FSH RP-1, 62 μg/l). Follicle-stimulating hormone was added on days 1 or 3 of culture.

### Assay of inhibin-like activity using dispersed pituitary cells in culture

Anterior pituitary cells were obtained and cultured as described by Hermans *et al.* (1982*b*) without modification. In short, anterior pituitary glands were minced, treated with Dispase II and finally dispersed in a dounce homogenizer. The cell number and viability, determined with trypan blue exclusion, were $3{\cdot}5 \pm 0{\cdot}3 \times 10^6$ cells/ml and $69 \pm 2\%$ ($n = 17$) respectively, and $1{\cdot}5$–$2 \times 10^5$ cells/dish were used. Culture conditions were as described for granulosa cells.

After 3 days of pre-culture the spent culture medium was discarded and replaced by 2 ml fresh culture medium containing either test substances (two to three doses) or internal standard (three doses, charcoal-treated bovine follicular fluid (bFF) diluted 1 : 1000 with culture medium) each in three to four dishes. After a further 3 days of culture the spent media were collected and stored at −20 °C until assayed for FSH and luteinizing hormone (LH).

In the bioassay, calculation of relative potencies and 95% confidence limits and analysis of linearity and parallelism were based on the method of Finney (1964) as elaborated by Borth (1976), using a computer program kindly provided by Dr H. W. G. Baker, Howard Florey Institute of Experimental Physiology and Medicine, Parkville, Victoria, Australia. Unknown inhibin-like activities, expressed as relative potencies, were calculated relative to the inhibin-like activity of the standard preparation of bFF, as published and discussed earlier (de Jong, Smith & van der Molen, 1979; de Jong, Jansen & van der Molen, 1981; Hermans *et al.* 1982*a*). This standard preparation of charcoal-treated bFF is a pool which has been diluted and stored at −20 °C in aliquots. Samples from this pool were always used. The FSH secretion suppressing activity of this pool is 300 times higher (per mg protein) than that of the ovine testicular lymph protein described by Eddie, Baker, Higginson *et al.* (1979) and de Jong *et al.* (1981). The index of precision ($\lambda$) of the bioassay ranged from 0·06 to 0·14 (mean 0·09 ± 0·01 (S.E.M.)), while significant deviations from linearity and parallelism were not observed.

**Removal of steroids**

Rat granulosa cell conditioned medium from pro-oestrous cells on days 1 and 2 of culture, dioestrous-1 cells on day 1 of culture, dioestrous-2 cells on day 2 of culture, and dioestrous-3 cells on day 2 of culture were treated with dextran-coated charcoal: 25 mg dextran (T-500; Pharmacia Fine Chemicals, Uppsala, Sweden) were dissolved in 100 ml phosphate buffer (10 mmol/l, pH 7·0) and to this 250 mg charcoal (Sigma) were added. The mixture was diluted to give a final concentration of 0·18% (w/v) charcoal in rGCCM.

**Hormone determinations**

Concentrations of FSH and LH in spent pituitary cell media were estimated in duplicate, using the radioimmunoassay described by Welschen, Osman, Dullaart *et al.* (1975). Spent media from one pituitary cell culture were always assayed in the same assay. Intra- and interassay variations were 3·9 and 20·8% for FSH and 8·2 and 31·8% for LH. Results are expressed as means ± S.D. in terms of NIADDK-rat-FSH RP-1 and NIADDK-rat-LH RP-1 (μg/l) or as percentages of mean control values found for spent media from cells cultured without addition of test substances.

In order to rule out possible effects of the administered FSH in the pituitary cell bioassay, FSH-containing control medium was tested simultaneously with rGCCM. Concentrations of progesterone and oestrogen were estimated by radioimmunoassay, using the methods of de Jong, Baird & van der Molen (1974) and de Jong, Hey & van der Molen (1973) respectively.

**Statistical procedures**

The significance of differences between gonadotrophin concentrations found after addition of test substances or after addition of control medium was assessed with Student's *t*-test. Differences were considered to be significant when $P < 0{\cdot}05$ (two-tailed tests).

## RESULTS

**Inhibin-like activity**

A pilot experiment with granulosa cells obtained from pro-oestrous, oestrous or dioestrous-2 follicles kept in culture for 6–8 days yielded the following results. On the first day of culture the relative potency of conditioned medium was highest for all three stages of the cycle (0·5, 0·25 and 0·07 respectively) after which the relative potency declined to a rather steady low level for the three stages of the cycle (between 0·1–0·2, 0·05–0·15 and 0·02–0·07 respectively). In further experiments only media obtained during the first 4 days of the granulosa cell cultures were studied. Data from this pilot experiment are included in the final results. In the bioassays we did not find a systematic, dose-dependent suppression of LH secretion after addition of bFF or rGCCM, but statistical evaluation (Student's paired *t*-test) of 30 bioassays with respect to the difference between control values and the values after addition of bFF, but not of rGCCM, revealed a significant reduction in LH release with the highest dose (11% with 225 nl bFF; $P < 0{\cdot}001$) and the middle dose (6% with 75 nl bFF; $P < 0{\cdot}05$).

When the relative potency of charcoal/dextran treated and untreated rGCCM was determined simultaneously a highly significant correlation between the relative potencies was found ($P < 0{\cdot}0005$; $y = -0{\cdot}06 \pm 0{\cdot}99x$; $n = 7$; $r = 0{\cdot}99$, where $y$ is the relative potency after charcoal/dextran treatment and $x$ the relative potency before this treatment). Removal efficiencies were > 97·5% for progesterone and > 99% for testosterone and oestradiol. From these data it was concluded that the steroids in rGCCM did not interfere with the measurement of inhibin-like activity using dispersed pituitary cells. Consequently the rest of the media was not treated with dextran-coated charcoal.

Results of duplicate experiments with cell material from all days of the cycle are presented in Fig. 1. On day 1 of culture the relative potency of the media from granulosa cells obtained on the various days of the cycle was higher than on the subsequent days of culture; a significant ($P < 0{\cdot}025$; 95% confidence limits) decrease was observed on day 2 of culture, except for the dioestrous-1 cultures which showed no significant change in relative potency over the entire culture period, and for one pro-oestrous culture which showed

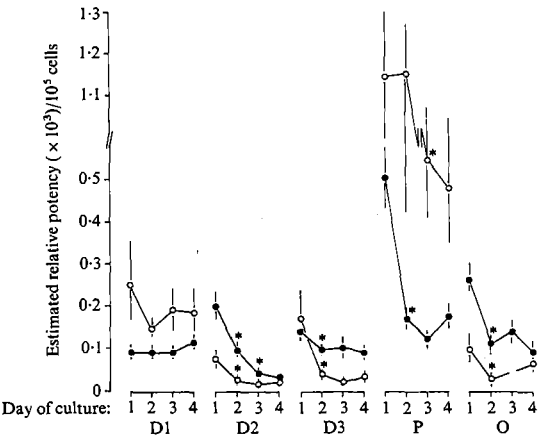



FIGURE 1. Estimated relative potencies ( $\times 10^3$) $\pm$ 95% confidence limits of media conditioned by approximately $10^5$ granulosa cells isolated from rats on various days of the oestrous cycle. The experiment was performed in duplicate: the first experiment is shown by the solid symbols and the second by the open symbols. The cells were cultured for 4 days in medium with 10% fetal calf serum. Media were harvested and renewed every 24 h and their potency to suppress the release of FSH was estimated relative to a standard bovine follicular fluid preparation (potency≡1). D1, dioestrus-1; D2, dioestrus-2; D3, dioestrus-3; P, pro-oestrus; O, oestrus. * $P < 0{\cdot}025$ (no overlap in confidence limits) compared to the previous culture day ($t$-test). Note the break in the axis for one of the pro-oestrous cultures.

a significant decline only from days 2 to 3. After this decline only insignificant changes in relative potency occurred, except in one dioestrous-2 culture in which a further significant decline was found from days 2 to 3.

On day 1 of culture the relative potency of rGCCM from both pro-oestrous cultures was significantly higher than the relative potency observed in media from all other cultures ($P < 0{\cdot}025$; 95% confidence limits).

**In-vitro progesterone and oestrogen secretion**

The concentration of progesterone was measured in rGCCM on all days of culture of a series of cultures from each stage of the oestrous cycle (Fig. 2). Maximum progesterone concentrations were found at pro-oestrus (3·75 nmol/l). Generally progesterone levels were higher on day 1 of culture than on the subsequent days of culture. The only exception was seen on dioestrus-1, when levels were consistently below the detection limit of the assay (0·3 nmol/l). On days 2–4 of culture the levels were all below the detection limit except for day 2 of a pro-oestrous culture (1·65 nmol/l). Oestrogen levels were estimated in rGCCM from day 1 of culture on all days of the cycle, except dioestrus-1, and were 279, 286, 367 and 308 nmol/l for dioestrus-2, -3, pro-oestrus and oestrus respectively. Thus progesterone secretion showed a pattern comparable to that of inhibin-like activity, and secretion of both progesterone and oestrogen was highest on day 1 of a pro-oestrous culture.

**Release of inhibin-like activity in the presence of FSH and/or FCS**

When FSH (62 μg/l) was added from day 3 onwards to a dioestrous-2 or pro-oestrous culture in the presence of FCS (10%), no significant change in relative potency of rGCCM over the next 3 days of culture was found as compared to controls cultured without the addition of FSH (data not shown).

The influence of FCS on release of inhibin-like activity was studied as follows. Medium with 10% FCS was used in all cultures on the first day of culture to enhance attachment and subsequent spreading of the cells in the culture wells. Over a 2-day period culturing was then continued in media with and without FCS. In cultures of pro-oestrous cells without FCS, the inhibin-like activity of rGCCM was consistently lower than that observed in the presence of FCS. After addition of a 250 μl sample the decrease in FSH secretion in the bioassay (as a percentage of control) was: day 1, 81% for all cultures; day 2, 67% for cultures with serum vs 55% without serum; day 3, 77% with

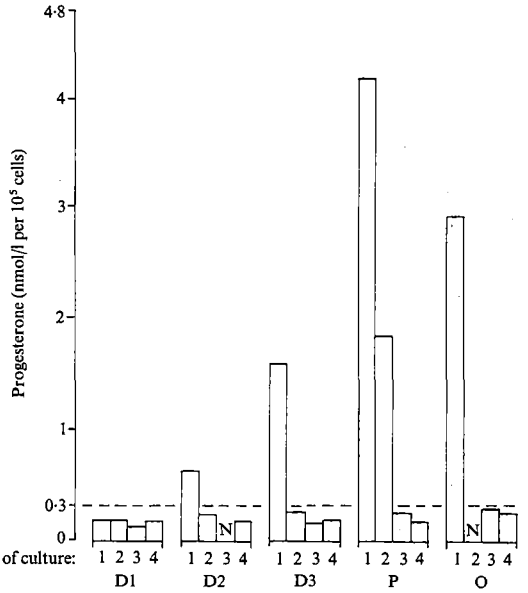



FIGURE 2. Progesterone concentration in rat granulosa cell medium conditioned by $10^5$ granulosa cells. Concentrations were estimated on each day of the cycle during 4 days of culture, D1, dioestrus-1; D2, dioestrus-2; D3, dioestrus-3; P, pro-oestrus; O, oestrus. The detection limit (0·3 nmol/l) is shown by the broken line. N, not determined.

serum vs 37% without serum. Levels of LH in the pituitary cell bioassay were not influenced by the presence or absence of 10% FCS in the rGCCM.

In a subsequent experiment pro-oestrous granulosa cells were allowed to attach to the culture dish in medium containing 10% FCS for 1 day. On the next 3 days of culture FSH was added in serum-free medium (Fig. 3). The suppression of FSH in the medium of pituitary cells, caused by rGCCM from granulosa cells which were cultured in the presence or absence of FSH, was not significantly different. Control culture medium with or without FSH did not affect the release of FSH from the pituitary cells.

## DISCUSSION

This study extends earlier observations that culture medium conditioned by ovarian granulosa cells of adult, untreated rats contains a factor (inhibin) that preferentially suppresses FSH secretion in the dispersed pituitary cell culture assay (Erickson & Hsueh, 1978; Hermans *et al.* 1982*b*). This factor will be called inhibin in this discussion. In the present study it was shown that the amount of inhibin released by cultured granulosa cells varied with the day of the cycle on which the cells were collected. A comparison of inhibin levels in medium collected between days 1 and 4 of culture of pro-oestrous cells in this study with the data of Hermans *et al.* (1982*b*) showed clear similarities: after initially high values on days 1 and 2 levels decreased by 50–70% to rather constant levels on subsequent days.

The pattern of inhibin secretion of granulosa cells during the oestrous cycle agrees with the results of Chappel (1979) who found that in the hamster only extracts from pro-oestrous ovarian homogenates caused a significant suppression of FSH release in long-term ovariectomized animals. Furthermore Fujii *et al.* (1983) reported that pooled rat follicular fluid, collected daily from all surface follicles throughout the oestrous cycle, generally contained constant inhibin levels per unit volume, except on pro-oestrus when significantly higher levels were found.

Therefore all studies concerning the ovary as a source of inhibin-like activity (granulosa cells in culture, follicular fluid, ovarian homogenates) point to a maximum in ovarian inhibin production and content

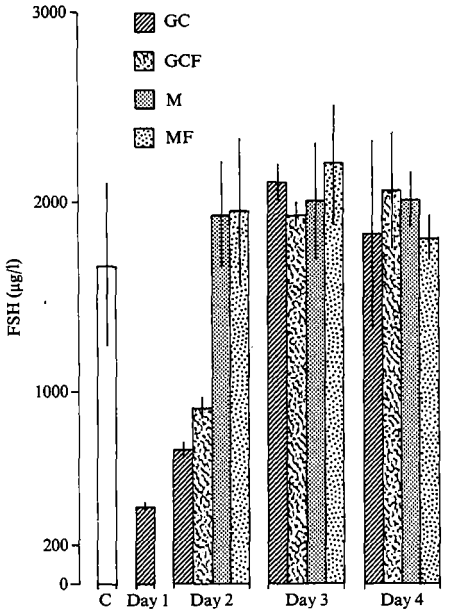



FIGURE 3. Concentration of FSH (means ± S.D.; $n = 4$) in medium from a dispersed pituitary cell culture bioassay to which 250 μl rat granulosa cell conditioned medium (rGCCM) were added. Granulosa cells were plated in the presence of 10% fetal calf serum (FCS). After 24 h the medium was collected (day 1) and replaced with fresh medium without FCS, and to half of the culture wells FSH was added at a final concentration of 62 μg/l. Control wells (M and MF) without cells were treated identically. On days 2, 3 and 4 of culture (day 2, day 3, day 4) culture medium was handled in the same way. C, control dishes of the bioassay; GC, rGCCM; GCF, rGCCM with FSH added; M, culture medium alone; MF, culture medium with FSH added.

on the day of pro-oestrus. In contrast to these data obtained with ovarian material, Kimura, Katoh, Taya & Sasamoto (1983), studying inhibin activity in OVP, did not find significantly higher values of FSH-suppressing activity on the day of pro-oestrus, compared to the overall dioestrous levels. The temporary decrease of inhibin levels in OVP from 14.00 h on pro-oestrus until 09.00 h on the day of oestrus reported by DePaolo *et al.* (1979), compared to the peak in in-vitro granulosa cell inhibin secretion at pro-oestrus, as found in this study, may reflect ovarian changes induced by the LH surge. Luteinization of the mature follicles and the loss of a large number of granulosa cells at ovulation on the morning of oestrus may result in a reduction of inhibin output in the ovarian vein.

Another topic which warrants discussion is the failure of granulosa cells *in vitro* to respond to FSH stimulation with a change in inhibin secretion. This has been reported before for cultured bovine granulosa cells (Henderson & Franchimont, 1981). A complicating factor in experiments with stimulation by FSH is that both rat and bovine (Henderson & Franchimont, 1981) granulosa cells had to be cultured in the presence of serum (10% FCS) to obtain inhibin secretion. On the other hand, Orly, Sato & Erickson (1980) reported that serum prevents hormonally induced functional changes of cultured granulosa cells from immature, hypophysectomized and diethylstilboestrol-treated rats.

Other observations made in this study showed that material present in bFF, but not in rGCCM, suppressed LH secretion (see also Sasamoto, Otani & Shirota, 1981). An explanation has not yet been found, but this phenomenon could be a result either of varying sensitivity of the female rat pituitary cells used in the present study, due to cyclic changes in hormonal priming *in vivo*, or of the presence of other substances, which affect gonadotrophin secretion, in follicular fluid (de Jong *et al.* 1979).

The secretion of progesterone by granulosa cell cultures from various days of the oestrous cycle was studied to validate the quality of the culture system with respect to granulosa cell steroidogenesis. In dioestrous cultures no increase in secretion of progesterone was found, in contrast to the findings of maximal secretion of progesterone at dioestrus shown by high levels in ovarian venous plasma (16.00 h, dioestrus-1; DePaolo *et al.* 1979) and peripheral plasma (03.00 h, dioestrus-2; Butcher, Collins & Fugo, 1974). This is easily explained by the fact that no corpora lutea were involved in the cultures. The gradually increasing level of progesterone in rGCCM seen in this study (Fig. 2) on the first day of culture from dioestrus-1 to pro-oestrus may reflect the increasing follicular steroidogenic activity, which is also reflected in oestrogen levels in rGCCM (this study) and in follicular fluid (Fujii *et al.* 1983), and is presumably caused by increased LH binding of the granulosa cells (Uilenbroek & Richards, 1979).

The following conclusions may be drawn. In cultures started on the day of pro-oestrus, secretion of inhibin as well as of progesterone and oestrogen is higher than on other days of the cycle. This suggests parallel states of activity for steroid and peptide synthesis by the granulosa cells collected on this day. Armstrong & Dorrington (1976), Orly *et al.* (1980) and Hermans *et al.* (1982*b*) also reported the inability of rat granulosa cells in culture to maintain progesterone secretion. In contrast, Henderson & Franchimont

(1981) reported an inverse relationship between inhibin and progesterone content in bovine granulosa cell conditioned medium.

Furthermore, granulosa cells collected from oestrus until the end of dioestrus secrete inhibin *in vitro* in nearly constant amounts. This may cause suppressed FSH levels *in vivo* during the days of oestrus and dioestrus. On pro-oestrus, granulosa cells are capable of the secretion of large amounts of inhibin. Surprisingly this is not reflected by a greater suppression of plasma FSH levels, which suggests that inhibin may be retained in the follicles (Fujii *et al.* 1983).

## ACKNOWLEDGEMENTS

Thanks are due to Drs H. M. A. Meijs-Roelofs and J. Moll for their critical reading of the manuscript. We are grateful to the NIADDK, Bethesda, Maryland, U.S.A., for the supply of the gonadotrophins used in the radioimmunoassays.

## REFERENCES

Anderson, L. D. & Hoover, D. J. (1982). Hormonal control of inhibin secretion. In *Intraovarian control mechanisms*, pp. 53–78. Eds C. P. Channing & S. J. Segal. New York: Plenum Press.

Armstrong, D. T. & Dorrington, J. H. (1976). Androgens augment FSH induced progesterone secretion by cultured rat granulosa cells. *Endocrinology* **99**, 1411–1414.

Borth, R. (1976). Statistics of parallel line assay. In *Methods of hormone analysis*, pp. 500–513. Eds H. Breuer, D. Hamel & H. L. Krüskemper. Stuttgart: Georg Thième Verlag.

Butcher, R. L., Collins, W. E. & Fugo, N. W. (1974). Plasma concentrations of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4 day estrous cycle of the rat. *Endocrinology* **94**, 1704–1708.

Channing, C. P., Tanabe, K., Guthrie, H. D., Batta, S. K. & Hoover, D. J. (1981). Changes in inhibin-F activity (folliculostatin) in human, porcine, rat and monkey ovarian follicular fluid and ovarian vein blood during various reproductive states. In *Intragonadal regulation of reproduction*, pp. 365–376. Eds P. Franchimont & C. P. Channing. London: Academic Press.

Chappel, S. C. (1979). Cyclic fluctuations in ovarian FSH-inhibiting material in the golden hamster. *Biology of Reproduction* **21**, 447–453.

DePaolo, L. V., Anderson, L. D. & Hirshfield, A. N. (1981). Possible existence of a long loop feedback system between FSH and inhibin in female rats. *American Journal of Physiology* **240**, E544–E549.

DePaolo, L. V., Shander, D., Wise, P. M., Barraclough, C. A. & Channing, C. P. (1979). Identification of inhibin-like activity in ovarian venous plasma of rats during the estrous cycle. *Endocrinology* **105**, 647–654.

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

Erickson, G. F. & Hsueh, A. J. W. (1978). Secretion of "inhibin" by rat granulosa cells *in vitro*. *Endocrinology* **103**, 1960–1963.

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

Fujii, T., Hoover, D. J. & Channing, C. P. (1983). Changes in inhibin activity, and progesterone, oestrogen and androstenedione concentrations, in rat follicular fluid throughout the oestrous cycle. *Journal of Reproduction and Fertility* **69**, 307–314.

Henderson, K. M. & Franchimont, P. (1981). Regulation of inhibin production by bovine ovarian cells *in vitro*. *Journal of Reproduction and Fertility* **63**, 431–442.

Hermans, W. P., Debets, M. H. M., van Leeuwen, E. C. M. & de Jong, F. H. (1981). Time related secretion of gonadotrophins after a single injection of steroid-free bovine follicular fluid in prepubertal and adult female rats. *Journal of Endocrinology* **90**, 69–76.

Hermans, W. P., Debets, M. H. M., van Leeuwen, E. C. M. & de Jong, F. H. (1982*a*). Effects of single injections of bovine follicular fluid on gonadotrophin concentrations throughout the oestrous cycle of the rat. *Journal of Endocrinology* **92**, 425–432.

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

de Jong, F. H., Baird, D. T. & van der Molen, H. J. (1974). Ovarian secretion rates of oestrogen, androgen and progesterone in normal women and in women with persistent ovarian follicles. *Acta Endocrinologica* **77**, 575–587.

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

de Jong, F. H., Jansen, E. H. J. M. & van der Molen, H. J. (1981). Purification and characterization of inhibin. In *Intragonadal regulation of reproduction*, pp. 229–250. Eds P. Franchimont & S. J. Segal. London: Academic Press.

de Jong, F. H. & Sharpe, R. M. (1976). Evidence for inhibin-like activity in bovine follicular fluid. *Nature* **263**, 71–72.

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

Kimura, J., Katoh, M., Taya, K. & Sasamoto, S. (1983). An inverse relationship between inhibin and follicle-stimulating hormone during the period of ovulation induced by human chorionic gonadotrophin in dioestrous rats. *Journal of Endocrinology* **97**, 313–318.

Lee, V. W. K., McMaster, J., Quigg, H., Findlay, J. & Leversha, L. (1981). Ovarian and peripheral blood inhibin concentrations increase with gonadotropin treatment in immature rats. *Endocrinology* **108**, 2403–2405.

Nequin, L. G., Alvarez, J. & Schwartz, N. B. (1979). Measurement of serum steroid and gonadotropin levels and uterine and ovarian variables throughout 4 day and 5 day estrous cycles in the rat. *Biology of Reproduction* **20**, 659–670.

Orly, J., Sato, G. & Erickson, G. F. (1980). Serum suppresses the expression of hormonally induced functions in cultured granulosa cells. *Cell* **20**, 817–827.

Sasamoto, S., Otani, T. & Shirota, M. (1981). Delay of the selective surge of follicle-stimulating hormone by bovine follicular fluid during the period of ovulation induced by human chorionic gonadotrophin in dioestrous rats. *Journal of Endocrinology* **89**, 205–212.

Schwartz, N. B. & Channing, C. P. (1977). Evidence for ovarian 'inhibin': suppression of the secondary rise in serum follicle-stimulating hormone levels in pro-oestrus rats by injection of porcine follicular fluid. *Proceedings of the National Academy of Sciences of the U.S.A.* **74**, 5721–5724.

Uilenbroek, J. Th. J. & Richards, J. S. (1979). Ovarian follicular development during the rat estrous cycle: gonadotropin receptors and follicular responsiveness. *Biology of Reproduction* **20**, 1159–1165.

Welschen, R., Hermans, W. P., Dullaart, J. & de Jong, F. H. (1977). Effects of an inhibin-like factor present in bovine and porcine follicular fluid on gonadotrophin levels in ovariectomized rats. *Journal of Reproduction and Fertility* **50**, 129–131.

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

Papers related to this thesis.

De Jong,F.H., Grootenhuis,A.J., Sander,H.J., Steenbergen,J., Timmerman,M.A. & Van Dijk,S. (1987). Comparison between inhibin from bovine follicular fluid and rat Sertoli cell culture medium. In: Inhibin - non-steroidal regulation of follicle-stimulating hormone secretion. Editors: H.G.Burger, J.K.Findlay, M.Igarashi & D.M.De Kretser. Raven Press, New York. pp.35-46

De Jong,F.H., Grootegoed,A.J., Grootenhuis,A.J., Sander,H.J., Steenbergen,J., Toebosch,A.M.W. & Ultee-Van Gessel,A.M. (1988). The role of inhibin in reproductive physiology in the rat. Proceedings of The British Endocrine Society 41, 1823-1830

# PART THREE

## Samenvatting.

Beheersing van de voortplanting is van groot belang : in de veeteelt vanuit economisch oogpunt en bij de mens vanwege sociaal en persoonlijk belang. Enerzijds is wetenschappelijk onderzoek van voortplanting er op gericht deze te perfectioneren, terwijl anderzijds onderzoek verricht wordt naar mogelijkheden van vrijwillige beperking van de vruchtbaarheid.

Bij de mens en ook bij andere zoogdieren is, tot op heden, het voortbrengen van nakomelingen voorbehouden aan het vrouwelijke organisme. Het ovarium van het vrouwtje levert daartoe een rijpe eicel (oocyt) die na samensmelting met een mannelijke zaadcel (spermatocyt) in de baarmoeder uitgroeit tot een nieuw individu. De produktie en de afgifte (ovulatie) van eicellen verloopt volgens een regelmatig weerkerend patroon (voortplantings-cyclus). Dit alles maakt dat bestudering van het vrouwelijke voortplantingsmechanisme niet alleen eenvoudiger is, maar tevens waardevoller voor de samenleving, dan bestudering ervan bij de man.

Hoewel regulatie van de voortplantings-activiteit bij vrouwtjes van verschillende zoogdiersoorten op veel punten overeenkomst vertoont, zijn er ook vele punten van verschil. Deze Samenvatting van het Proefschrift heeft - tenzij anders vermeld - betrekking op de vrouwelijke laboratorium rat.

Na een draagtijd van 22 dagen wordt de jonge rat geboren op, wat genoemd wordt, dag nul (0), en daarna gespeend (van de moeder gescheiden) op dag 22. De overgang van het onvolwassen naar het volwassen stadium vindt plaats rondom dag 40.

Tijdens het volwassen stadium vindt een regelmatige opeenvolging van ovulaties plaats, de zogeheten oestrus cyclus. Deze cyclus duurt bij de hier gebruikte rattestam vijf dagen, en is als volgt ingedeeld :
1) oestrus, de dag van ovulatie; 2) di-oestrus dag-1 (of metoestrus), de dag na oestrus; 3) en 4) di-oestrus dag-2 en -3; 5) pro-oestrus, de dag voorafgaand aan (de volgende) oestrus. Aan de hand van vaginaal uitstrijkjes is het cyclus-stadium waarin het vrouwtje zich bevindt (meestal) nauwkeurig vast te stellen.

Na zes maanden beginnen de eerste tekenen van veroudering van de voortplantingsorganen op te treden en, na een jaar zijn zij niet meer geschikt voor de voortplanting.

Bij het pasgeboren vrouwtje zijn in het ovarium de eicellen voor de rest van het leven aanwezig, maar voordat de eicellen bij ovulatie het ovarium verlaten moeten ze een lang, nauwkeurig gekontroleerd rijpingsproces ondergaan. Dit vindt plaats binnen de beschermende omhulling van een follikel. Zo'n follikel bestaat uit een wand van cellen (granulosa-type en theca-type) die, in een verder gevorderd stadium van de rijping, een met follikel-vloeistof gevulde ruimte (antrum) gaan omsluiten, terwijl de eicel in een heuveltje van cellen (cumulus) aan de binnenwand van de follikel gehecht zit. Het geheel van de rijpende oocyt binnen de follikel is enigszins vergelijkbaar met de manier waarop een vrucht zich bevindt binnen de baarmoeder. Een rijpe rattefollikel heeft vlak voor het moment van ovulatie (een zogeheten ovulatoire- of Graafse follikel) een doorsnede van 0.5-1 mm (bij de vrouw is dat 10-30 mm).

Het verloop van de eicel rijping wordt gestuurd vanuit drie organen, te weten de hersenen (met name de hypothalamus), de hypofyse (een kliertje dat onder tegen de hersenen aan ligt en er mee vergroeid is) en, het ovarium zelf. Samen vormen zij de Hypothalamus-Hypofyse-Ovarium as, de HHO as. Voor controle van de rijping worden langs de HHO as signalen uitgewisseld die zorg dragen voor normale rijping van een normaal aantal

eicellen (de rat ovuleert per cyclus 9-15 levensvatbare eicellen, de vrouw meestal 1). De signalen bestaan hoofdzakelijk uit stoffen (hormonen) die het ene orgaan maakt, in de bloedstroom uitscheidt (endocriene secretie) en die zodoende de activiteit van andere (doel-)organen kunnen reguleren. Deze organen geven op hun beurt ook weer een (terugkoppelend-) signaal af. In hoeverre er onderlinge beïnvloeding via zenuwbanen plaatsvindt is nog niet geheel duidelijk.

De belangrijkste hormonen (signalen) die actief zijn langs de HHO as worden hieronder kort beschreven.

De hypofyse scheidt hormonen uit die regulerend werken op het ovarium (bij de man op de testis), maar niet op de hypothalamus. Tot deze zogeheten gonadotrope hormonen (of gonadotrofinen) behoren het luteïniserend hormoon (LH, of lutropine) en het follikel-stimulerend hormoon (FSH, of follitropine). Een lage, strikt geregelde, basale bloedspiegel van FSH garandeert de rijping van een normaal aantal follikels (met eicel). Een plotselinge, verhoogde afgifte van FSH tijdens de ovulatie (= de ovulatoire FSH piek) zorgt er voor dat een groep jonge (of primaire) follikels aan de rijpingsgroei voor de volgende cyclus begint (recrutering).

Een zeer lage, basale bloedspiegel van LH stimuleert de aanmaak en secretie van hormonen door het ovarium, terwijl een piek van LH (+FSH) circa 8 uur voorafgaand aan de feitelijke ovulatie (= de pre-ovulatoire LH+FSH piek) het ovulatie-proces op gang brengt. (De betekenis van FSH voor de ovulatie is niet duidelijk.)

De hypothalamus produceert het gonadotrofinen-regulerend hormoon (GnRH, ook wel LH-regulerend hormoon (LHRH) genoemd) dat de afgifte van LH en FSH door de hypofyse regelt. Een piek in de afgifte van GnRH veroorzaakt de pre-ovulatoire LH+FSH piek. Of de hypothalamus het ovarium rechtstreeks kan beïnvloeden is onduidelijk.

Het ovarium produceert steroid-hormonen (oestrogenen, progestagenen, androgenen) en eiwit-hormonen (inhibines, activines). Beide worden gemaakt door rijpende follikels die daarin door FSH en LH gestimuleerd kunnen worden. Het bestaan van steroid-hormonen is al lang bekend : deze hormonen kunnen, afhankelijk van de fase van de cyclus zowel de hypothalamus als de hypofyse, stimuleren of remmen . Daarnaast bepalen ze de secundaire geslachtskenmerken. Ovariële eiwit-hormonen vormen een relatief jonge, nog tamelijk onbekende groep, met uitzondering van het hormoon inhibine.

Al in 1923 publiceerden Mottram & Cramer dat er een eiwit-hormoon van gonadale (testiculaire) oorsprong moest zijn, dat de secretie van FSH (niet van LH) door de hypofyse onderdrukt. In 1932 noemde McCullagh dit hormoon "inhibin", ofwel inhibine. Het vermoede bestaan van inhibine werd in de daarop volgende jaren bevestigd aan de hand van, zowel, klinische ziektebeelden, als van gegevens uit dierexperimenteel onderzoek : een injectie met een inhibine bevattend preparaat verlaagt (o.a. bij ratten) de bloedspiegel van FSH binnen 8 uur en dit effect houdt 12-16 uur aan. Een injectie met steroiden werkt trager en langduriger.

Inhibine is in 1987 gezuiverd uit follikel-vloeistof van pre-ovulatoire, antrale follikels van diverse diersoorten, en is in 1988 officieel gedefinieerd als "een eiwit-hormoon dat bestaat uit twee ongelijke, met disulfide-bruggen verbonden eenheden, dat hypofysaire productie en/of afgifte van gonadotrofinen remt, voornamelijk die van FSH"

(Burger & Igarashi). Fysiologische effecten van inhibine op de hypothalamus zijn nog niet gevonden.

De voortplantings activiteit van de vrouwelijke rat kan verdeeld worden in drie levensfasen. De eerste fase begint als de pasgeborene geheel op eigen kracht moet gaan leven. De organen van de HHO as groeien geleidelijk, en funktioneren met een toenemende mate van onderlinge beïnvloeding. Dit vindt zijn hoogtepunt in het optreden van eerste ovulatie, waarna de tweede- of volwassen fase inzet, gekenmerkt door een cyclisch patroon van follikelgroei en ovulatie (oestrus cycli). De tweede fase gaat geleidelijk over in de derde- of verouderings fase, waarin de HHO as in toenemende mate niet meer in staat is om normale, regelmatige oestrus cycli te bewerkstelligen. Uiteindelijk gaat de mogelijkheid tot voortplanting verloren (reproductieve senescentie).

In dit Proefschrift worden de resultaten beschreven van onderzoek naar de mogelijke rol van inhibine tijdens de sexuele rijping (fase 1), bij de regulatie van de oestrus cyclus (fase 2), en tijdens de verouderingsfase (fase 3).

De Hoofdstukken 1 en 2 bevatten een uitgebreidere versie van de hierboven staande inleiding.

Hoofdstuk 3 bevat de gegevens over de mogelijke rol van inhibine bij de regulatie van de voortplanting van de vrouwelijke rat.

Hoofdstuk 3.1 en de Appendix Papers I, II, III en IV beschrijven onderzoek dat heeft geleid tot meer gegevens over de aanwezigheid en fysiologische betekenis van inhibine tijdens de onvolwassen fase.

Vanaf de geboorte tot ongeveer dag 18 speelt inhibine geen rol van betekenis bij de regulatie van bloedspiegels van FSH. Na dag 18 groeien de eerste primaire follikels uit tot het klein-antrale stadium, kan inhibine aangetoond worden in het ovarium, en neemt inhibine geleidelijk in belangrijkheid toe. Naast steroid hormonen wordt inhibine tot een noodzakelijke factor voor de juiste regulatie van FSH. Na dag 28, het moment dat de eerste follikels het groot-antrale stadium bereiken, stijgt het ovariële inhibine nivo naar een hoger pijl. Tijdens de laatste 5 dagen voorafgaande aan de eerste ovulatie stijgt het ovariële inhibine nivo verder volgens een patroon dat ook gevonden wordt bij volwassen dieren tijdens de cyclus. Deze laatste stijging komt geheel voor rekening van de uitgroei van een groep grote, pre-ovulatoire follikels tot het punt van ovulatie. Tijdens deze periode is inhibine essentieel voor het voorkómen van grote fluctuaties in de hypofysaire secretie van FSH, waardoor ovulatie van een normaal aantal follikels zeker gesteld wordt.

Hoofdstuk 3.2 en Appendix Papers V en VI beschrijven de rol van inhibine tijdens de ovariële of oestrus cyclus van de volwassen rat.

De oestrus-cyclus kan globaal in tweeën worden gedeeld :
het eerste deel duurt van 12.00 uur op oestrus tot 12.00 uur op pro-oestrus, en wordt gekarakteriseerd door geleidelijke veranderingen, zowel wat hormoonspiegels als follikelgroei betreft. Gedurende deze periode is de inhibine produktie en secretie tamelijk gelijkmatig, wat

verklaard kan worden uit de eveneens gelijkmatig verlopende follikelgroei. Dit resulteert in een gelijkmatige, lage FSH spiegel die groei van een normaal aantal pre-ovulatoire follikels garandeert. Als inhibine op di-oestrus dag-1 met behulp van een antiserum weggehaald wordt uit de bloedstroom, resulteert dit in een piek van FSH die de ovulatoire piek evenaart wat hoogte betreft, en, het aantal geovuleerde follikels op de eerstvolgende en volgende dag van oestrus doet toenemen.

het tweede deel duurt van 12.00 uur op pro-oestrus tot 12.00 uur op oestrus. Het omvat snelle veranderingen van de hormoonspiegels (pre-ovulatoire LH+FSH- en ovulatoire FSH-piek) en van het aantal en de types groeiende follikels in het ovarium (ovulatie!). De pre-ovulatoire piek (14.00 uur pro-oestrus) start het ovulatie-proces, hetgeen (via LH) ondermeer een sterke daling van inhibine spiegels na 16.00 uur in het bloed teweegbrengt. Dit zal resulteren in verminderde suppressie van hypofysaire FSH secretie, wat op zijn beurt resulteert in de ovulatoire FSH piek op oestrus-ochtend. Deze piek wordt weer onderdrukt door inhibine uit drie mogelijke bronnen, 1) inhibine dat bij de ovulatie buiten het ovarium terecht komt en vandaar mogelijk in de bloedstroom, 2) inhibine afkomstig van de groep kleine, antrale follikels die juist met de groei zijn begonnen, en, 3) inhibine uit grote, niet geovuleerde follikels die nu ten gronde gaan (atresie).

Er lijkt een onderlinge afhankelijkheid te bestaan tussen, aan de ene kant, ovulatie van de huidige cyclus en, aan de andere kant, recrutering door FSH (ovulatoire piek, inhibine afhankelijk) van een nieuwe groep jonge follikels voor de volgende cyclus.

In Hoofdstuk 3.3 staan de gegevens die betrekking hebben op de periode van veroudering van de voortplantings functies.

Aangezien veroudering niet, zoals bij rijping en cyclus wel het geval is, volgens een bekend tijdschema verloopt, zijn de gegevens over de rol van inhibine hierbij nog schaars. Veroudering op het nivo van de hypothalamus, begint bij 7-9 maand met afbrokkeling van de stimulerende werking van ovariële steroiden op de afgifte van GnRH. Daardoor worden de pre-ovulatoire gonadotrofinen pieken verlaagd. Bij 7 maanden blijkt de ovulatoire piek van FSH met ca. 4 uur vertraging op te treden, terwijl pas bij 9 maanden ook de pre-ovulatoire piek vertraagd is, en vervloeit met de ovulatoire piek. Dit wijst er op dat tijdens het verouderingsproces de inhibine geregelde secretie van FSH eerder verandert dan de GnRH afhankelijke secretie. Bij 2 jaar oude ratten is de bloedspiegel van FSH verhoogd, het inhibine-nivo van het ovarium verlaagd, en lijkt de hypofyse minder gevoelig te zijn geworden voor inhibine. Daarnaast is bij deze dieren de bloedspiegel van LH zeer laag.

Er is (tot nu toe) geen reden om aan te nemen dat inhibine een leidende rol zou spelen bij het gehele proces van veroudering van voortplantingsfunkties.

Conclusie.

De gegevens in dit Proefschrift, samen met de gegevens uit de literatuur (Hoofdstuk 6), wijzen erop dat inhibine een belangrijke rol speelt bij het endocriene mechanisme dat de voortplanting bij de vrouwelijke rat regelt. Tijdens de sexuele rijping worden aanwezigheid en

rol van inhibine onmiskenbaar tussen dag 18 en dag 23. De belangrijkste rol lijkt inhibine echter te spelen tijdens de cyclische, volwassen periode waarin de feitelijke voortplanting kan plaatsvinden. De terugval in voortplantingscapaciteit tijdens het proces van veroudering valt samen met het geleidelijke verdwijnen van de rol van inhibine.

Tijdens iedere levensfase is inhibine, en de rol die het speelt bij de voortplanting, nauw verbonden met de ovariële follikel populatie en tevens met de bloedspiegel van FSH. Dit rechtvaardigt het inpassen van inhibine bij de toekomstige bestudering van de fysiologie van de voortplanting, en afwijkingen daarvan.

## Dankwoord.

Succesvol wetenschappelijk onderzoek en ander werk zijn sterk afhankelijk van stimulerende samenwerking met anderen, meestal vele anderen. Zo ook in dit geval.

Het is allemaal begonnen met mijn promotor, Prof.Dr. H.Moll (daarna volgden nog 4 hoogleraren).
Beste Han, formeel bestuurd heb je me maar kort, gestuurd echter des te langer, tijdens de periode waarin ik het proefschrift ("het boekje") geschreven heb. Als post- en spoor-promotor heb je je op een bijzonder goede manier van je taak gekweten, daarbij niet vergetend altijd enig opbeurend en/of verrijkend gedachtengoed mee te geven.
Bedankt.

Waar mijn promotor als verre vriend optrad, fungeerde mijn co-promotor, Dr.H.M.A.Meijs-Roelofs, als goede buur.
Beste Jiet, met je part-time aanstelling en met je niet geringe zorgen op het thuisfront en op het werk, kon je toch altijd weer een gaatje vinden voor het corrigeren van weer een versie van weer een hoofdstuk en voor het bespreken van de opzet en de uitslag van een experiment. Dat we daarnaast vaak van gedachten wisselden over andere aspecten van het dagelijkse leven heb ik als zeer positief ervaren.
Bedankt.

De overige leden van de promotie-commissie (de "lees commissie"), Prof.Dr.S.W.J. Lamberts, Prof.Dr.J.J. van der Werff ten Bosch en Dr.F.H. De Jong, waren bereid het uiteindelijke manuscript te beoordelen en het (in een persoonlijk gesprek) van nuttige wenken en kanttekeningen te voorzien.
Bedankt.

De mensen van de werkvloer bepalen in hoge mate de dagelijkse gang van zaken en het werkplezier.
*Liesbeth van Leeuwen, je hebt altijd een accurate manier van werken gekoppeld aan enthousiasme en doorzettingsvermogen. Daarnaast beschik je tot mijn vreugde over een schier onverwoestbaar optimisme en een opgewekt humeur. Ik vond het een genoegen met je samen te werken en je als paranymf te hebben. *Piet Kramer, de dierexperimentele kant van de experimenten kwam vaak bij of via jou tot stand, inclusief het weinig begeerlijke nacht-of weekend-werk. Ook histologisch werk gaat je goed af. Je bent een waardevolle bron van informatie, terwijl je ook volop meedenkt over opzet en resultaat van de experimenten. Ook voor jou geldt, dat ik blij ben dat je als paranymf wilt op treden. *Gert van Cappellen, jij bent degene die mij ervan overtuigd heeft dat ook een computer een apparaat is dat mensen nodig heeft. Waar het de toepassingen van de computer betreft, ben jij degene die onze onderzoeksgroep weer geheel bij de tijd heeft gebracht, met name wat betreft follikelmeting. *Cobie Steenbergen, jij bent eigenlijk onze "vaste relatie" met Biochemie II, onderdeel Inhiberia. Altijd in voor een praatje over werk of thuis, bij thee of koffie.
Bedankt.

Bij vrijwel alle onderzoek hangt het dier-experimentele deel af van goed georganiseerde kweek en verzorging van de proefdieren.
Voor de hier beschreven experimenten werd dit uitgevoerd door *George Ketting, *Jan Velkers en *Piet Kramer.
Bedankt.

Het Histo-lab is al die jaren niet alleen een bron van thee, koffie en gebak geweest, vaak heeft het ook plotseling benodigde informatie of goederen kunnen aangedragen.
*Richard Hawkins, *Henk Visser, *Ellen Voigt, *Henk van der Heyden (vooral ook voor de talloze coupes die je voor me gesneden hebt) en anderen tekenden hiervoor. Op deze plaats moeten de nogal heftige, soms diepgravend-principiële, vaak verwarrende, en meestal gelach opwekkende discussies gememoreerd worden, die daar over de tafel rolden.
Bedankt.

Uiteraard is het mede verzorgen van (anatomie-)onderwijs een fundamentele taak van een universiteits-medewerker.
Ik heb met plezier lering getrokken uit de anatomische kennis en didaktische kwaliteiten van *Werner Klein, *Pingjan Osman, *Rob Stoeckart en *Harry Jansen. Ook hier bleek dat goed functionerende onderwijs-ondersteuning onontbeerlijk is : het werk van *Cees Entius en *Jan Velkers.
Bedankt.

Veel profijt heb ik gehad van de door de afdeling Biochemie II (Prof.Dr.H.J.van der Molen, Dr.F.H.De Jong) geboden gastvrijheid en faciliteiten, met name voor het kweken van hypofyse- en granulosa-cellen.
Bedankt.

Een nadere toelichting is op zijn plaats waar het het onderdeel Inhiberia betreft, de groep die zich op zuivering van inhibine gestort heeft.
Discussies over inhibine met, onder anderen, de (ex-) Inhiberianen, *Frank de Jong, *Cobie Steenbergen, *Simon van Dijk, *Annemarie Ultee-van Gessel, *Arijan Grootenhuis en *Marianna Timmerman heb ik zeer stimulerend gevonden Een prettig samenwerkingsverband, dat hopelijk word gecontinueerd.
Bedankt.

*Frank de Jong wil ik hier nog eens vermelden. Je was altijd bereid om uit te leggen hoe diverse vorken aan hun stelen zaten, en hoe ze gebruikt konden en hoorden te worden. Hoewel mijn inhibine-resultaten niet altijd in jouw tempo op tafel kwamen, ging je interesse niet verloren.
Bedankt.

Helma Hermans en Til Debets gaven hun toestemming voor opname van Appendix Paper V; Pingjan Osman de zijne voor Appendix Paper I.
Bedankt.

Ariëtta en de kinderen, mijn moeder, mijn broer en zus, vrienden en kennissen, allen hebben met hun voortdurende belangstelling bijgedragen aan mijn motivatie om door te gaan, en het geheel in de vorm van dit boekje af te ronden. Hun vraag naar het "wat hierna" viel niet eenduidig te beantwoorden.
Bedankt.

Hoewel mijn vader de uiteindelijke afronding helaas niet meer heeft mogen meemaken, was hij het die mijn belangstelling wekte voor al wat groeit en bloeit (en ons altijd weer boeit), en die met gretige belangstelling mijn vorderingen op het pad der wetenschap volgde.
Bedankt Pa.

Voor degene die mogelijkerwijs het gevoel heeft te kort gedaan te zijn : in ieder geval bedankt.

## Curriculum Vitae.

De schrijver werd in 1951 geboren te Jelsum (Friesland) en verhuisde op z'n zesde naar Haren (Groningen). In 1969 behaalde hij het diploma H.B.S.-b aan de Dalton HBS te Groningen. In dat jaar werd tevens begonnen met de Biologiestudie aan de Rijksuniversiteit te Groningen. Het doctoraal examen Biologie, met als specialisatie Electronenmicroscopie (Endocrinologie; Dr. S.J.Wendelaar-Bonga) en de bijvakken Neuroanatomie (Dr. P.G.M.Luiten) en Neurofysiologie (Dr. C.M.Ballintijn), inclusief de aantekening voor eerste-graads onderwijsbevoegdheid in de Biologie, werd in december 1977 afgelegd. Tijdens de doctoraalfase was de schrijver gedurende meerdere jaren student-assistent bij de vakgroep Zoölogie.

Van januari 1978 tot januari 1981 was schrijver dezes werkzaam (in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek, ZWO/FUNGO) bij de Rijksuniversiteit te Utrecht, faculteit der Geneeskunde, afdelingen Haematologie (Prof.Dr. J.J.Sixma) en Electronenmicroscopie (Prof.Dr. J.Geuze). Vanaf januari 1981 volgde de aanstelling tot wetenschappelijk medewerker (universitair docent) aan de Erasmus Universiteit Rotterdam, faculteit der Geneeskunde en Gezondheidswetenschappen, afdeling Anatomie, waar de in dit proefschrift beschreven studie werd uitgevoerd.