REGULATION OF THE NUMBER OF FOLLICLES MATURING AND OVULATING DURING THE 5-DAY CYCLE IN THE RAT

PROEFSCHRIFT

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CONTENTS

EFFECT OF UNILATERAL OVARIECTOMY ON FOLLICULAR GROWTH IN HYPOPHYSECTOMIZED RATS TREATED WITH PREGNANT MARE SERUM GONADOTROPHIN

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GENERAL INTRODUCTION

In mammals the number of follicles, rupturing and extruding their ovum during an oestrous cycle (ovulation rate, Land, 1970), varies widely from species to species. In many species, including man, bats and elephants, as a rule only one ovum is extruded from the ovaries at each oestrus. On the other hand, in rodents, insectivores, pigs and carnivores a number of eggs is released simultaneously at each oestrus. Within any given species there is a tendency to release a constant number of ova during each cycle (Brambell, 1966).

The study of the mechanisms controlling the number of ova shed per cycle is not only of academic interest. It may also lead to practical applications in at least two important fields. Firstly, it might provide further improvements in treatment of that category of infertile women, in which follicular maturation and ovulation fails to occur. Treatment of these patients with exogenous gonadotrophins results, at present, rather frequently in overstimulation of the ovaries, followed by ovarian cyst formation or multiple pregnancies (Gemzell & Roos, 1967; Gemzell & Johannson, 1970). Secondly, control of the number of eggs ovulated has potential uses in animal husbandry, such as the multiplication of offspring from selected animals (Hammond, 1961).

So far, most studies of the mechanism regulating ovulation rate in the rat followed the experimental approach of either hemispaying or induction of superovulation. In both cases ovulation rate per ovary is about twice the normal values, providing possibilities to study factors involved in the regulation of ovulation rate. Hemispaying results in a compensatory increase of the number of maturing and rupturing follicles in the remaining ovary. Our first studies were aimed at the regulation of this phenomenon (Welschen,

1970; 1971; 1972 see appendix). They contributed to the view that the compensatory processes are exclusively due to the increase of gonadotrophin levels induced by hemispaying. Superovulation, the simultaneous release of an unusual high number of ova, can be induced by the administration of additional amounts of gonadotrophic hormones. Our studies on this phenomenon (Welschen & Rutte, 1971 see appendix) suggested that in the rat during an oestrous cycle a maximum of about 10 follicles per ovary have, in principle, the capacity to come to full maturation and ovulation. Moreover, it was found that the administration of additional gonadotrophins resulted in a high incidence of abnormal follicles. The data summarized here, as well as the many data on the same subject reported in the literature (for references see Welschen & Rutte, 1971), suggest that normal maturation of a normal number of follicles during the cycle depends primarily on the presence of adequate gonadotrophin levels in the blood. Moreover, other studies (reviewed by Young, 1961) indicate that direct effects of ovarian hormones on the follicular population have to be considered also. Therefore, we decided to study whether or not the gonadotrophic and ovarian hormone levels present during the cycle are indeed indispensable for normal maturation of the normal number of follicles. The first results of this study are reported in this thesis.

CHAPTER I

INTRODUCTION

1. OVULATION RATE IN RATS

The number of ova released from the ovary during each oestrous cycle in the rat and in related species as the mouse and the hamster is rather constant (Table 1). However, variations of the mean are met with, not only in different individuals, but also in the same individual during consecutive oestrous cycles (Brambell, 1966). Ovulation rate depends on genetic characters, as was demonstrated in selection experiments (Land, 1969), and can be influenced by such internal factors as metabolism (Ingle, 1951), emotionality (Yeaker & Rhoades, 1941), age (Nalbandov, 1964) and external factors such as temperature (Pennycuick, 1964; Sod-Moriah, 1971), crowding (Cristian et al., 1965; Fuller et al., 1968) and possibly, the male present (Finn, 1964; Zarrow et al., 1971). Other factors such as nutrition and light may be expected to influence ovulation rate, since they exert clear effects on the secretion of gonadotrophins (nutrition: Leathem, 1961; light: Daane & Parlow, 1971).

Selection experiments have been performed in mice. Selection for large body size resulted in an increase of ovulation rate, due to increased levels of gonadotrophins in the blood (Fowler & Edwards, 1960; Edwards, 1962). Selection for litter size also resulted in changes in ovulation rate, but in this case, due to changes in ovarian responsiveness to gonadotrophins (McLaren, 1962). In rats no specific selection experiments have been preformed, but a number of data suggest that conclusions drawn from the experiments in mice are valid in rats too. For instance, it was found

that differences in gonadotrophin levels and in ovarian sensitivity exist between different strains of rats and between different maternal lines of a single strain (see Mauleon & Pelletier, 1964 for gonadotrophin secretion; Chapman, 1946; Hamburger, 1950; Courrier et al., 1961 and Mauleon & Rao, 1963, for ovarian sensitivity). Moreover, Aron et al. (1967) showed that ovulation rate is correlated with bodyweight in rats too.

TABLE 1

Mean number of eggs per two ovaries ovulated at natural oestrus in various strains of mice, hamsters and rats.

species	strain	no of ova	author
mouse	N, large bodyweight	10.1	Fowler & Edwards, 1957
	N, small bodyweight	7.1	
	C, large bodyweight	15.2	ibid
	C, small bodyweight	9.0	
	J	9.6	ibid
	C 57	8.3	ibid
	L	12.8	ibid
	Swiss albino	11.0	Greenwald & Choudary, 1969
hamster		11.4	Greenwald, 1960
		13.6	Printz & Greenwald, 1970
rat	Wistar	10.3	Peppler & Greenwald, 1970[a]
	Wistar	9.4	Asch & Roos, 1965
	Sprague-Dawley	11.5	Rodgers, 1971
	Wistar substrain R-Amsterdam	11.4	Welschen, 1970

In rats, mice and hamsters kept under laboratory conditions the variations in the number of ovulations may be expected to be rather small, because of the standardization of factors such as temperature, light, crowding, nutrition etc. and the intentional or unintentional selection usually performed. Nevertheless, considerable differences in ovulation rate were found between individual rats of our highly inbred colony living under standard conditions (range from 7 to 14).

2. MECHANISM OF CONTROL

During the oestrous cycle of the rat[1] - under normal laboratory conditions lasting 4 or 5 days: consecutively dioestrus (2 or 3 days), prooestrus (1 day) and oestrus (1 day) - follicular maturation takes place during the period from oestrus to prooestrus and ovulation during the night between prooestrus and oestrus. Ovulation rate could, in principle, be regulated in two ways: a) via control of the number of maturing follicles and b) via control of the percentage of mature follicles, which ruptures and extrudes the ovum during the night between prooestrus and oestrus. Data of Peppler & Greenwald (1970) and of Welschen & Rutte (1971) show that in the rat almost all mature follicles present in the ovaries during late dioestrus and prooestrus have extruded their ovum at oestrus. This suggests that control of ovulation rate occurs via control of the number of maturing follicles. The same seems to be true in mice (Pedersen, 1970) and hamsters (Greenwald, 1961). The question whether or not the second way of control suggested above (b) is, in addition to the first way, operative during the cycle has not been answered up till now. This possibility can only be excluded, when it is demonstrated that the amount of ovulation inducing gonadotrophic hormones, normally released during late prooestrus, is sufficiently large to induce not only rupture and ovum extrusion of a normal number of mature follicles but also of numbers exceeding the normal one.

In intact rats a sudden release of large amounts of FSH, LH and prolactin occurs about ten hours prior to ovulation (Schwartz & Caldarelli, 1965; Anderson & McShan, 1966; McClintock & Schwartz, 1968; Kamioka, 1970; Gay et al., 1970). Of these hormones only prolactin has not been reported to be effective in inducing ovulation. In rats, in which the spontaneous ovulatory release of gonadotrophins is blocked by hypophysectomy or by nembutal, injections of LH induce ovulation. FSH is found to synergize with LH in inducing luteinization (Browning & Larke, 1965; Aron et al., 1969) and ovulation (Harrington & Bex, 1970; Labhsetwar, 1970[a]) and is also able to induce ovulation by itself (Carter et al., 1961; Lohstroh and Johnson, 1966; Goldman & Mahesh, 1968, 1969; Harrington & Elton, 1969; Labhsetwar, 1970[a]; Stern & Schutz, 1970; Ying & Greep, 1971[a]). During the normal cycle LH plays a predominant role in the induction of ovulation as was shown by Schwartz (1969), who observed that ovulation was prevented by an injection of anti-LH given at 13.00 h on the day of prooestrus but not by a similar injection of anti-FSH.

1) In rats, mice and hamsters the cycle is very short. The follicular phase culminates in ovulation, which is immediately followed by a new follicular phase. A luteal phase is usually not present.

The view that the amount of LH released during prooestrus is considerably larger than that required for full ovulation and therefore does not limit ovulation rate, has been defended by Labhsetwar (1970[b]). He estimated the LH contents of the pituitary in the morning of prooestrus and of oestrus in rats and concluded that the amount released in the period between both measurements was at least 2 or 3 times greater than the minimal amount of LH required, as a single dose, for the induction of full ovulation. Moreover, Kalra et al. (1971) found in nembutal treated prooestrous rats that electrochemical stimulation of the medical preoptic area resulted in full ovulation, although in these rats LH levels were considerably less elevated than during normal prooestrus and FSH levels were not elevated at all.

Both findings show that the amount of LH released during normal prooestrus is larger than the minimal amount required to induce full ovulation. When, in addition, the ovulation inducing capacity of FSH is taken into consideration, it seems clear that the prooestrous peaklevels of gonadotrophins do not limit ovulation rate during the normal cycle. However, strict evidence on this subject is still lacking.

Incidental findings suggesting that the ovulatory amount of LH is limiting ovulation rate during the normal cycle are reported by a number of authors, but are not very convincing. Mitchell & Yochim (1968) found in rats signs of an additional ovulation after injection of 50 IU HCG during oestrus. Rodgers (1971) observed that a coitus after the critical time of LH release during prooestrus increased ovulation rate in rats, probably via an additional LH release. Moreover, Greenwald & Choudary (1969) found in mice that an HCG injection on the sperm positive day (oestrus) resulted in a second ovulation in 30 percent of the animals. However, in all these cases the number of ova released in addition to the first set was rather small, even when the amount of ovulation inducing hormone injected was 25 to 50 times greater than the minimal amount required for full ovulation in nembutal blocked or hypophysectomized rats.

The data suggest that in rats, the control of ovulation rate occurs via maturation of a limited number of follicles. Therefore, it is of interest to study the development of the follicular population during the cycle and its control mechanisms.

3. DEVELOPMENT OF FOLLICLES DURING THE CYCLE

The early development of the ovarian follicles in mammals has been extensively reviewed by Young (1961) and Brambell (1966). Nevertheless a short description may be useful:

In mammals oogonia have completed their proliferative activity and have become primary oocytes before or shortly after birth. The possibility that oogenesis occurs also during phases later in life has been extensively studied (Zuckermann, 1951, 1956; Thung, 1958; Franchi et al., 1961) and recently definitely excluded by Peters (1969), who found that postnatally injected ^{3}H thymidine was never incorporated in oocytes, whereas other newly formed cells picked up the label. Arai (1920) estimated the number of ova in the ovaries of the rat at about 35.000 at birth, 10.000 at puberty and 2.000 at the end of the reproductive period. In rats and mice 50-70 percent of the oocytes are atretic already shortly after birth (Ingram, 1961, for references).

As an oocyte starts to grow, the flat investing cells proliferate and form a membrane granulosa. Soon after the oocyte has attained its maximal size, a theca interna is formed from the contiguous connective tissue around the granulosa cells (Brambell, 1928; Peters & Pedersen, 1968). About the same time small fluid-filled cavities are seen between the granulosa cells. These cavities will later enlarge and coalsce to form the antrum folliculi.

In a number of functional aspects clear differences between follicles with and without antrum can be observed: 1) after hypophysectomy all antrum containing follicles degenerate, whereas follicles without antrum are maintained (Smith, 1930; Lane & Greep, 1935; Paesi, 1949), 2) during the cycle no large changes in the number of follicles without antrum are observed, whereas the number of larger follicles show clear and significant changes (Lane & Davis, 1939; Mandl & Zuckermann, 1952; Pedersen, 1970) and, 3) the mitotic activity and growth rate are considerably smaller in follicles without antrum than in larger follicles (Lane & Davis, 1939; Pedersen, 1970). These data suggest that the follicles without antrum form a pool of relatively quiescent follicles from which during each cycle a crop of follicles starts further development leading, within one or two cycles, to ovulation or degeneration (see also Peters & Levy, 1966).

Mandl & Zuckermann (1952) found in ovaries of rats during consecutive phases of the cycle significant changes in the numbers of follicles with a diameter $> 350\ \mu$. Their results are in agreement with and add to the earlier observations of Boling et al. (1941). They are confirmed by results of Peppler & Greenwald (1970[b]) and of Welschen & Rutte (1971).

These studies show a biphasic development of the group of follicles that enters the ultimate maturation phases during each cycle. During a 5 day

cycle the first phase lasts from prooestrus to dioestrus 2. The number of follicles with a diameter $> 350\ \mu$ (corresponding to a volume $> 200 \times 10^5\ \mu m^3$) increases steadily as does the size of individual follicles. By the end of this phase a number of about 10 of these follicles reach a diameter $> 450\ \mu$ (corresponding to a volume $> 500 \times 10^5\ \mu m^3$). It is these follicles that are apparently destined to ovulate. They even are already capable of ovulating at that time (Mitchell & Yochim, 1968; Peppler & Greenwald, 1970; Holsinger & Everett, 1970; Ying & Greep, 1971[a]; Welschen & Rutte, 1971). The second phase lasts from dioestrus 2 to prooestrus. During this phase the follicles destined to ovulate show a further increase in size and finally nearly all ovulate, whereas the follicles in a size range of $> 350\ \mu$ and $< 450\ \mu$ degenerate.

A similar biphasic development of the group of large follicles during each cycle is seen in hamsters (Greenwald, 1961) and in mice (Pedersen, 1970).

This pattern of development of the follicular population suggests that the number of follicles destined to ovulate at the next oestrus is fixed at the dioestrus 2 stage of the cycle. In good agreement with this assumption, Peppler & Greenwald (1970[a]) found that unilateral ovariectomy only results in a doubling of ovulation rate in the remaining ovary if performed before 14.00 h during dioestrus 3.

4. HORMONES INVOLVED IN THE REGULATION OF FOLLICULAR DEVELOPMENT

It has generally been accepted, that the cyclic growth of ovarian follicles is regulated by hypophyseal and ovarian hormones (Hisaw, 1947). At least four different hormones are involved: the hypophyseal gonodotrophic hormones FSH (follicle stimulating hormone) and LH (luteinizing or ovulation inducing hormone) and the ovarian steroids: oestrogen and progesterone.

In a number of experiments other gonadotrophins have been used: PMS, produced in the endometrial cups in pregnant mares, and HCG, secreted by the placenta in women. Both preparations have a combined FSH and LH like activity; in PMS the FSH effect is predominant at the level of the ovaries, in HCG the LH effect (Nalbandov, 1964; Louwerens, 1970). Oestrogens are often replaced by the non steroidal stilbestrol.

All four hormones probably exert direct actions on the follicular population. In addition, they are able to exert indirect actions by influencing each others' secretion rate. First, the direct action will be discussed.

Data on the effects of individual hormones on the follicular development come from a variety of experiments. The most direct evidence results from experiments in which the release of endogenous hormones is avoided, i.e. in animals that are hypophysectomized for some days. In these animals gonadotrophin and steroid levels are undectable, whereas the ovaries contain only healthy follicles of the preantrum stage (Rowlands & Parkes, 1966).

FSH and LH

Highly purified FSH preparations induce in hypophysectomized rats a growth of follicles to medium size (Li et al., 1962; Lohstroh & Johnson, 1966; Gemzell & Roos, 1966 for references). Follicles stimulated with less purified FSH grow to larger volumes, but do not luteinize (Inoué, 1965) and have a low capacity to ovulate after a ovulatory dose of LH (Carter et al., 1961). LH preparations in a purified form do not exert any growth stimulating activity on smaller follicles but may be active on FSH primed larger ones (Lohstroh & Johnson, 1966). Combinations of both hormones are very effective in stimulating follicular growth and making follicles capable of ovulating (Carter et al., 1961; Lohstroh & Johnson, 1966; Rowlands & Parkes, 1966 for references). These data from hypophysectomized rats are confirmed by data of experiments in which anti-FSH or anti-LH was injected in intact rats. Anti-FSH completely blocked follicular growth (Talaat & Laurence, 1969), whereas anti-LH did not (Laurence & Ichikawa, 1968).

PMS and HCG

At the level of the ovary, PMS exerts exclusively an FSH effect if given in small quantities; given in larger quantities an LH effect asserts itself (Nalbandov, 1964, for references). Accordingly, PMS induces follicular growth but, in general, follicles stimulated by PMS have a subnormal capability of rupturing and extruding their ovum (Callantine & Humphrey, 1965). Effects of HCG on a non-PMS pretreated follicular population in hypophysectomized rats have to the author's knowledge, not been reported. Additional evidence for the effect of gonadotrophins on the follicular population

TABLE 2 Some recent studies on precocious ovulation and superovulation in prepuberal or adult rats, mice and hamsters.

species	prepuberal or adult	amount and type of gonadotrophin	maximal number of tubal ova induced by endogenous LH	maximal number of tubal ova induced by HCG	
rat	prepub	FSH 4 mg[1)]	42.5		Zarrow & Gallo, 1966
"	"	" 1.3 mg[2)]	66		Meyer & McCormack, 1967
"	"	PMS 10 IU		40	Wilson & Zarrow, 1962
"	"	" 30 IU	70	60	Zarrow & Quinn, 1963
"	"	" 30 IU		50	Weifenbach, 1965
"	"	" 40 IU	24		Ying & Meyer, 1969
"	"	HCG 12.5 IU	3.5		Lunn & Bell, 1968
"	"	" 20 IU	9		Sugawara & Takeuchi, 1970
mouse	"	PMS 6 IU	20		Bell, Cristie & Parkes, 1971
"	"	" 4 IU		30	Purshottam, Mason & Pincus, 1961
rat	adult	PMS 40 IU		20	Sato, 1962
"	"	" 30 IU		20	Weifenbach, 1965
"	"	" 50 IU		27	Husain & Saucier, 1970
"	"	" 50 IU	2	43	Welschen & Rutte, 1971
mouse	"	PMS 3 IU		25	Edwards, Wilson & Fowler, 1963
"	"	" 4 IU		30	Land, 1970
hamster	"	PMS 30 IU	70		Greenwald, 1962

1) Exact preparation not stated.

2) Preparation S141A of their laboratory, probably contaminated with LH.

comes from experiments with intact animals in which injections of gonadotrophins induced precocious ovulation or superovulation, suggesting an increase of the growth rate of follicles and of the number of developing follicles (references in Table 2).

Oestrogens

Oestrogens injected into hypophysectomized rats show some clear direct effects on the follicular population. First of all they stimulate growth of the small follicles to medium size by inducing proliferation of the granulosa cells (Pencharz, 1940 and Williams, 1940; 1945 using stilbestrol; Gaarenstroom & de Jongh, 1946 using stilbestrol or oestradiol; De Wit, 1953 and Croes-Buth et al., 1959 using oestradiol benzoate). Secondly, oestrogens induce a decrease of the rate of atresia, normally occurring after hypophysectomy (Pencharz, 1940; Williams, 1944, 1945[a,b]; Desclin, 1949; De Wit, 1953; Payne & Hellbaum, 1955; Ingram, 1959[a,b]). Moreover, in hypophysectomized and in intact rats oestrogens induce an increased responsiveness of the ovaries to gonadotrophins (Bradbury, 1961; Smith & Bradbury, 1963; Husain & Pincus, 1969). However, as yet no clear evidence has been presented suggesting a direct action of oestrogens on the number of follicles maturing and ovulating during a normal or artificial cycle (Krähenbuhl & Desaulles, 1964; Callantine & Humphrey, 1965).

Progesterone

Progesterone injected into hypophysectomized immature or adult rats, in which follicular growth and ovulation was induced by well-timed FSH (or PMS) and LH (or HCG) injections, exerted no effect on the number of ova shed (Krähenbuhl & Desaulles, 1964; France & Pincus, 1964; Callantine & Humphrey, 1965; Smith & Bradbury, 1966; Gallo & Zarrow, 1970; Kaasjager, 1970). Moreover, progesterone did not make ovaries of rats more responsive to gonadotrophins (Smith & Bradbury, 1961). However, in other species progesterone has direct effects on the ovary (Wallach & Noriega, 1970).

Interactions between the hormones involved

It is generally accepted that the gonadotrophins are able to stimulate

ovarian steroid secretion. However, the present state of purity of the preparations casts some doubt on the effects described to FSH or LH on the secretion of estrogens or progesterone (Gemzell & Roos, 1965; Segaloff, 1965). The experimental data suggest that neither oestrogen nor progesterone secretion can be stimulated by purified FSH only (Greep, 1961; Lohstroh & Johnson, 1966; Schwartz, 1969). On the other hand, LH seems to have a clear effect on both oestrogen and progesterone secretion (Greep, 1961; Solod et al., 1966). FSH in combination with LH is also very effective.

On the other hand ovarian steroids may have an inhibitory as well as a stimulatory effect on the synthesis and release of gonadotrophins from the pituitary (see reviews by Everett, 1964; 1969). An inhibitory action of oestrogen on gonadotrophin release has been demonstrated in gonadectomized rats. In such animals pituitary and serum levels of both FSH and LH are elevated but return to normal after daily injections of oestrogen (review by van Rees, 1964). Progesterone injections were less effective in decreasing post-gonadectomy levels of LH (Kaufman & Rothchild, 1963) and appeared completely ineffective with regard to FSH levels (van Rees, 1964). Similar findings have been reported in hamsters (Keever & Greenwald, 1967). Data of Rothchild & Schwartz (1965) showing that follicular growth continued after injections of progesterone, also indicate that this hormone exerts an only feeble inhibitory effect on the basic gonadotrophin release (Rothchild, 1965). On the other hand it has been found that the prooestrous peak levels of gonadotrophins can be negatively influenced by progestins (Rothchild, 1965; Arimura & Schally, 1970; Davidson et al., 1970; Schally et al., 1971). A facilitatory action of both types of ovarian hormones on gonadotrophin release has been demonstrated in ovariectomized rats, where injections of oestrogen (Swelheim, 1965) or progesterone (Caligaris et al., 1968) given after pretreatment with small dosages of oestrogen , increased the gonadotrophin output from the pituitary.

5. THE RELATION BETWEEN HORMONE LEVELS AND FOLLICULAR DEVELOPMENT DURING THE CYCLE

In the previous paragraph it has been discussed that at least three or four hormones (FSH, LH, oestrogens and, probably, progesterone) have direct effects on follicular development. During the cycle the levels of these hormones in the blood show patterns, which may be described as follows.

While studies of gonadotrophins have documented that both FSH and LH show peak levels during late prooestrus, they have not fully agreed as to the pattern of secretion during the other days of the cycle. Gay et al. (1970) using hundreds of rats, demonstrated by radio-immuno-assay methods (RIA) that FSH levels gradually decreased during the first three days (5 day cycle) after prooestrus, whereas LH levels were essentially constant. Other RIA studies on LH also showed an essentially constant level during oestrus and dioestrus (Monroe et al., 1969; Goldman et al., 1969; McDonald et al., 1969; Piacsek et al., 1971). However, data of bioassay studies are less uniform. Some studies showed a second FSH peak during mid-dioestrus (Peppler, 1972) and a second LH peak during early (Anderson & McShan, 1966) or late (Peppler, 1972) dioestrus; other studies showed patterns identical to those obtained in RIA studies (Kamioka, 1970).

Oestrogen levels, in ovarian venous plasma (bioassay), appeared low and constant during oestrus and early dioestrus, rising during late dioestrus and showing a peak during early prooestrus (Hori et al., 1968; Yoshinaga et al., 1969). The ovarian oestrogen secretion rate, as determined by RIA, showed a more gradual increase during the period from oestrus to late dioestrus and a sharp increase during the morning of prooestrus (Shaikh, 1971).

Systemic blood levels of progestins, determined by a gas-chromatographic technique appeared high during late prooestrus and early oestrus, and somewhat elevated during dioestrus (Feder et al., 1967). Ovarian secretion rates of progesterone showed a similar pattern. There are some discrepancies as to the timing of the elevation during dioestrus. Piacsek et al. (1971) and Uchiuda et al. (1969) found an elevation during early dioestrus, Roser, Benoit Bloch (1969) during mid-dioestrus, whereas McDonald et al. (1969) found higher secretion rates during late dioestrus, possibly due to the fact that measurements were performed in rats with differences in cycle-length.

The pattern of follicular development during the cycle has been discussed earlier and is given in Fig. 1. in combination with the pattern of hormone levels probably present at the level of the ovary during the cycle.

The data depicted in fig. 1 suggest a number of relationships between changes in the hormone levels and events in the follicular population, following these changes. The most obvious one is the relation between the peak levels of gonadotrophins at prooestrus and ovulation, a relationship which has been affirmed in many experiments. However, other possible relationships, such as that between the prooestrous peak levels of gonadotrophins

and the start of development of a new crop of follicles or that between the rising oestrogen or high progesterone levels during dioestrus and the induction of atresia in smaller follicles or of the capacity to rupture of larger follicles are less well studied. Nevertheless, these possible relationships may play a crucial role in the regulation during the cycle of follicular development and of the number of maturing and rupturing follicles. Therefore, we started experiments to test these possible relationships. The first results are reported in chapter II, the experimental part of this thesis.

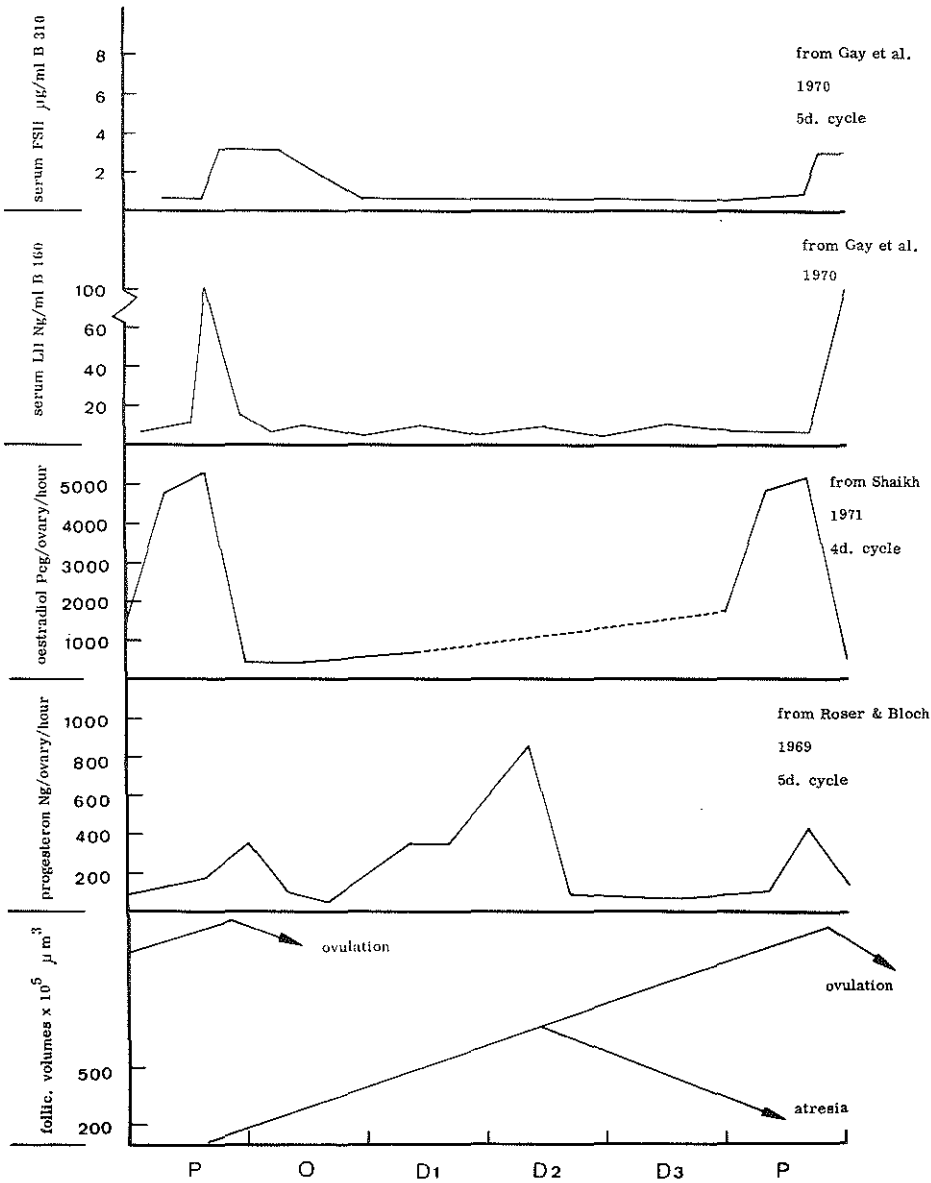

Fig. 1.

FSH, LH, oestrogen and progesterone levels and follicular development during the 5-day cycle in the rat.

CHAPTER II

EXPERIMENTS

1. AMOUNTS OF GONADOTROPHINS REQUIRED FOR NORMAL FOLLICULAR GROWTH IN HYPOPHYSECTOMIZED ADULT RATS*

ABSTRACT

Rats were hypophysectomized at oestrus, at days 1, 2 and 3 of dioestrus or at prooestrus. They were injected with various doses of PMS only, or of combinations of PMS and HCG, in such a way that rather constant blood levels could be expected, and killed 24 hrs later.

It was found that the minimal doses of substitutional gonadotrophins, required to maintain normal follicular growth, were considerably higher during the period from prooestrus to oestrus (when a new generation of follicles starts its development) than during all other periods. This peak requirement concerned primarily PMS. The minimal requirements during other periods showed minor variations only. The maximal doses which still caused normal follicular growth were higher during the period from dioestrus -1 to -3 than during the period from oestrus to dioestrus -1 on the period dioestrus -3 to prooestrus.

The data suggest that during prooestrus very high amounts of FSH are required to recruite a new crop of follicles, whereas during other phases low levels of both FSH and LH are generally a requirement for normal follicular growth.

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The growth of follicles during the ovarian cycle of the rat shows a very constant pattern (Mandl & Zuckerman, 1952[a,b]; Peppler & Greenwald, 1970[a,b]; Welschen & Rutte, 1971). During each cycle a constant number of follicles becomes capable of ovulating and does ovulate. The mechanisms regulating this constancy are not fully understood.

A large number of investigations indicates that both FSH and LH levels in the blood influence follicular development (reviews: Young, 1961; Rowlands & Parkes, 1966). During the cycle both plasma FSH and plasma LH levels have been shown to be rather constant except for the well-known ovulation inducing prooestrous peaks (FSH: Mc Clintock & Schwartz, 1968; LH: Ramirez & Mc Cann, 1964; Schwartz & Caldarelli, 1965; both hormones: Kamioka, 1970; Gay, Rees-Midgley & Niswender, 1970).

The aim of this study was to estimate amounts of gonadotrophins required for inducing normal follicular growth in acutely hypophysectomized rats during consecutive days of the 5 day cycle. It then should be possible to deduce to what extent the gonadotrophin levels, present during the cycle, play a role in the regulation of the dynamics of the follicular population. Thus, doses of Pregnant Mare Serum Gonadotrophin (PMS) and/or Human Chorionic Gonadotrophin (HCG), required to maintain normal follicular growth during a period of 24 hrs after hypophysectomy, performed at oestrus, dioestrus -1 (= metoestrus), dioestrus -2, dioestrus -3 or prooestrus, were estimated.

MATERIAL AND METHODS

The experiments were performed on adult female rats (180-200 g. bodyweight) of the highly inbred R-Amsterdam strain. The rats were kept in groups of 5 rats per cage and given food and water ad libitum. The rat room was illuminated from 5.00 to 19.00 h. Vaginal smears were taken daily for at least 3 weeks and only rats with 3 consecutive 5 day cycles were used. Experimental procedures included hypophysectomy and treatment with PMS and HCG. Hypophysectomies were performed between 12.00 and 13.00 h by the transauricular approach, using a Hoffman-Reiter H-200 hypophysectomy apparatus (H. Neuman & Company, Skokie, Illinois). PMS* (Gestyl ^{R}, Organon) and HCG* (Pregnyl ^{R}, Organon) were dissolved in 0.1 ml 0.9% NaCl and injected

* The hormones were kindly supplied by Organon, Oss, The Netherlands, through the courtesy of Dr. G.A. Overbeek.

intramuscularly. In order to obtain rather constant blood levels of PMS and HCG (biological half-life times 26 and 4.9 hrs respectively, see Parlow & Ward, 1961), PMS was given in 2 injections per 24 hrs (a first dose of x IU at 12.00 h followed 13 hrs later by a second dose of 0.25 x IU). HCG was given in 5 injections per 24 hrs (a first dose of y IU at 12.00 h followed by doses of 0.5 y IU every 5 hrs later). We will refer to these regimens by mentioning only the initial dose of PMS or HCG.

Follicular volumes in the right ovary were determined after routine histological procedures by the method of Boling, Blandau, Soderwal & Young (1941), modified in two ways: (1) two diameters were measured in the section in which the nucleolus of the ovum was found; (2) the 3rd diameter was substituted by the mean of the other diameters. In an additional experiment, tubal eggs were counted by the method of Rowlands (1944). For statistical analysis of the results Wilcoxon's two sample test was used. A difference was considered as statistically significant if the double tail probability was < 0.02.

EXPERIMENTAL DESIGN

In order to determine the pattern of gonadotrophin levels required for normal follicular development during the 5 day cycle, we estimated the amounts of PMS and HCG required immediately after hypophysectomy during various stages of the cycle. Such estimations are only possible on the following conditions: 1) significant changes in the follicular population take place on consecutive days of the cycle; 2) these changes can be prevented by hypophysectomy; 3) these changes can be re-established by certain doses of PMS and/or HCG. These conditions were all fulfilled in our experiments.

A. Changes in the follicular population on subsequent days of the cycle.

Rats were killed in groups of 7-10 at 12.00 h at various stages of the cycle. Since Mandl & Zuckerman (1952[b]) showed that clear cyclic changes occur only in the numbers and volumes of follicles larger than 200 x 10^5 μm^3, we limited our counts to such follicles. These were further subdivized in three classes: 200-499; 500-999 and larger than 1000 x 10^5 μm^3 respectively.

B. Prevention of follicular growth by hypophysectomy.

Rats were hypophysectomized at various stages of the cycle and killed 24 hrs later.

C. Re-establishment of follicular growth by PMS and HCG immediately after hypophysectomy during various phases of the cycle.

In a first series, rats at various stages of the cycle were hypophysectomized and subjected to regimens of PMS with an initial dose of 4, 6, 8, 12, 16 or 32 IU. Rats in prooestrus received

an additional injection of 5 IU HCG at 16.00 h in order to induce ovulation in large follicles.

In a second series rats were treated with a combination of PMS and HCG after hypophysectomy. PMS was given with an initial dose of 4 IU; HCG was given with an initial dose of 0.5, 1, 2, 3, 4, 5 or 25 IU.

In a third series, some other combinations of PMS and HCG were tested. During the phase from prooestrus to oestrus we gave 8 IU PMS combined with 5 or 25 IU HCG, and also 12 IU PMS combined with 1, 2, 5 or 10 IU HCG. During the other phases 8 IU PMS combined with 1 IU HCG was tested.

In an additional experiment the capacity of follicles to ovulate was tested after hypophysectomy performed at dioestrus -2 and earlier stages, followed by administration of gonadotrophins, adequate for maintaining normal follicular growth.

RESULTS

A. Changes in the follicular population on consecutive days of the cycle (Table 1).

In ovaries of rats killed at oestrus only follicles $< 500 \times 10^5$ μm^3 were found, apart from an occasional larger atretic follicle. The number of these small follicles was significantly larger than in prooestrous ovaries, indicating that the first development of a new generation of follicles took place during the period from prooestrus to oestrus. During the three days following prooestrus the follicles increased in size and many more follicles reached a volume $\geq 200 \times 10^5$ μm^3. During dioestrus -2 a maximum in the total number of follicles $\geq 200 \times 10^5$ μm^3 was found.

From dioestrus -2 onwards, two remarkable events were observed: 1) the follicles $\geq 500 \times 10^5$ μm^3 increased in volume, but their number did not increase significantly during the next days of the cycle; 2) smaller follicles became atretic, smaller and significantly less numerous. Earlier studies have shown that follicles $>$ about 500×10^5 μm^3 are not only destined to ovulate at the end of the cycle but are also capable of ovulating precociously (van Rees et al. 1968; Peppler & Greenwald, 1970[a]; Welschen & Rutte, 1971), whereas smaller follicles show only some luteinization after an ovulatory stimulus (Chateau, 1969).

TABLE 1

Numbers of follicles in various size classes in the right ovary of untreated adult rats with a 5 day cycle.

Volume range of follicles x 10^5 μm^3	MEAN NO. Follicles ± S.E. at: pro-oestrus	oestrus	dioestrus 1	dioestrus 2	dioestrus 3	pro-oestrus
≥ 1000	- [1]	-	0.1 ± 0.1	0.1 ± 0.1	3.1 ± 0.3	4.3 ± 0.6
500-999	-	0.4 ± 0.3	1.3 ± 0.3	5.6 ± 0.8	3.4 ± 0.5	1.9 ± 0.6
200-499	1.4 ± 0.5	8.5 ± 0.4	12.6 ± 1.4	11.5 ± 1.2	6.1 ± 0.5	1.4 ± 0.5
total ≥ 200	1.4 ± 0.5 (9)[2]	9.0 ± 0.8 (9)	14.0 ± 1.7 (9)	17.3 ± 1.8 (7)	12.4 ± 0.8 (10)	7.7 ± 0.7 (9)

1) Numbers of large follicles omitted to focus attention to the new generation of follicles; data identical to those of last column.

2) Number of animals.

3) Underlined numbers are significantly different from numbers in the same size-class on the previous day ($P < 0.02$).

B. Prevention of follicular growth by hypophysectomy (Table 2).

It was found that, with one exception, significant increases or significant decreases in the numbers or volumes of follicles did not occur within 24 hrs after hypophysectomy. The exception was: hypophysectomy during dioestrus -3 resulted in a significant decrease in the number of follicles $\geq 200 \times 10^5$ μm^3. Histologically it was observed that atresia had started in all follicles $\geq 200 \times 10^5$ μm^3.

TABLE 2

Numbers of follicles in various size classes in the right ovary of rats with a 5 day cycle, 24 hours after hypophysectomy on the various stages of the cycle.

Volume range of follicles $\times 10^5$ μm^3	MEAN NO. OF FOLLICLES ± S.E. 24 hrs after hypophysectomy at: oestrus	dioestrus 1	dioestrus 2	dioestrus 3	pro-oestrus
≥ 1000	0.1 ± 0.1			2.7 ± 1.2	3.3 ± 1.1
500-999	0.1 ± 0.1	2.7 ± 0.4	4.4 ± 1.0	4.7 ± 1.2	3.8 ± 0.6
200-499	11.7 ± 0.6	12.7 ± 0.5	9.8 ± 1.2	1.3 ± 0.7[2]	0.9 ± 0.4
total ≥ 200	11.9 ± 1.0 (7)[1]	15.5 ± 0.6 (4)	14.2 ± 0.9 (5)	8.7 ± 2.3 (5)	8.0 ± 0.3 (6)

1) Number of animals.

2) Underlined number is significantly different from number in the same size-class on the previous day in intact animals ($P < 0.02$) (see table 1).

C.1- Effect of PMS treatment on follicular growth in rats hypophysectomized at various phases of the 5 day cycle (Table 3 and fig. 1a).

For comparison with data on normal growth, the reader is referred to Table 1. Doses of 4 IU of PMS were consistently ineffective, as is shown by comparison of the data presented in Table 3 and these on hypophysectomized untreated animals shown in Table 2.

TABLE 3 Numbers of follicles in various size classes found in the right ovary of hypophysectomized rats, treated for 24 hrs with various regimens of PMS, during the phases of the 5 day cycle.

Period of [1] treatment	Volume range of follicles x 10^5 μm^3	MEAN NO. FOLLICLES ± S.E.					
		24 hrs after start of treatment: Hpx [1] +					
		4 IU PMS	6 IU PMS	8 IU PMS	12 IU PMS	16 IU PMS	32 IU PMS
P to O	≥ 1000			0.3 ± 0.3		0.4 ± 0.4	0.3 ± 0.3
	500-999			0.0 ± 0.0		0.2 ± 0.2	0.0 ± 0.0
	200-499	1.6 ± 0.4 [3]	no data	2.0 ± 0.8	4.6 ± 0.3	9.2 ± 0.9	8.8 ± 1.4
	total ≥ 200	1.6 ± 0.4 (6) [2]		2.3 ± 1.2 (5)	4.6 ± 0.3 (5)	9.8 ± 1.0 (5)	9.0 ± 2.3 (5)
O to D1	≥ 1000		-	1.0 ± 0.7	-	-	
	500-999	1.0 ± 0.3	0.7 ± 0.7	1.0 ± 0.7	3.0 ± 1.0	4.6 ± 1.0	
	200-499	8.5 ± 0.5	9.6 ± 0.8	13.8 ± 1.2	16.6 ± 0.3	16.4 ± 2.0	no data
	total ≥ 200	9.5 ± 0.4 (6)	10.3 ± 1.5 (8)	14.8 ± 1.7 (8)	19.6 ± 1.6 (5)	20.9 ± 2.3 (5)	
D1 to D2	≥ 1000	0.5 ± 0.3	-	-	1.3 ± 1.0	0.3 ± 0.3	
	500-999	0.5 ± 0.3	2.3 ± 0.6	3.0 ± 0.7	5.3 ± 0.8	7.0 ± 3.5	
	200-499	12.8 ± 0.9	11.2 ± 1.0	12.9 ± 1.1	11.0 ± 0.8	15.7 ± 2.2	no data
	total ≥ 200	13.8 ± 0.6 (4)	13.7 ± 1.2 (4)	15.9 ± 1.7 (8)	17.6 ± 2.1 (8)	23.0 ± 4.6 (5)	
D2 to D3	≥ 1000	0.7 ± 0.3	1.3 ± 0.3	3.4 ± 0.8	4.3 ± 0.8	3.2 ± 0.9	
	500-999	4.6 ± 0.9	5.0 ± 2.0	2.4 ± 0.6	3.0 ± 0.9	3.2 ± 0.8	
	200-499	9.3 ± 1.3	6.0 ± 0.5	7.0 ± 1.5	6.7 ± 1.0	7.9 ± 1.9	no data
	total ≥ 200	14.6 ± 1.5 (5)	12.3 ± 1.2 (5)	12.4 ± 1.3 (8)	14.0 ± 0.7 (5)	14.2 ± 1.6	
D3 to P	≥ 1000	2.5 ± 0.6	4.3 ± 0.5	5.0 ± 1.1	4.0 ± 1.0	4.8 ± 0.2	
	500-999	3.7 ± 1.1	1.7 ± 0.7	1.8 ± 0.4	1.0 ± 1.0	1.2 ± 0.2	
	200-499	3.6 ± 0.6	3.4 ± 0.9	3.6 ± 0.6	5.9 ± 1.0	5.4 ± 2.7	no data
	total ≥ 200	9.8 ± 1.1 (6)	9.2 ± 1.4 (6)	10.4 ± 1.0 (5)	10.9 ± 0.8 (5)	11.4 ± 2.0 (5)	

1) P: prooestrus, O: oestrus, D: dioestrus, Hpx: hypophysectomized.

2) Number of animals.

3) The underlined values are significantly different from corresponding values in intact rats (Table 1) $P < 0.02$.

From prooestrus to oestrus a regimen of 12 IU PMS induced a significant follicular growth. After 16 IU the ovarian response was increased and apparently maximal since 32 IU did not increase it further. During the corresponding phase of the normal cycle the follicular growth was not significantly different from that seen after 16 and 32 IU PMS (see Table 1).

From oestrus to dioestrus -1, a dose of 8 IU PMS induced a significant follicular growth. After 12 IU the number of follicles $\geq 200 \times 10^5$ μm^3 was further increased and apparently maximal since 16 IU did not cause a further increase. The latter dose of PMS, however, resulted in an increased growth rate of the follicles $\geq 200 \times 10^5$ μm^3. During the corresponding phase of the normal cycle the growth was not significantly different from that seen after 8 IU PMS.

From dioestrus -1 to -2, a dose of 12 IU PMS induced a significant follicular growth. A higher amount did not induce significant changes in the number of follicles $\geq 200 \times 10^5$ μm^3 or in the distribution of follicles in the various size classes. During the corresponding phase of the normal cycle the development was similar to that seen after 12 and 16 IU PMS.

From dioestrus -2 to -3, a dose of 8 IU PMS induced a significant follicular growth. Higher amounts neither increased the growth rate of follicles $\geq 500 \times 10^5$ μm^3 nor prevented atresia in smaller follicles. During the corresponding phase of the normal cycle the development was similar to that seen after 8, 12 and 16 IU PMS.

From dioestrus -3 to prooestrus a dose of 6 IU PMS induced a significant growth of follicles $\geq 500 \times 10^5$ μm^3. Amounts of 12 and 16 IU PMS significantly reduced the normally occurring decrease in the number of smaller follicles, whereas 6 and 8 IU PMS did not. During the corresponding phase of the normal cycle the development was similar to that after 6 and 8 IU PMS.

C.2- Effect of combined PMS/HCG treatment on follicular growth in rats hypophysectomized at various stages of the 5 day cycle.

The most interesting results of this second experimental series are given in Table 4 and fig. 1b. All regimens of HCG were given in addition to a basic regimen of 4 IU PMS, which, given alone, does not increase follicular growth (Table 3).

TABLE 4 Numbers of follicles of various size classes found in the right ovary of hypophysectomized rats, treated for 24 hrs with a regimen of 4 IU PMS combined with 1/2, 1, 2, 25 IU HCG, during the phases of the cycle.

Period of treatment [1]	Volume range of follicles x 10^5 μm^3	MEAN NO. FOLLICLES ± S.E.					
		24 hrs after start of treatment: Hpx [1] + 4 IU PMS +					
		no HCG	0.5 IU HCG	1 IU HCG	2 IU HCG	5 IU HCG	25 IU HCG
P to O [1]	≥ 1000						0.3 ± 0.3
	500-999		no data	no data	2.2 ± 1.0 [3]	no data	3.0 ± 1.2
	200-499	1.6 ± 0.4 [3]					
	total ≥ 200	1.6 ± 0.4 (6) [2]			2.2 ± 1.0 (7)		3.3 ± 1.8 (7)
O to D1	≥ 1000						
	500-999	1.0 ± 0.3		0.2 ± 0.2	2.7 ± 1.3	7.3 ± 2.0	
	200-499	8.5 ± 0.5	no data	9.4 ± 1.2	10.5 ± 1.8	3.2 ± 1.2	no data
	total ≥ 200	9.5 ± 0.4 (6)		9.6 ± 1.7 (5)	13.2 ± 1.5 (5)	20.6 ± 2.6 (5)	
D1 to D2	≥ 1000	0.5 ± 0.3			0.5 ± 0.3	0.3 ± 0.3	
	500-999	0.5 ± 0.3		2.1 ± 1.1	4.5 ± 1.2	5.8 ± 0.9	
	200-499	12.8 ± 0.9	no data	12.9 ± 1.2	9.2 ± 1.5	11.0 ± 1.7	no data
	total ≥ 200	13.8 ± 0.6 (4)		15.0 ± 1.6 (5)	14.2 ± 1.8 (7)	17.0 ± 2.3 (4)	
D2 to D3	≥ 1000	0.7 ± 0.3		0.2 ± 0.2	1.9 ± 1.2	2.8 ± 0.9 [4]	
	500-999	4.6 ± 0.9		4.9 ± 1.2	4.8 ± 0.8	1.8 ± 0.6	
	200-499	9.3 ± 1.3	no data	10.6 ± 1.0	5.2 ± 1.2	13.0 ± 1.3	
	total ≥ 200	14.6 ± 1.5 (5)		15.7 ± 1.6 (5)	11.9 ± 1.7 (8)	17.7 ± 0.7 (6)	
D3 to P	≥ 1000	2.5 ± 0.6	2.3 ± 0.5	6.0 ± 0.3	3.4 ± 1.1 [4]	0.3 ± 0.2 [4]	
	500-999	3.7 ± 1.1	4.0 ± 0.4	0.6 ± 0.2	1.2 ± 0.6	0.7 ± 0.3	no data
	200-499	5.6 ± 0.6	1.6 ± 0.5	2.8 ± 1.5	4.2 ± 1.3	5.7 ± 1.4	
	total ≥ 200	9.8 ± 1.1 (6)	7.8 ± 1.1 (5)	9.4 ± 2.1 (5)	8.8 ± 0.6 (5)	6.7 ± 1.7 (5)	

1) P: procestrus, O: oestrus, D: dioestrus, Hpx: hypophysectomized.

2) Number of animals.

3) The underlined values are significantly different from corresponding values in intact rats (Table 1) $P < 0.02$.

4) Some or all large follicles had ovulated.

From prooestrus to oestrus even the highest regimen of HCG tested (25 IU) induced follicular growth, which was significantly less than that seen during the corresponding phase of the cycle in intact rats.

From oestrus to dioestrus -1, doses of 2 and 5 IU HCG induced a significant follicular growth. During the corresponding phase of the normal cycle a follicular development was seen similar to that after 2 or 3 IU HCG.

From dioestrus -1 to -2, a dose of 2 IU HCG induced a significant follicular growth. The number of follicles $\geq 200 \times 10^5$ μm^3 was probably maximal after this treatment, since it was not significantly higher after 5 IU HCG. During the corresponding phase of the normal cycle the development was similar to that seen after 2 or 5 IU.

From dioestrus -2 to -3 1 IU HCG induced no significant growth of follicles $\geq 500 \times 10^5$ μm^3, whereas 2 and 5 IU HCG did. The doses of 2 IU HCG did not prevent the loss of small follicles. After 5 IU HCG some of the larger follicles ovulated and no loss of smaller follicles was seen. During the corresponding phase of the normal cycle the development was similar to that seen after 2 IU HCG.

From dioestrus -3 to prooestrus 0.5 IU HCG induced no growth of follicles $\geq 500 \times 10^5$ μm^3 and did not prevent a loss of smaller follicles. After 1 and 2 IU HCG a significant growth of large follicles, and an increasing reduction of loss of smaller follicles was observed. After 2 IU HCG ovulations were observed in part of the animals, whereas after 5 IU HCG all animals ovulated. During the corresponding phase of the normal cycle the development was similar to that after 1 IU.

In the next experimental series (no Table) rats were hypophysectomized and treated with a combination of 8 IU PMS/1 IU HCG. This regimen maintained normal follicular growth during the periods from oestrus to dioestrus -1, dioestrus -1 to -2, dioestrus -2 to -3 and dioestrus -3 to prooestrus, but not from prooestrus to oestrus. During the latter phase no follicular growth was observed.

The experiments discussed so far all indicate that to maintain normal follicular development after hypophysectomy, higher gonadotrophin levels are required during the period from prooestrus to oestrus than during the other phases of the cycle. To find out whether this peak requirement concerns primarily gonadotrophins with FSH-like activity or LH-like activity, PMS/HCG regimens with varying ratio's were tested (no Table). It was found that 8 IU PMS/25 IU HCG did not fully re-establish normal follicular growth

whereas 12 IU PMS/2 IU HCG was fully effective in this respect, suggesting that primarily FSH-like gonadotrophins are required.

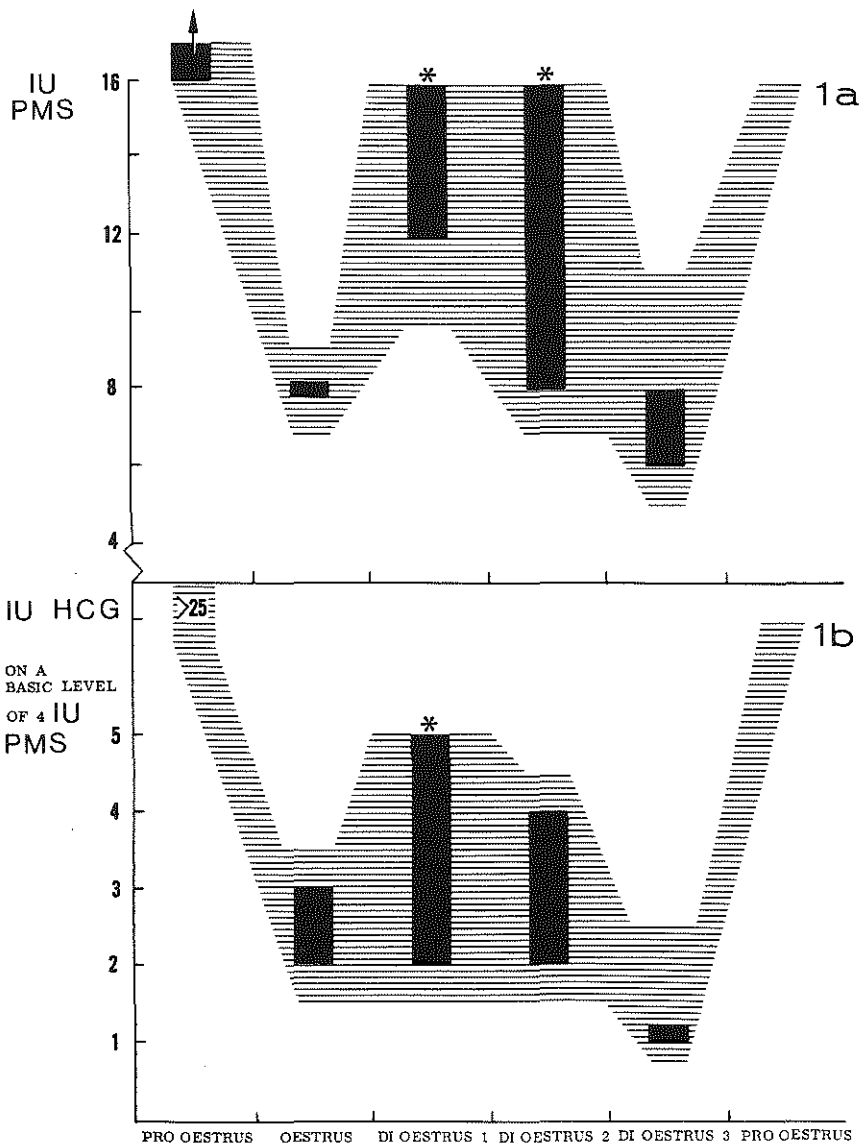

Fig. 1.

Pattern of doses of gonadotrophins able to maintain normal follicular development during the 5 day cycle, as suggested by the data of the tables 3 and 4.
The solid bars represent the range of levels that actually maintained normal follicular growth after hypophysectomy.
The marked values do not represent maximal values, since higher values were not tested.

C.3- Capacity of follicles to ovulate after treatment with gonadotrophin regimens with a predominantly FSH-like (1); LH-like (2) or FSH and LH-like activity (3).

The previous experiments dealt with the effect of substitutional gonadotrophins on only one parameter of follicular development: follicular size. In this additional experiment the effect on a second parameter: the capability of ovulating was tested. Moreover, the effect of treatment of hypophysectomized rats with substitutional gonadotrophins during 1 to 4 days was tested (Table 5). The question, which levels of gonadotrophins are required by developing follicles to become capable of ovulating, was only tested before the dioestrus -2 stage, since follicles spontaneously reached the capacity to ovulate during this phase and maintained this capacity after hypophysectomy and treatment with 8 IU PMS and 1 IU HCG for some days. When rats were hypophysectomized at 12.00 h at dioestrus -2 and treated with substitutional gonadotrophins (8 IU PMS/1 IU HCG) full ovulation was also induced (group 2). This enabled us to test different hormone treatments during dioestrus -1: 12 IU PMS (group 3), 4 IU PMS/2 IU HCG (group 4) and 8 IU PMS/1 IU HCG (group 5). As already observed, these treatments all induced normal follicular growth during that phase. As table 5 shows, a significantly subnormal capacity of follicles to ovulate was seen after treatment with PMS only, whereas both PMS/HCG combinations induced a normal capability of ovulating. Histological control of the ovaries showed corpora lutea atretica and follicles with a partially luteinized wall after treatment with PMS only and no abnormal features after combined PMS/HCG treatment.

When hypophysectomy was performed at oestrus and substitutional gonadotrophins were given during three days, a normal number of ovulations could be induced (group 6). However, after hypophysectomy during prooestrus followed by substitutional gonadotrophins during 4 days, ovulation occurred in a minority of the rats only. These data suggest that the treatment during prooestrus was inadequate. More animals ovulated after giving at pro-oestrus 12 IU PMS/15 IU HCG* than after giving 12 IU PMS/2 IU HCG (groups 7 and 8).

* (15 IU HCG during the first 10 hrs only, since the level had to be decreased to about 1 IU at 12.00 noon during oestrus when the 8 IU PMS/1IU HCG treatment started).

TABLE 5

Number of tubal ova in hypophysectomized rats treated with substitutional doses of PMS and HCG.

Group	Treatment during				number of tubal ova ± S.E. on D3	
	P to O[1]	O to D1	D1 to D2	D2 to D3		
1	none	none	none	15 IU HCG	10.9 ± 0.4 ($^{10}/_{10}$)[3]	9-11[4]
2	none	none	none	hpx[2] 8 IU PMS 1 IU HCG 15 IU HCG	10.3 ± 0.6 ($^{8}/_{8}$)	9-11
3	none	none	hpx 12 IU PMS	8 IU PMS 1 IU HCG 15 IU HCG	4.1 ± 1.3 ($^{4}/_{9}$)	2-6
4	none	none	hpx 4 IU PMS 2 IU HCG	8 IU PMS 1 IU HCG 15 IU HCG	10.8 ± 2.2 ($^{5}/_{5}$)	5-15
5	none	none	hpx 8 IU PMS 1 IU HCG	8 IU PMS 1 IU HCG 15 IU HCG	12.6 ± 1.1 ($^{8}/_{8}$)	8-14
6	none	hpx 8 IU PMS 1 IU HCG	8 IU PMS 1 IU HCG	8 IU PMS 1 IU HCG 15 IU HCG	9.6 ± 2.1 ($^{7}/_{8}$)	5-19
7	hpx 12 IU PMS 2 IU HCG	8 IU PMS 1 IU HCG	8 IU PMS 1 IU HCG	8 IU PMS 1 IU HCG 15 IU HCG	- ($^{0}/_{12}$)	-
8	hpx 12 IU PMS 15 IU HCG	8 IU PMS 1 IU HCG	8 IU PMS 1 IU HCG	8 IU PMS 1 IU HCG 15 IU HCG	8.8 ± 2.5 ($^{6}/_{13}$)	3-21

1) P = procestrus, O = oestrus, D = dioestrus.

2) hpx: hypophysectomy.

3) Number of ovulating rats / total number of rats in that group.

4) Range of numbers of tubal ova in the group of ovulating rats.

Histological inspection of ovaries of rats of these groups showed in the non-ovulating rats: no follicles $\geq 350 \times 10^5$ μm^3 and no fresh corpora lutea; in the ovulating rats: fresh corpora lutea corresponding to the number of ova found and no large follicles. Since it was found (see above) that 12 IU PMS/2 IU HCG induced "normal" growth of small follicles during prooestrus, the histological findings indicate that, in spite of continued treatment, in these non-ovulating rats follicles regressed during the 3 days following prooestrus. The initial stimulus probably has to be stronger, even stronger than 12 IU PMS/15 IU HCG, after which doses follicular growth is maintained in only 50% of the rats. However, higher gonadotrophin levels during prooestrus were not tested since, because of the biological half-life of PMS and HCG, also high bloodlevels at oestrus would occur.

DISCUSSION

The aim of this study was to estimate the pattern of exogenous gonadotrophin levels required for normal follicular development during the 5 day cycle in the rat, and to compare it with the pattern of endogenous gonadotrophin levels measured in intact rats. On the basis of these data it would then be possible to determine to what extent the gonadotrophin levels normally present during the cycle play a role in the regulation of the dynamics of the follicular population.

The estimations could be made since significant changes in the follicular population were found to occur during consecutive days of the cycle in the intact rat, since these changes could be prevented by hypophysectomy and since they could be re-established by certain doses of substitutional gonadotrophins. PMS and HCG were chosen as substitutional gonadotrophins since their biological half-life (Parlow & Ward, 1961) made them more suitable to obtain a rather constant bloodlevel than FSH and LH. However, it is realized that PMS and HCG are not fully comparable with FSH and LH respectively (Schmidt-Elmendorf & Buchholz, 1965; Lunenfeld & Eshkol, 1967; Louwerens, 1970; Northcutt & Albert, 1970). Therefore, changes in gonadotrophin doses required during consecutive days are of greater interest than the absolute amounts.

The data depicted in fig. 1 show the range of gonadotrophin doses required to maintain a normal development in the follicular population after

hypophysectomy during various phases of the cycle.

The minimal levels required during the period from prooestrus to oestrus were in all experiments considerably higher than during other phases. This peak requirement concerned primarily PMS since peak amounts of PMS were able to induce normal follicular growth with or without HCG, whereas modest or low levels of PMS, even in combination with very high levels of HCG were unable to do so (table 5). These results indicate that a peak level of FSH-like gonadotrophins is required to activate a new generation of follicles in adult cycling rats; LH exerting possibly a synergistic action.

The minimal doses found to be required during the three following periods of 24 hrs were approximately constant, except for the peak requirement from dioestrus -1 to -2 during treatment with PMS only. However, this peak requirement is probably not of biological significance, since treatment with PMS only is clearly inadequate during this phase. It resulted in an abnormal low capacity of follicles to ovulate, whereas this was not the case following treatment with a combination of PMS and HCG (Table 5).

The minimal levels required during the period from dioestrus -3 to prooestrus were lower than during all other phases.

The range of the doses able to maintain normal follicular development after hypophysectomy was small during the period from oestrus to dioestrus -1 and the period from dioestrus -3 to prooestrus but it was rather large during other phases.

From these data it can be concluded that the gonadotrophin levels in cycling rats have to be high from prooestrus to oestrus and low from oestrus to dioestrus 1 and from dioestrus -3 to prooestrus. During the other phases they may be in the range of oestrus and dioestrus -3 values but also higher.

The first of these possibilities seems to be realized in the intact rat, for measurements of plasma levels of gonadotrophins in intact rats (Gay et al., 1970) show that a FSH and LH peak is present during prooestrus, whereas a rather low and constant level is present during the other days of the cycle.

Taken in conjunction, these data indicate that the plasma FSH and LH levels present during the cycle in the rat do indeed regulate the dynamics of the follicular population.

The significance of the prooestrous peaks of gonadotrophins apparently is a double one: they simultaneously induce the end of the maturation phase of the present generation of follicles (i.e. ovulation) and the start of the

maturation phase of a next generation of follicles. This finding is in line with that of Pedersen (1970), who found during oestrus a higher growth rate of follicles and a larger number of growing follicles than during any other phase of the cycle.

The present finding is probably also valid for mammalian species in which the cycle includes a luteal phase, since in a number of such species a similar pattern of gonadotrophin levels is found (Gay et al., 1970) and since also in these species the start of development of a new generation of follicles seems to occur around ovulation (see Everett, 1961, for references).

Attention should be given to the atresia of follicles in a size range of $\geq 200 \times 10^5$ μm^3 and $< 500 \times 10^5$ μm^3, which normally occurs during the second half of the cycle. From dioestrus -2 to -3 a loss of follicles occurs in intact rats but, in the reported experiments, not in hypophysectomized rats (tables 1 and 2). However, the latter finding could not be reproduced in later hypophysectomy experiments (unpublished) and possibly has no significance.

A final comment should be made on the differences between individual rats in ovulatory responsiveness, especially following combined PMS/HCG treatment over 4 days and starting at prooestrus. Although the rats used were from the same highly inbred strain, had the same age, bodyweight, cycle length and were living under standard conditions, large differences in responses to the same PMS/HCG treatment were seen (table 5, group 8). In contrast, intact rats of this strain showed only small differences in the number of tubal ova (Welschen, 1970; 1971). This suggests a very precise homeostatic control of follicular growth by a gonadotrophin secretion adapted to ovarian sensitivity in the intact rat. The large differences of the levels of LH and FSH of individual rats seen during prooestrus (Gay et al., 1970) may reflect this mechanism.

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2. DIRECT EFFECTS OF OESTRADIOLBENZOATE AND PROGESTERONE ON MATURING FOLLICLES IN THE ADULT RAT

ABSTRACT

Acute direct effects of oestradiolbenzoate (OB) and progesterone (P) on follicular growth and on the capacity of follicles to ovulate, were studied in the rat. During each of the days of the 5 day cycle a group of rats was hypophysectomized at 12.00 h (noon) and treated with regimens of Pregnant Mare Serum Gonadotrophin (PMS) or of PMS combined with Human Chorionic Gonadotrophin (HCG), which in previous studies proved able to maintain follicular growth. In addition, the rats received either OB (10 or 500 µg), P (2 or 20 mg) or oil. Twenty-four hours later a first series of rats was killed to study the effect of the steroids on follicular growth, and a second series was injected with 15 IU HCG, to study the effect on the capacity of follicles to ovulate.

Follicular growth was usually not influenced by OB or P. A marginal stimulatory effect of P was observed during the period from dioestrus 1 (metoestrus) to dioestrus 2 only. In contradistinction to this, the processes leading to the capacity of follicles to ovulate were stimulated by the low dosages of both OB and P. In rats, hypophysectomized during oestrus and treated with gonadotrophins and oil, only an average of 2.1 (in 8 out of 17 rats) follicles capable of ovulating were present during "artificial" dioestrus 1. Additional OB or P increased these values significantly to 9.7 (in 10 out of 18 rats) and 6.9 (in 18 out of 19 rats) respectively. During later phases of the cycle no effect of OB or P on the number of follicles capable of ovulating could be demonstrated.

Oestrogen and progesterone act on the ovary via the hypothalamo-hypophyseal system. In addition, they are able to exert direct effects on the ovary: in hypophysectomized rats oestrogens are able to slow down the process of follicular atresia (Ingram 1959[a, b]) and are able to stimulate growth of small follicles to medium size by inducing proliferation of granulosa cells (de Wit, 1953; Croes-Buth et al., 1959). Moreover, under certain experimental conditions the responsiveness of the ovaries to gonadotrophins was found to be increased by oestrogen in both intact and hypophysectomized rats (Bradbury, 1961; Smith & Bradbury, 1963). In the rat direct effects of progesterone on the ovaries have not been demonstrated (Smith & Bradbury,

1963), but progesterone has both stimulatory and inhibitory direct influences on the follicular population in other species (Rothchild, 1965; Wallach & Noriega, 1970). In prepuberal rats, in which after hypophysectomy follicular maturation was induced by PMS (or FSH) and ovulation by HCG (or LH), no effect of either oestrogen or progesterone on ovulation rate could be observed (Krähenbuhl & Desaulles, 1964; Callantine & Humphrey, 1965; Gallo & Zarrow, 1970). It seemed of interest to investigate in greater detail the possible direct effects of both types of steroids on other parameters, such as: the growth-rate of follicles and the capacity of follicles to ovulate.

In the present experiment the acute effect of oestradiolbenzoate (OB) and of progesterone (P) on these particulars was studied in hypophysectomized adult rats', in which normal follicular growth, i.e. growth comparable to that during the cycle, was maintained by "physiological" doses of exogenous gonadotrophins (see previous paragraph).

MATERIAL AND METHODS

Rats of 180-200 g bodyweight of a Wistar substrain were used. They were kept in rooms with 14 hrs of light and 10 hrs of darkness and given a commercial diet (Hope Farms) and tap water ad lib. Days of the cycle were determined from vaginal smears and only rats with three consecutive 5 day cycles were used. Experimental procedures included hypophysectomy with a Hoffman-Reiter H-200 hypophysectomy apparatus (H. Neuman & Co, Skokie, Ill.) and treatment with Pregnant Mare Serum Gonadotrophin (HCG, Pregnyl, Organon), oestradiolbenzoate and progesterone. The gonadotrophins were dissolved in 0.1 ml saline and injected intramuscularly. In order to obtain rather constant bloodlevels, PMS was given in two injections per 24 hrs (a first injection of X IU followed by injections of 0.25 X IU every 13 hrs later) and HCG in five injections per 24 hrs (a first injection of Y IU, followed every 5 hrs later by 0.5 Y IU). We will refer to the PMS and HCG regimens by mentioning the intitial dose only. The capacity of follicles to ovulate was tested by means of a single injection of 15 IU HCG at 16.00 h. Oestradiolbenzoate (OB) and progesterone (P) were dissolved in 1 ml oil and injected subcutaneously. Large (500 µg OB, 20 mg P) and relatively small (10 µg OB, 2 mg P) doses were used. All four doses caused a significant increase of uterine weight within 24 hrs. Follicular volumes were determined as described earlier (see previous paragraph) and tubal ova were counted by the method of Rowlands (1942). For statistical analysis of the results Wilcoxon's two sample test was used.

EXPERIMENTAL DESIGN

Exp. I: Effect of steroids on follicular growth.

Rats were hypophysectomized on the day of prooestrus, oestrus, dioestrus 1 (metoestrus), dioestrus 2 or dioestrus 3 between 12.00 and 13.00 h. Immediately afterwards the first dosages of the PMS (1^a) or of the PMS-HCG (1^b) regimens both capable of maintaining normal follicular growth, were injected (see Table 1 for exact doses). Simultaneously OB, P or oil was injected. In rats of group 1^a both the large and the small doses of OB and P were tested, in rats of group 1^b only the small doses. The rats were killed 24 hrs later.

Exp. II: Effect of steroids on the capacity of follicles to ovulate.

Rats were hypophysectomized on the day of oestrus, dioestrus, 1, 2, 3 or prooestrus between 12.00 noon and 1.00 p.m. and treated with the PMS or PMS-HCG regimens, which proved capable of maintaining normal follicular growth (see Table 2 for exact doses). A first injection of OB, P, or oil was given immediately after hypophysectomy, a second 24 hrs later. Only the small doses of OB and P were tested. Twenty-eight hours after hypophysectomy a single dose of 15 IU HCG was given. Ova were counted the next morning.

RESULTS AND DISCUSSION

Exp. 1: Effect of oestradiolbenzoate and progesterone on the growth-rate of follicles.

The substitutional regimens of PMS (group 1^a) and PMS combined with HCG (group 1^b), maintained follicular growth at a rate comparable to that during the cycle (see previous paragraph). Moreover, atresia in follicles of a volume range from 200 to 499 x 10^5 μm^3, characteristic for intact rats in the period from dioestrus 2 to prooestrus, was also observed in these experimental rats.

Results of additional OB or P injections are given in Table 1. Data of group 1^a are given completely, whereas of group 1^b only data of the period from dioestrus 1 to dioestrus 2 are included. OB, given in addition to the gonadotrophins, affected neither follicular growth nor atresia during any phase of the cycle. P was ineffective during most phases, but caused

TABLE 1

Effect of oestradiolbenzoate and of progesterone on follicular growth, maintained by a PMS or PMS/HCG treatment during 24 hours after hypophysectomy on the different days of the oestrous cycle.

Period of treatment (regimens of PMS or PMS/HCG)	Exper. group	Volume range of follicles x 10^5 µm^3	MEAN NO. FOLLICLES ± SE in the right ovary				
			Oil	10 µg OB	500 µg OB	2 mg P	20 mg P
P[1] to O		≥ 1000	0.2 ± 0.2	0.1 ± 0.2	0.3 ± 0.3	0.2 ± 0.2	1.0 ± 0.4
(12 IU PMS + add	1[a]	500-999	0.0 ± 0.0	0.6 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3
inject. of 5 IU HCG		200-499	3.8 ± 0.6 (5)	5.6 ± 0.7 (10)	4.3 ± 1.0 (4)	3.8 ± 1.0 (5)	5.0 ± 1.0 (8)
O to D1		≥ 1000	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
(8 IU PMS)	1[a]	500-999	1.2 ± 0.7	1.0 ± 0.4	1.0 ± 0.6	1.8 ± 0.6	1.0 ± 0.6
		200-499	12.4 ± 1.5 (6)	14.0 ± 1.5 (6)	14.2 ± 1.0 (4)	12.0 ± 1.0 (4)	14.2 ± 2.0 (4)
D1 to D2		≥ 1000	1.7 ± 0.9	1.0 ± 1.0	1.2 ± 0.6	1.4 ± 0.6	1.5 ± 1.0
(10 IU PMS)	1[a]	500-999	5.7 ± 1.3	4.6 ± 0.8	6.6 ± 1.3	5.0 ± 0.8	10.8 ± 1.8[2]
		200-499	9.7 ± 1.7 (6)	11.8 ± 1.2 (4)	10.8 ± 1.8 (5)	12.3 ± 1.4 (4)	6.3 ± 1.9 (8)
D1 to D2		≥ 1000	0.0 ± 0.0	0.6 ± 0.6		0.6 ± 0.6	
(4 IU PMS/2 IU HCG)	1[b]	500-999	7.3 ± 1.8	7.0 ± 1.0		10.8 ± 1.0[2]	
		200-499	11.3 ± 1.7 (6)	11.4 ± 1.4 (5)		7.6 ± 0.6[2] (6)	
D2 to D3		≥ 1000	4.6 ± 1.1	4.0 ± 1.2	4.5 ± 0.6	3.6 ± 1.0	2.0 ± 1.3
(8 IU PMS)	1[a]	500-999	3.4 ± 0.4	3.8 ± 1.3	4.0 ± 1.0	3.0 ± 0.8	2.4 ± 0.7
		200-499	6.3 ± 1.3 (8)	5.6 ± 0.9 (4)	4.3 ± 0.5 (5)	6.4 ± 1.3 (5)	6.0 ± 1.3 (5)
D3 to P		≥ 1000	5.3 ± 0.9	5.6 ± 1.3	4.8 ± 1.1	4.0 ± 0.4	5.8 ± 1.3
(5 IU PMS)	1[a]	500-999	1.3 ± 0.9	1.0 ± 0.5	0.8 ± 0.3	2.3 ± 0.6	1.3 ± 0.6
		200-499	2.7 ± 1.2 (10)	1.1 ± 0.3 (4)	3.0 ± 1.1 (5)	1.4 ± 0.2 (4)	3.5 ± 1.0 (5)

1) P: prooestrus; O: oestrus; D: dioestrus.

2) Underlined values are significantly different from values of oil-treated rats ($P < 0.05$).

3) During prooestrus an additional amount of 5 IU HCG was injected to induce ovulation in the large follicles.

in both experimental groups during the period from dioestrus 1 to dioestrus 2 a significant shift of follicles from the volume range of 200 to 499 x 10^5 μm^3 to that $\geq$ 500 x 10^5 μm^3. For the induction of this phenomenon more P was required during PMS treatment than during PMS/HCG treatment, suggesting a synergism of the P injected with LH-like components of the exogenous gonadotrophins.

Exp. 2: Effect of oestradiolbenzoate and progesterone on the capacity of follicles to ovulate.

In untreated rats of our strain about 10 follicles reach a volume $\geq$ 500 x 10^5 μm^3 and become capable of ovulating during the period from dioestrus 1 to dioestrus 2 (Welschen & Rutte, 1971). In rats, hypophysectomized during oestrus and treated with such regimens of PMS that normal follicular growth was maintained, a similar number of follicles became capable of ovulating during this period: 13.6 $\pm$ 1.2 follicles in 9 out of 10 rats ovulated after HCG during "dioestrus 2", whereas one day earlier, during "dioestrus 1" only 2.4 $\pm$ 1.3 follicles in 3 out of 10 rats ovulated after HCG. Apparently, the substitutional gonadotrophins maintained normal follicular development. OB or P administered simultaneously with the dosis of 15 IU HCG, given to test the capability of ovulating, was ineffective in these hypophysectomized rats (data not included in Table 2). In contrast, OB and P given in addition to the follicular growth maintaining regimens of gonadotrophins, exerted a clear effect (Table 2), when given from oestrus onwards. Then, both steroids induced a significant advancement of the moment when follicles became capable of ovulating, since ovulation of a considerable number of ova was found after HCG given during "dioestrus 1" (Table 2, group 1). The data given in Table 1 show that the ovulating follicles had a volume $<$ 500 x 10^5 μm^3. During the normal cycle follicles of that volume are never capable of ovulating. Moreover, P induced a marginal increase of the number of follicles capable of ovulating during "dioestrus 2" (Table 2, groups 2 and 3). This increase reached statistical significance in group 3 only. This phenomenon paralells the effect of P on the follicular growth rate during this period (exp. 1). OB or P given in addition to follicular growth maintaining regimens of substitutional gonadotrophins during later phases of the cycle did not influence the number of follicles capable of ovulating (data not given in the Table).

TABLE 2

Effect of OB (10 μg) and of P (2 mg) on the capacity of follicles to ovulate.

	TREATMENT				MEAN NO. OF TUBAL OVA ± SE in ovulating animals		
	O [1]	D1	D2	D3	oil	OB	P
group 1	hpx [1] 8 IU PMS oil, OB or P	10 IU PMS oil, OB or P 15 IU HCG	≠ [4]		2.1 ± 1.5 (8/12) [2]	9.7 ± 2.0 (10/18) P < 0.01 [3]	6.9 ± 1.2 (18/19) P < 0.01 [3]
group 2		hpx 10 IU PMS oil, OB or P	8 IU PMS oil, OB or P 15 IU HCG	≠	12.6 ± 1.2 (14/14) [5]	12.3 ± 2.1 (13/13) NS [3]	16.0 ± 1.4 (11/12) NS [3]
group 3		hpx 4 IU PMS 2 IU HCG oil, OB or P	8 IU PMS oil, OB or P 15 IU HCG	≠	13.8 ± 1.6 (6/6)	14.0 ± 0.3 (6/6) NS [3]	19.2 ± 1.4 (6/6) P < 0.05 [3]

1) O: oestrus; D: dioestrus; hpx: hypophysectomy.

2) Number of ovulating rats/total number

3) Significance of differences compared with oil treated control rats.

4) Day of autopsy.

5) This result is considerably different from that of a similar experiment described in the previous paragraph, probably because of the use of a new batch of PMS.

The present experiments show that OB and P are able to exert an acute stimulatory action on processes leading to the capacity to ovulate. Moreover, P appeared able to stimulate follicular growth to volumes $\geq 500 \times 10^5$ μm^3 during dioestrus 1. Whether or not the presence of oestrogen and progesterone is indispensable during these phases of follicular maturation could not be determined during these experiments, since the gonadotrophin treatment induced the secretion of endogenous oestrogen and progesterone. However, the present data taken in conjunction with data showing increasing levels of oestrogen (Shaikh, 1971) and even peak levels of progesterone (Hashimoto et al., 1968; Roser & Bloch, 1969) in ovarian venous plasma near the time when follicles reach volumes $\geq 500 \times 10^5$ μm^3 and become capable of ovulating, might suggest that both types of steroids are involved in this part of follicular maturation.

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CHAPTER III

GENERAL DISCUSSION

GENERAL DISCUSSION

In this chapter we will discuss a) data obtained in the personal studies described and b) a concept of the regulation of follicular development and ovulation rate during the cycle.

A 1.

During the first part of the cycle a certain number of follicles was found to be recruited from the pool of small ($< 200 \times 10^5\ \mu m^3$) follicles. These follicles reached volumes $\geq 200 \times 10^5\ \mu m^3$ during oestrus, dioestrus 1 or dioestrus 2. In the first experiments reported in chapter II, the amounts of gonadotrophins required to induce a similar follicular development in acutely hypophysectomized rats were estimated. The results indicated that high amounts especially of FSH were required in the period from prooestrus to oestrus and much smaller amounts during the days from oestrus to dioestrus 2. Moreover, it was found that high amounts of FSH-like gonadotrophins are required during the whole night between prooestrus and oestrus, since 16 IU PMS, injected at 12.00 noon during prooestrus in acutely hypophysectomized rats, was only effective if followed 13 hours later by an additional injection of at least 4 IU (unpublished results). In intact rats, indeed, high FSH levels are present during the whole night between prooestrus and oestrus (Gay et al., 1970). Taken in conjunction these data indicate that these levels recruite the new crop of ripening follicles. With regard to this hypothesis,

it would be of interest to study the effect on the follicular development of an injection of anti-FSH given during prooestrus. In the present experiments considerable differences were found between individual rats in the amounts of gonadotrophins required for full activation of follicles during prooestrus. This suggests differences in the ovarian sensitivity to gonadotrophins. On the other hand Gay et al. (1970) observed considerable differences between prooestrous gonadotrophin levels in individual rats. The combined evidence of these findings suggests that the amount of FSH secreted in an intact animal might be adapted to the ovarian requirements.

A 2.

The number of follicles recruited during the normal cycle by the peak-levels of FSH, and reaching a volume $\geq 200 \times 10^5$ μm^3 during the period from prooestrus to dioestrus 2, appeared about 20 per ovary. Neither additional gonadotrophins (Welschen & Rutte, 1971) nor high doses of gonadotrophins given after hypophysectomy were able to induce an increase of this number within 24 hours. This suggests that

a) the number of follicles of this type is limited at the level of the ovary,
b) the stimulus normally occurring in intact rats is sufficiently large to recruite the maximal number of follicles capable to respond at that moment, and
c) during each cycle a number of about 40 follicles becomes capable of being recruited during the start of the next cycle.

With regard to these points, two series of observations may be of interest:

1) The number of follicles maturing and ovulating simultaneously after an optimal superovulation treatment (a dose of PMS followed 48 hours later by a dose of HCG) is usually higher in prepuberal than in adult rats (chapter I, 4, Table 2). This suggests a decrease of the number of follicles capable of being recruited simultaneously, with age, possibly due to the continuously occurring atresia in all types of follicles.
2) A superovulation inducing treatment in adult rats was more effective after pretreatment with oestrogen for some days (Husain & Pincus, 1968; Husain, 1969). This can be interpreted as an increase of the number of follicles capable of being recruited simultaneously, due to a direct stimulatory effect of oestrogen on the smaller follicles.

These findings suggest that the number of follicles able to start their final ripening at the beginning of a cycle is primarily determined by the number

of oocytes and small follicles present in the pool. However, this number can possibly be modified by factors such as changing steroid hormone levels.

A 3.

Of the 20 follicles recruited per ovary about 10 are, in principle, able to come to full maturation and ovulation, when adequately stimulated. This was shown in superovulation experiments (Welschen & Rutte, 1971) and after unilateral ovariectomy. The data available at present, do not provide conclusive evidence as to the potency to become capable of ovulating of the other 10 follicles. They might from the beginning be destined to degenerate.

A 4.

During the normal 5 day cycle the period during which growth is observed in all activated follicles lasts from prooestrus to dioestrus 2. From prooestrus to oestrus growth rate is maximal (cannot be increased by additional gonadotrophins) but during the following two days distinctly submaximal, due to relatively low levels of gonadotrophins. The relatively low levels of gonadotrophins probably limit the number of follicles reaching volumes of $\geq 500 \times 10^5\ \mu m^3$ and becoming capable of ovulating.

A 5.

During the normal cycle there is a relation between the volume of the follicles and their capability of ovulating (Peppler & Greenwald, 1970[a,b]; Welschen & Rutte, 1971). Usually all follicles in a volume range of $\geq 500 \times 10^5\ \mu m^3$ are capable of ovulating, whereas smaller follicles are not. On the other hand it has to be emphasized that the capacity to ovulate is not causally determined by the volume. We found that after oestrogen or progesterone treatment follicles $< 500 \times 10^5\ \mu m^3$ were capable of ovulating (chapter II, paragraph 2), whereas in intact rats receiving additional gonadotrophins usually only half the number of follicles $\geq 500 \times 10^5\ \mu m^3$ ovulated after 15 IU HCG (Welschen & Rutte, 1971). Apparently, under physiological conditions qualitative changes leading to capacity to ovulate are accompagnied by increases of the volume to values $\geq 500 \times 10^5\ \mu m^3$.

A 6.

During the normal cycle about 10 follicles reach volumes $\geq 500 \times 10^5\ \mu m^3$ and become capable of ovulating during dioestrus 2. The data of

the presented experiments suggest that progesterone might be directly involved in these processes.

A 7.

A dramatic parting of the ways of follicles $\geq 500 \times 10^5$ μm^3 and of follicles ranging from 200 to 499×10^5 μm^3 during the period from dioestrus 2 to dioestrus 3, the larger ones growing further, the smaller ones degenerating, has been described by other authors (chapter I for references) and has also been observed during the present experiments. This differential response of the two types of follicles points to qualitative differences of, up till now, unknown nature. In the first experiment described in chapter II the degeneration (atresia) of the smaller follicles was prevented by hypophysectomy and could be re-established by a substitutional treatment with exogenous gonadotrophins. The amounts of gonadotrophins required to induce this type of atresia were in the same range as those needed to re-establish normal growth in the larger follicles after hypophysectomy during this phase. These data, suggesting that gonadotrophins are required to induce atresia fit well with those of other studies, showing that atresia is accelerated directly or indirectly by gonadotrophins (Williams, 1956; Talbert, 1968; Eshkol et al., cited by Peters, 1969). Probably LH is involved (Greenwald, 1967). However, in later experiments (unpublished) we could reproduce the effect of hypophysectomy on atresia in 50% of the cases only. Furthermore, the present experiments suggest that acute changes in the level of either oestrogen or progesterone are not directly responsible for the induction of atresia. Further investigations are necessary to elucidate the regulation of this proces of physiological atresia.

A 8.

During the period from dioestrus 3 to prooestrus the growth rate of the large follicles seems to be maximal (it could not be increased by larger amounts of gonadotrophins). Since gonadotrophin levels are unaltered or even decrease during this period, the responsiveness of the follicles might be increased, probably because of the increased steroid levels. Data of van Rees et al. (1968) showing an increasing responsiveness to HCG of these follicles are in agreement with this view.

The data discussed so far are summarized in Fig. 3.

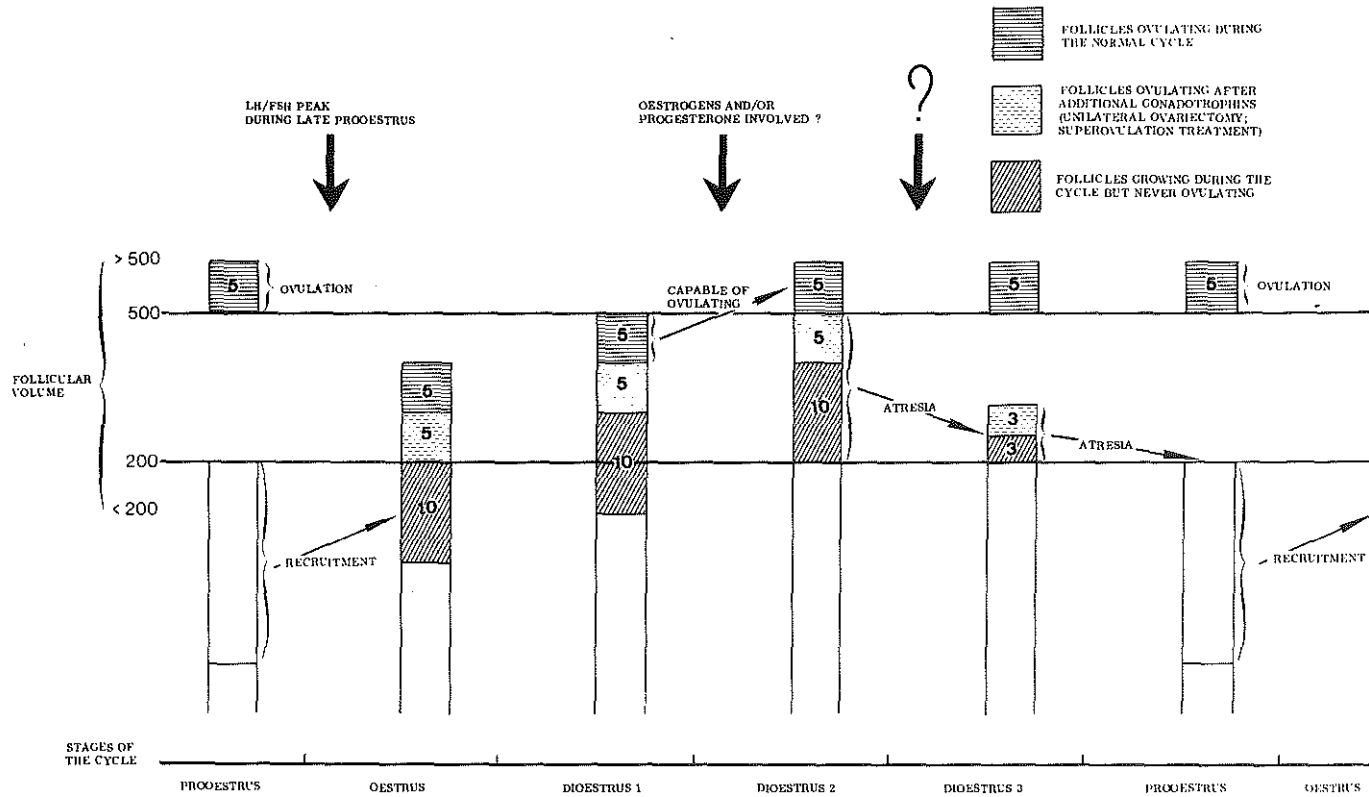

Fig. 3.

Follicular development during the 5-day cycle in the rat, as suggested by the findings discussed in the parts A 1 to A 7 of this general discussion.

B. The data presently available, suggest the following concept of the regulation of the number of follicles destined to ovulate during the 5 day cycle.

At the end of an oestrous cycle the ovaries contain about 10 fully mature follicles and about 40 follicles that are capable of starting their final development during the next cycle. The mature follicles produce increasing amounts of oestrogen , which trigger the release of peak-amounts of LH and FSH. The resulting peaklevels of LH and FSH seem to be the adequate stimulus for both the induction of rupture of mature follicles and for the activation of the 40 smaller follicles. By early oestrus the gonadotrophin levels decrease and maintain follicular growth at a distinctly submaximal rate until dioestrus 2. As yet, the regulation of this decrease of gonadotrophin secretion is not fully understood. It might be induced by the disappearance of trigger amounts of oestrogen , by a negative influence of the relatively high amounts of oestrogen still present during the peak release of gonadotrophins, by a short loop feedback system of the type described by Szontagh & Uhlarik (1964), David et al. (1966), Corbin (1966), Corbin & Story (1967), Fraschini et al. (1968), Hirono et al. (1970), by the high progesterone levels (Davidson et al., 1970) or simply by a relative exhaustion of hypothalamic stores of releasing factors or hypophyseal stores of gonadotrophins. The maintainance of the relatively low levels of gonadotrophins during oestrus, dioestrus 1 and dioestrus 2 seem to be due to the negative feedback action of, at least, oestrogen . This can be concluded from the following findings:

a) An intact negative feedback system is present during this period as was indirectly demonstrated by the occurrence compensatory phenomena after unilateral ovariectomy (Greenwald, 1968; Benson et al., 1969; Welschen, 1970; Welschen, 1972) and by the effect of injections of small amounts of oestradiolbenzoate during these days, which resulted in a decrease of follicular growth rate and a prolongation of the cycle (Gilmore & McDonald, 1969; Welschen, unpublished results).
b) Recent RIA measurements of FSH (Gay et al., 1970) and of oestrogen (Shaikh, 1971) show patterns which suggest a negative feedback relation.

Due to the relatively low levels of gonadotrophins and, consequently, of ovarian steroids, both types of hormones exerting a direct stimulatory effect on the ovaries, only 10 follicles out of the crop of 40 ripening follicles become capable of rupturing during dioestrus 2.

From dioestrus 2 onwards the 30 less developed follicles become atretic and cannot be brought to ovulation any more. There is no evidence available suggesting that the degeneration of smaller follicles is induced by the 10 larger ones. As has been discussed (A_7) the mechanism regulating this wave of atresia is rather obscure. However, after the wave of atresia the number of follicles destined to ovulate at the end of the cycle is fixed. Then, the larger follicles produce increasing amounts of oestrogen -, which trigger the prooestrous gonadotrophin release, and the chain of events described above, starts again.

The hormonal regulation of follicular development and ovulation rate during an oestrous cycle includes

1) the start the final development of 40 follicles,
2) the subsequent maintenance of this development during some days leading to the capacity to ovulate of ten of these follicles and
3) the end of this development, by induction of atresia in the 30 smaller follicles and, two days later, ovulation in the 10 larger ones.

Of the outlined chain of events many data are lacking and have to be available before the regulatory mechanisms can be fully understood. This holds especially for the regulation of gonadotrophin secretion, the effect of ovarian steroids on the ovary and the induction of the wave of atresia during the cycle. When the hormonal regulation is more fully understood, the way is open to study the influence of genetical and environmental factors on follicular development and ovulation rate in greater detail.

SUMMARY

1. Data from the literature show that in the rat the number of ova released from the ovaries during an oestrous cycle depends exclusively on the number of mature follicles present. The mechanism regulating the number of maturing follicles is not yet fully understood; however, the hypophyseal hormones FSH and LH and the ovarian hormones oestrogen and progesterone are certainly involved. For that reason, a study of the effects of these hormones on follicular development during the cycle was undertaken.

2. During the 5 day cycle the following development in the follicular population has been observed: during the period from prooestrus to oestrus about 40 follicles start a rather fast development, which in principle, may lead to full maturation (capacity to rupture) in at least 20 of these follicles, the others seem from the beginning to be destined to degenerate. Normally, of these 20 follicles the 10 largest become capable of ovulating during dioestrus 2, whereas the 10 smaller follicles become atretic at the same time. Therefore, the number of follicles destined to ovulate at the end of the cycle, is fixed during dioestrus 2.

3. The relation between doses of gonadotrophins and follicular development was studied in hypophysectomized rats, treated with PMS (a preparation with a predominant FSH-like activity) and HCG (predominant LH-like activity). During each of the days of the 5 day cycle rats were hypophysectomized and treated with regimens of PMS ranging from 4-32 IU or with regimens of 4 IU PMS combined with 0.5-25 IU HCG. The animals were killed 24 hrs later and the ovaries were studied after routine

histological procedures.

The most important results were:

- The follicular development during the cycle seems to require high gonadotrophin levels (especially FSH) during the period from prooestrus to oestrus (after hypophysectomy a dose of at least 16 IU PMS was required), whereas during other phases low levels are sufficient (8 IU PMS or 4 IU PMS combined with 2 IU HCG).
- During the normal cycle the follicular population is maximally activated from prooestrus to oestrus but submaximally from oestrus to dioestrus 2.
- A relation between gonadotrophin levels and the atresia occurring in smaller follicles from dioestrus 2 onwards, could not be demonstrated convincingly.

4. A first step to study possible direct effects of oestrogen and progesterone on follicular development was made in rats, that were hypophysectomized during various phases of the cycle and, in which follicular growth was maintained by an adequate treatment with PMS or PMS combined with HCG. In addition to the gonadotrophins these rats were given: oil, oestradiolbenzoate (OB. 10 or 500 μg) or progesterone (P, 2 or 20 mg). Twentyfour hours later, the rats were either killed to study the effect of the steroids on follicular growth or injected with 15 IU HCG to study the effect of the steroids on the capacity of follicles to ovulate. The most important results were:

   - OB did not influence follicular growth at all, whereas P seemed to induce an increase of the number of large follicles during the period from dioestrus 1 to dioestrus 2,
   - Both OB and P accelerated processes leading to the capacity of follicles to ovulate.
   - Neither OB nor P exerted any acute effect on atresia of smaller follicles.

5. With regard to the regulation of the number of maturing follicles, the results obtained show that during prooestrus and early oestrus the high levels of FSH present recruite a number of follicles determined at the ovarian level (about 40 in our rats) to start a development to final maturation or atresia. Due to the relatively low gonadotrophin levels present

from oestrus to dioestrus 2 only about 10 follicles of this group became capable of ovulating. This number is possibly also influenced by progesterone levels. The other maturing follicles degenerate. The present experiments were not specifically aimed at this process of degeneration and they did not provide conclusive evidence on a possible role of FSH, LH, oestrogen or progesterone in the induction of atresia. Consequently, the mechanism regulating the number of follicles ovulating during the oestrous cycle in the rat could not be completely elucidated.

SAMENVATTING

1. Uit literatuurgegevens blijkt dat bij de rat het aantal eicellen, dat per ovariële cyclus vrijkomt vrijwel uitsluitend afhangt van het aantal tot rijping gekomen follikels. Het mechanisme dat het aantal tot rijping komende follikels reguleert is nog onduidelijk; van de hypophyse hormonen FSH en LH en van de ovarium hormonen oestrogeen en progesteron mag worden aangenomen dat ze een belangrijke rol spelen. Effecten van deze hormonen op de follikelrijping tijdens de cyclus werden nagegaan.

2. De ontwikkelingen in de follikelpopulatie tijdens de 5-daagse cyclus verlopen als volgt: in de periode van prooestrus tot oestrus starten ongeveer 40 follikels een betrekkelijk snelle ontwikkeling die kan leiden tot volledige rijpheid (ovuleerbaarheid) in tenminste 20 van deze follikels; de andere schijnen al direct voorbestemd te zijn te degenereren. Tijdens dioestrus 2 blijken de 10 grootste follikels in staat te zijn tot ovulatie; ongeveer gelijktijdig gaan de 30 andere tekenen van degeneratie vertonen. De grote follikels groeien door en ovuleren, de kleine degenereren volledig. Het aantal follikels dat zal gaan ovuleren blijkt bepaald tijdens dioestrus 2.

3. De relatie tussen gonadotrophine spiegels en follikelontwikkeling werd bestudeerd in gehypophysectomeerde ratten, die behandeld werden met PMS (een preparaat met voornamelijk FSH-achtige activiteit) en HCG (voornamelijk LH-achtige activiteit). Tijdens elk van de stadia van de 5-daagse cyclus werden ratten gehypophysectomeerd en behandeld met regimens van 4 tot 32 IU PMS of 4 IU PMS gecombineerd met 0.5 tot 25 IU HCG. De dieren werden 24 uur later gedood waarna de ovaria histologisch wer-

den bestudeerd. De belangrijkste resultaten waren:

- De tijdens de cyclus optredende follikelontwikkeling vereist hoge gonadotrophine spiegels (met voornamelijk FSH-activiteit) in de periode van prooestrus naar oestrus (minstens 16 IU PMS), terwijl gedurende de andere fases kan worden volstaan met lage spiegels (8 IU PMS of 4 IU PMS/2 IU HCG).
- In de periode van prooestrus naar oestrus en vanaf dioestrus 2 is de follikelpopulatie in intacte ratten vrijwel maximaal geactiveerd, maar tijdens oestrus, dioestrus 1 en dioestrus 2 submaximaal.
- Een effect van gonadotrophines op de degeneratie van follikels kon niet overtuigend worden aangetoond.

4. Een eerste stap om mogelijke directe effecten van oestrogeen en progesteron op de follikelontwikkeling te bestuderen werd als volgt gemaakt: ratten werden gehypophysectomeerd tijdens verschillende fases van de cyclus en daarna werd de normale follikelontwikkeling onderhouden door adequate hoeveelheden PMS of PMS gecombineerd met HCG. Gelijktijdig werd olie, oestradiobenzoaat (OB, 10 of 500 µg) of progesteron (P, 2 of 20 mg) toegediend. Na 24 uur werden de dieren gedood om de follikels histologisch te bestuderen of werd HCG ingespoten om de ovuleerbaarheid van de follikels te testen. De belangrijkste resultaten waren:
   - OB heeft geen effect op follikelgroei, terwijl P alleen in de periode van dioestrus 1 tot dioestrus 2, een marginale toename van het aantal grote follikels veroorzaakt.
   - OB en P bevorderen beide de snelheid waarmee rijpende follikels ovuleerbaar worden.
   - OB noch P heeft enig acuut effect op follikeldegeneratie.

5. Met betrekking tot de regulatie van het aantal ovuleerbare follikels tijdens de cyclus kan uit de resultaten worden afgeleid dat in prooestrus o. i.v. de hoge FSH spiegel een op ovarium niveau bepaald maximum aantal follikels (ongeveer 40 in onze ratten) een ontwikkeling start die tot volledige rijping of degeneratie kan leiden. Van deze groep wordt in de periode van oestrus tot dioestrus 2 door de relatief lage gonadotrophine spiegels slechts een tiental ovuleerbaar. Mogelijk wordt dit aantal ook beinvloed door progesteron spiegels. De andere follikels degenereren. In de gebruikte proefopstelling, die niet specifiek op het bestuderen van deze

degeneratie gericht was, kon niet worden aangetoond of uitgesloten dat hierbij gonadotrope of ovariumhormonen een rol spelen. Daardoor kon het probleem van de regulatie van het aantal ovulaties per cyclus slechts ten dele opgelost worden.

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APPENDIX

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SHORT COMMUNICATIONS

CORPORA LUTEA ATRETICA IN OVARIAN GRAFTS

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Detection of ovulation or processes resembling ovulation in ovarian grafts is regularly used to demonstrate ovulatory discharge of gonadotrophins. However, corpora lutea atretica (CLA), i.e. corpora lutea with entrapped ova, are often found (Noyes, Yamate & Clewe, 1958; Moll & Zeilmaker, 1966; Quinn, 1966). Since CLA occur not only in grafts (Deanesley, 1956; Jones & Krohn, 1960) but also in ovaries *in situ* (Jones & Krohn, 1961), especially after an inadequate ovulatory stimulus (Rowlands, 1944), they may reflect both abnormal responsiveness of the tissue after grafting and abnormal discharge of gonadotrophins. Therefore, the occurrence of CLA in ovarian grafts functioning under normal hormonal control was studied.

In a first experiment neonatal rats were ovariectomized unilaterally (15 rats) or bilaterally (12 rats) and received one ovarian autograft under the left kidney capsule. Normal (19) and unilaterally ovariectomized rats (16) served as controls. The rats were killed during the first, fifth or tenth oestrus and all ovarian tissues studied histologically. In the cycles studied, the three operated groups showed a number of fresh corpora lutea ranging from $9{\cdot}0 \pm 1{\cdot}9$ (S.E.M.) to $12{\cdot}6 \pm 1{\cdot}7$, values not significantly different from those of intact controls (range of $10{\cdot}7 \pm 1{\cdot}0$ to $12{\cdot}1 \pm 1{\cdot}4$). Neither the control nor the experimental groups showed CLA. Furthermore, the distribution of ovulations between the ovaries *in situ* and the grafts changed in unilaterally ovariectomized rats from 9·6 (ovary *in situ*) *v.* 3·0 to 8·6 *v.* 1·8 and to 11·5 *v.* 0·7 at the first, fifth and tenth oestrus respectively.

In a second experiment adult rats underwent similar operations during oestrus. They were killed on the day immediately after the operation or after 1–30 cycles. Normal (24) and unilaterally ovariectomized rats (24) served as controls. The mean number of ovulations in the control groups was $11{\cdot}0 \pm 0{\cdot}9$ for the normal and $11{\cdot}5 \pm 1{\cdot}2$ for the unilaterally ovariectomized rats, values not significantly different from those in the experimental groups. In the control groups no CLA were found. The experimental results (Table 1) show that, in rats in which the absence of CLA in the ovary *in situ* proved a normal hormonal balance, at least 25 % of the fresh corpora lutea in the grafts were CLA. This percentage was larger immediately after operation, suggesting its dependence on the length of the reorganization period of the grafted tissue.

The number of ovulations in the grafts of unilaterally ovariectomized graft-bearing rats decreased postoperatively, whereas it increased in the ovaries *in situ*, like the first experiment. The reduction of the number of ovulations in the grafts was

Table 1. *Total number of fresh corpora lutea (CL, means* ± S.E.M.*), and the % corpora lutea atretica (CLA), in ovaries* in situ *and in ovarian grafts 1–30 cycles after the operation in adult female rats*

Number of postoperative cycles	Bilateral ovariectomy: Grafted ovary		Unilateral ovariectomy: Ovary *in situ*		Unilateral ovariectomy: Grafted ovary	
	CL	% CLA	CL	% CLA	CL	% CLA
1	0* (8)	–	10·3 ± 0·9 (18)	0	0†	–
2	9·8 ± 1·9 (8)	85	8·4 ± 1·9 (12)	2	2·9 ± 1·8	52
3	9·1 ± 2·1 (6)	34	8·6 ± 1·8 (16)	2	2·6 ± 1·4	28
5	9·9 ± 1·8 (8)	28	8·9 ± 1·1 (16)	0	1·4 ± 1·2	20
10	9·7 ± 2·7 (8)	20	9·7 ± 2·1 (8)	0	1·0 ± 1·0	about 25
30	9·1 ± 2·1 (8)	24	10·0 ± 2·0 (8)	4	0·6 ± 0·6	about 25

* First postoperative oestrus started on day 8 or 9 after operation and was prolonged (2–4 days); grafts showed follicles of all sizes, sometimes with a partially luteinized wall.

† First postoperative oestrus on day 5 after operation; grafts showed only small follicles.

Number of animals in parentheses.

not due to loss of oocytes caused by transplantation (Jones & Krohn, 1960). In an additional experiment we found that grafts that had been in unilaterally ovariectomized rats for 10 cycles showed 11·8 ± 1·4 fresh corpora lutea after removal of the ovary *in situ*. Therefore, a competition between ovaries *in situ* and grafts, leading to inactivity of the grafts (Biskind, Kordan & Biskind, 1950), seems likely. This could be due to suboptimal reorganization of the grafted tissue.

Our results indicate that even under normal hormonal conditions CLA are to be expected in ovarian autografts. This can be explained by a decreased responsiveness of the grafts due to a suboptimal reorganization. The absence of CLA in ovarian grafts with a long reorganization period (neonatal grafts) and the gradually decreasing percentage of CLA in adult grafts may reflect progressive reorganization of the grafted tissue.

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COMPENSATORY OVARIAN GROWTH AND COMPENSATORY OVULATION AFTER UNILATERAL OVARIECTOMY IN RATS WITH AN OVARIAN AUTOGRAFT IN THE REGION OF THE PORTAL VEIN

By

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ABSTRACT

Compensatory growth and the compensatory increase of the number of ovulations in the remaining ovary after unilateral ovariectomy were compared in three groups of rats:
I. Rats with no ovarian graft. II. Rats with an ovarian autograft in the region of the portal vein. III. Rats with an ovarian autograft in a region draining into the general circulation.
Compensatory growth was not inhibited by ovarian grafts placed in the region of the portal vein, whereas this was inhibited with grafts of the same volume but draining into the general circulation. This finding supports the view that compensatory ovarian growth after unilateral ovariectomy is at least partially the result of an increase in the gonadotrophin level in the blood, following the decrease in oestrogen level.
In contrast, the total number of ovulations did not differ significantly in the three groups. The number of ovulations in the ovary *in situ* was in most cases decreased, when ovulations had occurred in the grafts, even in animals with a graft in the region of the portal vein. These findings are discussed.

After unilateral ovariectomy the remaining ovary shows compensatory growth and a compensatory increase in the number of ovulations. Two mechanisms have been suggested to explain these phenomena: (1) consumption or inactivation of an increased proportion of an unchanged amount of circulating gonadotrophins by the one remaining ovary. This has been defended by

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McLaren (1966), who found no increase in the gonadotrophin concentration of the hypophysis after unilateral ovariectomy, and *Zarrow et al.* (1965) (2) a compensatory increase in the output of gonadotrophins from the pituitary gland as the result of a decreased oestrogen secretion following the removal of one ovary (*Edgren et al.* 1965). Since such an increase in the output of gonadotrophins from the pituitary gland has recently been reported (*Grady & Greenwald* 1968; *Benson et al.* 1969), it was considered of interest to test the consumption theory again. The compensatory phenomena have been compared in three groups of unilaterally ovariectomized rats. I. Rats with no ovarian graft. II. Rats with an ovarian graft in the kidney or ovarian bursa. III. Rats with an ovarian graft in the vena porta region. This last type of graft in the adult rat contributes only a very small amount of oestrogens to the general circulation because of the metabolizing activity of the liver (*Donovan & O'Keeffe* 1966; *Ber* 1968). If the consumption theory is correct, a similar inhibition of the compensatory phenomena would be expected in rats with both types of grafts. *McLaren*'s (1966) data from a comparable experiment in mice did not provide any conclusive evidence. This she ascribed to the rapid degeneration of the grafts.

MATERIALS AND METHODS

The experiments were performed on adult female rats of the inbred R strain of the Netherlands Cancer Institute Amsterdam. Rats were maintained in groups of five per cage with food and water *ad lib.* The rat room was illuminated from 6.00 a. m. to 8.00 p. m. Vaginal smears were taken daily for at least two weeks before the operations until the animals were killed. Operations and autopsies were always performed on the day of vaginal oestrus (almost all cells were cornified). All animals had a regular 5 day cycle before and after operation.

Most of the rats were unilaterally or bilaterally ovariectomized and received an ovarian autograft. In a first experiment the grafts were placed either under the kidney capsule or in the spleen. Since ovarian grafts in the spleen had a somewhat abnormal appearance with distinct fibrosis and a reduced number of small follicles and corpora lutea, a second experiment was performed in which the grafts were placed in the ovarian bursa instead of under the kidney capsule and in the greater omentum instead of in the spleen. These grafts proved to be histologically comparable. The term ovary *in situ* is used only for untouched *in situ* ovaries, not for grafts in the ovarian bursa.

In preliminary experiments it was found that during the first three cycles after unilateral ovariectomy the weight of the remaining ovary increased by 50 % (see also *Peterson et al.* 1964). During the following cycles the increase in weight was small. On the other hand, a decrease in the number of ovulations (eventually leading to a complete absence of ovulations) was observed in ovarian grafts of unilaterally ovariectomized rats during the first five cycles after the operation. Three cycles after the operation there was both a distinct compensatory ovarian growth and an active graft. Therefore the animals were killed in the third vaginal oestrus after operation.

After killing the ovaries *in situ* and uteri were weighed wet. The weight of the grafts could not be determined accurately since it was very difficult to clean them from the adjacent tissue without causing damage which might cause difficulties in the histological studies. Ovaries *in situ* and grafts were fixed in Bouin's fluid, embedded in paraffin and sectioned. The sections were stained with haematoxylin and eosin and used for counting the number of fresh corpora lutea.

All data of animals in which adhesions were found between the grafts in the vena porta region and the body wall or periovarian fat, were discarded.

The statistical analysis of the results was performed using Wilcoxon's two sample test. A difference was considered as statistically significant, if the double tail probability ≤ 0.01. The ovarian and uterine weights in the tables are given per 100 g bodyweight. Statistical evaluation of the results, however, leads to the same conclusions when absolute weights are compared.

OBSERVATIONS

Experiment I: Comparison of the effect of ovarian grafts of different sizes in the spleen or the kidney on compensatory ovarian growth and ovulation

After the survival period of three cycles, the ovaries of unilaterally ovariectomized rats showed a mean increase in weight of about 50 %. The mean number of corpora lutea showed a two fold increase (Table 1, groups 1 and 2).

The inhibitory effect of ovarian grafts in the kidney on compensatory growth of the ovary *in situ* was very marked, when 1/1 or 1/2 ovary was grafted. When, however, 1/10 ovary was grafted to the kidney we found no inhibition. Absence of inhibition was also found with grafts in the spleen independently of their volume. Rough estimations of the weight of the grafts yielded values of 8–11 mg when 1/1 or 1/2 ovary was grafted and 0.5–2 mg when 1/10 ovary was grafted. We did not find a tendency for kidney ovaries to have a higher weight than spleen ovaries. Rough estimates of the volume of the grafts from histological sections too did not show any difference between spleen and kidney ovaries.

The total number of fresh corpora lutea per animal (in *in situ* and, if present, in grafted ovaries) did not differ significantly between the control and experimental groups, although a considerable variation was observed. The number of fresh corpora lutea in the ovaries *in situ* was significantly higher without grafted ovaries than with 1/1 or 1/2 ovary grafted to the kidney or 1/1 ovary grafted to the spleen (Table 1, groups 2, 3, 4 and 6).

The mean uterine weight was significantly higher in the groups with spleen ovaries than in the other groups.

Experiment II: Comparison of the effect of ovarian grafts in ovarian bursa and greater omentum on compensatory ovarian growth and ovulation

The histological appearance of ovarian grafts approached the normal con-

Table 1.

Inhibitory effects of ovarian autografts (of different sizes) in the kidney or in the spleen on compensatory growth and ovulation of the right ovary 3 cycles after removal of the left ovary.

Group	Number of animals	Mean weight ± SE of the right ovary (mg per 100 g body-weight[1])	Mean number of fresh corpora lutea ± SE in the right ovary *in situ*	in the graft	Mean weight ± SE of uterus (mg per 100 g bodyweight[1])
1) Intact animals[2])	10	19.6 ± 0.8	5.7 ± 0.9	—	238 ± 8
2) Unilaterally ovariectomized animals	10	29.4 ± 2.6	10.6 ± 1.2	—	231 ± 6
3) Unilaterally ovariectomized animals with 1/1 ovary grafted to the kidney	6	23.3 ± 2.3[3])	7.2 ± 1.8[4])	3.2 ± 1.1	231 ± 11
4) Same, 1/2 ovary grafted to the kidney	6	23.5 ± 2.5[3])	6.7 ± 3.1[4])	2.0 ± 1.9	235 ± 9
5) Same, 1/10 ovary grafted to the kidney	6	28.0 ± 2.8	10.5 ± 1.9	1.8 ± 1.6	242 ± 7
6) Same, 1/1 ovary grafted to the spleen	6	31.4 ± 4.1	8.0 ± 1.1[4])	1.5 ± 1.0	257 ± 7[5])
7) Same, 1/2 ovary grafted to the spleen	6	29.2 ± 4.8	9.8 ± 1.4	0.5 ± 0.8	268 ± 7[5])
8) Same, 1/10 ovary grafted to the spleen	6	28.9 ± 4.9	10.0 ± 1.4	0.6 ± 0.3	272 ± 12[5])

1) All animals weighed 130–150 g.
2) No significant differences were found between weights and ovulation rates of right and left ovaries.
3) Significantly different from data of groups 1, 2, 5, 6, 7 and 8.
4) Significantly different from data of groups 1, 2, 5, 7 and 8.
5) Significantly different from data of groups 1, 2, 3, 4 and 5.

dition more closely in the greater omentum than in the spleen. Nevertheless the number of fresh corpora lutea was somewhat lower in the ovaries in the omentum than in the ovaries grafted to the ovarian bursa. This difference, however, was not significant. Moreover rough estimates of the weight and volume of both types of grafts showed no significant differences. The results are given in Table 2. This experiment confirmed the three major results of the first experiment: (1) an ovarian graft in the vena porta region did not inhibit compensatory ovarian growth, whereas this occurred with ovarian grafts draining into the general circulation. This effect was quantitatively small, but statistically convincing. (2) Both types of grafts are able to »inhibit« to some extent the increase in the number of ovulations in the ovary *in situ* after unilateral ovariectomy. (3) The mean uterine weight is significantly higher in unilateral ovariectomized animals bearing an ovarian graft in the vena porta region than in animals with no ovarian graft or with a graft draining into the general circulation.

In order to get more information about the oestrogen metabolizing activity of the liver we included two groups of bilaterally ovariectomized rats, one with no graft and one with a graft in the greater omentum. The grafts were large and looked healthy after a survival period of 15 days. Nevertheless, none of the animals showed vaginal cornification during the postoperative period. On the other hand, the mean uterine weight in bilaterally ovariectomized rats bearing an ovarian graft in the vena porta region was higher than in bilaterally ovariectomized rats with no graft. In both groups, however, the mean uterine weight was markedly decreased in comparison with the other groups in Table 2.

DISCUSSION

As pointed out in the introduction we tried to decide between the two suggested mechanisms of compensatory growth and ovulation after unilateral ovariectomy. It seemed reasonable to assume that otherwise comparable ovarian grafts in the vena porta region and in a region draining into the general circulation would have the same inhibitory effect on the compensatory growth and ovulation providing that the consumption theory was correct but a different inhibitory effect if the feedback theory was correct.

Firstly it is desirable to make some remarks on the testsystem used. The system has to fulfil two conditions (1) that both types of grafts are fully comparable and (2) that grafts in the vena porta region contribute little or no oestrogens to the general circulation, and in consequence do not exert a major feedback action on the hypothalamo-hypophyseal system. The first condition is not fulfilled in the case of ovarian grafts in the kidney and spleen since

Table 2.

Effects of ovarian autografts (of a whole ovary) in the ovarian bursa or greater omentum on uterine weight, compensatory growth and ovulation of the right ovary 3 cycles after removal of the left ovary or 15 days after removal of ovaries.

Group	Number of animals	Mean weight ± SE of the right ovary (mg per 100 g body-weight[1])	Mean number of fresh corpora lutea ± SE in the right ovary *in situ*	in the graft	Mean uterine weight ± SE (mg per 100 g bodyweight[1])
1) Unilaterally ovariectomized animals	10	24.9 ± 1.9	10.8 ± 0.7[4])	—	223 ± 7
2) idem + ovary grafted to the ovarian bursa	10	21.1 ± 1.5[3])	8.6 ± 0.4	1.9 ± 1.7	220 ± 6
3) idem + ovary grafted to the greater omentum	10	24.8 ± 2.1	9.4 ± 0.4	1.1 ± 1.0	247 ± 10[5])
4) bilaterally ovariectomized[2])	10				75 ± 4[6])
5) bilaterally ovariectomized[2]) + ovary to the greater omentum	10				98 ± 6[6 7])

1) All animals weighed 155–180 g.
2) Since these animals were not cycling, they were killed on the 15th day after operation (comparable with 3 cycles).
3) Significantly different from values of the groups 1 and 3.
4) Significantly higher than the numbers of groups 2 and 3.
5) Significantly higher than values of groups 1 and 2.
6) Significantly lower than values of groups 1, 2 and 3.
7) Significantly higher than that of group 4.

ovaries in the spleen showed a distinct fibrosis (see also *Biskind et al.* 1950). Omentum and bursa ovaries, on the other hand, seemed to be fully comparable in this respect. With regard to the second point it may be remarked that *Ber* (1968) and many other investigators found an effect of ovarian grafts in the vena porta region of bilateral ovariectomized rats on uterine weight, indicating that some oestrogens pass through the liver. We found the same effect in both bilaterally and unilaterally ovariectomized rats. Some of the investigators mentioned by *Ber* (1968), however, conclude from the progressive tumour-formation in ovarian grafts in the spleen of bilaterally ovariectomized rats, that there is no major feedback action by these oestrogens on the hypothalamo-hypophyseal system. This may be due to two factors, i. e. either the higher threshold of this system for oestrogens or the fact that the oestrogens which pass through the liver produce metabolites which influence the uterus but not the hypothalamo-hypophyseal system. *Byrnes & Meyer* (1951*a,b*), *Maekawa & Imai* (1954) and *Miyake* (1961) showed that in immature rats more oestrogen was required to produce positive stimulation of the uterus than to inhibit the pituitary gland; the evidence for a similar differential in adult rats, however, was equivocal (*Byrnes & Meyer* 1951*b*). On the basis of these considerations we believe that the test system allows of valid conclusions.

In this system the ovarian weight seems to be a much more sensitive parameter for the gonadotrophin level than the number of ovulations. The ovarian weight adds the responses of all types of ovarian tissue over a period of many days, whereas the number of ovulations gives information on the gonadotrophin secretion over a few days only. Compensatory growth will therefore first be considered. Ovarian grafts in the vena porta region did not inhibit compensatory growth of the ovary *in situ*, whereas inhibition occurred with comparable grafts draining into the general circulation. This indicates that compensatory ovarian growth depends on diminution of the amount of oestrogen producing ovarian tissue and not on the diminution of the amount of gonadotrophin consuming ovarian tissue. This excludes the first concept, mentioned in the introduction, as a plausible mechanism of compensatory ovarian *growth*. It is in agreement with the findings of *Benson et al.* (1969) that unilateral ovariectomy induces an increase in gonadotrophin levels and ovarian weight, which can be reduced by oestrogen injections.

The total number of fresh corpora lutea, indicating the number of ovulations, is the same in unoperated and unilaterally ovariectomized rats. From this it can be argued that the increase in gonadotrophin levels after unilateral ovariectomy is too small to cause more ovulations than occurs normally.

The distribution of the ovulations between the ovaries *in situ* and grafts is the same in unilaterally ovariectomized animals, whether they bear an ovarian graft in the vena porta region or in a region draining into the general circulation. The most plausible explanation for this finding is that the hormonal

feedback agent of large preovulatory follicles is not important for the determination of the number of ovulations. This last suggestion may be correct, provided that *Greenwald*'s (1962) finding in the golden hamster, that the number of ovulations depends on the FSH level in the blood in the first days of the cycle, is also true for rats.

This interpretation of our findings comes very close to the view of *McLaren* (1966) and suggests that the increase in gonadotrophin levels after unilateral ovariectomy is not responsible for all the compensatory phenomena.

In conclusion we may say that in rats, the decrease in the oestrogen level of the blood after unilateral ovariectomy results in an increase in gonadotrophin levels, sufficiently large to cause an increase in ovarian weight but probably too small to cause an increase in the total number of ovulations.

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OVULATION IN ADULT RATS AFTER TREATMENT WITH PREGNANT MARE SERUM GONADOTROPHIN DURING OESTRUS

By

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ABSTRACT

Treatment of the adult rat with pregnant mare serum gonadotrophin (PMS) followed by human chorionic gonadotrophin (HCG) is the standard procedure for inducing superovulation. Experiments were performed on rats with a 5 day cycle to determine why treatment with PMS only does not produce superovulation.

I. In *untreated* animals all follicles in a range of $\geqq 55 \times 10^6$ μm^3 take part in ovulation. Similarly, in precocious ovulation induced by HCG in otherwise untreated animals, all follicles in this size range produce ovulations.

II. After the injection of *5 IU of PMS* into rats during oestrus the number of follicles in the size range of $\geqq 55 \times 10^6$ μm^3 is doubled, but only half of them take part in spontaneous ovulation, which occurs one day earlier than in untreated animals. An additional ovulating stimulus by means of treatment with HCG causes no increase in the number of ovulations. Data from hypophysectomized animals receiving HCG indicate that the ovulatory release of luteinizing hormone (LH) is not subnormal following treatment with 5 IU of PMS.

III. After the administration of *10–35 IU PMS* in oestrus, spontaneous ovulation does not occur. Data on hypophysectomized animals receiving HCG indicate that at this dose level of PMS, the ovulatory release of LH is subnormal. Indirect evidence suggests that this is due to high oestrogen levels in the blood, blocking the ovulatory release of LH.

IV. After *50–80 IU of PMS* spontaneous ovulation of a small number of ova occurs on day 3. The ovulatory release of LH, estimated as in

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the previous experiments, is not distinctly subnormal. Therefore at this dose level of PMS a diminished responsiveness of the ovaries is responsible for the subnormal number of ovulations.

The number of follicles that ripen in the two ovaries of an adult mammal during an oestrous cycle and consequently the number of ovulations, is said to depend primarily on the levels of follicle stimulating hormone (FSH) in the blood (*Fowler & Edwards* 1957). As part of a study on the mechanisms that regulate the constancy of the number of ovulations per cycle in the rat, it seemed of interest to test the effect of administration of pregnant mare serum gonadotrophin (PMS), which has predominantly an FSH-like activity, on the number of ovulations.

It has already been demonstrated that PMS injections increase both the rate of follicular development and the number of developing follicles (*Greenwald* 1962, golden hamster). This may result in spontaneous superovulation (*Greenwald* 1962; *Ying & Meyer* 1969). However, in most experiments, in which adult mice and rats were injected with PMS in order to induce superovulation, additional treatment with human chorionic gonadotrophin (HCG) was apparently required (*Fowler & Edwards* 1957; *Edwards & Fowler* 1960; *Husain & Pincus* 1968; *Husain* 1969; *Husain & Saucier* 1970). It may be assumed that in these animals the release of endogenous luteinizing hormone (LH) is insufficient to induce superovulation or even ovulation.

The present experiments were performed to verify this assumption. More specifically we tried to answer the following questions: (1) does ovulation induced by endogenous LH occur after PMS treatment at the start of the cycle? When this turned out to be the case to a minimal extent only, the next question was: (2) is this minimal ovulatory activity due to a subnormal release of endogenous LH or to a decreased responsiveness to LH of the PMS stimulated ovaries?

MATERIALS AND METHODS

The experiments were performed with adult female rats (150 g body weight) of the R-Amsterdam strain bred in this laboratory. The rats were kept in groups of 5 animals per cage and given food and water *ad libitum*. The rat room was illuminated from 5.00 a. m. to 7.00 p. m. Vaginal smears were taken daily for at least two weeks before the animals were used. Only rats with two consecutive 5 day cycles were used. Experimental procedures included treatment with PMS, with HCG, and also hypophysectomy. PMS (Gestyl®, Organon) and HCG (Pregnyl®, Organon) were always administered at 2.00 p. m., dissolved in 0.1 ml saline. PMS was given when the animals were in oestrus. Hypophysectomies were performed by the trans-auricular approach (*Falconi & Rossi* 1964). Tubal eggs were counted by the method of *Rowlands* (1944). Three separate experiments were performed.

I. Ovulation after a combined PMS-HCG treatment and after PMS treatment only

Series a. – The rats received PMS at dose levels of 0, 5, 10 or 50 IU and were killed on the 1st to 6th day after injection.

In the ovaries of these animals the follicle sizes were determined by the method of *Boling et al.* (1941) using routine histological procedures.

Series b. – The rats received PMS at the same dose levels as in series a, but in addition received 15 IU HCG on the 1st, 2nd, 3rd or 4th day after PMS treatment. They were killed on the day after HCG administration. In the group, which received 50 IU PMS, doses of HCG both below and above the standard dose of 15 IU were also tested.

II. Effects of hypophysectomy on ovulation following PMS treatment

Rats treated with 50 IU PMS were hypophysectomized on the day before the expected ovulation at 12.00 noon, 2.00 p. m. or 4.00 p. m.

III. Estimation of endogenous LH release in PMS-treated rats

These animals received 0, 5, 10, 20, 35, 60, 80 or 100 IU PMS respectively. For each dose level, one group of rats was killed without any further treatment on the day of the expected ovulation. The remaining animals were hypophysectomized and treated with saline or with substitutional doses of 0.5–5 IU HCG on the day before the expected ovulation and killed on the next day.

RESULTS

Experiment 1: Ovulation after a combined PMS-HCG treatment and after PMS treatment only

In untreated rats with a 5 day cycle, only 10–12 follicles reached a volume of $\geqq 55 \times 10^6$ μm^3. On the day after ovulation, of 10–12 ova, the largest follicles had a volume of $\leqq 54 \times 10^6$ μm^3 (Table 1). These data suggest that in these rats all the follicles that reached a volume of $\geqq 55 \times 10^6$ μm^3 were destined to ovulate. An injection of 15 IU HCG given to normal rats on the 2nd, 3rd or 4th day of the cycle resulted in ovulation of an approximately normal number of ova. A similar injection given on the 1st day of the cycle was ineffective (Table 3, first line). This showed that in normal rats a positive response to HCG was only found when the ovaries contained follicles of $\geqq 55 \times 10^6$ μm^3 in a number corresponding to the normal number of ovulations. Thus suggests that in normal rats follicles of this volume not only ovulate at the end of the cycle but are also capable of ovulating as soon as a sufficiently large ovulatory stimulus is given.

A single injection of 5–50 IU PMS during oestrus induced an acceleration of the development of the individual follicles and also an increase in the number of follicles of $\geqq 55 \times 10^6$ μm^3 up to 20–60 per animal (Table 2). However, the injection of 15 IU HCG resulted in a number of ovulations that

Table 1.
Volumes and numbers of follicles per two ovaries during the oestrous cycle of the intact rat with a 5 day cycle.

Volume range of follicles ($\times 10^6$ μm^3)	Mean No. follicles ± SE[1]				
	Day 1	Day 2	Day 3	Day 4	Day 5
201–250	–	–	–	0.6 ± 0.3	–
151–200	–	–	1.0 ± 0.0	4.3 ± 1.3	–
101–150	–	0.3 ± 0.3	4.3 ± 0.3	4.0 ± 1.0	–
55–100	2.0 ± 0.0	10.0 ± 1.5	7.3 ± 1.2	2.5 ± 1.5	–
40–54	7.3 ± 1.2	5.7 ± 0.3	2.7 ± 0.9	0.6 ± 0.3	3.0 ± 0.3

[1] All numbers given are the mean of groups of 3 rats.

Table 2.
Mean number of follicles of $\geq 55 \times 10^6$ μm^3 per two ovaries of rats after an injection with saline or PMS during oestrus.

PMS dose	Mean No. follicles ± SE[1]				
	Day 1	Day 2	Day 3	Day 4	Day 5
0 IU	2.0 ± 0.0	10.3 ± 1.3	12.7 ± 0.7	11.3 ± 0.5	0.0
5 IU	7.3 ± 3.4	20.0 ± 3.7	18.0 ± 3.5	8.0 ± 0.0	–[2]
10 IU	7.0 ± 2.1	58.3 ± 6.1	49.7 ± 4.2	57.3 ± 5.9	–[2]
50 IU	21.7 ± 0.7	44.3 ± 3.8	46.0 ± 2.6	43.7 ± 3.1	–[2]

[1] All numbers given are the mean of groups of 3 rats.
[2] In animals of these groups no follicles were measured or counted.

was much smaller than the number of large follicles (Table 3). The possibility that 15 IU HCG was insufficient to induce ovulation in all the follicles was eliminated by the finding that the numbers of ovulations induced by 5, 15 or 45 IU HCG in 3 groups each of 10 rats, pretreated with 50 IU PMS, showed no significant differences. These data show that in PMS stimulated ovaries a large number of follicles of $\geq 55 \times 10^6$ μm^3 are unable to ovulate. This notwithstanding the fact that they have reached a size, which, in normal animals, is typical for follicles that are able to ovulate. Nevertheless, the ovaries of rats

Table 3.

Ovulation inducing effect of an injection of 15 IU HCG given at 2.00 p. m. on the 1st – 4th day after an injection of PMS given during oestrus.

PMS dose	Mean No. ovulations ± SE after HCG on			
	Day 1	Day 2	Day 3	Day 4
0 IU	0 (0/8)[1]	8.5±1.6 (7/8)	8.3±2.1 (7/8)	10.4±1.2 (8/8)
5 IU	3 (1/8)	8.4±1.1 (8/8)	9.2±1.0 (8/8)	–[2]
10 IU	1 (1/8)	21.0±3.8 (8/8)	29.4±4.9 (8/8)	26.2±6.7 (8/8)
50 IU	21.9±5.0 (8/8)	23.6±7.6 (8/8)	43.4±5.8 (8/8)	26.6±6.1 (8/8)

[1] number of ovulating animals/number of animals in that group.
[2] no data.

treated with 5, 10 or 50 IU PMS contained approximately 9, 25 and 25 follicles that were able to ovulate after a strong ovulatory stimulus.

If no HCG was given to PMS rats, spontaneous ovulation was found to occur in many cases (Table 4): on day 4 after an injection of 5 IU PMS and on day 3 after injection of 50 IU PMS. After the injection of 10 IU PMS only 1 out of 5 animals ovulated. The number of ova was normal after 5 IU PMS but very small after 10 or 50 IU PMS.

Histological sections of ovaries of rats injected with saline or 5 IU PMS showed normal follicles and corpora lutea. After the injection of 10 IU PMS the follicles were normal but the corpora lutea had histological features charac-

Table 4.

Spontaneous ovulation in animals during oestrus treated with saline or PMS.

PMS dose	day after PMS injection					
	1	2	3	4	5	6
0 IU	–[1]	–	–	–	10.8±1.2[2] (5/5)	–
5 IU	–	–	–	9.2±0.2 (4/5)	–	–
10 IU	–	–	1 (1/5)	–	–	–
50 IU	–	–	2.6±1.4 (4/5)	–	–	–

[1] no ovulation.
[2] mean number of ovulations ± SE.
[3] number of ovulating animals/total number of animals in this group.

teristic of (pseudo) pregnancy. After the injection of 50 IU PMS the ovaries on day 2 showed small numbers of follicles with a partially luteinized wall. On day 3, i. e. the day after ovulation, some fresh corpora lutea and large numbers of cystic follicles with a luteinized wall and entrapped ova were found. These data suggest a release of endogenous LH after 5 and 50 IU PMS, but an inhibition of this release after 10 IU PMS.

Experiment 2: Effect of hypophysectomy on ovulation following PMS treatment

Since the ovulation found on day 3 after the injection of 50 IU PMS could be due to a direct ovulation-inducing effect of (components of) the PMS injected, the effect of hypophysectomy on day 2 was determined in 3 groups of 10 rats. Hypophysectomy on that day prevented ovulation if performed at 12.00 noon or 2.00 p. m. but not if performed at 4.00 p. m. The ovulation on day 3 is therefore due to endogenous LH, released between 2.00 and 4.00 p. m. on day 2.

Experiment 3: Estimation of endogenous LH release in PMS treated rats

The minimal spontaneous ovulatory activity found after a single injection of doses of 10 and 50 IU of PMS during oestrus could be due to a decreased ovulatory LH release or to a decreased ovarian response to LH. Therefore the amount of LH released on the day before ovulation was estimated and the ovarian response to HCG was tested both in untreated and in PMS treated rats (Table 5).

After a single injection of saline or PMS during oestrus, spontaneous ovulation was found after saline (10.8 ova) and 5 IU PMS (9.0 ova), ovulation did not occur after 10, 20 and 35 IU PMS, but was observed again after 50, 65 and 80 IU PMS (2–4 ova), and was again absent after 100 IU PMS.

In rats pretreated with saline or 5 IU PMS, an amount of 2 IU HCG was required to restore normal ovulation after hypophysectomy performed on the day before the expected ovulation. The ovulatory amount of endogenous LH in untreated rats is therefore equivalent to *at least* 2 IU HCG. In rats injected with 5 IU PMS a similar ovulatory LH release occurs.

After the injection of 10 or 20 IU PMS no spontaneous ovulation occurred, whereas an injection of 2 IU HCG after hypophysectomy on day 2 induced ovulation of respectively 8.0 and 16.8 ova. This suggests that the absence of spontaneous ovulation in these rats is due to a subnormal ovulatory LH release.

After the injection of 50 or 80 IU PMS, 2 IU HCG was required to restore normal ovulation after hypophysectomy (2–4 ova). Thus no decrease in the ovulatory LH release could be demonstrated in these rats. However, the ovulatory response to HCG was decreased, since 2 IU HCG induced ovulation of only 2–4 ova in these rats, but of 9.8 ova in the untreated animals.

Table 5.

Ovulation induced by endogenous LH or after hypophysectomy by various doses of HCG in animals treated with saline or various doses of PMS during oestrus.

PMS dose	Mean No. ova ± SE induced by endogenous LH	Mean number of ova ± SE ovulated after hypophysectomy and a substitutional injection of various doses HCG[5]			
		1.0 IU	2.0 IU	3.5 IU	5.0 IU
0	10.8 ± 0.3 (8/8)[1][3]	9.0 ± 0.0 (4/8)	9.8 ± 1.2 (7/8)	10.6 ± 0.2 (8/8)	9.8 ± 1.2 (8/8)
5	9.0 ± 0.3 (6/8)[2]	6.3 ± 0.3 (4/8)	8.0 ± 1.0 (6/8)	8.0 ± 1.2 (7/8)	8.4 ± 0.8 (7/8)
10	0 (0/8)	0 (0/8)	8.0 ± 2.1 (6/8)	12.7 ± 1.6 (8/8)	15.2 ± 2.1 (8/8)
20	0 (0/8)	1 (1/8)	16.8 ± 2.6 (6/8)	16.0 ± 2.3 (8/8)	23.4 ± 4.1 (8/8)[4]
35	0 (0/8)	–	–	–	–
50	2.2 ± 0.6 (5/8)	1.0 ± 0.0 (3/8)	2.4 ± 0.4 (5/8)	18.0 ± 1.8 (7/8)	31.6 ± 5.4 (8/8)[4]
65	1.5 ± 0.3 (2/8)	–	–	–	–
80	3.7 ± 0.8 (6/8)	6 (1/8)	4.6 ± 2.6 (5/8)	7.0 ± 3.7 (8/8)	18.7 ± 3.8 (7/8)[4]
100	0 (0/8)	–	–	–	–

[1] ovulation on day 5.

[2] ovulation on day 4.

[3] number of ovulating animals/total number of animals in this group.

[4] no data.

[5] Doses of 0.0 and 0.5 IU HCG were also tested in animals pretreated with saline or 5, 10, 20, 50 or 80 IU PMS. No ovulation was observed after such treatment.

DISCUSSION

A single injection of from 5 to 100 IU PMS during oestrus into adult rats led to an accelerated development of the individual follicles and to an increase in the number of large follicles. However, spontaneous superovulation was not found and in some cases even ovulation did not occur.

An injection of *5 IU PMS* during oestrus resulted in ovulation of a normal number of ova one day earlier than in the untreated rats. However, superovulation could have been expected on the basis of our data on follicular development.

In normally cycling animals follicles of $\geqq 55 \times 10^6$ μm^3 are destined to ovulate on the expected day of ovulation and these follicles are almost without exception also capable of ovulating following HCG treatment in earlier phases of the cycle (see also *van Rees et al.* 1968; *Peppler & Greenwald* 1969). Following treatment with 5 IU PMS the number of such follicles is greatly increased, but a large percentage of them did not ovulate spontaneously and could not be brought to ovulation with HCG. Therefore, the absence of superovulation after treatment with 5 IU PMS is most probably due to the fact that of the increased number of large follicles, only $\pm$ 9 follicles respond to LH.

In all the rats injected with from *10–80 IU PMS*, superovulation could be induced by an overdose of HCG. However, spontaneous ovulation was absent (after 10, 20 or 35 IU PMS) or only moderate (after 50, 65 or 80 IU PMS). Since such ovulations were found to be induced by endogenous LH, the question arose whether the moderate spontaneous ovulatory activity after PMS treatment was due to a decreased LH release or to a decreased ovarian response to LH.

Estimations of the ovulatory amount of LH released in untreated rats showed that this was equivalent to at least 2 IU HCG. It cannot be excluded from these experiments, however, that a larger amount than the equivalent of 2 IU HCG is released (see also *Labhsetwar* 1970). Thus it could be shown that after treatment with 10 or 20 IU PMS, a subnormal amount of endogenous LH is released. Since it was also found that the ovaries of these animals contained corpora lutea with features characteristic of corpora lutea of (pseudo) pregnancy, an inhibition of the FSH an LH output from the pituitary induced by the increased oestrogen secretion may be the explanation.

In the groups in which small numbers of ova were ovulated (after 50–80 IU PMS) the ovulatory release of LH was not distinctly subnormal. The inhibition of the release of LH by high oestrogen levels is probably overcome by rising progesterone levels (*Ying & Meyer* 1969). Consequently, at this dose level of PMS a diminished response of the ovaries is responsible for the subnormal number of ovulations.

It is likely therefore that the absence of ovulation after 10–35 IU PMS is due to an inhibition of the ovulatory LH release, whereas the ovulation of small numbers of ova after 50–80 IU PMS is due to a decreased ovarian response to LH.

One last remark should be made: the results of these experiments are in agreement with the results obtained in prepuberal rats treated with PMS (*Ying & Meyer* 1969) but not with the findings of *Weifenbach* (1965) in adult rats treated with another FSH preparation. The effects described here could therefore be dependent on the type of FSH preparation used.

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EFFECT OF UNILATERAL OVARIECTOMY ON FOLLICULAR GROWTH IN HYPOPHYSECTOMIZED RATS TREATED WITH PREGNANT MARE SERUM GONADOTROPHIN[1)]

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Unilateral ovariectomy results in compensatory follicular growth and doubling of the number of ovulations in the remaining ovary. It has been suggested that this is (at least partly) due to the increased availability of circulating gonadotrophins to the remaining ovary (McLaren, 1966). If this view is correct, it could be expected that in hypophysectomized rats, treated with fixed regimens of Pregnant Mare Serum Gonadotrophin (PMS) follicular development in one ovary is stimulated by removal of the other ovary. This was investigated.

Wistar-Rats (180-200 g bodyweight) which had had at least three consecutive 5 day cycles were used. In a first experiment the effect of unilateral ovariectomy on follicular growth in rats with an intact hypophysis was determined. The animals were unilaterally ovariectomized during oestrus or 2 days later, during dioestrus 2. Results are given in Table 1. In these rats a significant increase of follicular growth in the remaining ovary is seen on the second postoperative day after removal of one ovary during oestrus and on the first postoperative day after removal of one ovary during dioestrus 2. In a second experiment rats were hypophysectomized transauricularly, unilaterally ovariectomized or sham operated and injected with 0.5, 1 or 2 times a dose of PMS known to maintain normal follicular growth (Welschen, in press). Again all manipulations were performed either during oestrus or during dioestrus 2. The rats were killed 24 hrs later. The results, given in Table 2, show that in these hypophysectomized rats no significant compensatory follicular growth occurs after unilaterally ovariectomy. This is in agreement with results of Greenwald (1968), who found no com-

1) J. Endocr. 1972, 55, in press.

pensatory ovulation in unilaterally ovariectomized hypophysectomized prepuberal rats after treatment with PMS and Human Chorionic Gonadotrophin.

The results indicate that changes in the gonadotrophin levels are required for compensatory follicular growth after unilateral ovariectomy. Moreover, changes in gonadotrophin levels have been demonstrated after unilateral ovariectomy (Benson, Sorrentino & Evans, 1969; Peppler, 1969). Therefore, as was already defended previously (Welschen, 1970) the compensatory phenomena after unilateral ovariectomy seem to be exclusively due to the secretion of additional gonadotrophins.

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TABLE 1

Effect of unilateral ovariectomy on follicular growth in rats with an intact hypophyse.

	Foll. Volume[3] x 10^5 µm^3	MEAN NO. FOLLICLES ± S.E. in the right ovary at[2] oestrus	dioestrus 1	dioestrus 2	dioestrus 3	prooestrus
left ovary	≥ 1000				3.7 ± 0.7	4.3 ± 1.3
in situ	500-999		1.0 ± 0.6	5.7 ± 0.9	2.8 ± 0.8	1.6 ± 0.8
	200-499	9.7 ± 0.7 (5)[1]	13.3 ± 1.2 (5)	10.7 ± 0.6 (5)	7.0 ± 0.6 (5)	2.6 ± 1.5 (4)
left ovary[2]	≥ 1000			0.8 ± 0.8	2.3 ± 0.9	8.6 ± 2.0[4]
removed	500-999	-	1.0 ± 0.6	11.0 ± 0.7[4]	7.3 ± 0.6[4]	3.7 ± 2.2
during oestrus	200-499		12.7 ± 0.3 (5)	8.0 ± 1.6 (5)	8.0 ± 0.5 (5)	3.7 ± 1.3 (5)
left ovary	≥ 1000				4.8 ± 0.9	6.6 ± 1.1
removed	500-999	-	-	-	5.2 ± 1.2[4]	7.0 ± 2.1[4]
during dioestrus 2	200-499				8.4 ± 1.6 (8)	7.6 ± 2.3 (5)

1) The number of rats is indicated in parentheses.

2) Operations and autopsies were always performed between 12.00 and 13.00 h.

3) Follicular volumes were determined as described elsewhere (Welschen, in press).

4) The underlined values are significantly different from corresponding values in non-ovariectomized rats ($P < 0.02$).

TABLE 2

Effect of unilateral ovariectomy on follicular growth in PMS-treated[2] hypophysectomized rats.

	Foll. Volume x 10^5 µm^3	MEAN NO. OF FOLLICLES ± S.E. in the right ovary. treatment from oestrus to dioestrus 1 with PMS			treatment from dioestrus 2 to dioestrus 3 with PMS		
		4 IU	8 IU	16 IU	4 IU	8 IU	16 IU
left ovary	≥ 1000		1.0 ± 0.7		2.0 ± 0.3	3.3 ± 1.5	4.4 ± 1.0
in situ	500-999		1.6 ± 0.6	4.6 ± 1.0	3.3 ± 0.6	3.3 ± 0.9	6.0 ± 0.7
	200-499	9.0 ± 0.8 (5)[1]	13.8 ± 1.2 (5)	16.4 ± 2.0 (4)	6.0 ± 1.3 (5)	7.0 ± 3.1 (5)	12.8 ± 1.4 (5)
left ovary	≥ 1000				2.4 ± 0.6	3.2 ± 0.1	4.7 ± 0.7
removed	500-999		2.0 ± 1.1	4.7 ± 1.3	2.7 ± 0.7	4.0 ± 2.1	6.9 ± 0.5
	200-499	8.5 ± 1.5 (5)	12.3 ± 0.9 (5)	14.0 ± 2.3 (5)	6.9 ± 1.9 (5)	7.8 ± 1.2 (5)	11.9 ± 0.5 (7)
Significance of differences		NS	NS	NS	NS	NS	NS

1) The number of rats is indicated in parentheses.

2) PMS (Gestyl, Organon), dissolved in 0.1 ml saline was injected intramuscularly, a first dose of 4.8 or 16 IU immediately after hypophysectomy and a second dose of resp. 1, 2 or 4 IU 13 hours later.

3) Operations and autopsies were always performed between 12.00 and 14.00 h.

CURRICULUM VITAE

(op verzoek van de faculteit)

De schrijver werd geboren in 1941 te Breda, waar hij in 1959 aan het O.L.Vr. Lyceum het diploma gymnasium β behaalde. In datzelfde jaar begon hij met de studie in de biologie aan de Rijks Universiteit te Utrecht. In 1963 werd het kandidaatsexamen afgelegd. Het praktisch werk voor het doktoraalexamen werd gedaan aan het Genetisch Laboratorium onder leiding van Prof. Dr. G. van Arkel en Dr. G. van Nigtevecht en aan het Zoologisch Laboratorium onder leiding van Dr. L. Timmermans (Prof. Dr. Chr. Raven) en Dr. A. Burgers (Prof. Dr. P.W.G.J. van Oord). Het doktoraalexamen werd afgelegd in 1965 met als bijvak Paedagogie en Didactiek (Prof. Dr. N. Perquin). Vanaf 1962 tot 1971 is de schrijver (meestal voor een beperkt aantal lesuren per week) achtereenvolgens verbonden geweest aan het Titus Brandsma College te Dordrecht, het gymnasium Ypelaar te Breda en het Laurens College te Rotterdam. In 1966 werd hij benoemd tot wetenschappelijk medewerker bij de afdeling anatomie van de Medische Fakulteit Rotterdam, waar het onderzoek beschreven in dit proefschrift werd verricht.