EFFECTS OF EXOGENOUS PROLACTIN AND GROWTH HORMONE ON THEIR SECRETION BY THE PITUITARY AND ON OTHER HORMONES

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ABSTRACT

EFFECTS OF EXOGENOUS PROLACTIN AND GROWTH HORMONE ON THEIR SECRETION BY THE PITUITARY AND ON OTHER HORMONES

By

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1. Changes in serum and anterior pituitary levels of prolactin were determined in female rats from age 21 days until maturity. Serum prolactin levels rose sharply on the day of vaginal opening whereas pituitary prolactin levels increased significantly soon after puberty. Daily injections of 0.01-5.0 ug of estradiol benzoate for 4 days beginning at 26 days of age (prior to puberty) significantly increased both serum and pituitary prolactin levels above those in similar rats injected with corn oil. These results suggest that the sharp increase in serum prolactin levels on the day of vaginal opening and subsequent estrous phases of each cycle is due to estrogen secretion.

2. Serum and pituitary levels of prolactin and luteinizing hormone (LH) were measured during the estrous cycle in 3-month-old female rats and in old female rats in constant estrus or repeated pseudopregnancies. Serum prolactin and LH were low during metestrus and diestrus. A

sharp rise in serum LH and prolactin was noted during the late afternoon of proestrus. Thereafter serum LH decreased rapidly, but serum prolactin remained relatively high during the morning of estrus. Pituitary prolactin and LH decreased during the afternoon of proestrus. Serum prolactin levels in old constant estrous rats were similar to prolactin levels during estrus. Old rats showing repeated pseudopregnancies had serum prolactin levels similar to young mature rats in diestrus. Serum LH levels were about twice as high in constant estrous old rats as in old pseudopregnant rats. Both these levels in old rats are very low as compared to levels during proestrus in young mature rats. These results show that there is great fluctuation in serum and pituitary prolactin and LH during the estrous cycle, with peak levels of these hormones being found in the serum during the late afternoon of proestrus. The consistently high prolactin levels in the old constant estrous rats are probably due to persistent estrogen secretion, and may play a role in the onset of spontaneous mammary tumors.

3. The effect of an implant of prolactin in the median eminence (ME) of cycling female rats on serum prolactin and LH during the cycle, especially on the afternoon of proestrus, was measured. Serum prolactin was significantly lower during estrus after the ME implantation of prolactin. The proestrous prolactin peak in rats implanted with prolactin on the morning of proestrus

was completely inhibited as compared to controls. Serum LH was increased during both diestrus and estrus following the ME implantation of prolactin. These results support the hypothesis that prolactin acts back on the hypothalamus to inhibit its own release, and to stimulate LH release.

4. The effects of an implant of prolactin in ME of pseudopregnant (PP) rats on serum and pituitary LH, FSH, and prolactin was measured. In the PP rats implanted with prolactin, 28 of the 31 rats came into estrus 2 or 3 days later whereas 26 of 28 rats implanted with cocoa butter remained PP. Serum LH and FSH increased significantly following ME implantation of prolactin, whereas serum prolactin remained unaffected. Pituitary prolactin and FSH decreased significantly in the prolactin implanted rat. Mammary gland development was greatly decreased in these rats, and a definite stimulation of ovarian follicular development and uterine epithelial and endometrial layers was noted. These results demonstrate that ME implants of prolactin inhibit prolactin secretion and stimulate LH and FSH release.

5. The effects of ME implant of prolactin on serum prolactin following suckling in postpartum rats was studied. Control lactating rats with an ME implant of cocoa-butter had serum prolactin levels greater than 300 ng/ml serum following a 1-hour suckling period. Rats implanted with prolactin had serum prolactin levels less than 50 ng/ml serum following 1-hour of suckling. This large reduction

in serum prolactin was paralleled by decreased lactation, evidenced by reduced litter weight gain. These results indicate that the suckling stimulus is not capable of overcoming the inhibitory effect of an ME implant of prolactin on pituitary prolactin release.

6. ME implants of human and ovine growth hormone (GH) decreased pituitary GH concentration in cycling female rats. Rats implanted with either human or ovine GH also had reduced hypothalamic content of growth hormone releasing factor (GHRF), indicating that the mechanism of inhibitory feedback of GH on its own secretion was by reducing GHRF content in the hypothalamus. Serum prolactin levels were reduced in rats implanted with human GH and the mammary glands were atrophied. Thus human GH inhibits both GH and prolactin release, presumably because it contains the activities of both hormones in its molecule.

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Dedicated to my wife Mary

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INTRODUCTION

Since the birth of neuroendocrinology some 30-40 years ago, many large strides have been made on clarifying the nervous control of the anterior pituitary gland. Only the most hardened skeptics still doubt that the brain, primarily the hypothalamus, plays a very important role in regulating hormone secretion by the pituitary gland.

The anterior pituitary hormone, prolactin, is uniquely regulated by a prolactin inhibiting factor (PIF) in the hypothalamus. This factor, of unknown structure, is capable of inhibiting synthesis and release of prolactin from the pituitary. The other five anterior pituitary hormones are controlled, in large part, by substances produced in the brain called releasing factors. These releasing factors stimulate the synthesis and release of all anterior pituitary hormones except prolactin. Each anterior pituitary hormone apparently has its own specific releasing factor.

Very recently, a breakthrough of great dimensions occurred in neuroendocrinology. The chemical structure of one releasing factor, thyrotropin releasing factor (TRF) was determined to be a simple 3 amino acid compound,

pyro-glutamic acid, histidine, proline amide. It is easily synthesized and available commercially. This molecule is also called thyrotropin releasing hormone (TRH) by Dr. Schally and his collaborators. The laboratories of Guillemin and Schally, working independently, are responsible for this advancement. It is hoped that the structures of the remaining hypothalamic factors, including PIF, will be forthcoming soon.

There is no doubt that control of hypothalamic releasing factors, and therefore of anterior pituitary hormone secretion, is mediated in large part by the conventional long feedback loop from target organ hormones. More recent evidence indicates that this classic negative (positive under some conditions) feedback mechanism of target organ hormones is not the sole internal control for secretion of anterior pituitary hormones. Anterior pituitary hormones may regulate their own secretion through the hypothalamus. This hypothesis is especially interesting for explaining control of prolactin and growth hormone since neither of these two hormones have classic target organs. Therefore, one aspect of this thesis is an investigation of the role prolactin and growth hormone have in controlling their own secretion and possibly that of other hormones of the pituitary gland.

Recent technical advances in the area of protein hormone assay has made it possible to detect and measure hormones in the blood using small amounts of serum. These

radioimmunological methods have made it possible to delineate many of the natural endocrine changes which occur in the organism during its life cycle, including such events as puberty, cycling, pregnancy, lactation, and old age. This thesis describes some of this natural history, especially as it relates to prolactin. Perhaps more importantly, it attempts to determine the effects on the neuroendocrine system when the hormone balance is upset during these natural processes.

REVIEW OF LITERATURE

I. Anatomy of the Hypothalamo-Pituitary Axis

A. Anatomy of the Hypothalamus

A thorough understanding of the physiological relationships between the hypothalamus and anterior pituitary gland requires at least a brief review of their anatomy. The hypothalamus of the rat is composed of many nuclear masses and fiber tracts (Krieg, 1932). The use of many terms by different authors for the same nuclear structure in the hypothalamus has caused considerable confusion: therefore, this thesis will follow the more general terminology established by Rioch, Wislocki, and O'Leary (1940), applicable to all mammalian species.

According to Netter (1962), the hypothalamus is bordered anteriorly by the lamina terminalis, posteriorly by the interpeduncular fossa, dorsally by the hypothalamic sulcus in the third ventricle and ventrally by the tuber cinereum. The hypothalamus can be divided into four sections. The preoptic area is composed of several preoptic nuclear masses. More caudally is the anterior hypothalamus, composed of the paraventricular, supraoptic

and suprachiasmatic nuclei, as well as a small medial anterior nucleus, which gives way to the well-defined dorsomedial and ventral medial nuclei. Also located in this medial region are the periventricular nuclei, which form the walls of the third ventricle. At the lower end they become the periventricular arcuate nuclei. The large lateral nuclei extend along the entire length of the hypothalamus, reaching the most posterior nuclear masses, the mammillary complex. An excellent stereotaxic atlas of the rat hypothalamus, describing the exact location of all nuclei and their nerve pathways, has been developed by deGroot (1959). This atlas was used in the present studies.

B. Anatomy of the Hypothalamo-Pituitary Connections

No direct neural connections between the hypothalamus and the anterior pituitary are evident; rather, the connection is by way of hypophysial portal blood vessels. These vessels originate in the infundibular stem (stalk) and in the median eminence (anterior portion of the beginning of the infundibular stem). The anatomists, Popa and Fielding (1930a,b) first described the relation of the hypothalamus to the portal vessels. However, they erroneously described the flow of blood as going from the pituitary towards the hypothalamus. Morphological evidence in the cat, monkey, and man (Wislocki and King, 1936) and physiological evidence in the toad (Houssay et al., 1935)

and rat (Green, 1947) indicated that the flow was from the hypothalamus to the pituitary.

More recently, this question has been raised again. Observations by Torok (1954, 1962, 1964), Szentagothai et al. (1957), and Duvernoy (1960), indicate that there may be a minor amount of blood flowing from the median eminence and pars tuberalis to the hypothalamus. The finding of anterior pituitary hormones in the pars tuberalis indicates that blood could bring pituitary hormones from here to the hypothalamus. Torok (1954) has also described blood flowing upward in surface veins on the pars distalis. If these findings are corroborated, it could provide an anatomical basis for a direct feedback of anterior pituitary hormones on the hypothalamus.

II. Physiology of the Hypothalamo-Pituitary Axis

The first clear postulation that the hypothalamus exerted a control over the anterior pituitary gland was by Harris (1937) in which he induced ovulation in rabbits by electrical stimulation of the hypothalamus. Hinsey (1937) postulated that this control was by a neurohumoral substance produced in the hypothalamus and carried by way of the portal vessels to the anterior pituitary gland. A landmark experiment was done by Harris and Jacobsohn (1952), in which they transplanted pituitary tissue to the median eminence area of hypophysectomized rats, and the rats showed normal reproductive functions.

A. Releasing and Inhibiting Factors of the Hypothalamus

In addition to much indirect evidence for the existence of releasing factors controlling follicle stimulating hormone (FSH), luteinizing hormone (LH), thyrotropic hormone (TSH), growth hormone (GH), adrenocorticotropic hormone (ACTH), and prolactin (by an inhibiting factor), some direct evidence is available. I will review evidence for only those factors which are concerned with this thesis.

Existence for growth hormone releasing factor (GHRF) was first clearly demonstrated by Deuben and Meites (1963) using an acid extract of hypothalamus. (This was later confirmed by Deuben and Meites, 1964; Schally et al., 1965.)

Direct evidence of the presence of a follicle-stimulating hormone-releasing factor (FSH-RF) in rat hypothalamus was reported by Mittler and Meites (1964) and an in vitro assay for FSH-RF was established. Igarashi and McCann (1964) demonstrated FSH-RF by an in vivo method.

Luteinizing hormone releasing factor (LRF) was first shown to exist by McCann et al. (1960) and by Campbell and Harris (1960) in crude acid extracts of the hypothalamus of rats. Later McCann (1962) demonstrated a dose response curve for LRF in vivo. Courrier et al. (1961) confirmed these results in rats and also sheep.

Definitive evidence for a prolactin inhibiting factor (PIF) was presented by Meites and co-workers. Neutralized

acid extracts of rat hypothalami inhibited the release of prolactin from the pituitary (Meites et al., 1961; Talwalker et al., 1963). Other animal species including sheep, cattle, and swine, were also shown to contain PIF activity in the hypothalamus (Talwalker et al., 1963; Schally et al., 1965; Pasteels, 1962).

Until very recently, the chemical structure of none of these factors was known. However, two independent reports by Bowers et al. (1970) and Burgus et al. (1969) described the chemical structure of the releasing factor for TSH. The structure of thyrotropin releasing factor (TRF) or hormone (TRH) is pyro-glutamyl-histidine-proline-NH_2.

It is probable that the structure of the other hypothalamic factors will be forthcoming soon.

B. Feedback of Hormones on the Hypothalamo-Pituitary Axis

1. Classic Endocrine Feedback System

The classical endocrine feedback is that of target organ hormones exerting a negative or positive feedback on the hypothalamus and/or pituitary gland to affect further secretion of their tropic hormones. It has not yet been completely established whether the hypothalamus or pituitary is the major site of steroid hormone feedback. It appears that estrogen may inhibit FSH (Bogdanove, 1963) and LH (Ramirez et al., 1964) by direct action on the

pituitary. However, most experimental evidence indicates that the action of estrogen and progesterone on FSH and LH secretion is via the hypothalamus. Many reviews have been written on this subject in recent years (Everett, 1964; Bogdanove, 1964; Flerko, 1966; Davidson, 1966; McCann et al., 1968), and the reader is referred to these. The most recent and very excellent review, by Davidson (1969), attempts to show the areas of the hypothalamus which are either stimulated or inhibited by ovarian or testicular steroids.

2. "Short" Feedback Systems

The second general type of hormonal feedback regulating the pituitary has been demonstrated more recently. This system is called the "short," "auto," or "internal" feedback type, in which the anterior pituitary hormones themselves act to control their own secretion. There is some evidence for the existence of a "short" feedback mechanism for all anterior pituitary hormones. This review will consider the feedback of ACTH, LH, FSH, prolactin and GH on themselves, mediated through the hypothalamus.

Many different experimental techniques have been used to demonstrate the existence of a short feedback loop. One approach has been the use of hypophysectomy, and subsequent appearance of hitherto undetectable levels of releasing factors in the blood. Administration of

exogenous pituitary hormones have been shown to alter electrical activity of the brain, and of the hypothalamus in particular. Animals bearing pituitary tumors which secrete large amounts of pituitary hormones have altered hypothalami stores of releasing and/or inhibiting factors and produced changes as well in most pituitary hormones. Another approach widely used, and in much of the work described in this thesis, involves the implantation of minute amounts of pituitary hormones in certain areas of the central nervous system, particularly the hypothalamus, and observing changes in pituitary secretion of the hormones.

Before reviewing the work on short feedback systems, it is important to ask the question whether or not pituitary hormones can and do reach the hypothalamus. Since the median eminence area of the hypothalamus is relatively free from the blood brain barrier, it is possible that pituitary hormones can reach the hypothalamus via the general circulation. Several pituitary hormones have been found in the median eminence (Guillemin et al., 1962; Schally et al., 1962; Jacobowitz et al., 1963; Johnson and Nelson, 1966). It is also possible that pituitary hormones can reach the median eminence and the rest of the hypothalamus by reverse blood flow from the pituitary. Torok (1954, 1964) and Jazdowska and Dobrowalski (1965) have attempted to demonstrate blood passing from the ventral surface of the anterior pituitary to the capillary complex of the median

eminence. However, no later reports have confirmed or denied these results.

a. "Short" feedback control of ACTH.--The existence of a short feedback system involving ACTH has been more extensively investigated than for any other pituitary hormones. Blockage of the stress-induced fall in pituitary ACTH stores occurred following injection of exogenous ACTH (Kitay et al., 1959). Rats with pituitary tumors which secrete large amounts of ACTH do not show an increase in plasma or pituitary ACTH, which usually occurs after adrenalectomy (Vernikos-Danellis and Trigg, 1967).

Attempts have been made to determine whether the brain or pituitary is the site of ACTH feedback, and most evidence indicates the brain is most important. Halasz and Szentagothai (1960) found that transplantation of anterior pituitary tissue to the third ventricle of an intact rat reduced adrenal function. Motta et al. (1965) introduced the technique of placing small amounts of pituitary hormones in areas of the brain, and observed that placement of ACTH only in the median eminence and not elsewhere in the brain or pituitary, reduced the plasma corticosterone response to stress. However, Davidson et al. (1968) did not observe lower resting corticosterone level in male rats implanted with ACTH in the median eminence.

Direct measurements of cortiocotropin releasing factor (CRF) have also yielded valuable data on site of action of ACTH feedback. Adrenalectomy and hypophysectomy increased CRF content in the hypothalamus more than adrenalectomy alone (Motta et al., 1968). It is well established that hypophysectomy increases CRF in the circulation (Eik-Nes and Brizzee, 1958; Brodish and Long, 1962). Thus, by eliminating the inhibitory effect of ACTH, CRF synthesis and release both increase.

Changes in electrical activity of the brain have been associated with ACTH changes. Increased plasma ACTH depressed the electrical activity of neurons in the median eminence of the rabbit (Kawakami et al., 1966). However, it is important to note that both ACTH and corticoids have a role in regulating the pituitary. Sawyer (1969) has shown that ACTH, in the presence of corticoids, inhibits the electrical activity of the median eminence, whereas when ACTH is given to adrenalectomized rats, enhanced electrical activity of the lateral hypothalamus is observed.

b. "Short" feedback control of gonadotropins.--The first evidence that LH may be controlled in part by itself was presented by Sawyer and Kawakami (1959) and by Kawakami and Sawyer (1959). They observed EEG changes following coitus. They could mimic these postcoital changes in EEG by injecting purified LH, PMS, or HCG in spayed rabbits. These observations led others to

investigate LH and FSH feedback more intensely. Small amounts of LH placed into the median eminence of normal or castrated female and male rats resulted in a decrease in pituitary and plasma LH levels (David et al., 1966; Corbin and Cohen, 1966; Corbin, 1966a). Associated with the decline in LH secretion in intact female rats implanted with LH was irregular vaginal cycling, few corpora lutea, and normal follicular growth. Only one of nine implanted females became pregnant when exposed to a fertile male. It appears that there are specific LH receptors in the median eminence, since implants of FSH or ACTH had no effect on LH secretion (David et al., 1966; Corbin and Cohen, 1966; Corbin, 1966a).

Hypophysectomy increased release of luteinizing hormone releasing factor (LRF) into the blood, probably by eliminating the inhibitory LH effect (Naller and McCann, 1965; Frankel et al., 1965; Pelletier, 1965). Exogenous LH reduced these increased plasma LRF levels (McCann et al., 1968a,b).

Recently, more sophisticated experiments utilizing unit recording of discrete hypothalami areas have yielded more convincing data on LH short feedback loops. Terasawa and Sawyer (1968) found that exogenous LH activated neurons in the arcuate region, but depressed firing rates of neurons in the preoptic region of the hypothalamus. These two regions which have opposite electrical patterns in

response to LH injection also have been postulated to control "basal" and "cyclic" release of LH independently.

Similar types of experiments have been done to show that FSH can inhibit its own secretion through an action on the hypothalamus. Normal adult female rats implanted with FSH in the median eminence have lowered pituitary FSH and hypothalamic FSH-RF contents (Corbin, 1966b; Corbin and Story, 1967). In addition, the ovaries contained degenerate follicles and some corpora lutea. Implantation of LH was not effective in reducing FSH or FSH-RF, thus it appears FSH is specific in this role. Arai and Gorski (1968) found that median eminence implants of FSH or of PMS (a "pregnancy" gonadotropin with FSH activity) inhibited ovarian compensatory hypertrophy. Implants of ACTH, LH, HCG, or TSH were without effect. Administration of exogenous FSH resulted in a significant drop in pituitary FSH content and hypothalamic FSH-RF content in castrate adult male rats (Fraschini et al., 1968). These effects were not duplicated with ACTH or LH.

Following hypophysectomy in male rats, FSH-RF appears in the general circulation in detectable amounts (Negro-Vilar et al., 1968; Saito et al., 1967). Injection of FSH into hypophysectomized rats removed FSH-RF from the blood (Motta et al., 1969), and also decreased hypothalamic FSH-RF (Corbin et al., 1970). However, this has not been observed by others (Saito et al., 1967).

Less electrophysiological evidence favoring a feedback effect on the brain is available for FSH than LH. Kawakami and Terasawa (1967) stimulated the amygdala which evoked nerve potentials in the hypothalamus. FSH could inhibit this phenomenon. Conversely, Ramirez et al. (1967) did not find any effect of FSH on unit recording of the hypothalamus.

A positive short feedback loop for FSH has been postulated by Ojeda and Ramirez (1969) in immature female rats. Implantation of FSH into many areas of the basal hypothalamus induced precocious puberty, suggesting that FSH exerts an activating effect on its own secretion. This report awaits confirmation.

c. "Short" feedback control of prolactin secretion.--The hypothesis that prolactin can control its own secretion was first hypothesized by Sgouris and Meites (1953), when they found decreased pituitary prolactin following injection of exogenous prolactin in rats. However, no further work was done on this subject until recently. MacLeod et al. (1966, 1968) found that rats bearing pituitary tumors which secrete large amounts of prolactin as well as GH and ACTH, had reduced pituitary prolactin content as measured by disc electrophoresis methods. Chen et al. (1967) reported that similar tumor bearing rats have increased prolactin inhibiting factor (PIF) content in the hypothalamus, as well as reduced pituitary

prolactin, suggesting that the action of prolactin is on PIF. However, since these tumors did secrete not only prolactin, but ACTH and GH as well, it was difficult to separate the effects of prolactin from ACTH and GH.

Pituitary prolactin stores can be decreased by injecting purified prolactin into normal female rats (Sinha and Tucker, 1968) or by transplanting pituitary glands underneath the kidney capsule of ovariectomized rats (Welsch et al., 1968a). These grafts secrete mainly prolactin and only small amounts of TSH, FSH, LH, ACTH, and GH (Meites, 1966).

The laboratory of Meites is the only one to report effects of implanting prolactin into the hypothalamus. The first report (Clemens and Meites, 1968) showed that implantation of prolactin into the median eminence of either intact or ovariectomized rats reduced pituitary prolactin content. This decreased pituitary prolactin may indicate a decrease in both synthesis and release of prolactin, since there was probably less circulating prolactin as evidenced by definite regression of the mammary glands and fewer corpora lutea as compared to controls. The probable site at which prolactin acted in this experiment was the hypothalamus, where an increase in PIF content was observed in both ovariectomized and intact rats implanted with prolactin.

The experiment just described was the stimulus for many other similar experiments, in an attempt to show that prolactin can be a powerful inhibitor of its own secretion. Certain physiological states in female rats are accompanied by enhanced prolactin secretion, i.e., postpartum lactation and pseudopregnancy. Clemens et al. (1969a) found that a median eminence implant of prolactin during lactation significantly decreased lactation as judged by decreased litter weight gain. An implant of prolactin and ACTH together decreased lactation further, reflecting the need of both these hormones for lactation. Chen et al. (1968) found that by implanting prolactin into the median eminence of pseudopregnant (PP) rats, the length of pseudopregnancy was shortened. They also found that this prolactin implant prevented the formation of deciduomata after the uterus was traumatized on day 4 of PP. Neither LH nor FSH implants were effective in preventing deciduomata formation, or in shortening the length of pseudopregnancy. These results suggested, like the above experiment by Clemens and Meites (1968), that prolactin can act in the hypothalamus to decrease prolactin release from the pituitary.

Clemens et al. (1969b) found that pregnancy was terminated in the rat following implantation of prolactin into the median eminence, when the implant was performed before day 7 of pregnancy. Pregnancy could be maintained if progesterone was administered subcutaneously at a dose of 2 mg/day. Thus it appeared that prolactin implants

caused a decrease in pituitary prolactin release, resulting in luteal regression and reduced progesterone secretion, necessary for maintaining pregnancy.

The obvious conclusions drawn from the above experiments are that prolactin can inhibit its own secretion in a short feedback loop under several physiological states. The less obvious result of those experiments is that a prolactin implant in the hypothalamus can cause increased gonadotropin (LH and FSH) secretion from the pituitary. The evidence for this conclusion is that lactating rats implanted with prolactin began to cycle, and ovulated, shown by recovery of ova in the Fallopian tubes. Histology of the ovaries revealed a definite increase in follicular growth following prolactin implantation, indicating increased circulating FSH reaching the ovary. These data indicate that prolactin action is not completely specific, but can affect gonadotropin release as well as inhibiting prolactin release. To further test this hypothesis, 21-day-old female rats were implanted with prolactin. Since prolactin secretion in young female rats was believed to be very low (Minaguchi et al., 1968) any effect on FSH and LH could be interpreted as a direct effect of the hypothalamic prolactin. The female rats implanted with prolactin came into puberty about one week earlier than control rats. Similar effects were obtained by injecting prolactin systemically (Clemens et al., 1969). It was then determined that rats implanted with prolactin had increased

FSH (Voogt et al., 1969b) and LH (Voogt et al., 1969a) release, hastening the onset of puberty.

Thus it appears that prolactin is not entirely specific in its feedback effect. Prolactin implanted in the hypothalamus apparently inhibits one hormone, prolactin itself, and stimulates FSH and LH. It does not appear that this possibility is shared by LH, FSH, or ACTH. As mentioned previously, LH implants in the hypothalamus decreased pituitary LH, whereas ACTH and FSH implants had no effect on LH (David et al., 1966; Corbin and Cohen, 1966; Corbin, 1966a). Similarly, FSH implants in the median eminence reduced FSH release, but LH implants were ineffective (Corbin, 1966b; Corbin and Story, 1967). However, no one has reported the effects of LH or FSH implantation into the median eminence on prolactin release.

d. "Short" loop feedback control of growth hormone.--Several published reports have indicated that GH may feed back on the hypothalamo-pituitary axis to inhibit further GH secretion. Koneff et al. (1948) reported that long term exogenous GH reduced endogenous pituitary production of GH in the rat. Later Krulich and McCann (1966) found that several injections of bovine GH would reduce GH content of rat pituitaries. Müller and Pecile (1966) observed that exogenous GH prevented the decrease in pituitary GH normally seen following insulin injection in the rat. Rats which bear GH secreting tumors have reduced

pituitary GH content (MacLeod et al., 1966; MacLeod et al., 1968). Peake et al. (1967) using radioimmunoassay techniques in rats with M+TW15 pituitary tumors, demonstrated that GH in the in situ pituitary decreased to less than 30% of normal whereas serum GH values rose greatly. It is worth noting that Tashjian et al. (1968) have shown that GH produced by these pituitary tumors in rats is chemically and immunologically similar to normal pituitary rat GH.

An elegant study by Sakuma and Knobil (1970) demonstrated that growth hormone can inhibit its own release in response to acute stimuli in the rhesus monkey. These researchers found that infusion of exogenous human GH prior to Pitressin or insulin injection abolished the response to Pitressin and insulin. Both normally increase serum GH levels in the monkey. The important feature of this experiment is that circulating levels of GH inhibited the GH response to insulin and Pitressin. This contributes to the physiological significance of short feedback control of GH.

The site of GH feedback, pituitary or hypothalamus, is not entirely clear. Removal of GH by hypophysectomy stimulated release of growth hormone releasing factor (GHRF) into the general circulation (Müller et al, 1967a). Implantation of GH into the median eminence significantly reduced pituitary weight as well as pituitary GH concentration (Katz et al., 1969). In contrast to the above

observations suggesting that the hypothalamus is the site of GH feedback, Müller et al. (1967b) showed that exogenous GH blocks the decrease in pituitary GH observed after an intracarotid injection of ovine GHRF. They also reported that exogenous GH did not affect plasma or hypothalamic GHRF levels after long term hypophysectomy (Müller et al., 1967a,b).

It is important to realize that neither prolactin nor GH have target end organs in the classical manner, such as ACTH, TSH, FSH, or LH. Thus the classic feedback mechanism briefly described in the earlier section, in which end organ hormones, such as corticoids, act back on the hypothalamus and pituitary to inhibit ACTH release, is not a major part of the control factors for prolactin and GH. Thus it is reasonable to postulate a short or internal feedback for prolactin and GH as playing an important role in regulation of these two hormones.

III. Prolactin, LH, and FSH Levels During Different Physiological States

Since this dissertation is concerned mainly with experimental endocrine manipulation during several different physiological states, it is necessary to review the literature concerning normal endocrine physiology during these states, especially that literature pertaining to prolactin, LH, and FSH. Also, the first part of the experimental section in this dissertation reports values for LH, FSH, and prolactin during several different

physiological states, and found by the author to be valid under normal non-experimental conditions in this laboratory.

With the recent advent of radioimmunoassays, probably more useful and definitive data have been obtained on serum and pituitary LH, FSH, and prolactin in rats during puberty, the estrous cycle and different reproductive states, than in the preceding 20 years of bioassay. Therefore I will limit this section of the review mainly to what has been reported in the last 2 to 3 years. However, it should be noted that most radioimmunoassay data have mainly confirmed in more precise and accurate detail, what had been concluded from bioassays to be the normal endocrine changes in the life cycle of a female rat.

A. Prolactin, LH, and FSH Levels Before and During Puberty

Measurement of pituitary FSH before puberty in female rats revealed a peak at 21 days of age (Corbin and Daniels, 1967; Kragt and Ganong, 1967). Serum FSH reached a peak at 15 days of age in female rats (Kragt et al., 1970) and continued to decrease until the time of puberty or vaginal opening, at which time serum FSH rose again (Wilson et al., 1970). Pituitary LH is also high in the female rat before puberty, and declines rapidly at the time of puberty (Ramirez and Sawyer, 1965). Serum LH is low prepuberally, although relatively few values have been reported for this

hormone in very early life. Serum LH rises abruptly on the day of vaginal opening (Wilson et al., 1970). Pituitary prolactin is very low in female rats prior to puberty, and begins to rise several days after the onset of puberty (Minaguchi et al., 1968; Voogt et al., 1970). Serum prolactin is low on days 21, 26, 31, and 33 of life in female rats, and rises sharply on the day of vaginal opening on about day 37 (Voogt et al., 1970; Wilson et al., 1970).

B. Prolactin, LH, and FSH During the Rat Estrous Cycle

The availability of radioimmunoassays for LH, FSH, and prolactin have made it possible to measure these hormones during the estrous cycle in the female rat hour by hour. One of the first reports by the Midgley group in Ann Arbor (Monroe et al., 1969) indicated that LH content in the pituitary of 4- or 5-day cycling rats was maximal on the afternoon of diestrus and decreased very rapidly during the afternoon and evening of proestrus. Serum LH rose at least 50-fold at this time compared to any other stage of the cycle. Parlow et al. (1969) also reported that serum LH rose during the late afternoon of proestrus, and subsided by late evening of the same day. In addition, they reported that serum FSH rose about the same time as LH, but persisted in the blood at elevated levels for several more hours. At a symposium on the control of ovulation at the 54th Annual FASEB Meeting, 1970, Gay,

Midgley, and Niswender reported that, in addition to a rise in LH during the late afternoon of proestrus, FSH also rises, but only 4-5 fold. Serum prolactin increased about 10 fold at a similar time during proestrus, but was still high on the morning of estrus. This high serum prolactin during proestrus and estrus was also reported by Amenomori et al. (1970) and by Niswender et al. (1969).

A determination of half life for LH, FSH, and prolactin by measuring disappearance of these hormones from the blood of rats following hypophysectomy on the afternoon of proestrus was also reported (Gay, 1970). The half life for LH is 20 minutes, FSH is 110 minutes, and prolactin is 13 minutes.

C. Prolactin, LH, and FSH During Pseudopregnancy in the Rat

Although the question as to which pituitary hormones are responsible for luteal maintenance and progestin secretion in the rat remains unanswered, much evidence favors prolactin. In vivo results (Fajer and Barraclough, 1967) indicated that prolactin, and not LH could stimulate progesterone secretion by the rat ovary. More recently, MacDonald and Greep (1968) found that prolactin alone is luteotropic in the rat, and LH does not facilitate the prolactin effect. However, not much literature is available at present on serum LH or FSH levels during pseudopregnancy in the rat. One abstract (Spies and Niswender,

1970) indicates that there is a rise in serum levels of all three hormones (LH, FSH, and prolactin), 20 minutes after mating with sterile males. Thereafter these hormones decreased in the serum. Amenomori and Dickerman (personal communication) found low serum prolactin levels throughout pseudopregnancy induced by mating with sterile males. Kwa and Verhofstad (1967) observed a rise in serum prolactin for the first 3 days of pseudopregnancy and thereafter it declined. Similarly Amenomori et al., 1970, found an initial increase in serum prolactin for 2-3 days following matings with fertile males; thereafter serum prolactin decreased and remained low during pregnancy until near parturition.

D. Prolactin, FSH, and LH During Lactation

Pituitary prolactin has been shown to rise appreciably after parturition in rats (Grosvenor and Turner, 1960). Serum prolactin increased quickly after parturition and remained elevated during lactation (Amenomori et al., 1970). A short period of suckling (30 minutes to 3 hours) induced a sharp drop in pituitary prolactin, and significant elevation in serum prolactin (Amenomori et al., 1970).

Pituitary LH and FSH decreased 5-10 hours after parturition in the rat. Serum LH rose about 50 fold 3-7 hours after delivery, declined after 4 hours and was very low during lactation. FSH levels in the serum did not

change significantly and remained low during lactation (Diebel and Bogdanove, 1970). Wilson et al. (1970) reported a rapid rise in serum FSH and LH 3-12 hours after parturition in rats, and thereafter a decline to almost undetectable levels. Serum prolactin remained high during lactation. Thus it appears that soon after parturition, all 3 hormones rise. Thereafter, a reciprocal relationship develops between FSH and LH on one hand, and prolactin. A similar, but opposite reciprocal relationship follows ovariectomy in the rat and will be described in the next section.

E. Prolactin, LH, and FSH Following Castration

Following ovariectomy in rats prolactin secretion decreases and remains at a low level during long-term ovariectomy. Estrogen administration can effectively increase both pituitary and serum prolactin in ovariectomized rats (Chen and Meites, 1970; Kalra et al., 1970).

Serum FSH increased 10-20 fold 1 month after castration in both female and male rats. Serum LH increased 20-50 fold. These LH and FSH levels plateaued and were maintained by periodic increases in serum LH and FSH, occurring at 15-30 minute intervals, and declining at a rate approximately equal to the half life of each hormone. The periodic bursts of LH increase were 20-100%, whereas lower increases (5-10%) were noted for FSH (Gay, 1970).

Very similar rhythmic oscillations in plasma LH were found in ovariectomized rhesus monkeys (Dierschke et al., 1970). LH peaks, representing a 75% elevation, occurred every 75 minutes. These peaks were not observed in intact females. Estradiol-17B and testosterone abolished these oscillations, whereas progesterone had no effect.

It appears that after ovariectomy, prolactin decreases and LH-FSH increases. LH and FSH levels remain elevated throughout castration by periodic sharp bursts of hormones released from the pituitary. What signals these bursts is unknown.

F. Prolactin, LH, and FSH in Old Female Rats

It has been well established that fertility in mammals decreases with increasing age (Engle, 1944; Epstein, 1955). Vaginal cycles in rats as they become older show 3 basic patterns (Aschheim, 1964; Mandl, 1961). The most frequent pattern is one of repeated pseudopregnancies, evidenced by long periods of diestrus as indicated by vaginal smears and ability to produce a decidual reaction. The second most frequent pattern is constant estrus, in which rats show vaginal cornification for long, usually uninterrupted, periods. The third condition is one of irregularity in the cycles, with no definite pattern evident.

It appears that it is the hypophysis or hypothalamus, and not the ovaries, that lose their ability to function

normally. This is evidenced by the ability of exogenous LH to cause ovulation and regular cycles in old constant estrus rats (Aschheim, 1965).

There has not been, to my knowledge, any published data on serum gonadotropins or prolactin levels in old female rats. A few studies have been done on pituitary content of these 3 hormones as the rat becomes older. Bioassays for LH and FSH by several researchers (Saxton and Greene, 1939; Solomon and Shock, 1950; Duncan et al., 1952; Ceresa and Laeroix, 1951) who used rather crude methods, showed no differences between the content of gonadotropins of the hypophysis of young, mature, and old animals. Clemens (Ph.D. Thesis, 1968) found a 50% decrease in pituitary FSH, and a 60% decrease in pituitary LH concentration in old constant estrous rats compared to young adult cycling rats in estrus. Pituitary prolactin, however, was increased very significantly in old constant estrus rats. This finding of increased pituitary prolactin is in agreement with earlier work of Meites et al. (1961), who presented evidence that pituitary prolactin increased with age in rats.

It would appear, based on pituitary hormone data alone, that LH is not high enough in the old constant estrus rat to cause ovulation. This may be attributed to a malfunction in the hypothalamo-pituitary axis in which insufficient LRF for LH release is produced. FSH-RF

content of the hypothalamus is higher in old rats than in younger rats; PIF content is unchanged (Clemens, Ph.D. Thesis, 1968).

G. Prolactin, FSH, and LH in Males

Most observations indicate that prolactin is secreted in very low amounts in most male mammals (Meites and Turner, 1950). However, until advent of the radioimmunoassay, serum levels of prolactin were never measured, and comparison between male and female serum prolactin levels was impossible. One recent report, using a radioimmunoassay for rat prolactin, showed that serum prolactin levels in male rats is low and is about equal to serum levels in the female rat during diestrus. Also they found no cyclic changes in serum prolactin in the male rat (Amenomori et al., 1970).

The male rat pituitary contains more LH and FSH than female rat pituitaries (Kragt and Ganong, 1968). Pituitary FSH and LH peaks early in life in female rats and declines to a lower level thereafter. However pituitary LH content in male rats does not peak before puberty, but continues to rise as the rat gets older (Parlow, 1959; Ramirez and McCann, 1963). Pituitary FSH in the female decreases with age (Kragt and Ganong, 1967). Conversely, pituitary FSH in the male increases with age, so that adult male rats have greater pituitary content of FSH than female rats (Kragt and Ganong, 1968).

MATERIALS AND METHODS

I. Animals

All intact rats used for experiments were Sprague-Dawley rats, purchased from either Spartan Research Animals (Haslett, Michigan) or the Holtzman Company (Madison, Wisconsin). All hypophysectomized rats used for bioassays were purchased from Hormone Assay Labs (Chicago, Illinois). All rats received a standard diet of Wayne Lab Blox (Allied Mills, Chicago, Illinois) and tap water ad lib. In addition, hypophysectomized or old female rats received a supplement of oranges, carrots, and sugar cubes. Old rats also were given monthly injections of Bicillin (Wyeth Labs, Philadelphia, Pa.), a wide spectrum antibiotic. All animals were housed in metal wire cages in temperature controlled rooms at 75±1 F with light from 7 AM to 9 PM and darkness from 9 PM to 7 AM.

II. Stereotaxic Technique

A Stoelting, Stellar model, stereotaxic instrument, especially designed for small animal use was used in all studies involving the placement of hormones into the brain of rats (Fig. 1). By using a stereotaxic instrument, one

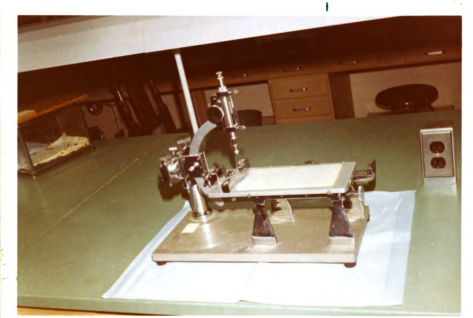


Figure 1. Stoelting, stellar model, stereotaxic instrument used in these studies to place hormones into the brain of rats.

can fix a rat's head in a location which has all three cartesian planes in space as reference points. The instrument is so constructed as to hold the rat's head at a fixed position, and the relative location of the head is constant from rat to rat.

The three planes used in stereotaxic work are the horizontal, vertical, and lateral (sagittal). The horizontal plane passes intracerebrally through the anterior and posterior commissures and is approximately parallel to the midportion of the corpus callosum. The vertical planes are perpendicular to the horizontal plane. The principal vertical plane is perpendicular to a line in the horizontal plane connecting the external auditory meatus, a line called the interaural line. The lateral or midsagittal plane is at a right angle to both the vertical horizontal planes and divides the brain into symmetrical halves. This plane passes through a reference landmark on the skull called bregma, the point where frontal and parietal bones meet at midline.

In most of the present studies, rats weighing 180 to 220 grams were used for stereotaxic work. Since rats of this size have a fairly constant head size and shape from one individual rat to another, it was very easy to accurately place hormone implants in the brain. An atlas was used to locate the various areas of interest in the brain (deGroot, 1959). This atlas requires that the upper incisor bar is at a height of 5.0 mm above the interaural

line. This is necessary in order for the rat's brain to be fixed in a constant plane coinciding with that of the brain illustrated in the atlas.

Rats were prepared for implantation by using ether as an anesthetic. The top of the head was shaved and washed with Nolvasan-S (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) a virucide. Ear plugs were inserted into each ear and seated against the rat's skull. The ear plugs were then placed into the ear bars and secured by tightening the set screws. The interaural line is the imaginary line connecting the tips of the ear plugs. If the head is properly placed, a line joining the two eyes must be parallel with the ear bar. This is very necessary to insure proper placement of the implant.

The incisor bar was next positioned behind the upper incisor so that it was 5 mm above the interaural line. The head was checked to be immobile before work was begun on it. The top of the skull was exposed and dried to enable a clear and distinct view of bregma. The electrode carrier which held the hormone implant within a glass tube was positioned directly above bregma. All readings were made from this zero reference point. It was determined, mainly by trial and error that implants were placed in the median eminence of the tuber cinereum if the electrode carrier which held the glass tube for implantation was moved posteriorly 0.5-0.7 mm from bregma and the tube lowered to 1.0 mm above the base of the skull.

III. Preparation and Implantation of Hormones

All hormones implanted into the rat were placed first inside a 23-gauge glass tube, approximately 2 cm in length. Hormones were prepared for implantation by mixing them thoroughly in equal quantities of cocoa butter, used as a vehicle. Cocoa butter alone was implanted into rats which served as controls. The hormone-cocoa butter mixture was tamped into the tip of the glass tube, which was weighed before and after addition of the mixture to determine the amount of hormone implanted. The end of the tube opposite the mixture was sealed with bone wax to prevent efflux of the hormone when lowered into the brain. The glass tube was placed into the chuck of the electrode carrier and lowered through a hole drilled in the skull until the tip of the tube touched the floor of the cranial cavity (Fig. 2). The tip was then raised 1.0 mm so that it was located in the anterior median eminence. Skull screws were placed into previously drilled holes adjacent to the tube. The entire area was then covered with dental cement, which hardened in 5-7 minutes. The glass tube was left in place, anchored by the cement, and the skull screws served to anchor the cement to the skull.

At the end of each experiment, the rats implanted with hormones in the brain, were killed and the ventral surface of the brain was exposed under a dissecting microscope. Only those rats which had properly placed implants

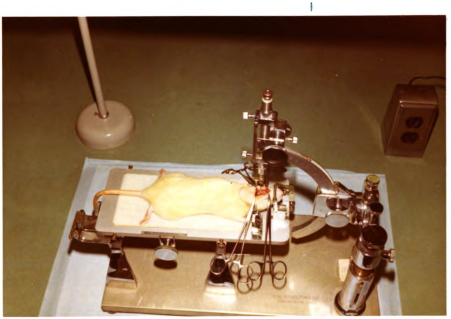


Figure 2. View of a rat held in place while glass tube filled with a hormone is readied for lowering into the brain through hole in skull.

in the median eminence were used for collection of data. Approximately 90% of the rats used in these studies had properly located implants.

IV. In Vitro Incubation Technique

The hypothalamus, including the stalk-median eminence area to be assayed for releasing or inhibiting factors, was dissected out of the brain using a small scissors and curved forceps. All hypothalami from each group were placed immediately into cold 0.1 N HCl (1.0 hypothalamus/0.1 ml) and frozen at -20 C until used in the incubation.

Just prior to incubation, which was always done within 3 weeks after collection of the hypothalami, the hypothalami were homogenized with a Sonifier Cell Disruptor (Heat Systems, Inc., Plainview, New York). The homogenates were centrifuged at 12,000 g for 40 minutes at 4 C in a Sorvall RC 2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The volume of supernatant fluid was measured and the fluid was placed in protein free medium 199 (Difco Labs, Detroit, Michigan). The pH was adjusted to 7.4 with 1.0 N NaOH immediately prior to addition of the hypothalamic extract to 25 ml Erlenmeyer flasks for incubation.

Adult male rats weighing 300-400 gms were used as donors of anterior pituitaries for the incubation. Males were chosen rather than females because of absence of

cyclic hormone changes. These rats were killed by rapid decapitation using a guillotine, and the anterior pituitaries were removed quickly, separated from the posterior lobe, and placed in a petri dish on filter paper moistened with medium 199. The anterior pituitaries were halved with a razor blade, and one half was placed in the control Erlenmeyer flask and the other half in the experimental flask, each flask containing 2 ml of medium 199, pH-7.4. The number of pituitary halves which were placed into each flask depended on which releasing or inhibiting factor was being assayed. Incubations for LRF or PIF utilized 2 pituitary halves/flask. Incubations for GHRF and FSH-RF utilized 12 pituitary halves/flask.

The incubation was done in a Dubnoff metabolic shaker at 60 cycles/minute under constant gassing with 95% O_2-5% CO_2 at 37$\pm$0.5 C. A pre-incubation period of one-half hour was done for each incubation in which the anterior pituitaries alone were incubated with the medium 199. At the end of this time period, the incubated medium was discarded and replaced by fresh medium. To this mixture of medium and anterior pituitary halves was added the neutralized hypothalamic extract. In general, control flasks received hypothalamic extracts from control animals and experimental flasks received hypothalamic extracts from experimental rats. Occasionally, additional control flasks received extract of cerebral cortex.

The incubation time depended on the particular releasing or inhibiting factor being assayed. The time for LRF or PIF was 2 hours, whereas the time for FSH-RF and GHRF was 6 hours. At the end of the incubation period, the pituitary halves were separated from the medium and weighed to allow one to equalize the relative amount of pituitary tissue found in each flask. The medium was centrifuged at 3,000 g for 20 minutes, and the supernatant was frozen at -20 C until assayed for pituitary hormones.

V. Assays of Pituitary Hormones

A. Bioassay

Growth Hormone was measured in rat pituitaries and incubation medium by the standard tibia test of Greenspan et al. (1949). All bioassays included 2 doses of the control pituitary tissue or incubation medium, and 2 doses of the experimental pituitary tissue or incubation medium. The reference standard was also assayed at 2 dose levels. Four female rats, hypophysectomized 17 days earlier at 26 days of age, were used for each assay point. Each rat was injected intraperitoneally with test material once daily for 4 days. On the 5th day the rats were killed, one tibia of each rat was split in the mid sagittal plane and stained with 2% silver nitrate. The width of the epiphysial cartilage was determined by taking the mean of 10 measurements on each tibia.

Pituitaries were bioassayed at 2 dose levels, 2 and 4 mg/assay rat/4 days. The 2 doses of incubation medium used were 0.3 and 1.2 equivalents of incubated anterior pituitary tissue. Thus, if a flask contained 12 pituitary halves weighing 60 mg in 4 ml of medium, .66 ml and 2.64 ml was used for the 2 dose levels.

Follicle stimulating hormone content in pituitaries was measured by the HCG ovarian augmentation method of Steelman and Pohley (1953) as modified by Parlow and Reichert (1963). Two dose levels of pituitary (15 mg and 30 mg) and standards were assayed, with 5 assay rats in each group. The FSH standard used was NIH-FSH-S4 with a stated potency of 36.44 IU/mg.

B. Radioimmunoassay

Radioimmunoassay techniques were used to measure serum, pituitary and incubation medium for prolactin, LH, and FSH. The radioimmunoassays for these 3 hormones all utilize a double antibody method widely used and accepted in other laboratories. The reader is directed to several books described below which have recently been published explaining the theories and application in basic and applied research of radioimmunoassays. A rather brief and understandable book on the subject is Radioisotopes in Medicine - In Vitro Studies by Hayes et al. (1968). A good book describing recent progress in immunoassay of gonadotropins is the First Karolinska Symposium on

Research Methods in Reproductive Endocrinology, edited by Diczfalusy (1969). Protein and Polypeptide Hormones, edited by Margoulies (1969), gives extensive coverage to radioimmunoassay theory and practical application, as well as many experimental results.

1. Radioimmunoassay of Rat Prolactin

The radioimmunoassay used to measure prolactin in serum, pituitary, and incubation medium was developed by joint effort of Dr. Midgley's laboratory in the Department of Pathology of the University of Michigan at Ann Arbor, Michigan and this laboratory of Dr. Meites' at Michigan State University, largely through the efforts of Dr. Chao-ling Chen. A description of this method and its validation is found in a recent publication by Niswender, Chen et al. (1969). The Ph.D. dissertation by Dr. C. L. Chen (Michigan State University, 1969) describes the development of the assay, including the production of first and second antisera. The antibody against rat prolactin used in the present experiments, produced in rabbit #625, and used at a working dilution of 1:4000, consistently bound 40-50% of the radioiodinated rat prolactin that had been incubated.

Purified rat prolactin (H1010 B and HIV-8-C) obtained from Dr. Ellis (NASA Research Center, Ames, California) was used for radioiodination with I^{131} (high specific activity, carrier free, for protein iodination, purchased from Cambridge Nuclear, Cambridge, Massachusetts).

Two and one-half ug of rat prolactin was iodinated with 1 millicurrie of I^{131} with 30 ug of Chloramine-T as the oxidizing agent, for a time period of 2 minutes. After stopping the reaction with sodium metabisulfite, the mixture was layered on a 1x15 cm Bio-gel P 60 column. One milliliter fractions were collected, and tubes with peak labelled prolactin were retained. These tubes were diluted in 0.1% BSA-PBS (bovine serum albumin-phosphate buffered saline) to concentrations of .4-.8 ng prolactin per 100 ug, which gave counts of 60,000-120,000 CPM in a Nuclear-Chicago autogamma counter. It was this amount that was pipetted to each test tube during incubation.

Production of sheep antiserum against rabbit gamma globulin (Anti-RGG) was done by immunizing mature female sheep with 3 subcutaneous injections of 100 mg rabbit gamma globulin (Nutritional Biochemical Corporation, Cleveland, Ohio), emulsified in Freund's complete adjuvant, at 3 week intervals. A blood volume of 800-1200 ml was obtained monthly, and the serum was titrated for the optimal dilution to precipitate the rabbit gamma globulin. The optimal dilution ranged from 1:3 to 1:8, dependent on time of booster injection and bleeding.

The incubation procedure for assaying prolactin was carried out at 4 C. Disposable culture tubes, 12x75 mm (Kimble Owens, Illinois, Ohio) were used for all assays. On day 1, first antibody, unlabelled prolactin (known or unknown amount) and 1% BSA-PBS as diluent were added to

each tube. Twenty-four hours later, on day 2, 0.4-0.8 ng of labelled rat prolactin was added to each tube and the tubes were shaken. On day 3, the second antibody (anti-RGG) was added to each tube and each tube was shaken. On day 6, or 72 hours after addition of anti-RGG, 3 ml of PBS was added to each tube and the tubes were centrifuged in an International Centrifuge RP-2 at 2,000 rpm for 30 minutes. The supernatant of all tubes was decanted and the precipitate was counted in a Nuclear-Chicago autogamma counter.

A standard curve was drawn on semi-logarithmic paper from 10 different doses of standard rat prolactin, each done in duplicate or triplicate. A range of 0.5-16.0 ng of rat prolactin (HIV-8-C with a relative potency of 0.66xNIH-P-B1) usually comprised the straight part of the curve and was usable for obtaining data. Unknowns done in duplicate or triplicate were read out from the standard curve and expressed as ng of prolactin per ml serum.

It was possible to measure serum prolactin in adult female rats using 20-100 ml serum for assay. More serum, 75-150 ul, was required in immature female rats or male rats. Only 5-20 ul of a 0.25 mg/ml concentration of pituitary tissue was needed for assay. All pituitaries were prepared for assay by homogenization in cold phosphate buffered saline, pH-7.0, using a cell disruptor.

2. Radioimmunoassay of Rat LH

Many of the procedures described for radioimmunoassay of rat prolactin are identical for assay of rat LH, and will not be repeated here. It is necessary to describe the various antisera and hormones used for radioiodination, however.

In one experiment reported in this thesis, number V, a radioimmunoassay for rat LH utilizing anti-rat LH for antiserum and purified rat LH for iodination was used. This assay is described in the literature (Monroe et al., 1968). This assay utilized the reagents made available by the National Institute of Arthritis and Metabolic Diseases (NIAMD) of the National Institutes of Health (NIH). Rat LH for radioiodination labelled NIAMD-Rat LH-I-1, had a biological potency of approximately 1.0 x NIH-LH-S1 and FSH contamination less than 0.04 x NIH-FSH-S1. The antisera to rat LH, prepared in rabbits, and labelled NIAMD-Anti-Rat LH Serum-1, was used at a working dilution of 1:32,000. This gave consistent binding of 35-45% with labelled LH. The same anti-RGG described previously in this section was used as the second antibody. The reference preparation used was crude pituitary extract labelled NIAMD-Rat LH-RP-1. This has a stated biological potency of 0.03 x NIH-LH-S1 and 0.54 x NIH-FSH-S1.

A second radioimmunoassay was used to obtain the rat serum and pituitary LH values found in experiments II, III, IV, and VI of this thesis. This assay, described

in detail in a recent publication (Niswender et al., 1968) utilizes ovine LH both for immunization and radioiodination. The antiserum to ovine LH was used at a dilution of 1:50,000 and bound 30-45% of the labelled hormone. The same reference preparation was used for this LH assay as for the rat LH assay.

In both of these LH radioimmunoassays, serum and pituitary samples were done in duplicate or triplicate. A similar procedure as described for radioimmunoassay of rat prolactin was used for calculation of actual levels of LH from a standard curve. All dilutions were done in egg white-PBS rather than BSA-PBS due to the LH contamination in BSA.

3. Radioimmunoassay of Rat FSH

Many of the procedures described for radioimmunoassay of rat prolactin are identical for assay of rat FSH, and will not be repeated here. It is necessary to describe the various antisera and hormones used for radioiodination, however.

The radioimmunoassay for rat FSH utilized anti-rat FSH serum and purified rat FSH for iodination. This assay was described at the 51st Endocrine Meetings (1969) by Parlow. This assay utilized the reagents made available by NIAMD. Rat FSH for radioiodination, labelled NIAMD-Rat FSH-I-1, had a biological potency of approximately 100xNIH-FSH-S1 and LH contamination was less than

0.002xNIH-LH-S1. The antisera to rat FSH, prepared in rabbits and labelled NIAMD-Anti-Rat-FSH-serum-1, was used at a working concentration of 1:625. This dilution bound 25-30% of the labelled FSH. The same anti-RGG described previously in this section was used as the second antibody. The reference preparation used was a crude pituitary extract labelled NIAMD-Rat FSH-RP-1. This had a stated biological potency of 2.1xNIH-FSH-S1. All assays for FSH were done in duplicate and actual concentration values in ng/ml serum were obtained directly from a standard curve.

VI. Methods of Statistical Analysis

Each data point for serum and pituitary hormone concentration obtained from radioimmunoassay was the mean of 2 or 3 assay values. Mean and the standard error of the mean were calculated from the average value of each sample for each group in the experiment. If only two groups of data, experimental and control, were obtained in one experiment, the "t" test was used to test for significance. If there were 3 or more groups, a test of significant differences was carried out by an "F" test for approximation. If the "F" test revealed significant differences, the data were subjected to Duncan's multiple range test of significance (1955). Bioassays for GH and FSH were analyzed according to Bliss (1952).

EXPERIMENTAL

I. Serum and Pituitary Prolactin Levels Before, During, and After Puberty in Female Rats

A. Objective

Sexually immature female rats have a much lower pituitary prolactin content than mature female rats (Minaguchi et al., 1968), as measured by bioassay. By contrast, pituitary FSH and LH was reported to be high prepuberally (Kragt and Ganong, 1967; Ramirez and Sawyer, 1965). Pituitary prolactin levels, therefore, show an opposite trend from pituitary gonadotropins prepuberally. The purpose of this investigation was to assay serum as well as pituitary prolactin levels by radioimmunoassay before, during, and after vaginal opening, and after estrogen injection.

B. Materials and Methods

1. Experimental Animals

A total of 156, 21-day-old female Sprague-Dawley rats (Spartan Research Animals, Haslett, Michigan) were used in these experiments. The rats were fed ad libitum

Wayne Lab Blox (Allied Mills, Chicago, Illinois) and maintained on a lighting schedule of 14 hr of light and 10 hr of darkness at 25±1 C.

2. Treatments

All rats were examined for vaginal opening beginning at 21 days of age. Four groups of rats were killed at 21, 26, 31, and 33 days of age. Nineteen rats with closed vaginas were killed at 36 days of age and separated into 2 groups: one group had obviously ballooned uteri indicating proestrus, and the other group had small, non-ballooned uteri. A seventh group consisted of rats killed within 16 hours after vaginal opening, while the eighth and ninth groups consisted of rats killed 1-3 days after vaginal opening. The tenth group was killed during the first estrus following the estrus which accompanied vaginal opening. The eleventh group consisted of 3-month-old cycling females killed during estrus.

In another experiment, 0.05 to 5.0 ug of estradiol benzoate (EB) dissolved in corn oil was injected once daily for 4 days into female rats, beginning at day 26 of life. These rats were killed at 30 days of age.

All rats were anesthetized with ether, bled from the abdominal vena cava and decapitated with a guillotine between 8 and 10 AM. The serum was separated and kept frozen at -20 C until the time of assay. The anterior pituitaries were individually weighed and frozen immediately at -20 C

until the time of assay. The ovaries and uteri were trimmed and weighed.

3. Prolactin Assay

Prolactin of individual serum samples and pituitaries were measured by radioimmunoassay (Niswender et al., 1969). Pituitaries were prepared in cold phosphate buffer saline by homogenizing with a Sonifier cell disruptor. Data from radioimmunoassay and organ weights were subjected to analysis of variance. Means of each group were analyzed further by Duncan's New Multiple Range Test (1955).

C. Results

1. Prolactin Levels

Serum prolactin levels were uniformly low (13-21 ng/ml) from day 21 to day 36 (Fig. 3, Table 1). There was twice as much serum prolactin (not statistically significant) in the 36-day-old rats with ballooned uteri as in rats of similar age with non-ballooned uteri. A sharp 3-4 fold increase in serum prolactin (to 76 mug/ml) was noted on the day of vaginal opening, at which time all the rats were in estrus. A precipitous drop in serum prolactin occurred 1-3 days after vaginal opening, at which time most rats were in diestrus. There was a significant increase in serum prolactin at the next estrus (41-47 days of age), comparable to that seen during the estrus of 3-month-old virgin rats (70-80 mug/ml).

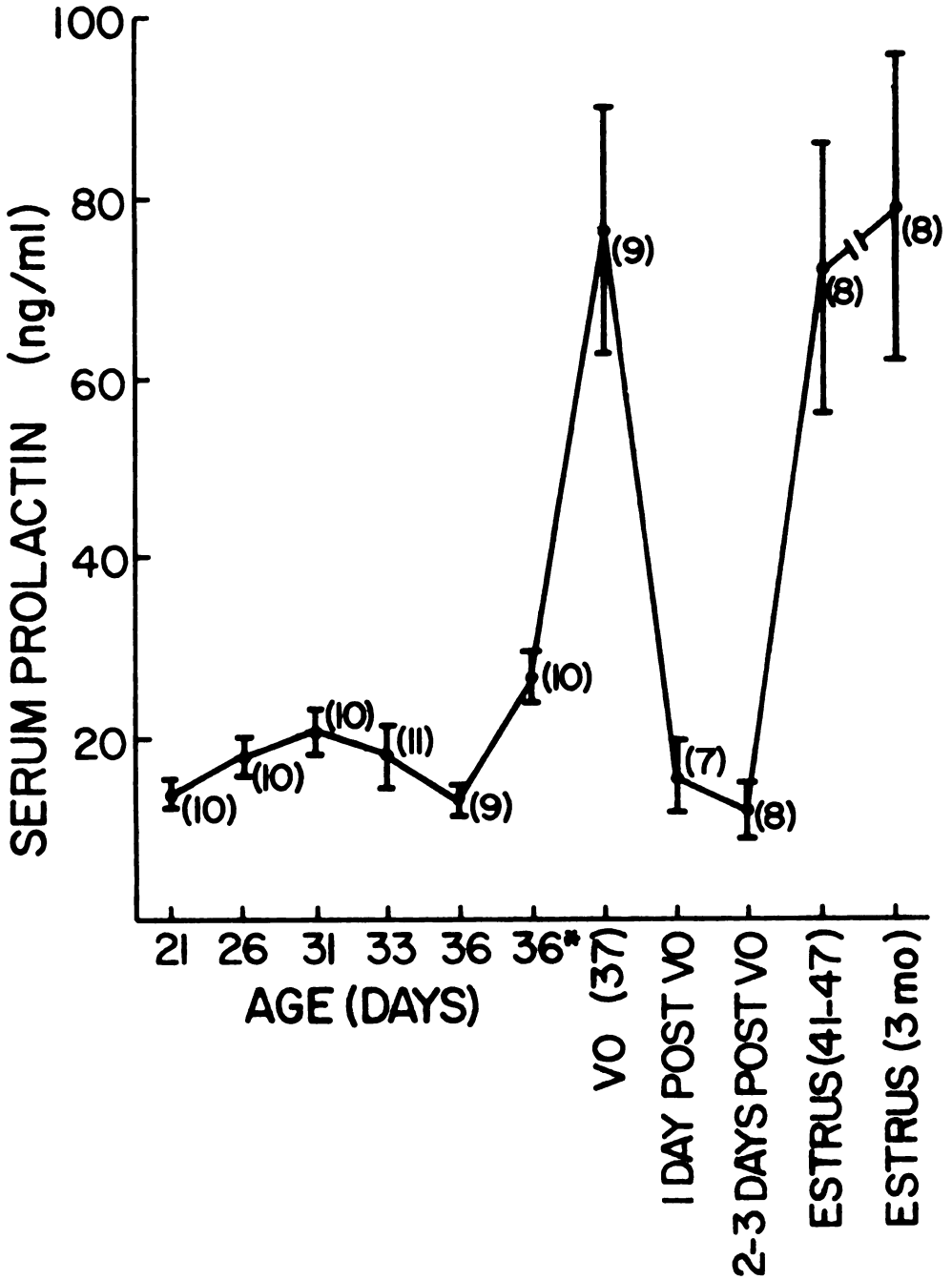


Figure 3. Serum prolactin concentration before, during, and after onset of puberty. VO = Vaginal Opening. *Indicates rats with ballooned uteri.

TABLE 1.--Organ weights and serum and pituitary prolactin levels before, during, and after the onset of puberty.

Age	No. of Rats	Body Wt. (mg)[1]	AP (mg)[1]	Ovaries (mg)[1]	Uterus (mg)[1]	Prolactin Levels		
						mug/ml serum[1]	ug/mg AP[1]	ug/AP[1]
21 days	10	60.6±0.8[3]	2.1±0.1[3]	18.0±0.7[3]	29.1±1.4[3]	14.0±1.4[3]	.54±0.03[3,4]	1.11±0.07[3]
26 days	10	73.8±1.1[4]	2.4±0.1[3]	23.7±1.5[3,4]	30.4±1.8[3]	18.2±2.2[3]	.61±0.05[4]	1.45±0.14[3]
31 days	10	106.1±1.6[5]	3.3±0.1[4]	30.1±1.7[4]	52.7±4.5[3,4]	20.7±2.4[3]	.65±0.09[4]	2.15±0.33[3]
33 days	11	118.0±1.8[6]	3.5±0.1[4,5]	29.3±1.4[4]	73.1±7.9[4]	18.4±3.6[3]	.39±0.03[3]	1.37±0.11[3]
36 days	9	131.8±3.6[7]	4.3±0.2[5]	30.2±1.7[4]	95.1±8.9[4,5]	13.6±1.5[3]	.32±0.03[3]	1.64±0.14[3]
36 days[2]	10	132.6±2.7[7]	4.4±0.2[5]	30.0±1.0[4]	169.6±7.2[6]	26.9±3.6[3]	.39±0.04[3]	1.34±0.21[3]
VO 37.0±0.27[1]	10	131.9±2.0[7]	4.9±0.2[5,6]	46.1±2.0[6]	150.9±6.1[6]	76.6±14.8[4]	.45±0.05[3,4]	2.34±0.19[3]
1 day post VO	7	126.8±2.2[6,7]	4.2±0.4[5]	34.8±4.3[4,5]	107.6±12.9[5]	16.4±4.2[3]	.37±0.06[3]	1.59±0.35[3]
2-3 days post VO	8	150.6±5.6[8]	5.2±0.3[6]	50.1±3.1[6]	116.8±7.5[5]	12.6±3.0[3]	.41±0.04[3,4]	2.20±0.35[3]
Estrus 44.8±1.4[1]	8	175.5±6.2[9]	6.7±0.4[7]	59.0±3.7[7]	245.1±20.7[7]	71.9±14.1[4]	.78±0.06[4,5]	5.27±0.57[4]
Estrus (2-3 months)	8	272.3±7.1[10]	11.8±0.6[8]	57.5±20.7[7]	71.9±9.1[7]	79.3±17.9[4]	1.43±0.18[6]	15.25±1.90[5]

[1]Mean ± Standard Error of the mean.

[2]36-day-old rats with ballooned uteri.

[3,4,5,6,7,8,9,10]Means with the same superscripts are not significantly different ($p > 0.05$) from each other.

Pituitary prolactin concentration was slightly higher in 26- and 31-day-old rats than in 21-, 33-, and 36-day-old rats (Fig. 4, Table 1). It was not until the first estrus following vaginal opening, however, that pituitary prolactin concentration began to rise markedly. Three-month-old female rats killed during the morning of estrus had even higher pituitary prolactin levels than estrous rats 41-47 days old. A similar pattern was seen in pituitary prolactin content. Prolactin remained relatively low until the first estrus after vaginal opening, at which time a 2-3 fold increase was noted. There was another 3 fold increase in serum prolactin in 3-month-old estrous rats.

Injections of 0.05 ug EB for 4 days beginning at 26 days of age did not increase serum prolactin levels above control rats injected with corn oil (Fig. 5). Rats injected with 0.10, 0.30, or 0.50 ug EB showed significant increments in serum prolactin concentration, whereas 5.0 ug was less effective than 0.30 or 0.50 ug in stimulating prolactin release. All of the rats injected with 0.10 ug or more EB had open vaginas and were in estrus at the time of killing. None of the controls or rats injected with 0.05 ug EB had open vaginas at the time of killing. Pituitary prolactin concentration and content increased similarly to serum levels, reaching a peak at a dose of 0.3 ug EB (Fig. 5).

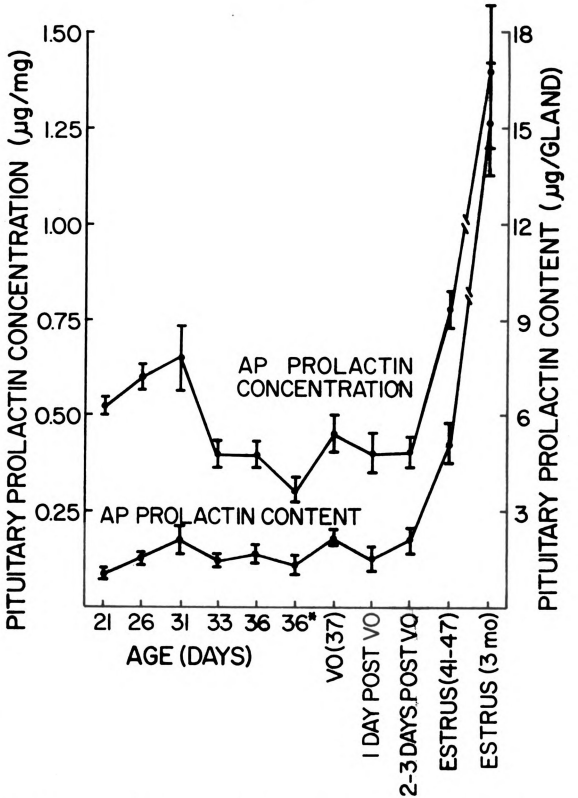


Figure 4. Pituitary prolactin concentration and content before, during, and after onset of puberty. VO = Vaginal Opening. *Indicates rats with ballooned uteri.

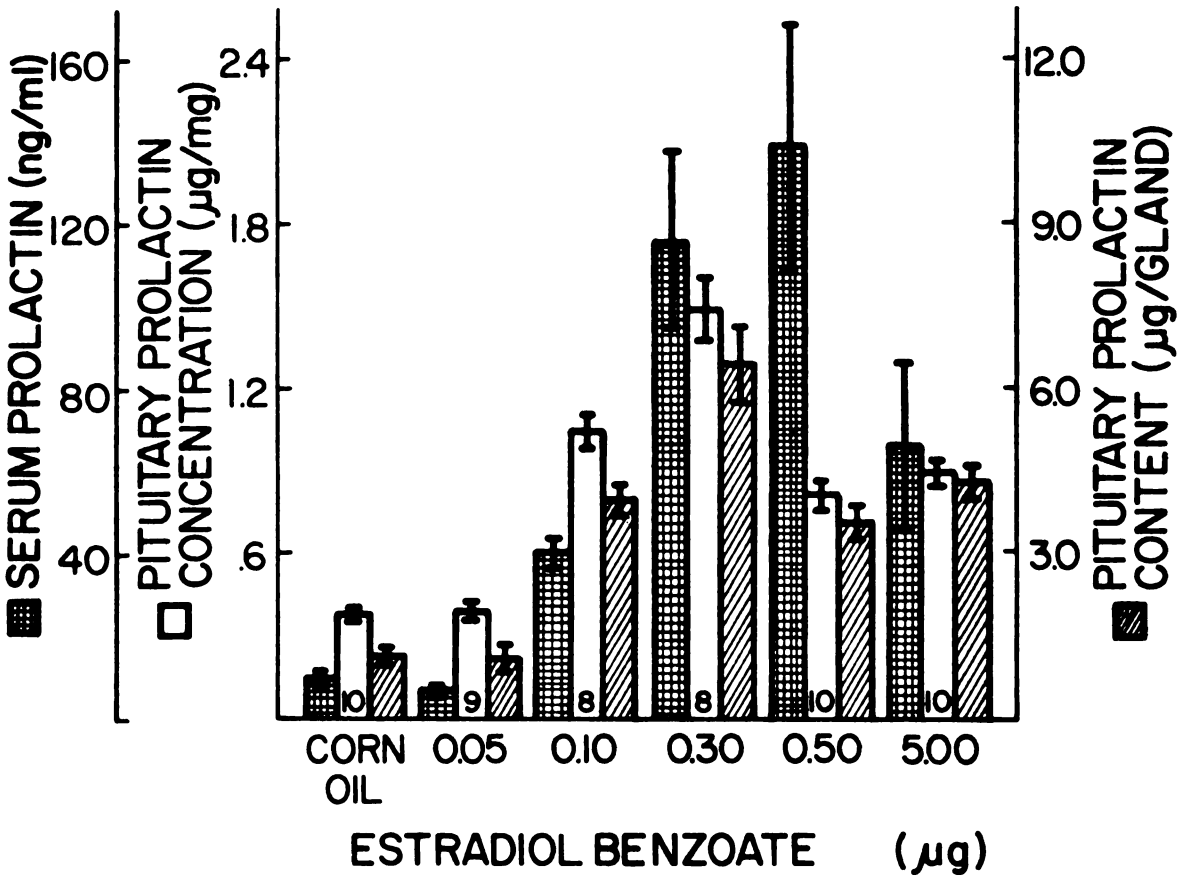


Figure 5. Serum and pituitary prolactin levels of 30-day old female rats following estradiol benzoate injections.

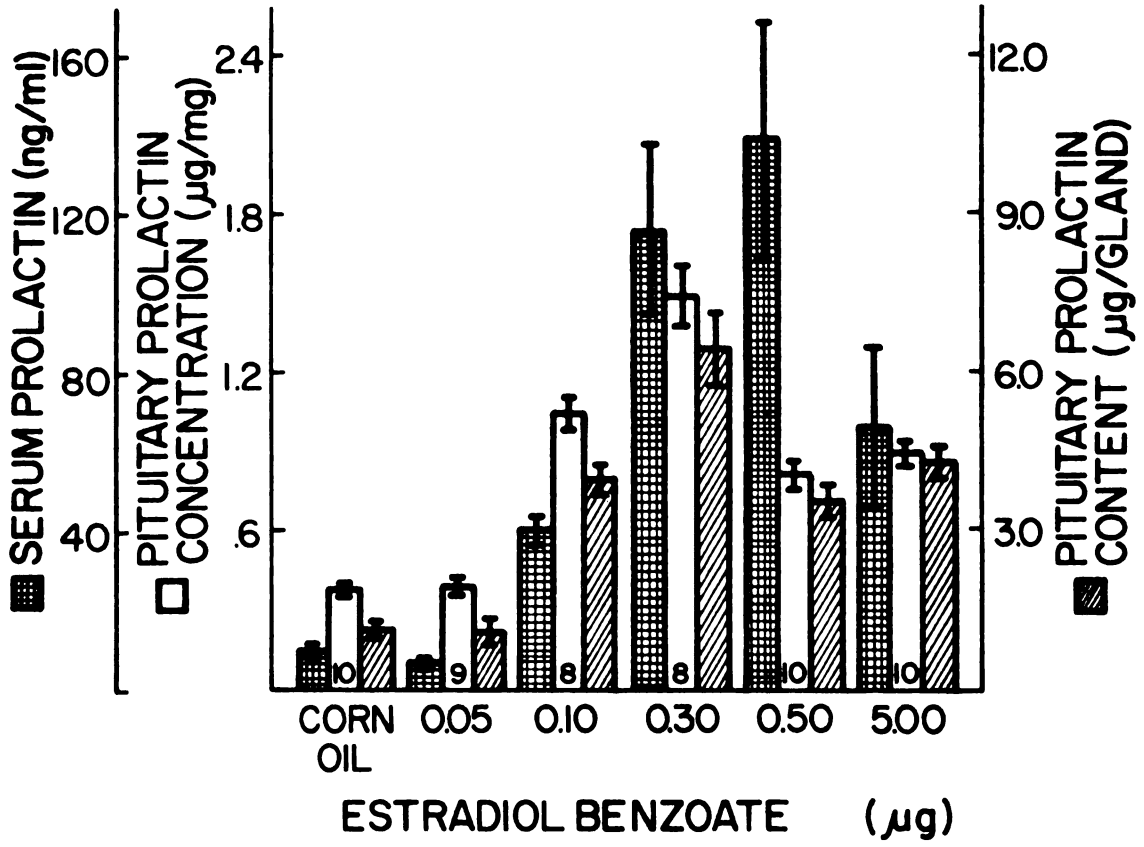


Figure 5. Serum and pituitary prolactin levels of 30-day old female rats following estradiol benzoate injections.

2. Organ Weights

Body weight and anterior pituitary, ovarian and uterine weights increased significantly from day 21 to the day of vaginal opening at 35-39 days of age (Table 1). The ballooned uteri of rats at 36 days of age were significantly heavier than the non-ballooned uteri of similar aged rats. However, ovarian weights of the 2 groups did not differ. There was a significant increase in ovarian weight on the day of vaginal opening compared to 36-day-old rats with closed vaginas. A significant increase in weight of the pituitary, ovaries, and uterus occurred at the first estrus following vaginal opening (41-47 days) as compared to rats at the onset of puberty.

Injections of 0.10 ug EB for 4 days beginning at 26 days of age significantly increased uterine and anterior pituitary weights (Table 2). Doses of EB up to 5.0 ug further increased pituitary weight, but none of the doses affected body or ovarian weight.

D. Discussion

The present study shows that serum prolactin levels are low in prepubertal rats, and increase sharply at the time of vaginal opening. A sharp decline occurs for 1-3 days after vaginal opening and serum prolactin remains low until the next estrus, at which time it rises to the level observed during estrus in 3-month-old virgin rats. Both pituitary prolactin concentration and content are relatively

TABLE 2.--Organ weights and serum and pituitary prolactin levels of 30-day-old female rats following estradiol benzoate administration.

Estradiol Benzoate (ug)	No. of Rats	Body Wt. (g)[1]	AP (mg)[1]	Ovaries (mg)[1]	Uterus (mg)[1]	Prolactin Levels		
						mug/ml serum[1]	ug/mg AP[1]	ug/gland[1]
0.00	10	101.9±1.6[2]	3.4 ±0.10[2,3]	26.8±1.2[2]	57.9±4.9[2]	11.02± 1.09[2]	0.39±0.03[2]	1.36±0.11[2]
0.05	9	97.0±3.2[2]	3.25±0.18[2]	22.7±2.8[2]	62.5±6.4[2]	7.34± 1.02[2]	0.39±0.03[2]	1.29±0.11[2]
0.10	8	101.5±3.2[2]	3.83±0.11[3]	31.6±2.7[2]	118.7±5.6[3]	41.36± 4.18[2,3]	1.04±0.04[4]	3.99±0.27[3]
0.30	8	99.8±3.3[2]	4.39±0.22[4]	34.5±3.4[2]	133.4±5.0[3]	117.45±23.96[4]	1.51±0.14[5]	6.60±0.67[4]
0.50	10	103.3±1.4[2]	4.37±0.16[4]	28.8±1.5[2]	129.4±6.5[3]	138.82±26.10[4]	0.82±0.05[3]	3.62±0.32[3]
5.00	10	96.4±1.8[2]	5.16±0.20[5]	34.9±2.0[2]	132.3±3.8[3]	66.99±20.34[3]	0.92±0.04[3,4]	4.39±0.68[3]

[1]Mean ± Standard Error of the mean.

[2,3,4,5]Means with the same superscripts are not significantly different ($p > 0.05$) from each other.

low before and at the time of puberty, and increase significantly soon after puberty, in agreement with Minaguchi et al. (1968). However, these higher pituitary prolactin levels are not reflected by similar changes in serum prolactin, and may have no physiological significance. The lack of change in pituitary prolactin levels on the day of vaginal opening, when all the rats are in estrus, is in sharp contrast to the marked rise in prolactin. It is possible that a significant increase in serum prolactin does not necessarily require release of large quantities of prolactin from the pituitary. The total amount of prolactin in the blood appears to be low compared to the total in the pituitary, and therefore notable changes in serum prolactin may not always be reflected by comparable changes in pituitary prolactin levels.

Several laboratories (Corbin and Daniels, 1967; Kragt and Ganong, 1967; Ramirez and McCann, 1963) reported that FSH and LH secretion increased as rats approached puberty. The increased gonadotropin secretion is believed to act on the ovaries to increase estrogen secretion, and estrogen is known to stimulate prolactin secretion. Estrogen injections increase both pituitary and serum prolactin levels in mature ovariectomized rats (Chen and Meites, 1970; Niswender et al., 1969). The present study shows that estrogen also increases serum and pituitary prolactin in intact immature female rats.

The 36-day-old rats with closed vaginas but with ballooned, proestrous uteri, had serum prolactin levels of 26.9 mug/ml as compared to 13.6 mug/ml serum in 36-day-old rats with non-ballooned uteri. Increased estrogen secretion by the ovaries of the former rats is believed to account for the 2 fold increase in serum prolactin in these rats. Injections of small doses of EB daily into immature rats have been shown to hasten puberty in rats (Corbin and Daniels, 1969; Ramirez and Sawyer, 1965). Ramirez and Sawyer (1966) observed a drop in LRF at the time of puberty and also following estrogen treatment in immature rats. Corbin and Daniels (1969) found a similar decline in FSH-RF and FSH after injections of estrogen into immature female rats. Both laboratories concluded that estrogen stimulated release of LRF and FSH-RF, respectively.

We have recently reported that subcutaneous injections, or implantation of prolactin into the median eminence hastens puberty about 6-7 days in female rats (Clemens et al., 1969c), and that a prolactin implant in the median eminence increases release of FSH (Voogt et al., 1969b) and LH (Voogt et al., 1969a). It is possible therefore, that small doses of EB hastened puberty by stimulating prolactin secretion which in turn acted back on the hypothalamus to stimulate release of LRF and FSH-RF. This does not exclude the possibility that estrogen may

also act directly on the gonadotropin releasing factors of the hypothalamus.

The low prolactin secretion in the immature rat is in direct contrast to the high gonadotropin secretion. A reciprocal pattern is frequently observed between prolactin and gonadotropin secretion in different reproductive states. However, a major exception to this pattern appears to be present on the day of vaginal opening, when increased serum LH levels are found as determined by bioassay (Ramirez and Sawyer, 1965) and more recently by radioimmunoassay (A. R. Midgley et al., personal communication).

One cannot conclude from these results that prolactin has a definite role in the initiation of puberty in rats. It is apparent, however, that prolactin is released in large amounts on the day of vaginal opening, and that estrogen is a potent stimulator of prolactin secretion in the immature as well as in the adult female rat. The secretion of gonadotropins by the pituitary may, therefore, be partly conditioned by prolactin secretion.

II. Serum and Pituitary Prolactin and LH During the Estrous Cycle, Following Ovariectomy, and During Lactation

A. Objective

Several reports have been published recently describing changes in pituitary and serum prolactin, LH, and FSH during the rat estrous cycle and following castration. Reports on LH (Monroe et al., 1969), FSH (Parlow et al., 1969), and prolactin (Niswender et al., 1969) indicate that all 3 hormones were elevated during the late afternoon of proestrus. Serum LH and FSH were very low at all other stages of the cycle, whereas serum prolactin remained relatively high during estrus (Amenomori et al., 1970). Pituitary LH decreased in late proestrus (Monroe et al., 1969). Following ovariectomy in the female rat, serum LH and FSH rose (Gay, 1970). Serum and pituitary prolactin levels declined significantly 3 weeks after ovariectomy (Amenomori et al., 1970).

The measurement of serum and pituitary LH and prolactin during the cycle and following ovariectomy was done by this investigator to confirm the above reports. In addition, tabulation of these data in terms of definite concentrations in the blood and pituitaries established reference points. Thus during experimental manipulation of different physiological states, the experimenter can determine whether his results are within the physiological

range, and their possible relation to various physiological functions.

B. Materials and Methods

Animals used in this study were 3-month-old virgin female rats from Spartan Research Animals (Haslett, Michigan). Vaginal smears were taken for 2 weeks to be certain that all rats used in the study were cycling normally. Only rats which showed a definite 4-day cycle were used. Eight groups of rats were used for the study on the estrous cycle, with 6-10 rats/group. Light ether anesthesia was used, and all rats were bled from the abdominal vena cava. Pituitaries and sera were assayed for LH and prolactin.

One group of rats was ovariectomized and bled 4 weeks later. The blood was pooled and assayed for prolactin and LH. Sera from adult male rats were pooled and assayed for LH and prolactin. Postpartum lactating rats, on the eighth day of lactation were also used as a source of serum for assay.

C. Results

In normally cycling female rats, serum prolactin was low during metestrus and diestrus (Fig. 6). A sharp rise in serum prolactin was noted during the late afternoon of proestrus, when values as high as 1000 ng/ml were recorded. Prolactin remained relatively high during the morning of

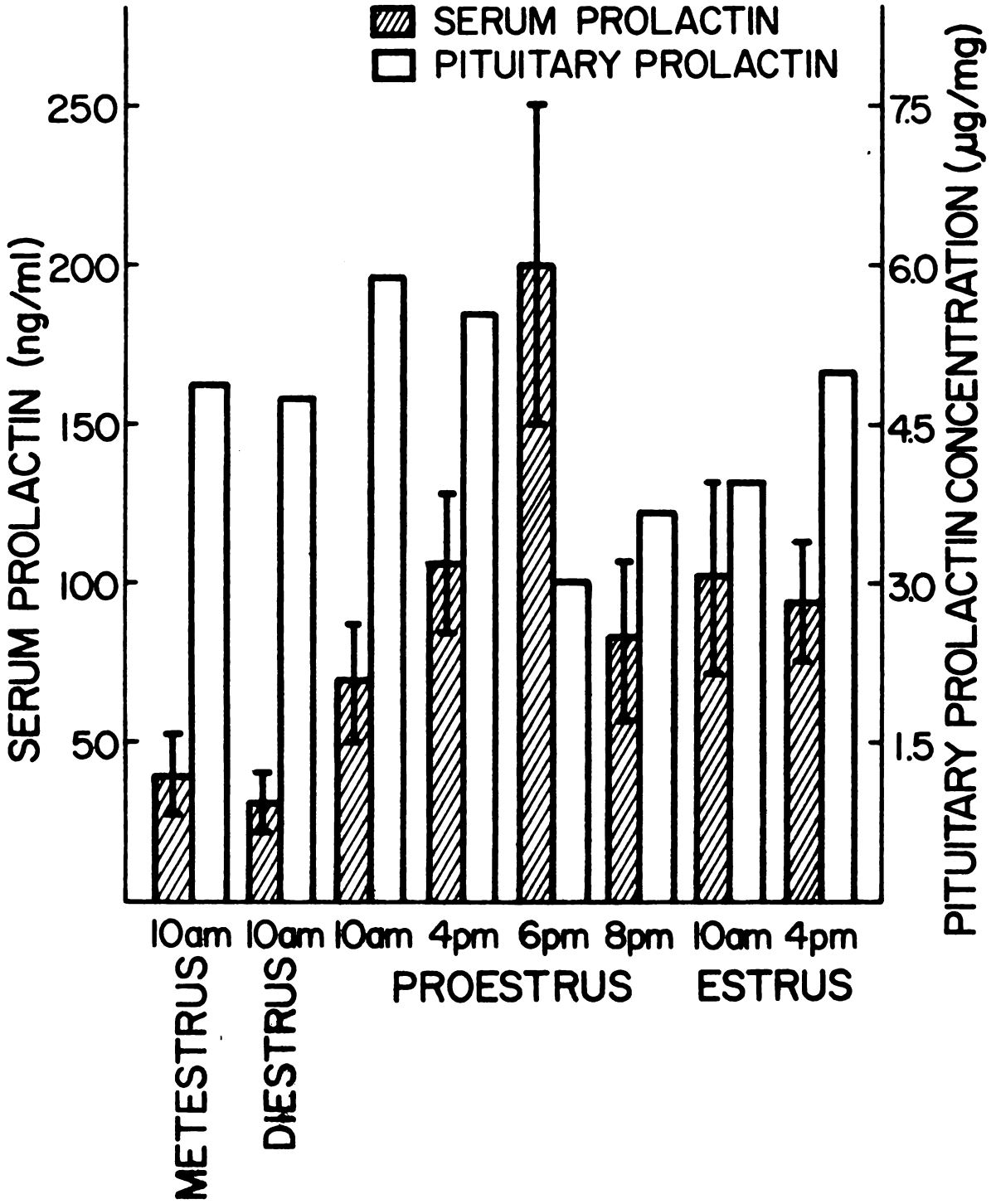


Figure 6. Serum and pituitary prolactin concentration during the estrous cycle in the rat.

estrus (10 AM), and then decreased. Pituitary prolactin concentration reached a peak during early proestrus, and then decreased from 5.8 ug/mg gland at 10 AM of proestrus, to 3.71 ug/mg gland at 8 PM of proestrus (Fig. 6). This rapid decrease in pituitary prolactin occurred at the same time as the large increase in serum prolactin. Thereafter, pituitary prolactin began to rise.

Serum LH showed a peak at the same time as the rise in serum prolactin during the late afternoon and evening of proestrus. However, at all other stages of the estrous cycle, serum LH was very low (Fig. 7). Pituitary LH did not fluctuate as greatly as pituitary prolactin (Fig. 7). A decrease from 4.7 ug/mg gland at 4 PM proestrus, to 3.8 ug/mg gland at 10 AM estrus was the largest change.

Rats which were ovariectomized 4 weeks earlier had a pooled serum prolactin level of 20.4 ng/ml, very similar to diestrus levels. During lactation, serum prolactin averaged 213 ng/ml serum, considerably higher than during estrus, and comparable to late proestrus peak values. Male rats had serum prolactin levels very similar to ovariectomized female rats or female rats in diestrus (20.2 ng/ml). Female rats previously hypophysectomized had serum prolactin values less than 5.0 ng/ml.

Serum LH levels following ovariectomy for 4 weeks rose to 686 ng/ml, comparable to peak proestrus levels. During lactation, serum LH was often undetectable unless

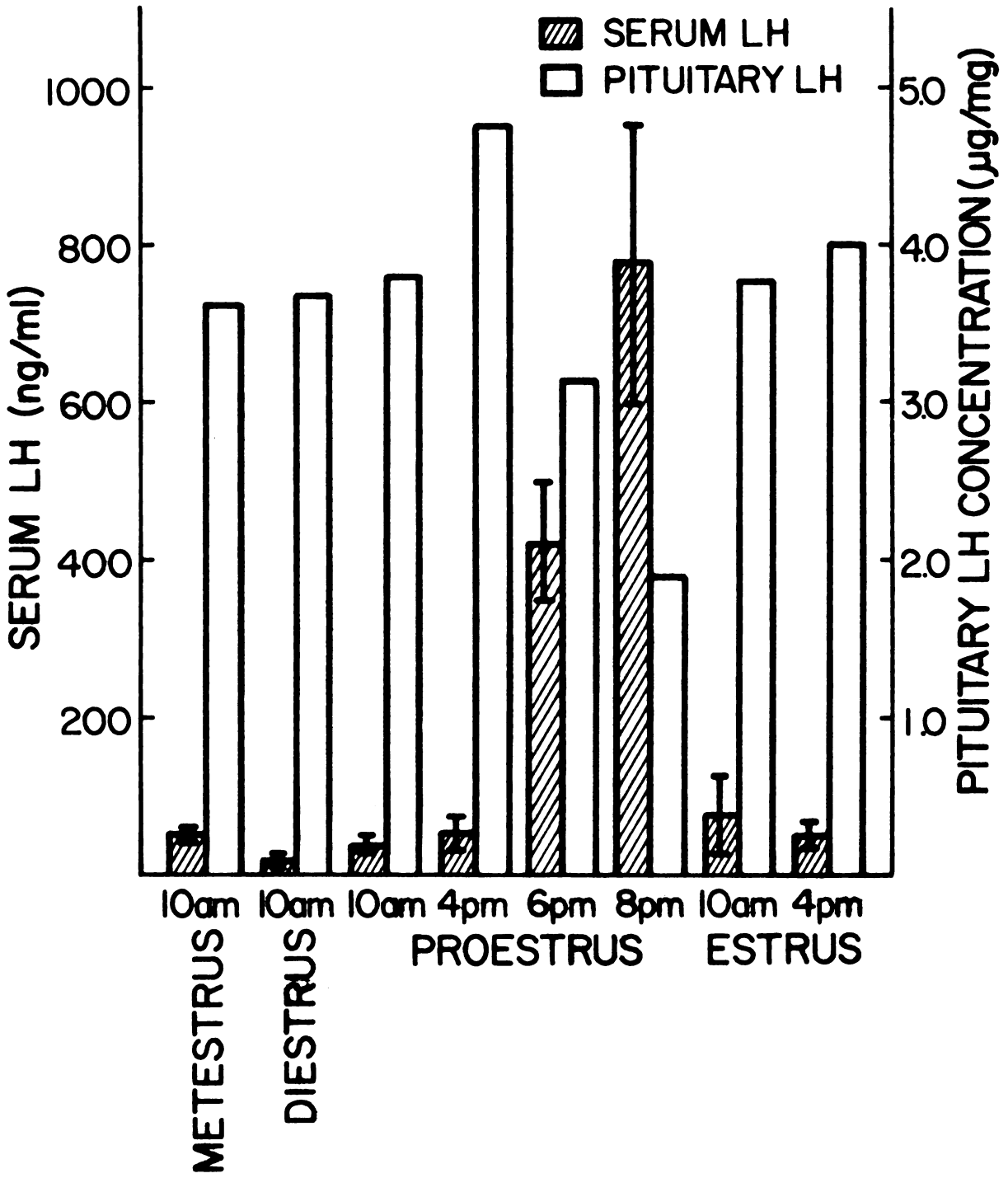


Figure 7. Serum and pituitary LH concentrations during the estrous cycle in the rat.

as much as 200 ul of serum was assayed. Then values were calculated to be less than 100 ng/ml. Male serum LH levels were 87.0 ng/ml, somewhat higher than diestrus levels. Pituitary LH levels in ovariectomized rats were about 100 ug/mg, much higher than at any other stage measured. During lactation, pituitary LH concentration was reduced to less than 2 ug/mg. Male pituitaries had somewhat more LH.

D. Discussion

The changes in serum prolactin and LH during the estrous cycle are in good agreement with previously reported observations (Monroe et al., 1969; Niswender et al., 1969). In this study, high concentrations of serum LH and prolactin were noted during the late afternoon and early evening of proestrus. Serum prolactin remained high until the afternoon of estrus, in agreement with results reported by Amenomori et al. (1970).

Pituitary prolactin concentration decreased rapidly during late proestrus, during the time serum prolactin rose to high levels. This decrease in pituitary prolactin is probably the result of a large discharge of prolactin from the gland into the circulation at this time. Soon thereafter, prolactin concentration in the pituitary rose, indicating an increase in synthesis to replace the depleted hormone probably stimulated by increased estrogen. Pituitary LH also decreased during late proestrus, at the time

that serum LH rose. This is in good agreement with Monroe et al. (1969) using radioimmunoassay, and Schwartz and Bartosik (1962) using bioassay.

Following ovariectomy, serum prolactin levels were considerably lower than during late proestrus or estrus. In this study serum prolactin levels ranged from 15-35 ng/ml, confirming reports by Niswender et al. (1969), and Amenomori et al. (1970). Conversely, serum and pituitary LH rose considerably following ovariectomy, in agreement with Gay, 1970. Another physiological state, that of post-partum lactation, showed prolactin and LH to be reciprocally related. Serum prolactin was high (162-403 ng/ml) during lactation, whereas serum and pituitary LH were low.

Male rats were found to have low prolactin and relatively low LH in the serum. Serum prolactin values in male rats were similar to ovariectomized rats and cycling rats in diestrus, in agreement with Niswender et al. (1969). Serum LH in male rats was somewhat higher than during diestrus in female rats, but not nearly as high as during proestrus.

The results presented and discussed in this study not only confirmed previous reports in the literature, but made it possible for me to compare these results with other experiments in which I experimentally manipulated these "natural" states. These results yielded some reference values, helpful not only to me, but hopefully, also to the reader. These confirmatory results also gave me

confidence in the radioimmunoassays themselves, and in my utilization of them. In addition, the rather large variation in hormone levels in the serum during different physiological states noted in this experiment indicated the need for large numbers of animals per group during subsequent experiments.

III. Serum Prolactin and LH in Old Female Rats

A. Objective

Few data are available in the literature regarding prolactin and gonadotropin secretion in the aged animal. In the Ph.D. thesis of Clemens, 1968 (Michigan State University, East Lansing, Michigan), it was reported that pituitary prolactin and FSH are higher in old constant estrous rats than in 3-month-old rats in estrus. Conversely, pituitary LH was reported to be lower in old constant estrus rats.

There are no reports of LH or prolactin levels in the serum of old female rats. The purpose of this study was to measure serum LH and prolactin in old female rats which were in constant estrus or experienced repeated pseudopregnancies.

B. Materials and Methods

The animals used in this study were discard Sprague-Dawley rats previously used for breeding by Spartan Research Animals (Haslett, Michigan). Daily vaginal smears were taken for 6 weeks before the rats were bled. Only rats which were 18-22 months old and showed either constant estrus or repeated pseudopregnancies were used in this study. Light ether anesthesia was used, and about 1 ml of blood was withdrawn via cardiac puncture from each rat.

Both prolactin and LH were measured by the radioimmunoassays described in the general Materials and Methods section. The LH assay used was that described by Niswender et al. (1968).

C. Results

Serum prolactin concentration, measured in ng/ml, averaged 113.6±31.2 in 18 old rats in constant estrus (Table 3). This prolactin level is slightly higher than levels seen during the morning of estrus. Fourteen different rats were pseudopregnant when bled, and had a mean serum prolactin level of 28.2±3.1, which was significantly lower than the serum prolactin in constant estrus rats. This prolactin level of 28.2 ng/ml is comparable to diestrous values in cycling rats.

Serum LH concentration in 8 rats in constant estrus averaged 24.4 ng/ml (Table 3). This was significantly higher than the LH levels in 12 old rats pseudopregnant when bled, which averaged 13.2 ng/ml. These levels are comparable to cycling female rats in estrus or diestrus. However, the levels of serum LH in both groups are relatively low compared to levels in proestrus or following ovariectomy.

D. Discussion

The results presented here suggest that there are no changes in serum levels of prolactin or LH in constant

TABLE 3.--Serum prolactin and LH in old rats.

Type of Vaginal Smear	Serum Prolactin ng/ml[1]	Serum LH ng/ml[1]
Constant Estrus	113.6±31.2 (18)	24.4±1.9 (8)
Repeat Pseudopregnancy	28.2± 3.1 (14)*	13.2±1.5 (12)**

( ) Number of rats

[1]Mean ± SE of the mean.

*p < .05

**p < .001

estrous old rats as compared to mature young rats on the day of estrus. Thus, the serum level of prolactin during estrus in normal mature rats, reported in experiment II of this thesis, is nearly the same as the serum prolactin level of old constant estrus rats reported in this experiment. However, the serum prolactin values of the old constant estrous rats are consistently high whereas the normal mature rats show cyclic fluctuation, with low values during each diestrous period (the latter usually about 48 hours duration during each cycle). These consistently high prolactin levels in the old constant estrous rats are believed to have a role in the onset of spontaneous mammary tumors. The persistent estrogen secretion is also believed to contribute to mammary as well as to pituitary tumorogenesis in old female rats (Welsch et al., 1970). The serum level of prolactin during diestrus of a cycling rat reported in experiment II of this thesis is comparable to the serum prolactin levels of old pseudopregnant rats.

Clemens in his Ph.D. thesis (1968), reported an increase in pituitary FSH and a decrease in pituitary LH in old constant estrous rats. He also reported high FSH-RF levels in the hypothalamus, and suggested that the constant estrous state observed often in old rats is a result of a malfunction arising in the hypothalamus. Thus, high FSH is secreted which increases estrogen secretion, resulting in constant estrus. Too little LH is secreted to cause ovulation, thus the rats remain in estrus.

Unfortunately serum FSH was not measured in the present experiment to determine whether this hypothesis is correct.

The high serum prolactin levels found in constant estrous rats is well correlated with the high pituitary prolactin content reported earlier (Meites et al., 1961; Clemens, Ph.D. Thesis, 1968). This increase in prolactin secretion observed during constant estrus is probably due to estrogen secretion. The pituitaries of old female rats in constant estrus are almost invariably enlarged, and occasionally these rats develop pituitary tumors (Clemens, Ph.D. Thesis, 1968). It is well established that estrogen is a potent stimulator of prolactin release (Chen and Meites, 1970).

More work utilizing radioimmunoassays to measure serum levels of LH, prolactin and FSH is needed to determine the nature of the changes in pituitary hormone secretion with age. Measures of hypothalamic hormone secretion are also needed. Only when these changes are clearly described, can exploration begin on the possible neural influence with age on the brain, particularly the hypothalamus.

IV. Effect of Median Eminence Implantation of Prolactin on Serum Prolactin and LH During the Estrous Cycle

A. Objective

Previously in this thesis I described variations in serum LH and prolactin levels during the estrous cycle in the rat. It has been noted in the literature review that implantation of prolactin into the median eminence decreased pituitary prolactin concentration and may stimulate LH release. Thus it was of interest to determine the effect of an implant of prolactin into the median eminence of cycling female rats on serum prolactin and LH, especially during the proestrous peak of these two hormones. The effects of an implant of prolactin, FSH, or FSH/LH in the median eminence of ovariectomized rats was also studied.

B. Materials and Methods

All rats used in this study were mature, cycling, virgin females obtained from Spartan Research Animals, Haslett, Michigan. Before the implantation of hormones, the cycles of all rats were followed by examining daily vaginal smears for at least 2 cycles, and only normally cycling rats were used for experimental purposes. Rats were also ovariectomized and used 3 weeks later.

The design of this experiment is comprised of three distinct parts. The first 2 groups of rats received median eminence implants of either prolactin-cocoa butter

or cocoa butter alone at random times during the cycle. These rats were bled via cardiac puncture under light ether anesthesia immediately prior to implantation, and daily thereafter for 5 days. All bleedings (1 ml each) and implantations were done before 11 AM, and vaginal smears were taken daily. After 5 days all rats were killed, and only those with properly placed implants were used.

The second 2 groups of rats received implants in the median eminence of either prolactin-cocoa butter or cocoa butter alone during the morning of proestrus. These rats were bled via heart puncture under light ether anesthesia 4 times on the same day that they were implanted. One ml of blood was removed each hour beginning at 4:30 PM. Vaginal smears were taken from all rats for several days after implantation, and only those rats which came into estrus the day after implantation were considered to be in proestrus when bled.

A third part of the experiment used 4 groups of female rats which were ovariectomized and used for experiments 3 weeks later. These rats received median eminence implants of cocoa butter alone, prolactin, FSH or FSH/LH cocoa butter mixtures on day 0. Bleedings were done via heart puncture on day 2, 4, and 6. All serum samples were assayed for prolactin.

The serum was separated from the blood and assayed for prolactin and LH. Prolactin and LH were measured by

the RIA described in the general Materials and Methods section. The reference preparations used were H 1010B prolactin and NIAMD-LH-RP-1.

C. Results

The effect of implanting prolactin into the median eminence of intact female rats during random stages of the cycle on serum prolactin is shown in Table 4. Only 2 stages, estrus and diestrus, are shown because these stages had large enough numbers of rats to give the data reliability. Prior to implantation, on day 0, serum prolactin during estrus was nearly the same in the two groups, 87.3 and 89.7 ng/ml. Following implantation of prolactin, the mean serum prolactin value during estrus during the 5 day post-implantation period was significantly lower (41.7 ng/ml) than on day 0. It also was lower than serum prolactin of control rats implanted with cocoa butter. There was no effect of the prolactin implant on serum prolactin during diestrus.

Figure 8 illustrates the mean serum prolactin levels daily beginning on day 0, for rats in estrus at the time of bleeding. These results are the same as shown in Table 4 except they show the daily mean serum prolactin levels. This figure illustrates that as early as 1 day after implantation of prolactin into the median eminence, serum prolactin levels did not rise to pre-implantation levels.

TABLE 4.--Effect of ME implant of prolactin on serum prolactin during the estrous cycle.

Treatment and no. of Rats	Stage of Estrous Cycle	Serum Prolactin (ng/ml)[1]	
		Before Implant	After Implant
Cocoa butter (10)	Diestrus	34.0± 2.9 (3)[a]	35.0± 3.1 (31)[a]
	Estrus	89.7±19.9 (7)[b]	98.8±23.4 (10)[b]
Prolactin (10)	Diestrus	29.5± 9.9 (4)[a]	23.0± 3.6 (28)[a]
	Estrus	87.3± 4.5 (6)[b]	41.7± 6.3 (18)[a]

[1]Mean ± SE of the mean.

[a,b]Means with the same superscript are not statistically different from each other ($p > .05$).

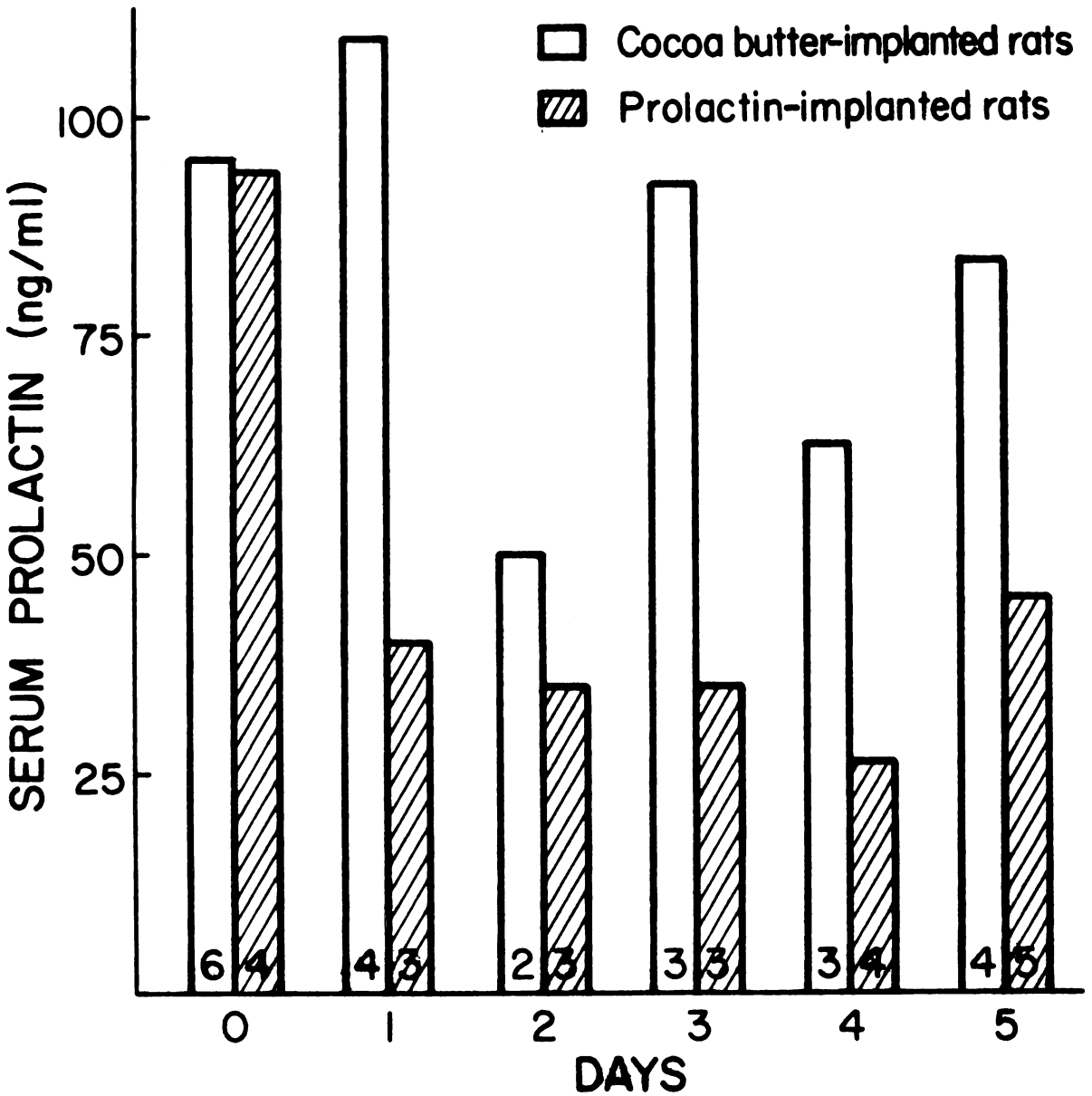


Figure 8. Effect of median eminence implant of prolactin on serum prolactin concentration during estrus in the rat.

The effect of the median eminence implantation of prolactin on serum LH levels in cycling rats is shown in Table 5. Rats implanted with cocoa butter alone had similar serum LH levels during diestrus and estrus, and these serum LH levels were similar to pre-implantation LH levels. Following implantation of prolactin into the median eminence, serum LH levels increased from 53.4 ng/ml to 114.7 ng/ml during diestrus. Serum LH values during estrus increased from 42.6 ng/ml before implantation, to 97.3 ng/ml 1-5 days after implantation. These increases are significant ($p < .05$) when compared to pre-implantation levels of LH, and when compared to rats implanted with cocoa butter.

Prolactin-cocoa butter or cocoa butter alone was implanted into the median eminence during the morning of proestrus, and the rats were bled at 1-hour intervals beginning at 4:30 in the afternoon. Rats implanted with prolactin had significantly lower serum prolactin levels compared to control rats implanted with cocoa butter (Table 6). Serum prolactin increased from 71.6 ng/ml at 4:30 PM to 186.7 ng/ml at 6:30 PM in controls. Rats given prolactin implants had less than 20 ng/ml prolactin in the serum at all bleeding times. This inhibition of serum prolactin was statistically significant at all times after prolactin implantation.

Thirty-five adult female rats were ovariectomized 3 weeks prior to implantation with either cocoa butter

TABLE 5.--Effect of ME implant of prolactin on serum LH during the estrous cycle.

Treatment and No. of Rats	Stage of Estrous Cycle	Serum LH (ng/ml)[1]	
		Before Implant	After Implant
Cocoa butter (10)	Diestrus	33.7±4.5 (3)[a]	49.7± 9.7 (22)[a]
	Estrus	41.1±6.4 (5)[a]	42.5± 4.2 (6)[a]
Prolactin (10)	Diestrus	53.4±6.1 (5)[a]	114.7±36.3 (23)[b]
	Estrus	42.6±8.7 (3)[a]	97.3±29.5 (19)[b]

[1]Mean ± SE of the mean.

[a,b]Means with the same superscript are not statistically different from each other ($p > .05$).

TABLE 6.--Effect of ME implant of prolactin on serum prolactin during proestrus.

Treatment	No. of Rats	Serum Prolactin (ng/ml)[1]			
		4:30 PM	5:30 PM	6:30 PM	7:30 PM
Cocoa Butter	8	71.6±19.4	114.0±22.1	186.7±33.3	100.9±14.9
Prolactin	8	14.1± 2.5*	11.0± 1.7**	18.0± 5.6***	11.6± 1.8****

[1]Mean ± SE of the mean.

*p < .02
**p < .001
***p < .0002
****p < .0001

alone, prolactin, FSH, or a FSH-LH mixture. All implants were placed in the median eminence area, and 200-250 ug of hormone was used in each implant. Serum prolactin levels were not affected by any of these hormone treatments as compared to cocoa-butter implanted rats (Table 7). Serum prolactin levels were low after ovariectomy, approximately the same as prolactin levels during diestrus.

D. Discussion

The results of these experiments show that implantation of prolactin into the median eminence of cycling female rats decreased serum prolactin during estrus, and increased serum LH during both diestrus and estrus for 5 days. Implantation of prolactin into the median eminence during the morning of proestrus inhibitied the rise in serum prolactin levels normally seen during the late afternoon and early evening of proestrus. This supports the hypothesis that prolactin acts back on the hypothalamus to inhibit its own release, and to stimulate LH release. Implantation of prolactin, FSH, or FSH-LH into the median eminence following ovariectomy had no effect on the already low serum prolactin concentrations.

Several articles have recently appeared indicating that a short-loop feedback for prolactin exists. One of the first reports, by Clemens and Meites (1968), indicated that median eminence prolactin implants in cycling female rats inhibited mammary growth and luteal function, and

TABLE 7.--Effect of median eminence implantation of prolactin, FSH, or FSH/LH on serum prolactin in ovariectomized rats.

Treatment	No. of Rats	AP wt. (mg)	Serum Prolactin (ng/ml)[1]			
			Day 0	Day 2	Day 4	Day 6
Cocoa Butter	9	11.9	24.2±4.7	21.5±2.5	16.6±1.7	23.3±4.4
Prolactin	8	11.2	23.4±4.0	26.5±3.1	15.8±3.8	19.9±1.9
FSH	8	12.7	22.0±2.1	23.7±4.7	18.4±4.1	24.9±5.0
FSH/LH	10	11.3	26.0±3.4	19.4±2.7	22.5±3.2	26.0±6.1

[1]Mean ± SE of the mean.

decreased pituitary prolactin concentration. They also reported that rats with prolactin implants had a higher PIF content in the hypothalamus, indicating that the site of prolactin feedback action was at the hypothalamic level. Similar experiments in ovariectomized rats showed the same results as in intact rats. Sinha and Tucker (1968) injected female rats with ovine prolactin for 10 days, and found decreased pituitary prolactin content in these rats. Female rats with pituitary transplants under the kidney capsule also had reduced pituitary prolactin content. Ovariectomy did not alter these effects, indicating that the autofeedback control of prolactin can function in the absence of the ovary. Another report (Welsch et al., 1968a) indicated that pituitary prolactin was increased, and hypothalamic PIF reduced, following pituitary transplantation in intact rats. They also reported that ovariectomy plus pituitary transplantation reduced pituitary prolactin. The experiment reported here indicated that prolactin implants into the median eminence of ovariectomized rats did not decrease serum prolactin. It is important to note that serum prolactin levels after ovariectomy alone are already low. Similarly, implants of prolactin did not reduce serum prolactin during diestrus compared to controls. Thus it appears that when serum levels of prolactin are low, prolactin implantation into the median eminence does not lower them further.

As noted in experiment II of this thesis, serum prolactin increases sharply during the late afternoon of proestrus. Results of this experiment IV indicate that the prolactin implant in the median eminence completely suppressed this rise in serum prolactin. Thus the prolactin in the hypothalamus blocked whatever signal normally reaches the hypothalamo-pituitary axis to stimulate prolactin release. It is possible that the signal is increased estrogen from the ovaries in response to LH and FSH. This increased estrogen may reach the hypothalamus and stimulate prolactin release by decreasing PIF. Nagasawa et al. (1969) showed that median eminence implants of estrogen significantly increased serum and pituitary prolactin levels. Evidence is already in the literature indicating that median eminence implants of prolactin can block the stimulatory effects of estrogen on pituitary prolactin (Welsch et al., 1968b). They reported that 1.0 or 5.0 ug estradiol benzoate injected subcutaneously for 5 days was ineffective in increasing pituitary prolactin content and concentration in rats implanted with prolactin in the median eminence, whereas rats implanted with cocoa butter had increased pituitary prolactin levels following estrogen injection. The mechanism by which prolactin in the hypothalamus can block the stimulatory effect of estrogen on prolactin release is not known, but it is likely that this blocking action of prolactin explains the elimination of the serum

prolactin peak during proestrus following median eminence implantation of prolactin.

Some indirect evidence has been published which suggested that prolactin may stimulate gonadotropin release. Clemens et al. (1969a), reported that implanting prolactin into the median eminence of lactating rats not only depressed lactation, but also stimulated ovulation. Stimulation of ovulation and termination of pregnancy also occurred in pregnant rats following implantation of prolactin into the median eminence (Clemens et al., 1969b). Implantation of prolactin into immature female rats hastened puberty (Clemens et al., 1969c) and stimulated FSH (Voogt et al., 1969b), and LH (Voogt et al., 1969a) release as measured by FSH and LH bioassay of pituitary tissue. The study reported here gives evidence that serum LH is increased even on the days of diestrus and estrus when it is normally low, following implantation of prolactin into the median eminence. The mechanism(s) by which a prolactin implant in the median eminence stimulates LH release is not clear.

V. Effects of an Implant of Prolactin in Median Eminence of Pseudopregnant Rats on Serum and Pituitary LH, FSH, and Prolactin

A. Objective

A short loop inhibitory feedback by anterior pituitary (AP) hormones on the secretion of AP hormones has been reported, with indications that each AP hormone inhibits only its own secretion (Corbin and Story, 1967). However this concept does not appear to apply to the "short loop" feedback of prolactin. Thus implantation of prolactin in the median eminence (ME) reduced the duration of pseudopregnancy (Chen et al., 1968) and pregnancy (Clemens et al., 1969b) by causing ovulation.

None of the above work reported serum levels for LH, FSH, and prolactin after implanting prolactin into the ME. It was the purpose of the present investigation to determine the effects of an implant of prolactin in the ME of pseudopregnant rats on serum and pituitary LH, FSH, and prolactin. LRF, FSH-RF and PIF levels in the hypothalamus also were measured.

B. Materials and Methods

1. Animals

Mature virgin female Sprague-Dawley rats weighing 200-250 g each were obtained from Spartan Research Animals (Haslett, Michigan) and used for these experiments. Two estrous cycles were followed by taking vaginal smears

on all rats before the experiments began. Only rats with normal 4-5 day cycles were used. Pseudopregnancy (PP) was induced by mechanical stimulation of the cervix with a glass rod on the day(s) of vaginal cornification (day 0). The appearance of leucocytes in the vaginal smear was designated as day 1 of PP. The day a proestrous or estrous smear was found was designated as the final day of PP.

2. ME Implantation

Prolactin (NIH-P-S8) supplied by the Endocrinology Study Section, NIH, was mixed with equal amounts of cocoa butter. The prolactin-cocoa butter mixture (approximately 250 ug prolactin) was tamped into one end of a 23-gauge glass tube, implanted into the ME with a Stoelting stereotaxic instrument and left in situ. All rats were implanted on the fourth day of PP. Vaginal smears were taken daily and all rats were killed by guillotine on the morning 3 days after implantation (7 days after induction of PP). Only rats with proper ME implants, determined by examination under a dissecting microscope, were used for obtaining data.

3. Collection and Preparation of Serum, Pituitaries, and Hypothalami for Assay

Blood was collected under ether anesthesia via the abdominal vena cava. Serum was separated and kept frozen at -20 C until assayed. AP's from the implanted rats were individually weighed, placed in cold .01 M phosphate buffer

in 0.14 M NaCl (pH=7.0), homogenized with a Sonifier cell disruptor and frozen until assayed. The hypothalami were removed immediately after killing the rats, homogenized in 0.1 N HCl, centrifuged at 12,000 g for 40 minutes at 4 C and neutralized with 1 N NaOH just prior to use. LRF (Piacsek and Meites, 1966), FSH-RF (Mittler and Meites, 1964), and PIF (Kragt and Meites, 1967) were measured by incubation of pituitary halves from mature male rats with pooled hypothalamic extracts. Incubation time for measuring LRF and PIF was 2 hours after one-half hr pre-incubation, whereas incubation time for FSH-RF was 6 hrs after one-half hr pre-incubation. The incubation medium 199 (Difco Labs, Detroit, Michigan) was stored at -20 C until assayed for LH, FSH, and prolactin.

4. Collection of Ovaries, Uteri, and Mammary Glands for Histological Examination

Ovaries and uteri were removed, weighed and fixed in Bouin's fluid after killing the rats. They were stained with hemotoxylin and eosin and examined histologically. The left inguinal mammary gland was removed from each rat, fixed in Bouin's fluid and stained with Harris' hematoxylin. These glands were then rated for degree of development on a scale of 1-6. Ratings of 1-3 indicate progressive increases in ductal development and ratings of 4-6 indicate progressive increases in lobulo-alveolar development, as described previously (Welsch *et al.*, 1968a).

5. Radioimmunoassays (RIA)

Prolactin from individual serum samples, pituitaries, and incubation media were measured at 3 dose levels by a double antibody RIA for rat prolactin described previously (Niswender et al., 1969). Purified rat prolactin was used as a reference preparation (HIV-8-C, biological potency = .77 x NIH-P-B1, kindly supplied by Dr. S. Ellis, NASA, Ames Research Center, Moffett Field, California).

LH and FSH from individual serum samples, pituitaries, and incubation medium were measured at 3 dose levels. LH was measured by a double antibody technique described by Monroe et al. (1968). The reference preparation for rat LH was NIAMD-LH-RF-1 with a biological potency equal to 0.03 x NIH-LH-S1. FSH was measured by a double antibody method described by Parlow et al. (1969). The reference preparation for rat FSH was NIAMD-FSH-RP-1 with a biological potency equal to 2.1 x NIH-FSH-S1. Pituitary FSH also was measured by the Steelman-Pohley method (Steelman and Pohley, 1953) as modified by Parlow and Reichert (1963). The reference standard used was NIH-FSH-S4, 36.44 IU/mg. Since all calculations compared only two means, controls and experimental groups, Student's "t" test was used to determine significance of differences between groups.

C. Results

1. Effect of a ME Implant of Prolactin on Duration of PP

Of a total of 28 rats implanted with cocoa butter alone (controls), 26 were still PP when they were killed 7 days after induction of PP. Among 31 rats implanted with prolactin, 28 came into estrus 2 or 3 days later.

2. Effect of a ME Implant of Prolactin on Serum LH, FSH, and Prolactin

Serum prolactin concentration was unaffected by implantation of prolactin (Table 8). Duplicate measurements of serum prolactin by RIA indicated that during the seventh day of PP, prolactin values for control rats were 14-17 ng/ml serum, approximately equal to normal serum values during the diestrous phase of the estrous cycle. Similar serum prolactin values of 13-17 ng/ml were noted after prolactin implantation, at which time 93% of the rats were in estrus. Since normal serum prolactin values during the morning of estrus range from 80-120 ng/ml, it is evident that the prolactin implant blocked the increase in serum prolactin which normally occurs at estrus.

Serum LH concentration increased ($p < 0.005$) from 109 ng/ml in the controls to 226 ng/ml following implantation of prolactin (Table 8). Serum FSH concentration showed a rise similar to LH, increasing from 199 ng/ml in

TABLE 8.--Effect of ME implantation of prolactin in PP rats on serum prolactin, LH, and FSH.

Treatment	No. of Rats	Assay No.	Prolactin Conc. as ng/ml Serum[1,2]	LH Conc. as ng/ml Serum[1,3]	FSH Conc. as ng/ml Serum[1,4]
Cocoa Butter	24	1	14.5±1.0	109.2± 5.2	199.4±34.4
		2	17.5±1.0		
Prolactin	25	1	13.8±1.5	226.1±31.9*	385.9±62.7**
		2	17.4±2.0		

[1]Mean ± SE of the mean.

[2]Reference preparation = HIV-8-C.

[3]Reference preparation = NIAMD-LH-RP-1.

[4]Reference preparation = NIAMD-FHS-RP-1.

*$p < .005$

**$p < .05$

the controls to 386 ng/ml in the rats implanted with prolactin ($p < 0.05$).

3. Effect of implant of Prolactin in the ME on Pituitary LH, FSH, and Prolactin

Pituitary prolactin concentration decreased 47% following prolactin implantation, from 2.57 ug/mg AP in control rats to 1.35 ug/mg AP in rats implanted with prolactin (Table 9). Pituitary LH concentration, although slightly reduced, was not significantly affected by implantation of prolactin. Pituitary FSH concentration decreased from 5.87 ug/mg AP in the controls to 4.06 ug/mg AP after prolactin implantation ($p < .001$) (Table 9). Bioassay of pituitary FSH revealed a similar decrease following prolactin implantation.

4. Effect of a ME Implant of Prolactin on Hypothalamic PIF, LRF, and FSH-RF Content

Hypothalamic PIF content was increased only slightly following implantation of prolactin for 3 days, as indicated by decreased release of prolactin from the AP tissue during incubation with hypothalamic extract (Table 10). However this 14% decrease in prolactin release was not significant. The 21% decrease in LH release in response to hypothalamic extract from rats implanted with prolactin was not statistically significant. There was no effect on hypothalamic FSH-RF content after prolactin implantation.

TABLE 9.--Effect of ME implantation of prolactin in PP rats on pituitary prolactin, LH, and FSH concentration.

Treatment	No. of Rats	Prolactin Conc. as ug/mg AP[1,2]	LH Conc. as ug/mg AP[1,3]	FSH Conc. as ug/mg AP[1,4,5]	Type of Assay
Cocoa Butter	24	2.57±0.25	63.52±3.48	5.87±0.23	RIA
Prolactin	25	1.35±0.14*	53.61±4.94	4.06±0.26*	RIA
Cocoa Butter	24	-----	-----	4.58(3.81-5.43)	Bioassay
Prolactin	25	-----	-----	3.07(2.24-3.62)	Bioassay

[1]Mean ± SE of the mean.

[2]Reference Preparation = HIV-8-C.

[3]Reference Preparation = NIAMD-LH-RP-1.

[4]Reference Preparation = NIAMD-FSH-RP-1 for RIA data.

[5]Expressed as ug NIH-FSH-S1 for bioassay data.

*$p < .001$

TABLE 10.--Effect of ME implantation of prolactin in PP rats on hypothalamic PIF, LRF, and FSH-RF content.

Treatment	No. of Rats	Prolactin Released in Med. (ng/mg AP)[1,2]	LH Released in Med. (ng/mg AP)[1,3]	FSH Released in Med. (ng/mg AP)[1,4]
Cocoa Butter	24	280±32	3383±344	1086±238
Prolactin	25	241±40	2646±147	902±106

[1]Mean ± SE of the mean.
[2]Reference Preparation = HIV-8-C.
[3]Reference Preparation = NIAMD-LH-RP-1.
[4]Reference Preparation = NIAMD-FSH-RP-1.

5. Effect of a ME Implant of Prolactin on Organ Weights and Mammary Gland Development

Table 11 indicates that ovarian weight is unchanged following prolactin implantation. However, a distinct increase in follicular development and a decrease in luteal tissue was seen in ovaries from prolactin implanted rats (Fig. 9B) as compared with ovaries from cocoa butter implanted controls which remained in PP (Fig. 9A). The disappearance of corpora lutea may be due not only to a reduction in prolactin release by the AP, but also to luteolytic activity resulting from increased LH release. The uterine weights of rats implanted with prolactin and cocoa butter were significantly greater ($p < .00005$) than in rats implanted with cocoa butter alone. Microscopic examination of the uterus from prolactin implanted rats (Fig. 10B) revealed a definite stimulation of the epithelial and endometrial layers when compared with the uterus of rats implanted with cocoa butter (Fig. 10A).

AP weights did not differ 3 days after prolactin implantation. There was a significant atrophy of the mammary glands following prolactin implantation as seen by the mammary gland ratings (Table 11). The mammary glands of the control PP rats were given an average rating of 4.25 and showed a well developed lobulo-alveolar system (Fig. 11A), as compared with mammary glands from prolactin-implanted rats which were given an average rating of only

TABLE 11.--Effect of ME implantation of prolactin in PP rats on organ weights and mammary gland ratings.

Treatment	No. of Rats	Av. Ovarian Weight (mg)[1]	Av. Uterine Weight (mg)[1]	Av. AP Weight (mg)[1]	Av. Mammary Gland Ratings[1]
Cocoa Butter	22	84.9±2.4	318.9±20.1	9.68±0.23	4.25±0.26
Prolactin	25	87.7±4.5	513.5±22.8**	9.96±0.26	2.70±0.28*

[1]Mean ± SE of the Mean

*p < .005
**p < .00005

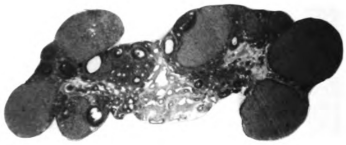


Figure 9A. Photomicrograph of ovary from PP rat implanted with cocoa butter in ME. Note predominance of well-developed corpora lutea. x15.

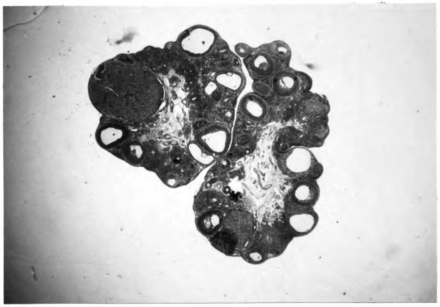


Figure 9B. Photomicrograph of ovary from PP rat implanted with prolactin. Note numerous follicles and few corpora lutea. x15.

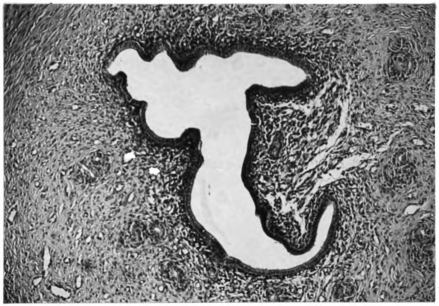


Figure 10A. Photomicrograph of uterus from PP rat implanted with cocoa butter. Note relative lack of estrogen stimulation. x110.

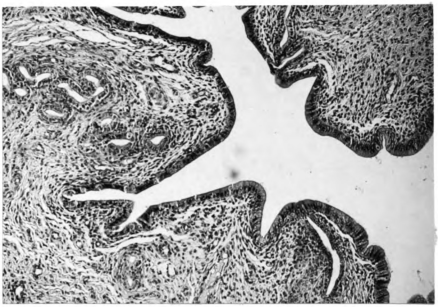


Figure 10B. Photomicrograph of uterus from PP rat implanted with prolactin. Note stimulation of epithileum and endometrium. x110.

Figure 11A. Well-developed mammary gland from PP rat implanted with cocoa butter, showing extensive branching and lobulo-alveolar development. x10.

Figure 11B. Mammary gland from PP rat implanted with prolactin, showing branching and end buds but little lobulo-alveolar development. x10.

2.7 ($p < .005$) and showed only a ductular system (Fig. 11B).

D. Discussion

These results demonstrate that a prolactin implant in the ME interrupted PP, stimulated release of LH and FSH, and blocked the increase in serum prolactin normally observed during estrus. This provides further evidence that prolactin can inhibit its own secretion, and extends earlier work from our laboratory showing that a prolactin-cholesterol pellet placed in the ME shortens the length of PP and inhibits deciduoma formation (Chen et al., 1968). When a prolactin-cholesterol pellet was placed in the ME, PP was shortened only 3-4 days, whereas a prolactin-cocoa butter implant in the ME resulted in termination of PP within 3 days in almost all rats. A difference in rate of absorption of prolactin from the 2 types of implants may account for the differential effects on length of PP.

The increase in serum LH and FSH noted 3 days after prolactin implantation, as well as the fall in pituitary FSH, indicate that the primary or initial effect may be on release rather than on synthesis of gonadotropins. The lack of a significant change in pituitary LH concentration in face of a 100% increase in serum LH may be due to the large amounts of pituitary LH compared to the amounts present in the blood. Similar observations have been made by others (Gay, 1970).

The mechanism whereby prolactin in the ME stimulates gonadotropin release is not clear. There was no definite effect of prolactin on hypothalamic LRF or FSH-RF content. However this does not exclude the possibility that prolactin stimulated the rate of both synthesis and release of these RF's to an equal degree, thereby resulting in no net change in content. It may be argued that prolactin may pass down the portal vessels and act directly on the pituitary to stimulate LH and FSH. However, in an in vitro experiment, we were unable to observe any stimulatory effect by prolactin on release of FSH from the pituitary gland (unpublished data).

It is possible that prolactin does not act directly on the hypothalamus to stimulate LH and FSH release but may operate indirectly through other mechanisms. Since pituitary prolactin is decreased following prolactin implantation in the ME, this may allow more of the cellular machinery in the AP to synthesize and release LH and FSH. Also, as a result of the inhibition of prolactin release, the corpora lutea of PP cease to function, and this could remove any inhibition by progesterone to LH and FSH secretion by the AP. Whether the presence of the ovaries is a factor in these interactions between the 3 pituitary hormones remains to be determined.

VI. Inhibition of Lactation and Serum Prolactin in Postpartum Rats by Median Eminence Implantation of Prolactin

A. Objective

The hypothesis that prolactin can inhibit its own secretion has been postulated by researchers in this laboratory. Implantation of prolactin into the median eminence (ME) of postpartum lactating rats reduced lactation as measured by pup weight gains (Clemens et al., 1969a). Since prolactin is essential for maintenance of lactation (Meites, 1959, 1966), it was concluded that the reduced lactation was a result of reduced serum prolactin levels following the median eminence implantation of prolactin. It was of interest, therefore, to determine the effects of ME implants of prolactin on serum prolactin following suckling in postpartum lactating rats. Serum LH also was measured to determine whether the ME implant of prolactin stimulated LH release.

B. Materials and Methods

Three-month-old female rats (Spartan Research Animals) were bred and housed in individual cages near the end of pregnancy. On the day after parturition all litters were adjusted to 8 pups and the lactating rats were divided into 4 groups. The first group (9 rats) received subcutaneous injections of saline twice daily beginning 1 day after delivery and continuing for 11 days. The

second group (9 rats) received subcutaneous injections of 1 mg ovine prolactin (NIH-S8) twice daily for the same time period. These 2 groups of rats were bled via cardiac puncture under light ether anesthesia on day 4, 8, and 11 postpartum after a 3 hour non-suckling-1 hour suckling period.

The third and fourth groups of rats received stereotaxic implants in the anterior ME of either cocoa butter alone (controls) or a prolactin-cocoa butter mixture containing approximately 250 ug of NIH-S8 ovine prolactin. The implants were made using 23 gauge glass tubes, which were fixed to the skull and left in place. All implants were done on the fourth day of lactation (day 0). At this time and every day thereafter, litter weights were recorded. The litters of the latter 2 groups of rats were removed for 3 hours and then returned for 1 hour of suckling prior to obtaining a blood sample with subsequent implantation on day 0. On day 2, 4, and 6, a similar non-suckling-suckling design was carried out, always followed immediately by heart puncture to provide a 1 ml sample of blood.

C. Results

Effect on serum prolactin levels of injecting 2 mg of ovine prolactin each day into lactating rats is shown in Table 12. Although serum prolactin levels in rats which received the prolactin injections were depressed as

TABLE 12.--Effect of injecting exogenous ovine prolactin on serum prolactin levels following suckling.

Treatment	No. of Rats	Serum Prolactin (ng/ml)[1]		
		Day 4	Day 8	Day 11
Saline	9	317.6±60.1	391.4±41.3	438.2±80.5
Prolactin	9	389.1±48.3	298.1±68.4	274.3±87.3

[1]Mean ± SE of the mean.

compared to saline injected controls after 1 hour of suckling, the differences were not significant due to very large variations among rats. Effect of exogenous prolactin on serum LH levels following suckling is shown in Table 13. Serum LH levels following suckling were very low in both saline and prolactin injected rats at all 3 times measured.

The second 2 groups of rats received either prolactin or cocoa butter implants in the ME on the fourth day of lactation, referred to as day 0 in Tables 14 and 15 and Fig. 12. The litters (8 pups/litter) of control lactating mother rats gained 10-21 gms/day for 6 days. Litters from mother rats implanted with prolactin gained much less, from 0.2-13.7 gms/day. Even 6 days after implantation of prolactin, these pups gained less than half as much as litters nursing control mother rats. Thus the prolactin implant was effective in reducing lactation for at least 6 days.

All rats were bled 1 hour after suckling prior to ME implantation of prolactin or cocoa butter on day 0, the fourth day of lactation. Serum prolactin levels averaged 275 and 288 ng/ml in the 2 groups (Fig. 12). On day 2, ME prolactin completely inhibited the rise in serum prolactin usually observed following suckling. This inhibition lasted at least 6 days, similar to the period of time that lactation was decreased in rats with ME implants of prolactin.

The effect of these ME implants of prolactin on serum LH following suckling is shown in Table 15. There

TABLE 13.--Effect of exogenous ovine prolactin on serum LH levels following suckling.

Treatment	No. of Rats	Serum LH (ng/ml)[1]		
		Day 4	Day 8	Day 11
Saline	9	26.7±2.4	14.5±2.9	15.5±3.1
Prolactin	9	21.2±3.7	19.4±1.4	13.0±2.3

[1]Mean ± SE of the mean.

TABLE 14.--Effect of a ME implant of ovine prolactin in lactating rats on daily litter weight gain.

Treatment	No. of Litters	Av. Daily Litter Wt. Gain (gms)					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Cocoa Butter	19	17.4	11.5	10.2	19.7	19.2	21.5
Prolactin	18	3.2	0.2	13.7	12.1	10.3	8.3

TABLE 15.--Effect of median eminence implantation of prolactin in lactating rats on serum LH.

Treatment	No. of Rats	Serum LH (ng/ml)[1]			
		Day 0	Day 2	Day 4	Day 6
Cocoa Butter	13	20.5±2.7	14.8±3.4	9.3±3.7	12.0±3.1
Prolactin	14	13.6±5.1	19.8±2.9	10.7±1.9	14.8±2.0

[1]Mean ± SE of the mean.

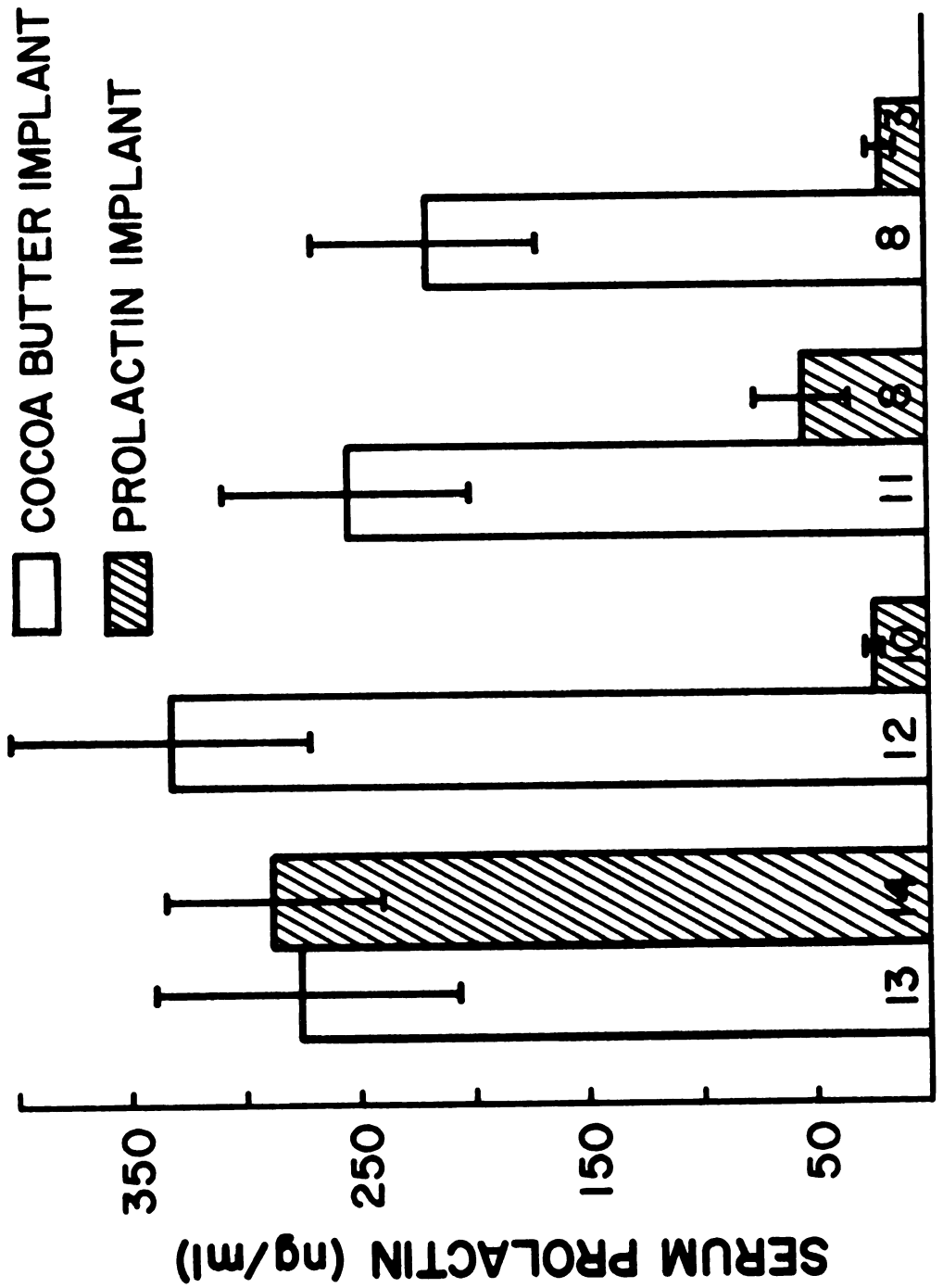


Figure 12. Serum prolactin levels in lactating rats with ME implants of prolactin.

was no difference in serum LH levels following suckling in rats implanted with prolactin in the ME compared to cocoa-butter implanted controls. Serum LH was very low at all times in both groups.

D. Discussion

This study demonstrates that placement of prolactin into the median eminence of postpartum lactating rats significantly impairs lactation as measured by litter weight gains. This decrease in lactation is probably the result of an inhibition of the serum prolactin rise which normally occurs during suckling (Amenomori et al., 1970). As early as 2 days after implantation of prolactin into the ME, serum prolactin levels were less than 20 ng/ml after 1 hour of suckling, whereas control rats had serum prolactin levels of more than 300 ng/ml after 1 hour of suckling. This significant inhibition of serum prolactin continued for the duration of the experiment, 6 days.

It appears that a glass tube filled with 250 ug prolactin and placed in the ME area of the hypothalamus, can release prolactin from its site for at least 6 days and inhibit prolactin secretion from the pituitary. About 40 ug of prolactin are calculated to be released daily, assuming that all of the prolactin is released in 6 days and the same amount is released each day. It is probable that lactating rats have an average serum prolactin level around 200 ng/ml serum, based on studies by Amenomori

et al. (1970) and in this thesis. This means there may be about 2000 ng (200 ng/ml x 10 ml serum in lactating rats) of prolactin in the serum of a lactating rat. Since prolactin has a calculated half life of only 13 minutes (Gay et al., 1970), it is possible that during lactation prolactin is secreted in much greater quantities per day than 40 ug, which may be the amount released/day from the glass tube. Thus this amount of prolactin acting on the ME may be within a physiological range.

These results confirm work by Clemens et al. (1969a) who found that similar ME implants of prolactin decreased lactation. They also found decreased mammary gland weight and reduced secretory activity of the mammary gland. Similar to my results, they found that lactating rats implanted with prolactin came into estrus and began to cycle. This was accompanied by ovaries which had large follicles and smaller corpora lutea than controls.

These results and others from our laboratory described in the literature review suggest that an implant of prolactin in the ME not only inhibits prolactin release, but also stimulates gonadotropin release. As described in experiments IV and V of this thesis, ME implants of prolactin increased serum LH and FSH levels. Similar serum LH increases were not found in the present experiment despite a return to estrus. One possible explanation is that the suckling stimulus temporarily blocks or inhibits LH release almost completely. As

noted in the results, serum LH in control lactating rats is very low, almost undetectable, following suckling.

The suckling stimulus is necessary to maintain postpartum lactation by evoking release of prolactin (Meites, 1959; Amenomori, 1970) and ACTH (Voogt et al., 1969c). It is believed that nerve impulses beginning at the nipples travel by way of the spinal cord to the brain and to the hypothalamus. These impulses are important for releasing prolactin and ACTH, presumably by inhibiting or lowering PIF levels, and stimulating CRF (corticotropin releasing factor) levels. The mechanism by which the prolactin implant in the ME interferes with this signal is unknown. It is possible that PIF activity is enhanced and prevents prolactin release during suckling, when prolactin is present in the ME. Clemens et al. (1968b) found a significant increase in hypothalamic PIF content and a significant decrease in pituitary prolactin concentration in cycling female rats implanted with prolactin in the ME. Minaguchi and Meites (1967) found that suckling reduced hypothalamic PIF content. It is possible that the constant presence of prolactin in the ME can overcome the stimulus evoked by suckling which normally would reduce PIF and increase prolactin release.

VII. Pituitary GH and Hypothalamic GHRF After Median Eminence Implantation of Ovine and Human GH

A. Objective

This study attempts to elucidate the site where GH inhibits its own secretion by measuring the effects of implants of human or ovine GH into the median eminence area on pituitary GH and hypothalamic GHRF (growth hormone releasing factor) content. It was also of interest to determine whether human GH, which has prolactin activity, could act to inhibit prolactin secretion, similar to the feedback properties of ovine prolactin. Serum prolactin, mammary development, pituitary weight, and body growth were measured in rats.

B. Materials and Methods

1. Experimental Animals

Immature 21-day-old female rats and mature 10-12-week-old female rats of the Sprague-Dawley strain (Spartan Research Animals, Haslett, Michigan) were used for the implantation studies. Mature male rats weighing 350-400 gms were used as pituitary donors. For GH bioassays, immature female rats (Hormone Assay Labs, Chicago, Illinois) were hypophysectomized at 26 days of age and shipped 7 days later. Bioassays were begun 9-10 days later. All animals were maintained on a schedule of 14 hrs. of light and 10 hrs. of darkness at 25±1 C. The rats were fed Wayne Lab

Blox pellets (Allied Mills, Chicago, Illinois). The diet of the hypophysectomized rats was supplemented with carrots, orange slices, and sugar cubes.

2. Treatments

Immature 21-day-old female rats were implanted stereotaxically in the median eminence with a single pellet containing approximately 100 ug ovine GH mixed in cholesterol. A Stoelting stereotaxic instrument was used together with DeGroot's atlas (1959). Control rats were implanted with an equivalent amount of cholesterol alone in pellet form. The animals were weighed and head to tail measurements were recorded daily for 2 weeks.

In two separate experiments, mature cycling female rats were given a single stereotaxic implantation of approximately 250 ug ovine GH (OGH) or human GH (HGH) in cocoa butter in the median eminence area, while control rats received cocoa-butter (CB) alone. The GH-cocoa butter mixture was tamped into the tip of 23-gauge glass tubing, implanted and left *in situ*. Implants were fixed in place by using dental cement and skull screws, as described previously by Clemens and Meites (1968b). Vaginal smears were taken daily prior to and after implantation. All rats were killed by guillotine 7 days after implantation and examined under a dissecting microscope for site of the implant. Only rats with properly located implants were used for obtaining data.

3. Preparation of Hypothalamic Extracts and Pituitaries

The hypothalami were removed immediately after killing the rats, homogenized in 0.1 N HCl, centrifuged at 12,000 g for 40 minutes at 4 C and neutralized with 1 N NaOH just prior to use. GHRF was measured by incubating pituitary halves from male rats for 6 hours with hypothalamic extract, as described previously by Dickerman et al. (1969a). The incubation medium was stored at -30 C until assayed for GH. Anterior pituitary glands (AP) from implanted rats were placed in cold saline, homogenized with a Sonifier cell disruptor and assayed for GH.

4. GH Bioassay

Growth hormone activity was assayed by the standard tibia test of Greenspan et al. (1949). All bioassays included 2 doses of the control pituitary tissue or incubation medium, and 2 doses of the experimental pituitary tissue or medium. The reference standard (NIH-GH-S8) was also assayed at 2 dose levels. Four rats were used for each assay point. Statistical treatments included analysis of the symmetrical 4-point parallel line assays, calculated mean tibial responses, standards errors, analysis of variance, and relative potencies of experimental versus control test preparations. Student's "t" test was used to compare differences between 2 groups of organ weights, mammary gland ratings, etc., whereas one-way

analysis of variance was used when means of more than 2 groups were compared.

C. Results

1. Pituitary GH Concentration After Median Eminence GH Implantation

Pituitaries were bioassayed at 2 dose levels, 2 and 4 mg/assay rat/4 days (Table 16). Pituitary GH decreased 25% in experiment I and 64% in experiment II, 7 days after HGH implantation in adult female rats, as compared to controls implanted with cholesterol or cocoa butter. Similar decreases in pituitary GH were observed following OGH implantation. The higher GH values in experiment I as compared to experiment II may be due to more sensitive bioassay rats.

2. Hypothalamic GHRF Following Median Eminence GH Implantation

One dose (.5 hypothalamic equivalents/incubated pituitary) was used to assay hypothalamic content of GHRF *in vitro*. Table 17 shows that implantation of HGH reduced hypothalamic GHRF 48% in both experiments I and II. A similar decrease in hypothalamic GHRF was observed in experiment II following implantation of OGH. Hypothalamic GHRF was not measured in OGH implanted rats in experiment I.

TABLE 16.--Effect of GH implant in the median eminence of adult female rats for 7 days on pituitary GH concentration.

Exp. No.	Implant	No. of Rats	Dose (mg AP/ Assay Rat)	Av. Tibial Width (u) (Mean±SE)	GH (ug/mg AP)[1,2]	Relative Potency[3]	λ[4]
1	Controls	8	2	261±9			
			4	314±7	113.6±10.5		
	Human GH	7	2	248±3			
			4	275±6	84.2± 4.8	.633(.405-.838)	.124
	Controls	8	2	172±3			
			4	201±7	28.9± 1.9		
	Ovine GH	10	2	138±4			
			4	175±3	17.7± 1.2	.532(.359-.685)	.088
2	Controls	10	2	233±4			
			4	262±7	45.5± 3.8		
	Human GH	10	2	186±10			
			4	200±8	16.5± 2.7	.166(.057-.441)	.250
	Ovine GH	10	2	173±7			
			4	224±8	17.5± 2.0	.428(.196-.620)	.121

[1]Expressed as ug equivalents of NIH-GH-S8.

[2]Mean ± SE of the mean.

[3]Mean and 95% confidence limits.

[4]Index of precision.

TABLE 17.--Effect of GH implanted into the median eminence of adult female rats for 7 days on hypothalamic GHRF activity.

Exp. No.	Implant	No. of Rats	Dose AP Equivalents/ Assay Rat	Av. Tibial Width (u) (Mean±SE)	GH Released (ug/mg AP[1,2])	Relative Potency[3]	λ[4]
1	Controls	8	0.3	196±4			
			1.2	243±6	52.9±4.3		
	Human GH	7	0.3	168±3			
			1.2	197±4	27.4±1.7	.508(.347-.650)	.083
2	Controls	10	0.3	180±7			
			1.2	210±6	11.4±1.9		
	Human GH	10	0.3	161±4			
			1.2	191±5	5.9±0.6	.647(.357-.905)	.132
	Ovine	10	0.3	172±4			
			1.2	185±5	6.0±0.7	.578(.148-.924)	.185

[1]Expressed as ug equivalents of NIH-GH-S8.

[2]Mean ± SE of the mean.

[3]Mean and 95% confidence limits.

[4]Index of Precision.

3. Serum Prolactin Levels in Rats with Median Eminence HGH Implant

Serum prolactin levels were measured by the radioimmunoassay of Niswender et al. (1969). Blood samples were taken by heart puncture under light ether anesthesia just prior to and 3, 5, and 7 days after implantation of HGH in the median eminence. A significant ($p < .002$) decrease in serum prolactin was observed in rats in estrus following HGH implantation as compared to controls (Table 18). There also was a decrease in serum prolactin 3, 5, and 7 days after HGH implantation in rats in estrus as compared to pre-implantation estrous values (Day 0). There was no effect on serum prolactin in rats during diestrus.

4. Body Growth in 21-Day-Old Female Rats Implanted with GH in the Median Eminence

Table 19 shows the effect of an OGH implant into the median eminence of 21-day-old female rats on body weight and length. Body weight gain 7 days after implantation averaged 21.8 gms in OGH group and 14.7 gms in the cholesterol group, representing a significant increase in body weight. However, subcutaneous injections of 200 ug OGH had no effect on body weight as compared to saline injected controls. Three rats in this latter group failed to gain more than 2 gms during the first 7 days. By day 14, average body weight gains were equal in both groups. There were no differences in body length after 7 or 14 days. The average age at the time of vaginal opening was

TABLE 18.--Effect of GH implant in the median eminence of adult female rats on serum prolactin concentration.

Implant	No. of Rats	Stage of Estrous Cycle	Serum Prolactin[1] (ng/ml)	
			Day 0[2]	Days 3,5,7
Controls	18	Diestrus	19.5± 1.7[a]	25.1± 4.0[a]
		Estrus	97.8±29.5[b]	125.6±36.3[b]
Human GH	18	Diestrus	11.5± 2.1[a]	17.7± 5.9[a]
		Estrus	99.7±30.5[b]	32.9± 8.1[a]

[1]Reference Preparation = HIV-8-C with a stated biological potency = 0.77xNIH-P-B1.

[2]Mean ± SE of the mean.

[a,b]Means with different superscripts are significantly different ($p < .05$).

TABLE 19.--Effect of GH implant in the median eminence on body growth of 21-day-old female rats.

Implant	No. of Rats	Initial Body Wt. (g)[1]	Body Wt. Gain by Day 7 (g)[1]	Body Wt. Gain by Day 14 (g)[1]	Initial Length, Head to Tail (cm)[1]	Length Increase by Day 7 (cm)[1]	Length Increase by Day 14 (cm)[1]
Cholesterol	10	50.8±0.3	14.7±1.2	54.5±2.6	17.9±0.3	4.3±0.3	9.4±0.3
Ovine GH[2]	10	50.7±0.5	21.8±2.6*	55.1±4.3	18.2±0.4	4.4±0.3	9.5±0.3

[1]Mean ± SE of the mean.
[2]NIH-GH-S8
*$p < .05$

36.2±1.1 days for the OGH group and 36.4±1.0 days for the cholesterol group.

5. Organ Weights and Mammary Development of Adult Female Rats 7 Days After OGH or HGH Implantation

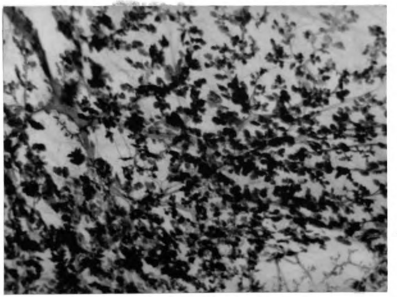
Table 20 summarizes data from 2 separate experiments. It shows that implantation of either OGH or HGH into the median eminence of adult cycling female rats had no effect on body, ovarian, or uterine weights by 7 days after implantation. A very significant decrease in AP weight in the HGH group was observed in both experiments. A less significant decrease in AP weight occurred in the OGH group.

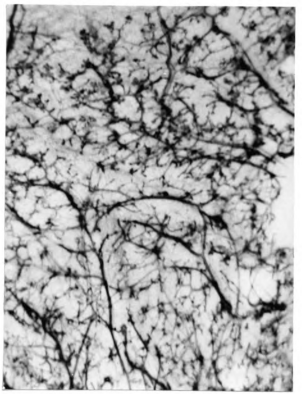
The mammary glands were fixed, stained, and rated for degree of development by a method described previously by Meites (1959). Mammary glands from control rats implanted with cocoa butter showed well-developed ducts and some lobulo-alveolar development (Fig. 13A). Implantation of HGH resulted in marked atrophy of the mammary glands, with only ducts evident (Fig. 13B). Implantation of OGH caused slight atrophy of the mammary glands when compared with the controls, but this was not nearly as significant as in the HGH group (Fig. 13C). Average mammary gland ratings (Table 20) of control rats were 3.8±0.1 in experiment I and 3.7±0.2 in experiment II, whereas the HGH groups had ratings of 2.8±0.1 and 2.3±0.1 in experiments 1 and 2 respectively, representing a very significant

TABLE 20.--Effect of GH implant in the median eminence of adult female rats on organ weights and mammary gland development.

Expt. No.	Implant	No. of Rats	Initial Body Weight(g)	Final Body Weight(g)	AP Weight (mg)	Ovarian Weight (mg)	Uterine Weight (mg)	Mammary Gland Rating
1	Control	8	278.8±3.1	269.9±3.4	11.1±0.3	---	---	3.8±0.1
	Human GH	7	275.5±3.4	258.5±5.7	8.3±0.3**	---	---	2.8±0.1**
2	Control	10	240.1±4.7	239.5±4.2	8.7±0.6	83.8±3.2	297.1±34.9	3.7±0.2
	Human GH	10	242.8±2.6	237.4±4.4	6.8±0.3*	83.7±4.1	306.1±21.6	2.3±0.1***
	Ovine GH	10	243.6±5.3	236.7±6.5	7.4±0.4	71.4±3.7	333.8±39.5	3.1±0.2*

*$p < .02$
**$p < .0005$
***$p < .00005$





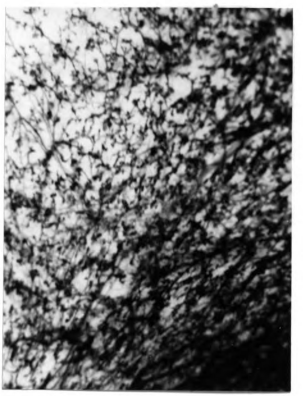


Figure 13. A. Well-developed mammary gland from control rat with cocoa butter implant in ME, showing numerous ducts, branching, and some lobulo-alveolar growth. B. Mammary gland from rat with human GH implant in ME, showing thin atrophic ducts and few end buds. C. Mammary gland from rat with ovine GH implant in ME, showing numerous ducts, branching, and slight lobulo-alveolar growth.

decrease ($p < .0005$) in mammary development. The mean mammary gland rating of 3.1 ± 0.2 in the OGH group was significantly less (at the 2% level) than the 3.7 ± 0.2 rating in the control group.

D. Discussion

These results demonstrate that either human or ovine GH can exert a negative feedback on the hypothalamus to regulate its own production. This action of GH apparently is mediated through a decrease in synthesis and release of hypothalamic GHRF, thereby depressing pituitary GH secretion.

The decrease in pituitary weight observed in our experiments after GH implantation in the median eminence was associated with a reduction in pituitary GH concentration. It is not possible on the basis of the evidence presented in these experiments to state conclusively that GH release from the pituitary was decreased or that GH synthesis was decreased, or whether both release and synthesis were decreased. To elucidate this would require measurement of GH secretion rates in addition to pituitary GH concentration. However, it is believed that the decrease in pituitary GH concentration after GH implantation in the ME probably reflects decreased circulating GH. Previously it was shown that reduced pituitary levels of GH were associated with decreased serum GH values in starved rats (Dickerman *et al.*, 1969b).

The sharp decrease in hypothalamic GHRF following either human or ovine GH implantation in the median eminence indicates that the hypothalamus is the primary site of feedback of GH on its secretion. The decrease in GHRF is reflected by a similar decline in pituitary GH concentration. Possible passage of the implanted GH to the pituitary by way of the portal vessels cannot be entirely ruled out in these experiments. However, the hypothalamus appears to be the major site of feedback for GH since GHRF activity can be altered either by a decrease in circulating GH (Müller et al., 1967a) or by an increase in hypothalamic GH as shown in the present work.

The reduction in AP weight 7 days after ovine GH implantation in adult female rats in good agreement with the findings of Katz et al. (1969), who used male rats. The even greater reduction in AP weight following HGH implantation may be due to both the GH and prolactin activity present in this preparation. Additional evidence for prolactin activity of the HGH is indicated by the extensive regression of the mammary glands in the rats implanted with HGH. Less mammary gland regression was noted in rats implanted with OGH, reflecting the lesser importance of GH as compared to prolactin for maintaining mammary development. The decrease in serum prolactin in these HGH implanted rats provides direct evidence that it inhibits prolactin release.

In contrast to the findings of Katz et al. (1969) indicating a slight decrease in body weight after OGH implantation, no significant changes in body growth were observed in our experiments after human or ovine GH was implanted in immature or adult female rats of the Sprague-Dawley strain. Katz et al. (1969) used adult male rats of the Sherman strain, and this may account for the differences in our observations. The failure to inhibit body growth in 21-day-old female rats with OGH implants in the median eminence may have several explanations. Young immature rats may not be as sensitive to GH feedback as adult rats. It is also possible that less GH is needed for body growth in rats until after 30 days of age (Walker et al., 1952), and that depression of GH secretion prior to that time has less effect on body growth. It is also possible that any inhibition of body growth due to decreased GH secretion may have been partially counteracted by the systemic effect of the 100 ug GH implant in the median eminence. This appears doubtful since an injection of 200 ug GH had no effect on body weight.

It is believed that GH has a role in regulating its own secretion since it lacks a specific peripheral target organ which secretes hormones to act back on the hypothalamus or pituitary. Thus administration of GH to monkeys prior to injection with vasopressin or insulin prevented an increase in serum GH (Sakuma and Knobil, 1970). A similar "short loop" feedback for prolactin

also is believed to exist, since prolactin lacks a specific target organ hormone. Hypothalamic implants of prolactin have been shown to increase PIF, decrease pituitary (Clemens and Meites, 1968b) and serum prolactin levels (Niswender et al., 1969) and to inhibit the ability of systemically administered estrogen to increase pituitary prolactin (Welsch et al., 1968b).

GENERAL DISCUSSION

The evidence presented in this thesis suggests that serum prolactin concentrations vary greatly with age and during several different physiological states, i.e., pseudopregnancy, ovariectomy, puberty, old age, and estrous cycle. Results summarized in this thesis also demonstrate that prolactin and GH can inhibit their own secretion from the anterior pituitary gland. It was found that by placing prolactin in the median eminence area of the hypothalamus, serum prolactin concentrations were significantly reduced during proestrus and estrus, and following a short period of suckling. Prolactin implants also quickly terminated pseudopregnancy. GH implantation in the median eminence reduced pituitary GH concentration and hypothalamic GHRF content.

There may be specialized cells in the hypothalamus which are receptors for prolactin and GH, and can accumulate prolactin and GH until their concentration reaches a threshold, at which time they begin to stimulate PIF synthesis and/or release and reduce GHRF secretion. No one has yet determined whether prolactin or GH are

normally found in the rat hypothalamus, and if present, whether their concentration changes with changing serum prolactin or GH concentrations. Reichlin (personal communication) found the greatest concentration of GH in the hypothalamus following intravenous GH injection. Other anterior pituitary hormones have been found in the hypothalamus.

It cannot be concluded from the results in this thesis that prolactin has a physiological role in inhibiting its own secretion. However, it is possible that during the afternoon of proestrus, when serum prolactin may reach levels as high as 1000 ng/ml, prolactin reaches the hypothalamus in relatively large amounts and acts to inhibit its own secretion. Already there is some evidence in the literature that indicates a physiological role of prolactin in inhibiting its own secretion. Chen et al. (1970) reported that transplantation of 1 anterior pituitary (AP) under the kidney capsule of hypophysectomized, ovariectomized female rats increased serum prolactin to 115 ng/ml, comparable to serum prolactin levels observed during estrus. Transplantation of 2 or 4 AP's increased serum prolactin to 178 and 250 ng/ml serum, respectively. The highest prolactin level is comparable to that seen during proestrus or after suckling. Four weeks after AP transplantation, all rats showed a decrease in serum prolactin levels. Thus it appears that the circulating serum prolactin may feedback on the hypothalamus to

increase PIF release which in turn inhibited prolactin release by the transplanted AP's. Similar results were observed in hypophysectomized rats with intact ovaries. Other reports (Sinha and Tucker, 1968; Welsch et al., 1968) showed that ovariectomized rats with 1 AP transplant show a decrease in pituitary prolactin content of the in situ pituitary. These results suggest that physiological levels of circulating prolactin (115-250 ng/ml) are capable of decreasing release of prolactin from the in situ pituitary. No one knows how much prolactin or GH may be required to inhibit their own secretion, nor whether the degree of inhibition increases with increasing amounts of prolactin or GH reaching the hypothalamus.

One can only speculate on whether the rapid fall in serum prolactin during the evening of proestrus, after it reaches a peak level in the afternoon, is due to an inhibitory feedback from the previously high serum prolactin levels. It would be of interest to determine the effect of graded doses of prolactin before the peak rise in serum prolactin during the afternoon of proestrus. Would an intact rat with a pituitary transplant have a normal prolactin peak during the afternoon of proestrus, or would the relatively high serum prolactin in this rat from the AP transplant inhibit the sharp rise in prolactin? Would the high serum prolactin levels found during continuous intense suckling reduce pituitary prolactin release due to prolactin feedback, or would the suckling stimulus

overcome any inhibitory prolactin feedback? The present results suggest that the normal rise in serum prolactin during proestrus or suckling may be inhibited if sufficient amounts of prolactin are present in the circulation.

There are several naturally occurring states in the life cycle of the rat in which prolactin secretion is reciprocally related to LH and FSH secretion. One good example is during postpartum lactation, when serum prolactin levels are very high and LH and FSH levels are almost undetectable. A similar relationship exists in the prepubertal state, although on the day of puberty both LH and prolactin levels rise. Removal of the ovaries stimulates LH and FSH release, whereas serum prolactin levels decrease to values below diestrus. On the day of estrus serum prolactin is high compared to LH and FSH in the rat. Similarly, estrogen stimulated rats have high prolactin levels and low LH and FSH levels. There are only a few instances in which all three hormones appear to rise and fall together, i.e., proestrus, immediately postpartum, on the day of vaginal opening.

The results in this thesis demonstrate that exogenous prolactin can be the stimulus to promote gonadotropin release and inhibit prolactin release at the same time. Increased serum LH and FSH levels were observed following prolactin implantation in cycling and pseudopregnant rats. It also has been reported that prolactin implants increase LH and FSH release in immature rats

(Voogt et al., 1969a,b). Lactating rats returned to estrus following prolactin implantation, although increased serum LH levels were not observed in these animals. It is important to consider that implanting prolactin into the median eminence is a different method for increasing the amount of prolactin in the hypothalamus than the suckling stimulus or estrogen administration when serum prolactin increases as the result of some external environmental stimulus (suckling) or some end organ hormone (estrogen). These stimuli may inhibit LH and FSH release directly rather than through first increasing prolactin secretion, which in turn may decrease FSH and LH secretion. Implantation of prolactin directly into the hypothalamus bypasses these stimuli and can cause the reverse reciprocal relationship to occur, that is, prolactin levels decrease and LH and FSH levels increase.

It is not understood how prolactin in the median eminence can stimulate LH and FSH release. It is possible that prolactin directly stimulates LRF and FSH-RF release into the portal vessels, thereby increasing pituitary LH and FSH release. However, no evidence is available to support this theory. Another possibility is that prolactin implants, by decreasing pituitary content of prolactin, allows a greater number of pituitary cells to synthesize and release LH and FSH. This may occur because more blood containing the necessary precusors for gonadotropin synthesis reach the basophile cells, since less

blood needs to go to the inhibited prolactin cells. An additional possibility involves the ovarian hormones. A decrease in the amount of prolactin which reaches the ovary after prolactin implantation in the ME may result in decreased progesterone secretion. Removal of progesterone inhibition on pituitary LH and FSH could allow an increase in LH and FSH release. It is also possible that serum prolactin levels increase earlier than LH or FSH on the afternoon of proestrus (Gay, 1970), and that prolactin may help initiate the peak rise in serum LH and FSH observed at this time.

Results presented in this thesis show that human and ovine GH can inhibit rat GH secretion, apparently by decreasing hypothalamic GHRF content. Whether the secretion of pituitary hormones other than GH can be influenced by exogenous GH remains to be determined. Apparently exogenous rat GH does not influence prolactin secretion, nor does exogenous prolactin influence GH secretion (MacLeod et al., 1966). However human GH apparently has prolactin properties within its molecule, since it can elicit many responses typical of prolactin and not typical of rat GH, i.e., initiate lactation in rabbits. This is also demonstrated in the short loop feedback of human GH, when human GH inhibited prolactin release and mammary development. There is some evidence that prolactin in combination with GH may affect secretion of TSH. MacLeod (1967) showed that rats bearing pituitary tumors which secrete large

amounts of both prolactin and GH have depressed thyroid and TSH secretion. Actions of one anterior pituitary hormone on other pituitary hormones deserves further study.

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APPENDIX

CURRICULUM VITAE

Curriculum Vitae

NAME: Voogt, James Leonard

DATE OF BIRTH: February 8, 1944

PLACE OF BIRTH: Grand Rapids, Michigan, U.S.A.

NATIONALITY: American Sex: Male

MARITAL STATUS: Married

PRESENT ADDRESS: Department of Physiology
Michigan State University
East Lansing, Michigan 48823

FUTURE ADDRESS: Department of Physiology
University of California
San Francisco Medical Center
San Francisco, California 94101

EDUCATION:

Degree	Year	Institution	Major Field of Study
None	1962-1964	Calvin College	General
B.S.	1964-1966	Michigan Technological University	Biological Sci.
M.S.	1966-1968	Michigan State University	Physiology
Ph.D.	1968-1970	Michigan State University	Physiology

HONORS:

(a) NSF Undergraduate Trainee, 1966
(b) Elected member of Phi Kappa Phi Society, 1966
(c) Graduated with "Honor" from Michigan Technological University, 1966
(d) Elected associate member of Sigma Xi, 1968
(e) Elected full member of Sigma Xi, 1969
(f) Selected outstanding Graduate Student in Physiology, 1969
(g) Sigma Xi Graduate Student Award, 1970

POSITIONS HELD:

(a) Technician, The Upjohn Company, Summer of 1966.
(b) National Institutes of Health Trainee, Michigan State University, 1966-March-1969.
(c) National Institutes of Health Predoctoral Fellow, Michigan State University, April 1969-September 1970.
(d) Postdoctoral Fellow, University of California, October 1970.

TALKS PRESENTED AT SCIENTIFIC MEETINGS:

Meetings	Date	Topic
Annual Meeting of Michigan Acad. of Science, Grand Valley, Mich.	1968	Effect of various reproductive states on ACTH and corticosterone levels
53rd Meeting of Federation of American Societies for Experimental Biology	1969	Effect of subcutaneous injections or implants of prolactin into the median eminence on onset of puberty and gonadotropin release in immature female rats.
54th Meeting of Federation of American Societies for Experimental Biology	1970	Effects of implant of GH into median eminence of cycling female rats on pituitary GH and hypothalamic GRF content.
52nd Meeting of The Endocrine Society	1970	Serum prolactin, LH and FSH in female rats implanted with prolactin in median eminence.
Spring Meeting of Michigan Society for Experimental Biology and Medicine	1970	Recent studies on Hypothalamic Control of Prolactin, LH, FSH and GH.

RESEARCH PUBLICATIONS:

1. Chen, C. L., J. L. Voogt and J. Meites. (1968). Effect of median eminence implants of prolactin, LH and FSH on luteal function in the rat. Fed. Proc. 27:269.

2. Chen, C. L., J. L. Voogt and J. Meites. (1968). Effect of median eminence implants of LH, FSH and prolactin on luteal function in the rat. Endocrinology. 83:1273-1277.

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4. Voogt, J. L., M. Sar and J. Meites. (1969). Influence of cycling, pregnancy, labor and suckling on corticosterone-ACTH levels. Am. J. Physiology. 216:655-658.

5. Voogt, J. L., J. A. Clemens and W. D. Collings. (1969). Effect of subcutaneous injections or implants of prolactin into the median eminence on onset of puberty and gonadotropin release in immature female rats. Fed. Prod. 28:437.

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7. Clemens, J. A., H. Minaguchi, R. Storey, J. L. Voogt and J. Meites. (1969). Induction of precocious puberty in female rats by prolactin. Neuroendocrinology. 4:150-156.

8. Chen, C. L., Y. Amenomori, J. L. Voogt and J. Meites. (1969). Serum prolactin levels in rats bearing pituitary transplants. The Endocrine Society, 51st Meeting. P. 48.

9. Voogt, J. L., C. L. Chen and J. Meites. (1970). Serum and pituitary prolactin levels before, during and after puberty in female rats. Am. J. Physiology. 218:396-399.

10. Chen, C. L., Y. Amenomori, K. H. Lu, J. L. Voogt and J. Meites. (1970). Serum prolactin levels in rats with pituitary transplants or hypothalamic lesions. Neuroendocrinology. 6:220-225

11. Voogt, J. L., J. A. Clemens and C. K. Whitehair. (1970). Effects of implant of growth hormone into median eminence of cycling female rats on pituitary GH and hypothalamic GRF content. Fed. Proc. 29: 377.

12. Voogt, J. L. and J. Meites. (1970). Serum prolactin, LH and FSH in female rats implanted with prolactin in median eminence (ME). The Endocrine Society, 52nd Meeting. P. 124.

13. Voogt, J. L. and J. Meites. (1970). Effects of an implant of prolactin in median eminence of pseudo-pregnant rats on serum and pituitary LH, FSH and prolactin. Endocrinology. In press.

14. Voogt, J. L., J. A. Clemens, A. Negro-Vilar, C. Welsch and J. Meites. (1970). Pituitary GH and Hypothalamic GHRF after median eminence implantation of ovine and human GH. Endocrinology. In press.