ROLE OF THE HYPOTHALAMUS IN PITUITARY HORMONE FEEDBACK, MAMMARY CANCER, PUBERTY AND AGING IN THE RAT

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This is to certify that the

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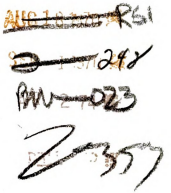

ABSTRACT

ROLE OF THE HYPOTHALAMUS IN PITUITARY
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by James Allen Clemens

1. Single stereotaxic implants of a prolactin-cocoa butter mixture were made in the median eminence of 10 mature and 6 ovariectomized female Sprague-Dawley rats. A similar number of control rats were implanted with cocoa butter alone. The animals were killed 6 days after implantation. The prolactin implanted rats showed marked mammary regression when compared with those of the control intact or control ovariectomized rats, nearly a 3 fold increase in hypothalamic content of prolactin inhibiting factor (PIF) in both intact and ovariectomized rats, and a 40% decrease in pituitary prolactin concentration in the intact rats. The ovaries of intact prolactin-implanted rats were characterized by many well developed follicles and few corpora lutea while the ovaries from the corresponding controls showed mainly well developed corpora lutea. The former showed regular cycling while the latter appeared to be pseudopregnant, as judged by daily vaginal smears. These

observations suggest that implanted prolactin inhibits pituitary prolactin synthesis and release by a direct feedback action on hypothalamic PIF, and this results in depressing pituitary prolactin concentration, mammary growth and luteal function.

2. Twenty-five lactating female Sprague-Dawley rats were divided into 3 groups on the 4th day postpartum, and were each implanted stereotaxically with 23 gauge glass tubing containing the following substances: group I, controls, cocoa butter; group II, mixture of prolactin and cocoa butter; group III, prolactin, ACTH and cocoa butter. All implanted tubes were fixed rigidly in place by means of dental cement and skull screws. On the day of implantation (day 0) litter size was reduced to 6 pups. On the 4th day after implantation, average weight gains for 6 pups were as follows: group I, 40.4±2.7g; group II, 18.8±3.5g; group III, 8.7±3.6g. Daily vaginal smears on all implanted rats showed that rats in group I remained in diestrus, whereas the rats in group II and group III came into estrus and began to cycle. These results demonstrate that prolactin implants into the median eminence can counteract the stimulating effects of suckling on prolactin release by the pituitary, thereby causing a significant impairment of lactation and regression of the corpora lutea of lactation. Combining

prolactin with ACTH resulted in an even greater impairment of lactation, by inhibiting pituitary secretion of these two most essential hormones for the lactation process.

3. Median eminence (ME) lesions were placed before and after carcinogen (DMBA) administration in female rats. Intact and ovariectomized Sprague-Dawley rats with ME lesions placed prior to treatment with DMBA, showed a 30 and 0% mammary tumor incidence, respectively, in contrast to a 95 and 54% mammary tumor incidence in the respective non-lesioned control groups. Intact and ovariectomized rats lesioned 75 days after DMBA treatment showed a 120 and 80% increase, respectively, in the number of palpable mammary tumors by 10 days after treatment. In contrast the non-lesioned intact and ovariectomized control groups showed a 19% increase and a 27% decrease in number of palpable mammary tumors, respectively. After 25 days, stimulation of mammary tumor growth was still marked in the intact-lesioned group but regression occurred in the ovariectomized-lesioned group. These results indicate that ME lesions placed before DMBA treatment inhibit mammary tumorigenesis, presumably by increasing prolactin secretion and stimulating mammary gland development, thereby rendering it refractory to the carcinogen. The dramatic increase in number of tumors and tumor growth when rats were lesioned after carcinogen administration,

is believed to be due primarily to stimulation of already formed tumors by increased prolactin secretion.

4. Administration of prolactin (NIH-P-B1 and NIH-P-S7) significantly advanced the onset of puberty in immature rats in 3 separate trials. Treatment of immature rats with anterior pituitary hormone impurities specified to be present as contaminants in the NIH prolactin preparations did not advance the onset of puberty. Median eminence implants of prolactin were found to hasten puberty by an average of 6.6 days, whereas subcutaneous implants containing the same amount of prolactin or median eminence implants of prolactin contaminants had no effect on puberty. The observed effect of prolactin on the advancement of puberty is believed to be mediated through the hypothalamus, probably as a result of increased pituitary FSH secretion.

5. Changes in anterior pituitary hormone secretion and hypothalamic releasing factor content in aged constant estrous rats were determined. Anterior pituitary halves incubated with hypothalamic extracts from aged constant estrous rats released significantly more FSH into the medium than the corresponding pituitary halves incubated with hypothalamic extract from young animals killed in estrous. This indicates that FSH-RF content of the hypothalamus increases in these old rats. Pituitary FSH

concentration was higher in old constant estrus than in young estrous rats. No change was observed in hypothalamic PIF content with aging; however, pituitary prolactin content was increased in old constant estrous rats. Pituitary LH concentration in old constant estrous rats was very low. These observations suggest that ovarian dysfunction in old rats may be the result of hypothalamic-induced changes in pituitary hormone secretion.

6. The effects of an antiandrogen (Cyproterone acetate), progesterone, epinephrine, cervical stimulation, reserpine and preoptic stimulation on the estrous cycle and on ovulation were determined in the old constant estrous rat. Treatment with progesterone, which is known to act centrally in facilitating ovulation, caused 6 out of 10 animals to ovulate. Epinephrine, which also can act centrally, produced ovulation in 11 out of 22 rats. In 3 out of 5 old rats, electrical stimulation of the preoptic hypothalamus evoked ovulation. Antiandrogen administration, cervical stimulation, reserpine treatment and thyroxine injections had no effect on ovulation. These results suggest that changes in brain function as a result of aging may be at least partially responsible for the decline of ovarian function in the old rat.

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Dedicated

to

My Wife

Clare

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INTRODUCTION

One of the milestones in the science of endocrinology was the finding that the pituitary gland, considered to be the master gland of the body, was controlled by the brain. This early work was only definitely established in recent years and laid the foundation for the modern science of neuroendocrinology.

Since the early studies, the hypothalamus has been shown to secrete regulatory neurohormones for each of the six anterior pituitary hormones. In the case of prolactin, the neurohormone inhibits release and synthesis of this hormone by the pituitary and is appropriately named prolactin inhibiting factor (PIF). In the case of all other anterior pituitary hormones, the hypothalamic neurohormones have been shown to elevate release and may also stimulate synthesis of these hormones--these have appropriately been termed "releasing factors."

The production of releasing factors seems to be partially dependent upon circulating levels of target organ hormones. If for some reason there is excessive production of a target organ hormone, the hormone may act upon the hypothalamus to decrease the production of the releasing

factor specific for its trophic hormone. Some target organ hormones may act directly on the pituitary. Recent evidence suggests that anterior pituitary hormones may also exert a feedback action directly upon the hypothalamus to regulate their own secretion. One of the objectives of this thesis was to investigate the possibilities of a hypothalamic feedback for prolactin and to determine to what extent the operation of this feedback could influence the prolactin dependent processes, mammary growth, lactation and pregnancy.

Even though the brain is the primary regulator of the endocrine system, no studies have yet been reported on its possible relationship to hormone dependent cancers. One such cancer is the chemically induced rat mammary cancer. Previous reports from our and other laboratories have demonstrated the importance of prolactin in mammary cancer development, and have strongly suggested that prolactin is a primary factor in development of mammary cancer. Since it has been well established that lesions in the median eminence area severly impair secretion of all anterior pituitary hormones except prolactin secretion, I decided to produce median eminence lesions in rats before and after carcinogen administration. With this type of lesion the only pituitary hormone secreted in significant quantities is prolactin. By this means we can see what

effect prolactin alone has on mammary cancer development and growth.

Another physiological process under brain control is the estrous cycle. Currently it is thought some neural event in the CNS initiates the estrous cycle at puberty; however, the mechanism responsible for initiation of cyclicity remains unknown and is currently the subject of much controversy. Even less is known about the mechanisms concerned with the loss of the estrous cycle and concomitant infertility in old female rats. Since many of the experimental treatments that cause precocious puberty also cause prolactin release it was of interest to determine the effects of exogenous prolactin on the onset of puberty. It also seemed possible that loss of cyclicity in aging animals may be due to changes in the brain and not to direct failure of the ovaries. Therefore, studies were also initiated in infertile, old rats to try to understand why reproductive functions were lost in these animals.

REVIEW OF LITERATURE

I. Anatomy of the Hypothalamus

Many of the early studies on the hypothalamus have been devoted to studying the configuration of some of its nuclear masses (Malone, 1910, 1914; Sutkowaja, 1928; Spiegel, 1928; Grunthal, 1929, 1930; Rioch, 1929; Morgan, 1930; Loo, 1931). All of these studies were performed using animals other than the rat. One of the first studies linking the pituitary gland with the hypothalamus was the discovery of the supraopticohypophysial tract by Greving (1928). Later, Krieg (1932) made a thorough study of the albino rat hypothalamus defining its nuclei and outlining its fiber tracts.

The nomenclature of the various hypothalamic nuclei has varied greatly from one species to another, but even within one and the same species various authors employ different terms. To alleviate the resulting confusion, Rioch, Wislocki and O'Leary (1940) have established a uniform terminology applicable to all mammalian species. All the following work in this thesis uses this terminology.

According to Zeman and Innes (1963) the hypothalamus can be divided into an anterior part containing a

few myelinated fibers and many unmyelinated fibers, represented by the tuber cinereum and the nuclei in the lateral walls of the third ventricle as well as the preoptic area. Included in this part of the hypothalamus are the infundibulum and the posterior lobe of the pituitary. In contrast, the posterior wall of the hypothalamus, comprising the mammillary complex contains a wealth of myelinated fiber systems. The poorly myelinated part of the hypothalamus appears to be the final common pathway for neural influences on the pituitary, whereas mammillary bodies apparently exert no direct control over this endocrine gland. This poorly myelinated part is termed the vegetative hypothalamus. An excellent stereotaxic atlas for the rat hypothalamus has been developed by de Groot (1959). This atlas shows the location of all nuclei and most of the fiber pathways in the hypothalamus and was the atlas used in these studies.

II. Physiology of the Hypothalamo-Hypophyseal System

A. Anatomical connections of the anterior pituitary and their functional significance

The anterior pituitary is connected to the hypothalamus by means of portal blood vessels which originate in the capillary beds in the median eminence and upper infundibular stem. Popa and Fielding (1930a,b) described portal vessels running along the pituitary stalk and

connecting it with nervous tissue of the hypothalamus. They concluded from their studies that blood flowed from the pituitary gland upward to the hypothalamus. These studies led to much work on the blood supply to the pituitary, and it soon became apparent that the blood in the portal vessels of the pituitary stalk did not flow from the gland to the brain, but in the opposite direction. Wislocki and King (1936) came to the conclusion on morphological grounds alone, that the direction of blood flow must be from the capillary bed to the pars distalis. The direction of blood flow along the portal vessels of the stalk was observed in living animals first by Houssay et al. (1935) and later by Green (1947). However, as pointed out by Flerko (1966), the pituitary circulation is not as simple as revealed in most textbooks and monographs on neuroendocrinology.

On the basis of observations by Torok (1954, 1962, 1964), Szentagothai et al. (1957) and Duvernoy (1960), a backflow of blood from the dense vascular plexus between the median eminence and pars tuberalis toward the hypothalamus has been postulated. The pars tuberalis contains significant amounts of anterior pituitary hormones and shows clear signs of secretory activity. Blood could bring pituitary hormones from here to the hypothalamus. In addition Torok (1954) has described veins on the posterior

surface of the pars distalis which have an upward direction of flow. These observations provide the anatomical basis for a direct feedback of anterior pituitary hormones on the hypothalamus.

B. Hypothalamic releasing and inhibiting factors

There are many indirect lines of evidence for hypothalamic releasing and inhibiting factors, which have been reviewed scores of times. Here I will present only direct evidence, obtained by the use of hypothalamic extracts, for releasing and inhibiting factors concerned with this thesis.

Definitive evidence for the existence of a prolactin inhibiting factor (PIF) in acid extracts of hypothalamus was presented by Meites and his co-workers (Meites et al., 1961, 1962; Talwalker et al., 1963). Acid extracts of beef, sheep and pig hypothalami were also shown to have PIF activity (Talwalker et al., 1963; Shally et al., 1965).

Convincing in vitro evidence for the existence of a growth hormone releasing factor (GRF) was first presented by Deuben and Meites (1963) and later confirmed (Deuben and Meites, 1964; Shally et al., 1965).

Igarashi and McCann (1964) presented evidence for the presence of a follicle-stimulating hormone-releasing factor (FSH-RF) in rat hypothalamus using the HCG augmented

mouse uterine weight assay which later proved to be invalid as a specific bioassay. Therefore, the first direct, valid, demonstration of an FSH-RF was by Mittler and Meites (1964). A luteinizing hormone releasing factor (LRF) was probably first demonstrated by McCann et al. (1960) by using intravenous infusions of acid extracts of rat median eminence tissue. These results were confirmed later by Courrier et al. (1961). They found that hypothalamic extracts from both rats and sheep can induce release of LH from the pituitary.

C. Hormone feedback

1. Conventional type-target organ hormone feedback

a. Gonadotrophin-sex steroid

The conventional feedback is that of target organ hormones exerting a negative feedback on the hypothalamus and/or pituitary gland to inhibit secretion of their trophic hormones.

Estrogen may inhibit FSH release by a direct action at the pituitary level. Rose and Nelson (1957) perfused estrogen into the hypophyseal fossa and inhibited the castration reaction. Bogdanove (1963) concluded that there could be a direct inhibitory effect of estrogen on the pituitary. However, these experiments do not demonstrate unequivocally the existance of such a direct

inhibitory action. Most experimental evidence supports the view that gonadal steroids feed back on the hypothalamus to reduce gonadotrophin secretion.

Flerko (1957) demonstrated the importance of the anterior hypothalamus as a site of estrogen feedback. He inhibited the effects of estrogen on the castration induced rise of FSH in rats with anterior hypothalamic lesions. Flerko and Szentagothai (1957) showed that estrogen released from small fragments of ovarian tissue autotransplanted into the anterior hypothalamus inhibits FSH secretion. Similar ovarian implants into the anterior pituitary or liver tissue grafts into the anterior hypothalamus did not produce this effect. Hohlweg and Daume (1959) found that injections of estrogen into the anterior hypothalamus of rats has an anti-FSH effect 125 times that of estrogen administered subcutaneously.

The above data support the hypothesis that nervous elements located in the anterior hypothalamus play some role in the feedback mechanism by which FSH release is inhibited by a slight elevation in blood sex steroid level. Meites et al. (1966) reported that gonadal steroids can alter hypothalamic FSH-RF and LH-RF, and indicate that this is the major pathway by which gonadal steroids exert their negative feedback on anterior pituitary release of gonadotrophins.

There is much data which indicate that neural structures in the medial basal hypothalamus are affected by sex steroids in a negative feedback fashion. Davidson and Sawyer (1961) implanted estradiol benzoate into various regions of the brain in the female rabbit. Failure of copulation-induced ovulation and ovarian atrophy followed implants in the basal tuberal area near the posterior median eminence. Implants in the anterior pituitary and mammillary bodies had no effect. These findings were extended by Kanematsu and Sawyer (1963). Kanematsu and Sawyer found also that these implants caused decreased amounts of LH in the plasma of ovariectomized rabbits. Similarly Ramirez et al. (1964) found that implants of estradiol in the median eminence prevented the rise in plasma LH which occurs after ovariectomy in rats.

Lisk (1960) implanted estradiol benzoate into the hypothalamus in male and female rats. The sensitive area, in which implants caused atrophy of male and female tracts and gonads included the arcuate nucleus, surrounding ventral hypothalamus and mammillary bodies. Lisk (1963) found that estradiol implants into the arcuate nucleus of spayed rats prevented the characteristic changes in pituitary cytology which occur following gonadectomy. Implants in the mammillary bodies and anterior lobe of the pituitary did not alter the castration reaction. Control substances

also were ineffective. Lisk and Newlon (1963) found that estradiol implants in the arcuate nucleus caused decreased size of nucleoli in the cell bodies accompanied by atrophy of ovaries, uteri, testes, etc.

Lisk (1962) implanted testosterone into brains of male and female rats. Atrophy of ovaries, uteri, testes, seminal vesicles and prostates was obtained 30 days later from implants into the median eminence and other parts of the basal tuberal region. Davidson and Sawyer (1961) implanted testosterone propionate into the brains of male dogs. Testis atrophy resulted from implants in the posterior median eminence and posterior tuberal regions.

These findings conflict sharply with those of Bogdanove (1963) who found that ovarian or estradiol dipropionate implants were effective directly on the pituitary. Such implants produced localized regression of castration cells in ovariectomized female rats. In addition, Ramirez et al. (1964) found that estradiol implants in the anterior pituitaries of female rats prevented the rise in plasma LH after ovariectomy. Kanematsu and Sawyer (1964), in contrast to some of their earlier work reported an increase in plasma LH following estradiol implants in the anterior pituitaries of ovariectomized rabbits.

b. Corticotrophin-adrenal-cortical steroids

The dilemma of site of action of adrenal steroids in the feedback control mechanism of pituitary ACTH secretion is perhaps as inconclusive as that of the sex steroids just mentioned. A major function of circulating adrenal steroids is the feedback control of pituitary ACTH secretion, and there is strong evidence that this action of the corticoids is exerted at least in part indirectly via the hypothalamus. In a careful study on dogs Eik-Nes and Brizzee (1965) discovered that intravenously injected radioactive cortisol was rapidly concentrated in and slowly released from hypothalamic nuclei; only small amounts of the steroid were taken up by the pituitary gland or cerebral cortex.

The question of the site of action in the feedback control mechanism has also been investigated extensively with micro-injections or implantations of the steroids directly into the hypothalamus and pituitary gland. Endroczi, Lissak and Tekeres (1961) reported that intrahypothalamic and mid-brain implants of cortisone in agar depressed pituitary-adrenal activity in rats and that similarly directed injections of soluble cortisone in the cat exerted the same influence. Although the intrahypothalamic cortisone might have reached pituitary cells to exert a direct action it seemed unlikely that the mid-brain

injections could have done so. Similar interpretations were later made by Corbin et al. (1965) on their finding that dexamethasone implanted into the mid-brain lateral reticular formation lowered adrenal and plasma corticosterone levels in rats. Dexamethasone injections directly into the rat pituitary were no more effective in suppressing adrenal venous corticosterone secretion than were equivalent dosages given subcutaneously (Kendall, 1962).

Davidson and Feldman (1963) found that median eminence implants of cortisol were highly effective, and mid-brain implants partly effective, in blocking compensatory adrenal hypertrophy after unilateral adrenalectomy. Chowers et al. (1963) observed that cortisol implants into the rat median eminence prevented depletion of adrenal ascorbic acid following the stress of unilateral adrenalectomy. In Slusher's (1966) experiments implants of cortisol into the rat hippo-campus or mid-brain inhibited the diurnal changes in adrenal and plasma corticosteroids, but only implants into the median eminence depressed adrenal steroid levels and induced adrenal atrophy. Atrophy of the adrenals has also been observed following median eminence implants of cortisol by Davidson and Feldman (1963) and Chowers et al. (1963). In the larger hypothalamus of the rabbit, Smelik and Sawyer (1962) noted that implants of cortisol into the

anterior median eminence and postoptic area blocked the stress-induced rise in adrenal steroids without causing adrenal atrophy.

If the negative feedback action of the corticoids is exerted primarily at the hypothalamic level one would expect a rise in hypothalamic corticotrophin releasing factor (CRF) following adrenalectomy. Such was found to be the case by Vernikos-Danellis (1965). However, DeWeid (1964) has found that pretreatment with dexamethasone prevents the ACTH releasing effect of hypothalamic extracts containing CRF; probably the steroids exert both direct and indirect effects on the pituitary. Support for this view is provided by experiments by Chowers et al. (1967) in which they found that both median eminence and pituitary implants of dexamethasone reduced pituitary ACTH release, while only median eminence dexamethasone implants decreased CRF content.

In view of these different observations it does not seem unreasonable to propose three levels of feedback for the adrenal corticosteroids. Corticosteroids may act on structures of the limbic system to modify diurnal rhythms; they may act on the hypothalamus to decrease base levels of CRF, and they may act directly on the pituitary to decrease ACTH.

c. Special case of prolactin and growth hormone

The question that immediately poses itself is how are prolactin and growth hormone regulated by a feedback control when there are no target organ hormones to exert a feedback? It would appear that the answer to this is that prolactin and growth hormone exert a feedback action upon the hypothalamus to control their own production, however this is an instance of hypothalamic feedback which will be discussed in the next section. Although there are no target organ hormones for prolactin there exist numerous hormones and pharmacological agents which stimulate prolactin secretion (see Meites and Nicoll, 1965, 1966).

2. Feedback of pituitary hormones on the hypothalamus

a. Corticotrophin-ACTH

There is considerable evidence that ACTH can exert a feedback influence on its own secretion by acting on the hypothalamus. The existence of this hypothalamic feedback mechanism is suggested by the observation that treatment of adrenalectomized animals with exogeneous ACTH may reduce pituitary weight (Eriksson, 1959; Gemzell and Heijkenskjold, 1957) and block the fall of pituitary corticotrophin usually induced by stress (Kitay et al., 1959; Stark et al., 1963). Furthermore, in adrenalectomized rats submitted to stressful stimuli, a greater increase in blood ACTH occurs

when the initial blood levels of the hormone are low than when they are high (Hodges and Vernikos, 1958, 1959). More direct evidence for hypothalamic feedback is provided by Halasz and Szentagothai (1960) who implanted anterior pituitary tissue in the infundibular recess of the third ventricle of the rat and found a depressing effect of such implants on adrenal function. Motta et al. (1965) made stereotaxic implantations of solid ACTH into several areas of the brain and into the pituitary of normal male rats. In these studies, ACTH implants into the median eminence were effective in significantly depressing blood corticosterone levels. A significant decrease in pituitary weight occurred, but there was no change in adrenal weight. Implants of ACTH elsewhere were completely ineffective. Legori et al. (1965) have shown that implants of ACTH in adrenalectomized rats significantly reduce pituitary ACTH content.

The work of Torok (1954, 1964) showing the existence of a two directional blood flow in the hypophyseal portal system from and to the hypothalamus, has demonstrated one anatomical route by which pituitary hormones might pass from the pituitary to the hypothalamus.

b. Gonadotrophins--FSH and LH

A hypothalamic feedback in gonadotrophic functions has independently been suggested by Sawyer and Kawakami

(1961) based on indirect evidence. They noted EEG changes associated with the behavorial after-reaction following coitus. At first it was thought that the EEG after-reaction was in fact a correlate of the nervous activation of the hypophysis (Sawyer and Kawakami, 1959). It soon became apparent, however, that the time of these events was too much delayed for such a relationship. Therefore, it seemed more probable that the EEG after-reaction was due to the discharge process of the pituitary hormones, or perhaps a direct feedback action of the released hormones on the nervous system. Since the reaction also occurred in spayed rabbits it was evident that ovarian steroids could not be concerned with the feedback (Sawyer and Kawakami, 1959a). Attempts were made to induce the spontaneous EEG after-reaction in the absence of vaginal stimulation or coitus (Kawakami and Sawyer, 1959b). This was successful with purified preparation of pituitary LH, chorionic gonadotrophin (HCG) and pregnant mare's serum (PMS) and also with prolactin and neurohypophyseal hormones. Negative results were obtained with other hormones. It is quite interesting to note that all of the pituitary hormones which produced an EEG after-reaction are released in response to coitus in the rabbit. Thus, it is evident that the pituitary hormones, released after coitus, exert a feedback action on the hypothalamus. Recent evidence by

Corbin and Cohen (1966a, 1966b) and David, et al. (1966), lend the concept of short loop feedback strong support by showing that median eminence implants of tubes containing LH in a cholesterol or cocoa butter vehicle depress the LH content of rat pituitary and plasma. Recently Corbin and Story (1967) have demonstrated a hypothalamic feedback for FSH by using the above mentioned implant technique, and for the first time have shown that a pituitary hormone can decrease production of its own releasing factor by acting directly on the hypothalamus.

c. Prolactin and growth hormone

At the time research work was begun on this thesis there was no direct evidence for a hypothalamic feedback for prolactin, except for the hypothesis of Meites and Sigouris (1953) that prolactin injections decrease pituitary prolactin content. Recently indirect evidence for a hypothalamic feedback influence by prolactin and growth hormone was provided by MacLeod, et al. (1966). In these studies they found that subcutaneous transplants into intact rats of $MtTW_5$ pituitary tumors secreting large amounts of prolactin and growth hormone result in reduced pituitary weight and decrease the prolactin and growth hormone content of the animal's own pituitary. These observations have recently been confirmed by MacLeod, et al. (1968), who in addition found a decrease in

pituitary TSH content. The initial findings of MacLeod regarding pituitary prolactin content were confirmed using a more quantitative technique by Chen et al. (1967) and extended to include for the first time evidence that suggests prolactin secreted from the $WtTW_5$ tumor increases hypothalamic PIF content by a hypothalamic feedback action.

The first definitive evidence for a hypothalamic feedback action for prolactin was presented in the observations of Clemens and Meites (1967, 1968). Their results demonstrate that direct implantation into the median eminence of tubes containing prolactin in a cocoa butter carrier increase hypothalamic PIF content and reduce pituitary prolactin secretion. In addition they observed mammary gland regression and loss of luteal function in prolactin-implanted rats. These observations can be regarded as the most definite proof for the hypothalamic feedback and its possible physiological significance, because target organ responses were observed which showed for the first time that the lowered pituitary hormone levels meant decreased hormone release into the systemic circulation.

In further pursuit of the hypothesis of a hypothalamic feedback for prolactin, Clemens et al. (1968) found that median eminence implants of prolactin inhibit postpartum lactation in rats. In addition they found

that when ACTH was added to the prolactin implant there was a greater inhibition of lactation noted than with the prolactin alone.

The only published evidence regarding a hypothalamic feedback for growth hormone is the abstract by Katz et al. (1967) indicating that median eminence implants of growth hormone in a cholesterol pellet reduce pituitary growth hormone content.

III. Experimental Production of Mammary Tumors in Rats

A. Methods of induction

Mammary tumors may be induced readily in a high percentage of animals by a number of means. The mammary tissue of the rat appears to be highly susceptible to malignant changes following treatment with aminofluorene compounds, benzpyrine, 3-methylcholanthrene (3-MCA) and 7, 12-dimethylbenz (a) anthracene (DMBA). Noble and Cutts (1959) presented an extensive review of mammary tumors of the rat and methods of tumor induction. The two most widely used carcinogens are 3-MCA and DMBA. The induction of mammary tumors by these carcinogens apparently require both anterior pituitary hormones and ovarian hormones. Thus, rats ovariectomized or hypophysectomized prior to carcinogen administration develop few or no mammary tumors (Dao, 1964).

B. Hormonal dependence

The carcinogen induced mammary tumors are generally adenocarcinomas which are sensitive to hormone manipulation. The hormonal dependency of mammary tumors in rats was described by Noble and Collip (1941) after treatment with estrogen pellets. The adenocarcinomas rapidly regressed in the rats when the estrogen pellet was surgically removed.

Most of the studies demonstrating the hormone dependency of rat mammary adenocarcinomas used ablation of certain endocrine organs. In some cases endocrine organs are removed before carcinogen administration in order to ascertain endocrine influences on tumor induction, and in other cases endocrine organs are ablated some period of time after carcinogen administration to ascertain effects on tumor growth. In other experiments, hormones are administered either before or after appearance of the mammary tumors.

Tumor induction and growth were studied in ovariectomized and in hypophysectomized Sprague-Dawley rats by Huggins and Briziarelli (1959) and Huggins et al. (1959). Their findings indicate that ovariectomy delays the onset and inhibits tumor growth, while hypophysectomy completely inhibits the formation of tumors. Hypophysectomy after tumor formation caused regression of tumors. The effects

of ovariectomy were largely mediated through estrogen, since administration of 0.1-1.0 ug estradiol per day completely nullified the effects of ovariectomy on tumor formation and growth.

According to the findings of Dao (1962) ovariectomy sufficiently in advance of carcinogen administration (30 days) completely inhibits tumor formation. Dao concluded from his study that the presence of estrogen is necessary for the formation of mammary tumors. This conclusion may be premature, since it is entirely possible that the observed inhibition of tumor incidence may be due to lack of prolactin. Estrogen has been shown to maintain or stimulate growth or mammary tumors only in the presence of the pituitary (Sterental et al., 1963). In an excellent study Talwalker et al. (1964) have shown that even in the absence of the ovaries treatment with the pituitary hormones, prolactin and growth hormone, can induce mammary tumors in rats given a single feeding of DMBA. This study suggests that the pituitary hormones, prolactin and growth hormone, are of primary importance in mammary carcinogenesis in rats.

IV. Neural Control of Puberty in Female Rats

A. General review

There is agreement that increased pituitary gonadotrophin secretion is responsible for the onset of

puberty (Donovan and Van der Werff ten Bosch, 1965; Van der Werff ten Bosch, 1966; Critchlow, 1967). The majority of recent investigations concerning control of puberty are centered around "trigger" mechanisms responsible for this surge in gonadotrophin secretion before puberty.

Puberty occurs in female Sprague-Dawley rats at approximately 38 days of age. In the young rat the ovaries have been shown to be refractory to gonadotrophin stimulation until about the twenty-second day (Leonard and Smith, 1934; Gaarenstroom et al., 1960; McCormack and Meyer, 1964). In order to obtain an understanding of the neural mechanisms, investigators have tried to hasten or delay puberty by various manipulations. These studies will be reviewed in the ensuing sections.

B. Experimental production of precocious puberty

Thirty-six years ago Hohlweg and Junkmann (1932) ingeniously arrived at the conclusion that a neural sex center was involved in the control of puberty and reproductive cycles. Unfortunately this brilliant theory was almost totally obscured by the popular theory of Moore and Price (1932) which considered pituitary-gonadal interactions sufficient to explain phenomena associated with reproductive functions. It was 20 years later that Harris and Jacobsohn (1952) demonstrated that the immature rat

hypophyses transplanted under the median eminence of hypophysectomized adult female rats restored vaginal cyclicity and ovulation. This study furnished the major stimulus for once again considering the nervous system in relation to puberty, and demonstrated the passive role of the pituitary in the initiation of puberty. This study also suggested that the portion of the prepubertal period which follows the attainment of gonadal responsitivity to gonadotrophins may result from inhibition or inactivity of hypothalamic mechanisms regulating pituitary-gonad functions in the adult. Donovan and Van der Werff ten Bosch (1956, 1959) found that electrolytic lesions in the anterior hypothalamus of rats resulted in precocious puberty as ascertained by vaginal opening and ovulation. These studies supported the concept of neural inhibition of gonadotrophin secretion in the prepubertal animal and have been confirmed (Bogdanove and Schoen, 1959; Elwers and Critchlow, 1960; Horowitz and Van der Werff ten Bosch, 1962; Schiavi, 1964).

Attempts to localize and identify the hypothalamic structures involved in the control of puberty have not been fruitful. In addition to anterior hypothalamic lesions advancing puberty Schiavi (1964) found that posteriorly placed electrolytic lesions were equally effective. In contrast to the above findings Gellert and Ganong (1960)

found posterior tuberal, not anterior hypothalamic destruction, effective in advancing puberty.

In addition to hypothalamic mechanisms controlling sexual development, experimental evidence by Elwers and Critchlow (1960) demonstrated that lesions placed in the amygdaloid complex advanced puberty. Other treatments which are probably mediated through the central nervous system and hasten onset of puberty are mild stress (Morton et al. 1963) and electrical stimulation of the uterus (Swingle et al. 1951).

C. Experimental delay of puberty

Whereas the treatments described above resulted in acceleration of sexual development in female rats, destruction localized to the region of the ventromedial and arcuate nuclei prevented or delayed puberty in rabbits (Bustamante, 1942; Bustamante et al., 1942). A similar delay in sexual maturation was recently reported in conjunction with electrolytic lesions placed in the region of the ventromedial-mamillary body area of female rats (Corbin and Schottelius, 1960, 1961). These observations were interpreted as evidence for the presence of a gonadotrophin-stimulating area in this part of the brain. Recently Bar-Sela and Critchlow (1966) reported that stimulation of the amygdaloid nuclear complex significantly delayed puberty in female rats.

D. Possible neural mechanism involved in the onset of puberty

The original idea of Hohlweg and Junkmann (1932), that neural structures are involved in control of reproductive functions are in accord with present day concepts. Thus Donovan and Van der Werff ten Bosch (1959), while considering the effects of neural lesions, suggested that the main difference between the immature and adult animal is the extreme sensitivity of the brain of the former to gonadal hormones. Similarly, Ramirez and McCann (1963) and McCann and Ramirez (1964), on the basis of differences in the supressive effects of estrogen and androgen on pituitary and plasma LH levels in gonadectomized rats, suggested that the onset of puberty results from resetting of a hypothalamic "gonadostat." Because of this proposed change in threshold, circulating levels of gonadal hormones which are effective in restraining gonadotrophin secretion in the immature animal are rendered ineffective at puberty.

Thus, the current concept is that gonadotrophin-regulating mechanisms in the CNS of the immature animal are markedly more sensitive to the negative feedback influences of the sex steroids, and that a decrease in sensitivity may be responsible for the onset of puberty.

V. Loss of Reproductive Cycling Activity With Aging

A. Age changes in the pituitary

The first detailed study of the senile decline of the human pituitary gland was made by Cooper (1925). She studied the pituitary from fetal life to old age and pointed out that in the early stages of life the pituitary was oval in sagittal section but in old age it became spherical. The cleft between the anterior pituitary and posterior that could be seen in fetal life became much smaller after puberty, and in early adult life it disappeared from view. She also noted that the maximum vascularity in the gland occurred at puberty and that this vascularity persisted until the fifth or sixth decade; after that period vascularity became reduced, at least in the anterior lobe. In the case of the posterior pituitary, without such a rich blood supply, there was no apparent change with increasing age.

At birth the epithelial cells of the anterior lobe were well separated and as age advanced they became more densely packed. According to Cooper, the acidophil cells were the first ones to be differentiated, and she found that they were present in the third month of intra-uterine life. She found that they rapidly increased in childhood until puberty. The basophilic cells, she said, differentiated a little later and occurred in the greatest number

in old subjects, and it is possible that this correlates with the increased gonadotropin secretion in older people. The chromophobes did not appear until about 7 months. She also noted that Herring bodies were absent in the fetus but were present in the second year of age and that they increased steadily in number and size until puberty they ceased to increase any further.

Weiss and Jansing (1953) made an electron microscopic study of the aging pituitary gland. They used Swiss albino mice and found that in the pituitary of normal young animals the chromophobes, acidophils, and basophils contained granules, double membrane systems and mitochondria in varying amounts, and that the nuclear membrane appeared to be double. With the onset of aging, however, the nuclear membrane was found to become irregular in outline, the mitochondria enlarged and became vacuolated and the double membrane system became fragmented. Thus, it is clear that there are fundamental cytophysiological changes taking place in the pituitary cells with the onset of aging.

Spagnoli and Charipper (1955) found that in old male golden hamster pituitaries, there was an increase in degranulation and an occurrence of pyknotic nuclei and vacuolated basophils. Chromophobic and colloid-filled acini appeared among the cell cords in both males and

females and these increased slightly in number with increasing age. Chromophobic networks appeared in the glands of the animals over 12 months of age. There was a decrease in the number of acidophils in older males and an increase in basophils in older females. There seemed also to be some increase in the density, and some disorganization of the reticular framework.

Assays of pituitary hormones reflect the functioning of the aging gland. In the human, Becker and Albert (1965) found urinary excretion of FSH increased 11 times and LH increased 9 times indicating high production of these hormones in post-menopausal women. In the rat evidence concerning FSH and LH levels is scanty and very unconvincing. Lausen et al. (1939) studied pituitaries of rats of different ages and measured gonadotrophic activity by the uterine assay. Throughout sexual life the activity remained at the puberal level, however in three females 2.5 years of age a remarkable increase in total gonadotrophic content was found. Similar assays were carried out by Saxton and Greene (1939), Solomon and Shock (1950), Duncan et al. (1952), Blumenthal (1955) and Ceresa and Lacroix (1951) and all concluded that there were no differences between the content of gonadotrophin of the hypophyses of young, mature and old animals. This view was adopted by Korenchevsky (1961), Hollandbeck et al.

(1956), and Jones and Krohn, (1959). Evidence to the contrary will be presented in this thesis.

Meites et al. (1961) presented evidence indicating that as rats become older the pituitary prolactin content increases and Aschheim and Pasteels (1963) have indirect and scanty evidence that prolactin secretion may be increased in old, senile constant estrous rats. No significant differences have been reported in STH, TSH and ACTH content of pituitaries from young and old animals.

B. Age changes in the ovary

Since it appears that the decline of ovarian function is of a different etiology in humans than in other mammals, age changes in human ovaries will be reviewed separately.

1. Human ovary

In women the decline of fertility and of endocrine ovarian function are associated with a decrease in number of follicles, which has led to the assumption that menopause occurs when the last oocyte is spent (Engle, 1951, 1955; Zuckerman 1956). This view is contradicted by post-menopausal ovaries which still contain follicles and probably produce estrogen as well. Yet such cases may not be incompatible with the primary role of follicular depletion in ovarian senesence (Comfort 1956). The

menopause marks only a relative failure in ovarian function, the decline of which had started long before this point is reached.

In addition to menopause there are other events that accompany the gradual downhill slope of ovarian involution. An early symptom is the occurrence of anovulatory cycles, i.e. the failure of ovulation and corpus luteum formation. Anovulatory cycles may, of course, occur at all ages but their frequency is highest in young adolescent and near-climacteric women. At both age levels, moreover, even when corpora lutea are formed, they may be less stable functionally than those formed between the ages of 25 and 40, as was demonstrated by Collett, Wertenberger and Fiske (1954), using basal temperature curves. It appears that both the period during which ovarian function gets into its stride, and the years when it is slowly regressing, are characterized by deficient progestational activity. Follicular growth, however, and the concomitant production of estrogens are manifest over a much longer period, from before puberty to long after the menopause. The urinary excretion of estrogens has been shown to decrease gradually and does not reach its lowest level until about the age of 60 (Pincus, 1955). Some estrogens are still excreted, although the site of their elaboration is disputed. Vaginal smears may also

demonstrate this estrogenic activity (Berger and Keller, 1954) which has been claimed to be cyclic (Bourg and Pundel, 1951).

Continued estrogenic activity during the climacteric, unopposed by progesterone secretion, has been related to the frequency, about this age, of endomentrial hyperplasia (Novack and Richardson, 1941). It is of considerable interest that even after 60 such hyperplasia is not uncommon. This again raises the question, as did the continued urinary estrogen excretion, whether at this age these hormones can still be ascribed to the ovaries. Except in cases of exogenous hormone influences of hormone-producing ovarian tumors, most authors tend to ascribe late estrogenic activity to the adrenal gland, which has even been called "the gonad of the aged" (Hamblen, 1945). Although the adrenals may be the main source of sex hormones in senile women, the ovaries should not be ruled out as a contributory factor. Besides follicles which may be found up to 6 years after the menopause, patches of ovarian stromal hyperplasia (Hertig, 1944) and hilus cells have been suggested as possible sources of estrogens in senile ovaries. In this connection the work of Paulsen et al. (1955) deserves attention. These authors found that, in post-menopausal women, the administration of gonadotropic hormones could lead to an increase in urinary estrogen

excretion, while ovariectomy could be followed by a decrease. A recent study (Becker and Albert, 1965) has shown that compared to normal men and women, in post-menopausal women FSH and LH secretions were increased 11 and 9 fold respectively due to the decreased feedback from the ovary.

2. Other mammals

Information on ovarian changes in old animals is scarce, but it is well known that fertility decreases with increasing age (Ekstein, 1955; Engle, 1944). The general impression is that ovarian involution in most animals takes a slower course, relative to the life span, than in women (Ekstein, 1955) and that consequently estrogenic activity and follicular growth still occur in many senile animals, even in monkeys (Engle, 1944; Krohn, 1955). Most available data are concerned with laboratory or domesticated animals. More is known about mice and rats than about any other species. The literature indicates that in mice fertility declines with increasing age (Bittner, 1935; Murray, 1934) but follicular growth and ovulation may still occur in senile animals, although the number of ovulated oocytes is low and most of these are abnormal. Vaginal cycles may also continue up to extreme ages but there are quantitative strain differences in this respect. Cycles may become irregular with increasing diestrous intervals which merge

into a final anestrous condition, while in other cases periods of continuous estrogenic stimulation intervene (Thung et al. 1956). It has been suggested that in rats and mice the decline in ovarian function is secondary to hypophyseal failure, while in man ovarian involution precedes the senile changes of other organs (Krohn, 1955).

The unimpaired sensitivity of old mouse ovaries to hypophyseal hormones was demonstrated by Zondek and Aschheim (1927). Similar experiments (Hoffman, 1931; Romeris, 1931; Zondek, 1929) reported reactivation of the ovaries of old mice by hypophyseal implants or extracts. Similar results have been reported for rats (Engelhart and Hauser, 1937; Weisner, 1932). The above results would seem to indicate that there may be changes in the hypophysis with age or more fundamentally changes in the central nervous system. These results suggest that the effects produced by pituitary implants were due to the residual amount of gonadotropin remaining in the pituitary, and that the effects of aging on the ovaries in rats and mice are initiated by fundamental changes in the hypothalamus or higher centers, possibly the limbic forebrain system. This center has been shown to be intimately connected with activity of the hypothalamus (Nauta, 1963).

C. Changes in the estrous cycle with age

Estrous cycles in aging rats exhibit several different patterns. Studies by Aschheim (1961) and Mandl (1961) demonstrate 3 different patterns of cycling in senile rats. The most prevalent pattern was repeated periods of pseudo-pregnancy evidenced by prolonged periods of diestrus and ability to produce a decidual reaction. The second most frequent pattern was manifested by persistent vaginal cornification. This constant estrous condition lasted for many months, usually uninterrupted. Finally the third pattern was characterized by total irregularity in the pattern of cycles; no regular, cyclic sequence of events could be established. Aschheim, (1965) suggested that it was not the ovary that failed in aging but possibly the hypothalamus or the portal circulation, since LH injections into old constant estrous rats caused regular estrous cycles.

MATERIALS AND METHODS

I. Animals

Mature and immature intact female Sprague-Dawley rats from Spartan Animal Farms (Haslett, Michigan) and Holtzman Company (Madison, Wisconsin) were used in all experiments. The animals were housed in a temperature controlled (75±1°F) and light controlled (14 hr/day) room. The animals were maintained on a diet of Wayne Lab Blox (Allied Mills, Chicago, Illinois) and tap water. Female hypophysectomized rats, 21 days old, were obtained from Hormone Assay Labs., Chicago, Illinois, for the puberty study. White King Squabs (Cascade Squab Farm, Grand Rapids, Michigan) 4 to 8 weeks old, were used for prolactin assays.

II. Stereotaxic Technique

The stereotaxic instrument used in these studies was the Stoelting, Stellar (Fig. 1) model, obtained from C. H. Stoelting Company, Chicago, Illinois. The stereotaxic instrument is constructed in such a manner that any given point in the brain may be located with reference to the three cartesian planes in space. The animal's head is so held in the instrument that these three planes are relatively constant from animal to animal.

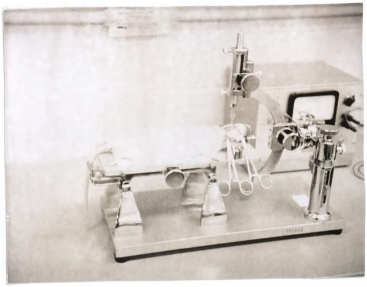

Fig. 1. Stoelting, Stellar model, stereotaxic instrument used in implant studies.

The three planes are called the horizontal, coronal and sagittal. The horizontal planes are parallel to that plane which passes through the inferior orbital margins and the centers of the external auditory meatus. The coronal planes are perpendicular to the horizontal planes, the principal one of which corresponds to a line connecting the centers of the two external auditory meatuses. The sagittal planes are perpendicular to both the horizontal and coronal planes and parallel to the mid-sagittal plane to the animals head, i.e., corresponding to the interhemispheric fissure.

Rats used in these studies were adult female rats of the Sprague-Dawley strain (Holtzman Co., Madison, Wisc.) weighing about 200 grams. Rats of this size have a fairly constant head shape and size. At about 200 grams there is little change in the brain or head growth; since mature albino rats were used, modifications were not necessary.

The best anesthetic for all types of work turned out to be ether. It was difficult to establish a standard dose of pentobarbital. Some rats were much more sensitive to it than others. Anesthesia was regarded as sufficiently deep when the animal just failed to respond to a paw pinch. The top and the sides of the head were shaved, and the head was washed with 70% ethanol. The ear plugs were then inserted.

Rats' ears are usually clean and the ear plugs are pointed, so that the necessity of cleaning the ears is

probably not as great as in other animals. The ears must be inspected for abnormalities, however, so as to prevent any unnecessary error. Cold sterilization of the ear plugs in a cold non-corrosive germicidal solution was carried out periodically. The ear plug on one side is inserted into the rat's ear and seated against the side of the rat's skull. The head is then held firmly against this plug while the other is placed in the opposite canal. The ear plugs are then placed into the ear bars (Fig. 2) and are secured by tightening the set-screws. The head is observed to assure that a line joining the two eyes is parallel to the ear bars which hold the two plugs. If the lines are not parallel the rat is not in the instrument properly and, the entire procedure must be repeated.

Great care must be taken in placing the ear plugs in the rat's ears since it is possible to puncture and fracture the rat's skull and irreparably damage the animal for experimental work. The same precautions are necessary when the ear bars are placed in the ear plugs; however a firm setting must be obtained if accuracy is to be maintained.

The incisor bar was positioned behind the upper incisor so that its top surface was 5.0 mm above the interaural line. This measurement was necessary in order for the rat's brain to be fixed in the same plane as the

Fig. 2. Ear plugs held in place by the ear bars.

brain in the brain atlas. The atlas used was that of de Groot (1959). A cut is made from the occiput forward to a line between the eyes. The skin is retracted and the galea and the periosteum are scraped from the bone. All ruptured blood vessels are then cauterized so that the skull becomes dry allowing one to see the suture lines.

The bregma of the rat's skull was used to determine the mid-sagittal plane. This is the point of intersection of the sagittal suture and the coronal suture. Since the suture lines in the rat, as in other animals, are not straight lines, it was frequently necessary to estimate the center of this point. The horizontal bar of the electrode carrier was moved until the electrode tip was exactly over the bregma. The reading thus obtained on the horizontal bar was used as the zero reference for all para-sagittal settings. After the coordinates were adjusted according to those in the atlas for the median eminence and recorded, a hole was drilled through the skull with a dental drill. Care was taken not to damage the brain.

The stereotaxic instrument used in the lesion studies was a Neuman and Lab-Tronics Stereotaxic instrument (Fig. 3) (Neuman Company, Chicago, Illinois). Much of the preparation of the animal and operational techniques are the same for use of this instrument and the Stoelting instrument previously described. There are some critical differences however and these will now be mentioned.

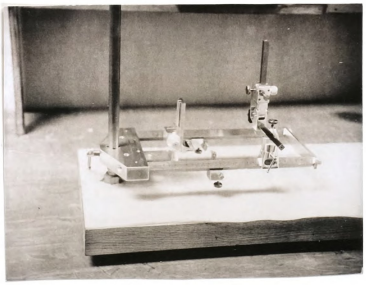

Fig. 3. Neuman and Lab Tronics stereotaxic instrument used in lesion studies.

The major part of the instrument consists of a yoke with two horizontal side bars. A removable posterior cross brace is placed between these to prevent any angular deviation after the animal is in place. Attached to the undersurface of the yoke is a plate containing the jaw clamps. On the undersurface of each horizontal side bar, is an ear bar support, in each of which is an ear bar. The ear plugs are fastened to the bar that joins them by set-screws. It is easier to place the rat between the ear plugs if one plug remains fixed on the bar. This plug is inserted into the rat's ear and seated against the side of the rat's skull. The head is then held firmly against this plug while the other is placed in the opposite canal and external auditory meatus by sliding it along the connecting bar. It is secured by tightening the set-screws. The head is observed to assure that a line joining the two eyes parallel to the bar which holds the two plugs. The relative position of the ear plugs from the end of the bar is not essential, since the bar serves only as a support and has nothing to do with aligning the rat's head in the mid-sagittal plane. Once the ear plugs are seated, they and the rat can be placed in position between the two ear bars of the instrument. The ear bars can be slipped into the appropriate holes in the plugs and adjusted to align the rat's head in the center between the two horizontal side bars.

Examination of the upper incisor bar reveals that there is an expanded area in the center with a sharp ridge around its circumference. This expanded area goes directly behind the upper incisors and the sharp ridge is fitted between the two teeth. The assembly is moved back toward the animal until the rat's upper incisor teeth can just be placed over the bar with the expanded area located as above. When the incisor bar clamp is tightened care should be taken that undue pressure is not exerted against the upper incisor teeth as it is possible to crush them. The vertically adjustable incisor bar should be set at the approximate position which is applicable for the atlas employed to avoid any undue motion of the head up and down after the plate and the incisor bar have been firmly fixed. Any undue deviation of the head after this may pull the animals head forward and cause distortion. The atlas used was that of de Groot. This atlas calls for placing the top of the incisor bar 5 mm above the line between the ear plugs. Other atlases differ.

III. Making Lesioning Electrodes

Most of the lesioning electrodes are made from #00 insect pins which can be purchased from any book store. The black coating was scraped off the pins using fine steel wool. The pins were then washed in xylene to remove any

grease. The pins were dipped in epoxylite resin very slowly and then baked in a vacuum oven at 190°C for one hour. The dipping and subsequent baking is repeated 3 more times. Before the electrodes can be used insulation is scraped off at the tip.

IV. Production of Lesions

The electrode tips are usually sharp and will readily pierce the dura very easily. The lesion is made with the anode and the indifferent cathode is placed in the rectum. The amount of current used depends on the size and location of the lesion to be produced. Using 0.15 mm stainless steel electrodes and a current of 5 milliamperes for 15 seconds, a 1.0 mm diameter lesion can be produced in the hypothalamus. A Stoelting direct current lesion maker with a regulated power supply was used to produce all neural lesions.

V. Preparation of Implantation and Securing Hormone Implants

In this study all substances to be implanted were placed inside 23-gauge glass tubing. Measured portions of hormones and cocoa butter were thoroughly mixed, and used to implant into the experimental groups. Cocoa butter alone was implanted into the control groups. Tubes to be implanted were prepared by tamping the glass tubes

into the mixture to be implanted, and after an adequate amount entered the tube the opposite end was sealed with bone wax in order to prevent a back pressure from pushing the hormone mixture up and out of the tube when it was lowered into the brain. The glass tubes were placed into the chuck of the stereotaxic instrument (Fig. 4) and lowered into the brain through the hole previously drilled (Fig. 5). The glass tube was lowered until the tip touched the floor of the cranial cavity and then raised 1.0 mm so that the tip would lie in the middle of the anterior median eminence. Skull screws were placed into holes previously drilled along side of the hole into which the implant was lowered. Silk thread was tied around the implanted tube and fastened to the skull screws. The entire area was then covered with dental cement. The skull screws served to anchor the implanted tube and cement to the skull and the silk thread serves a purpose analogous to the steel rods in reinforced concrete.

VI. Incubation Technique

Rats used in these experiments were killed by guillotine, and the anterior pituitaries and hypothalami were removed quickly. The posterior lobe was discarded and the anterior lobe was weighed, frozen and stored at -20°C until assayed. The hypothalamus and median eminence

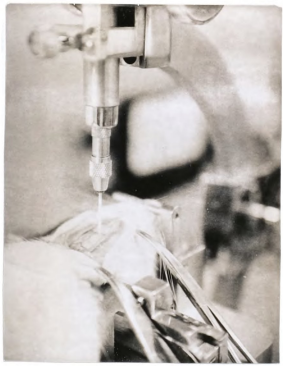

Fig. 4. Glass tube, filled with hormone, secured in chuck of stereotaxic instrument.

Fig. 5. Glass tube, filled with hormone, being lowered through hole drilled in skull.

were collected in chilled o.lN HCl (1.0 hypothalamus/0.1ml) and frozen at -20°C until used for incubation.

A. Preparation of hypothalamic extract

On the day of incubation the hypothalami were thawed and homogenized in a ground glass homogenizer, and centrifuged at 12,000 g for 40 minutes at 4°C. The supernatant was placed in protein free medium 199 (Difco Lab., Detroit, Michigan), and the pH was adjusted to 7.4 by adding drop by drop 1N NAOH and testing with glass electrodes. The hypothalamic extract was neutralized immediately before use.

B. Incubation

Adult male rats of the Sprague-Dawley strain were killed by guillotine, the anterior pituitaries were removed immediately and placed in a petri dish over a filter paper moistened with medium 199. Each pituitary was hemisected, one-half being placed in a control flask (25 ml Erlenmeyer) containing 2.0 ml of medium 199 and the other half in the experimental flask also containing 2.0 ml of medium 199. The equivalent of 4 anterior pituitaries (8 halves) was placed into each flask. The neutralized hypothalamic extract (equivalent of 0.25 or 1 hypothalamus per pituitary incubated) was added rapidly to each flask.

Incubations were carried out in a Dubnoff metabolic shaker (60 cycles/min.) under constant gassing with 95%

O_2-5% CO_2 at 37±0.5°C for 4 hours. At the termination of the incubation, the pituitary halves were weighed and the medium was centrifuged at 2,500 g for 10 minutes, and the supernatant was frozen at -20°C until assayed.

VII. Bioassays

A. Prolactin

The incubation medium was assayed for prolactin activity by the intradermal pigeon-crop technique of Lyons (1937) as modified by Reece and Turner (1937). A dose of 0.2 ml was injected daily for 4 days into each bird. A direct comparison was made between samples by injecting one sample over one side of the crop sac and the other sample over the other side of the crop of the same bird. Anterior pituitaries were homogenized in normal saline. Each injection was in a 0.1 ml volume and an equivalent of 3.0 mg of pituitary was injected into each bird for 4 days.

The prolactin responses in each bird were expressed in Reece-Turner units (RTU), and these units were converted into international units (IU) by use of a standard dose response curve established in the same breed of pigeons with NIH prolactin (Nicoll and Meites, 1963).

B. Follicle stimulating hormone (FSH)

The method of Steelman and Pohley (1953) as modified by Parlow and Reichert (1963) was used to measure

FSH. Pituitaries were ground in a glass homogenizer and extracted with physiological saline solution. The solutions were centrifuged and stored at 4°C during the assay.

C. Lutenizing hormone (LH)

LH assays were done by the ascorbic acid depletion method of Parlow (1961). All materials were assayed at four-fold differences in concentration. The media were assayed in doses equivalent to 2.5 and 10.0 mg of incubated pituitary. Pituitary LH content was assayed by injecting 1.0 and 4.0 mg of pituitary tissue, respectively.

VIII. Statistical Analysis

Statistical analysis for prolactin was performed by the "t" test for paired observations. Bioassays for FSH and LH were analyzed according to Bliss (1952).

EXPERIMENTAL

I. Direct Hypothalamic Feedback for Prolactin

A. Effects of median eminence implants of prolactin on hypothalamic PIF content, pituitary prolactin concentration, mammary glands and ovaries

Objectives

There is evidence that anterior pituitary hormones participate in their own feedback control by acting directly on the hypothalamus. Thus it has been reported that FSH (Corbin and Story, 1967), ACTH (Legori et al., 1965), LH (Corbin and Cohen, 1966) and GH (Katz et al., 1967) implants into the hypothalamus reduce pituitary concentration of each of these hormones in the rat. The present experiment was designed to determine the effects of prolactin implants in the median eminence of rats on hypothalamic content of prolactin inhibiting factor (PIF), pituitary prolactin concentration and on the mammary glands and ovaries.

Materials and Methods

A total of 35 mature female Sprague-Dawley rats, approximately 3 months old, were used in this study. Single stereotaxic implantations of approximately 250 ug prolactin (NIH-P-S7) in cocoa butter were made in the

median eminence of 13 mature intact and 6 ovariectomized rats. Implants in the intact rats were all made on the day of estrus. A total of 10 intact and 6 ovariectomized control rats were implanted with cocoa butter alone. Glass 23-gauge tubing was tamped into the prolactin-cocoa butter mixture, implanted and left *in situ*. In intact rats, vaginal smears were taken daily beginning 10 days prior to implantation and continued until the rats were sacrificed. Ovariectomy was performed 7 days prior to implantation.

All animals were killed 6 days after implantation, and the ventral surface of the brain was exposed. The exact location of the implant was ascertained with the aid of a dissecting microscope. The hypothalami were removed and placed in 0.1 N HCl, frozen and stored at -20°C. On the day of PIF assay, the hypothalami were homogenized in 0.1 N HCl and the homogenate was boiled for 12 minutes in order to destroy any prolactin which may have been present, although prolactin has not been detected in rat hypothalamic extracts Talwalker *et al.*, (1963). The hypothalamic extract was incubated with male rat pituitary according to the method of Kragt and Meites (1965), and the medium was assayed for prolactin by the method of Lyons (1937) as modified by Reece and Turner (1937).

Results

The results in Table 1 show that pituitary incubated with hypothalamic extract from intact prolactin implanted rats released significantly less prolactin ($p < .05$) than that from sham-implanted rats. A similar difference was noted between the prolactin-implanted and sham operated ovariectomized groups ($p < .01$). This indicates that hypothalamic PIF content was increased by the prolactin implants. There was also a significant reduction in pituitary prolactin concentration in the intact ($p < .01$) and ovariectomized ($p < .05$) prolactin-implanted rats when compared with the non-prolactin implanted controls. Anterior pituitary weight was also significantly depressed by the prolactin implants ($p < .05$).

Mammary glands from intact control rats implanted with cocoa butter alone showed well developed ducts and some lobulo-alveolar development (Fig. 6). Implantation of prolactin in the hypothalamus produced marked mammary gland atrophy (Fig. 7) characterized by only bare ducts and no alveolar elements. These glands resembled those from long term ovariectomized rats even though the rats were still cycling. The control ovariectomized rats with cocoa butter implants also showed much better structural maintenance of the mammary glands (Fig. 8) than the ovariectomized rats with prolactin implants (Fig. 9).

TABLE 1. Effects of prolactin implants into the median eminence on pituitary prolactin concentration and hypothalamic PIF content

Treatment	No. of rats	AP Weight mg	AP Prolactin Conc. IU/100 mg	PIF Content (AP Prolactin released in vitro IU/100 mg AP)
Intact controls	13	8.1±0.2	2.36±0.50	1.77±0.30*
Intact prolactin implanted	10	6.2±0.4	1.32±0.20	0.81±0.14
OVX[1] controls	6	11.3±0.6	3.31±0.23	1.09±0.40
OVX prolactin implanted	6	9.5±0.5	2.35±0.56	0.32±0.15

[1]OVX = Ovariectomized.

*Mean ± SE.

Fig. 6. Mammary gland of intact control rat implanted with cocoa butter. Note well developed ducts and some alveolar growth (x30).

Fig. 7. Mammary gland of intact rat implanted with prolactin. Note marked regression of ducts and absence of alveoli (x30).

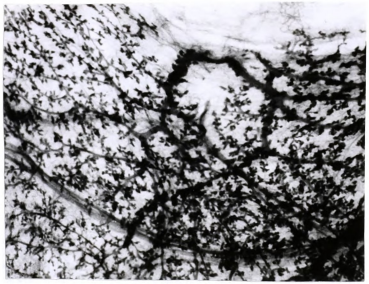

Fig. 8. Mammary gland from control ovariectomized rat implanted with cocoa butter. Less development is evident as compared to intact control rats (x30).

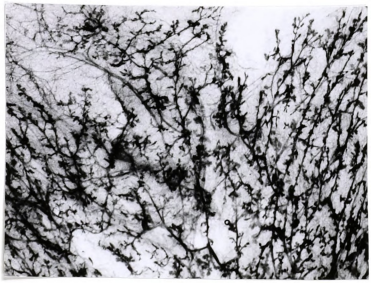

Fig. 9. Mammary gland from ovariectomized rat with a prolactin implant. Note marked regression and breaking up of ductal system (x30).

Ten of the 13 cocoa butter-implanted control rats showed persistent diestrous smears indicative of pseudopregnancy, whereas they all exhibited regular 4 or 5 day cycles prior to implantation. The ovaries of these rats showed mainly well developed corpora lutea (Fig. 10). The ovaries of the intact prolactin-implanted rats had many well developed follicles and corpora lutea in various stages of development (Fig. 11), typical of cycling rats.

Discussion

These observations demonstrate that prolactin can exert a feedback action on the hypothalamus to regulate its own production. This negative feedback by the hypothalamic implants of prolactin apparently is exerted by increasing PIF synthesis and release in the hypothalamus, which in turn depresses prolactin secretion by the pituitary. The possibility that prolactin may act directly on the pituitary cannot be ruled out at this time.

It is interesting that most of the rats given sham implants became pseudopregnant presumably due to leaving the glass tube in the median eminence and perhaps also to the stress of the operation. On the other hand all of the prolactin-implanted animals demonstrated normal estrous cycles indicating that the hormone implant exerted a primary influence on pituitary prolactin secretion. This

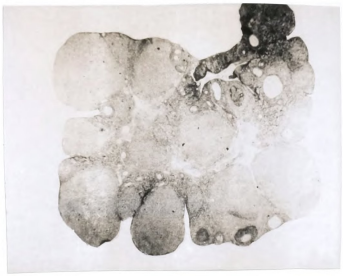

Fig. 10. Ovary from intact control rat implanted with cocoa butter. Note predominance of well developed corpora lutea (x15).

Fig. 11. Ovary from prolactin implanted rat. Note well developed follicles (x15).

inhibition of prolactin secretion can be attributed to the enhanced PIF in the hypothalamus.

The rapid regression of the mammary glands observed in this study indicates that constant secretion of prolactin is necessary to maintain the growth and structural integrity of the mammary gland. The findings in the ovariectomized rats indicate that even in the absence of ovarian hormones, pituitary prolactin still helps to maintain some structural integrity of the mammary glands, and that inhibition of prolactin secretion results in mammary regression. We have observed mammary regression as early as 4 days after prolactin implantation into the median eminence (Unpublished observations).

Recent reports indicate that transplants into intact rats of pituitary tumors secreting large amounts of prolactin and GH result in reduced pituitary weight and prolactin concentration (MacLeod et al., 1966; Chen et al., 1967), and increased hypothalamic PIF content (Chen et al., 1967). These observations are in agreement with the results on pituitary weight, pituitary prolactin concentration and hypothalamic PIF content found in this experiment. To what extent prolactin in the circulation under normal physiological states may exert a negative feedback on pituitary prolactin secretion remains to be determined.

B. Effects of median eminence implants of prolactin and ACTH on postpartum lactation in rats

Objectives

Since prolactin and the ACTH-adrenal cortical system are both essential for maintenance of lactation (Meites, 1959, 1966), it was of interest to determine the effects of ME implants of prolactin and ACTH on postpartum lactation in rats.

Materials and Methods

Fifty lactating Sprague-Dawley rats (Spartan Animal Farms, Haslett, Michigan) approximately 3 months old, were divided into 3 groups on the 4th day postpartum and each received stereotaxic implants in the anterior ME of the following substances: group I, controls, cocoa butter; group II, a mixture of NIH-P-S8 prolactin and cocoa butter; group III, a mixture of prolactin, ACTH (Nutritional Biochemicals) and cocoa butter. The cocoa butter or hormone mixtures were tamped into the ends of 23 gauge glass tubing, implanted into the ME and fixed rigidly in place by means of dental cement and skull screws. It is estimated that the rats were implanted with about 250-300 ug of each hormone.

On the day of implantation (day 0) litter size was reduced to 6 pups, and individual pup weights were recorded on days 1, 3, 5 and 7 after implantation. Some

of the rats were killed on the 5th day after implantation, and the mammary glands were removed for weighing and histological examination. The other rats were killed 7 days post implantation in order to more adequately ascertain the effects of the implants on the estrous cycle. When the animals were killed the ventral part of the skull was removed to expose the hypothalamus, and the exact location of the implant was determined with a dissecting microscope. When the implants were not found in the anterior ME, the rats were not included in the experiments presented here. The data were subjected to analysis of variance, and significance of differences between means were assessed by Duncan's (1956) multiple range test.

Results

Prolactin implants elicited a significant decrease in litter weight gains when compared with the controls (Table 2), whereas implants containing both ACTH and prolactin resulted in litter weight gains significantly below those of rats given prolactin alone. The effects of the implants on litter weight gains were essentially similar at 5 and 7 days after implantation.

The mammary glands from rats 5 days after implantation of prolactin showed a significant decrease in weight as compared to the controls, whereas a combination of prolactin and ACTH reduced mammary gland weights even below

TABLE 2. Effects of implants of prolactin and ACTH in the median eminence on litter weight gains and mammary gland weights. Values are means ± standard errors

Treatment	Av. Wt. gain by 6 pups 5 days post implantation g	Av. Wt. gain by 6 pups 7 days post implantation g	Av. mammary gland Wt. 5 days post implantation g
Controls, cocoa butter	40.4 ± 2.7^{a} (11)	70.7 ± 6.2^{a} (5)	2.87 ± 0.12^{a} (6)
Prolactin + cocoa butter	18.8 ± 3.5^{b} (14)	32.6 ± 5.9^{b} (9)	1.96 ± 0.16^{b} (5)
Prolactin, ACTH + cocoa butter	8.7 ± 3.6^{c} (9)	16.9 ± 7.0^{c} (3)	1.42 ± 0.14^{c} (6)

a,b,c Values having different superscripts are significantly different from each other $P < .05$.

( ) = No. of animals.

the values obtained with prolactin alone. A significant regression was observed in the mammary glands of the prolactin implanted rats (Fig. 12) as compared to the controls (Fig. 13).

Control lactating rats were all in diestrus, whereas all animals implanted with prolactin or prolactin and ACTH came into estrus and began to cycle. The ovaries of the control rats showed follicular inhibition and large corpora lutea (Fig. 14), whereas the ovaries from both experimental groups showed follicles in different stages of development (Fig. 15). Ova were detected in the oviducts of the cycling rats.

Discussion

The present study shows that implants of prolactin or prolactin and ACTH into the anterior ME resulted in a significant impairment of lactation. The implanted hormones decreased their own secretion apparently by acting on the hypothalamic neurons responsible for secreting PIF and CRF. We recently reported that ME implants of prolactin into mature cycling female rats significantly increased PIF content in the hypothalamus, decreased pituitary prolactin concentration and induced involution of the mammary glands (Clemens and Meites, 1967, 1968). ME implants of ACTH were reported to decrease pituitary ACTH secretion (Legori *et al.*, 1965).

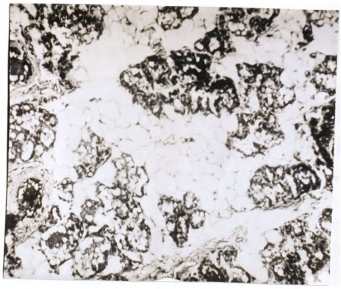

Fig. 12. Mammary gland from lactating rat implanted with prolactin and cocoa butter. Note atrophy and little milk in alveoli (x70).

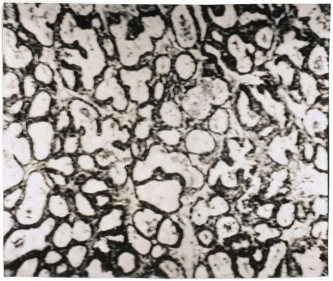

Fig. 13. Mammary gland from lactating control rat implanted with cocoa butter alone. Note compact alveoli filled with milk (x70).

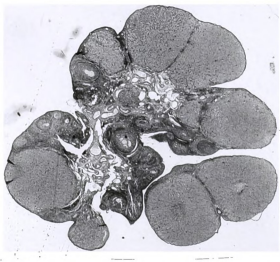

Fig. 14. Ovary from lactating control rat implanted with cocoa butter alone. Note predominance of corpora lutea of lactation (x15).

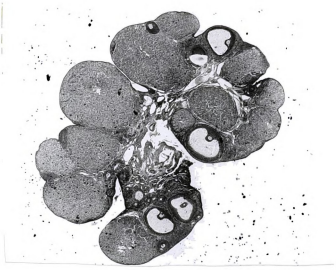

Fig. 15. Ovary from lactating rat implanted with prolactin and cocoa butter. Note large preovulatory follicles as well as corpora lutea (x15).

In the rat, prolactin stimulates mammary growth and milk secretion, and also maintains the corpora lutea of lactation. The suckling stimulus serves to maintain lactation by evoking release of prolactin and ACTH-adrenal cortical hormones (Meites, 1966). ME implants of prolactin and ACTH apparently counteracted the ability of the suckling stimulus to elicit release of these two hormones from the pituitary, and resulted in reduced milk secretion and loss of luteal function. This provides further evidence that prolactin is essential in maintaining milk secretion as well as luteal function in the rat, and that the feedback of ME implants of prolactin and ACTH are powerful enough to suppress lactation.

C. Effects of median eminence implants of prolactin on pregnancy in rats

Object

Recent experiments by Clemens and Meites (1967, 1968) have demonstrated the existence of a hypothalamic feedback for prolactin, and have been extended by Clemens et al. (1968) to show that the prolactin dependent process of lactation can be inhibited by median eminence prolactin implants. In addition to inhibition of lactation they found that the corpora lutea of lactation disappeared as a result of the prolactin implant. Since these implants appear to terminate luteal function, it was of interest

to determine the effects of a prolactin implants in the median eminence on pregnancy in the rat.

Materials and Methods

Adult, female, 3 month old Holtzman rats (Madison, Wisc.) were used in this study. The rats were housed with males until pregnancy had occurred as ascertained by the appearance of spermatozoa in the vaginal smear. The day spermatozoa were detected in the vaginal smear was designated as day 1 of pregnancy.

Single glass tubes containing a prolactin (NIH-P-S7)-cocoa butter mixture were stereotaxically implanted into the median eminence of each of one group of pregnant rats. Another group (controls) received implants of glass tubes containing cocoa butter alone into the median eminence. Six rats were implanted with tubes containing prolactin and cocoa butter, and 6 control rats were implanted with cocoa butter alone each day from day 1 of pregnancy until day 8 of pregnancy. A total of 48 rats received implant of the prolactin-cocoa butter mixture, and 48 rats received cocoa butter alone.

On the 4th day of pregnancy 12 rats were divided equally into 2 groups. The first group of rats served as controls and received implants of cocoa butter and daily injections of 2 mg of progesterone from day 4 until day 11 (midpregnancy). The second group received implants of the

prolactin-cocoa butter mixture on the 4th day of pregnancy and injections of progesterone from day 4 until day 11.

Results

Table 3 shows that when prolactin was implanted into pregnant rats from days 1-6, pregnancy was terminated in all cases. Laporatomies were performed on day 15 and no fetuses were found in these rats. Control implants of cocoa butter had no effect regardless of the day of pregnancy on which they were implanted, and at laparotomy both number and size of fetuses appeared to be normal. When prolactin was implanted on the 4th day of pregnancy and 2 mg progesterone were administered daily, pregnancy was maintained. However, when progesterone treatment was withdrawn on day 11, all of the prolactin-implanted rats aborted by day 15 while cocoa butter-implanted control rats remained pregnant (Table 4).

Discussion

The results of this experiment demonstrate that prolactin secretion is necessary for maintenance of pregnancy, at least for the first 6 days. The probably decreased prolactin secretion from the implant after the 6th day did not appear to have any effect on pregnancy. This may be due to secretion of luteotrophin by the newly formed placenta. The prolactin implant appeared to exert

TABLE 3. Effects of prolactin implants in the median eminence on pregnancy in rats

Treatment	No. rats	Day of pregnancy implant placed	No. rats remaining pregnant
Prolactin implant	6	1	0
"	6	2	0
"	6	3	0
"	6	4	0
"	6	5	0
"	6	6	0
"	6	7	5
"	6	8	6
Cocoa butter implant	6	1	6
"	6	2	6
"	6	3	6
"	6	4	6
"	6	5	6
"	6	6	6
"	6	7	6
"	6	8	6

TABLE 4. Effects of prolactin implants placed on the 4th day of pregnancy and daily progesterone administration on pregnancy

Treatment	Day of implantation	No. rats pregnant on day 11	No. rats pregnant on day 15 after withdrawal of progesterone on day 11
Prolactin implant and 2 mg progesterone per day	4	6	0
Cocoa butter implant and 2 mg progesterone per day	4	6	6

its effect by causing luteal regression, resulting in a decrease in progesterone production. Evidence to support this hypothesis stems from the observation that daily administration of 2 mg of progesterone maintained pregnancy in rats given implants of prolactin. When the progesterone treatments were discontinued the animals immediately aborted. This indicates that the prolactin implant caused the corpora lutea to regress and resulted in a concomitant decrease in progesterone secretion, while the exogenous progesterone maintained the pregnant state. Since the rats aborted when progesterone was withdrawn after the 11th day, it appears that once the corpora lutea have regressed as a result of the prolactin implant they cannot be reactivated by placental luteotrophin. Thus, it appears that pituitary prolactin secretion is essential during the early stages of pregnancy and that prolactin exerts its effects through the corpus luteum by stimulating progesterone secretion.

II. Hypothalamic Control of Mammary Carcinogenesis and Tumor Growth

Object

The onset and growth of mammary adenocarcinomas in female rats given carcinogens apparently require both anterior pituitary and ovarian hormones. Thus rats ovariectomized or hypophysectomized prior to carcinogen treatment develop few or no mammary tumors (Dao, 1964), and

ovariectomy or hypophysectomy after mammary tumor development can result in tumor regression (Huggins et al., 1959). Further evidence of the importance of anterior pituitary hormones, particularly prolactin, is provided by experiments showing that single or multiple pituitary transplants can increase the incidence of mammary tumors in mice (Muhlbock and Boot, 1959). Prolonged injections of prolactin into intact female mice also resulted in mammary tumors (Boot et al., 1962), as did transplantation of pituitary "mammotropic" tumors in mice (Haran-Ghera, 1961) and rats (Meites and Sinha, 1966).

Placement of lesions in the median eminence (ME) of the tuber cinereum results in enhanced release of prolactin and in marked reduction in release of all other anterior pituitary hormones (Meites, et al., 1963). It was of interest therefore, to study the effects of ME lesions on the induction and growth of mammary tumors in intact and ovariectomized rats treated with a carcinogen.

Materials and Methods

Placement of ME lesions before DMBA

Seventy-five virgin female Sprague-Dawley rats (Holtzman, Madison, Wisc.) all uniform in body weight were divided randomly into four groups. At 56 days of age all animals were given a single intravenous injection of 1 ml of a lipid emulsion containing 5 mg of 7,

12-dimethylbenzanthracene (DMBA). Additional treatments were as follows: group I, intact controls, no treatment; group II, intact, bilateral lesions placed in the ME at 50 days of age; group III, ovariectomized at 64 days of age and no further treatment; group IV, placement of bilateral lesions in the ME at 50 days of age and ovariectomized at 64 days of age. After carcinogen treatment all animals were examined once weekly for a period of 6 months for development of mammary tumors. All ME lesions were produced by passing a direct current of 2 mA for 7 sec through epoxylite coated steel electrodes prepared from insect pins. The ME was located with the aid of a Stoelting sterotaxic instrument and de Groot's atlas of the rat brain. At the time of autopsy the brains were removed, fixed in neutral buffered formalin, sectioned at 10 μ and stained with cresyl violet and luxol fast blue. The location of the ME lesions (Fig. 16) was confirmed.

Placement of ME lesions after DMBA

Fifty-seven virgin female Sprague-Dawley rats were divided randomly into four groups. At 55 days of age all animals were given a single intravenous injection of 5 mg of DMBA. Additional treatments were as follows: group I, controls, no treatment; group II, bilateral

Fig. 16. Histological section through the hypothalamus of a rat with a median eminence lesion. Arrow points toward lesion (x25).

lesions placed in the ME 75 days after DMBA treatment; group III, ovariectomized 75 days after DMBA treatment; group IV, ovariectomized and bilateral lesions placed in the ME 75 days after DMBA treatment. All animals were examined at 0, 10 and 25 days after ovariectomy and/or placement of lesions for palpable mammary tumors. Tumors larger than 1 cm in diameter were measured with a vernier caliper. All tumors were removed at the end of each experiment, fixed in Bouin's fluid, sectioned at 4 μ and stained with hematoxylin and eosin. They were all adenocarcinomas, similar to those described by Huggins et al. (1959) in Sprague-Dawley rats treated with DMBA.

Results

Placement of ME lesions before DMBA (Table 5)

The results show that ME lesions had a definite inhibitory effect on mammary tumor induction. In the intact control rats (group I), 95% of the rats had mammary tumors when the experiment was terminated 6 months after DMBA treatment, whereas intact rats with bilateral lesions in the ME (group II) had only a 30% incidence of mammary tumors. The ovariectomized controls (group III) showed a 54% tumor incidence 6 months after DMBA treatment whereas ovariectomized rats with bilateral lesions of the ME (group IV) were completely free of mammary tumors. The average number of tumors per tumor bearing rat was

TABLE 5. Effects of median eminence lesions placed in rats before treatment with DMBA

Group and treatment	Total No. of rats	No. and % of rats with tumors	Av. No. of tumors/ tumor-bearing rat	Range and mean latency period (days)
I. Control intact	22	21 (95)	3.1 ±0.11[b]	40-175 (78± 5)[b]
II. Lesioned intact[e]	20	6 (30)	1.16±0.03[d]	65-177 (104±14)[d]
III. Control ovariectomized[f]	13	7 (54)	2.33±0.42[c]	102-186 (156± 7)[c]
IV. Lesioned ovariectomized	20	0 (0)	0	0 (0)

[a]DMBA administered on day 56.

[b,c,d], Groups possessing different superscripts are significantly different from each other (p = .05). Standard error of the mean is indicated for each group.

[e]ME lesion placed on day 50.

[f]Ovariectomy performed on day 64.

significantly lower in the lesioned groups than in either of the control groups. The number of rats with tumors and average number of tumors per rat were significantly fewer in the ovariectomized controls (group III) than in the intact controls (group I).

Placement of ME lesions after DMBA (Table 6)

Prior to placement of the ME lesions most tumors were barely palpable. The ME lesions resulted in rapid growth stimulation and in appearance of a greater number of mammary tumors. At the end of 10 days, average mean diameter of measurable tumors in the lesioned intact group (group II) increased by 7.1 mm as compared to an increase of 3.3 mm in the control rats (group I). Ten days after ovariectomy (group III) the average mean diameter of measurable tumors was reduced by 6.5 mm, whereas there was an increase of 2.1 mm in the average mean diameter of tumors in the lesioned ovariectomized rats (group IV). The lesioned intact rats (group II) showed a 120% increase in number of palpable mammary tumors 10 days after treatment, whereas the intact controls (group I) had only a 19% increase in tumors during the same period. The stimulatory action of the ME lesions was still apparent 25 days after the lesions were placed.

Ovariectomy alone resulted in significant tumor regression 10 days after surgery, and in even greater

TABLE 6. Effects of median eminence lesions placed in rats 75 days after treatment with DMBA

Group and treatment[a]	Total No. of rats	Av. No. of palpable tumors per rat: Initial	10 days after treatment	Change (%)	25 days after treatment	Change (%)
I. Control intact	13	3.2±0.7[b]	3.8±0.9[b]	+ 19	4.3±0.8[b]	+ 33
II. Lesioned intact	12	3.5±1.2	7.7±2.3	+120	10.1±1.7	+189
III. Control ovariectomized	16	4.0±0.7	2.9±0.5	- 27	1.4±0.5	- 56
IV. Lesioned ovariectomized	16	4.6±1.1	8.3±1.1	+ 80	2.8±0.6	- 39

[a]DMBA was administered at 55 days of age. The rats were ovariectomized and/or lesioned approximately 75 days after DMBA treatment.

[b]SE of the mean.

regression 25 days after surgery (group III). When ovariectomy was combined with ME lesions (group IV), there was a striking increase (80%) in number of palpable mammary tumors during the first 10 days after treatment. However, the stimulatory effects of the ME lesions in the ovariectomized rats did not persist, since marked tumor regression was observed by the end of 25 days.

Discussion

The results of this study indicate that when ME lesions were placed in intact and ovariectomized rats prior to DMBA treatment, mammary tumor incidence was significantly decreased. On the other hand, similar lesions placed in the ME after the appearance of small, palpable mammary tumors, rapidly stimulated tumor growth and increased the total number of tumors.

The decreased incidence of mammary tumors in rats with ME lesions placed prior to DMBA treatment may be partly due to the increased release of prolactin, stimulating mammary growth but making the mammary tissue relatively refractory to the action of DMBA. We have observed that transplantation of pituitaries into intact rats prior to tumorigenesis stimulates mammary growth (Welsch and Meites, 1967). In the intact rats increased prolactin release as a result of ME lesions may stimulate progesterone secretion by the ovaries, and the combined

action of the 2 hormones could promote mammary growth. Ovariectomy was not performed until 14 days after ME lesions were placed and 8 days after DMBA was administered. Hence the mammary glands were in an active growth phase during a critical period before and after DMBA was given. Dao (1967) showed that the critical period for the presence of the ovaries occurred within 2 weeks after DMBA treatment. In intact mice without ME lesions transplants of normal pituitaries for prolonged periods resulted in increased incidence of mammary tumors by providing continuous prolactin stimulation. However, DMBA was not given to these mice, and continuous hormonal stimulation alone may have evoked mammary tumorigenesis.

ME lesions placed after the appearance of mammary tumors resulted in a rapid and striking increased growth and development of these tumors. In addition, many new mammary tumors arose. This can be attributed to increased prolactin stimulation of the mammary glands. In agreement with these results, transplantation of pituitary "mammosomatotrophic" tumors (W5 or W15) into inbred Wistar female rats was found to induce development of mammary tumors and to markedly enhance growth of these tumors upon transplantation (Meites and Sinha, 1966). The rapidity with which the mammary tumors grew in size and number in the ME lesioned rats was remarkable. This was as apparent in the

ovariectomized as in the intact rats during the first 10 days after placing the ME lesions. The subsequent regression of the mammary tumors in the ovariectomized ME lesioned rats, suggests that enhanced prolactin secretion not by itself is not sufficient to maintain mammary tumor development. Apparently estrogen is also necessary for optimal growth of mammary tumors in these rats. This is in agreement with other reports that both pituitary and ovarian hormones are necessary to maintain mammary tumor growth in rats (Huggins, 1965). Estrogen is believed to act in part by promoting pituitary prolaction secretion (Nicoll and Meites, 1962).

III. Effects of Prolactin on the Onset of Puberty

Object

Donovan and Van der Werff ten Bosch (1965) and Critchlow and Bar Sela (1967) reviewed the factors which influence the onset of puberty in a variety of species. Treatments which advance puberty include lesions of the ventromedial hypothalamus (Gellert and Ganong, 1960; Donovan and Van der Werff ten Bosch, 1965), amygdala lesions (Elwers and Critchlow, 1960), mild stress (Morton et al., 1963) and electrical stimulation of the uterus (Swingle et al., 1951). Since some of these treatments also stimulate prolactin secretion in rats (Meites and Nicoll, 1966; also unpublished observations), it appeared

that increased prolactin secretion might have some role in advancing puberty in these animals. Therefore, the effect of prolactin injections and median eminence implants of prolactin on the onset of puberty in immature female rats was studied.

Materials and Methods and Results

All rats used were immature female Sprague-Dawley rats obtained from Spartan Animal Farms, Haslett, Michigan. Twenty-five day old hypophysectomized Sprague-Dawley female rats (Hormone Assay Lab., Chicago, Illinois) were used to study the direct effects of prolactin on the ovaries and uteri. All rats were housed in a constant temperature room (25±1°C) with automatically controlled lighting (14 hrs. light daily). The diet consisted of Wayne Lab Blox pellets (Allied Mills, Inc., Chicago, Illinois) and tap water. In addition, the hypophysectomized rats received orange slices, carrots and sugar cubes as a supplement. Results were analyzed statistically by Kramer's (1956) multiple range test and the "t" test.

In experiment 1, sixteen 25 day old rats were divided into 2 groups. One group received subcutaneous injections of 1.0 mg NIH-P-B1 prolactin twice daily in 0.1 ml saline, and the other group was given daily injections of physiological saline. Each animal was injected from the 25th day of age until the day of puberty, as

determined by vaginal opening and first proestrus or estrus.

The results (Table 7) indicate that injections of NIH-P-B1 beginning on the 25th day of age (group I) hastened the onset of puberty by about 2 days when compared with saline-treated controls (group II).

In the second experiment 42 female rats, each 20 days of age, were divided into 3 groups. Group I received subcutaneous injections of 1.0 mg of NIH-P-B1 prolactin twice daily, the second group received saline injections alone and the third group received injections of 8.18 ug of NIH-FSH-S3 and 0.55 ug of NIH-LH-S8 twice daily. These latter hormones constituted the maximum amounts of gonadotrophins reported to be present in 1.0 mg of NIH-P-B1 prolactin.

In this experiment (Table 7), injections of prolactin beginning at 20 days of age, advanced the onset of puberty by about 6 days (group III) as compared to the saline-injected controls (group I) or in controls injected with FSH and LH (group II) in amounts equal to those contained as impurities in the NIH-P-B1 prolactin.

In experiment 3 one group of 11 rats received no treatment. A second group of 21 rats received injections twice daily of 0.61 ug of NIH-FSH-S3, 0.09 ug of NIH-LH-S8, 0.14 ug of NIH-TSH-B3 and 3.70 ug of NIH-GH-S8, all

TABLE 7. Effects of prolactin and other hormones on the time of puberty and on ovarian and uterine weights of immature female rats

Exp. No.	Group and twice daily treatment	Age treatment began (days)	Av. age at onset of puberty (days)	Av. ovarian weight (mg)	Av. uterine weight (mg)
1	I. Saline	25	35.0±0.4 (8)+		
	II. 1.0 mg NIH-P-B1		33.0±0.4 (7)**		
2	I. Saline	20	37.0±0.5 (11)		
	II. 8.18 ug NIH-FSH-S3 0.55 ug NIH-LH-S8		36.9±0.5 (11)		
	III. 1.0 mg NIH-P-B1		30.8±0.1 (15)*		
3	I. No treatment			20.2±1.1 (11)	44.1±2.7
	II. 0.61 ug NIH-FSH-S3 0.09 ug NIH-LH-S8 0.14 ug NIH-TSH-B3 3.70 ug NIH-GH-S8	20	38.4±1.2 (14)	22.0±0.7 (10)	40.8±2.7
	III. 0.75 mg NIH-P-S7		32.5±1.6 (15)*	23.6±0.9 (10)	93.3±6.3*

( ) Number of animals.

*P .001.

**P .02 + Mean ± SE.

reported to be the maximum amounts of these hormones present as contaminants in the amount of NIH-P-S7 prolactin injected into the third group. Group III contained 11 rats which received injections of 0.75 mg of NIH-P-S7 prolactin twice daily. When the rats reached 30 days of age, all rats from group I, 6 rats from group II and 6 rats from group III were killed and the ovaries and uteri were removed and weighed.

The results of this experiment (Table 7) show that injections of prolactin beginning at 20 days of age advanced the average time of puberty by about 6 days (group III) when compared to control rats given the maximum amounts of other pituitary hormones present in NIH-P-S7 prolactin (group II). This experiment also shows that prolactin injections resulted in a significant increase in uterine but not in ovarian weight (group III) when compared with the untreated controls (group I) or animals given the prolactin impurities (group II).

The fourth experiment was performed in order to test any possibility of a direct action of prolactin on the ovaries and uterus. Eighteen hypophysectomized rats were divided into two groups. Group II received 0.75 mg of NIH-P-S7 twice daily and group I received twice daily injections of the maximum quantities of anterior pituitary hormone contaminants calculated to be present in NIH-P-S7 prolactin.

Table 8 indicates that hypophysectomized rats treated with prolactin contaminants (group I) had ovaries and uteri which were not significantly different in weight from those of similar rats treated with prolactin (group II).

The fifth experiment was performed to ascertain if prolactin might act centrally to advance puberty. A total of 43 immature rats used in this experiment were divided into 3 groups. Group I consisted of 18 rats with median eminence implants of a prolactin (NIH-P-S7)-cholesterol mixture consisting of equal quantities of each. The mixture was packed into a 21 gauge stainless steel tube slightly flaired at the tip. The tube was lowered into the brain stereotaxically, and the pellet was ejected from the tube into the anterior median eminence and the tube was then withdrawn. Group II consisted of 18 rats implanted in an identical fashion with a cholesterol pellet containing anterior pituitary hormones reported to be present as impurities in the NIH prolactin preparations. All median eminence implants were performed at 21 days of age. To eliminate the possibility of any systemic effects, a prolactin-cholesterol pellet of the same size was implanted subcutaneously in a third group of 7 rats.

Table 9 demonstrates that anterior median eminence implants of prolactin advanced puberty 6 days (group I)

TABLE 8. Effects of prolactin and other hormones on ovarian and uterine weights in hypophysectomized rats

Group and twice daily treatment	Age treatment began (days)	Av. ovarian weight (mg)	Av. uterine weight (mg)
I. Hypophysectomized 0.61 ug NIH-FSH-S3 0.09 ug NIH-LH-S8 0.14 ug NIH-TSH-B3 3.70 ug NIH-GH-S8	21	4.7±0.6 (6)	15.5±1.7
II. Hypophysectomized 0.75 mg NIH-P-S7	21	4.6±0.6 (6)	15.7±0.4

( ) Number of animals.

TABLE 9. Effects of anterior median eminence and subcutaneous implants of hormones on onset of puberty in female rats

Group and treatment	No. of rats	Av. age at onset of puberty (days)
I. Prolactin implant	18	31.6±1.9*
II. Prolactin impurities implant	18	38.2±2.1+
III. Subcutaneous prolactin implant	7	39.1±2.0

*P < .01.

+Mean ± SE.

when compared with rats receiving median eminence implants of prolactin impurities (group II) or given subcutaneous prolactin implants (group III).

Discussion

The results of this study demonstrate that NIH-P-B1 and NIH-P-S7 prolactins significantly advanced the onset of puberty in rats in 3 separate trials. A direct action on the ovaries can be ruled out because of the inability of prolactin to stimulate the ovary or uterus of the hypophysectomized rats. The significant increase in uterine weight in intact rats injected with prolactin suggests that this may have been a consequence of estrogen production by the ovaries. However, the amounts of FSH and LH reported to be present in the 2 prolactin preparation had no effect on the onset of puberty in these rats.

Uterine stimulation by prolactin in the intact immature rats and the inability of prolactin injections to stimulate the uterus in hypophysectomized rats suggests that prolactin acted centrally in the immature rats to stimulate production of gonadotrophins by the pituitary. This hypothesis is strengthened by the implantation experiment, since median eminence implants of prolactin were found to hasten puberty by an average of 6.6 days. Since subcutaneous implants of pellets containing the same

amount of prolactin as the median eminence implants elicited no effect on the onset of puberty, the observed advancement of puberty in the rats with median eminence prolactin implants is believed to be due to the local effect on the hypothalamus. Prolactin implants may stimulate FSH-RF release by the hypothalamus and result in an increased pituitary FSH production. Recently our laboratory obtained evidence that median eminence prolactin implants in immature rats resulted in increased FSH secretion. It is possible that the decrease in pituitary prolactin level which results from prolactin implants (Clemens and Meites, 1967, 1968) may allow the pituitary to utilize more of its cellular machinery in synthesis of gonadotrophins. Clemens et al., (1968) recently observed that in postpartum lactating rats with suckling litters, median eminence implants of prolactin induce regression of corpora lutea of lactation and reinitiate follicular growth and cycling.

Studies in our laboratory by Minaguchi et al., (1968) on the changes in pituitary prolactin content and concentration in female rats before and after the onset of puberty showed that prolactin levels remained uniformly low prior to puberty and increased significantly only after the onset of puberty. The increase in prolactin after puberty was attributed to estrogen secretion. This

suggests that prolactin may have no role in the onset of puberty in the rat except in a negative sense.

There are many physiological states in which there appears to be a divergence between prolactin and gonadotrophin secretion. These data suggest that there may be a complex interaction among the anterior pituitary hormones whereby secretion of one hormone may influence the production and/or release of another. Some investigators who implanted anterior pituitary hormones into the hypothalamus to demonstrate a hypothalamic feedback (Corbin and Cohen, 1966; David et al., 1966) suggested that a specific anterior pituitary hormone influences pituitary production of that hormone only. Our present results suggest that this hypothesis may not be correct, since hypothalamic implants or injection of prolactin appears to stimulate pituitary FSH release in the pre-pubertal rat.

IV. A Neuroendocrine Profile of Reproductive Functions in the Old Rat

A. Analysis of pituitary prolactin, FSH, LH and hypothalamic FSH-RF and PIF in old constant estrous rats

Objective

In general there is agreement in the literature regarding gonadotrophin secretion in the aged animal. Unlike the senile human, in which gonadotrophin secretion is high, most animals appear to show little change in

gonadotrophin secretion as they become old (Korenchevsky, 1961). Since the hormone assay methods used in arriving at these conclusions are now outdated and considered to be unreliable, the question of anterior pituitary hormone secretion in the aged animal once again poses itself.

It is well known that reproductive cycling activity is a good index of anterior pituitary function, and in old rats normal estrous cycles rarely occur (Aschheim, 1961; Mandl, 1961). Vaginal smears from old rats indicate 3 different abnormal patterns of cyclic activity. The first is characterized by constant vaginal cornification (constant estrus), the second by repeated pseudopregnancies and the third by no pattern at all (Mandl, 1961). Changes in pituitary gonadotrophin secretion could account for the age changes observed in the estrous cycle and ovary; however Zuckerman (1956) argues that loss of reproductive function is due merely to exhuastion of the ovary. In spite of this argument, ovaries from old anovulatory rats nevertheless contain mature follicles capable of ovulating (Aschheim, 1965).

The present study was undertaken to determine the changes in anterior pituitary LH, prolactin and FSH levels and in hypothalamic FSH-RF and PIF content in the senile constant estrous rat.

Materials and Methods

The animals used in this study were discard Sprague-Dawley breeding rats obtained from Spartan Animal Farms, Haslett, Michigan and from Holtzman Co., Madison, Wisc. Upon arrival in our laboratory the rats were approximately 12-15 months of age. The rats were housed in steel cages, fed the standard lab diet and received oxytetracycline in their drinking water (0.2 mg/ml) once weekly as a prophylactic measure against respiratory infections which are common in old rats. Experiments were not begun until the rats reached 20 months of age. At this time daily vaginal smears were taken and recorded. When it was ascertained that a rat was not cycling, but in constant estrus for 1 month or more, it was selected for experimentation. Rats found to be in constant estrus were killed by guillotine and the hypothalami and anterior pituitaries were removed. The hypothalami were placed in ice cold 0.1N HCl (0.1 ml per hypothalamus) and stored at -20°C. The pituitaries were weighed individually and stored at -20°C. A few days later the hypothalami were assayed for FSH-RF and PIF, and the pituitaries were assayed for FSH, prolactin and LH by methods described previously. The values obtained for the above hormones were compared with values obtained from 3 month old, normally cycling, female rats killed on the day of estrus.

The results of the FSH-RF, FSH and LH assays were analyzed for statistical significance by the methods of Bliss (1952), while the "t" test for paired observations was used for PIF and prolactin. Ovaries and uteri were cleaned, weighed and processed for histological study. Differences in organ weights were analyzed by Student's "t" test.

Results

Effect of aging on hypothalamic FSH-RF and pituitary FSH levels

Anterior pituitary halves incubated with hypothalamic extracts from aged rats released significantly more FSH into the medium than the corresponding anterior pituitary halves incubated with hypothalamic extracts from young control animals killed in estrus (Table 10). This indicates that the FSH-RF content of the hypothalamus increases with age. It can be seen in Table 11 that pituitary content and concentration of FSH were also higher in old than in young rats.

Effect of aging on hypothalamic PIF and pituitary prolactin and LH levels

When the male rat pituitary was incubated with hypothalamic extracts from old and young rats, similar amounts of prolactin were released into the media, indicating no difference in hypothalamic PIF content between young and old rats (Table 12). In contrast Table 12 shows

TABLE 10. Effect of aging on hypothalamic FSH-RF content

Condition and No. of rats	Hypothalamic equivalents/incubated pituitary	FSH released (ug/mg of pituitary)[ab]	λ[c]
Senile constant estrous rats (20)	0.25	10.50 (7.94 - 13.90)	
			0.128
Young adult rats in estrous (20)	0.25	5.18 (3.75 - 6.85)	

[a]Expressed as ug equivalents of NIH-FSH-S3.

[b]Mean and 95% confidence limits.

[c]Index of precision.

TABLE 11. Effect of aging on pituitary FSH concentration

Condition and No. of rats	FSH (ug/mg of wet pituitary)[ab]	λ[c]
Senile constant estrous rats (30)	2.50 (1.75 - 3.56)	.128
Young adult rats in estrus (30)	4.98 (3.66 - 6.76)	

[a]Expressed as ug equivalents of NIH-FSH-S3.

[b]Mean and 95% confidence limits.

[c]Index of precision.

TABLE 12. Pituitary prolactin concentration and hypothalamic PIF content of senile constant estrous rats versus young adult rats on the day of estrous

Exp. No.	Type rat	No. of rats	AP[1] prolactin conc. IU/100 mg	PIF content (AP prolactin released in vitro IU/100 mg AP)
1	Old constant estrus	(10)	5.62±1.27[2]	1.80±0.40
	Young cycling in estrus	(10)	1.89±0.44	1.61±0.21
2	Old constant estrus	(10)	6.13±0.74[3]	
	Young cycling in estrus	(10)	3.30±0.80	

[1]AP = anterior pituitary. Mean and standard error.

[2]P < .001 computed by using "t" test for paired experiments.

[3]P < .05.

that the pituitary prolactin content of old rats was significantly higher than that of young rats. Mammary glands from the old rats showed varying degrees of stimulation or regression, however most glands showed hyperplasia and many contained hyperplastic alveolar nodules (Figs. 17, 18), possibly a result of continued prolactin stimulation.

Table 13 shows that in old rats there was a significant increase in pituitary and uterine weights and a significant decrease in ovarian weight. The ovaries of senile constant estrous rats had a large number of well developed follicles and no corpora lutea (Fig. 19) which indicated that these rats were anovulatory. The ovaries of old rats showing a series of repeated pseudopregnancies had an abundance of large corpora lutea (Fig. 20) and showed evidence of follicular inhibition. The uteri of the old constant estrous rats were much larger than those of the controls (Table 13).

It can be seen in Table 14 that pituitary concentration of LH is significantly lower in old constant estrous rats than in the corresponding controls. LRF assays were not done in these rats.

Discussion

The results presented here demonstrate that there are changes in anterior pituitary hormone levels with

Fig. 17. Mammary gland showing many hyperplastic alveoli from an old constant estrous rat (x30).

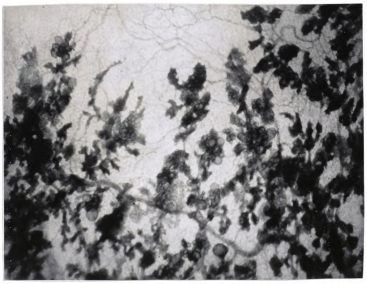

Fig. 18. Mammary gland showing many hyperplastic alveolar nodules from an old pseudopregnant rat (x30).

TABLE 13. Pituitary, ovarian and uterine weights, of old constant estrous rats and old pseudopregnant rats versus young adult rats in estrus

Condition of rat	No. of rats	Pituitary* Wt. (mg)	Ovarian Wt. (mg)	Uterine Wt. (mg)
Old, pseudopregnant	19	13.68±0.58[a]	93.26±7.16[a]	493±44.7[a]
Old, constant estrous	40	15.70±0.19[b]	39.30±2.33[b]	700±25.6[b]
Young, estrous	43	9.25±0.07[c]	68.40±2.1[c]	320±11.4[c]

*Mean ± SE.

[a,b,c]Values possessing different superscripts are significantly different from each other. $P < .01$.

Fig. 19. Ovary from old constant estrous rat. Note absence of corpora lutea (x15).

Fig. 20. Ovary from old rat which exhibited repeated periods of pseudopregnancy. Note predominance of corpora lutea (x15).

TABLE 14. Pituitary LH concentration of old constant estrous rats versus young adults on the day of estrus

Condition of rat	No. of rats	LH (ug/mg of wet pituitary)[ab]	λ[c]
Old constant estrous rats	(15)	3.07 (2.04 - 4.60)	0.179
Young adult cycling in estrus	(15)	8.51 (5.60 - 12.82)	

[a]Expressed as ug equivalents of NIH-LH-S1.

[b]Mean and 95% confidence limits.

[c]Index of precision.

aging, which may account for the loss of reproductive function observed at this period of life. Since Aschheim (1965) could reactivate the ovaries of senile rats with gonadotrophin injections, we can no longer accept the previous conclusion that loss of reproductive cycles is due to aging of the ovary.

These results suggest that the constant estrous state observed in old rats is a result of a malfunction arising in the hypothalamus, since the FSH-RF content of the hypothalamus was observed to be very high. The lack of ovulation in these animals can be attributed to the very low levels of LH. The low level of LH observed in these constant estrous rats may be due to death of neurons in the brain which are excitatory to LRF production or else possibly due to a lack of the neural transmitter substance responsible for stimulating neurons that secrete LRF. Such a lack of transmitter substance in old rats has been demonstrated by Frolkis (1966). Since these animals are unable to ovulate, they will remain in constant estrus. The high pituitary prolactin content paralleled the hyperplasia noted in many of the mammary glands. Although no hormone assays were performed on old rats demonstrating repeated periods of pseudopregnancy, the many corpora lutea found in the ovaries of these rats and the well developed

mammary glands suggest that prolactin secretion may have been high.

The high prolactin levels found in the pituitaries of the old rats is in agreement with the previous observations reported by Meites et al., (1961). The increase in pituitary prolactin is probably due, at least in part, to the estrogen secreted by the follicles of the constant estrous rats. This increased prolactin together with the continuous estrogen secretion by the ovaries may account in part for the increased incidence of mammary tumors found in old rats.

More work must be done in this area to determine changes in pituitary hormone secretion in rats with advancing age. In addition it would be helpful to explore possible changes in other age related endocrine changes from the standpoint of their neural control centers.

B. Effects of antiandrogen, progesterone, epinephrine, cervical stimulation, resperine and preoptic stimulation on the estrous cycle and on ovulation in the old constant estrous rat

Objective

Observations by Aschheim (1964-65) demonstrated that transplantation of young ovaries into old rats did not result in initiation of estrous cycles, providing evidence that the loss of reproductive cycling in old rats did not originate at the ovarian level. Furthermore,

Aschheim showed that ovulation and vaginal cycles could be restored in old constant estrous rats by means of LH administration. This suggested that the primary cause of ovarian dysfunction was a change in anterior pituitary hormone secretion.

Since anterior pituitary hormone secretion has been shown to be controlled by the hypothalamus, it is possible that changes within the hypothalamus may account for the loss of reproductive cycles. In the present study this possibility was investigated by the use of centrally acting drugs and hormones and direct electrical stimulation of the preoptic area of the hypothalamus. The preoptic hypothalamus was stimulated, since Everett (1966) has shown that this area controls ovulation. In addition, thyroxine, and antiandrogen and castration were used in an attempt to ascertain their effects on reproductive functions in old rats.

Materials and Methods

Rats similar to those used in the previous study were used in this study. General treatment of the animals was also the same. Progesterone, antiandrogen (Cyproterone acetate, Schering) and epinephrine were dissolved in sesame oil. Reserpine and thyroxine were injected in aqueous solutions. Progesterone, 4.0 mg was administered subcutaneously daily for 3 days. Other hormones were

administered subcutaneously daily for 14 days at the following dosages: antiandrogen, 10 mg; epinephrine, 0.25 mg; reserpine 50 mg and thyroxine at 1, 5 and 10 µg.

The preoptic hypothalamus was electrolytically stimulated by passing monophasic square wave pulses, 150 µ amperes intensity, 1.0 msec. duration and 100 per sec. for 2 minutes through stainless steel electrodes. While stimulation was in progress, current and waveform were continuously monitered on Tektronics 564 oscilloscope. The electrodes were composed of wire 0.05 mm thick and were bipolar. After electrodes were implanted a laparotomy was performed, and the ovaries were examined for corpora lutes. No corpora lutea were observed in any of the senile constant estrous rats. One week after stimulation a laparotomy was again performed, and the ovaries were again checked for corpora lutea to determine whether ovulation had occurred.

Results

The results of all treatments are shown in Table 15. Treatment with progesterone, which is known to act centrally in facilitating ovulation, caused 6 out of 10 animals to ovulate. Epinephrine which is also known to act centrally in many aspects of neuroendocrine function, produced ovulation in 11 out of 22 rats. Unlike progesterone, which only produced a few days of diestrus

TABLE 15. Effects of various drugs, hormones, castration and stimulation of preoptic area of the brain on ovulation and estrous cycles in old, anovulatory constant estrous rats

Treatment	No. of rats	Ovulation	Observed estrous cycles
Progesterone 4 mg daily for 3 days	10	6	Few days diestrus followed by constant estrus (8/10)
Epinephrine 0.25 mg daily	22	11	Many normal cycles (16/22)
Ovariectomy	3	0	Constant anestrus (3/3)
Reserpine 50 mg daily	6	0	Constant estrus (6/6)
Cervical stimulation	6	0	Constant estrus (6/6)
Antiandrogen 10 mg daily	10	0	Constant estrus (6/6)
Stimulation of preoptic hypothalamus	5	3	Few days diestrus (5/5) followed by irregular pattern
Thyroxine 1, 5 or 10 µg daily	18	0	Constant estrus (18/18)

after administration, epinephrine administration caused many normal appearing estrous cycles in 16 out of 22 rats.

Treatments which exerted no observable effects were Cyproterone acetate administration, cervical stimulation, reserpine treatment and daily administration of various doses of thyroxine.

Ovariectomy resulted in a permanent anestrous condition demonstrating that estrogen secretion from the ovaries was responsible for the constant estrus. In 3 out of 5 very old females (2 years old) with atrophic appearing ovaries and a history of 3 months of constant estrus, stimulation of the preoptic area of the hypothalamus caused ovulation. Implantation of electrodes without stimulation did not cause ovulation.

Discussion

The production of ovulation in old rats by the administration of centrally acting hormones and by electrical stimulation of one of the neural centers controlling ovulation, suggests that changes in brain function as a result of aging, possibly in the preoptic area, are at least in part responsible for the senile decline of ovarian function. We cannot entirely rule out aging of the ovary as a contributory factor to its own senescence. However, the fact remains that old ovaries in rats can be reactivated

and that ovarian failure is at a level higher than the ovaries.

The inability of the antiandrogen (Cyproterone acetate) to counteract the constant estrus observed in these animals eliminated the possibility of androgens causing the constant estrus. The anestrous condition observed after ovariectomy showed that the adrenals, which may be capable of secreting estrogens with advancing age (Verzar, 1966) was not responsible for the estrogen secretion. In addition it does not appear that decreased thyroid function can be the cause of ovarian failure, since administration of thyroxine in this experiment had no effect. Thus, it is evident that possible changes in other endocrine organs have little to do with the decline of ovarian function.

Since the ovary is entirely dependent upon pituitary gonadotrophin secretion for its functions, it is easy to conceive how a change in gonadotrophin secretion could severely impair its function. If a loss of gonadotrophin secretion were allowed to continue for a long period of time, severe ovarian atrophy could result. This may explain why not all animals ovulated in response to treatment with progesterone, epinephrine or preoptic stimulation.

It appears from this and the related study in the preceeding experiment that the altered secretion of

releasing factors by the hypothalamus and changes in anterior pituitary gonadotrophin secretion are probably due to some fundamental change in neural activity in the brain. It is not at all clear what the nature of this neural change is that accompanies the "menopause" in the rat or if it is located in the hypothalamus or in areas impinging on the hypothalamus. It may be possible that a fundamental neural change takes place with aging similar to that which takes place at puberty. The possibility exists that there may be a decrease in a neural transmitter substance for the release of LRF. Frolkis (1966) has observed a decrease in neural transmitter substances with aging. This possibility appears likely since electrical stimulation caused ovulation. Dead neurons could not respond. The exact nature of these neural changes awaits further investigation.

GENERAL DISCUSSION

In this thesis an attempt was made to determine the role of the hypothalamus in the feedback of prolactin and in various other physiological states. The evidence presented in the implant studies demonstrates that the hypothalamus is a site of pituitary prolactin feedback. The negative feedback effect of a prolactin implant in the hypothalamus is great enough to inhibit many normal physiological processes. The processes of pseudopregnancy, pregnancy, mammary development and lactation have been shown to be either terminated or severely impaired by these prolactin implants. From the data presented in this thesis there is little doubt that this negative feedback action by prolactin on pituitary prolactin secretion arises from increased hypothalamic PIF production. A rise in hypothalamic PIF content was noted in both intact and ovariectomized rats receiving prolactin implants. Since prolactin implants could produce an increase in hypothalamic PIF production in the absence of the ovaries, it is reasonable to assume that estrogen and progesterone are not needed for the operation of this feedback of prolactin upon the hypothalamus.

There have been numerous experiments demonstrating that the sex steroids, estrogen, testosterone and progesterone stimulate prolactin secretion; however none of these hormones has been shown to increase hypothalamic PIF content and on the contrary, they decrease PIF content. There are no reports suggesting that any hormone, with the exception of prolactin can increase hypothalamic PIF content and act as a negative feedback control for prolactin. The results of these studies therefore indicate that prolactin may constitute the major negative feedback control for prolactin secretion.

Anatomical basis for the short loop feedback is provided by the observations of Torok (1964) who reported that some portal vessels actually carry blood from the pituitary to the hypothalamus. It is easy to see how these vessles could play a role in the hypothalamic feedback, because they would contain a higher concentration of prolactin in the blood than in any other vessels. This upward flow of portal blood has not yet been confirmed by other workers. We do not yet know whether the prolactin levels in the systemic circulation normally become high enough to exert a negative feedback action upon the hypothalamus. Further work is necessary to establish to what extent the normal circulating levels of prolactin act as a negative feedback on pituitary prolactin secretion and

interfere with agents which may stimulate pituitary prolactin secretion, i.e. steroids, drugs, etc.

Another hypothalamic mechanism which may involve prolactin is that which determines the time of onset of puberty. At this time we can only speculate regarding this mechanism. The current hypothesis is that the hypothalamus becomes less sensitive to sex steroid feedback, and this results in greater quantities of gonadotrophin released and leads to the onset of puberty. This is an attractive theory, but it does not reveal the root of the problem. The basic, theoretical question is: what causes the hypothalamus to become less sensitive to sex steroid feedback? The results presented in this thesis indicate that when prolactin is implanted into the hypothalamus of immature female rats, the onset of puberty is significantly advanced. This may explain why many nonspecific mildly stressful stimuli are able to advance puberty, since they can also elicit prolactin secretion. These results also indicate that there is a complex interaction of anterior pituitary hormones at the hypothalamic level, because the prolactin implant also resulted in stimulation of gonadotrophin secretion. Therefore, it is possible that interactions among anterior pituitary hormones at the level of the hypothalamus may be partially responsible for puberty. The possibility cannot be ruled out that pituitary hormones may also act directly on the pituitary.

Neural changes responsible for the onset of puberty may be similar in some ways to those occurring at the time of the loss of cycling activity. From the experiments reported in this thesis it appears that loss of reproductive functions in rats is due to changes occurring in the hypothalamus. Much work remains to be done in elucidating the mechanisms producing these changes. It appears that these mechanisms are neural in origin since centrally acting drugs caused ovulation in the old rats with constant estrus. The fundamental site of failure to ovulate in old rats may be located in the preoptic area, since this area appears to control LH secretion and electrical stimulation of this area caused ovulation. Thus, it is apparent that the brain plays a major role in the initiation of reproductive activity in the young immature animal, and in loss of reproductive functions in old animals.

The results on the effects of hypothalamic lesions on development and growth of carcinogen induced mammary tumors in rats emphasizes the importance of the CNS in this process. It is probable that the increased release of prolactin resulting from the median eminence lesions was the principal factor influencing mammary tumorogenesis and growth. The fact that transplantation of extra pituitaries before and after carcinogen treatment of rats results in essentially similar decreases and increases in

mammary tumorogenesis, respectively, is in agreement with this view. Pituitaries transplanted to extra-hypothalamic sites are known to secrete mainly prolactin, and only small amounts of other hormones. The possibility cannot be excluded however, that other effects arising from the median eminence lesions may also influence mammary tumorogenesis. This appears to be particularly evident in the rats given median eminence lesions after tumors were already established by the carcinogen, when the tumors grew extremely rapidly. Further work with brain lesions is indicated.

The inhibitory effects of prolactin implants in the median eminence on mammary development also suggests that this may be a fertile approach for treating mammary tumors. In other words, for such experiments to be successful, it will be necessary to devise methods to insure that the effects of prolactin implants will be long-lasting. At the present time, the implants produce effects which last up to 10 days at the most. When means are devised for prolonging the prolactin effects, it should be possible to inhibit growth and development of mammary tumors. As an alternative, when purified PIF becomes available, administration of this neurohormone should similarly result in mammary tumor regression.

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APPENDIX I

CURRICULUM VITAE AND LIST OF PUBLICATIONS DURING GRADUATE STUDIES AT MICHIGAN STATE UNIVERSITY IN WHICH WRITER WAS AUTHOR OR CO-AUTHOR

CURRICULUM VITAE

NAME: Clemens, James Allen

DATE OF BIRTH: February 4, 1941

PLACE OF BIRTH: Windsor, Pennsylvania, U.S.A.

NATIONALITY: American SEX: Male

MARITAL STATUS: Married

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

FUTURE ADDRESS: Department of Anatomy
and Brain Research Institute
University of California at
Los Angeles.

EDUCATION:

Degree	Year	Institution	Major Field of Study
B.S.	1963	Pennsylvania State University	Zoology - Biochemistry
M.S.	1965	Pennsylvania State University	Zoology
Ph.D.	1968	Michigan State University	Physiology

HONORS:

(a) Elected associate member Sigma Xi, 1964.
(b) Elected full member Sigma Xi, 1967.
(c) Elected member of Phi Sigma Society, 1964.
(d) Outstanding graduate student award, Dept. of Physiology, Michigan State University, 1967.
(e) Graduate Student Research Award, Sigma Xi, 1967.
(f) NIH Postdoctoral Fellowship Award to begin September, 1968.

POSITIONS HELD:

(a) Teaching Assistant, Department of Zoology, Pennsylvania State University, 1963-1965.
(b) Instructor in Anatomy and Physiology, Pennsylvania State University, 1965.
(c) National Institutes of Health Trainee, Michigan State University, 1965-1968.

Curriculum Vitae
Clemens, James Allen

TALKS PRESENTED AT SCIENTIFIC MEETINGS:

	Meeting	Date	Topic
1.	Ann. Meeting of Mich. Acad. of Science, Brooklodge, Michigan	1967	Effects of hypothalamic lesions on DMBA induced mammary tumorigenesis in rats.
2.	51st Ann. Meeting of Fed. Am. Soc. Exptl. Biol., Chicago, Illinois	1967	Inhibition by hypothalamic lesions of mammary tumorigenesis induced by DMBA in rats.
3.	Fall Meeting of Am. Physiol. Soc., Washington, D.C.	1967	Prolactin implant into the median eminence inhibits pituitary prolactin secretion mammary growth and luteal function
4.	52nd Ann. Meeting of Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.	1968	Inhibition of lactation by median eminence implant of prolactin and ACTH.
5.	Co-author of paper presented at 3rd Internat. Cong. of Endocrinol., Mexico City, Mexico	1968	Growth and development of carcinogen induced mammary tumors as influenced by hypothalamic lesions
6.	24th Internat. Cong. of Physiological Sciences, Washington, D.C.	1968	Termination of pregnancy by median eminence implants of prolactin.

Curriculum Vitae
Clemens, James Allen

RESEARCH PUBLICATIONS
(During graduate studies at Michigan State University)

1. Clemens, J. A., and J. Meites. (1968). Inhibition by hypothalamic prolactin implants of prolactin secretion, mammary growth and luteal function. Endocrinology. 82: 878-881.

2. Minaguchi, H., J. Clemens and J. Meites. (1968). Changes in pituitary prolactin levels in rats from weaning to adulthood. Endocrinology. 82: 555-558.

3. Clemens, J. A. and J. Meites. (1967). Prolactin implant into the median eminence inhibits pituitary prolactin secretion, mammary growth and luteal function. The Physiologist. 10: 144, (Abstract).

4. Clemens, J. A., C. W. Welsch and J. Meites. (1968). Effects of hypothalamic lesions on incidence and growth of mammary tumors in carcinogen treated rats. Proc. Soc. Exp. Biol. Med. 127: 969-972.

5. Clemens, J. A., and J. Meites. (1967). Inhibition by hypothalamic lesions of mammary tumorigenesis induced by DMBA in rats. Fed. Proc. 26: 366, (Abstract).

6. Clemens, J. A., M. Sar and J. Meites. (1968). Termination of pregnancy by prolactin implants in median eminence of rats. To be presented at XXIV International Congress of Physiological Science, August, 1968. (Abstract in proceedings of Society).

7. Clemens, J. A., M. Sar and J. Meites. (1968). Inhibition of lactation by median eminence implants of prolactin and ACTH. Fed. Proc. 27: 269, (Abstract).

8. Welsch, C. W., J. A. Clemens and J. Meites. (1968). Effects of multiple pituitary homografts or progesterone on DMBA induced mammary tumors in rats. J. Natl. Cancer Inst. in press.

9. Welsch, C. W., J. A. Clemens and J. Meites. (1968). The effects of hypothalamic lesions on growth and development of DMBA induced mammary tumors in rats. Proc. Amer. Assoc. Cancer Res. 9: 76.

10. Welsch, C. W., J. A. Clemens and J. Meites. (1968). Growth and development of carcinogen induced mammary tumors as influenced by hypothalamic lesions. Experta Medica. 157: 203.

11. Welsch, C. W., M. Sar, J. A. Clemens and J. Meites. (1968). Effects of estrogen on pituitary prolactin levels of female rats bearing median eminence implants of prolactin. Proc. Soc. Exp. Biol. Med. In Press.

12. Clemens, J. A., H. Minaguchi and J. Meites. (1968). Relation of the local circulation between the ovaries and uterus on the life span of corpora lutea. Proc. Soc. Exp. Biol. Med. 127: 1248-1251.

Curriculum Vitae
Clemens, James Allen

MANUSCRIPTS IN PREPARATION

1. Clemens, J. A., M. Sar and J. Meites. (1968). Inhibition of lactation by prolactin and ACTH implants in the median eminence.

2. Clemens, J. A., M. Sar and J. Meites. (1968). Termination of pregnancy in rats by prolactin implants in the median eminence.

3. Clemens, J. A., H. Minaguchi, R. Storey, J. Voogt and J. Meites. (1968). Advancement of puberty in rats by prolactin injections and hypothalamic prolactin implants.

4. Voogt, J., J. Clemens and J. Meites. (1968) Stimulation of FSH secretion in immature rats by median eminence prolactin implants.

5. Welsch, C., J. Clemens and J. Meites. (1968). The effect of median eminence, preoptic and amygdaloid lesions on development and growth of carcinogen induced mammary tumors in female rats.

6. Clemens, J. A., Y. Amenomori and J. Meites. (1968). Analysis of pituitary prolactin, FSH, LH and hypothalamic FSH-RF and PIF in senile constant estrous rats.

7. Clemens, J. A., Y. Amenomori, T. Jenkins and J. Meites. (1968). Effects of drug and hormone administration and preoptic stimulation on estrous cycles and ovulation in senile constant estrous rats.

8. Clemens, J. A., T. Jenkins, C. Welsch and J. Meites. (1968). Effects of estradiol implants in the median eminence and constant light on carcinogen induced mammary tumors.

APPENDIX II

COMPUTER PROGRAM USED FOR STATISTICAL ANALYSIS OF DATA OBTAINED FROM HORMONE ASSAYS

EXPLANATION OF SYMBOLS USED IN

THE COMPUTER PROGRAM

Computer Language	Statistic
BB	B^2
s	SP (XY)
b	B
XXCOMB	(X^2)
SCOMB	ΣSP
BCOMB	b combined
BBCOMB	B^2 combined
SUMBB	ΣB^2
PREC	λ (index of precision)
PARAL	parallism

FORTRAN IV COMPUTER PROGRAM USED IN CALCULATING

THE RESULTS OF HORMONE ASSAYS

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'JOB, 302143,417274,5                    3                    CLEMENS, JAMES
'FTN,L,X,
      PROGRAM BLISS
      READ(60,1)AA,AB,AC,AD,AE,BA,BB,BC,BD,BE,CA,CB,CC,CD,CE,DA
      READ(60,2)DB,DC,DD,DE,EA,EB,EC,ED,EE,FA,FB,FC,FD,FE,GA,GB
      READ(60,3)GC,GD,GE,HA,HB,HC,HD,HE,OA,OB,OC,OD,OE,PA,PB,PC
      READ(60,4)PD,PE,QA,QB,QC,QD,QE,RA,RB,RC,RD,RE,SA,SB,SC,SD
      READ(60,5)SE,TA,TB,TC,TD,TE,A,B,C,D,E,F,G,T
    1 FORMAT(16F5.0)
    2 FORMAT(16F5.0)
    3 FORMAT(16F5.0)
    4 FORMAT(16F5.0)
    5 FORMAT(13F5.0,F6.0)
      SY1=AA+AB+AC+AD+AE
      SY2=BA+BB+BC+BD+BE
      SY3=CA+CB+CC+CD+CE
      SY4=DA+DB+DC+DD+DE
      SY5=EA+EB+EC+ED+EE
      SY6=FA+FB+FC+FD+FE
      SY7=GA+GB+GC+GD+GE
      SY8=HA+HB+HC+HD+HE
      SY9=OA+OB+OC+OD+OE
      SY10=PA+PB+PC+PD+PE
      SY11=QA+QB+QC+QD+QE
      SY12=RA+RB+RC+RD+RE
      SY13=SA+SB+SC+SD+SE
      SY14=TA+TB+TC+TD+TE
      SYY1=AA**2+AB**2+AC**2+AD**2+AE**2
      SYY2=BA**2+BB**2+BC**2+BD**2+BE**2
```

```
 SYY3=CA**2+CB**2+CC**2+CD**2+CE**2
 SYY4=DA**2+DB**2+DC**2+DD**2+DE**2
 SYY5=EA**2+EB**2+EC**2+ED**2+EE**2
 SYY6=FA**2+FB**2+FC**2+FD**2+FE**2
 SYY7=GA**2+GB**2+GC**2+GD**2+GE**2
 SYY8=HA**2+HB**2+HC**2+HD**2+HE**2
 SYY9=OA**2+OB**2+OC**2+OD**2+OE**2
 SYY10=PA**2+PB**2+PC**2+PD**2+PE**2
 SYY11=QA**2+QB**2+QC**2+QD**2+QE**2
 SYY12=RA**2+RB**2+RC**2+RD**2+RE**2
 SYY13=SA**2+SB**2+SC**2+SD**2+SE**2
 SYY14=TA**2+TB**2+TC**2+TD**2+TE**2
 AVE1=SY1/5.
 AVE2=SY2/5.
 AVE3=SY3/5.
 AVE4=SY4/5.
 AVE5=SY5/5.
 AVE6=SY6/5.
 AVE7=SY7/5.
 AVE8=SY8/5.
 AVE9=SY9/5.
 AVE10=SY10/5.
 AVE11=SY11/5.
 AVE12=SY12/5.
 AVE13=SY13/5.
 AVE14=SY14/5.
 SSY=SY1+SY2+SY3+SY4+SY5+SY6+SY7+SY8+SY9+SY10+SY11+SY12+SY13+SY14
 SSYY=SYY1+SYY2+SYY3+SYY4+SYY5+SYY6+SYY7+SYY8+SYY9+SYY10+SYY11+SYY1
12+SYY13+SYY14
 SSYSY=SY1**2+SY2**2+SY3**2+SY4**2+SY5**2+SY6**2+SY8**2+SY9*
1*2+SY10**2+SY11**2+SY12**2+SY13**2+SY14**2
 XY1=0.301*(SY2-SY1)
 XY2=0.301*(SY4-SY3)
```

```
 XY3=0.301*(SY6-SY5)
 XY4=0.301*(SY8-SY7)
 XY5=0.301*(SY10-SY9)
 XY6=0.301*(SY12-SY11)
 XY7=0.301*(SY14-SY13)
 B1=XY1/0.90601
 B2=XY2/0.90601
 B3=XY3/0.90601
 B4=XY4/0.90601
 B5=XY5/0.90601
 B6=XY6/0.90601
 B7=XY7/0.90601
 BB1=B1*XY1
 BB2=B2*XY2
 BB3=B3*XY3
 BB4=B4*XY4
 BB5=B5*XY5
 BB6=B6*XY6
 BB7=B7*XY7
 XXCOMB=(A+B+C+D+E+F+G)*0.90601
 XYCOMB=XY1+XY2+XY3+XY4+XY5+XY6+XY7
 BCOMB=XYCOMB/XXCOMB
 BBCOMB=XYCOMB*BCOMB
 SUMBB=BB1+BB2+BB3+BB4+BB5+BB6+BB7
 AVERS=SQRT((SSYY-SSYS/5.)/(8.*(A+B+C+D+E+F+G)))
 PREC=AVERS/BCOMB
 PARAL=(SUMBB-BBCOMB)*(A+B+C+D+E+F+G)*8./((SSYY-SSYSY/5.)*(A+B+C+D+
1E+F+G-1))
 AVERA=(AVE1+AVE2)/2.
 AVERB=(AVE3+AVE4)/2.
 AVERC=(AVE5+AVE6)/2.
 AVERD=(AVE7+AVE8)/2.
 AVERE=(AVE9+AVE10)/2.
 AVERF=(AVE11+AVE12)/2.
```

```
      AVERG=(AVE13+AVE14)/2.
      DELTAB=AVERA-AVERB
      DELTAC=AVERA-AVERC
      DELTAD=AVERA-AVERD
      DELTAE=AVERA-AVERE
      DELTAF=AVERA-AVERF
      DELTAG=AVERA-AVERG
      RELPOTB=10.**(DELTAB/BCOMB)
      RELPOTC=10.**(DELTAC/BCOMB)
      RELPOTD=10.**(DELTAD/BCOMB)
      RELPOTE=10.**(DELTAE/BCOMB)
      RELPOTF=10.**(DELTAF/BCOMB)
      RELPOTG=10.**(DELTAG/BCOMB)
      D=BBCOMB-AVERS**2*T**2
      CONLIMB=10.**(T*PREC*SQRT((BBCOMB/D)*(0.2+DELTAB**2/D)))
      CONLIMC=10.**(T*PREC*SQRT((BBCOMB/D)*(0.2+DELTAC**2/D)))
      CONLIMD=10.**(T*PREC*SQRT((BBCOMB/D)*(0.2+DELTAD**2/D)))
      CONLIME=10.**(T*PREC*SQRT((BBCOMB/D)*(0.2+DELTAE**2/D)))
      CONLIMF=10.**(T*PREC*SQRT((BBCOMB/D)*(0.2+DELTAF**2/D)))
      CONLIMG=10.**(T*PREC*SQRT((BBCOMB/D)*(0.2+DELTAG**2/D)))
      WRITE(61,6)B1,B2,B3,B4,B5,B6,B7,BCOMB,AVERS,PREC,RELPOTB,RELPOTC,R
     1ELPOTD,RELPOTE,RELPOTF,RELPOTG,CONLIMB,CONLIMC,CONLIMD,CONLIME,CON
     2LIMF,CONLIMG,PARAL
    6 FORMAT(*0*,16F7.3,/,7F7.3)
      END
RUN
```