STUDIES ON PROLACTIN SECRETION IN VITRO

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This is to certify that the

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STUDIES ON PROLACTIN SECRETION

IN VITRO

By

Charles Samuel Nicoll

AN ABSTRACT OF A THESIS

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ABSTRACT

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By Charles Samuel Nicoll

Prolactin secretion in vitro by explants of anterior pituitary (AP) glands from rats and other species was investigated. The AP explants were cultured by modified watch glass techniques in medium 199 for 3 to 6 days. The cultures were incubated in air or 95% O_2 - 5% CO_2 atmosphere at 35°C. The prolactin activities in the culture medium samples were determined by the local "micro" pigeon crop method. The results of these studies were as follows:

1. Explants from the APs of rats actively secreted prolactin in vitro in synthetic 199 medium. Prolactin activity was detectable in the medium of cultures which were incubated for periods up to 21 days. The AP glands from lactating rats produced about twice as much prolactin in vitro as the glands from non-lactating mature females. The AP fragments which were incubated in air atmosphere synthesized about as much prolactin during each day of culture as they contained at the

beginning of culture. The AP fragments incubated in the 95% O_2 - 5% CO_2 atmosphere produced from 3 to 11 times their initial prolactin content during each day of *in vitro* life and explant survival was much better than in cultures in air atmosphere. These results demonstrate that AP glands can actively secrete prolactin in a system which is virtually free of hypothalamic or other *in vivo* influences. This indicates that prolactin secretion is an autonomous characteristic of the AP.

2. Explants from the APs of female guinea pigs, rabbits and mice and of male mice and Cynomalogus monkeys actively secreted prolactin *in vitro*. The glands of male and female pigeons released very little prolactin into the culture medium. These results demonstrate that the APs of male and female mammals can secrete prolactin autonomously and indicate that there may be a basic physiological difference in prolactin secretion by mammalian and avian pituitaries.

3. Medium incubated without any tissue or with several non-pituitary tissues did not have prolactin activity and the culture medium did not potentiate the pigeon

crop response to prolactin.

4. Cutting the AP glands of rats into 6 explants resulted in about a 20% reduction in wet weight indicating a loss of 1/5 of the tissue. Incubation in 199 medium for 3 days caused a further loss in wet weight of 8%. At the end of 3 days of incubation, the explants contained about 84.5% of their initial prolactin content.

5. Incubation of rat AP fragments with hypothalamic or cerebral explants, or with homogenates of rat hypothalamic or cerebral tissue added to the medium, greatly reduced the prolactin activity in the medium samples. An acid extract of rat hypothalamic tissue significantly reduced prolactin secretion <u>in vitro</u> whereas a similar extract of cerebral tissue had no effect. The results with the extracts indicate that hypothalamic tissue may contain an acid extractable material which specifically inhibits prolactin secretion.

6. Neither oxytocin nor pitressin influenced prolactin secretion when added to the medium at concentrations of 0.1 U and 1.0 U per ml respectively.

7. Addition of estradiol to the 199 medium at concentrations of 0.05 and 0.5 μgm per ml increased prolactin secretion *in vitro*. No stimulation occurred with 2 μgm estradiol per ml. Thyroxine and triiodothyronine stimulated prolactin production *in vitro* at concentrations of 0.1 μgm per ml. Cortisol, at a concentration of 10 μgm per ml depressed prolactin secretion *in vitro* and 20 μgm/ml of corticosterone had no effect on prolactin production. Neither testosterone nor progesterone influenced prolactin secretion *in vitro* at a concentration of 2 μgm/ml. Concentrations of the latter steroids of 5 and 10 μgm per ml were toxic to the AP explants as was corticosterone at concentrations of 30 and 50 μgm per ml. These results indicate that estradiol, thyroid hormones and cortisol can influence prolactin secretion by a direct action on the AP cells. The lack of an effect of progesterone and testosterone *in vitro* indicates that the effects of these steroids on prolactin secretion *in vivo* may be indirect.

8. Insulin did not affect prolactin secretion *in vitro* indicating that insulin is not essential for prolactin synthesis.

9. A dose-response relationship was established for NIH prolactin in White King squabs for prolactin doses from 2 to 60 μgm. With 10 pigeons per dose a regression equation was obtained in which $y = 0.13 + 1.64\ x$, where y represents the pigeon crop response to x μgm of prolactin. The index of precision of the assay method was 0.34 and the variance ratio was highly significant ($F = 190.4$) thus demonstrating the high degree of acceptability of the assay method.

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Dedicated

to

my Mother

and

my Father

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INTRODUCTION

Considerable research in the last three decades has been devoted to evaluating the endocrine function of the anterior pituitary gland. It is becoming increasingly apparent that the function of the pars anterior is regulated to a considerable extent by the central nervous system (CNS). The hypothalamus is evidently the portion of the CNS which is most intimately associated with governing adenohypophysial secretion.

The modus operandi of hypothalamic control of the anterior pituitary gland has been shown to be of a neurohumoral nature rather than by direct innervation of the adenohypophysial cells. Neurosecretory cells in the hypothalamus liberate chemical mediators which are conveyed by the hypothalamic-hypophysial portal blood to the pars anterior where they influence the secretion of the endocrine products of the gland.

It is now generally accepted that the secretion of adrenocorticotropin (ACTH), thyrotropin (TSH), follicle stimulating hormone (FSH), luteinizing hormone (LH) and somatotropin (STH) is contingent upon humoral stimulatory

agents of hypothalamic origin. The secretion of prolactin by the pars anterior is exceptional in this respect since it seems to be independent of hypothalamic stimulation. Neural regulation of prolactin secretion appears to be accomplished by an hypothalamic engendered neurohumor which inhibits prolactin secretion. Procedures which prevent the hypothalamic neurohumors from gaining access to the adenohypophysis, such as pituitary transplantation, severance of the hypothalamic-hypophysial stalk or destruction of regions of the hypothalamus by electrolytic lesions, result in a pronounced reduction in the secretion of all of the hormones of the pars anterior except prolactin. Indeed, prolactin secretion is apparently favored by such measures.

It has not been established, however, whether prolactin secretion is in fact an autonomous characteristic of the anterior pituitary or whether secretion of the hormone depends upon some extrahypothalamic stimulation which can manifest itself when hypothalamic inhibition is removed. It also remains undetermined whether the hormones which alter anterior pituitary function, such as estrogens and thyroid hormones, accomplish this by an action on the adenohypophysis, on the hypothalamus or on both locations.

It was of interest, therefore, to determine whether anterior pituitary fragments could secrete prolactin in an organ culture system where *in vivo* influences could be virtually eliminated. Since a number of hormones are known to influence prolactin secretion *in vivo*, the effects of these hormones on prolactin production *in vitro* were also examined to determine if they influenced prolactin secretion by a direct action on the adenohypophysis.

REVIEW OF THE LITERATURE

The outlook of early endocrinologists was considerably influenced by the remarkable advances in the field of neurophysiology which occurred in the late nineteenth and early twentieth centuries. To establish the endocrine function of an organ, endocrinologists were compelled to eliminate all possible neural influences from their experimental situations. They became accustomed, therefore, to regarding the functions of the nervous and endocrine systems as being quite independent of one another. The paramount possibility of interrelation and interaction between these two co-ordinative systems consequently eluded the attention of physiologists generally.

Many of the early researchers who investigated the functional activity of adenohypophysial transplants in hypophysectomized animals erroneously concluded that normally functioning transplants were obtained (see Harris, 1955). This supported the concept, which was generally accepted for a time, that the pituitary performed its several functions without significant influence by the other organ systems of the body. A number of observations,

however, indicated that the pars anterior is influenced by the central nervous system.

I. Evidence for Neural Influence on Adenohypophysial Function

Marshall (1942) was one of the first to emphasize the relationships between the external environment and reproductive processes. He drew attention to the fact that a variety of environmental factors, such as food, light and temperature, are of importance in conditioning sexual periodicity and suggested that they operated through the central nervous system on the anterior pituitary. The influence of exteroceptive stimuli on thyroid and adrenal function was also recognized, and the mechanism by which certain psychic states influenced the activity of the target organs of the adenohypophysial trophic hormones remained unexplained. These observations, together with other phenomena, such as reflex induction of ovulation in rabbits consequent to the stimulus of coitus, and numerous endocrinopathies associated with hypothalamic disease such as Frohlichs adiposo-genital syndrome, prompted the investigation of the possibility that the activity of the pars distalis is influenced by the CNS.

II. Evidence for Hypothalamic Regulation of the Pars Distalis

Since the adenohypophysis originates as an evagination of the Rathke's pouch, which migrates from the roof of the mouth to become attached to the floor of the third ventricle, it seemed probable that the hypothalamus is the part of the CNS most directly concerned with the regulation of the adenohypophysis (Harris, 1955). Control of the function of the adenohypophysis by direct innervation of the cells was discounted due to the paucity of nerve fibers in the pars anterior (Rasmussen, 1938). On the other hand, there exists a network of fine blood vessels in the hypothalamic area at the base of the brain which coalesces into larger vessels leading down the pituitary stalk from the hypothalamus to the pituitary gland, where they again ramify to supply the blood sinuses in the adenohypophysis. This portal circulation, first described by Popa and Fielding (1930), is ideally suited to convey materials of hypothalamic origin directly to the anterior pituitary. The possibility was therefore investigated that the neurosecretory cells of the hypothalamus release humoral agents which are conveyed by the portal blood vessels to the anterior pituitary

where they activate the secretion of hormones from the gland. This possibility was first proposed by Harris (1937) and Brooks (1938).

The investigation of hypothalamic regulation of the adenohypophysis has employed a variety of procedures including pituitary transplantation, sectioning of the pituitary stalk, electrical stimulation of the CNS and hypothalamus, induction of hypothalamic lesions and administration of drugs. Electrical stimulation of the hypothalamus can induce increased secretion of adrenocorticotropin (ACTH), thyrotropin (TSH) and gonadotropins (FSH-LH) (Harris, 1955). Stimulation of the pituitary gland directly is ineffective in this respect. The electrical stimuli are believed to activate the release of humoral agents from the neurosecretory cells of the hypothalamus into the portal circulation. The neurohumors are then conveyed to the pars distalis where they stimulate the secretion of the tropic hormones.

The procedure of inducing relatively discrete electrolytic lesions in the hypothalamus has disclosed that ACTH (deGroot and Harris, 1950), TSH (Greer, 1952) and gonadotropin (Dey, 1943) secretion can be impaired. Massive

lesions depress growth hormone (STH) secretion (Reichlin, 1961). The lesions studies indicate that localized centers in the hypothalamus may be concerned with the regulation of ACTH, TSH and FSH-LH secretion. Injury to these centers either destroys the neurosecretory cells specifically concerned with regulating the secretion of each trophic hormone, or impairs the neural areas which govern the activity of the neurosecretory elements.

Transplantation of the adenohypophysis to an ectopic site in hypophysectomized animals or severence of the pituitary stalk, with measures to prevent re-establishment of the vascular connections between the hypothalamus and the pituitary (e.g., by insertion of a wax paper barrier), are procedures which divorce the pituitary gland from hypothalamic neurohumoral regulation. These measures have been found to cause a profound reduction in ACTH, TSH, FSH-LH and STH secretion (Harris, 1955).

Harris (1950) demonstrated that severence of the pituitary stalk of female rats resulted in a cessation of estrous cycles. Many of the rats, however, showed a resumption of cyclic activity after a few days. When serial sections of the hypothalmo-hypophysial region of these rats

were examined histologically after perfusion of the vascular system with India ink, it was found that the portal circulation had become re-established in the rats which showed a restoration of estrous cycles. The animals which did not return to estrous activity had more or less permanently disrupted portal connections. When pituitary grafts are placed beneath the median eminence of the hypothalamus of hypophysectomized rabbits (Jacobsohn, 1954) or rats (Harris and Jacobsohn, 1952), thus enabling the portal blood supply to become re-established, regular estrous cycles return. The animals became pregnant when mated and delivered normal young. Similar grafts in other sites did not support reproductive function. Nikitovitch-Winer and Everett (1958) showed that when pituitary transplants were re-transplanted from the kidney capsule to the median eminence of rats, estrous cycles were resumed and thyroid and adrenal function returned to normal. When the grafts were re-transplanted under the temporal lobe of the brain or were permitted to remain on the kidney there were no indications of a return of FSH-LH, ACTH or TSH secretion. It is therefore apparent that when the adenohypophysis is separated from its hypothalamic connnections, and then placed in a position which

enables regeneration of the hypothalamo-hypophysial portal supply, normal functional activity of the gland returns. These observations provide some of the most cogent evidence that hypothalamic regulation of the adenohypophysis is accomplished by a humoral mechanism.

Secretion of the adenohypophysial hormone, prolactin, is rather unusual in certain respects. Prolactin does not appear to participate in a feedback mechanism with its target organs, a feature which it shares with STH. In addition, prolactin secretion is apparently not depressed by the procedures which disrupt hypothalamic regulation of the pars distalis, such as transplantation, pituitary stalk section or hypothalamic lesions. In contrast, the production of all the other anterior pituitary hormones is greatly impaired by such disruptive measures. Indeed, prolactin secretion is apparently favored by measures which remove hypothalamic control from the adenohypophysis. The hypothalamic neurohumoral mechanism controlling prolactin secretion is therefore considered to be of an inhibitory nature, whereas all of the other adenohypophysial hormones apparently require neurohumoral stimulators. Hypothalamic regulation of prolactin secretion is considered in more detail in the following sections.

III. Evidence for Hypothalamic Regulation of Prolactin Secretion

A. The Suckling Stimulus

The first experimental indications that an exteroceptive stimulus influences prolactin secretion emerged from the observations of Selye (1934). He found that continued suckling of rats with ligated galactophores maintained the secretory activity and structural integrity of their mammary glands. Secretory activity in the mammary glands of unsuckled rats ceased rapidly and involution occurred shortly thereafter. Retardation of mammary involution in rats with excised nipples when other intact glands were nursed gave further evidence that suckling influenced prolactin secretion. This led to the suggestion that prolactin was released in response to suckling (Selye et al., 1934). Further evidence of the role of suckling in prolactin secretion was gained from studies on the effects of suckling on non-lactating cyclic female rats (Selye and McKeown, 1934). Induction of pseudopregnancy, mammary growth and initiation of lactation was observed in these virgin females. These observations on suckling and the maintenance of mammary integrity have been amply

confirmed by a number of studies (see Cross, 1961). The influence of exteroceptive stimuli on prolactin secretion is further illustrated by the observations that irritation of the nipples with turpentine retards mammary involution in rats and mice (Hooker and Williams, 1940; Mixner and Turner, 1941) and electrical stimulation of the nipples of estrogen primed rats initiates lactation (Maqsood and Meites, 1961).

More cogent evidence that suckling stimulates prolactin secretion was provided by studies on the effects of suckling on the pituitary prolactin content of lactating rats. After a period of nonsuckling, a rapid reduction of pituitary prolactin content from the prenursing level was observed in response to a brief period of suckling (Reece and Turner, 1937; Grosvenor and Turner, 1957). Regular suckling increases the pituitary prolactin content and maintains it at a higher level than in glands of nonsuckled postpartum rabbits (Meites and Turner, 1948). The observation that suckling causes degranulation of pituitary acidophils is further evidence that this stimulus influences prolactin secretion (Desclin, 1947) since the acidophils are generally associated with prolactin secretion (see Purves, 1961).

The suckling stimulus apparently influences the secretion of other anterior pituitary hormones. Gregoire (1947) and Tabachnick and Trentin (1951) presented evidence that nursing stimulates ACTH discharge. Ovariectomy does not result in the appearance of castration cells in the pituitaries of lactating rats when they are suckled (Desclin, 1947), suggesting that nursing inhibits gonadotropin secretion. Resumption of postpartum estrous cycles is hastened when litter size, and consequently the intensity of the nursing stimulus, is reduced in mice (Parks, 1926) and rats (Rothchild, 1960a). Clap (1937) observed that the interval from parturition to first estrous was prolonged in cows with increased frequency of milking or suckling. Evidence has been presented for a reciprocal relation between the secretion of FSH-LH and prolactin (see Meites et al., 1962), and it has been suggested that suckling promotes prolactin secretion primarily by depressing gonadotropin secretion (Donovan, 1960). In addition to the well-established fact that suckling stimulates oxytocin release from the neurohypophysis (Petersen, 1942; Cross and Harris, 1952), there is evidence that nipple stimulation promotes antidiuretic hormone (ADH) secretion

in a number of species (see Cross, 1961), as judged by inhibition of urine flow during application of the milking stimulus. It is thus apparent that the exteroceptive stimulus of suckling can considerably alter anterior and posterior pituitary function and these effects are presumably mediated via the CNS.

B. Effects of Drugs, Hormones and Nonspecific Agents on Prolactin Secretion

Many drugs, hormones and nonspecific factors have been tested in our laboratory to determine whether they can induce prolactin secretion, as indicated by (a) initiation of mammary secretion in estrogen-primed rats and rabbits and pseudopregnant rabbits and (b) maintenance of secretory activity and mammary integrity in postpartum rats after litter removal. The tranquilizing drugs reserpine, chlorpromazine and meprobamate were found to be effective in promoting prolactin secretion in rats and rabbits (see Meites et al., 1962). These drugs have occasionally been observed to promote breast growth and lactation in women (Sulman and Winnik, 1956; Winnik and Tennenbaum, 1955) and are reported to induce pseudopregnancy in rats (Barraclough and Sawyer, 1959). Kanematzu et al. (1961) recently

reported that reserpine produced a depletion of pituitary prolactin content three days after a single injection into rabbits, whereas Meites (1958a) found an increase seven days after injection. This suggests that reserpine increases both the synthesis and release of prolactin, but the latter effect diminishes by the seventh day. The action of reserpine may depend on its demonstrated ability to inhibit hypothalamic function (Gaunt et al., 1954; Bein, 1956), thereby removing the inhibition on prolactin secretion. The other tranquilizers may operate in a similar manner. Reserpine and chlorpromazine can inhibit FSH-LH release (Gaunt et al., 1954; Barraclough and Sawyer, 1959) and stimulate prolactin and ACTH discharge (Harwood and Mason, 1957).

The neurohormones epinephrine, nor-adrenaline, acetylcholine and serotonin were effective in stimulating prolactin secretion (Meites, 1959a; Meites et al., 1959b, 1960b). Anti-adrenergic and cholinergic drugs were also effective in this respect as were numerous other agents such as morphine sulfate, hypothalamic extracts and Guillemin's corticortropin releasing factor (CRF) (Meites, Nicoll and Talwalker; 1962). CRF is a polypeptide of hypothalamic

origin which Guillemin and colleagues have isolated and which they believe to be the principal neurohumoral agent responsible for ACTH secretion (Guillemin, 1962). The mechanism by which most of these agents elicit prolactin secretion is unknown. The materials may have acted directly on the pituitary gland, on the hypothalamus or elsewhere in the CNS.

Most of these drugs and other agents have been reported to cause ACTH discharge (Ganong and Forsham, 1960) and induce pseudopregnancy in rats (Swingle et al., 1951b). Since nonspecific stresses can induce prolactin secretion, as judged by initiation of lactation (Nicoll et al., 1960) and pseudopregnancy (Swingle et al., 1951b), it is quite likely that most of the factors which induce prolactin and ACTH secretion were operating as stressful agents.

C. Hypothalamic Lesions

The studies on the effects of hypothalamic lesions on prolactin secretion have disclosed that lesions do not depress production of this hormone. Indeed when prolactin secretion was affected by hypothalamic lesions, augmented production of the hormone was evidenced. Electrolytic lesions in the supraoptico-hypophysial tract (Cross and

Harris, 1952) or in the hypothalamo-pituitary area (Donovan and van der Werff ten Bosch, 1957) of lactating rabbits results in failure of milk ejection without interference with milk synthesis. Injection of posterior pituitary extracts or oxytocin enabled the young to obtain normal milk yields as judged by litter growth rate. Lesions in the dorsal and posterior regions of the hypothalamus did not interfere with either milk synthesis or removal (Cross and Harris, 1952).

The effects of hypothalamic lesions on prolactin secretion, as judged by lactational performance in post-partum rats, have been studied by several investigators. Yokoyama and Ota (1959a, 1959b, 1960) observed that the litters of lactating rats bearing hypothalamic lesions died within seven days after the operation despite vigorous suckling, and no milk was found in the stomachs of the young. Nevertheless, milk could be expressed from the mother's nipples even 10 days after lesioning, indicating a selective impairment of the regulation of oxytocin secretion without disturbing the production of milk. In a preliminary study McCann, Mack and Gale (1959) asserted that hypothalamic lesions in the supraoptico-hypophysial

tract inhibited the release of both prolactin and oxytocin. Since injections of oxytocin partially restored lactation in these rats, they suggested, in concurrence with the view of Benson and Folley (1956, 1957a), that oxytocin is the neurohumor which stimulates the release of lactogenic hormone from the pars distalis. The mammary glands of these rats were apparently not examined, however, to determine whether milk secretion had in fact been suppressed. In a subsequent report Gale et al. (1961) re-examined the effects of median eminence lesions in lactating rats. They observed that prolactin injections did not restore lactation, whereas cortisol was partially effective. They also observed a significant decrease in adrenal weight and concluded that the lesions interfered with ACTH rather than with prolactin secretion.

McCann and Friedman (1960) reported that lesions induced in the hypothalami of rats during proestrus or estrus resulted in pseudopregnancy, indicating increased prolactin or luteotropin (LTH) secretion. They concluded that the role of oxytocin in LTH discharge is a doubtful one since the secretion rate of LTH is augmented in the presence of lesions which abolish oxytocin secretion. Degranulation of

pituitary acidophils, subsequent to the placement of hypothalamic lesions in rats has been observed by Soulairac and Desclaux (1947). This can be considered as indirect evidence for increased prolactin release since the acidophils are associated with prolactin secretion (Purves, 1961).

Lactation has been induced in rabbits and cats by induction of hypothalamic lesions thus indicating augmented prolactin secretion. Haun and Sawyer (1960) placed lesions in several regions of the hypothalamus of overiectomized rabbits whose mammary glands had been developed by estrogen treatment. Lactation occurred only in animals with lesions in the medial basal tuberal region, suggesting that this area may inhibit release of prolactin in rabbits. Since this same area apparently controls the release of LH (Haun and Sawyer, 1960), they suggested, in agreement with the view of Everett (1954, 1956), that the neurohumoral factor(s) which traverses the hypophysial portal system to induce discharge of LH, also inhibits prolactin release. In postpartum cats whose mammary glands had regressed for a period of up to 2 months after litter removal, Grosz and Rothballer (1961) sectioned the tuber cinereum transversely just behind

the optic chiasma and observed the onset of milk secretion within 3-12 days. Since stimulation of this area induces estrus in the cat (Sawyer, 1960), these authors suggested that this region exerts a reciprocal control on the release of FSH and prolactin.

D. Pituitary Stalk Section

A number of investigators have studied hypothalamic regulation of the hypophysis by transecting the pituitary stalk, which connects the pituitary gland to the hypothalamus. This procedure disrupts the pathway of communication between the diencephalon and the pars distalis without disturbing the anatomical location of the gland. Studies on the effects of pituitary stalk section on prolactin secretion have been inconclusive and sometimes contradictory, and the degree of regeneration of the hypophysial portal vessels was usually not determined. Herold (1939) and Desclin (1940) reported that pituitary stalk section in rats during late pregnancy or early lactation resulted in failure of milk secretion as judged by growth of the suckling young. Dempsey and Uotila (1940) observed no inhibition of milk secretion in stalk sectioned rats. Jacobsohn and Westman (1945) found that mammary involution

after stalk section in lactating rats was slower than after weaning or hypophysectomy. Secretory activity was found to persist in the mammary alveoli even though most of the parenchyma had regressed. Retardation of mammary involution and maintenance of secretory activity are indicative of augmented, or at least, persistent prolactin secretion (Meites, 1959b).

Evidence that pituitary stalk section does not interfere with prolactin secretion in rabbits is provided by the studies of Jacobsohn (1949). Transection of the stalk of pregnant and lactating rabbits disclosed that mammary secretory activity was maintained and involution was retarded for up to 33 days after surgery. Despite vigorous suckling of the young, most died within 6-8 days of the operation. The completeness of stalk severence was determined by examining serial sections of the midbrain, pituitary and adjacent areas. When hypophysial stalk section was incomplete, the mammary glands were similar to those of unoperated rabbits and the young nursed normally. In a similar study, Donovan and van der Werff ten Bosch (1957) observed no reduction in milk volume provided oxytocin was administered to ensure milk ejection. A decrease

in litter growth rate was noted, however, which was attributed to a change in milk composition. Augmented prolactin secretion consequent to pituitary stalk section, as evidenced by induction of lactation, has been observed in women with breast cancer (Eckles et al., 1958).

It may be concluded from the above studies that removal of hypothalamic regulation of the pituitary gland, by severence of the pituitary stalk, does not depress prolactin secretion and may even foster the production of this hormone. Lactation failure consequent to pituitary stalk section appears to be primarily due to oxytocin deficiency rather than to inadequacy of prolactin.

E. Pituitary Transplantation

The clearest evidence that an organ is an endocrine gland is that the organ will maintain its normal function if transplanted to a distant site in the body. Many of the early studies with pituitary transplants in hypophysectomized animals claimed that functionally active glands were obtained. This supported the concept that the pars distalis functioned without major influence from the other organ systems of the body. Several reports, however, noted that hypophysectomized animals bearing pituitary

transplants showed reduced adrenal cortical, gonadal and thyroidal activity (Harris, 1955; Everett, 1962). When more thorough studies were performed, in which the completeness of hypophysectomy was carefully checked at autopsy, it was found that the pituitary gland differed markedly from other endocrine glands with respect to transplantation. Adenohypophysial transplants in completely hypophysectomized animals show a marked reduction in TSH, ACTH, STH and gonadotropin secretion (Harris, 1955; Everett, 1962). The earlier claims of functionally active transplants in hypophysectomized animals are therefore probably in error due to incompleteness of hypophysectomy.

1. Luteotropic activity of pituitary transplants. Prolactin is the only pituitary hormone which is known to exert a luteotropic action in rats and mice (Astwood, 1941; Evans et al., 1941). Induction of pseudopregnancy is therefore an excellent indicator of prolactin secretion by the adenohypophysis. Desclin (1949) first studied the effects of pituitary grafts on the luteal bodies of rats. He reported that administration of estrogen to hypophysectomized rats with single pituitary grafts under the kidney capsule evoked hypertrophy of the corpora lutea and

mucification of the vagina comparable to that seen in pregnancy. Everett (1954, 1956) confirmed Desclin's observation and further demonstrated that prolactin secretion was independent of estrogen treatment. He observed that the transplants could maintain continuous secretory function of the corpora lutea for up to 90 days in hypophysectomized rats, and suggested that prolactin secretion was actually favored by such isolation. In a subsequent study Desclin (1950) noted that estrogen induced more intensive luteinization of the ovaries of hypophysectomized rats bearing pituitary grafts, and the grafts showed a greater degree of degranulation of the acidophils and disappearance of the basophils. He concluded that estrogen increased prolactin secretion by the pituitary grafts. The luteotrophic action of pituitary transplants in rats has been thoroughly confirmed (Alloiteau, 1958; Quilligan and Rothchild, 1960; Sanders and Rennels, 1957).

Pituitary transplants in intact mice (Muhlbock and Boot, 1959) and rats (Quilligan and Rothchild, 1960) change the normal sequence of estrous cycles into a series of pseudopregnancies. Nikitovitch-Winer (1960) recently observed that massive destruction of the hypothalamus did not

interfere with LTH secretion by rat pituitary autografts. This indicates that materials of hypothalamic origin, which may be conveyed to the transplant by the systemic circulation, are not responsible for prolactin secretion by the grafts. Montemurro and Gardner (1961) recently noted that an increase in LTH secretion does not occur when the hypothalamus is transplanted together with the pituitary to the kidney capsule of intact mice. Most of the mice maintained normal estrous cycles for up to 54 days following transplantation. This study provides further evidence that the hypothalamus inhibits prolactin secretion.

2. Pituitary transplants and mammary growth and secretion. Desclin (1956a, 1956b) observed that single pituitary transplants to the kidney capsule induced lobulo-alveolar growth of the mammary glands of hypophysectomized rats; however mammary secretion was not observed. Meites and Hopkins (1960) compared the effects of single pituitary grafts on mammary secretion and lobulo-alveolar development in estrogen primed, intact and hypophysectomized rats. The grafts induced a much higher incidence of mammary secretion and better lobulo-alveolar development in the intact as

compared with the hypophysectomized animals. The low incidence of mammary secretion in the hypophysectomized rats was attributed to a deficiency of ACTH and this suspicion was confirmed by subsequent studies (Meites, Nicoll and Talwalker, 1962).

The effects of pituitary transplants on lactation in postpartum rats have been studied by several workers. Benson et al. (1958, 1959) implanted intact lactating rats with 3-6 pituitaties (derived from the mother's own 7-day-old pups) under the kidney capsule. The rats were mated and on the fourth day of their subsequent postpartum lactation, they were hypophysectomized and injected with oxytocin to allow the young to obtain milk. Some secretion was obtained, and this was slightly enhanced by STH injected and even more so by ACTH. In a similar experiment Cowie et al. (1960) transplanted pituitaries from 7-8-day-old rats to the anterior eye chamber or under the kidney capsule of lactating rats. The rats were mated and then hypophysectomized after parturition. Lactation could be partially maintained in these rats if the mothers were injected with oxytocin. Substantial increases in milk production occurred when ACTH was injected, while STH had

a lesser effect. Rothchild (1960a) also reported partial maintenance of lactation and litter growth rate when hypophysectomized rats with single pituitary transplants were injected with oxytocin 2-6 times daily. Milk production, as estimated by litter growth rate, was not returned to normal in any of these experiments. In a related study, Meites and Hopkins (1960) reported that single pituitary transplants to the kidney capsule retarded mammary involution in intact postpartum rats after litter removal. Since mammary growth, initiation and maintenance of lactation, and retardation of mammary involution are all dependent upon prolactin, these studies provide further evidence that pituitary transplants secrete prolactin and that secretion of this hormone appears to be an autonomous characteristic of the mammalian pituitary gland.

IV. Hormone Production by Pituitary in Vitro

Although there have been numerous culture studies of pituitary tissue, only a few investigators have attempted to study adenohypophysial hormone secretion and its regulation by tissue culture techniques. Anderson and Haymaker (1935) cultured anterior and posterior lobe tissue from

young rats and found that only melanophore stimulating hormone (MSH) showed evidence of increased activity in the culture medium. Definite hormonal restorative effects were noted upon the thyroids, ovaries and adrenals of hypophysectomized rats when material from a large number of cultures was assayed. The effects, however, were no greater than those produced by an extract of fresh, non-cultured pituitaries. Cutting and Lewis (1938) cultured pituitaries of rats for periods of 50-80 days and assayed the nutrient fluid for trophic hormone activity at various intervals of culture. Evidence of TSH, ACTH and gonadotropin activity was obtained only within the first 10 days of culture and was accounted for by release of the gland's initial content. Anterior pituitary explants, when cultured adjacent to osteogenic cells, were observed to promote growth of the cells suggesting STH activity (Gaillard, 1937). Substituting liver or salivary gland for adenohypophysial explants was ineffective in this respect.

More recently, Guillemin and Rosenberg (1955) and Guillemin (1956) reported that explants of dog and rat pituitary tissue failed to produce detectable quantities of ACTH after the 4th day in culture. Addition of hypothalamic

explants or homogenates to the cultures re-established detectable ACTH activity in the medium. Substituting cerebral cortex, liver or spleen for hypothalamic tissue did not stimulate ACTH secretion. Although pitressin preparations had ACTH-hypophysiotrophic activity in this in vitro system, purified vasopressin was ineffective. These observations provide further evidence for the existence of a specific material of hypothalamic origin which is necessary to stimulate and sustain ACTH secretion. Schaberg and DeGroot (1958) cultured adrenals from 5 day old rats in contact with anterior pituitary fragments. The cultured adrenals exhibited morphological changes and produced corticosteroids in a manner characteristic of ACTH stimulation. Evidence of ACTH activity by this system was demonstrated even after 12 days in culture. The discrepancies between the latter work and that of Guillemin and Rosenberg (1955) and Guillemin (1956) may be due to differences in the sensitivities of the systems employed.

Florsheim et al. (1957) attempted to demonstrate the existence of an hypothalamic stimulator of thyrotropin secretion by organ cultures of mouse adenohypophysis. TSH secretion was not detected in the medium after the first week of

culture and addition of hypothalamic fragments did not reinstate discernable TSH production. The hypothalamic fragments did, however, stimulate corticotropin secretion as was observed by Guillemin and Rosenberg (1955) and Guillemin (1956). Suspension cultures of human pituitary cells have been studied for hormonal activity. Samples of the suspensions were found to contain detectable quantities of STH, ACTH and gonadotropins (Thompson et al., 1959). Kobayashi et al. (1961) have reported that explant and monoloyer cultures of rat pituitary produce little FSH in vitro. Addition of hypothalamic extracts to the cultures increased FSH levels in the cells and culture medium. Extracts of the cerebral cortex were ineffective in this respect. This study provides convincing evidence for the existence of a material of hypothalamic origin which is necessary to maintain FSH secretion.

In all of the above studies on in vitro secretion of adenohypophysial hormones, hormonal activity, when detected, was found only for the first few days of culture. With the possible exception of MSH (Anderson and Haymaker, 1935), significant in vitro hormonal synthesis was not demonstrated in any of the studies. This is in accord with the abundant

evidence that secretion of most of the anterior pituitary hormones requires hypothalamic mediated stimulation. Prolactin is a notable exception in this respect since removal of hypothalamic regulation from the pituitary apparently favors prolactin secretion. The unusual *in vivo* behavior of prolactin might be expected to be manifested *in vitro* where the pars distalis can be maintained in an environment free of hypothalamic or other *in vivo* influence. Studies were therefore undertaken to determine if anterior pituitary fragments, from rats and other species, would secrete detectable quantities of prolactin into a synthetic medium when the explants were maintained by organ culture techniques. The effects of other hormones on prolactin secretion *in vitro* were also studied since the endocrine products of several glands apparently influence prolactin secretion *in vivo*. The objectives of these studies, in more detail, were as follows:

1. To determine if explants of anterior pituitary (AP) tissue from rats and other species could actively secrete prolactin *in vitro* and to compare the prolactin secreting capacities of explants from the APs of lactating and non-lactating rats.

2. To investigate the influence of explants, homogenates and acid extracts of hypothalamic and cerebral tissue on prolactin secretion *in vitro*.
3. To determine the effects of oxytocin and vasopressin on prolactin secretion *in vitro* since the neurohypophysial hormones are considered by many investigators to be the neurohumoral regulators of AP function.
4. To study the effects of the endocrine products of the gonads, thyroid and adrenal cortex on prolactin secretion *in vitro*.
5. To determine if insulin affects prolactin secretion by rat AP explants.
6. To evaluate the local, "micro" method of prolactin bioassay in the pigeon crop to determine the acceptability and precision of the assay procedure.

MATERIALS AND METHODS

I. Animals

Most of the 550 rats used in these experiments were of the Carworth CFN strain. In some of the preliminary studies, multiparous females weighing 270-300 grams were used. Three to four-month-old females weighing 180-220 grams were employed for most of the remaining experiments. Description of other animals used for the culture studies appears with each experiment where necessary. The animals were housed in an air conditioned room under uniform temperature (76 $\pm 1^{o}$ F) and lighting conditions.

Mature or 6-10 week old White Carneau pigeons (Palmetto Pigeon Plant, Sumpter, N.C.) were used as assay animals in the initial studies. The remaining assays were performed in 4-8 week old White King squabs (Cascade Squab Farm, Grand Rapids, Mich.). A total of 963 pigeons were used for these studies.

II. Culture Methods

The watch glass procedure of Fell and Robison (1929), as modified by Chen (1954), was used in the initial studies. This preparation consists of a 3 inch watch glass supported

by moist cotton or paper pulp inside a 4 inch Petri dish. The culture medium is placed in the watch glass and a raft of siliconized lens paper or cellulose acetate is placed on the medium. The raft supports the tissue explants at the medium-atmosphere interface. This enables the tissues to obtain nutrients from the culture medium and maintains them in a moist condition while providing optimal conditions for respiratory exchange. Although the Petri dish is closed in this system, an airtight seal is not obtained; therefore, the atmosphere inside the dish can exchange readily with the external gases.

In the preliminary studies rafts of cellulose acetate or urethane foam sponge were used. These did not prove to be satisfactory since the cellulose acetate rafts frequently sank in the medium and the urethane foam rafts often floated too high on the medium. Explant survival consequently suffered. A modification of the method of Trowell (1959) was therefore adopted. This consisted of using rafts of #46 grid stainless steel mesh (United Surgical Supply Co.) instead of lens paper or cellulose acetate. The rafts were made by cutting 1 cm x 2.6 cm rectangles from the sheets of stainless steel mesh. When in contact with the sides of

the watch glasses, the ends of the rectangles supported the raft at the surface of the medium. The explants were placed on sterile, washed lens paper rectangles (1 cm x 2 cm) and the lens paper was then placed upon the rafts. The ends of the lens paper draped over the sides of the rafts into the medium where they could act as wicks in the event of evaporative loss of the culture fluid. This system proved to be highly satisfactory from the standpoint of explant survival and hormone production; however, the size of the Petri dishes limited to 24 the number of cultures which could be simultaneously incubated in the culture chamber. In addition to this objection, the process of cleaning and sterilizing the watch glasses and Petri dishes was rather laborious, and the problem of residual contamination from the watch glasses could not be eliminated with absolute certainty. Further modifications were necessary to eliminate these problems.

Instead of using the watch glass - Petri dish preparations to hold the medium, rafts and tissues, sterile, disposable plastic Petri dishes alone were used. These plastic Petri dishes (Falcon Plastics, Inc.) have a diameter of 3.5 cm and a height of 1 cm. The stainless steel mesh

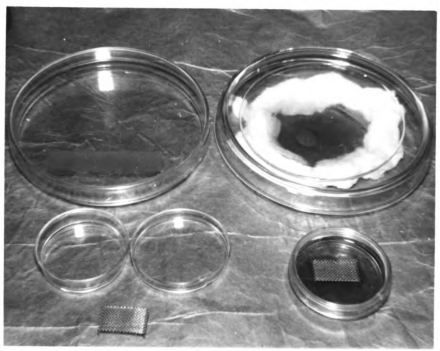


Figure 1. The glass and plastic Petri dish preparations. Upper left: a 10 cm x 1.5 cm sterile plastic Petri dish used for cutting AP glands into 6 explants. Upper right: the standard watch glass-Petri dish preparation for explant culture. Lower left: an open 3.5 cm x 1 cm sterile plastic Petri dish with a stainless steel mesh platform in front. Lower right: a 3.5 cm x 1 cm sterile plastic Petri dish containing 3 ml of medium and the stainless steel mesh platform.

rectangles were modified for use in the plastic dishes by bending each end at right angles to form a platform 1 cm wide, 1.8 cm long and 4 mm high. When these platforms were placed inside the plastic Petri dishes with 3 ml of medium they supported the explants, which were again placed on the lens paper rectangles, at the surface of the medium. This system proved to be highly satisfactory in all respects. Explant survival and hormone production was excellent; as many as 120 cultures could be incubated at one time and the dishes were discarded after use. The platforms were not discarded. The glass and plastic Petri dish preparations are shown in Figure 1 along with one of the 10 cm x 1.5 cm plastic Petri dishes which were used for cutting the pituitary glands.

A few of the initial cultures were incubated in air atmosphere; however, explant survival and hormone production left much to be desired. The remainder of the cultures were therefore performed in an atmosphere of 95% O_2 - 5% CO_2 in a plastic chamber. The chamber was constructed from a plastic mouse cage (10 x 10 x 8.5 inches) with an open top. The cage was inverted and placed on a sheet of 1/4 inch thick glass plate which was covered with a thin sheet of

polyethylene plastic. This provided a fairly good gas tight seal. The chamber was equipped with two gas inlets and two outlets. The O_2 - CO_2 gas mixture was passed through the chamber at a rate of about 200 cc per minute. Before entering the chamber the gas mixture was humidified by passing through a sintered glass filter which was immersed in distilled water in a glass cylinder. The cylinder was equipped with ground glass joints. The sintered glass filter caused the gas to form innumerable small bubbles which greatly enhanced humidification of the gas mixture. The filter also trapped any particulate material present in the gas.

The culture dishes were placed on square platforms of 1/4 inch thick plywood (9 1/4 x 9 1/4 inches) which were equipped with 3/4 inch legs on each corner. The plywood platforms were covered with Saran Wrap (Dow Chemical Corp.) and 6 were stacked one upon another in the culture chamber. Each of these platforms can accommodate 4 of the glass or 20 of the plastic Petri dishes. A plexiglass chamber was later constructed for gassing the cultures. This chamber measured 12.5 inches across the front, 9.5 inches in height and was 12 inches deep. The chamber was equipped with a

door which was held in place with screw clamps. The door had rubber weatherstripping insulation around the edges. The plexiglass used for this chamber was 1/4 inch thick. The shelves inside the chamber were constructed from 1/8 inch plexiglass and were perforated with numerous 1/4 inch diameter holes to facilitate thorough mixing of the gas.

The shelves were supported inside the chamber by strips of 1/4 x 1/4 inch plexiglass which were attached to the inside walls. They could therefore be removed and replaced readily by sliding them along the strip supports. Each of the shelves in this new chamber could accommodate up to 30 of the small sterile plastic Petri dishes. The chamber was equipped with 2 gas inlets and 2 outlets. One of the outlets was attached to a water manometer and when the chamber was in operation the gas inflow and outflow was adjusted to give an inter-chamber pressure of 3-5 mm Hg above atmospheric pressure. Difficulties have been experienced with the door and insulation of the chamber; therefore further modification is warranted. This chamber is shown in Figure 2. All cultures were incubated at 35 $\pm$ 1^{o}C.

Standard recommendations were followed in washing and sterilizing all glassware and instruments (Merchant et al.,

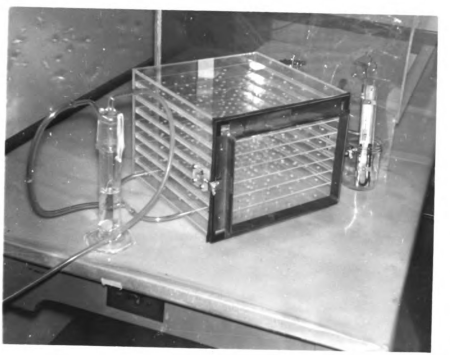


Figure 2. The plexiglass chamber used for gassing the cultures with the 95% O_2 - 5% CO_2 gas mixture. The cylinder containing water and the sintered glass filter, which was used for humidifying the gas mixture, is shown at the left. The water manometer is shown on the right of the chamber.

1960; Paul, 1960). The water used for the final rinses of the washed glassware and instruments was purified by distillation, passing through a deionizing resin column, then deorganified by percolation through another resin column. For preparation of the culture medium this water was purified further by a single distillation from glass. The stainless steel platforms were cleaned by soaking in 1/2 concentrated hydrochloric acid for 15 minutes, then washed in running tap water for 30 minutes. The rafts were then rinsed 4 times in the purified water, immersed in absolute ethanol for 30 minutes, immersed in diethyl ether for a further 30 minutes and finally allowed to dry in air. The platforms were placed in a Petri dish and sterilized.

All materials were sterilized by autoclaving except those which would be destroyed by the high temperatures, such as the insulin and antibiotic stocks. Such labile materials were sterilized by passing them through a Millipore bacteriological filter with a pore size of 0.45μ (Millipore Filter Corp.).

III. The Culture Medium

Synthetic Medium "199" was used exclusively for these studies. This medium contains all of the known essential

metabolites for cellular nutrition and maintenance in vitro but usually does not support cellular proliferation (Merchant et al., 1960). The "199" was obtained from Difco or Microbiological Associates, Inc. in bottles containing 100 ml of sterile, 10-fold concentrated stock solution. The ingredients used for the preparation of 100 ml of culture medium, with a pH of 7.4, for use in O_2 - CO_2 atmosphere, are shown in Table 1.

Table 1

PREPARATION OF 100 ML OF "199" CULTURE MEDIUM

Component	Volume
199 10x stock	10 ml
Antibiotic stock (5.0 mg streptomycin and 2,500 U penicillin per ml)	2 ml
Zn-free insulin stock (200 U/ml)	1 ml
5.6% $NaHCO_3$	6 ml
Purified water	81 ml

The final medium therefore had a concentration of 2U Zn-free insulin, 0.1 mg streptomycin sulfate and 50 U penicillin per ml. Insulin was used in the medium for most of the culture studies; however, it was found to be unnecessary

for prolactin secretion *in vitro* and was therefore omitted from the medium in the last few experiments. The $NaHCO_3$, Zn-free insulin and antibiotic stocks were sterilized by millipore filtration and stored in sterile, plastic 25 ml bottles until used. The antibiotic stock was frozen at $20^{\circ}C$ and the insulin and bicarbonate solution were stored in a refrigerator at $4^{\circ}C$. The Zn-free insulin stock was prepared by dissolving the insulin in a minimum of 0.002 N HCl and diluting to volume with purified water. Steroid hormones were added to the medium by first preparing concentrated stock solutions of the steroids in absolute ethanol. The ethanol stock was then added to give the desired concentration of the hormone with an alcohol concentration of 0.5% in the medium. Control medium for these studies contained an equivalent concentration of ethanol. The streptomycin sulfate and penicillin G potassium used in the culture medium were obtained from Nutritional Biochemicals Corporation.

IV. The Culture Procedure

The animals were decapitated and their heads were dropped for a few seconds into a beaker containing 70% ethanol. This moistens the hair of the heads and reduces

the chances of contamination of the cultures with air-borne rat hair. Usually 4 rat heads were handled together. The skull was exposed by cutting the skin with scissors and the top of the skull was removed with bone cutting scissors. The scissors were pre-soaked in 70% ethanol before use. The pituitary gland was then exposed by reflecting the brain with a pair of sterile forceps. A second pair of sterile forceps was used to separate and discard the posterior lobe of the pituitary and then the anterior lobe was removed. The gland was then placed in a sterile plastic 10 cm x 1.5 cm Petri dish containing a few drops of "199" medium.

Each anterior lobe was halved longitudinally and each half cut into 3 explants of about 2 mm diameter. Scalpels with #11 disposable blades (Crescent Mfg. Co.) were used for the dissection. The plastic Petri dishes were found to be much more satisfactory for dissecting the pituitary glands than glass Petri dishes containing moist filter paper. When the latter method was used, fragments of the paper often adhered to the explants and handling of the tissue was consequently encumbered. The explants were then placed on the

lens paper rectangles and transfered to a raft inside a culture dish into which the medium had previously been added. The culture dishes containing the explants were then transfered to the gassing chamber inside the incubator. The entire procedure, from removal of the glands to placing the tissues in the culture dishes, was performed inside a plexiglass hood (Figure 3). Sterility inside the hood was maintained by a 15 watt ultraviolet germicidal lamp which was usually left on when the hood was not in use. Immediately before use the inside of the hood was washed with 70% ethanol.

The medium from the few initial cultures which were incubated in air atmosphere was usually changed daily and only 1 ml of medium was placed in each dish. The duration of these cultures was usually 6 or 7 days. Medium from the cultures in O_2 - CO_2 atmosphere was changed every 3 days and 3 ml of medium was used in each dish. Many of the cultures in O_2 - CO_2 were incubated for 6 days; however, it was found that a 3 day incubation period was entirely satisfactory for most experiments. The medium from each culture dish was collected separately and stored in a freezer until used for assay. Medium from the cultures in air atmosphere

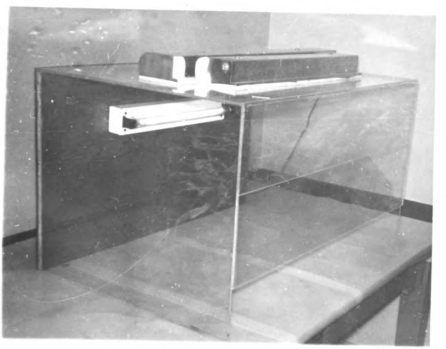


Figure 3. The plexiglass hood wherein the AP glands were removed from the animals heads and prepared for incubation. The ultraviolet lamp is shown on the inside rear wall of the hood. Two flourescent lamps are shown on the top.

was lyophilized and redissolved in water to a final volume suitable for administration of the entire quantity to 3 or 5 pigeons. A 2 ml aliquot of the medium from most of the cultures in the O_2 - CO_2 atmosphere was assayed in 5 birds and the total activity of the medium sample was calculated. The medium was pooled for assay in only a few of the cultures in air atmosphere. The medium samples from all of the remaining cultures were assayed separately without pooling. These studies involved a total of 542 cultures; however, the medium was not assayed from all of these.

Upon termination the explants from most of the cultures were weighed on a Mettler balance and fixed in Bouin's fluid or 10% neutral buffered formalin. The tissues were then embedded in paraffin, sectioned at 6μ and stained with hematoxylin and eosin. Attempts were made to differentially stain the explants by using several different procedures; however none of these gave satisfactory results.

V. The Assay Procedure

The culture medium was assayed for prolactin activity by the sensitive pigeon crop method of Lyons (1937) as modified by Reece and Turner (1937). The samples were injected daily in 0.1 ml volumes for 4 days in the same

intradermal site. Two samples were assayed in the same pigeon by injecting one sample over the left crop half and the other over the right side. The pigeon crop responds to prolactin by a proliferation of the mucosal epithelial cells. When prolactin is injected intradermally the mucosa thickens under the site of injection. The area and thickness of the response is a linear function of the logarithm of the dose of prolactin (see Experiment No. XV). The degree of the response is rated by a subjective method in units from 0.25 to 4.00 at intervals of 0.25. This subjective rating system takes both the area and the thickness of the response into consideration.

A dose-response standard curve was not established for the initial studies in which White Carneau pigeons were used. Prolactin standards were injected, however, which gave responses within the range observed with the responses from the medium samples. A rough estimate of the potency of the medium samples in International Units was therefore calculable. This procedure does not give results which are quantitatively exact; however, the relative differences are of value for comparative purposes.

A dose-response standard curve was established with

N.I.H. prolactin for the White King squabs and the values for the remainder of the assays were determined from this standard. At frequent intervals the birds were injected with prolactin standards to check for variations in sensitivity. As shown in Experiment XV, the sensitivity of the pigeons did not vary appreciably from that observed when the standard dose-response relationship was established. The same standard curve can therefore be used with reasonable certainty for conversion from Reece-Turner to International Units in all of the cases where this was performed.

Periodically the prolactin content of fresh rat anterior pituitary tissue was determined. Pituitary glands from the same type of rats as used in the culture studies were homogenized with a ground glass homogenizer in physiological saline or medium "199" and injected into assay pigeons.

VI. The Matched Pair Culture and Assay Procedure

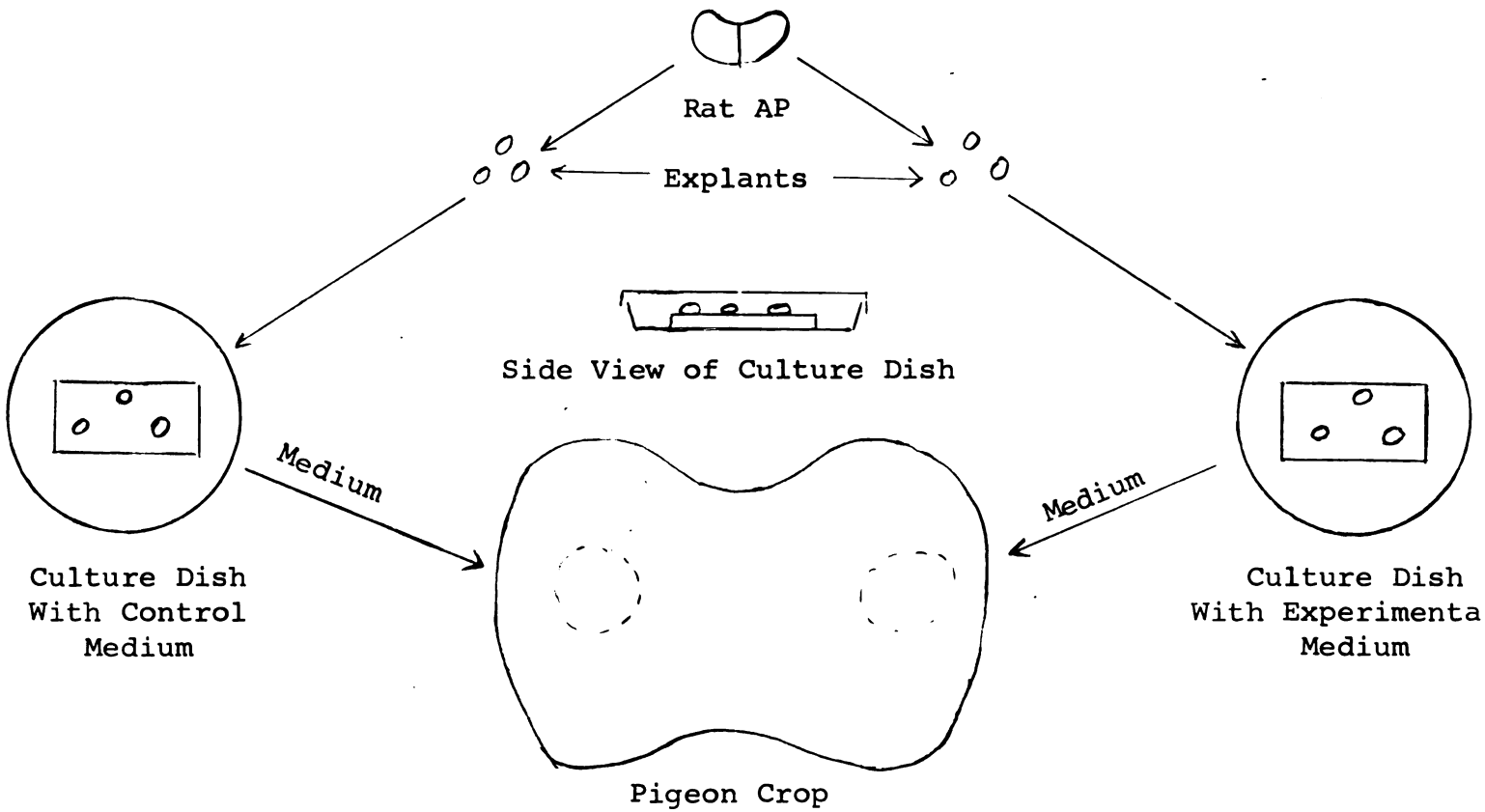
When the effect of a particular agent on *in vitro* prolactin secretion was investigated, a paired culture and assay procedure was used. This consisted of placing the explants from one-half of each anterior pituitary gland in a culture dish containing control medium and the explants

from the remaining halves in a dish containing the experimental medium. The experimental medium contained the material which was being tested. The control and experimental media were otherwise identical in composition. The control medium was assayed by injection over the right crop half of the assay pigeons and the experimental medium was injected over the left crop half. This permitted the influence of the agent on prolactin production by an equivalent amount of pituitary tissue from the same rats, cultured under the same conditions, to be ascertained in the same assay animals. A schematic representation of the paired culture and assay procedure is shown in Figure 4. When there was a difference in the degree of explant survival between the control and the experimental cultures, as judged by the histological appearance of the tissues, the media samples were not assayed.

VII. Hormone Preparations

The sources, forms, trade names and potencies of the hormones used in these studies are shown as follows:

Figure 4. Schematic representation of the paired culture and assay procedure.



Hormone	Source, Trade Name, Form and Potency
Prolactin	Endocrine Study Section, National Institutes of Health. Powder form. 15 IU/mg. Ovine.
Thyroxine	Smith, Kline and French Labs., Philadelphia, Pa. Elythrin: Sodium -L-thyroxine pentahydrate powder.
Triiodothyronine	Smith, Kline and French Labs. Powder form.
Pitressin	Parke, Davis and Co., Detroit, Mich. Powder form. 55 pressor and 4 oxytocic units per mg.
Oxytocin	Parke, Davis and Co. Pitocin. Aqueous solution in sterile ampuole. 10 IU/ml.
Insulin	Eli Lilly and Co., Indianapolis, Ind. Zn-free powder. 20 U/mg.
Estradiol	American Steroids Co., Puerto Rico. Non-esterified crystalline powder.
Testosterone	American Steroids Co. Non-esterified crystalline powder.
Progesterone	Nutritional Biochemical Corp. Non-esterified crystalline powder.
Cortisol	Merck and Co., Rahway, N.J. Non-esterified crystalline powder. Hydrocortisone.
Corticosterone	The Upjohn Co., Kalamazoo, Mich. Non-esterified crystalline powder.

VIII. Statistical Procedures

The students "t" test and the "t" test for matched observations were used for analysis of the data from these studies (Snedecor, 1956). The two-sided evaluation was used to determine the level of significance in all cases. Standard procedures for the analysis of bioassay data (Bliss, 1952) were used for the dose-response relationship established with standard N.I.H. prolactin preparations in the assay pigeons. The means and standard errors of the means are shown in the usual manner ($\bar{X} \pm S\bar{x}$) and the standard error of the mean was calculated by the formula:

$$S\bar{x} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n(n - 1)}}$$

EXPERIMENTAL

EXPERIMENT I. COMPARISON OF *IN VITRO* PROLACTIN PRODUCTION BY PITUITARY EXPLANTS FROM MATURE FEMALE AND POSTPARTUM LACTATING RATS.

In order to determine if anterior pituitary tissue from rats would secrete prolactin *in vitro*, the glands from mature female non-lactating and postpartum lactating rats were cultured by the watch glass technique. Six cultures were incubated in air atmosphere with the explants on cellulose acetate rafts. Explants from the pituitaries (APs) of 3 mature female rats (180-200 gm body weight) were placed in each of 4 culture dishes. An additional culture dish contained explants from 3 APs and another from 4 glands from 15 day postpartum lactating rats. The medium in each dish was changed daily and collected separately over the 7 day culture period. Medium from the first day of culture was discarded to remove possible contamination from cellular debri. The medium from the 4 cultures of mature female rat APs was pooled to give 2 samples each containing medium from 2 culture dishes. All of the culture fluid samples were lyophilized and redissolved in purified water to give a final volume suitable for administration to 3 or

5 pigeons. The medium from the cultures of APs from mature female rats was assayed by injection over the left crop sac of the pigeons and the culture fluid from the APs of lactating rats was injected over the right crop half. Each pooled sample of medium from the mature female rat APs was thus matched with a sample from the lactating rat APs.

In conjunction with the above assays, medium samples from similar cultures of rat AP tissue, obtained from Dr. R. H. Kahn*, were also tested for prolactin activity by the same assay procedure. Kahn used rats of the Sprague-Dawley (S.D.) strain and cultured the APs in Trowell T-8 medium by a watch glass procedure which utilizes stainless steel rafts (Merchant et al., 1960). He incubated the pituitaries for 3 days in an atmosphere of 95% O_2 - 5% CO_2. The number of APs per culture dish in Kahn's experiment is shown in Table 2 with the other assay results. Mature White Carneau pigeons were used for these assays.

Results and Discussion

The data presented in Table 2 show that prolactin activity was detected in the medium of all of the cultures.

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Furthermore, the assay results disclose that the medium samples from lactating rat pituitaries produced substantially more prolactin than the glands from the mature female animals. Prolactin production by the APs from lactating rats, on a per pituitary per day basis, was 100% greater than that of the mature females. This difference was significant ($P < 0.05$) and cannot be attributed entirely to a difference in the quantity of tissue per pituitary. Anterior pituitaries from similar mature female rats weighed about 8.7 mg and pituitary glands from similar lactating Carworth rats have previously been found to weigh about 11 mg (Johnson and Meites, 1958), or approximately 26% greater than glands from mature female rats. It is therefore apparent that about 74% of the excess prolactin production by the APs from the lactating rats, must be accounted for by factors other than a difference in the quantity of pituitary tissue.

Everett and Baker (1945) have reported that the pituitary glands of lactating rats show about a 60% increase in acidophil count above the prelactating level around the 16th day postpartum. Assuming that the same relative differences in acidophil count existed in the pituitary glands of the

non-lactating and lactating rats used in this culture experiment, these data indicate that most of the difference in the *in vitro* prolactin secreting capacities of the pituitaries is due to a difference in the number of prolactin producing cells. The pituitary acidophils are believed to be the cells which produce prolactin (see Purves, 1961). The 60% increase in acidophils can account for all but 14% of the difference which cannot be attributed to a difference in the quantity of tissue. This 14% difference may be due to a difference in the prolactin synthesizing capacity of the individual pituitary acidophil cells and suggests that the acidophils from the pituitary glands from lactating rats secrete more prolactin per cell than those of the non-lactating animals.

Meites and Turner (1948) reported that the pituitary glands of lactating rate contain 140% more prolactin at the 16th day postpartum than the glands from non-lactating females. The 100% difference in prolactin secretion between non-lactating and lactating rat pituitaries observed in this *in vitro* study supports the belief that the pituitary prolactin content can be indicative of *in vivo* prolactin secretion rate.

Histological examination of the explants which were cultured for 7 days disclosed that the tissue fragments had large areas of central necrosis. All of the explants possessed a thin outer rim of viable cells (Figure 5) and there was no obvious difference in the degree of cellular survival between the explants from the mature female or the lactating rats.

The prolactin levels found in the medium from Kahn's cultures, which were incubated in Trowell's medium in an atmosphere of 95% O_2 - 5% CO_2, are not appreciably greater than the levels reported here with 199 medium in air atmosphere. When we incubated rat AP explants in 199 medium in O_2 - CO_2 atmosphere, the explants produced about 8 times more prolactin than similar cultures in air atmosphere (see Experiment III). Since the APs in Kahn's cultures produced only a fraction of the amount of prolactin found in our cultures under similar conditions, it appears that Kahn's culture conditions were less satisfactory for prolactin secretion than our methods. Since explant survival was equally good in both instances, and it seems unlikely that the difference in the strain of rats could account for the difference in prolactin production, the difference may be

due to the composition of the culture media. Trowell's medium contains the antibiotic, chloramphenicol. Streptomycin and penicillin were used in the cultures with 199 medium. Since chloramphenicol has been shown to exert a pronounced depressant action on protem synthesis (Rendi, 1959), it is possible that this antibiotic is responsible for the lower levels of prolactin synthesis in Kahn's cultures.

Table 2

COMPARISON OF IN VITRO PROLACTIN PRODUCTION BY PITUITARIES OF MATURE FEMALE AND POSTPARTUM LACTATING RATS

Culture No.	Strain of Rat	No. of Pituitaries	No. of Birds per Assay	Type of Rat	Reece-Turner Prolactin Units in Medium			
					Total	Per AP	Per AP/Day	Difference
1	CFN*	6	3	Non-lactating	3.25	0.55	0.09	
	CFN	4	3	Lactating	8.75	2.19	0.36	+0.27
2	CFN*	6	5	Non-lactating	7.75	1.29	0.22	
	CFN	3	5	Lactating	7.25	2.42	0.40	+0.18
3	S.D.	4	3	Non-lactating	4.00	1.00	0.33	
	S.D.	4	3	Lactating	8.80	2.20	0.73	+0.40
4	S.D.	3	3	Non-lactating	3.00	1.00	0.33	
	S.D.	3	3	Lactating	3.90	1.30	0.43	+0.10

*Medium from 2 culture dishes pooled

Average of mature female rat pituitaries = 0.24

Average of lactating rat pituitaries = 0.48

Average difference = 0.24 ± 0.063 $t = 3.8$

$P < 0.05$

EXPERIMENT II. PROLACTIN SECRETION IN VITRO BY LONG TERM CULTURES OF RAT PITUITARY TISSUE.

Three cultures were incubated for 14 days and another 3 for 21 days in air atmosphere to determine if explants of rat anterior pituitary tissue could secrete prolactin for prolonged periods in vitro. All of the cultures contained explants from 3 mature female rat APs except one of the 14-day dishes which contained tissue from 3 lactating rat APs. One of the 14-day cultures contained estradiol in the medium at a concentration of 0.5 per ml. The medium was changed daily in the 14-day cultures. Each dish contained 1 ml of culture fluid. The medium used in the 21 day culture was buffered with Na_2HPO_4 and was changed every 3 days. Three ml of nutrient fluid was placed in each dish in these cultures. The explants were supported by cellulose acetate rafts in all of these cultures.

The medium from days 2-7 of the 14-day cultures was collected separately from each dish for assay. Medium from days 7-14 was similarly collected. The medium collected during the last 3-day culture interval (days 19-21) of the 3-week culture was pooled for assay. All medium samples were lyophilized, redissolved in water and each was assayed in 3 mature White Carneau pigeons.

Results and Discussion

The results of the 14-day culture are presented in Table 3. The pituitary explants obviously secreted considerably less prolactin during the second 7 day culture period than they produced during the first week of culture. The average prolactin production per pituitary per day during the first 7 days was 0.29 Reece-Turner units (RTU) for the 3 cultures. During the second week the cultures produced an average of 0.15 RTU per AP per day. This amounts to about a 50% reduction in prolactin production and is probably the result of death of the cells in the explant. Survival of the cells at the end of the 14-day culture was not as good as at the end of 7 days. The pooled medium sample from the last 3 days of the 21-day culture gave a total response of 4.4 RTU. The APs therefore produced 0.63 RTU/gland or 0.21 RTU per AP per day.

Histological examination of the explants from the 21-day culture revealed that very few viable cells remained and that tissue maintenance was even poorer than that observed at the end of 14 days. This was somewhat surprising since the prolactin activity of the 21-day cultures is intermediate between the values obtained at the end of 7

and 14 days of culture. With only one sample, however, no valid conclusions can be drawn from this observation regarding the relative quantities of prolactin produced. This experiment demonstrates that rat AP tissue can secrete prolactin for prolonged periods in vitro without participation of hypothalamic or other in vivo influences.

Recently Pasteels (1961a) reported the results of his study on prolactin in vitro during a three week culture period. He cultured the pituitary from one male rat in natural, growth promoting medium and observed that the cellular elements proliferated vigorously. The prolactin level in the medium was found to increase linearly with time. This observation of increasing prolactin activity in the medium with the duration of culture is probably due to the continued increase in the number of cells which produce the hormone rather than to a change in the prolactin secreting capacity per cell. No data were presented, however, which would permit the increase in prolactin secretion with the duration of culture to be compared with the increase in the cell population.

Table 3

IN VITRO PROLACTIN PRODUCTION BY RAT PITUITARY EXPLANTS OVER TWO SUCCESSIVE SEVEN-DAY PERIODS

No. of APs	Type of Rat	Medium	FIRST 7 DAYS				SECOND 7 DAYS			
			Days of Culture	Prolactin Units in Medium			Days of Culture	Prolactin Units in Medium		
				Total	Per AP	Per AP/Day		Total	Per AP	Per AP/Day
3	Mature female	199	6	2.75	1.29	0.22	7	1.25	0.42	0.06
3	Mature female	199 + estradiol	6	6.50	2.17	0.36	7	4.25	1.42	0.23
3	Lactating	199	6	4.75	1.80	0.30	7	3.50	1.17	0.17

EXPERIMENT III. EFFECTS OF 95% O_2 - 5% CO_2 ATMOSPHERE ON EXPLANT SURVIVAL AND PROLACTIN SECRETION IN VITRO.

Since explant survival in air atmosphere was not entirely satisfactory (Figure 5), most of the remaining cultures were incubated in an atmosphere of 95% O_2 - 5% CO_2. This procedure greatly improved explant survival and prolactin production by the pituitary explants. Assay results from 7 cultures in air atmosphere and 5 in O_2 - CO_2 are shown in Table 4. Each of the cultures contained explants from the pituitaries of 3 mature female rats on cellulose acetate rafts. The medium from 4 of the air atmosphere cultures was pooled to give 2 samples, each containing medium from 2 cultures. All of the medium samples were concentrated by lyophilization and redissolving in water for injection. Each sample was injected into 3 birds.

All of the cultures were incubated for 6 days. The medium from the air atmosphere cultures was changed daily and 1 ml of medium was used per dish. Medium from the cultures in O_2 - CO_2 was changed after 3 days and each dish contained 3 ml. Medium from the first day of culture was not discarded in any of these cultures. The medium from 2 of the O_2 - CO_2 cultures was collected separately over

the first and second three day culture periods and the medium from the second 3 days of culture (days 4-6) was assayed. In all other cases all of the medium from the 6 days of culture was assayed.

The anterior pituitary glands from 10 mature female rats were assayed for the determination of AP prolactin content by homogenizing in physiological saline and injecting into 5 assay birds. Mature White Carneau pigeons were used for these assays.

Results and Discussion

The assay results shown in Table 4 demonstrate that the pituitaries incubated in O_2 - CO_2 atmosphere secrete considerably more prolactin into the culture medium than the explants cultured in air atmosphere. The air cultures produced an average of 0.13 RTU per AP per day, whereas the O_2 - CO_2 cultures secreted 1.05 RTU/AP/day. This difference was highly significant ($P < 0.001$). Explant survival in the O_2 - CO_2 atmosphere was excellent (Figure 6) and the explants compared favorably with fresh, uncultured pituitary tissue in histological appearance (Figure 7). The beneficial effects of gassing on explant survival are thus reflected in the 8-fold greater prolactin production by the

pituitary tissue.

The assay results on the adenohypophyses of mature female rats disclosed that the glands contained an average of 1.25 $\pm$ 0.21 RTU per anterior lobe. When compared to the daily prolactin production of the glands in O_2 - CO_2 atmosphere, which averaged 1.05 RTU/gland/day, it is evident that the pituitary explants in vitro synthesized about as much prolactin during each day of culture as they contained when introduced into the culture system. A fair degree of net hormone synthesis by the glands in O_2 - CO_2 atmosphere was therefore realized.

The procedure of dissecting the pituitary glands was found to result in about a 20% loss in glandular tissue as judged by difference in pre- and post-dissection weight (see Experiment V). This indicates that in vitro prolactin production, on a per pituitary basis, should actually be 1/5 higher than reported since only about 4/5 of each gland was introduced into the culture dishes. When this difference is taken into consideration, it is evident that prolactin production each day, on a per pituitary basis, would actually be equivalent to the initial content of the tissue. This represents a substantial degree of hormone synthesis.

Table 4

COMPARISON OF PROLACTIN PRODUCTION BY PITUITARIES FROM MATURE FEMALE RATS CULTURED IN AIR AND 95% O_2 - 5% CO_2 ATMOSPHERE

6 DAY CULTURES IN AIR ATMOSPHERE						O_2 - CO_2 ATMOSPHERE						
Culture No.	No. of APs	Birds per Assay	Prolactin Units in Medium			Culture No.	No. of APs	Days Cultured	Birds per Assay	Prolactin Units in Medium		
			Total	Per AP	Per AP/ Day					Total	Per AP	Per AP/ Day
1	3	3	4.25	1.42	0.24	1	3	6	5	16.25	5.42	0.90
2	3	3	2.50	0.83	0.14	2	3	6	5	17.25	5.75	0.96
3	3	3	3.50	1.17	0.19	3	3	6	5	19.00	6.33	1.06
4	6*	3	3.25	0.55	0.09	4	3	3	5	9.63	3.21	1.07
5	6*	3	7.75	1.29	0.22	5	3	3	5	11.55	3.85	1.28

*Medium from 2 cultures pooled

Av. = 0.13 $\pm$ 0.06

Av. = 1.05 $\pm$ 0.065

t air vs. O_2 - CO_2 = 10.33

$P < 0.001$

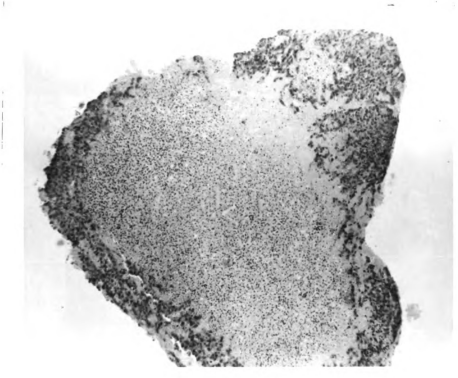

Figure 5. Photomicrograph of an explant of rat AP tissue cultured in air atmosphere for 6 days. The tissue consists of a large, lightly stained area of central necrosis with a thin peripheral shell of viable cells. Hematoxylin and eosin X 130.

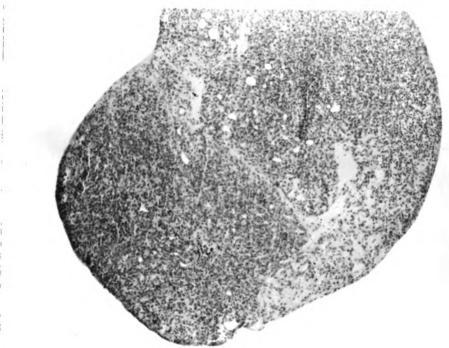

Figure 6. Photomicrograph of an explant of rat anterior pituitary tissue cultured in 95% O_2 - 5% CO_2 atmosphere for 6 days. Most of the cells are viable and normal in appearance. Only a few small areas of necrosis are present. The condition of the explant compares favorably with that of fresh, uncultured tissue in Figure 7. Hematoxylin and eosin X 130.

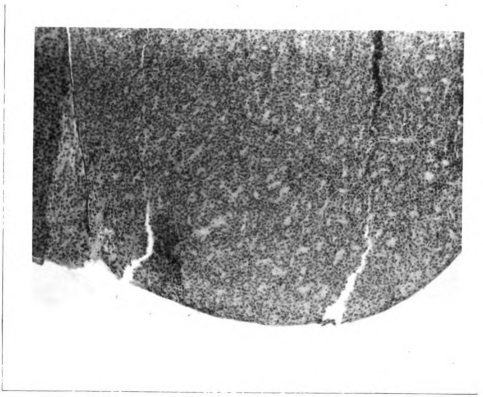

Figure 7. Photomicrograph of fresh, uncultured rat AP tissue. Hematoxylin and eosin X 130.

EXPERIMENT IV. ASSAYS TO DETERMINE IF 199 MEDIUM INCUBATED WITH AND WITHOUT TISSUES HAD PROLACTIN ACTIVITY AND THE EFFECTS OF 199 MEDIUM WITH INSULIN ON THE PIGEON CROP RESPONSE TO PROLACTIN.

Since substantial prolactin levels were found in the medium from all of the cultures with pituitary tissue, medium which was incubated in air atmosphere without any tissue was assayed to determine if the medium alone had pigeon crop stimulating properties. Medium which was used for culturing pigeon crop, guinea pig mammary or rabbit mammary tissue in O_2 - CO_2 atmosphere was also assayed. The cultures of pigeon crop and mammary tissues each had 6 explants per dish. Medium from 3 dishes of each tissue type was pooled for each assay. The medium incubated in air was lyophilized and redissolved to a final concentration equivalent to that used in the previous studies. Medium from the O_2 - CO_2 cultures was assayed without lyophilization. To determine if the 199 medium influenced the pigeon crop response to prolactin, the effects of the medium on the response to a standard dose of prolactin was determined. A solution of purified prolactin containing 25 μgm per ml was prepared in physiological saline. A solution was prepared in 199 medium with the same concentration of prolactin.

These standards were administered to 10 pigeons to give a total dose of 10 μgm of each preparation per bird. The saline solution was injected over the left crop of the pigeons and the 199 solution over the right. Each bird received 0.1 ml of each solution daily for 4 days. Young White Carneau birds were used for these assays.

Results and Discussion

The results of this study are shown in Table 5. None of the samples of medium incubated with or without tissue had prolactin activity. This demonstrates that the pigeon crop responses obtained from the cultures of pituitary tissue were due to something contributed to the medium by the AP explants. This material is undoubtedly prolactin.

The results of the pigeon crop responses to the standard prolactin solutions demonstrate that the insulin containing 199 medium had no effect on the pigeon crop response to prolactin. Both solutions gave results which were almost identical. The slight difference is not statistically significant. It may be concluded from these observations that the results from the assays of medium incubated with pituitary explants can be regarded as valid estimates of the prolactin activity of the culture fluid.

Talwalker and Meites (unpublished) have determined that prolactin is not inactivated by the incubation procedure used in these culture studies. In parallel studies on _in vitro_ prolactin secretion by pituitary "mammatropic" tumor tissue, they incubated solutions of ovine prolactin in 199 medium. The incubated material was then assayed for prolactin activity and compared with a portion of the same solution which was not incubated and stored in the frozen state. No difference in potency was found. This demonstrates that incubation per se does not alter the prolactin activity of the medium. The possibility exists that some of the prolactin secreted into the culture fluid by the pituitary explants is destroyed by cathepsins released by the explant cells, especially those which were dead or dying. This possibility cannot be satisfactorily verified, however, since the AP explants contribute prolactin to the medium and would therefore complicate attempts to determine the effect of AP tissue on a standard prolactin preparation. The use of non-pituitary tissues would not be strictly valid for this determination since other tissues may utilize the prolactin or release destructive cathepsins in a qualitatively and quantitatively different manner than AP explants.

Sgouris and Meites (1952) have demonstrated that prolactin is inactivated *in vitro* by the tissue of several different organs.

Table 5

ASSAYS TO DETERMINE IF 199 MEDIUM INCUBATED WITH AND WITHOUT TISSUES HAD PROLACTIN ACTIVITY AND THE EFFECTS OF 199 MEDIUM ON THE PIGEON CROP RESPONSE TO PROLACTIN

Days of Incubation	Atmosphere	Tissue	No. of Pigeons	Total Response
6	Air	None	3	0
6	O_2 - CO_2	Pigeon crop	3	0
6	O_2 - CO_2	Guinea pig mammary	3	0
6	O_2 - CO_2	Rabbit mammary	3	0

Sample	Solvent	No. of Pigeons	Average Response RTU
10 μgm prolactin	Saline	10	1.52 ± 0.09
10 μgm prolactin	199	10	1.50 ± 0.10

EXPERIMENT V. DETERMINATION OF THE QUANTITY OF GLANDULAR TISSUE LOST IN THE PROCEDURE OF DISSECTING THE ANTERIOR PITUITARIES OF RATS INTO 6 EXPLANTS.

In order to determine the quantity of AP tissue that is destroyed by cutting the APs of rats into 6 explants, the glands of 11 mature female rats were subjected to the dissection procedure. Each whole anterior lobe was weighed on a Roller-Smith balance before being placed in a plastic Petri dish which contained a few drops of 199 medium. Each gland was cut into 6 pieces and the fragments were blotted on Whatman No. 1 filter paper, which was moistened with 199 medium, before being reweighed.

Results and Discussion

The average weight of the 11 APs before dissection was found to be 9.52 $\pm$ 0.43 mg. After dissection the average weight of the 6 explants of each gland was 7.61 $\pm$ 0.47 mg. This represents an average weight loss of 1.90 $\pm$ 0.17 mg which is a highly significant reduction in tissue wet weight ($t = 11.2$; $P < 0.001$). The average percent loss in tissue weight was 20.47 $\pm$ 2.04 mg ($t = 10.01$; $P < 0.001$) which was also highly significant. The loss of 1/5 of the AP wet weight is probably due to crushing some of the cells

by the scalpel blade as the glands are cut into 6 explants. The blade may also tear some of the cells from the organ and rupture others causing the lost tissue to be left in the Petri dish. Manipulation of the tissue may also result in a loss of water which would contribute to the weight difference.

EXPERIMENT VI. EFFECTS OF IN VITRO INCUBATION ON THE WEIGHT AND PROLACTIN CONTENT OF ANTERIOR PITUITARY EXPLANTS.

An experiment was performed with the APs of 16 mature female rats to ascertain the effects of culturing on explant wet weight and prolactin content. Two APs were paired to provide tissue for determination of fresh gland prolactin content and for culturing. Each gland of each AP pair was halved and one-half was cut into 3 explants. The 6 explants from the 2 AP halves of each AP pair were cultured for 3 days in the plastic Petri dish preparations in O_2 - CO_2 atmosphere. The remaining 2 AP halves of each AP pair were weighed on a Mettler balance and prepared for assay. At the end of the culture period the 6 explants from each dish were weighed on the Mettler balance and prepared for assay. The explants from 2 of the culture dishes were combined in one case and the uncultured AP halves from the same pituitaries were also combined. In all other cases the explants from each culture dish and the fresh AP halves were treated separately.

The fresh and cultured AP tissue was prepared for assay by homogenizing in 199 medium at a concentration of 3 mg of AP per 0.4 ml of medium. The homogenates of the explants

from each AP pair were injected over the left crop half of 3 White King squabs and the homogenates of the 2 uncultured AP halves of the same AP pair were injected over the right crop half. The fresh tissue and explant content assays were, therefore, paired. Each bird received 3 mg of AP tissue by this assay procedure. The results of this study are shown in Tables 6 and 7. The standard dose-response plot (Experiment XV) was used to determine the potencies of these assays in IU.

Results and Discussion

The two pituitary halves of the 6 pituitary pairs had an average weight of 8.17 mg. After dissection and incubation the explants were found to have an average weight of 5.84 mg per cultured pituitary. Each AP therefore lost an average of 2.33 mg which represents an average weight reduction of 28.5 percent. This loss in weight is highly significant ($P < 0.001$). The previous study demonstrated that the dissection procedure caused a tissue loss which amounted to 20.47 percent. It is evident, therefore that incubation per se in 199 medium results in a wet weight loss of 8.03%. This slight difference is significant ($t = 2.13$; $P < 0.05$). The change in wet weight is probably

due to death of some of the explant cells with a consequent loss of the intracellular contents into the medium. Loss of red blood cells from the explants into the medium and a change in the degree of hydration of the explants may also have contributed to the weight reduction.

The assay results disclosed that the uncultured AP samples contained an average of 1.24 IU of prolactin whereas the explants contained 1.02 IU on the basis of 100 mg of tissue wet weight. This represents a reduction in prolactin content of 15.53% which is significant at the 1% level ($t = 3.33$). When the loss in weight of the explants due to incubation in 199 medium is considered, the reduction in prolactin content is increased by 7.47% and amounts of 16.69%.

Meites, Nicoll and Talwalker (1962) reported that AP transplants in the kidney capsule of intact female rats contain only about 1.5% as much prolactin as the <u>in situ</u> APs of the engrafted hosts. Since the AP transplants were secreting substantial quantities of prolactin, as judged by mammary growth and lactation, it is apparent that the transplant cells were releasing prolactin at such a high rate that the intracellular prolactin pool could only be maintained at a low level by the synthetic capacity of the cells. The results of the prolactin content of the explants are of

interest since one would expect the explants to behave in a manner similar to the transplanted AP with respect to prolactin release. The prolactin which escaped from the transplant cells must diffuse for a distance of only a few microns before being carried away by the circulation. Diffusion problems are therefore relatively unimportant in the transplant. In the cultured explants, however, the prolactin must diffuse through an extracellular distance which may amount to as much as 1 mm, after being released from the explant cells, before escape into the medium is possible. Since diffusion is not facilitated by circulation in the explants, the resistance to prolactin diffusion would presumably be rather large and consequently the synthetic capacity of the cells would be able to establish a relatively large intracellular, or perhaps more correctly, intra-explant prolactin pool. The permeability properties of the explant cells to prolactin, therefore, may not be appreciably different from those of the transplant cells. Part of the reduction in prolactin content of the AP explants and transplants is undoubtedly due to death of some of the cells; however it is not feasible to ascertain to what extent cellular death contributes to the lowered prolactin levels.

Table 6

WET WEIGHT OF UNCULTURED AND INCUBATED RAT AP TISSUE

No. of AP Halves	No. of APs	Total AP Weight mg	Weight/ AP mg	No. of AP Explant Halves	No. of APs	Explant Weight/ AP
8	4	32.3	8.1	4	2	4.3
4	2	15.2	7.6	2	1	5.8
4	2	15.4	7.7	2	1	5.7
4	2	17.4	8.7	2	1	6.8
4	2	14.6	7.3	2	1	5.8
4	2	20.4	10.2	2	1	7.1
4	2	15.1	7.6	2	1	5.6
			$\Sigma = 57.1$			$\Sigma = 40.9$
			$\bar{x} = 8.17 \pm 0.38$			$\bar{x} = 5.84 \pm 0.34$

Table 7

EFFECT OF IN VITRO INCUBATION ON THE PROLACTIN CONTENT OF RAT AP EXPLANTS

Pair No.	IU Prolactin per 100 mg Tissue		Percent Difference
	Fresh	Explants	
1	1.57	1.33	- 15.3
2	1.67	0.97	- 41.9
3	1.40	1.17	- 16.4
4	0.90	0.93	+ 3.3
5	1.17	0.96	- 17.9
6	1.10	0.96	- 12.7
7	0.90	0.83	- 7.8

$\Sigma = 108.7$

$\bar{x} = 15.53 \pm 4.66$

$t = 3.33$

$P < 0.01$

EXPERIMENT VII. PROLACTIN SECRETION IN VITRO BY PITUITARY GLANDS FROM DIFFERENT SPECIES.

Since prolactin secretion in vitro was so readily demonstrable with rat AP tissue, it was decided to examine the pituitary glands from a number of different species to determine if they could liberate detectable quantities of prolactin into 199 medium. Glands from 2 male Cynomalogus monkeys (Macacus Irus), 3 female guinea pigs, 3 nonpregnant multiparous female rats, 2 mature female rabbits, 5 male and 10 female mice and 12 pigeons of undetermined sex were used. The APs from monkeys, guinea pigs and rats were cultured one gland per dish. The 5 male mouse glands were placed in one dish, and 5 of the 10 female APs were cultured in each of 2 dishes. Four of the 12 glands from pigeons were cultured in each of 3 dishes. The explants were placed on rafts of urethane foam sponge and cultured for 6 days. One of the monkey and all of the rabbit AP cultures were incubated in air atmosphere. All of the other cultures were incubated in O_2 - CO_2 atmosphere.

At the end of the 6 day culture period, the explants from most of the cultures were weighed before being fixed for histological examination. As before, the medium from

the culture in air atmosphere was changed daily and each culture dish contained 1 ml of 199. The O_2 - CO_2 cultures were incubated with 3 ml of medium which was changed after 3 days. The medium samples from the guinea pig, rat and mouse cultures were each assayed in 5 pigeons. The samples from the monkey and pigeon cultures were assayed in 3 birds. Mature White Carneau pigeons were used for these assays.

Results and Discussion

The results from the assays of the medium samples are shown in Tables 8 and 9, together with data on gland content studies from earlier investigations (Meites and Turner, 1950). In Table 8 the results are averaged for the three pigeon cultures, 2 female mice culture dishes and the 3 rat guinea pig cultures. Prolactin activity for the culture data was approximated to International Units by using the relationship 1 IU = 8 RTU. This relationship was found in the response range of these assays in mature White Carneau pigeons. Although this conversion does not give values in IU which are quantitatively exact, the estimates are useful for the comparisons made with the data.

Prolactin activity was found in the medium from the cultures of all of these species. Although the culture data are of a rather limited nature, it will be noted that when the prolactin contents per mg of AP tissue and the prolactin activities of the medium samples per mg of explant tissue are ranked in increasing order of magnitude, an identical order is obtained. This constitutes additional evidence that the prolactin content of the AP can be a meaningful, though not an invariable indicator of the secretion rate of this hormone. It is also evident from Table 8 that the pituitaries from mice, rats and guinea pigs produced in 6 days from 9.5 to 15.8 times as much prolactin per mg of tissue in vitro as was contained in the freshly excised glands. This represents substantial net hormone production. The pigeon pituitaries, on the contrary, produced only 1.57 times as much prolactin as is present in the fresh gland. This indicates that little or no net synthesis of hormone was effected by the avian glands, and may reflect a basic physiological difference between avian and mammalian species. Nalbandov (1962) has recently reported that transplants of anterior pituitary glands of chickens do not secrete prolactin (luteotropin). This is

in contrast to the results from mammalian AP transplants (see review of literature) and is in agreement with the present studies.

The observation that anterior pituitary tissue from male mice and monkeys can actively secrete prolactin in vitro is of interest since no function for prolactin has been established in male mammals. This comparative study illustrates that pituitary tissue from both male and female mammals are capable of producing considerable quantities of prolactin in vitro. Photomicrographs of fresh rat AP tissue and of rat, monkey, guinea pig, and pigeon AP explants are shown in Figures 8, 9, 10, 11 and 12 respectively.

Table 8

COMPARISON OF PITUITARY PROLACTIN CONTENT AND IN VITRO PROLACTIN SECRETING CAPACITY OF ANTERIOR PITUITARIES FROM DIFFERENT SPECIES (6 DAY CULTURES IN 95% O_2 - 5% CO_2 ATMOSPHERE)

Species	IU* Prolactin per Mg AP	Rank	Total IU Prolactin in Medium per Mg/AP Explant	Rank	Prolactin Activity in Medium/Mg Explant / Prolactin Content/Mg of Fresh Tissues
Pigeon Male & female	0.014	-	0.022 (3)+	-	1.57
Male mouse	0.005	1	0.079 (1)	1	15.80
Female mouse	0.011	2	0.105 (2)	2	9.54
Female rat	0.021	3	0.212 (3)	3	10.09
Female guinea pig	0.029	4	0.337 (3)	4	11.62

*From Meites and Turner, 1950

+ = number of culture dishes

Table 9

PROLACTIN CONTENT OF MEDIA FROM 6-DAY CULTURES OF MONKEY AND RABBIT AP TISSUE

Species	Atmosphere	Total IU
Male monkey	Air	0.34
Male monkey	O_2 - CO_2	0.41
Female rabbit	Air	1.00
Female rabbit	Air	1.18

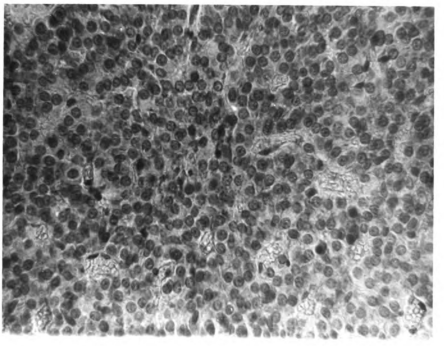

Figure 8. Photomicrograph of fresh, uncultured rat AP tissue. Hematoxylin and eosin X 450.

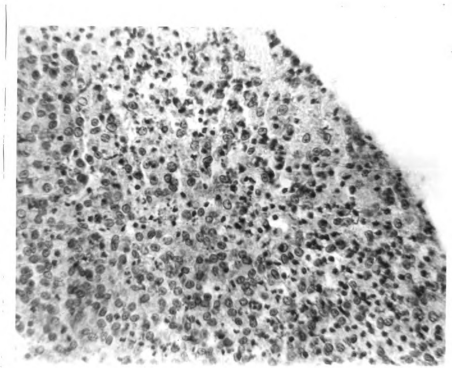

Figure 9. Photomicrograph of an explant of rat AP tissue cultured in 95% O_2 - 5% CO_2 atmosphere for 6 days. Most of the cells are viable and normal in appearance and compare favorably with the cells of fresh, uncultured tissue in Figure 8. Hematoxylin and eosin X 450.

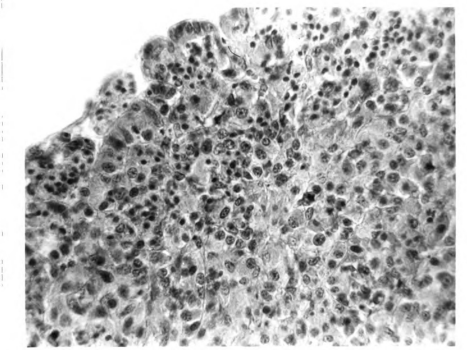

Figure 10. Photomicrograph of an explant of AP tissue from a male Cynomalogus monkey cultured for 6 days in 95% O_2 - 5% CO_2 atmosphere. Hematoxylin and eosin X 450.

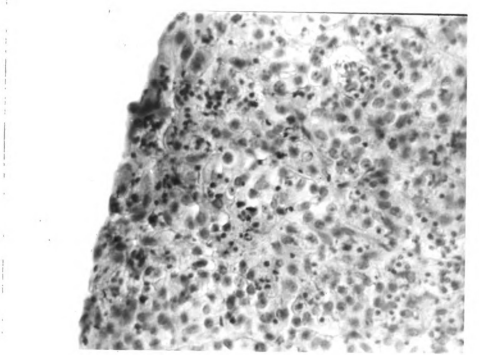

Figure 11. Photomicrograph of an explant of AP tissue from a mature female guinea pig cultured for 6 days in 95% O_2 - 5% CO_2 atmosphere. Hematoxylin and eosin, X 450.

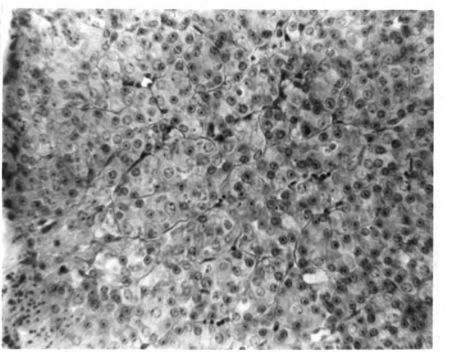

Figure 12. Photomicrograph of an explant of pigeon AP tissue cultured for 6 days in 95% O_2 - 5% CO_2 atmosphere. Hematoxylin and eosin, X 450.

EXPERIMENT VIII. EFFECTS OF HYPOTHALAMUS AND CEREBRUM ON PROLACTIN SECRETION IN VITRO.

Prolactin secretion is apparently augmented by procedures which disrupt hypothalamic regulation of the pars distalis, such as AP transplantation, pituitary stalk section and induction of hypothalamic lesions (see review of literature). The concept has emerged from these observations that prolactin secretion is usually inhibited by the hypothalamus. This was first proposed by Everett (1954, 1956). An hypothalamic neurohumoral agent is believed to be conveyed to the AP by the portal circulation, where it acts on the AP cells to block secretion of prolactin. This hypothetical neurohumor of hypothalamic origin will be called the prolactin inhibiting factor (PIF). When circumstances prevail which warrant active prolactin secretion, as during lactation or pseudopregnancy, the release of PIF from the hypothalamic neurosecretory elements would be reduced and consequently inhibition of prolactin secretion diminished. Elevated secretion of prolactin by the AP would ensue consequently.

Considerable evidence has accumulated from in vivo and in vitro studies which supports the notion that secretion of

anterior pituitary hormones, other than prolactin, is dependent upon, and regulated by, neurohumoral agents of hypothalamic origin. Guillemin and colleagues (see Guillemin, 1962) have isolated a polypeptide from the hypothalami of a number of species which they believe is responsible for stimulating and sustaining ACTH secretion. This material is called corticotropin releasing factor (CRF) and is effective in promoting ACTH release in animals in which ACTH secretion has been blocked by procedures such as morphine or glucocorticoid treatment or hypothalamic lesions. Short term incubation studies with AP tissue have shown that CRF will increase ACTH release in vitro when added to the incubation fluid.

McCann and Taleisnik (1961) and McCann (1962a) have studied the effects of acid extracts of rat median eminence tissue on the release of LH from rat AP glands. Ovariectomy was found to greatly increase the plasma LH levels of the blood of rats and estrogen administration depressed the LH levels in these gonadectomized animals. Intravenous injection of hypothalamic acid extracts increased the plasma LH levels in the ovariectomized rats in which LH secretion was blocked by estrogen. Acid extracts of cerebral tissue

did not increase the plasma LH levels in similar rats. These workers concluded that the median eminence area of rat hypothalami contained the specific acid extractable material responsible for stimulating LH secretion. They call this substance LH releasing factor (LH-RF).

The presence of a specific substance of hypothalamic origin which stimulates the secretion of gonadotropins in vivo is also indicated by the studies of Campbell et al. (1961) and Nikitovitch-Winer (1962). Campbell and colleagues (1961) infused acid extracts of median eminence from rats and steers into the pituitary glands of conscious rabbits by a procedure employing chronically implanted platinum canulea. This treatment resulted in a high incidence of ovulation in the rabbits. Intrahypophysial infusion of acid extracts from cerebral cortex or caudate nucleus, or intravenous infusion of the extract of median eminence, were essentially ineffective in promoting ovulation in the rabbits. Nikitovitch-Winer (1962) blocked ovulation during proestrus in rats with pentobarbitol and studied the effects of intrapituitary infusion and intravenous injection of extracts of bovine and rat hypothalamic and median eminence tissue. She found that intrapituitary infusion of

hypothalamic or median eminence extracts resulted in a high incidence of ovulation in these rats whereas intravenous injection of the same quantities of the hypothalamic materials were ineffective.

Harris (1962) has recently reported that acidic extracts of bovine or rabbit hypothalamus will augment TSH secretion when infused into the AP glands of rabbits. The I^{131} levels of the blood of rabbits were measured after a tracer dose of the radioisotope had been administered and allowed to accumulate in the thyroid glands. Slow intrapituitary infusion of the hypothalamic extracts resulted in a prompt and extensive elevation in plasma I^{131} levels indicating increased thyroid secretion presumably due to promotion of TSH release.

Several culture studies have provided evidence which supports the concept that hypothalamic control of the AP is mediated by a neurohumoral mechanism. Guillemin and Rosenberg (1955) and Guillemin (1956) have shown that ACTH activity in the medium of tissue and organ cultures of dog and rat AP is only detectable during the first 4 days of *in vitro* incubation. When fragments of hypothalamic tissue were added and cultured along with the AP tissue, the ACTH

activity in the medium returned to detectable levels. Similar co-cultures of AP and cerebral explants did not re-instate discernible ACTH secretion in this system. This observation suggests that a specific material was diffusing from the hypothalamic fragments and acting upon the AP explants to affect ACTH release. Guillemin and co-workers believe that this material is the CRF which they have isolated.

Florsheim et al. (1957) attempted to demonstrate the presence of a specific hypothalamic neurohumor which governs TSH secretion. They employed cultures of mouse AP and used co-culture procedures similar to those of Guillemin and Rosenberg (1955). Although Florsheim and colleagues were able to confirm Guillemin and Rosenberg's findings on the ACTH hypophysiotropic effects of their co-culture system, they were unable to stimulate detectable TSH secretion. TSH activity in the medium of this study with mouse AP was only discernible for the first 7 days of culture.

Explant and monolayer cultures have been observed to produce little gonadotropin, as judged by assays on the culture medium and cells (Kobayashi, et al., 1961). Addition of hypothalamic extracts to these cultures increased

the FSH levels in the medium and cells whereas cerebral extracts had no effect. These results provide further evidence that the hypothalamus contains a specific material necessary to stimulate and maintain FSH secretion.

The existence of the hypothetical PIF has thus far only been inferred from indirect evidence. It was therefore of interest to ascertain if hypothalamic explants, homogenates and acid extracts could influence prolactin secretion in vitro in a system where evidence for the existence of PIF, of a more direct and definitive nature, may be obtainable.

Methods

Anterior pituitary glands from multiparous breeder female rats, with a body weight of 270 - 300 gms, were used for this study. All of the cultures were incubated for 6 days in O_2 - CO_2 atmosphere with 3 ml of medium. The rafts consisted of urethane foam sponge disks which supported lens paper rectangles. For controls the results of 13 cultures in 199 medium alone are shown in this study. Three of the control culture dishes each contained explants from 2 APs, 7 dishes contained explants from 1 AP and the remaining 3 cultures were incubated with fragments of 1/2 of an AP.

To test the effects of hypothalamic and cerebral explants on prolactin secretion in vitro, 6 culture dishes each containing explants from 2 rat APs, were incubated. Two rat hypothalami were cut into 4 pieces and added to each of 3 of these cultures. Eight explants of rat cerebral cortical tissue, of about the same size as the hypothalamic explants, were added to each of the 3 remaining culture dishes. Since it was thought that cutting the rat hypothalami into 4 fragments may have been unduly injurious to the neural tissue, another experiment with hypothalamic-AP co-cultures was performed in which whole mouse hypothalami were used. The mouse hypothalami are sufficiently small to be cultured in one piece. Four culture dishes were incubated for this study and each dish contained explants from 1 rat AP. Four whole hypothalami from male and female mice were added to each of 2 of these cultures and hypothalamic-size pieces of mouse cerebral tissue were added to each of the remaining 2 dishes. In all cases with hypothalamic and cerebral co-cultures, the hypothalamic and cerebral explants were arranged around the outer edges of the rafts and the AP explants were in the center. The AP explants were not in direct contact with the neural fragments in any of these cultures.

Six culture dishes, each containing explants from 2 rat APs were used to determine the effects of homogenized hypothalamic and cerebral tissue on prolactin secretion *in vitro*. Twenty-seven rat hypothalami were removed aseptically and homogenized with a ground glass homogenizer with a quantity of 199 medium which produced a total volume of 9.5 ml. Three ml of this material was added to each of 3 culture dishes. An equivalent number of cerebral fragments, of about hypothalamic size, were homogenized with 199 medium in the same manner and 3 ml of this preparation was added to each of 3 culture dishes.

Six culture dishes, each containing explants from 1/2 of a rat AP were incubated with acid extracts of hypothalamus and cerebrum to ascertain if PIF was extractable with dilute acid. Twenty rat hypothalami (weighing 246 mg) were homogenized with 5 ml of 0.1 N HCl in a ground glass homogenizer. The homogenate was then centrifuged and the supernatant removed and sterilized by passing through a millipore filter. The acid extract was then incorporated into 25 ml of 199 medium in place of an equivalent quantity of water. Twenty pieces of rat cerebral tissue weighing 246 mg were treated in a similar manner and the acid extract was added to 199 at

the same concentration. Three ml of 199 containing the hypothalamic acid extract were placed in each of 3 culture dishes. The medium with cerebral extract was similarly dispensed.

At the end of the 6 day culture period the medium samples from all of these cultures were assayed for prolactin activity by injection over the crop sacs of 5 White Carneau squabs. The data were not converted to IU and are presented as RTU. The data from the co-cultures of rat and mouse hypothalamic and cerebral explants were combined for the sake of brevity.

Results and Discussion

The data presented in Table 10 show the results of these studies. The prolactin activities in the medium samples of the 13 control cultures averaged 18.0 $\pm$ 0.96 RTU per cultured AP. The medium samples from the 5 hypothalamic co-cultures had an average prolactin content of 9.2 $\pm$ 1.68 RTU/AP. This represents a 48.9% reduction in prolactin activity which is highly significant ($t = 4.54$; $P < 0.001$). The prolactin activity in the medium from the cerebral co-cultures averaged 11.0 $\pm$ 1.92 RTU/AP and this reduction of 38.9% in prolactin activity is also highly significant ($t = 3.26$; $P < 0.01$).

The prolactin levels in the medium samples from the cerebral and hypothalamic co-cultures were not significantly different from each other. It is evident therefore, that hypothalamic and cerebral explants are about equally effective in depressing the prolactin levels of the medium in this system.

The prolactin activities in the medium samples from the cultures which contained the hypothalamic and cerebral homogenates averaged 9.4 $\pm$ 3.48 and 9.0 $\pm$ 0.42 RTU/AP, respectively. These levels are significantly lower than the control value of 18.0 $\pm$ 0.96 RTU/AP ($P < 0.005$ and 0.001 respectively). The reduction in the prolactin levels in the medium samples by hypothalamic and cerebral homogenates, of 47.8% and 50% respectively, did not differ from each other nor from the values found in the study with hypothalamic and cerebral explants. These data demonstrate that hypothalamic and cerebral homogenates are about equally effective in reducing the prolactin activity of the culture medium and the homogenates are as effective in this respect as the explants.

The medium samples from the cultures with acid extract of hypothalamus contained an average of 7.8 $\pm$ 1.56 RTU/AP. This 56.7% reduction in prolactin activity from the control

level is significant at the 0.1% level (i.e. $P < 0.001$). The prolactin activities from the cultures with acid extract of cerebral tissue were found to be 17.5 $\pm$ 3.74 RTU/AP. This represents a reduction of only 2.8% and the difference in prolactin activity from the control value is not significant. It is apparent that the acid extract of cerebral tissue was ineffective in altering prolactin secretion by the AP explants. The acid extract of hypothalamic tissue reduced the prolactin activities of the medium samples to a degree equivalent to that found with hypothalamic and cerebral explants or homogenates since none of these values differed significantly from one another.

The hypothalamic and cerebral explants did not survive through the culture period, as judged by their histological appearance at the end of incubation. Possibly the lower levels of prolactin found in the medium samples from these cultures were due to destruction of the prolactin in the medium by exuded cellular contents from the dead neurons in these explants. It is also possible that the dead neural explants liberated materials which were inhibitory, in a non-specific manner, to prolactin secretion by the AP explants. The hypothalamic and cerebral homogenates may

have caused a reduction in the prolactin levels of the medium in a similar manner, i.e. destruction of the hormone or non-specific inhibition of prolactin secretion. It is evident, in any case, that the studies with hypothalamic and cerebral explants and homogenates provide no evidence for the presence of a specific hypothalamic PIF.

The study with the acid extracts presents some interesting indications that the non-specific materials, which were inhibiting prolactin secretion and/or destroying the hormone, can be eliminated by the extraction procedure. No evidence of inhibition of prolactin secretion or destruction of the hormone in vitro was obtained from the cultures with acid extracts of cerebral tissue. This indicates that the agents responsible for the low medium levels of prolactin, in the cultures with cerebral explants and homogenate, were either eliminated or destroyed by the procedure of acid extraction. The acid extract of hypothalamic tissue was still highly effective in depressing the prolactin activity of the medium samples. It seems reasonable to assume that the effect of hypothalamic acid extract may have been due to the specific PIF, since the acid extraction procedure would probably have eliminated any non-specific

inhibitors of prolactin secretion, or materials which would inactivate the hormone, as apparently occurred with the acid extracts of cerebral tissue. Guillemin's CRF (see Guillemin, 1962), the hypothalamic agents which seem to be responsible for stimulating TSH (Harris, 1962) and gonadotropin secretion (Campbell et al., 1961; Nikitovitch-Winer, 1962), and McCann's LH-RF (1962) are all found in similar crude acid extracts of the hypothalamus but not in acid extracts of cerebral tissue. This supports the notion that the depressant effect of the hypothalamic acid extract on the prolactin levels of the medium observed in this study, are due to the PIF. It seems reasonable to expect that the PIF would also be acid extractable. In this study, as in the investigations with the other hypothalamic neurohumors, no evidence of an inhibitory substance was found in the acid extract of cerebral tissue.

It is therefore concluded that the co-cultures with hypothalamic and cerebral explants, and the cultures with homogenates of these neural tissues, provide no evidence for the existence of a specific hypothalamic PIF. The cultures with the acid extracts, however, present rather strong evidence that an acid extractable PIF does exist in the hypothalamus which is not present in the cerebral tissues.

Table 10

EFFECTS OF HYPOTHALAMUS AND CEREBRUM ON PROLACTIN SECRETION IN VITRO

Culture	No. of Cultures	Average Prolactin Activity in Medium, RTU/AP			
		Hypothalamus	% Reduction from Control Value	Cerebrum	% Reduction from Control Value
Co-cultures	5	9.2 ± 1.68	48.9	11.0 ± 1.92	38.9
Homogenate	3	9.4 ± 3.48	47.8	9.0 ± 0.42	50.0
Acid extract	3	7.8 ± 1.56	56.7	17.5 ± 3.74	2.8

Average of 13 control cultures = 18.0 ± 0.96 RTU/AP

Conclusions

Haun and Sawyer (1960) observed that lesions in the medial basal tuberal region of the hypothalami of rabbits were effective in inducing lactation in the animals thus indicating augmented prolactin secretion. Since lesions in the same area depress gonadotropin secretion, they suggested that the same hypothalamic center(s) were involved in regulating FSH-LH and prolactin secretion. They also proposed, in agreement with the view of Everett (1954, 1956), and the same hypothalamic neurohumoral factor was responsible for regulating LH and prolactin secretion. When this material is liberated by the hypothalamic neurosecretory cells, into the portal blood vessels, it is conveyed to the AP, and LH release is stimulated. The same material could conceivably depress prolactin secretion by the AP while stimulating LH release. This same view has recently been expressed by McCann (1962a). Donovan (1960) has proposed that stimulation of prolactin secretion is a secondary consequence of suppression of FSH-LH secretion. He suggests that factors, such as the suckling stimulus, depress FSH-LH and promote prolactin secretion by an action on the same neural centers. Such a reciprocal regulatory mechanism

would prove economical for the process of hypothalamic control of the AP, since one neurohumoral agent could control 2 AP hormones; however further research is required before this concept can be accepted.

Administration of homogenates of hypothalamic tissue to estrogen-primed rats has been observed to initiate lactation indicating that prolactin secretion was increased (Meites et al., 1960a). This observation cannot be considered as evidence, however, that the hypothalamus contains a specific material which stimulates prolactin secretion. Stressful procedures have been observed to promote prolactin secretion in rats (Swingle et al., 1951b; Nicoll et al., 1960). It seems reasonable, therefore, to assume that the injected hypothalamic material promoted prolactin secretion via stress effects.

Pasteels (1961b) has recently reported that the medium from co-cultures of AP and hypothalamic explants contained less prolactin activity than the medium from co-cultures of AP and cerebral fragments. His results are in contrast with those of the present study with hypothalamic and cerebral co-cultures. This discrepancy may be due to the different types of culture media used in the two studies.

Pasteels (1961b) employed a natural, growth promoting medium for these studies with 3 rat APs whereas we used synthetic, defined medium. The growth promoting medium may have maintained the neural explants in a better condition than the synthetic medium.

Talwalker, Ratner and Meites (unpublished) have studied the effects of hypothalamus and other neural tissue on prolactin release in a short term in vitro incubation system. With this method they have demonstrated that hypothalamic fragments and acid extracts exert a profound depressing effect on prolactin release in vitro. Cerebral fragments or acid extracts were relatively ineffective in altering prolactin release. These extensive studies provide further evidence, of a rather convincing nature, that the hypothalamus contains a specific PIF. Final proof, however, that the PIF is contained in these hypothalamic acid extracts will require isolation, purification and identification of the material and further studies to establish its specificity.

EXPERIMENT IX. EFFECTS OF NEUROHYPOPHYSIAL HORMONES.

A possible relationship between the posterior pituitary and prolactin secretion was first advanced by Peterson (1942). He suggested that release of oxytocin might trigger the rapid discharge of prolactin which occurs in response to the suckling stimulus (Reece and Turner, 1937; Grosvenor and Turner, 1957). Benson and Folley (1956, 1957a, 1957b) demonstrated that oxytocin injections partially inhibited mammary involution in postpartum rats after litter removal. This observation has been confirmed by Meites (1958b), Meites and Nicoll (1959), McCann et al. (1959) and Ota and Yokoyama (1958). Since prolactin also inhibits mammary involution in rats, Benson and Folley (1956, 1957a, 1957b) interpreted these findings as evidence that oxytocin induces the release of prolactin from the anterior pituitary.

Meites and Turner (1942, 1948) tested Petersen's (1942) hypothesis by giving multiple injections of a posterior pituitary preparation (Pituitrin) to guinea pigs and rabbits to determine the effects on pituitary prolactin content. In contrast to the effects of suckling, Pituitrin failed to alter pituitary prolactin levels. This observation has been confirmed by Grosvenor and Turner (1958a) with oxytocin.

Haun (1959) reported that oxytocin injections induced lactation in estrogen-primed rabbits and he concluded that this was the result of oxytocin stimulating prolactin secretion. This observation was not confirmed, however, in pseudopregnant rabbits (Benson et al., 1959; Meites, 1959b) nor in estrogen primed rabbits and rats (Meites et al., 1961). The positive finding of Haun (1959) is probably the result of inadequate control procedures. A slight degree of lactation often occurs in estrogen primed rabbits after withdrawal of the estrogen treatment and presumably this would account for the mammary secretion that Haun attributed to oxytocin.

Oxytocin has also been reported to induce LTH (prolactin) secretion in rats as evidenced by vaginal mucification and the induction of pseudopregnancy (Desclin, 1956a, 1956b; Cowie and Folley, 1956; Stutinski, 1957). However, Benson et al. (1959), McCann and Friedman (1960) and Rothchild and Quilligan (1960) were unable to confirm this finding.

Meites et al. (1960) investigated the possibility that oxytocin retards mammary involution in unsuckled postpartum rats by an action on the mammary gland. It was demonstrated that typical milk ejection responses could still be obtained

in the mammary glands of rats on the 5th and even the 9th day after nursing had been suspended.

Benson et al. (1960) reported that valyl oxytocin, which is about 5 times as potent as natural oxytocin in promoting milk ejection in rabbits, was no more effective than oxytocin in retarding mammary involution in rats. They concluded that this indicated that the beneficial effect of oxytocin on mammary integrity could not be explained on the basis of an action on the mammary gland, since the more potent valyl oxytocin might be expected to retard mammary involution to a greater degree than the less active oxytocin. Inspection of their data, however, reveals that all doses of valyl oxytocin and oxytocin used were equally effective in retarding mammary involution, indicating that even the lower doses were exerting a maximal effect. Furthermore, if oxytocin promotes prolactin release, the more potent valyl oxytocin would be expected to retard mammary disintegration to a greater extent than the weaker preparation since more prolactin should be discharged from the AP. The reasoning which Benson et al. (1960) used to conclude that oxytocin does not retard mammary involution by an action at the mammary

level, can therefore be applied with equal validity to arrive at the conclusion that oxytocin does not influence prolactin release.

Meites and Nicoll (1959) observed that injection of a combination of oxytocin and prolactin to unsuckled postpartum rats was more effective in retarding mammary involution than either hormone given alone. The possibility that oxytocin exerted this additive effect on the prolactin response by stimulating endogenous prolactin release from the in situ pituitaries of the rats seems unlikely since relatively large doses of prolactin were employed. Therefore an action of oxytocin on the mammary gland is again implicated.

The possibility that oxytocin may retard mammary involution by an action at the mammary level was further examined by Meites and Hopkins (1961). They demonstrated that oxytocin was highly effective in preventing mammary deterioration in hypophysectomized rats which were treated with prolactin and cortisol or prolactin and ACTH. Since these rats had no pituitary glands on which the injected oxytocin could act, it is obvious that the action of oxytocin in maintaining mammary integrity cannot be due to an hypophysial effect.

Since the AP organ culture system provides a method of directly evaluating the effects of oxytocin on prolactin secretion, a study was undertaken to determine if oxytocin could alter prolactin secretion *in vitro*. The effects of the neurohypophysial hormone vasopressin (ADH) on AP function has been given considerable attention and emphasis in the last few years. Investigators have claimed that ADH can promote the secretion of practically every AP hormone (see Symposium of Neuroendocrinology, Miami, Florida, 1961; University of Illinois Press, in press). It was also of interest, therefore, to test the effects of ADH on prolactin secretion *in vitro*.

Methods

The paired culture and assay procedure was used for these experiments with oxytocin and vasopressin. Each culture dish of each culture pair in the oxytocin study contained explants from 1/2 of an AP from a mature, female, 3 month old CFN rat. Pituitary glands of 4 month old CFN female rats were used for the study with pitressin and explants from 1/2 of a gland were incubated in each culture dish. Oxytocin was added to the 199 medium at a concentration of 0.1 U/ml. The oxytocin preparation used (Pitocin;

Parke, Davis and Co.) contained chlorobutanol at a concentration of 0.5%. This preservative was therefore added to the control medium of the oxytocin study to give an equivalent final concentration. Pitressin was added to the medium from an aqueous stock solution of pitressin powder (Parke, Davis and Co.) which contained 55 pressor and 4 oxytocic units per mg. The final concentration of ADH in the medium was 1.0 U/ml.

All of the cultures were incubated for three days in O_2 - CO_2 atmosphere in plastic Petri dishes with stainless steel platforms. The medium samples from each culture pair were assayed in 5 White Carneau squabs. One IU of NIH prolactin was found to equal about 12 RTU in these birds. The prolactin potencies of the medium samples were therefore estimated by using this relationship.

Results and Discussion

The results of the experiment with oxytocin are shown in Table 11 and summarized in Table 12. Oxytocin had no apparent effect on the weight of the AP explants. The prolactin levels in the oxytocin medium samples averaged 16.25% higher than the control values on the basis of IU/AP and 16.24% higher on the basis of total RTU. These differences

are significant at the same probability level ($P < 0.05$). On the basis of IU per 100 mg AP, however, no difference was found in the prolactin levels of the oxytocin and control medium samples. Since oxytocin did not influence explant weight, it is concluded that oxytocin probably did not affect prolactin secretion in vitro, as judged by the prolactin activities in IU/100 mg of explant tissue. The higher values of the oxytocin medium samples, obtained when the data are expressed as total RTU or IU/AP, are probably due to the unequal distribution of the AP tissue which occurred in favor of the oxytocin cultures.

The conclusion that oxytocin did not affect prolactin secretion in vitro is supported by the observations of Talwalker, Ratner and Meites (unpublished) on the effects of oxytocin on prolactin release during short term incubation (2 hours) of rat AP tissue. These investigators found that oxytocin did not increase prolactin release by the APs into the incubation fluid. It is apparent, therefore, that this AP culture study, in agreement with the results of the short term incubation of AP tissue, indicates that oxytocin has no role in altering prolactin synthesis or release.

The results from the study with pitressin are shown in Table 13 and summarized in Table 14. Vasopressin apparently had no effect on the weight of the AP explants and the prolactin activities in the ADH medium samples were not different from the levels found in the control medium samples as judged by any criterion. It is therefore concluded that pitressin did not alter prolactin secretion in vitro.

Table 11

EFFECT OF 0.1 U PER ML OF OXYTOCIN ON PROLACTIN SECRETION IN VITRO

Three Day Cultures with Explants from 1/2 AP per Dish.
Five Pigeons per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	2.1	12.2	1.02	2.03	48.6	
Oxytocin-1	2.9	15.0	1.25	2.50	43.1	- 11.3
Control-2	2.1	18.9	1.41	2.82	67.1	
Oxytocin-2	2.6	17.4	1.45	2.90	55.8	- 16.8
Control-3	2.0	22.2	1.85	3.70	92.5	
Oxytocin-3	2.2	23.6	1.97	3.93	89.5	- 3.2
Control-4	2.4	12.0	1.00	2.00	41.7	
Oxytocin-4	2.3	16.0	1.33	2.67	57.8	+ 38.6
Control-5	2.4	13.5	1.13	2.25	47.1	
Oxytocin-5	3.4	17.6	1.47	2.93	43.2	- 8.3
Control-6	3.0	26.6	2.22	4.43	74.0	
Oxytocin-6	3.0	27.0	2.25	4.50	75.0	+ 1.4

Table 12

SUMMARY OF THE IN VITRO EFFECTS OF 0.1 U PER ML OF OXYTOCIN

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Oxytocin				
Explant weight mg	2.33	2.73	+ 0.4 ±0.165	+ 17.17	2.42	N.S.
IU prolactin per AP	2.87	3.24	+ 0.37	+ 16.25 ±5.88	2.76	0.05
IU prolactin per 100 mg AP	61.83	60.70	- 1.10	+ 0.07 ±5.55	-	N.S.
Total RTU	17.23	19.43	+ 2.20	+ 16.24 ±5.88	2.76	0.05

Table 13

EFFECT OF 1.0 U PER ML OF PITRESSIN ON PROLACTIN SECRETION IN VITRO

Three Day Cultures with 1/2 AP per Dish.
Five Pigeons per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	% Difference IU/100 Mg
Control-1	3.1	22.1	1.84	3.68	59.4	+ 11.1
Pitressin-1	3.0	23.8	1.98	3.96	66.0	
Control-2	3.6	18.8	1.57	3.14	43.6	
Pitressin-2	2.8	14.3	1.19	2.38	42.5	- 2.5
Control-3	4.4	16.8	1.40	2.80	31.8	
Pitressin-3	3.9	14.3	1.19	2.38	30.5	- 4.1
Control-4	3.5	24.7	2.06	4.12	58.9	
Pitressin-4	3.0	25.1	2.09	4.18	69.7	+ 18.3
Control-5	3.0	21.3	1.78	3.56	59.3	
Pitressin-5	3.5	21.0	1.75	3.50	50.0	- 15.7
Control-6	5.1	31.5	2.63	51.6	51.6	
Pitressin-6	2.9	18.8	1.57	54.1	54.1	+ 4.8

Table 14

SUMMARY OF THE IN VITRO EFFECTS OF 1.0 U PER ML OF PITRESSIN

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Oxytocin				
Explant weight mg	3.78	3.18	- 0.60 ±0.30	-	2.00	N.S.
IU prolactin per AP	3.76	3.26	- 0.84	- 12.0 ±6.15	1.95	N.S.
IU prolactin per 100 mg AP	50.77	52.13	+ 1.37	+ 1.98 ±1.32	1.50	N.S.
Total RTU	22.53	19.55	- 2.98	- 11.86 ±6.13	1.93	N.S.

Conclusions

The failure of oxytocin to influence prolactin secretion in vitro is in agreement with the observations that oxytocin administration does not alter prolactin secretion in vivo, as judged by the effects of oxytocin on the AP prolactin content (Meites and Turner, 1942, 1948; Grosvenor and Turner, 1958a). The results of this in vitro study also corroborate the other evidence, cited previously, which indicates that oxytocin has no effect on prolactin secretion. It is therefore concluded that the hypothesis of Benson and Folley (1956, 1957a, 1957b) that oxytocin is responsible for stimulating prolactin release, is untenable.

Guillemin (1956) reported that addition of a vasopressin preparation to the medium of cultures of rat or dog AP tissue resulted in stimulation of ACTH release. When synthetic vasopressin was added to these cultures, no ACTH-hypophysiotropic effect was obtained. The ACTH stimulatory action of the vasopressin preparation was therefore attributed to contamination with CRF. Since no effect on prolactin secretion was realized with pitressin in this in vitro study, it is evident that ADH does not influence prolactin secretion. The results also suggest that the pitressin did not contain

detectable quantities of the hypothetical hypothalamic prolactin inhibiting factor (PIF) as a contaminant although, as shown in Experiment VIII, hypothalamic acid extracts may contain the PIF.

EXPERIMENT X. EFFECTS OF ESTROGEN.

In vivo experiments have established that estrogens increase prolactin secretion in a variety of species as judged by increased pituitary prolactin content, stimulation of mammary growth and the initiation of lactation (Reece and Turner, 1937; Meites and Turner, 1948). Estrogen also induces pseudopregnancy in rats (Wolf, 1935; Merckel and Nelson, 1940) indicating enhanced LTH or prolactin secretion.

Although moderate doses of estrogen induce a pronounced rise in pituitary prolactin content, higher doses are less effective in this respect (Meites and Turner, 1948). Large doses of estrogens also depress milk secretion in lactating animals (Meites, 1959b). These latter observations have been interpreted by Folley and Malpress (1948) as indicative of a differential effect of estrogen on prolactin secretion. They suggested that low doses of estrogen stimulate prolactin secretion whereas higher levels exert a depressant effect. Since it has not been established whether estrogen augments prolactin secretion by an action at the pituitary level, or indirectly through an effect on the hypothalamus, it was of interest to determine if estrogen

could influence prolactin secretion *in vitro* by a direct action on the pituitary cells. The effects of different levels of estrogen were also tested to determine if a differential effect could be demonstrated.

Methods

Rats of the Carworth (CFN strain) or hybrids of Carworth and Michigan State University Department of Chemistry rats were used for the first study. All of the rats were mature nulliparous females weighing 180-200 gms. Six APs were used to provide explants for the 2 culture dishes of each culture pair. Three of the 6 explants from each of the 6 AP halves were transferred to a raft containing control medium. The remaining 3 explants from each of the 6 APs were placed on a similar raft and cultured with medium containing estradiol. Thus each culture dish of each culture pair contained 18 explants equivalent to 3 rat APs obtained from 6 rats. Media from both dishes of each culture pair was assayed in the same pigeons by the paired assay procedure described previously. This permitted the effects of estrogen on prolactin production from an equivalent amount of pituitary tissue from the same rats, cultured under the same conditions, to be ascertained in the same assay animals.

Estradiol was added to the medium from a stock solution of the steroid in absolute ethanol. The final concentrations in the medium were 0.5 μgm estradiol per ml in 0.5% ethanol. The control medium contained an equivalent concentration of ethanol. Rafts of urethane foam sponge were used in culture pairs 1, 2 and 3. Lens paper supported by urethane foam sponge was used for rafts in pairs 6 and 7 and in pairs 8, 9 and 10 stainless steel rafts were employed. All cultures were incubated for 6 days in O_2 - CO_2 atmosphere. The medium which was collected after the second 3-day culture interval (days 4-6) of culture pairs 1-5 was used for assay. All of the medium from the 6 days of incubation from culture pairs 6-8 was used for assay. A 2 ml aliquot of medium from each culture dish was assayed in 5 mature White Carneau pigeons and the total activity of the medium sample calculated. The explants from this study were not weighed at the end of culture.

For the determination of the effects of different concentrations of estradiol on prolactin secretion <u>in vitro</u>, the plastic Petri dish - stainless steel raft preparations were used and the APs were obtained from 180-200 gm CFN female rats. Estradiol was added to the 199 medium in the

same manner as before at concentrations of 0.05, 0.5 and 2 μgm per ml. Eight culture pairs were used at each dose level. Essentially the same paired culture and assay procedure was used for this study as was used in the first study. Each culture dish at the low dose level contained one explant (1/6 of an AP) and the dishes with the intermediate concentration each contained 2 explants (1/3 of an AP). For the high dose level one-half of an AP was placed in each culture dish (3 explants). A different quantity of tissue was used in the culture dishes at each dose level in an attempt to ascertain the effects of the quantity of tissue cultured on prolactin secretion in vitro by comparison of the prolactin levels of the control medium samples. This was unsuccessful, however, as will be shown subsequently.

The cultures in this experiment were incubated for 3 days in O_2 - CO_2 atmosphere and 3 ml of medium was used in each dish. Insulin was not added to the 199 medium for this second study with estradiol although it was present in the medium used in the first study. The medium was assayed for prolactin activity by injection of 1.2 ml of the nutrient fluid from each dish into 3 White King squabs. The prolactin levels in the medium samples were converted to IU by use

of the standard dose-response plot (Experiment XV). To determine if estrogen influenced the pigeon crop response to prolactin, 10 mature White Carneau pigeons were injected over the right crop sac with a total dose of 20 μgm prolactin per bird in 0.5% ethanol solution. The left crop halves of the same pigeons were injected with the same prolactin dose in 0.5% ethanol containing 0.5 μgm estradiol per ml. Upon termination of the cultures in this second estradiol study, the explants were weighed before being fixed for histological examination.

Results and Discussion

The data from the first experiment, which are shown in Table 15, demonstrate that the estradiol medium samples from each culture pair contained higher prolactin activity then the control medium samples in all 8 cases. Prolactin production by the APs in estradiol medium averaged 0.89 RTU per AP per day of culture and the average of the control cultures was 0.74 RTU per AP per day. This difference of 0.15 RTU represents a 31.94 percent increment in prolactin activity and is significant at the 2% level. Histological examination of the explants disclosed that tissue survival was excellent and no differences were apparent in the degree

of survival of explants from cultures in estradiol medium when compared with their appropriate control explants. The standard 20 μgm dose of prolactin gave an average response of 2.38 ± 0.22 RTU per bird while the same standard with 0.5 μgm/ml of estradiol produced an average response of 2.2 ± 0.25 RTU per bird. This difference was not significant; therefore, estradiol did not influence the pigeon crop response to prolactin at the dose level used.

The data from the experiment with the low level of estradiol in the second study are shown in Table 16 and summarized in Table 17. It will be seen that estradiol had no apparent effect on the weight of the AP explants. The explants cultured in the control and estradiol cultures had an average weight of 0.66 and 0.63 mg respectively. The average difference in explant weight of 0.04 ± 0.06 mg was not significant. The prolactin activity of the estradiol medium samples was found to be significantly greater than that of the control samples on the basis of IU/100 mg or total RTU. Estradiol at a concentration of 0.05 μgm per ml increased prolactin secretion by an average of 36.90% on the basis of IU per 100 mg of explant weight ($P < 0.05$) and by 28.40% on the basis of total RTU ($P < 0.05$). The

difference in prolactin activities in the estrogen and control medium samples was not significant on the basis of IU/AP.

The results from the experiment with estradiol at a concentration of 0.5 μgm per ml are shown in Table 18 and summarized in Table 19. In this study, as in the previous experiment, the estradiol did not effect the weight of the pituitary explants. The average difference in explant weight of 0.01 $\pm$ 0.09 mg is not significant. The prolactin content of the estradiol medium samples was not significantly different from the control samples on the basis of IU per cultured AP. On the basis of IU per 100 mg of explant weight, the medium from the estradiol cultures averaged 58.10% higher in prolactin content than the control cultures ($P < 0.05$). When expressed as total RTU, the estradiol medium samples averaged 29.20% higher in prolactin activity than the control samples ($P < 0.05$).

Table 20 shows the data obtained from the experiment with estradiol at a concentration of 2 μgm per ml and the results are summarized in Table 21. At this dose level estradiol did not affect the weight of the explants. The average difference in explant weight between the control

and estradiol cultures was only 0.06 $\pm$ 0.05 mg and was not significant. Although the prolactin activity of the estradiol medium samples was higher than the levels found in the control medium samples by an average of 18.54% and 15.18% on the basis of IU/AP and IU per 100 mg explant weight respectively, these differences were not significant. On the basis of total RTU, however, the prolactin levels in the estrogen medium samples were significantly higher than the control samples by 7.44% ($P < 0.05$).

Conclusions drawn from the statistical analysis have been based primarily on the criterion of IU per 100 mg of explant weight, since this is probably the most definitive of the parameters for assessing the effectiveness of the experimental treatment. It is therefore concluded that estrogen significantly increased the prolactin levels in the medium samples at the 0.05 and 0.5 μgm per ml dose levels but was without effect at the 2 μgm/ml concentration.

Explant survival was excellent in all cases in this second study with estradiol at different concentrations and no differences were apparent in the histological appearance of explants from the control and estradiol cultures. It is evident, therefore, that the higher prolactin levels found

in the estradiol medium samples from the experiments with the 0.05 and 0.5 μgm/ml concentrations, cannot be attributed to differences in explant survival. Since estradiol did not influence the pigeon crop response to prolactin at the 0.5 μgm dose level, and no differences in explant survival were detectable, it seems evident that estradiol increased the prolactin activity of the medium samples by an action on the AP explants. Presumably the estradiol acted directly on the pituitary acidophils to effect an increase in prolactin synthesis. Support for this interpretation is provided by the observations that estrogens increase the respiratory metabolism of rat (Victor and Anderson, 1937) and human (Gaul and Villee, 1959) AP tissue *in vitro*.

The stimulatory action of the 0.05 μgm/ml level of estradiol on prolactin secretion *in vitro* in the second study was about the same as that found with 0.5 μgm per ml in the first trial. Although the increase in prolactin synthesis induced by 0.5 μgm/ml of estradiol in the second study was greater than that produced by the same level in the first trial or by the 0.05 μgm per ml concentration in the second experiment, these increases did not differ from one another. Since the high concentration of estradiol (2

μgm/ml) did not stimulate prolactin secretion in vitro, these data indicate that the lower dose levels of estradiol are more effective in promoting prolactin secretion than higher levels. The ineffectiveness of the higher level of estrogen, in augmenting prolactin synthesis, is in agreement with the in vivo observation that high doses of estrogen are less effective in increasing the pituitary prolactin content in guinea pigs, rats and rabbits (Meites and Turner, 1948).

The average prolactin levels in the medium samples of the control cultures in the second study, with the 0.05, 0.5 and 2.0 μgm/ml concentrations of estradiol, were found to be 39.7 ± 5.3, 13.6 ± 2.16 and 17.2 ± 2.5 IU per 100 mg of explant weight respectively. The prolactin levels of the control medium samples from cultures with the intermediate and high concentrations of estradiol were significantly lower than at the lower level ($P < 0.001$ and $P < 0.01$ respectively). The difference between the control averages in prolactin synthesis of the cultures from the studies with the intermediate and high levels of estradiol are not significant. The average weight of the explants from the control cultures from the experiments with the low, intermediate and high concentrations of estradiol were 0.66, 1.66 and 2.18 mg respectively.

It is felt, however, that the marked differences in prolactin production observed in these studies cannot be attributed to the difference in the quantity of cultured tissue.

The differences in prolactin synthesis by the controls in these culture experiments is probably due to some difference in the culture conditions. The culture with the 0.5 and 2.0 μgm/ml levels of estradiol were incubated one week after the cultures with 0.05 μgm/ml. If an undetected reduction in either incubation temperature or gas flow rate had occurred, even though slight in degree and of relatively short duration, during incubation of these cultures, a pronounced reduction in hormone synthesis would probably occur without influencing explant morphology. Another possible explanation for the lower levels of prolactin synthesis in the control cultures from the studies with 0.5 and 2.0 μgm/ml of estradiol would be the presence in the medium of a minute amount of a material which was toxic or otherwise inhibitory to protein synthesis. It is conceivable that such a toxic material could have gained entry into the medium and, although insufficient in amount to cause cellular death, could be adequate to suppress prolactin synthesis.

The results of Kahn's culture studies (Experiment I) illustrate this point. Kahn used antibiotic chloramphenicol in his cultures. Although his explants were incubated in O_2 - CO_2 atmosphere and they were well maintained, the AP fragments synthesized only about as much prolactin as the cultures in air atmosphere in the author's studies. Chloramphenicol exerts a pronounced depressant effect on protein synthesis (Rendi, 1959).

It is recognized that other possibilities exist which could account for these differences in prolactin synthesis; however, it is the opinion of the author that the suggestions advanced are the most probable. Despite this marked between-experiment difference in prolactin production the within-experiment comparisons which have been made between the control and estradiol cultures are valid since the conditions of culture, the composition of the medium (apart from the presence of estradiol) and the assay conditions were identical for each experiment.

An assay of AP tissue from 190-200 gm CFN female rats, which was performed at about the same time as the assays on the medium samples from the study with different doses of estradiol, disclosed that the tissue had a prolactin content

of 1.04 ± 0.17 IU per 100 mg wet weight. Comparison of this value with the average prolactin levels in the control culture medium samples from the low, intermediate and high concentrations of estradiol (39.7, 13.8 and 17.2 IU per 100 mg of explant weight respectively) reveals that in all three studies the pituitary explants synthesized substantial quantities of prolactin. The AP explants in the control cultures evidently synthesized, during each day of the 3 day culture, about 13, 4 and 5 times as much prolactin as the explants contained at the beginning of culture. The prolactin synthesizing capacity of the AP explants in the control cultures from the study with the 0.05 μgm/ml level of estradiol admirably illustrates the autonomous nature of prolactin secretion by the adenohypophysis. In 3 days of culture the AP explants synthesized about 38 times as much prolactin as they contained when introduced into the culture system.

TABLE 15

EFFECT OF 0.5 μGM PER ML OF ESTRADIOL IN 199 MEDIUM ON PROLACTIN SECRETION IN VITRO
Six Day Cultures with Explants from 3 Rat APs per Culture Dish

Culture Pair No. and Medium	Incubation Period from Which Medium Was Assayed	REECE-TURNER PROLACTIN UNITS IN MEDIUM			% Difference
		Total	Per AP	Per AP/Day	
Control-1	Days 4-6	4.00	1.33	0.22	
Estradiol-1		6.40	2.20	0.35	+ 59.1
Control-2	Days 4-6	5.70	1.90	0.31	
Estradiol-2		7.50	2.50	0.41	+ 32.3
Control-3	Days 4-6	5.10	1.70	0.28	
Estradiol-3		9.80	3.27	0.54	+ 92.9
Control-4	Days 4-6	9.63	3.21	1.07	
Estradiol-4		11.55	3.85	1.24	+ 15.9
Control-5	Days 4-6	10.07	3.36	1.12	
Estradiol-5		10.85	3.61	1.20	+ 7.1
Control-6	Days 1-6	16.25	5.41	0.90	
Estradiol-6		19.25	6.41	1.07	+ 18.9
Control-7	Days 1-6	17.25	5.75	0.96	
Estradiol-7		20.75	6.91	1.15	+ 19.8
Control-8	Days 1-6	19.00	6.33	1.05	
Estradiol-8		20.75	6.91	1.15	+ 9.5

Average RTU/AP/Day	Control	Estradiol	Difference	% Difference	t	P <
	0.74	0.89	+ 0.15	+ 31.94 ± 10.45	3.00	0.02

TABLE 16

EFFFECT OF 0.05 μGM PER ML OF ESTRADIOL ON PROLACTIN SECRETION IN VITRO
Three Day Cultures with 1/6 AP (1 Explant) per Dish.
Three Pigeons per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	0.9	5.63	0.27	1.62	30.0	+ 20.0
Estradiol-1	0.5	3.80	0.18	1.08	36.0	
Control-2	0.9	9.32	0.57	3.42	63.3	- 9.8
Estradiol-2	0.7	7.57	0.40	2.40	57.1	
Control-3	0.7	2.81	0.15	0.90	21.4	+ 80.4
Estradiol-3	0.7	5.83	0.27	1.62	38.6	
Control-4	0.5	5.83	0.27	1.62	54.0	+ 18.5
Estradiol-4	0.5	6.41	0.32	1.92	64.0	
Control-5	0.6	6.40	0.31	1.86	51.7	+ 37.3
Estradiol-5	1.0	10.50	0.71	4.26	71.0	
Control-6	0.7	3.94	0.19	1.14	27.1	+ 26.6
Estradiol-6	0.7	5.05	0.24	1.44	34.3	
Control-7	0.5	4.50	0.20	1.20	40.0	+116.7
Estradiol-7	0.3	5.62	0.26	1.56	86.7	
Control-8	0.5	2.82	0.15	0.90	30.0	+ 5.7
Estradiol-8	0.6	4.06	0.19	1.14	31.7	

TABLE 17

SUMMARY OF THE IN VITRO EFFECTS OF 0.05 μGM PER ML OF ESTRADIOL

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Estradiol				
Explant weight mg	0.66	0.63	- 0.04 ± 0.06	-	0.66	N.S.
IU prolactin per AP	1.58	1.93	+ 0.35	+ 30.92 ± 13.43	2.30	N.S.
IU prolactin per 100 mg AP	39.69	52.43	+12.74	+ 36.90 ± 14.80	2.49	0.05
Total RTU	5.16	5.63	+ 0.95	+ 28.4 ± 10.90	2.60	0.05

TABLE 18

EFFFECT OF 0.5 μGM PER ML OF ESTRADIOL ON PROLACTIN SECRETION IN VITRO
Three Day Cultures with 1/3 AP (2 Explants) per Dish.
Three Pigeons per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	1.6	6.41	0.31	0.93	19.4	+ 24.7
Estradiol-1	1.4	6.76	0.34	1.02	24.2	
Control-2	1.8	6.51	0.31	0.93	17.2	+197.7
Estradiol-2	1.7	11.39	0.87	2.61	51.2	
Control-3	2.1	3.38	0.17	0.51	8.1	+ 54.3
Estradiol-3	2.0	5.43	0.25	0.75	12.5	
Control-4	2.2	7.31	0.37	1.11	16.8	+ 16.7
Estradiol-4	2.4	8.44	0.47	1.41	19.6	
Control-5	1.7	4.34	0.20	0.60	11.8	+ 36.4
Estradiol-5	2.3	7.31	0.37	1.11	16.1	
Control-6	2.2	5.97	0.28	0.84	12.7	0
Estradiol-6	2.2	5.97	0.28	0.84	12.7	
Control-7	1.7	5.43	0.25	0.75	14.7	+ 30.6
Estradiol-7	1.2	4.88	0.23	0.69	19.2	
Control-8	1.8	3.79	0.18	0.54	10.0	+ 82.0
Estradiol-8	1.1	4.50	0.20	0.60	18.2	

TABLE 19

SUMMARY OF THE IN VITRO EFFECTS OF 0.5 μGM PER ML OF ESTRADIOL

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Estradiol				
Explant weight mg	1.66	1.65	- 0.01 ± 0.09	-		N.S.
IU prolactin per AP	0.78	1.13	+ 0.35	+44.07 ±21.6	2.04	N.S.
IU prolactin per 100 mg AP	13.84	27.71	+ 7.88	+55.96 ±21.8	2.56	0.05
Total RTU	5.39	6.84	+ 1.44	+29.20 ±10.8	2.70	0.05

TABLE 20

EFFECT OF 2.0 μGM PER ML OF ESTRADIOL ON PROLACTIN SECRETION IN VITRO
Three Day Cultures with 1/2 AP (3 Explants) per Dish.
Three Pigeons per Assay.

Culture Pair No. and Medium	Explant Weight MG	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	2.5	10.49	0.71	1.42	28.4	
Estradiol-1	2.2	11.81	0.93	1.86	42.3	+ 48.9
Control-2	1.7	8.16	0.45	0.90	26.5	
Estradiol-2	2.0	9.32	0.55	1.10	27.5	+ 3.8
Control-3	2.1	7.31	0.38	0.76	18.1	
Estradiol-3	2.1	8.64	0.50	1.00	23.8	+ 31.5
Control-4	2.0	7.57	0.38	0.76	19.0	
Estradiol-4	2.1	10.13	0.66	1.32	31.4	+ 65.3
Control-5	2.2	5.80	0.28	0.56	12.7	
Estradiol-5	2.4	5.80	0.28	0.56	11.7	- 7.9
Control-6	2.3	5.43	0.26	0.52	11.3	
Estradiol-6	2.3	4.66	0.22	0.44	9.6	- 15.0
Control-7	2.4	4.66	0.22	0.44	9.2	
Estradiol-7	2.4	4.00	0.20	0.40	8.3	- 9.8
Control-8	2.2	5.97	0.28	0.56	12.7	
Estradiol-8	2.4	6.51	0.32	0.64	13.3	+ 4.7

TABLE 21

SUMMARY OF THE IN VITRO EFFECTS OF 2.0 μGM PER ML OF ESTRADIOL

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Estradiol				
Explant weight mg	2.18	2.24	+ 0.06 ± 0.05	-	1.2	N.S.
IU prolactin per AP	0.74	0.92	+ 0.17	+18.54 ±7.93	2.34	N.S.
IU prolactin per 100 mg AP	17.24	20.98	+ 3.75	+15.18 ±8.1	1.87	N.S.
Total RTU	6.92	7.61	+ 0.69	+ 7.44 ±3.0	2.48	0.05

Conclusions

It has been recognized for many years that estrogens exert a pronounced influence on AP morphology and function *in vivo*. The acidophils of the *in situ* adenohypophysis are increased in number and degree of granulation by estrogen treatment (Baker and Everett, 1947). Desclin (1950) has shown that estrogen promotes acidophil degranulation in AP transplants located in the kidney capsule of hypophysectomized rats, suggesting that this effect of estrogen is exerted directly on the AP cells. Estrogens also prevent or reverse the post castration vacuolization and degeneration of the pituitary basophils (Purves and Griesbach, 1951).

It is generally accepted that estrogens in moderate to high doses depress FSH secretion in mature mammals although low doses may exert a brief enhancing effect on FSH release (see Greep, 1961). The secretion of LH may also be enhanced or depressed by estrogen depending on the dose administered, the duration of treatment and other factors (Greep, 1961). Gemzel (1953) has demonstrated that estrogen treatment increases the ACTH levels in the blood and APs of intact male rats, and causes adrenal enlargement indicating enhanced synthesis and release of ACTH. Low doses of estrogen can

apparently stimulate pituitary TSH secretion in rats as judged by increased thyroidal I^{131} uptake (Soliman and Reineke, 1955) and thyroid secretion rate (Grosvenor and Turner, 1959). Administration of stilbesterol to cattle induces a rise in the pituitary STH levels and results in metabolic alterations which are indicative of enhanced STH secretion (Struempler and Burroughs, 1959). Estrogen administration also increases prolactin secretion in vivo as discussed previously.

It has not been determined, however, whether estrogens induce these changes in AP hormone secretion by a direct action at the adenohypophysial level, or by an effect on the hypothalamus which in turn influences AP function, or otherwise. Rose and Nelson (1957) demonstrated that infusion of an estrogen solution into the hypophysial fossa of gonadectomized female rats, by a microinjection technique, is effective in depressing the post-castration changes in the AP basophils. Infusion of the same extrogen solution just outside the hypophysial fossa or subcutaneously was ineffective in preventing the formation of castration cells. The dose of estrogen necessary to depress the post-castration changes in the pituitary basophils,

when infused into the hypophysial fossa, was only 1/7 to 1/10 of the amount required to produce the same effects when given systemically. Rose and Nelson (1957) concluded therefore, that estrogens influence gonadotropin production and release by a direct effect on the AP. The observation of Desclin (1950) that estrogen promotes acidophil degranulation in AP transplants provides further evidence that estrogens can influence the AP by a direct action on the adenohypophysial cells.

Evidence from the studies of a number of investigators indicates that estrogens can also influence AP gonadotropin secretion by an action at the hypothalamic level. Flerko and Szentagothai (1957, see also Flerko, 1962) observed that transplantation of ovarian tissue into the hypothalami of female rats resulted in atrophy of the gonads and reproductive tracts. Similar ovarian implants in the AP, or implants of hepatic tissue in the hypothalamus, were ineffective in depressing FSH secretion. Flerko concluded that the minute amounts of estrogen, secreted by the intrahypothalamic ovarian fragments, acted upon the neural centers which govern FSH secretion and the secretion of the gonadotropin by the AP was consequently reduced. Implantation

of an estrogen-filled, 27 gauge needle into the hypothalamus of male and female rats results in a marked reduction of FSH and LH secretion as evidenced by atrophy of the reproductive organs (Lisk, 1960). Blank needle implants were without effect. Davidson and Sawyer (1961a) observed that similar estrogen implants in the posterior median eminence-basal tuberal region of female rabbits resulted in failure of copulation-induced ovulation and eventually to ovarian atrophy. Estrogen implants into the AP, mammillary body and other parts of the brain were without effect on reproductive function in the rabbits. It is evident from the studies cited that estrogen influences FSH-LH secretion by an action at the hypothalamic and probably at the adenohypophysial level also.

Kanematsu and Sawyer (1962) have recently reported the effects of intrahypothalamic and pituitary implants of estrogen on prolactin secretion in rabbits. Insertion of stainless steel tubes containing minute amounts of estradiol benzoate in the ends, into the posterior median eminence area of the hypothalamus, elevated the pituitary prolactin content of the APs of the rabbits but did not initiate lactation. Conversely, the AP prolactin content

of the rabbits with intra-pituitary estrogen implants was relatively low and lactation was initiated. These investigators also noted gonadal atrophy in the rabbits with intra-hypothalamic implants and they found that the adrenals were inlarged in these animals. This latter observation suggests that estrogens may increase ACTH secretion by an action at the hypothalamic level. Kanematsu and Sawyer interpreted the results from their studies as indicating that estrogen increases prolactin synthesis by an action on the hypothalamus, and the effect of estrogen on the pituitary is that of inducing prolactin release.

The results of the present studies which demonstrate that estradiol stimulates prolactin production by AP explants in vitro, indicate that the effect of estrogens at the AP level, in increasing prolactin secretion, is not merely the result of increased release of prolactin or to conditions which favor release of the hormone.

The relative importance of the estrogen effects at the hypothalamic or adenohypophysial levels, in promoting prolactin secretion, cannot be adequately ascertained at present. It is evident, however, that estrogens can influence prolactin synthesis and release by actions at the hypothalamic and pituitary levels.

EXPERIMENT XI. EFFECTS OF PROGESTERONE AND TESTOSTERONE.

Progesterone and testosterone are reported to stimulate prolactin secretion *in vivo*. Reece and Bivins (1942) observed that administration of large doses of progesterone to ovariectomized mature female rats increases the AP prolactin content and initiates lactation. Similar effects of progesterone on AP prolactin levels were reported by Meites and Turner (1948) in rats and guinea pigs. Selye et al. (1936), Laqueur (1943) and Marx (1945) reported that testosterone and testesterone propionate augment prolactin secretion, as evidenced by the initiation of lactation in female rats. Testosterone induces mammary secretion in gonadectomized female rats and increases the prolactin content of their AP glands (Reece and Mixner, 1939). Androsterone, however, was ineffective in these respects (Reece, 1941). Since it has not been demonstrated that progesterone and testosterone increase prolactin secretion by an action at the pituitary level or elsewhere, it was of interest to determine if these steroids could alter prolactin production in the *in vitro* organ culture system.

Methods

The paired culture and assay procedure was used for these studies with progesterone and testosterone. The medium samples were assayed in White King pigeons and the prolactin potencies in IU were calculated from the standard dose-response curve (Experiment XV). Five birds were used to assay the medium samples from each culture pair. Six culture pairs with 1/2 an AP per dish were incubated for the testosterone study. Each dish in 5 of the culture pairs of the progesterone study contained 1/2 an AP and each dish in the remaining 3 culture pairs of this study contained 2 AP halves from 2 different rats. Testosterone or progesterone was added to the 199 medium at a concentration of 2 μgm/ml. The steroids were added to the medium from ethanol stock solutions, as described previously, and the control medium contained an equivalent concentration of ethanol. The culture dishes from both studies were incubated for 3 days in O_2 - CO_2 atmosphere.

Three additional culture experiments were performed with progesterone at a concentration of 10 μgm per ml and one was conducted with this steroid at a concentration of 5 μgm per ml. Eight culture pairs (64 cultures) were used

for each of these studies; however the progesterone was apparently toxic to the AP explants at 5 and 10 μgm per ml; therefore, the medium samples were not assayed. One additional culture study involving 8 culture pairs (16 culture dishes) was performed with testosterone at a concentration of 5 μgm per ml. The explant survival at this dose of testosterone was judged to be unsatisfactory, therefore the prolactin levels of the medium samples were not determined.

Results and Discussion

The data from the study with progesterone are shown in Table 22 and summarized in Table 23. It may be seen that progesterone did not influence the weight of the AP explants and had no significant effect on the prolactin levels of the medium samples as indicated by all 3 parameters (total RTU, IU per cultured AP and IU per 100 mg explant weight). Table 24 presents the data from the testosterone study which are summarized in Table 25. It is evident from these data that testosterone was as ineffective as progesterone in influencing prolactin secretion *in vitro* and testosterone had no apparent effect on explant weight. It is thus indicated that *in vivo* augmentation of prolactin secretion by

progesterone and testosterone is probably the result of an action other than a direct effect on the AP.

The average prolactin levels in the control medium samples of the progesterone and testosterone studies were 34.2 and 24.3 IU per 100 gm of explant weight. An assay on fresh, uncultured AP tissue from 6 CFN rats of the same age as used in this study, as measured in 6 pigeons, disclosed that the fresh tissue contained 1.37 $\pm$ 0.28 IU of prolactin per mg. The explants in control medium from the progesterone and testosterone studies consequently produced about 24.9 and 17.7 times as much prolactin, respectively, as was introduced into the culture dishes at the beginning of culture. This again represents substantial net hormone synthesis and further illustrates the independence of prolactin secretion from hypothalamic or other <u>in vivo</u> influence.

Table 22

EFFECT OF 2.0 μGM PER ML OF PROGESTERONE ON PROLACTIN SECRETION IN VITRO
Three Day Cultures with 1/2 or 1 AP per Dish
Five Pigeons per Assay

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	2.2*	6.00	0.28	0.56	12.7	
Progesterone-1	2.0	4.89	0.23	0.46	11.5	- 9.5
Control-2	1.8*	8.63	0.49	0.98	27.2	
Progesterone-2	1.9	8.25	0.46	0.92	24.2	- 11.0
Control-3	1.7*	6.38	0.32	0.64	18.8	
Progesterone-3	1.9	6.00	0.28	0.56	14.7	- 21.8
Control-4	1.8*	5.13	0.24	0.48	13.3	
Progesterone-4	2.1	8.03	0.48	0.96	22.9	+ 72.2
Control-5	1.1*	5.18	0.24	0.48	21.8	
Progesterone-5	1.1	4.80	0.22	0.44	20.0	- 8.3
Control-6	3.5+	12.70	1.10	1.10	31.4	
Progesterone-6	2.4	8.70	0.49	0.49	20.4	- 35.0
Control-7	3.9+	15.00	1.68	1.68	43.1	
Progesterone-7	2.9	14.40	1.58	1.58	54.4	+ 26.2
Control-8	2.0+	15.90	2.10	2.10	105.0	
Progesterone-8	2.5	15.00	1.68	1.68	67.2	- 36.0

* 1/2 AP per dish. + 1 AP per dish.

TABLE 23

SUMMARY OF THE IN VITRO EFFECTS OF 2.0 μGM PER ML OF PROGESTERONE

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Progesterone				
Explant weight mg	2.25	2.10	- 0.15 ± 0.15	-	1.00	N.S.
IU prolactin per AP	1.00	0.89	- 0.12	- 3.27 ±11.7	0.28	N.S.
IU prolactin per 100 mg AP	34.17	29.40	- 4.70	- 2.90 ± 7.4	0.39	N.S.
Total RTU	9.37	8.83	- 0.53	- 1.22 ± 7.9	0.15	N.S.

TABLE 24

EFFECT OF 2.0 μGM PER ML OF TESTOSTERONE ON PROLACTIN SECRETION IN VITRO
Three Day Cultures with 1/2 AP per Dish.
Five Pigeons per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	2.3	13.00	1.18	2.36	51.3	
Testosterone-1	3.1	12.00	0.95	1.90	30.6	- 40.4
Control-2	2.4	8.63	0.47	0.94	19.6	
Testosterone-2	1.8	7.50	0.38	0.76	21.1	+ 7.7
Control-3	1.7	6.38	0.32	0.64	18.8	
Testosterone-3	2.4	9.75	0.60	1.20	25.0	+ 33.0
Control-4	2.1	8.25	0.45	0.90	21.4	
Testosterone-4	2.7	8.78	0.50	1.00	18.5	- 13.6
Control-5	1.9	6.38	0.32	0.64	16.8	
Testosterone-5	1.7	6.75	0.34	0.68	20.0	+ 19.0
Control-6	1.6	6.18	0.29	0.58	18.1	
Testosterone-6	2.2	5.85	0.27	0.54	12.3	- 32.2

TABLE 25

SUMMARY OF THE IN VITRO EFFECTS OF 2.0 μGM PER ML OF TESTOSTERONE

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Testosterone				
Explant weight mg	2.00	2.32	+ 0.12 ± 0.08	-	1.45	N.S.
IU prolactin per AP	1.01	1.01	- 0.003	+ 9.9 ±12.7	0.78	N.S.
IU prolactin	24.33	21.25	- 2.55	- 4.41 ± 5.2	0.85	N.S.
Total RTU	8.14	8.44	+ 0.31	+ 6.63 ± 7.7	0.86	N.S.

Conclusions

The dominant effect of androgens and progesterone on FSH - LH secretion is generally agreed to be depressant (Greep, 1961). ACTH secretion is apparently reduced by progesterone (Holub, 1961) and testosterone (Zizine, 1956) (see also Ganong, 1962). Davidson and Sawyer (1961b) demonstrated that implantation of minute amounts of solid testosterone propionate into the posterior median eminence - posterior tuberal region of the hypothalamus of male dogs led to aspermia and profound testicular and prostatic atrophy. Implants in the AP were essentially ineffective in this respect as were implants in other areas of the CNS. This observation indicates that the effects of androgens on gonadotropin secretion in vivo are mediated by an effect, which appears to predominate at least, at the hypothalamic level. It is of interest that similar studies with estrogen have shown that this same area of the hypothalamus is concerned with regulation of FSH - LH (Davidson and Sawyer, 1961a) and prolactin (Kanematsu and Sawyer, 1962) secretion in female rabbits.

Ralph and Fraps (1960) have shown that progesterone influences AP function in hens by an action at the

hypothalamic level. They injected progesterone into the hypothalamus of hens and noted that premature ovulation was induced. Intra-pituitary injection of progesterone was ineffective in promoting ovulation in the birds. It seems evident therefore that progesterone can also influence AP function via the hypothalamus.

Since neither progesterone nor testosterone influenced prolactin secretion by rat AP in vitro, an hypothalamic mediated effect on the AP may explain the observations that these steroids increase prolactin secretion in vivo. Another likely explanation of augmented prolactin secretion in vivo by progesterone and testosterone would be the conversion of these hormones into estrogenic steroids. Baggett et al. (1956) demonstrated the conversion of testosterone to estradiol in vitro by slices of human ovary. Slices of human adrenal cortical carcinoma also convert testosterone to estradiol and estrone in vitro (see Villee, 1961). West et al. (1956) found that administration of testosterone to castrated, adrenalectomized women resulted in elevated excretion of estrogen. This suggests that tissues other than adrenals and gonads, presumably the liver, can convert androgens to estrogenic steroids. Progesterone is an

intermediate product in the biosynthetic pathways of all of the steroid hormones. It has been demonstrated that this luteal steroid can also be converted into estrogens *in vivo* (Davis and Plotz, 1958) and *in vitro* (Ryan and Smith, 1961). It thus seems possible that elevation of prolactin secretion induced by progesterone and testosterone can be explained on the basis of *in vivo* conversion of these steroids into estrogens. Since estrogenic steroids are exceedingly potent in promoting prolactin secretion by the AP *in vivo* (Meites and Turner, 1948), and, as shown in Experiment X, *in vitro*, it is conceivable that small amounts of estrogens, arising from administered progesterone and testosterone, could effect increased prolactin secretion by an action at the AP and/or hypothalamic level(s).

Rothchild (1960b) has proposed that progesterone-LTH (prolactin) relationship in the rat is that of a positive feedback mechanism, which is the converse of the feedback mechanisms operative between the AP and the adrenal cortex, ovarian follicular apparatus and thyroid glands. He observed that neither hemiovariectomy nor progesterone administration influenced the size of the corpora lutea of pseudopregnant rats. The same lack of effect of unilateral ovariectomy on

luteal size was noted in hypophysectomized rats with transplanted APs. Large doses of progesterone were also ineffective in altering the quantity of luteal tissue in these AP transplanted rats. If the corpus luteum - LTH relationship was of the nature of a negative feedback mechanism, hemiovariectomy would have been expected to increase the size of the corpora lutea in the rats due to augmented LTH secretion. This compensatory hypertrophy probably would not be expected in the hypophysectomized rats bearing the transplanted APs since the engrafted AP apparently secretes prolactin (LTH) in maximal or near maximal quantities and progesterone evidently has no direct effect on prolactin secretion by the AP, as shown by this in vitro study.

Administration of large quantities of progesterone would be expected to reduce the mass of luteal tissue in the rats of Rothchild's study if the corpus luteum - LTH feedback mechanism was a negative one. Since luteal regression was not realized with large quantities of progesterone, it seems probable that the corpus luteum - LTH relationship is not of a negative feedback character. Rothchild's hypothesis (1960b) of a positive feedback mechanism between the AP and corpus luteum maintains that

progesterone promotes LTH secretion by the AP and the LTH fosters progesterone secretion by the corpus luteum. If this mechanism does exist in the rat the observations from the present *in vitro* study would indicate that the progesterone effect on LTH secretion may be via the hypothalamus. *In vivo* conversion of progesterone into estrogens, with consequent estrogenic effects on prolactin secretion, also remains a possibility in this connection.

The results of this *in vitro* study with progesterone and testosterone indicate that any effects of these steroids on prolactin secretion *in vivo* are not mediated by an action at the AP level. Possibly these hormones influence AP prolactin secretion indirectly via hypothalamic receptor sites or through conversion, by adrenal, ovarian or hepatic tissues, into estrogenic steroids.

EXPERIMENT XII. EFFECTS OF HYDROCORTISONE AND CORTICOSTERONE.

It has been quite firmly established that the function of adrenal glucocorticoids, in maintaining the homeostatic equilibrium of the hypothalamo-hypophysial-adrenalcortical mechanism, is that of holding ACTH secretion in balance (Sayers and Sayers, 1948). Glucocorticoids can apparently depress ACTH secretion by an action on the AP component of the hypothalamo-hypophysial apparatus. Rose and Nelson (1956) demonstrated that infusion of cortisol into the pituitary fossa of stressed and unilaterally adrenalectomized rats resulted in a significant inhibition of adrenal cortical hypertrophy, indicating depressed ACTH output. Infusion of cortisol elsewhere in the subarachnoid space was without effect on ACTH secretion. The observations that cortisol administration depresses the pituitary response to treatment with corticotropin releasing factor (Royce and Sayers, 1958; Rockfort et al., 1959) suggests that glucocorticoids interfere with the action of CRF at the AP level. Other evidence which indicates that glucocorticoids depress ACTH synthesis has been reviewed recently by Ganong (1962).

Johnson and Meites (1955) reported that administration of cortisone or hydrocortisone induced a moderate degree of mammary secretion and promoted a slight elevation in the pituitary prolactin content of intact female rats. These results were interpreted as indicating that the glucocorticoids increased prolactin secretion. Selye (1954) reported that glucocorticoids could initiate lactation in ovariectomized rats simultaneously treated with estrogen. This observation, however, cannot be considered as evidence that corticoids influence prolactin secretion since the estrogen treatment per se would presumably augment the secretion of prolactin (Meites and Turner, 1948). Since adrenal glucocorticoids depress ACTH secretion *in vivo*, by an action, which is exerted in part at least, on the AP gland, and since prolactin secretion may be increased by glucocorticoid treatment, it was of interest to determine whether cortisol and corticosterone could influence prolactin secretion *in vitro* by a direct action on the AP cells.

Methods

The paired culture and assay procedure was used for these studies with cortisol and corticosterone. The AP

explants were incubated on stainless steel rafts inside the plastic Petri dishes with 3 ml of medium in O_2 - CO_2 atmosphere. Anterior pituitary glands from CFN multiparous female rats were used in the cortisol study and each culture dish of 2 of the culture pairs contained explants from 2 AP halves (equivalent to 1 rat AP). Each dish of the remaining 4 culture pairs contained explants from 1/2 an AP gland. Cortisol was added to the 199 medium from an ethanol stock solution to give a final concentration of 10 μgm per ml. The cultures in this study were incubated for 6 days and the medium was changed on the third day of culture.

For the study with corticosterone, each culture dish from each of the 8 culture pairs contained explants from 1/2 of an AP from 3-month-old CFN female rats (180 - 200) gram body weight range). The cultures in this study were incubated for three days and the steroid was incorporated into the medium, from an ethanol stock solution, at a concentration of 20 μgm per ml. Two additional culture experiments, involving 16 culture pairs, were performed with corticosterone at concentrations of 30 and 50 μgm/ml. These levels proved to be toxic to the explants, however, therefore the medium samples were not assayed.

The medium samples from each culture dish of each culture pair were assayed for prolactin activity in White King squabs. The prolactin potency of the medium samples in IU was determined by use of the standard dose-response plot (Experiment XV). The effect of cortisol on the pigeon crop response to prolactin was determined by injecting 10 pigeons with a total dose of 10 μgm of NIH prolactin in 199 medium over the right crop sac. The left crops of the same birds were treated with the same prolactin preparation containing 10 μgm per ml of cortisol.

Results and Discussion

The results of the cortisol study are shown in Table 26 and summarized in Table 27. The prolactin activities in the cortisol medium samples were lower than the control samples in all 6 cases on the basis of total RTU, IU/AP or IU per 100 mg explant weight. The hydrocortisone medium samples had prolactin activities which were lower than the control medium samples by an average of 52.8 $\pm$ 7.5% on the basis of IU per 100 mg explant weight ($P < 0.001$), 61.6 $\pm$ 5.3% on the basis of IU/AP ($P < 0.001$) and 27.8 $\pm$ 5.7% on the basis of total RTU ($P < 0.01$). Hydrocortisone had no apparent effect on the weight of the explants. The pigeon crops

injected with the standard 10 μgm dose of prolactin gave an average response of 1.63 ± 0.15 RTU and the cortisol treated crops responded with an average of 1.53 ± 0.14 RTU. This difference was not significant. Since hydrocortisone did not influence the pigeon crop response to prolactin, it seems reasonable to conclude that the lower prolactin level in the cortisol medium samples was the result of an action of the steroid on the AP explant cells.

The data from the study with corticosterone are shown in Table 28 and summarized in Table 29. It can be seen that compound B had no apparent effect on the prolactin activities of the medium samples. The slightly higher average levels of prolactin found in the corticosterone medium samples are not significantly different from the values found in the control samples. The absence of an effect of corticosterone on prolactin production, when hydrocortisone exerted a pronounced depressant effect at half the dose level, is probably a further illustration of the relative biological impotency of corticosterone as compared to hydrocortisone (Dorfman, 1950). The explants incubated in the corticosterone medium had an average weight of 2.63 mg and the AP fragments from the control

cultures averaged 2.10 mg. The corticosterone-treated explants therefore averaged 0.53 mg higher in weight than the control explants, and this difference in wet weight of 22.9 ± 5.9% is highly significant ($P < 0.01$). Possibly the corticosterone caused a greater degree of hydration of the AP explants; however further investigation will be required to definitely establish the cause of this weight difference.

The prolactin content of the medium samples from the control cultures of the cortisol study averaged 62.1 ± 26.1 IU per 100 mg of explant weight. The prolactin content of 6 APs from the same old breeder female rats was found to be 1.71 ± 0.25 IU/100 mg as measured in 8 pigeons. The AP explants of the control cultures from the cortisol study therefore synthesized in 6 days about 36.3 times as much prolactin as was present in the culture system at the beginning of incubation. The medium samples from the 8 control cultures of the corticosterone study had an average prolactin content of 24.8 ± 4.6 IU per 100 mg of explant weight. An assay of APs from similar 3 month-old rats, which was determined in conjunction with the assays on the medium samples, disclosed that the AP tissue contained

1.26 ± 0.16 IU prolactin per 100 mg wet weight. The explants in the control cultures from this study with compound B, therefore, synthesized about 19.7 times as much prolactin in 3 days of culture as they contained initially. Both of these studies further illustrate the high degree of prolactin production which can be obtained in the *in vitro* culture system.

TABLE 26

EFFECT OF 10 μGM PER ML OF CORTISOL ON PROLACTIN SECRETION IN VITRO
Six Day Cultures with Explants from 1/2 or 1 AP per Dish.
Five Birds per Assay

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	8.1	14.4	1.55	1.55	19.1	
Cortisol-1*	7.3	12.0	0.97	0.97	13.3	- 30.4
Control-2	7.8	21.0	5.88	5.88	75.4	
Cortisol-2*	7.7	15.0	1.77	1.77	22.9	- 69.6
Control-3	3.8	16.5	2.36	4.72	62.1	
Cortisol-3+	3.9	9.8	0.62	1.24	15.9	- 74.4
Control-4	3.7	22.4	6.72	13.44	181.6	
Cortisol-4+	4.7	17.8	3.09	6.18	65.7	- 63.8
Control-5	4.7	8.8	0.50	1.00	10.6	
Cortisol-5+	5.1	4.6	0.22	0.44	4.3	- 59.4
Control-6	5.4	13.4	1.28	2.56	23.7	
Cortisol-6+	6.3	11.7	0.93	1.86	14.8	- 37.6

* 1 AP per dish. + 1/2 AP per dish.

TABLE 27

SUMMARY OF THE IN VITRO EFFECTS OF 10 μGM PER ML OF HYDROCORTISONE

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Cortisol				
Explant weight mg	5.58	5.83	+ 0.25 ± 0.16	-	1.56	N.S.
IU prolactin per AP	4.86	2.08	- 2.78	- 52.8 ± 7.5	7.04	0.001
IU prolactin per 100 mg AP	62.1	22.8	-39.30	- 55.9 ± 6.9	8.1	0.001
Total RTU	13.9	11.7	- 3.57	- 27.8 ± 5.7	4.89	0.01

TABLE 28

EFFECT OF 20 μGM PER ML OF CORTICOSTERONE (B) ON PROLACTIN SECRETION IN VITRO
3 Day Cultures with Explants from 1/2 AP per Dish.
Five Birds per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	1.9	7.5	0.38	0.76	20.0	
B-1	2.3	9.0	0.53	1.06	23.0	+ 15.0
Control-2	2.0	8.0	0.42	0.84	21.0	
B-2	2.8	10.5	0.71	1.42	25.4	+ 20.9
Control-3	2.3	9.38	0.57	1.14	24.8	
B-3	3.4	15.33	1.89	3.78	55.6	+124.2
Control-4	2.1	8.50	0.47	0.94	22.4	
B-4	2.9	10.50	0.71	1.42	24.5	+ 9.4
Control-5	2.7	14.10	1.47	2.94	54.4	
B-5	2.9	10.00	0.64	1.28	22.1	- 59.4
Control-6	1.9	6.10	0.28	0.56	14.7	
B-6	2.9	6.25	0.30	0.60	10.3	- 29.9
Control-7	1.9	9.10	0.53	1.06	27.9	
B-7	1.7	5.86	0.27	0.54	15.9	- 43.0
Control-8	2.0	5.73	0.27	0.54	13.5	
B-8	2.1	5.20	0.24	0.48	11.4	- 15.6

TABLE 29

SUMMARY OF THE IN VITRO EFFECTS OF 20 μGM PER ML OF CORTICOSTERONE

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Corticosterone				
Explant weight mg	2.10	2.63	+ 0.53	+ 22.9 ± 5.9	3.88	0.01
IU prolactin per AP	1.10	1.32	+ 0.23	+ 35.2 ±25.1	1.40	N.S.
IU prolactin per 100 mg AP	24.84	25.53	- 1.31	+ 2.7 ±13.4	0.20	N.S.
Total RTU	8.55	9.08	+ 0.65	+ 9.4 ± 5.8	1.62	N.S.

Conclusions

The *in vivo* secretion patterns of prolactin and ACTH are apparently identical in a wide variety of circumstances. The secretion of both AP hormones is promoted by such divergent conditions as stresses (Swingle et al., 1951b; Nicoll et al., 1960), estrogen treatment (Gemzell, 1953; Meites and Turner, 1948), the suckling stimulus (Gregoire, 1947; Reece and Turner, 1937) and adrenalectomy (Sydnor and Sayers, 1954; Swingle et al., 1951a). The demonstration in the present study, that hydrocortisone depresses prolactin secretion *in vitro* by a direct action on the AP cells, further illustrates the similarities in the secretion of these two AP hormones. As discussed previously, cortisol can apparently depress ACTH secretion also by an action at the AP level (Rose and Nelson, 1956; Royce and Sayers, 1958; Rockfort et al., 1959).

Since it has been firmly established that cortisone and cortisol depress ACTH secretion *in vivo* (Sayers and Sayers, 1948), and the secretion patterns of prolactin and ACTH are apparently identical under a wide variety of conditions, glucocorticoid administration might also be expected to depress prolactin secretion *in vivo*. The observation of

Johnson and Meites (1955), that cortisone and hydrocortisone may increase prolactin secretion in intact female rats, therefore seems rather anomalous. Possibly a lower dose of cortisol would alter prolactin secretion *in vitro* in a manner different from the depressant effect observed in this study; however the data on hand indicate that glucocorticoids probably do not increase prolactin secretion *in vivo* by an action at the AP level. Possibly cortisone can alter prolactin secretion *in vivo* by an action at the hypothalamic level of the hypothalamo-hypophysial apparatus, or through some other means. It is evident, however, that the *in vivo* and *in vitro* effects of glucocorticoids on prolactin secretion warrant further study in order that this apparent dichotomy may be clarified.

In the study with estradiol (Experiment X), support for the conclusion, that estrogen increased prolactin secretion by a direct action on the AP explant cells was recruited from the observations that estrogens stimulate the respiratory metabolism of AP tissue *in vitro* (Victor and Anderson, 1937; Gaul and Villee, 1959). The conclusion of this study, that hydrocortisone depressed prolactin production by an effect on the AP explant cells, is

also supported by a study on the respiratory activity of AP tissue. Roberts and Keller (1955) have shown that cortisone, although at a very high concentration, depresses the oxygen consumption of rat AP tissue *in vitro*.

EXPERIMENT XIII. EFFECTS OF THYROXINE AND TRIIODOTHYRONINE

Evidence that the hormones of the thyroid gland can influence prolactin secretion has been provided by a number of experimental observations. Weichert and Boyd (1933-34) reported that pseudopregnancy was induced in rats when they were fed desiccated thyroid, indicating enhanced prolactin (LTH) secretion. Hypothyroidism, as induced by thyroidectomy or thiouracil treatment, reduces the AP prolactin content of male (McQueen - Williams, 1935) and young female (Meites and Turner, 1947) rats. Large doses of thyroxine have been reported to reduce the AP prolactin levels in male rats (Reece and Turner, 1937) but to have no effect on the pituitary prolactin content of female mice (Hurst and Turner, 1942).

The galactopoetic action of thyroxine and thyroactive substances (e.g. thyroprotein) in ruminants (Blaxter et al., 1949) and rats (Grosvenor and Turner, 1959b) may be partially the result of augmented prolactin secretion, as suggested by Folley and White (1936). Grosvenor (1961) recently reported that the AP prolactin content of rats, which were rendered hypothyroid by tapazole treatment, was reduced below the levels of normal control animals.

Administration of high "euthyroid" doses of thyroxine to these hypothyroid rodents elevated the AP prolactin levels above the control values. Moon (1962) has confirmed Grosvenor's report in a study with ovariectomized rats treated with estrogen progesterone and tapazole. He observed that thyroxine administration to these steroid-treated, hypothyroid rats elevated the AP prolactin content and increased mammary lobulo-alevealor growth. Stimulation of mammary growth is suggestive of increased prolactin secretion (Talwalker and Meites, 1961).

These studies provide some evidence, of an indirect nature, that thyroid hormones may increase prolactin secretion in vivo. An investigation of the effects of thyroid hormones on prolactin secretion in vitro was therefore undertaken to determine if these hormones could alter prolactin production by an action at the AP level.

Methods

The AP glands of 3-month old CFN female rats, weighing 200 - 220 grams, were used for the study with thyroxine (T_4). Sixteen glands from 4 month old CFN female rats (180 - 200 gram body weight) were used for the study with triiodothyronine (T_3). The paired culture and assay

procedure was used in both of these studies and the AP explants were incubated in the plastic Petri dish-stainless steel platform preparations. All of the cultures were incubated for 3 days in O_2 - CO_2 atmosphere with 3 ml of insulin-containing 199 medium per culture dish. Thyroxine was added to the 199 medium at a concentration of 0.1 μgm per ml and T_3 was used at the same concentration. The explants from these cultures were weighed at the end of incubation and the medium samples were assayed in White King squabs. Five pigeons were used to assay the prolactin levels in the medium samples from each culture pair in the T_4 study and 3 birds were used for each assay in the T_3 experiment. The prolactin activities were converted to IU by use of the dose-response plot established with the standard prolactin preparations (Experiment XV).

In order to determine if thyroxine would influence the pigeon crop response to prolactin, 10 White King birds were injected over the right crop sac with a total dose of 5 μgm of NIH prolactin per bird in 199 medium. The same prolactin dose in 199 with a thyroxine concentration of 0.1 μgm per ml was administered over the left crop half of the same birds. Triiodothyronine was also tested for its effects on

the pigeon crop response to prolactin by the same procedure, except that each bird received a total dose of 20 μgm of NIH prolactin and 9 squabs were used.

Results and Discussion

The data from the thyroxine study are shown in Table 30 and summarized in Table 31. The results of the T_3 experiment are presented in Table 32 and abridged in Table 33. Neither T_4 nor T_3 altered the weight of the AP explants as evidenced by the insignificant weight differences between the controls and experimentals of 0.03 ± 0.18 and 0.04 ± 0.08 mg respectively. The prolactin levels in the T_4 medium samples, on the basis of IU/AP, were higher than the control values in all of the 9 culture pairs. This difference averaged 51.1 ± 12.2% higher in the T_4 medium samples and was highly significant ($P < 0.01$). On the basis of IU/100 mg of explant weight, the T_4 medium samples had higher prolactin activity than the control samples in 8 of the 9 culture pairs and this increased activity averaged 51.8 ± 14.4% ($P < 0.01$). The prolactin content of the T_4 medium samples, when expressed as total RTU, was significantly greater than the control values by 24.3 ± 5.7% ($P < 0.01$).

In the study with triiodothyronine, the prolactin levels in the T_3 medium samples were higher than the control levels in 7 of the 8 culture pairs on the basis of total RTU or IU/AP. On the basis of IU per 100 mg explant weight, the prolactin activity of the T_3 medium samples was higher than the control values in all 8 cases. The prolactin levels in the medium from the T_3 cultures, on the basis of total RTU, IU/AP and IU per 100 mg explant weight, averaged 24.3 $\pm$ 5.95%, 62.8 $\pm$ 21.2% and 59.3 $\pm$ 13.2% higher than the levels in the control medium samples, respectively. These differences are all highly significant at the same probability level ($P < 0.005$).

The pigeon crop sacs injected with the 5 μgm dose of prolactin gave an average response of 1.28 $\pm$ 0.07 RTU and the crops from the same birds treated with the same dose of prolactin with thyroxine gave an average response of 1.30 $\pm$ 0.10 RTU. This difference is not significant. The crop sacs treated with 20 μgm of prolactin gave an average response of 2.44 $\pm$ 0.29 RTU whereas the contralateral crops of these birds treated with same dose of prolactin with T_3, showed an average response of 2.31 $\pm$ 0.22 RTU. This difference was again not significant. It seems evident,

therefore, that neither thyroxine nor triiodothyronine, at the dose levels used (0.1 μgm per ml), influenced the pigeon crop response to prolactin.

Since neither T_4 nor T_3 influenced the pigeon crop response to prolactin, the higher prolactin levels observed in the cultures with these thyroid hormones are probably the result of a direct action of T_4 and T_3 on the AP explants. These results confirm and extend the observations of the previously cited studies which suggested that thyroid hormones increased prolactin secretion in vivo, and indicates that the galactopoetic action of thyroidal material can be explained, in part at least, by augmented prolactin production. The stimulatory action of T_4 and T_3 on prolactin secretion are obviously qualitatively and quantitatively similar at the dose levels used.

The control culture medium samples from the T_4 study had an average prolactin content of 13.4 ± 1.8 and those from the T_3 study averaged 22.4 ± 2.3 IU per 100 mg of explant weight. An assay of 6 AP glands from similar rats, as determined in 9 pigeons, disclosed that the fresh tissue contained 1.24 ± 0.12 IU/100 mg. It is therefore evident that the AP explants from the control cultures from the

T_4 and T_3 studies synthesized 10.8 and 18.0 times more prolactin respectively than was initially introduced into the culture system.

TABLE 30

EFFECT OF 0.1 μGM PER ML OF THYROXINE ON PROLACTIN SECRETION IN VITRO
Three Day Cultures with Explants from 1/2 AP per Dish. Five Pigeons per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	5.1	12.70	1.10	2.2	21.6	
Thyroxine-1	4.3	15.70	2.00	4.0	46.5	+115.3
Control-2	5.0	6.17	0.29	0.58	5.8	
Thyroxine-2	4.9	9.75	0.61	1.22	12.4	+115.5
Control-3	3.3	7.50	0.38	0.76	11.5	
Thyroxine-3	4.0	9.50	0.60	1.20	15.0	+ 30.4
Control-4	4.0	9.50	0.60	1.20	15.0	
Thyroxine-4	4.2	10.50	0.71	1.42	16.9	+ 12.7
Control-5	3.6	6.20	0.29	0.58	8.1	
Thyroxine-5	4.4	6.70	0.34	0.68	7.7	- 4.9
Control-6	3.4	7.00	0.35	0.70	10.3	
Thyroxine-6	3.2	7.10	0.36	0.72	11.3	+ 9.7
Control-7	2.3	8.70	0.49	0.98	21.3	
Thyroxine-7	2.8	11.60	0.88	1.76	31.4	+ 47.4
Control-8	1.9	6.00	0.28	0.56	14.7	
Thyroxine-8	1.9	8.10	0.44	0.88	23.2	+ 57.8
Control-9	3.1	7.20	0.37	0.74	11.9	
Thyroxine-9	2.3	8.80	0.50	1.00	21.7	+ 82.4

TABLE 31

SUMMARY OF THE IN VITRO EFFECTS OF 0.1 μGM PER ML OF THYROXINE

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Thyroxine				
Explant weight mg	3.52	3.56	+ 0.03 ± 0.18	-	0.17	N.S.
IU prolactin per AP	0.92	1.43	+ 0.51	+ 51.1 ±12.2	4.19	0.001
IU prolactin per 100 mg AP	13.35	20.68	+ 7.42	+ 51.8 ±14.4	3.57	0.001
Total RTU	7.89	9.75	+ 1.86	+ 24.3 ± 5.7	4.26	0.001

TABLE 32

EFFECT OF 0.1 μGM PER ML OF TRIIODOTHYRONINE (T_3) ON PROLACTIN SECRETION IN VITRO
Three Day Cultures with Explants from 1/2 AP per Dish.
Three Pigeons per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg AP
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	2.8	9.38	0.57	1.14	20.3	
T3-1	2.6	11.25	0.81	1.62	31.2	+ 53.7
Control-2	2.5	9.00	0.53	1.06	21.2	
T3-2	2.1	8.50	0.47	0.94	22.4	+ 5.6
Control-3	2.1	12.00	0.95	1.90	45.2	
T3-3	3.0	17.50	2.89	5.78	96.3	+113.0
Control-4	3.3	12.50	1.05	2.10	31.8	
T3-4	2.7	14.50	1.58	3.16	58.5	+ 83.9
Control-5	3.5	5.50	0.26	0.52	7.4	
T3-5	3.2	8.50	0.47	0.94	14.7	+ 98.6
Control-6	2.7	8.00	0.43	0.86	15.9	
T3-6	2.9	10.50	0.71	1.42	24.5	+ 54.1
Control-7	3.1	9.24	0.55	1.10	17.8	
T3-7	3.0	8.50	0.71	1.42	23.7	+ 33.1
Control-8	2.8	9.24	0.55	1.10	19.6	
T3-8	3.0	11.00	0.78	1.56	26.0	+ 32.7

TABLE 33

SUMMARY OF THE IN VITRO EFFECTS OF 0.1 μGM PER ML OF TRIIODOTHYRONINE (T3)

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	T3				
Explant weight mg	2.85	2.81	- 0.04 ± 0.08	-	0.5	N.S.
IU prolactin per AP	1.22	2.10	+ 0.88	+ 62.8 ± 21.2	2.96	0.001
IU prolactin per 100 mg AP	22.40	37.76	+14.0	+ 59.3 ±13.2	4.55	0.001
Total RTU	9.36	11.53	+ 2.17	+ 24.3 ± 5.95	4.08	0.001

Conclusions

The secretory products of the thyroid gland can apparently alter the cytology and function of the pars distalis in a profound manner. Thyroidectomy or hypothyroidism, as induced by a treatment with goitrogens such as thiouracil, not only cause degranulation of the AP thyrotropic basophils (see Purves, 1961), but also cause a marked reduction in the number and degree of granulation of the AP acidophils (Zeckwer et al., 1935; Purves and Griesbach, 1946; see also Purves, 1961). These changes in AP cytology can be reversed by thyroxine replacement therapy (see Purves, 1961). It is of interest in this connection that Levey and Roberts (1957) have observed that the AP glands of thyroidectomized or thiouracil-treated rats consume more oxygen *in vitro* than APs from untreated controls. Adrenalectomy, castration or castration with thiouracil treatment were without effect on the respiratory metabolism of the AP tissue. The higher rates of oxygen utilization by the AP glands of the hypothyroid animals could be returned to normal by parenteral administration of thyroxine. The observation that hypothyroidism increases AP oxygen consumption is of interest since this condition

is associated with reduced metabolic activity in most other tissues of the body.

Thyroidectomy or goitrogen-induced hypothyroidism also cause reproductive irregularities suggestive of reduced gonadotropin secretion (see Reineke and Soliman, 1953). Contopoulos et al. (1958) have observed that thyroidectomy results in atrophy of the adrenal cortex, ovaries and reproductive organs of rats and reduces the STH activity in the plasma and AP glands of the rodents. A reduction in AP STH content after thyroidectomy has also been observed by Knigge (1958). Hypothyroidism also reduces the pituitary ACTH levels (Lazo - Wasem, 1960) and causes adrenal cortical atrophy (Bauman and Marine, 1946; Lazo - Wasem, 1960; McCarthy et al., 1959), indicating reduced secretion of adrenocorticotropin. Evidence that thyroid deficiency depresses prolactin secretion was discussed previously.

Thyroxine administration to hypothyroid animals restores the reduced rate of somatic growth (Purves and Griesbach, 1946), increases the low levels of STH in the AP glands (Solomon and Greep, 1959) and reverses the changes in AP cytology (Purves, 1961). Thyroid treatment also causes adrenal cortical enlargement indicating increased ACTH

secretion (Wallach and Reineke, 1949; Kruskemper, 1958). The restoration of AP prolactin content by thyroxine, in hypothyroid rats, as discussed previously, further illustrates the extent by which the thyroid status can alter AP function.

It has been quite firmly established that the homeostasis of the hypothalamo-hypophysial-thyroid apparatus is maintained by a negative feedback mechanism (Greep, 1957; D'Angelo, 1962). Evidence is accumulating that thyroid hormones depress TSH secretion, in part at least, by an action on the AP component of the hypothalamo-hypophysial terminus. Von Euler and Holmgren (1956) examined the effects of intra-pituitary and intra-hypothalamic injections of minute amounts of thyroxine on TSH secretion in rabbits. They found that only intra-pituitary injections of thyroxine depressed TSH secretion in doses which were systemically ineffective. Yamada (1959) performed similar microinjection studies in rats and reported that injections of minute quantities of thyroxine into the anterior hypothalamus were about as effective in depressing TSH secretions as intra-pituitary injection. Bogdanove and Crabill (1961) implanted fragments of thyroid glands into the APs or hypothalami of thyroidectomized rats and studied the effects of the

autotransplants on pituitary cytology. The intrapituitary implants prevented the post-thyroidectomy changes in the pituitary cells in their immediate vicinity indicating that the thyroid hormones secreted by the implants were acting directly on the AP cells. The intra-hypothalamic thyroidal implants exerted a slight restorative action on the thyroidectomy induced changes in AP morphology. A number of other studies also indicate that thyroid hormones depress TSH secretion by an action at the pituitary level. These studies have been reviewed by D'Angelo (1962).

The results of the present study on the effects of thyroid hormones on prolactin secretion in vitro, provide further evidence that thyroid hormones can influence AP function by an action at the pituitary level. Since prolactin secretion is augmented in vitro by an action of thyroid hormones on the explant cells, it seems reasonable to conclude that the other alterations in AP function induced by thyroid hormones may also be due, in part at least, to an action of thyroid hormones at the pituitary level. Hypothalamic involvement in the in vivo situation can not be excluded, however, since thyroid hormones may alter AP hormone secretion to a greater or lesser degree via effects on the hypothalamic centers which regulate AP function.

EXPERIMENT XIV. EFFECTS OF INSULIN

Very little attention has been given to the possible role that insulin may have in influencing the function of the AP gland. Gregory et al. (1956) observed that the basophils of the APs of dogs enlarged, became granulated and vacuolated after pancreatectomy. These changes could not be demonstrated if the diabetes was controlled with insulin or if pancreatectomy was incomplete. Similar changes were noted in the basophils of the APs of rabbits which were randered diabetic by alloxan administration (Thomas and Emerson, 1945). It was not determined, however, whether these changes were a primary or secondary consequence of the diabetic condition. Warren and Le Compte (1952) concluded, after examining clinical cases and the literature on diabetics, that no constant and significant changes occur in the hypophyses of patients with diabetes mellitus.

More recently, Goodner and Freinkel (1961) studied the effects of insulin and diabetes mellitus on carbohydrate metabolism of AP tissue *in vitro*. They found that insulin added *in vitro* increased glucose utilization by AP tissue from rats and calves. The APs of pancreatectomized or alloxan diabetic rats showed a reduced rate of glucose

utilization and this effect could be reversed by insulin replacement therapy. These investigators concluded that the AP is responsive to insulin and that hypophysial carbohydrate metabolism in the intact animal may be conditioned by the availability of insulin.

Insulin is required for the maintenance of the structural integrity and functional activity of a number of tissues, such as mammary gland in vitro (Rivera and Bern, 1961). Insulin was therefore incorporated into the medium used in most of the studies reported in this thesis. It was of interest, however, to determine if insulin was actually necessary for prolactin secretion in vitro. Such a study might also cast additional light on the effects of insulin on AP function.

Methods

The paired culture and assay procedure was used for this study. Anterior pituitary glands from 4-month-old CFN female rats were used and explants from 1/2 AP were cultured in each dish. The stainless steel platform - plastic Petri dish preparations were used and each culture dish contained 3 ml of medium. The explants were incubated in O_2 - CO_2 atmosphere. The control medium contained no

insulin and the insulin medium had the hormone added at the usual concentration (2 U/ml). At the end of the culture period the medium samples were assayed in White King squabs and the prolactin potencies in IU were determined by reference to the standard dose response plot (Experiment XV). Three pigeons were used to assay the medium samples from each culture pair. The lack of an effect of insulin on the pigeon crop response to prolactin was demonstrated in Experiment IV. Therefore this possibility was not re-examined with this study.

Results and Discussion

The data from this study with insulin are presented in Tables 34 and 35 and summarized in Table 36. It can be seen that no difference was apparent in the weights of the AP explants, and the prolactin activities in the insulin medium samples were not different from the levels found in the control medium samples. The average prolactin activity in the medium samples from the control and insulin cultures were 30.6 ± 6.7 and 28.5 ± 3.65 IU per 100 mg explant weight respectively. An assay of 6 pituitary glands from similar 4 month old CFN rats, in 199 medium, disclosed that the glands contained 2.26 ± 0.63 IU/100 mg wet weight. It is

therefore apparent that the explants from the control and insulin cultures synthesized about 13.6 and 12.6 times as much prolactin, respectively, as they contained when introduced into the culture system.

The lack of an effect of insulin on prolactin secretion *in vitro* indicates that the pituitary acidophils do not require insulin to effect substantial prolactin synthesis. The possibility that the insulin in the medium was destroyed during incubation, and consequently rendered inactive, can be discounted since the medium contains a high level of insulin potency after 3 days of culture. Meites and Hopkins (unpublished) found that injection of insulin-containing 199 medium from similar cultures of rat AP explants, into hypophysectomized weanling rats, resulted in the prompt precipitation of insulin shock in the animals. The incubation of the AP fragments in 199 medium may alter the permeability of the explant cells such that glucose could pass more readily through the cell membranes. The insulin would probably be ineffective in altering glucose entry into the cells in this circumstance and might consequently be without effect on prolactin synthesis.

The observations that insulin deficiency apparently

results in degenerative alterations only in the AP basophils in dogs (Gregory et al., 1956) and rabbits (Thomas and Emerson, 1945) suggest that insulin may be necessary for normal function of the basophils. Since insulin appears to be unnecessary for prolactin synthesis, and is therefore probably not required for glucose utilization by the AP acidophils, it seems reasonable to conclude that the reported *in vitro* stimulatory effects of insulin on AP tissue is probably due to an action on the pituitary basophil cells or at least not due to an action on the acidophils. It is obvious, however, that further investigation on the effects of insulin on AP physiology will be necessary before complete assessment of its role can be realized.

TABLE 34

EFFECT OF 2.0 U PER ML OF INSULIN IN 199 MEDIUM ON PROLACTIN SECRETION IN VITRO

Three Day Cultures with Explants from 1/2 AP per Dish. Three Birds per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-1	3.9	13.02	0.67	1.34	17.1	
Insulin-1	3.8	14.97	0.97	1.94	25.4	+ 48.5
Control-2	3.4	8.68	0.27	0.54	7.9	
Insulin-2	2.7	12.82	0.62	1.24	22.9	+189.9
Control-3	3.5	17.49	1.63	3.26	46.6	
Insulin-3	4.5	18.57	2.06	4.12	45.8	- 1.7
Control-4	2.9	11.92	0.53	1.06	18.3	
Insulin-4	3.5	14.62	0.90	1.80	25.7	+ 40.4
Control-5	3.5	14.04	0.82	1.64	23.4	
Insulin-5	3.3	13.50	0.73	1.46	22.1	- 5.5
Control-6	3.5	14.64	0.90	1.80	25.7	
Insulin-6	3.0	11.40	0.48	0.96	16.0	- 37.7

TABLE 35

EFFECT OF 2.0 U PER ML OF INSULIN IN 199 MEDIUM ON PROLACTIN SECRETION IN VITRO (Con't)

Three Day Cultures with Explants from 1/2 AP per Dish. Three Birds per Assay.

Culture Pair No. and Medium	Explant Weight Mg	PROLACTIN ACTIVITY IN MEDIUM				% Difference IU/100 Mg
		Total RTU	Total IU	IU/AP	IU/100 Mg AP	
Control-7	2.7	11.40	0.48	0.96	17.8	
Insulin-7	2.6	12.24	0.57	1.14	21.9	+ 23.0
Control-8	2.8	11.94	0.53	1.06	18.9	
Insulin-8	2.7	11.26	0.46	0.92	17.0	- 10.1
Control-9	1.9	10.42	0.39	0.78	20.5	
Insulin-9	3.3	13.51	0.73	1.46	22.1	+ 7.8
Control-10	3.0	17.90	1.76	3.52	58.7	
Insulin-10	3.2	17.10	1.51	3.02	47.2	- 19.6
Control-11	4.2	21.30	3.41	6.82	81.2	
Insulin-11	3.4	15.00	1.47	2.94	47.4	- 41.6

TABLE 36

SUMMARY OF THE IN VITRO EFFECTS OF 2.0 U PER ML OF INSULIN

Average of:	Culture		Average Difference	Average % Difference	t	P <
	Control	Insulin				
Explant weight mg	3.21	3.25	+ 0.04 ± 0.44	-		N.S.
IU prolactin per AP	2.07	1.91	- 0.16	+ 21.3 ±11.2	1.90	N.S.
IU prolactin	30.6	28.5	- 2.05	+ 17.88 ±15.8	1.12	N.S.
Total RTU	13.89	14.09	+ 0.20	+ 5.73 ±4.25	1.35	N.S.

EXPERIMENT XV. THE BIOASSAY OF PROLACTIN

The presence of a lactogenic factor in the pars anterior of mammals was first demonstrated by Stricker and Grüeter (1928). They initiated lactation in the mammary glands of pseudopregnant rabbits by injections of anterior pituitary material and thus set the foundation for establishing that lactation was a humorally controlled process.

Several bioassay procedures have been developed which utilize the response of the mammary gland as an index of prolactin stimulation. The degree of lactation initiated in the mammary glands of suitably prepared animals is used to determine the prolactin potency of the materials being tested. These bioassay methods, however, are of limited value since they lack sensitivity, are very highly subjective and are rather cumbersome. The mammalian bioassay methods have been reviewed by Meites and Turner (1950).

Riddle et al. (1931) discovered that the secretion of pigeon "crop milk" was stimulated by prolactin, and Riddle and Bates (1939) developed a method which used this phenomenon as a bioassay end point for prolactin. The weight of the pigeon crop sac was found to be related to the dose of prolactin administered. Several different procedures

have been developed for assaying prolactin which utilize the pigeon crop weight response (see Meites and Turner, 1950); however all of these methods are very insensitive.

A highly sensitive bioassay for prolactin was developed by Lyons and Page (1935) when they observed that intradermal injection of prolactin over the crop sacs of pigeons resulted in a localized proliferation of the mucosal epithelium directly under the site of injection. The assay procedure involved giving 4 daily injections and killing the pigeons on the 5th day. The crop sacs were removed from the birds and examined for proliferative responses. The prolactin potency of the injected material was estimated from the proportion of the assay birds showing a positive response. This method permits comparison of the prolactin potencies of two different materials in the same pigeons by injecting one preparation over one side of the crop sac and the other over the contralateral side.

Reece and Turner (1937) found that the area and thickness of mucosal proliferation, directly beneath the site of injection, is a function of the dose of prolactin when the hormone is injected intradermally over the crop sac.

They therefore modified the local intradermal method of Lyons and Page (1935) to develop a highly sensitive and quantitative bioassay for prolactin. In this method, the pigeons were injected once daily for 4 days and necropsy is performed on the 5th day.

The stimulated regions of the crop sacs are examined by holding the crops in front of a light source and the magnitude of the responses is rated by a subjective method which takes the area and thickness of the proliferated regions into account. The responses are rated from 0 to 4 in intervals of 0.25. Use of this method is illustrated by the following example. A moderately thick stimulated region with a diameter of about 2 cm is given a rating of one Reece-Turner unit (RTU). A response of equal diameter but of lesser thickness is rated less than one (0.5 - 0.75). The diameters of 2, 3 and 4 RTU responses are about 3, 3.5 and 4 cm respectively when the stimulated regions are moderately thick. Corrections are made for thickness throughout the response range.

Since the Reece-Turner (1937) method was used for determining the prolactin potencies of the medium samples from the culture studies, a standard dosage-response

relationship for prolactin was established in the assay pigeons. This enables evaluation of the method as a biological assay and permits the prolactin activities to be converted from response units (RTU) to potency units (IU).

Methods

Sixty White King squabs (4 - 8 weeks old) of undetermined sex were divided into 6 groups of 10 each for this study. All of the birds were injected once daily for 4 days over the right crop half with 0.1 ml of a solution which contained 25 μgm of NIH prolactin per ml. Thus a total dose of 10 μgm of prolactin was delivered to the right crop half of all 60 pigeons. The left crop halves of the 6 groups of 10 pigeons were similarly treated with different doses of prolactin. Groups 1-6 received injections which delivered total doses of 2, 5, 10, 20, 40 and 60 μgm of prolactin respectively. All of the prolactin solutions were prepared in physiological saline.

The pigeons were killed on the day after the last injection and the crop sacs were removed, washed in tap water and rated. The crop sac halves were stretched in front of a light source and the diameter of each response was compared with wire discs which had diameters of 2, 3,

3.5 and 4 cm. Each crop response was then rated after the thickness had been taken into account. The data were analyzed by standard statistical procedures for bioassays (Bliss, 1952).

Results and Discussion

The results of these assay determinations are shown in Table 37. The pigeon crop responses to the different doses of prolactin were found to be linearly related to the logarithm of the dose of hormone (Figure 13). The same logarithmic relationship was found by Reece and Turner (1937). Regression analysis, by the method of least squares, disclosed that the regression coefficient (slope, b) of the log dose-response curve had a value of 1.64 ± 0.014 and the intercept (a) was 0.13 ± 0.072. The equation for the curve is shown in Figure 13 together with the 99% confidence limits of the curve and the index of precision of the assay method.

The index of precision (λ) is found by dividing the variance of the computed line (s) by the slope of the curve (b). For this study s/b equalled 0.554/1.64 which gives a λ value of 0.34. This value for the precision of the prolactin assay compares favorably with the value reported by Greenspan et al. (1949) for the tibial assay of growth

hormone ($\lambda = 0.31$) and with the precision of the assays of other AP hormones (see Emmens, 1950).

The work form for the analysis of variance of the log dose-response relationship for prolactin is shown in Table 38 and the results of this evaluation are shown in Table 39. To determine if the slope of the line, b, is significantly different from zero, the variance ratio was computed. This computation involves dividing the variation of the dosage response line (B^2) by the variation due to the scatter of dose means about the computed line (A). The F value for the variance ratio (41.88/0.22) of 190.36 is highly significant. This demonstrates that the slope of the line is different from zero and shows that the technique has a high degree of acceptability. Computation of the variance ratio using the composite error term (0.31) in the same way (41.88/0.31) gives an F value of 135.2. This is again highly significant.

The statistic A/S^2 determines if the dose means are scattered excessively about the computed line. This dose-response relationship for prolactin gave an F value for the A/S^2 ratio (0.22/0.31) of 0.71 which is not significant. The dose means for the different levels of prolactin

used (2-60 μgm) are therefore well fitted to the computed line and analysis for simple or quadratic curvature is unnecessary since no indication of curvature is present.

Analysis of variance of the responses from the 6 groups of 10 birds which were injected with the 10 μgm dose of prolactin is shown in Table 40. It can be seen that none of the means of the responses of the six 10 μgm dose levels differ from any of the others. This observation demonstrates the high degree of reproducability of the assay, at least at the 10 μgm dose level. The right and left crop halves of group No. 3 of Table 37 gave average responses of 1.58 ± 0.15 and 1.63 ± 0.16 RTU, respectively to the 10 μgm dose of prolactin. Since this difference is not significant it is apparent that both crop halves respond to an equivalent degree to the same prolactin dose, as administered by this procedure. This demonstrates the validity of using the paired assay procedure for the culture experiments.

The average responses of several groups of pigeons, which were injected with different doses of prolactin at various times during the course of the culture studies, are shown in Table 41. These assays were conducted to ascertain if the sensitivity of the pigeons varied during the course

of the *in vitro* experiments. It can be seen that none of the responses obtained with these prolactin standards differed significantly from those obtained with the same dosage levels when the standard dose-response curve was established. This indicates that the variation in the sensitivity of the pigeons, during the course of these investigations, was not significant. The same dose-response relationship can therefore be used with considerable validity for conversion of the prolactin potencies of the medium samples from RTU to IU, where this has been performed.

Talwalker, Ratner and Meites (unpublished) have also administered similar NIH prolactin standards to the same assay pigeons at various periods interspersed among those shown in Table 41. These determinations were performed in conjunction with investigations which they were conducting in concomitance with the author's *in vitro* studies. They also found that the sensitivity of the assay birds did not vary appreciably from one time to another. These observations further demonstrate the reproducability of the assay method.

TABLE 37

DOSE-RESPONSE DATA FOR NIH PROLACTIN (15 IU/MG) IN WHITE KING PIGEONS IN TERMS OF REECE-TURNER UNITS

Group No.	Date	No. of Pigeons	Prolactin Dose on Left Crop Halves μgm	REECE-TURNER PROLACTIN UNITS	
				Average Response of Left Crop Halves	Average Response of Right Crop Halves to 10 μgm Dose
1	10/16/61	10	2	0.73 ± 0.14	1.85 ± 0.09
2	10/13/61	10	5	1.23 ± 0.18	1.83 ± 0.20
3	10/12/61	10	10	1.63 ± 0.16	1.58 ± 0.15
4	10/11/61	10	20	2.35 ± 0.17	1.78 ± 0.19
5	10/10/61	10	40	2.60 ± 0.21	1.68 ± 0.19
6	10/ 9/61	10	60	3.20 ± 0.11	1.68 ± 0.11

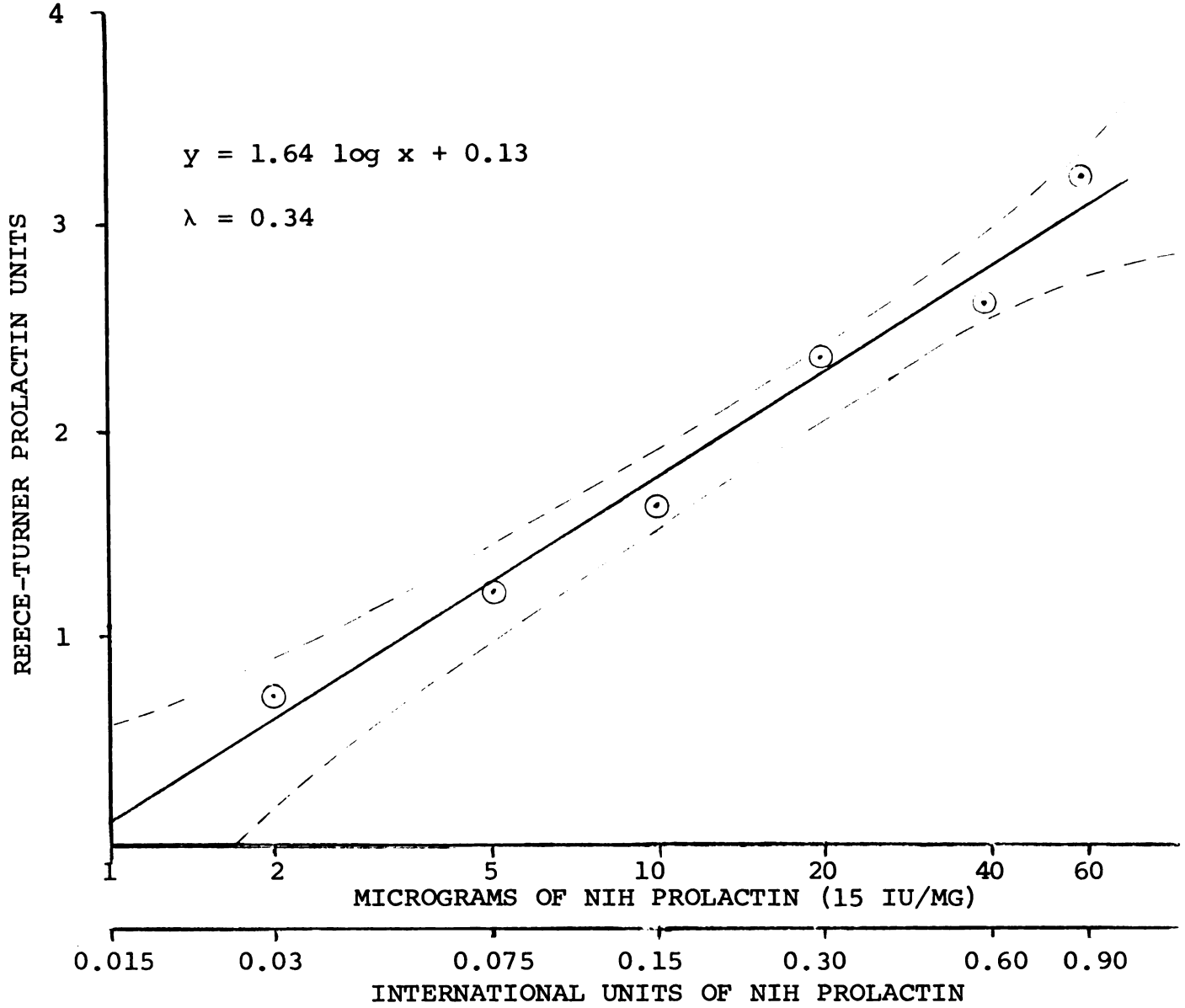

Figure 13. Dose-response curve of NIH prolactin showing the upper and lower 99% confidence limits.

TABLE 38

WORK FORM FOR THE ANALYSIS OF VARIANCE OF A DOSE-RESPONSE CURVE WITH TWO OR MORE RESPONSES AT EACH DOSE

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Slope of line, b	1	$B^2 = \dfrac{\Sigma(\log x)y - \dfrac{\Sigma \log x \, \Sigma y}{n}}{\Sigma(\log x)^2 - \dfrac{(\Sigma \log x)^2}{n}}$ (1)	B^2	B^2/A (variance ratio)
Scatter of dose means about line	k - 2	$\Sigma\left(\dfrac{Ty}{f}\right)^2 - C - B^2$ (2)	A	A/s^2
Deviations about dose means	n - k	Remainder (total - 1 - 2) (3)	s^2	
Total	n - 1	$\Sigma y^2 - (\Sigma y)^2/n$		
Correction for mean	1	$C = \dfrac{(\Sigma y)^2}{n}$		
Composite error	n - 2	2 + 3		

k = no. of dose levels n = total no. of observations x = dosage y = response

TABLE 39

ANALYSIS OF VARIANCE OF DOSE-RESPONSE CURVE FOR NIH PROLACTIN IN 60 WHITE KING PIGEONS

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P < ("2-sided Test")
Slope of line, b	1	41.88	41.88	190.36	.01
Scatter of dose means about line	4	0.88	0.22	0.71	N.S.
Deviations about dose means	54	16.92	0.31		
Total	59	59.68	1.01		
Correction for mean	1	229.13	229.13		
Composite error	58	17.80	0.31		

TABLE 40

ANALYSIS OF VARIANCE OF THE RESPONSES OF SIX GROUPS OF 10 PIGEONS WHICH WERE INJECTED WITH 10 μGM NIH PROLACTIN

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	F.95	P
Total	59	15.16				
Between means	5	2.65	0.530	2.28	2.37	N.S.
Within groups	54	12.51	0.232			

TABLE 41

PIGEON CROP RESPONSES TO PROLACTIN STANDARDS ADMINISTERED AT DIFFERENT INTERVALS DURING THE COURSE OF THE INVESTIGATIONS

Standard Trial No.	Date	Solvent	No. of Pigeons	Prolactin Dose μgm	Average RTU
1	7/24/61	Saline	10	10	1.85 ± 0.28
2	8/18/61	Saline	8	5	1.09 ± 0.16
3	9/20/61	199 medium	10	10	1.63 ± 0.15
4	10/3/61	Saline	8	10	1.67 ± 0.35
5	12/19/61	199 medium	10	5	1.28 ± 0.07
6	3/2/61	Saline	6	2	0.79 ± 0.08

Conclusions

This evaluation of the Reece-Turner (1937) procedure of prolactin bioassay demonstrates that the method has a high degree of acceptability, as indicated by the highly significant variance ratio and the relatively low numerical value of the precision index. Grosvenor and Turner (1958b) have reported on a modification of the Reece-Turner (1937) procedure which eliminates the element of subjectivity from the assay. They measured the diameter of the local crop responses by comparison with wire disks of known diameter and the thickness of the responses is ignored. An index of precision of 0.11 was obtained by this method in common pigeons and the regression coefficient (b) was 0.855.

It would appear, at first sight, that the method of Grosvenor and Turner (1958b) might be a better assay procedure than the Reece-Turner (1937) method. Measurement of the pigeon crop responses, obtained in this present evaluation of the Reece-Turner (1937) method, by the same procedure as used by Grosvenor and Turner (1958b), however, discloses that the two assay methods have about equivalent precision. The indices of precision of the Reece-Turner (1937) and Grosvenor-Turner (1958b) methods were 0.34 and

0.36, respectively and the respective regression coefficients of the two assay methods were 1.64 and 1.19.

The discrepancies in the values of the indices of precision and slopes obtained with the response diameter method by Grosvenor and Turner (1958b) and by the author in the present study may be due to a difference in the assay animals. Grosvenor and Turner (1958b) used common pigeons and we employed White King squabs. It is evident, however, that both the Reece-Turner (1937) and Grosvenor-Turner (1958b) methods are suitable procedures for the bioassay of prolactin.

General Discussion

It is apparent from these culture studies that explants of AP tissue from rats and other mammals can actively secrete prolactin *in vitro*. The explants in the control culture from the study with estradiol, at a concentration of 0.05 μgm per ml (Experiment X) synthesized about 40 times more prolactin in 3 days than they contained when introduced into the culture system. This is a dramatic illustration of the prolactin producing capacity of rat AP tissue *in vitro* in 95% O_2 - 5% CO_2 atmosphere. The explants in the control cultures from the experiment with thyroxine (Experiment XIII) synthesized only about 11 times their initial content of prolactin in 3 days. Despite the fact that this is the lowest level of prolactin production encountered in the 3 day culture studies, this 11-fold increase still represents a substantial degree of net hormone synthesis. The results of Experiment II, which demonstrated that prolactin activity was detectable in the medium of cultures which were incubated for 21 days, indicate that rat AP explants can produce prolactin *in vitro* for prolonged periods.

Previous studies on the *in vitro* secretion of AP hormones other than prolactin have shown the hormonal activity,

when detected, was present in the medium only for the first few days of culture (Anderson and Haymaker, 1935; Cutting Lewis, 1938; Guillemin and Rosenberg, 1955; Guillemin, 1956; Florsheim et al., 1957; Schaberg and De Groot, 1958; Kobayashi et al., 1961). Net hormonal synthesis was not demonstrated in any of these studies. These results stand in marked contrast with the present observations on prolactin secretion in culture and indicate that hormonal secretion by the AP in vitro manifests itself in a manner similar to, if not identical, with that of the AP deprived of hypothalamic regulation in vivo. Removal of neural regulation from the AP by transplantation, hypothalamic lesions or stalk section results in a pronounced diminution or absence of ACTH, TSH, FSH-LH and STH secretion while favoring prolactin production (see review of literature).

The disparity in the secretion of prolactin and the other AP hormones in vitro is well illustrated by the observations from our laboratory on STH secretion by rat AP explants. Meites, Hopkins and Deuben (1962) used the same culture procedures as were employed in these studies on prolactin secretion in vitro and found that rat AP fragments secreted only 80.9% of their initial STH content

into the 199 medium in 6 days of culture. It is therefore apparent that the capacity of rat AP explants to synthesize prolactin is much greater than their ability to produce STH in this *in vitro* system.

Differential staining of AP explants cultured in Trowell T-8 medium, under conditions similar to those used in these studies with 199 medium, disclosed that after 7 days, the explants were composed of degranulated acidophils, numerous chromophobes and only a few degenerate basophils (Kahn and Meites, unpublished). This cytological picture is similar to that observed by some investigators in rat APs which were transplanted to the kidney capsule (Desclin, 1956b; Quilligan and Rothchild, 1960), although it is at variance with the cytology of the AP transplanted to the anterior eye chamber where basophils are retained for prolonged periods (Martinovitch, 1950; Martini et al., 1959). The striking similarity in the physiology and cytology of the AP *in vitro* to that of the adenohypophysis deprived of neural regulation *in vivo*, illustrates the potential value of the organ culture approach for investigating the physiology of the AP and its interrelationships with the hypothalamus.

The average prolactin levels in the medium samples of the control cultures from the experiments with progesterone, testosterone, corticosterone, triiodothyronine and the cultures from the experiment with and without insulin were all found to be in fairly close agreement when expressed on the basis of IU per 100 mg of explant weight. An analysis of variance of the data from these 6 culture groups is shown in Table 42. It can be seen from this analysis that there is no significant difference between any of the groups of cultures. The within group (between culture) variation is evidently greater than the between group variation. We can therefore assume that the 6 groups of assay results were drawn from the same population and the data can be pooled for further analysis.

A scatter diagram from the data of these 52 cultures, showing the relationship between the explant weight and the total IU of prolactin in the medium samples, is presented in Figure 14. A correlation coefficient of 0.57 was found for the relationship between explant weight and total IU of prolactin in the medium samples. This correlation coefficient is highly significant ($t = 4.9$; $P < 0.001$) and demonstrates that relating the prolactin activity of each

TABLE 42

ANALYSIS OF VARIANCE OF THE ASSAY RESULTS FROM 6 GROUPS OF CULTURES

Source of Error	Degrees of Freedom	Sum of Squares	Mean Square	F
Total	51	16,601.98		
Between experiment	5	794.99	158.99	0.46
Within experiment (between culture)	46	15,806.91	343.60	

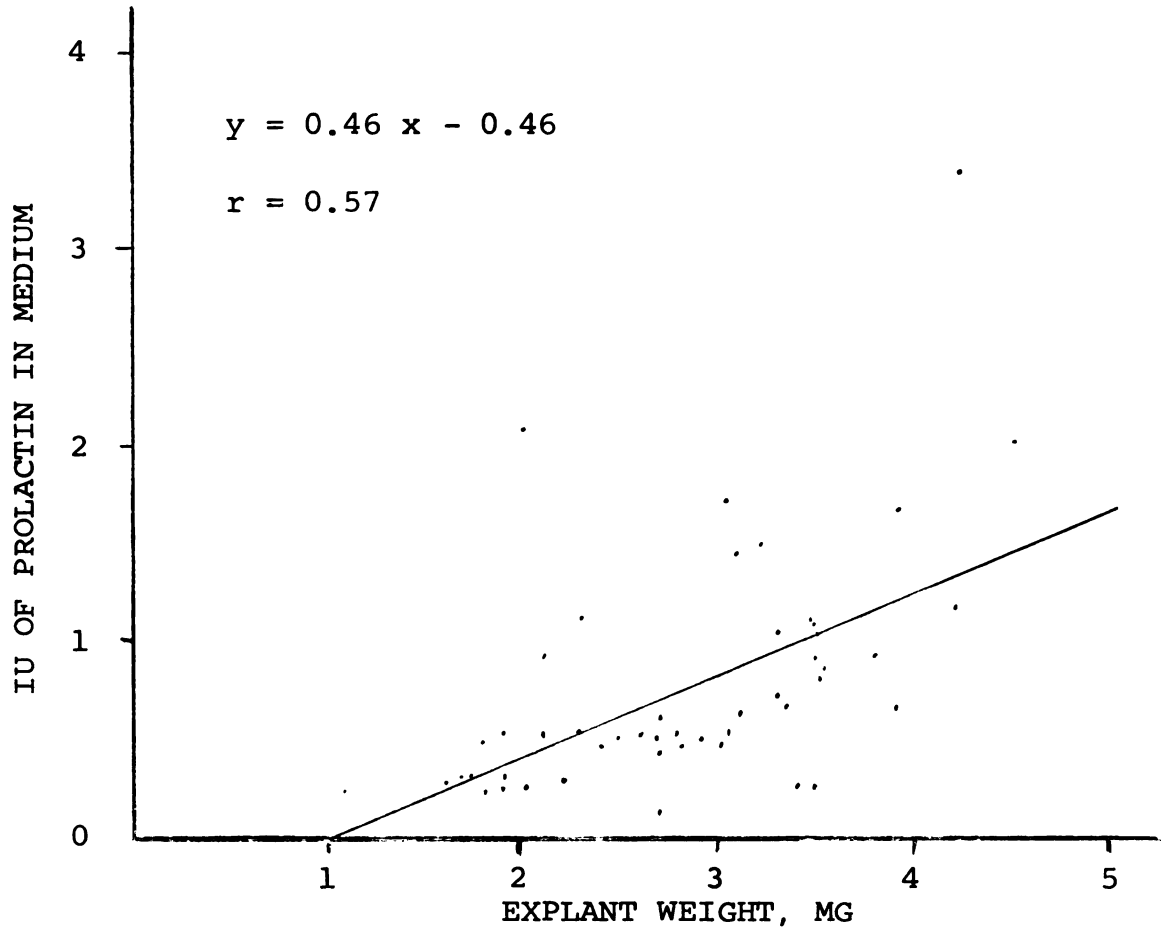

Figure 14. A scatter diagram showing the relationship between explant weight and total IU of prolactin in the medium samples.

medium sample to the amount of tissue present in the culture dish (i.e. explant weight) provides a valid index for analyzing the data.

The regression curve and equation for the relation between explant weight and total IU of prolactin in the medium samples are also shown in Figure 14. The regression equation, $y = 0.46 x - 0.46$, where y equals the total IU of prolactin and x equals the explant weight, indicates that with one mg of explant tissue, no prolactin would be detectable in the medium. Although this conclusion may be correct for these selected data, it cannot be extrapolated to all of the data from these studies. In Experiment X, substantial prolactin activity was found in medium samples from cultures which contained as little as 0.5 mg of AP tissue.

The average total prolactin levels in these 52 medium samples was 0.78 IU. Since each medium sample had a volume of about 2.8 ml at the end of the 3 day culture period, the average prolactin concentration in the medium samples was about 0.28 IU/ml. In Experiment VI, it was shown the rat AP explants contain 1.02 IU of prolactin per 100 mg of tissue wet weight. If we assume that rat AP tissue has a density of about 1, it is evident that the prolactin

concentration in the explants was approximately 10.2 IU per ml of AP tissue. It is therefore obvious from these estimates that there is no indication that the explants were secreting prolactin against a concentration gradient. The prolactin concentration differential of 10.2 IU/ml in the explant and 0.28 IU/ml in the medium indicates that the prolactin molecules could probably diffuse quite readily from the explants into the medium.

Since no data are available for comparison of the intracellular and extracellular prolactin concentrations within the explant, the intra-explant resistance to prolactin diffusion can not be determined. Diffusion of prolactin away from the AP cells is not facilitated by circulation in the explants, therefore a considerable extracellular concentration of prolactin might be present within the AP fragments. This might influence the rate of prolactin secretion by the explant cells by a mass action effect; however we cannot determine to what extent this phenomenon might have influenced prolactin secretion in this <u>in vitro</u> system.

Li and co-workers (see Li, 1961) have isolated a prolactin preparation from ovine AP glands which has a molecular

weight of about 24,000 and a maximum biological activity of 35 IU per mg. If it is assumed that this prolactin preparation is the "pure" hormone, and that the prolactin secreted by the rat AP explants has the same molecular weight and biological activity as the "pure" ovine preparation, then we can perform some rather interesting, though highly speculative, calculations.

The average amount of prolactin produced by the AP fragments in the 8 control cultures from the experiment with estradiol at a concentration of 0.05 μgm per ml, was 39.7 IU per 100 mg of explant weight. This is the highest degree of prolactin synthesis encountered in the 3 day culture studies. If the maximum biological activity of the rat prolactin is taken as 35 IU/mg, then each 100 mg of rat AP explant tissue synthesized about 1.14 mg of prolactin. Each gram of rat AP explant tissue therefore synthesized approximately 11.4 mg of the protein hormone. With a molecular weight of 24,000, each mole of prolactin would weigh 24×10^6 mg. The value of 11.4 mg of prolactin is therefore equivalent to 4.75×10^{-7} moles of the hormone. With Avogadro's No. of 6.02×10^{23} molecules/mole, it is evident that one gram of rat AP explant tissue

synthesized approximately 2.86×10^{17} molecules of the hormone in 3 days.

Thompson (personal communications) reported that one gram of human AP cells, derived from a suspension culture, contained about 3×10^{8} cells. If we further assume that rat AP parenchymal cells weigh about the same as these human cells, one gram of rat AP explant tissue would also contain 3×10^{8} cells. This estimate ignores the contribution of non-parenchymal elements (e.g. red blood cells) to the weight of the rat AP tissue; however it is not known to what extent the non-parenchymal elements contributed to Thompson's estimate of the number of cells per gram of human AP. For these highly conjectural estimates therefore, the value of 3×10^{8} cells per gram of AP tissue can probably be used with as much validity as any other figure. If it is assumed that approximately one-third of the rat AP cells were the prolactin producing acidophils, and that the acidophils account for 1/3 of the weight of the tissue, then one gram of rat AP explant tissue would have contained 1×10^{8} prolactin synthesizing cells.

It was previously estimated that one gram of rat AP explant tissue synthesized approximately 11.4 mg of

prolactin, and this was equivalent to 2.86×10^{17} molecules of the hormone. Using the value of 1×10^{8} acidophils per gram of AP explant, it can be estimated that each acidophil cell synthesized 11.4×10^{-8} mg of prolactin in 3 days and this is equivalent to 2.86×10^{9} molecules of the hormone. Each acidophil cell, therefore, produced an average of 1.58×10^{-9} mg of prolactin or 3.96×10^{7} molecules of the hormone per hour during the 3 days of *in vitro* incubation.

Setlow and Pollard (1962) have estimated that a bacillus of Eschericha Coli synthesizes 3.6×10^{6} protein molecules per hour during the process of replication. Corner (1947) estimated that each cell in the corpora lutea of pseudo-pregnant rabbits produces 5.4×10^{10} molecules of progesterone per hour. It is therefore evident that the estimated prolactin synthetic rate of 39.6 million molecules per acidophil cell per hour, in the *in vitro* system, is not at all unreasonable. When the molecular weight and biological activity of rat prolactin are known, and the number of functional acidophils in the *in vitro* system can be determined, a more precise estimate of the prolactin synthesizing capacity of the acidophil cells of the AP explants will be possible.

Reece and Turner (1937) and Meites and Turner (1942) have reported that the AP glands of lactating rats contain more prolactin than the glands of non-lactating mature female rats. They also observed that the APs of mature females contained higher prolactin levels than the glands of immature rats. The observation from Experiment I, that AP explants from lactating rats secrete more prolactin *in vitro* than the glands of non-lactating females, supports the conclusions of the *in vivo* studies that the prolactin content of the pars anterior can be a useful indicator of the amount of hormone secreted by the gland. Meites, Kahn and Nicoll (1961) have shown that APs from mature female rats secrete more prolactin *in vitro* than the glands of immature rats. This constitutes additional evidence that the AP prolactin content can reflect the secretion rate of the hormone. Further support for this concept was obtained from the comparative study (Experiment VII) where it was shown that the prolactin content of the APs of mice, rats and guinea pigs was reflected in their relative capacities to secrete the hormone *in vitro*.

It must be emphasized that the pituitary prolactin content per se merely reflects the intracellular hormone pool

which is the result of the differential between the rates of synthesis and release of the hormone. It is evident, however, that the AP prolactin content can be a useful, although not invariable, indicator of the secretion rate of the hormone.

Suppression of *in vitro* prolactin secretion by an acid extract of hypothalamic tissue (Experiment VIII), while a similar extract of cerebral tissue was without effect, provides rather convincing evidence for the existence of a material of hypothalamic origin which inhibits prolactin secretion. It cannot be ascertained as yet, however, whether the prolactin inhibiting factor (PIF) is also responsible for stimulating LH secretion as suggested by several investigators (Everett, 1954, 1956; Haun and Sawyer, 1960; McCann, 1962a).

Secretion of gonadotropins and prolactin (LTH) appears to be reciprocally related in many circumstances. During pseudopregnancy and lactation, when prolactin is presumably secreted actively, there is usually little or no indication of gonadotropin secretion, as evidenced by quiescence of the ovarian follicular apparatus. Injection of numerous agents, such as estrogen, tranquilizing drugs (Barraclough

and Sawyer, 1959) and morphine (Barraclough and Sawyer, 1955), suppresses FSH-LH secretion while stimulating prolactin release. Conversely, there are no physiological manifestations of prolactin secretion during normal estrous cycles of rats and mice. Several observations, however, indicate that secretion of gonadotropins and prolactin may not necessarily be mutually exclusive events under all conditions.

Electrical stimulation of the uterine cervix of immature rats promotes FSH secretion, as evidenced by hastened sexual maturation (Swingle et al., 1951c), and induces ovulation and pseudopregnancy in mature rats (Haterius, 1932), indicating that LH and prolactin secretion are increased. Cervical stimulation also initiates lactation in estrogen primed rats (Meites et al., 1959). Ovulation and pseudopregnancy are readily induced in rabbits by stimulation of the genitals indicating that LH and LTH secretion are increased. Epinephrine and acetylcholine are reported to induce LH release (Teubenhaus and Soskin, 1942; Sawyer et al., 1949) but can also induce mammary secretion in rats and rabbits (Meites, 1959; Meites et al., 1960), and pseudopregnancy in rats (Swingle et al., 1951b). Rats,

mice and guinea pigs ovulate within 1 or 2 days after parturition, indicating FSH and LH release (Asdell, 1946). At the same time these animals show a marked increase in prolactin secretion (Meites, 1959). Rabbits also enter a state of estrus shortly after parturition and readily ovulate in response to coitus.

Although these observations indicate that the secretion of prolactin and gonadotropins may occur simultaneously, other explanations are feasible for the apparent lack of reciprocal secretion of these hormones under these conditions. Induction of ovulation and pseudopregnancy could be the result of a concomitant increase of LTH and LH secretion for a brief period, followed by a diminished secretion of LH. It is also possible that LH secretion is first stimulated, and when ovulation occurs, LTH secretion commences concomitant with a reduction in LH secretion. The occurrence of ovulation shortly after parturition, when prolactin is apparently secreted actively, may be accomplished by a brief period of reduced LTH secretion during which sufficient LH is released to induce follicular rupture. Complete elucidation of this complex phenomena, however, will require considerable additional research.

The site of action of the endocrine products of the gonads, thyroid and adrenal cortex, in altering AP function, may be on the hypothalamus or the adenohypophysis or on both components of the hypothalamo-hypophysial apparatus. Thyroid hormones can apparently depress TSH secretion by an action on the AP (see D'Angelo, 1962). Other evidence indicates that estrogens depress gonadotropin secretion (Rose and Nelson, 1957) and cortisol inhibits ACTH secretion (Rose and Nelson, 1956) by acting directly on the AP. The stimulatory action of estradiol and thyroid hormones, and the depressant effect of cortisol, on prolactin secretion observed in these in vitro studies, provides further evidence that estrogenic, thyroidal and adrenal cortical hormones can alter adenohypophysial function by an action on the AP cells.

Endroczi et al. (1961) reported that injection of cortisone acetate in agar into the tuberal region of the hypothalamus of cats and rats depresses adrenal steroid secretion. Injections into the reticular formation and the top of the mammilary body had a slight inhibitory effect. Control injections into other parts of the hypothalamus were ineffective. These observations indicate

that the depressant action of corticoids on ACTH secretion may be mediated in part by an effect on the hypothalamus. Other experimental observations indicate that estrogens depress gonadotropin secretion (Flerko and Szentagothai, 1957; Flerko, 1962; Lisk, 1960; Davidson and Sawyer, 1961a) and thyroid hormones inhibit TSH release (Yamada, 1959; see also D'Angelo, 1962) by actions on the hypothalamus. It is possible, therefore, that the stimulatory action of thyroid hormones (Weichert and Boyd, 1933-34; Grosvenor, 1961; Moon, 1962) and estrogens (Reece and Turner, 1936; Meites and Turner, 1948) on prolactin secretion in vivo may be the result of an action of these hormones on the AP and the hypothalamus. The recent observation of Kanematsu and Sawyer (1962), that intrahypothalamic implants of estrogen increase the AP prolactin content of rabbits, indicates that estrogens can alter prolactin synthesis via the diencephalon. The relative importance of the hypothalamic and hypophysial actions of thyroidal and estrogenic hormones, in augmenting prolactin secretion in vivo, however, cannot be fully assessed at present.

The depressant action of cortisol on prolactin secretion in vitro does not conform with the conclusions of Johnson

and Meites (1955) that glucocorticoids increase prolactin secretion *in vivo*. In the *in vivo* situation, corticoids could conceivably stimulate prolactin secretion via the hypothalamus or by some other means. It seems unlikely, however, that corticoids could increase prolactin secretion by a direct action on the AP. The lack of an effect of corticosterone on prolactin secretion, even with a concentration of 20 μgm per ml, indicates that the depressant effect of cortisol is not due to nonspecific steroid toxicity.

Failure of progesterone and testosterone to alter prolactin secretion *in vitro*, while these steroids can apparently increase prolactin secretion *in vivo* (Reece and Mixner, 1939; Reece and Bivins, 1942; Meites and Turner, 1948) indicates that the *in vivo* effects of these steroids on prolactin secretion may be via the hypothalamus. Testosterone pellets in the hypothalami of dogs depress gonadotropin secretion (Davidson and Sawyer, 1961b) and injection of progesterone into the hypothalami of chickens (Ralph and Fraps, 1961) induces ovulation. It is apparent, therefore, that these gonadal steroids can influence AP function by actions on the hypothalamus. Hypothalamic

mediated stimulation of prolactin secretion by progesterone and testosterone is therefore quite conceivable. It would be of interest to determine if intra-hypothalamic implants of testosterone and progesterone could induce pseudopregnancy or initiate lactation in suitably prepared animals.

The *in vitro* procedures used in these studies on prolactin secretion have several advantages and disadvantages. The organ culture method permits the function of the AP to be studied in a highly simplified system where a great degree of experimental control is possible. The effects of numerous extraneous, and otherwise uncontrolable factors can be virtually eliminated from the experiments. Incubation of AP fragments in chemically defined medium, however, cannot be considered as physiological.

It is possible that the effects of the various hormones on prolactin secretion observed in these *in vitro* studies would be somewhat modified in the presence of physiological levels of the other hormones. For example, although progesterone and testosterone had no demonstrable effect on prolactin secretion *in vitro* when they were incorporated into the medium individually, if these steroids were tested in a system containing quantities of the other hormones

(estrogen, thyroxine, etc.) they may have influenced prolactin production. An *in vivo* illustration of such endocrine interaction is provided by a recent study of McCann (1962b). He observed that progesterone alone, even in very high doses, does not depress the high plasma LH levels in ovariectomized rats. When progesterone was administered along with a dose of estrogen, a greater depression of LH secretion was achieved than was obtained with either steroid alone. Thorough evaluation of the effects of hormones, individually at different concentrations, and in various combinations with other hormones, on prolactin secretion *in vitro* presents a task of herculean proportions for future endeavors.

It will be of interest to determine if interactions can be obtained from combinations of the hormones which were demonstrated to alter prolactin production in these *in vitro* studies. Would the stimulatory action of estrogen and thyroxine on prolactin secretion be synergistic when combinations of these two hormones are tested? Can the depressant effects of cortisol be overcome with estrogens and thyroid hormones? The observation that progesterone inhibits the estrogen-induced rise in AP prolactin content *in vivo*

(Meites and Turner, 1948) will be worthy of further investigation in this in vitro system. Possibly progesterone, though ineffective by itself, would block the estrogen-induced increase in prolactin secretion.

This in vitro system provides a convenient method for studying numerous other aspects of AP physiology. Cultures of adenohypophysial fragments could be used with much facility for further studies on the hypothalamic neurohumoral agents which apparently regulate AP function. Studies on the physico-chemical characteristics of the hormones which are actually secreted by the AP cells may be possible using culture techniques. Previously, such investigations relied on the use of the hormones contained within the AP cells and it is not known if the intracellular hormonal material is the same as that which is secreted by the cells.

Investigations on the apparently autonomous nature of prolactin synthesis and release may provide worthwhile information for further elucidation of the phenomena of protein biosynthesis and cellular secretion. Incorporation of isotopically labeled amino acids into the culture medium should permit the isolation of labeled prolactin which could

be used for numerous other studies. Tagged prolactin may permit the function of the hormone in male mammals and the lower vertebrates to be determined.

It is apparent from these few examples that the potential value of the organ culture procedure for future investigations is probably unlimited. The experimental observations recorded in this thesis have only scratched the surface, and possibly opened the door to vast new frontiers of endeavor. Further research using the in vitro approach should greatly advance our knowledge of the secretion of prolactin and the other hormones of the pars anterior.

SUMMARY

Prolactin secretion *in vitro* by explants of anterior pituitary (AP) glands from rats and other species was investigated. The AP explants were cultured by modified watch glass techniques in medium 199 for 3 to 21 days. The cultures were incubated in air or 95% O_2 - 5% CO_2 atmosphere at 35°C. The prolactin activities in the culture medium samples were determined by the local "micro" pigeon crop method. The results of these studies were as follows:

1. Explants from the APs of rats actively secreted prolactin *in vitro* in synthetic 199 medium. Prolactin activity was detectable in the medium of cultures which were incubated for periods up to 21 days. The AP glands from lactating rats produced about twice as much prolactin *in vitro* as the glands from non-lactating mature females. The AP fragments which were incubated in air atmosphere synthesized about as much prolactin during each day of culture as they contained at the beginning of culture. The AP fragments incubated in the 95% O_2 - 5% CO_2 atmosphere produced from 3 to 11 times their initial prolactin content during each day of

in vitro life and explant survival was much better than in cultures in air atmosphere. These results demonstrate that AP glands can actively secrete prolactin in a system which is virtually free of hypothalamic or other in vivo influences. This indicates that prolactin secretion is an autonomous characteristic of the AP.

2. Explants from the APs of female guinea pigs, rabbits and mice and of male mice and Cynomalogus monkeys actively secreted prolactin in vitro. The glands of male and female pigeons released very little prolactin into the culture medium. These results demonstrate that the APs of male and female mammals can secrete prolactin autonomously and indicate that there may be a basic physiological difference in prolactin secretion by mammalian and avian pituitaries.

3. Medium incubated without any tissue or with several non-pituitary tissues did not have prolactin activity and the culture medium did not potentiate the pigeon crop response to prolactin.

4. Cutting the AP glands of rats into 6 explants resulted in about a 20% reduction in wet weight indicating a

loss of 1/5 of the tissue. Incubation in 199 medium for 3 days caused a further loss in wet weight of 8%. At the end of 3 days of incubation, the explants contained about 84.5% of their initial prolactin content.

5. Incubation of rat AP fragments with hypothalamic or cerebral explants, or with homogenates of rat hypothalamic or cerebral tissue added to the medium, greatly reduced the prolactin activity in the medium samples. An acid extract of rat hypothalamic tissue significantly reduced prolactin secretion in vitro whereas a similar extract of cerebral tissue had no effect. The results with the extracts indicate that hypothalamic tissue may contain an acid extractable material which specifically inhibits prolactin secretion.

6. Neither oxytocin nor pitressin influenced prolactin secretion when added to the medium at concentrations of 0.1 U and 1.0 U per ml respectively.

7. Addition of estradiol to the 199 medium at concentrations of 0.05 and 0.5 μgm per ml increased prolactin secretion in vitro. No stimulation occurred with 2 μgm estradiol per ml. Thyroxine and triiodothyronine

stimulated prolactin production *in vitro* at concentrations of 0.1 μgm per ml. Cortisol, at a concentration of 10 μgm per ml depressed prolactin secretion *in vitro* and 20 μgm/ml of corticosterone had no effect on prolactin production. Neither testosterone nor progesterone influenced prolactin secretion *in vitro* at a concentration of 2 μgm/ml. Concentrations of the latter steroids of 5 and 10 μgm per ml were toxic to the AP explants as was corticosterone at concentrations of 30 and 50 μgm per ml. These results indicate that estradiol, thyroid hormones and cortisol can influence prolactin secretion by a direct action on the AP cells. The lack of an effect of progesterone and testosterone *in vitro* indicates that the effects of these steroids on prolactin secretion *in vivo* may be indirect.

8. Insulin did not affect prolactin secretion *in vitro* indicating that insulin is not essential for prolactin synthesis.

9. A dose-response relationship was established for NIH prolactin in White King squabs for prolactin doses from 2 to 60 μgm. With 10 pigeons per dose a regression

equation was obtained in which $y = 0.13 + 1.64x$, where y represents the pigeon crop response to x μgm of prolactin. The index of precision of the assay method was 0.34 and the variance ratio was highly significant ($F = 190.4$) thus demonstrating the high degree of acceptability of the assay method.

REFERENCES

Alloiteau, J. J. 1958. Sur la nature du controle de la fonction luteotrophe de l'hypophyse anterieure par l'hypothalamus chez la ratte. Compt. Rendu. Acad. Sci., Paris. 247: 1047.

Anderson, E. and Haymaker, W. 1935. Elaboration of hormones by pituitary cells growing in vitro. Proc. Soc. Exp. Biol. and Med. 33: 313.

Asdell, S. A. 1946. Patterns of Mammalian Reproduction. Comstock Publishing Co., Ithaca, New York.

Astwood, E. B. 1941. The regulation of corpus luteum function by hypophysial luteotropin. Endocrinology. 28: 309.

Barraclough, C. A. and Sawyer, C. H. 1955. Inhibition of the release of pituitary ovulating hormone in the rat by morphine. Endocrinology. 57: 329.

Barraclough, C. A. and Sawyer, C. H. 1959. Induction of pseudopregnancy in the rat by reserpine and chlorpromazine. Endocrinology. 65: 563.

Baker, B. L. and Everett, N. B. 1947. The effect of diethylstilbesterol on the anterior hypophysis of thyroidectomized rats. Endocrinology. 41: 144.

Baggett, B., Engel, L. L., Savard, K. and Dorfman, B. I. 1956. The conversion of testosterone 3 C^{14} to C^{14} - estradiol-17-beta by human ovarian tissue. J. Biol. Chem. 221: 931.

Bauman, E. T. and Marine, D. 1946. Involution of adrenal cortex in rats fed thiouracil. Endocrinology. 36: 400.

Benson, G. K. and Folley, S. J. 1956. Oxytocin as stimulator for the release of prolactin from the anterior pituitary. Nature, London. 177: 700.

Benson, G. K. and Folley, S. J. 1957a. Retardation of mammary involution in the rat by oxytocin. J. Endocrinol. 14: XL.

Benson, G. K. and Folley, S. J. 1957b. The effect of oxytocin on mammary gland involution in the rat. J. Endocrinol. 16: 189.

Benson, G. K., Cowie, A. T. and Tindal, J. S. 1958. The pituitary and the maintenance of milk secretion. Proc. Roy. Soc. B. 149: 330.

Benson, G. K., Cowie, A. T., Folley, S. J. and Tindal, J. S. 1959. Recent developments in endocrine studies on mammary growth and lactation. In Recent Progress in the Endocrinology of Reproduction. p. 457. Academic Press, Inc., New York and London.

Benson, G. K., Folley, S. J. and Tindal, J. S. 1960. Effects of synthetic oxytocin and valyl oxytocin on mammary involution in the rat. J. Endocrinol. 20: 106.

Bein, N. J. 1956. The pharmacology of rauwolfia. Pharm. Rev. 8: 435.

Blaxter, K. L., Reineke, E. P., Crampton, E. W. and Petersen, W. E. 1949. The role of thyroidal materials and of synthetic goitrogens in animal production and an appraisal of their practical use. J. Animal Sci. 8: 307.

Bliss, C. I. 1952. The Statistics of Bioassay. Academic Press, Inc., New York.

Bogdanove, E. M. and Crabill, E. V. 1961. Thyroid-pituitary feedback: direct or indirect? A comparison of the effects of intra-hypothalamic and intrapituitary thyroid autotransplants on pituitary thyroidectomy reactions in the rat. Endocrinology. 69: 581.

Brooks, C. McC. 1938. A study of the mechanism whereby coitus excites the ovulation-producing activity of the rabbit's pituitary. Am. J. Physiol. 121: 157.

Campbell, H. J., Feuer, G., Garcia, J. and Harris, G. W. 1961. The infusion of brain extracts into the anterior pituitary gland and the secretion of gonadotrophic hormone. J. Physiol. 157: 30.

Chen, J. M. 1954. The cultivation in fluid medium of organized liver, pancreas and other tissue of fetal rats. Exp. Cell. Res. 7: 518.

Clapp, H. A. 1937. A factor in breeding efficiency of dairy cattle. Am. Soc. Animal Prod., Proc. 30: 259.

Contopoulos, A. N., Simpson, M. E. and Koneff, A. A. 1958. Pituitary function in the thyroidectomized rat. Endocrinology. 63: 642.

Corner, C. W. 1947. The Hormones in Human Reproduction. Chapt. VII, p. 179. Princeton Univ. Press.

Cowie, A. T. and Folley, S. J. 1956. Neurohypophysial hormones and the mammary gland. In The Neurohypophysis. 8: 183. R. Heller (Ed.). Colston Papers, Butterworth Scientific Publications, London.

Cowie, A. T., Tindal, J. S. and Benson, G. K. 1960. Pituitary grafts and milk secretion in hypophysectomized rats. J. Endocrinol. 21: 115.

Cross, B. A. 1961. Neural control of lactation. In Milk: The Mammary Gland and Its Secretion. I: 229. S. K. Kon and A. J. Cowie (Eds.). Academic Press, New York and London.

Cross, B. A. and Harris, G. W. 1952. The role of the neurohypophysis in the milk ejection reflex. J. Endocrinol. 8: 148.

Cutting, W. C. and Lewis, M. R. 1938. Failure of the anterior lobe of the pituitary to produce hormones in tissue culture. Arch. Exp. Zellforsch. 21: 523.

D'Angelo, S. A. 1962. The central nervous system and the secretion and release of TSH. Chapt. 6. In Symposium of Neuroendocrinology. Miami, Florida, 1961. Univ. of Ill. Press, Urbana. (In Press.)

Davidson, J. M. and Sawyer, C. H. 1961a. Effects of localized intracerebral implantation of a estrogen on reproductive function in the female rabbit. Acta. Endocrinol. 37: 385.

Davidson, J. M. and Sawyer, C. H. 1961b. Evidence for an hypothalamic focus of inhibition of gonadotropin by androgen in the male. Proc. Soc. Exp. Biol. and Med. 107: 4.

Davis, M. E. and Platz, J. E. 1958. Hormones in human reproduction. Part II. Further investigation of steroid metabolism in human pregnancy. Am. J. Obstet. Gynecol. 76: 939.

Dempsey, E. W. and Uotila, U. U. 1940. The effect of pituitary stalk section upon reproductive phenomena in the female rat. Endocrinology. 27: 573.

Desclin, L. 1940. Influence de la section de la tige hypophysaire sur la lactation chez le rat blanc. Compt. Rendu. Soc. Biol., Paris. 134: 267.

Desclin, L. 1947. Concerning the mechanism of diestrum during lactation in the albino rat. Endocrinology. 40: 14.

Desclin, L. 1949. A propos de l'action combinee de la prolactin et des oestrogenes sur la structure de l'ovaire chez le rat. Compt. Rendu. Soc. Biol., Paris. 142: 115.

Desclin, L. 1950. A propos du mecahisme d'action des oestrogens sur le lobe anterieur de l'hypophyse chez le rat. Ann. d' Endocrinol. 11: 656.

Desclin, L. 1956a. L'ocytocine peut-elle declencher la liberation de luteotrophine hypophysiare chez le rat? Compt. Rendu. Soc. Biol., Paris. 150: 1489.

Desclin, L. 1956b. Hypothalamus et liberation d'hormone luteotrophique. Experiences de greffe hypophysaire chez le rat hypophysectomise. Action luteotrophique de l'ocytocine. Ann. d'Endocrinol., Paris. 17: 586.

Dey, F. L. 1943. Evidence of hypothalamic control of hypophysial gonadotropic function in the female guinea pig. Endocrinology. 33: 75.

Donovan, B. J. 1960. The inhibitory action of the hypothalamus upon gonadotropin secretion. In Memoirs of the Society for Endocrinology. No. 9, Part 1, p. 1, Cambridge Univ. Press, London.

Donovan, F. J. and van der Werff ten Bosch, J. J. 1957. The hypothalamus and lactation in the rabbit. J. Physiol. 137: 410.

Dorfman, R. I. 1950. Adrenal cortical hormones. Chapt. XIV, p. 325. In Hormone Assay. C. W. Emmens (Ed.). Academic Press Inc., Publishers, New York.

Eckles, M. E., Dhni, G. and Kirschbaum, A. 1958. Induction of lactation in human female by pituitary stalk-section. Anat. Rec. 130: 295.

Emmens, C. W. 1950. Hormone Assay. Academic Press Inc., Publishers, New York.

Endroczi, E., Lissak, K. and Tekeres, M. 1961. Hormonal "feedback" regulation of pituitary-adrenocortical activity. Acta Physiol. (Acad. Sci. Hungar.) 18: 291.

Euler, C. Von and Holmgren, B. 1956. The thyroxine "receptor" of the thyroid-pituitary system. J. Physiol. 131: 125.

Evans, H. M., Simpson, M. E., Lyons, W. R. and Tarpeinen, K. 1941. Anterior pituitary hormones which favor the production of traumatic uterine placentomata. Endocrinology. 28: 933.

Everett, J. W. 1954. Luteotrophic function of autografts of the hypophysis. Endocrinology. 54: 685.

Everett, J. W. 1956. Functional corpora lutea maintained for months by autographs of rat hypophysis. Endocrinology. 58: 786.

Everett, J. W. 1962. Physiology of the denervated anterior pituitary. Chapt. 9 in Symposium of Neuroendocrinology, Miami, Florida, 1961. Univ. of Ill. Press, Urbana (In Press).

Everett, N. B. and Baker, B. L. 1945. The distribution of cell types in anterior hypophysis during late pregnancy and lactation. Endocrinology. 37: 83.

Fell, H. B. and Robison, R. 1929. The growth, development and phosphate activity of embryonic avian femora and limb buds cultivated in vitro. Biochem. J. 23: 767.

Flerko, F. 1962. The central nervous system and the secretion and release of FSH and LH. Chapt. 7. In Symposium of Neuroendocrinology, Miami, Florida, 1961. Univ. of Ill. Press, Urbana. (In Press.)

Flerko, B. and Szentogothai, J. 1957. Oestrogen sensitive nervous structures in the hypothalamus. Acta Endocrinol. 26: 121.

Florsheim, W. H., Imagawa, D. T. and Greer, M. A. 1957. Failure of hypothalamic tissue to reinitiate thyrotopin production by mouse pituitaries in tissue culture. Proc. Soc. Exp. Biol. and Med. 95: 664.

Folley, S. J. and Malpress, F. H. 1948. Hormonal control of lactation. In The Hormones. G. Pincus and K. V. Thimann (Eds.). Chapt. XVI. Academic Press, Inc., New York.

Folley, S. J. and White, P. 1936. The effect of thyroxine on milk secretion and on the phosphatase of the blood and milk of the lactating cow. Proc. Roy. Soc. B. 120: 346.

Gaillard, P. J. 1937. Die glandula hypophysis von kanninchen in der gewebezuchtung, ihre struktur veranderungen und ihr einfluss auf das wachstum von met diesen zusammengesuchteten kulturen osteogen-etisher zellen. Protoplasma. 28: 1.

Gale, C.C., Taleisnik, S., Friedman, H. M. and McCann, S. M. 1961. Hormonal basis for impairment in milk synthesis and milk ejection following hypothalamic lesions. J. Endocrinol. 23: 303.

Ganong, W. F. 1962. The central nervous system and synthesis and release of ACTH. Chapt. 5. In Symposium of Neuroendocrinology, Miami, Florida, 1961. Univ. of Ill. Press, Urbana. (In Press.)

Ganong, W. R. and Forsham, P. H. 1960. Adenohypophysis and adrenal cortex. Ann. Rev. of Physiol. 22: 579.

Gaul, G. and Villee, C. A. 1959. Stimulation of respiration of slices of human adenohypophysis by estradiol-17 beta in vitro. J. Clin. Endocrinol. and Metabolism. 19: 1357.

Gaunt, R., Renzi, A. A., Antonchack, N., Miller, G. J. and Gilman, M. 1954. Endocrine aspects of the pharmacology of reserpine. Ann. N. Y. Acad. Sci. 59: 22.

Gemzel, C. A. 1953. Increase in the formation and secretion of ACTH in rats following administration of alpha-estradiol monobenzoate. Acta Endocrinol., 11: 221.

Goodner, C. J. and Freinkel, N. 1961. Studies of anterior pituitary tissue in vitro: effects of insulin and experimental diabetes mellitus upon carbohydrate metabolism. J. Clin. Invest. 40: 261.

Greenspan, F. S., Li, C. H., Simpson, M. E. and Evans, H. M. 1949. Bioassay of hypophysial growth hormone: the tibia test. Endocrinology. 45: 455.

Greep, R. O. 1961. Physiology of the anterior hypophysis in relation to reproduction. In Sex and Internal Secretion. Chapt. 4, p. 240. W. C. Young (Ed.). Williams and Wilkins Co., Baltimore.

Greer, M. A. 1952. The role of the hypothalamus in the control of thyroid function. J. Clin. Endocrinol. 12: 1259.

Greer, M. A. 1957. Studies on the influence of the central nervous system on anterior pituitary function. Recent Progress in Hormone Research. XIII: 67. Academic Press, New York.

Gregoire, C. 1947. Factors involved in maintaining involution of the thymus during suckling. J. Endocrinol. 5: 68.

Gregory, R. L., Drager, G. A., Tsai, S. Y. and May, L. G. 1956. The effect of diabetes on pancreatectomy on the pituitary cytology of dogs. Endocrinology. 58: 79.

Groot, J. de and Harris, G. W. 1950. Hypothalamic control of the anterior pituitary gland and blood lymphocytes. J. Physiol. 111: 335.

Grosvenor, C. E. 1961. Effect of experimentally induced hypo- and hyperthyroid states upon pituitary lactogenic hormone concentration in rats. Endocrinology. 69: 1092.

Grosvenor, C. E. and Turner, C. W. 1957. Release and restoration of pituitary lactogen in response to nursing stimuli in lactating rats. Proc. Soc. Exp. Biol. and Med. 96: 723.

Grosvenor, C. E. and Turner, C. W. 1958a. Effects of oxytocin and blocking agents upon pituitary lactogen discharge in lactating rats. Proc. Soc. Exp. Biol. and Med. 97: 463.

Grosvenor, C. E. and Turner, C. W. 1958b. Assay of lactogenic hormone. Endocrinology. 63: 530.

Grosvenor, C. E. and Turner, C. W. 1959a. Effect of estrogen upon thyroxine secretion rate in intact female rats. Proc. Soc. Exp. Biol. and Med. 101: 194.

Grosvenor, C. E. and Turner, C. W. 1959b. Thyroid hormone and lactation in the rat. Proc. Soc. Exp. Biol. and Med. 100: 162.

Grosz, H. J. and Rothballer, A. B. 1961. Hypothalamic control of lactogenic function in the cat. Nature, London. 190: 349.

Guillemin, R. 1956. Hypothalamic-hypophysial interrelationships in the production of pituitary hormones in vitro. In Hypothalamic-Hypophysial Interrelationships. W. S. Fields, R. Guillemin and C. A. Carton (Eds.), C. C. Thomas, Springfield, Illinois. p. 46.

Guillemin, R. 1962. Recent advances in the chemistry of neuroendocrine mediators originating in the central nervous system. Chapt. 10 in Symposium of Neuroendocrinology, Miami, Florida, 1961. Univ. of Ill. Press, Urbana. (In Press.)

Guillemin, R. and Rosenberg, B. 1955. Humoral hypothalamic control of anterior pituitary: A study with combined tissue cultures. Endocrinology. 57: 599.

Harris, G. W. 1937. The induction of ovulation in the rabbit by electrical stimulation of the hypothalamo-hypophysial mechanism. Proc. Roy. Soc. B. 122: 374.

Harris, G. W. 1950. Oestrous rhythm, pseudopregnancy and the pituitary stalk in the rat. J. Physiol. 111: 347.

Harris, G. W. 1955. Neural Control of the Pituitary Gland. Edward Arnold (Publishers) Ltd., London.

Harris, G. W. 1962. In discussion section of Chapt. 7. The central nervous system and the secretion and release of TSH. Symposium of Neuroendocrinology, Miami, Florida, 1961. Univ. of Ill. Press, Urbana. (In Press.)

Harris, G. W. and Jacobsohn, D. 1952. Functional grafts of the anterior pituitary gland. Proc. Roy. Soc. B. 139: 263.

Harwood, C. T. and Mason, J. W. 1957. Acute effects of tranquilizing drugs on the anterior pituitary ACTH mechanism. Endocrinology. 60: 239.

Haterius, H. O. 1932. Partial sympathectomy and induction of pseudopregnancy. Am. J. Physiol. 103: 97.

Haun, G. K. 1959. Induction of lactation in the rabbit with oxytocin. Anat. Rec. 133 286.

Haun, C. K. and Sawyer, C. H. 1960. Initiation of lactation in rabbits following placement of hypothalamic lesions. Endocrinology. 67: 270.

Herold, L. 1939. Einfluse der hypophysens tieldurchtrennung auf die lactation. Arch. Gynak. 168: 534.

Holub, D. A., Katz, F. H. and Jailer, J. W. 1961. Inhibition by 6-methyl-16-acetoxy progesterone of ACTH synthesis and release in the rat. Endocrinology. 68: 173.

Hooker, C. W. and Williams, W. L. 1940. Retardation of mammary involution in the mouse by irritation of the nipples. Yale J. Biol. Med. 12: 559.

Hurst, V. and Turner, C. W. 1942. Lactogenic hormone content of anterior pituitary gland of albino mouse as compared to other species. Endocrinology. 31: 334.

Jacobsohn, D. 1949. The effect of transection of the hypophysial stalk on the mammary glands of lactating rabbits. Acta. Physiol. Scand. 19: 10.

Jacobsohn, D. 1954. Regeneration of hypophysial portal vessels and grafts of anterior pituitary glands in rabbits. Acta Endocrinol. 17: 187.

Jacobsohn, D. and Westman, A. 1945. On the structure and function of the mammary glands after hypophysectomy and transection of the hypophysial stalk in rats. Acta. Physiol. Scand. 9: 284.

Johnson, R. M. and Meites, J. 1955. Effects of cortisone, hydrocortisone and ACTH on mammary growth and pituitary prolactin content of rats. Proc. Soc. Exp. Biol. and Med. 89: 455.

Johnson, R. M. and Meites, J. 1958. Effects of cortisone acetate on milk production and mammary involution in parturient rats. Endocrinology. 63: 290.

Kanematzu, S. and Sawyer, C. H. 1962. Effects of hypothalamic and intrahypophysial estrogen implants on pituitary prolactin content in the rabbit. Anat. Rec. 142: 311.

Kanematzu, S., Hillard, J. and Sawyer, C. H. 1961. Effects of reserpine and hypothalamic lesions on pituitary prolactin content in the rabbit. Program of the 43rd Meeting of the Endocrine Society, p. 3.

Knigge, K. M. 1958. Cytology and growth hormone content of rat's pituitary gland following thyroidectomy and stress. Anat. Rec. 130: 543.

Kobayashi, T., Kobayashi, T., Kigawa, T., Mizuno, M. and Amenomari, Y. 1961. Influence of the crude hypothalamic extract upon the cultivated rat anterior pituitary cells. Endocrinol. Japan. 8: 223.

Kruskemper, H. L. 1958. Veranderungen des relativgewichtes der nebennieren normaler und mit corticoiden behandelter ratten unter dem einfluss von thyroxin und trijodthyronin. Acta Endocrinol. 28: 373.

Laqueur, G. L. 1943. Effects of testosterone propionate on the mammary glands of female albino rats. Endocrinology. 32: 81.

Lazo-Wasem, E. H. 1960. Pituitary ACTH levels during adrenal involution following thiouracil. Proc. Soc. Exp. Biol. and Med. 103: 300.

Levey, H. A. and Roberts, S. 1957. Influence of thyroid function on the metabolism of the anterior pituitary gland. Am. J. Physiol. 189: 86.

Li, C. H. 1961. Biochemistry of prolactin. Chapt. 5, p. 205. In Milk: The Mammary Gland and Its Secretion. Vol. 2. S. K. Kon and A. T. Cowie (Eds.). Academic Press, New York and London.

Lisk, R. D. 1960. Estrogen sensitive centers in the hypothalamus of the rat. Jour. Exp. Zool. 145: 197.

Lyons, W. R. 1937. The preparation and assay of mamotropin. Cold Spring Harbor Symposium on Quantitative Biology. V: 198.

Lyons, W. R. and Page, E. 1935. Detection of mammotropin in the urine of lactating women. Proc. Soc. Exp. Biol. and Med. 32: 1049.

Maqsood, M. and Meites, J. 1961. Induction of mammary secretion in rats by electrical stimulation. Proc. Soc. Exp. Biol. and Med. 106: 104.

Marshal, F. H. A. 1942. Exteroceptive factors in sexual periodicity. Biol. Rev. 17: 68.

Martini, L., De Poli, A., Pecile, A., Saito, S. and Tani, F. 1959. Functional and morphological observations on the rat pituitary grafted into the anterior chamber of the eye. J. Endocrinol. 19: 164.

Martinovitch, P. N. 1950. Anterior pituitary explants of infantile rats grafted in the anterior eye chamber of hypophysectomized hosts. Nature, London. 165: 33.

Marx, L. 1945. Testosterone propionate induces secretion of mammary glands of suckling female rats. Endocrinology. 37: 380.

McCann, S. M. 1962a. An hypothalamic luteinizing-hormone releasing factor. Am. J. Physiol. 202: 395.

McCann, S. M. 1962b. Effect of progesterone on plasma luteinizing hormone activity. Am. J. Physiol. 202: 601.

McCann, S. M. and Friedman, H. M. 1960. The effect of hypothalamic lesions on the secretion of luteotropin. Endocrinology. 67: 597.

McCann, S. M. and Taleisnik, S. 1961. The effect of an hypothalamic extract on the plasma luteinizing hormone (LH) activity of the estrogenized, ovariectomized rat. Endocrinology. 68: 1071.

McCann, S. M., Mack, R. and Gale, C. C. 1959. The possible role of oxytocin in stimulating the release of prolactin. Endocrinology. 64: 870.

McCarthy, J. L., Corley, R. C. and Zarrow, M. X. 1959. Effect of goitrogens on adrenal gland of the rat. Am. J. Physiol. 197: 693.

McQueen-Williams, M. 1935. Decreased mammotropin in pituitaries of thyroidectomized (maternalized) male rats. Proc. Soc. Exp. Biol. and Med. 33: 406.

Meites, J. 1958a. Effect of reserpine on prolactin content of rabbit pituitary. Proc. Soc. Exp. Biol. and Med. 97: 742.

Meites, J. 1958b. Ability of hormones to extend lactation and prevent mammary involution in parturient rats after removal of litters. J. Animal Sci. 17: 1223.

Meites, J. 1959a. Induction and maintenance of mammary growth and lactation in rats with acetylcholine and epinephrine. Proc. Soc. Exp. Biol. and Med. 100: 750.

Meites, J. 1959b. Mammary growth and lactation. Chapt. 16, p. 539. In Reproduction in Domestic Animals. Vol. II. H. H. Cole and P. T. Cupps (Eds.). Academic Press, New York and London.

Meites, J. and Hopkins, T. F. 1960. Induction of lactation and mammary growth by pituitary grafts in intact and hypophysectomized rats. Proc. Soc. Exp. Biol. and Med. 104: 273.

Meites, J. and Hopkins, T. F. 1961. The mechanism of action of oxytocin in retarding mammary involution: a study in hypophysectomized rats. J. Endocrinol. 22: 207.

Meites, J. and Nicoll, C. S. 1959. Hormonal prolongation of lactation for 75 days after litter withdrawal in postpartum rats. Endocrinology. 65: 572.

Meites, J. and Turner, C. W. 1942. Studies concerning the mechanism controlling the initiationof lactation at parturition. III. Can estrogen account for the precipitous increase in the lactogen content of the pituitary following parturition? Endocrinology. 30: 726.

Meites, J. and Turner, C. W. 1947. Effect of thiouracil and estrogen on lactogenic hormone and weight of the pituitaries of rats. Proc. Soc. Exp. Biol. and Med. 64: 488.

Meites, J. and Turner, C. W. 1948. Studies concerning the induction and maintenance of lactation. Missouri Agri. Exp. Sta. Res. Bulls. Nos. 415 and 416.

Meites, J. and Turner, C. W. 1950. Lactogenic hormone. Chapt. X, p. 237 in Hormone Assay. C. W.Emmens (Ed.). Academic Press Inc., New York.

Meites, J., Hopkins, T. F. and Deuben, R. 1962. Growth hormone production by rat pituitary in vitro. Fed. Proc. 21: 196.

Meites, J., Kahn, R. H. and Nicoll, C. S. 1961. Prolactin production by rat pituitary in vitro. Proc. Soc. Exp. Biol. and Med. 102: 127.

Meites, J., Nicoll, C. S. and Talwalker, P. K. 1959b. Effects of reserpine and serotonin on milk secretion and mammary growth in the rat. Proc. Soc. Exp. Biol. and Med. 101: 563.

Meites, J., Nicoll, C. S. and Talwalker, P. K. 1960. Local action of oxytocin on mammary glands of postpartum rats after litter removal. Proc. Soc. Exp. and Med. 103: 118.

Meites, J., Nicoll, C. S. and Talwalker, P. K. 1962. The central nervous system and the secretion and release of prolactin. Chapt. 8 in Symposium of Neuroendocrinology, Miami, Florida, 1961. Univ. of Ill. Press, Urbana. (In Press.)

Meites, J., Talwalker, P. K. and Nicoll, C. S. 1960a. Initiation of lactation in rats with hypothalamic or cerebral tissue. Proc. Soc. Exp. Biol. and Med. 103: 298.

Meites, J., Talwalker, P. K. and Nicoll, C. S. 1960b. Induction of mammary growth and lactation in rabbits with epinephrine, acetylcholine and serotonin. Proc. Soc. Exp. Biol. and Med. 104: 192.

Meites, J., Talwalker, P. K. and Nicoll, C. S. 1961. Failure of oxytocin to initiate mammary secretion in rabbits or rats. Proc. Soc. Exp. Biol. and Med. 105: 567.

Merchant, D. J., Kahn, B. H. and Murphy, Jr., W. H. 1960. Handbook of Cell and Organ Culture. Burgess Publishing Co., Minneapolis.

Merckel, C.O. and Nelson, W. O. 1940. Relation of estrogenic hormone to formation and maintenance of corpora lutea in mature and immature rats. Anat. Rec. 76: 391.

Mixner, J. P. and Turner, C. W. 1941. Influence of local applications of turpentine on mammary gland growth and involution. Proc. Soc. Exp. Biol. and Med. 46: 437.

Montemurro, D. J. and Gardner, W. U. 1961. The effect of subcutaneous transplants of hypothalamus and pituitary gland on the estrous cycle of the mouse. Fed. Proc. 20: 188.

Moon, R. C. 1962. Influence of thyroxine upon pituitary lactogenic hormone content. Fed. Proc. 21: 193.

Muhlbock, O. and Boot, L. M. 1959. The mechanism of hormonal carcinogenesis. In Ciba Foundation Symposium on Carcinogenesis, Mechanisms of Action. G. E. Walstenholme (Ed.). Little, Brown and Co., Boston.

Nalbandov, A. V. 1962. Symposium of Neuroendocrinology. Miami, Florida, 1961. Univ. of Illinois Press, Urbana. (In Press.)

Nicoll, C. S., Talwalker, P. K. and Meites, J. 1960. Initiation of lactation in rats by nonspecific stresses. Am. J. Physiol. 198: 1103.

Nikitovitch-Winer, M. B. 1960. The influence of the hypothalamus on luteotropin secretion in the rat. In Memoirs of the Society for Endocrinology, No. 9, Part I, p. 70. Cambridge Univ. Press, London.

Nikitovitch-Winer, M. B. 1962. Induction of ovulation in rats by direct intrapituitary infusion of median eminence extracts. Endocrinology. 70: 350.

Nikitovitch-Winer, M. and Everett, J. W. 1958. Functional restitution of pituitary grafts retransplanted from kidney to median eminence. Endocrinology. 63: 916.

Ota, K. and Yokoyama, A. 1958. Effect of oxytocin administration on respiration of lactating mammary tissue in rats. Nature, London. 182: 1509.

Parks, A. S. 1926. Observations on the estrous cycle of the albino mouse. Proc. Roy. Soc. B. 100: 151.

Pasteels, J.-L. 1961a. Secretion de prolactine par l'hypophyse en culture de tissus. Compt. Rend. Acad. Sci., Paris. 253: 2140.

Pasteels, J.-L. 1961b. Premiers resultats de culture combines in vitro et d'hypothalamus, dans le but d'en apprecier la secretion de prolactine. Comp. Rend. Acad. Sci., Paris. 253: 3074.

Paul, J. 1960. Cell and Tissue Culture. 2nd ed. Williams and Wilkins Co., Baltimore.

Petersen, W. E. 1942. New developments in the physiology and biochemistry of lactation; a review. J. Dairy Sci. 25: 71.

Popa, G. T. and Fielding, U. 1930. A portal circulation from the pituitary to the hypothalamic region. J. Anat. 65: 88.

Purves, H. D. 1961. Morphology of the hypophysis related to its function. Chapt. 3, p. 161. In Sex and Internal Secretions. Vol. I. W. C. Young (Ed.). The Williams and Wilkins Co., Baltimore.

Purves, H. D. and Griesbach, W. E. 1946. Acidophil degranulation in relation to goitrogenic agents and extrathyroidal thyroxine synthesis. Brit. J. Exper. Path. 27: 170.

Purves, H. D. and Griesbach, W. E. 1951. The site of thyrotropin and gonadotropin production in the rat pituitary studied by McManus-Hotchkiss staining for glycoprotein. Endocrinology. 49: 244.

Quilligan, E. J. and Rothchild, I. 1960. The corpus luteum-pituitary relationship: the luteotrophic activity of homotransplanted pituitaries in intact rats. Endocrinology. 67: 48.

Ralph, C. L. and Fraps, R. M. 1960. Induction of ovulation in the hen by injection of progesterone into the brain. Endocrinology. 66: 269.

Rasmussen, A. T. 1938. Innervation of the hypophysis. Endocrinology. 23: 263.

Reece, R. P. 1941. Androsterone effect on pituitary and mammary gland. Proc. Soc. Exp. Biol. and Med. 46: 265.

Reece, R. P. and Bivins, J. A. 1942. Progesterone effect on pituitary lactogen content and on mammary glands of ovariectomized rats. Proc. Soc. Exper. Biol. and Med. 49: 582.

Reece, R. P. and Mixner, P. J. 1939. Effect of testosterone on pituitary and mammary glands. Proc. Soc. Exp. Biol. and Med. 40: 66.

Reece, R. P. and Turner, C. W. 1936. Influence of estrone upon galactin content of male rat pituitaries. Proc. Soc. Exp. Biol. and Med. 34: 402.

Reece, R. P. and Turner, C. W. 1937. The lactogenic and thyrotropic hormone content of the anterior lobe of the pituitary gland. Missouri Agric. Exp. Sta. Bull. No. 266.

Reichlin, S. 1961. Growth hormone content of pituitaries from rats with hypothalamic lesions. Endocrinology. 69: 225.

Reineke, E. P. and Solimon, F. A. 1953. Role of thyroid hormone in reproductive physiology of the female. Iowa State College Journal of Science. 28: 67.

Rendi, R. 1959. The effect of chloramphenicol on the incorporation of labeled amino acids into proteins by isolated subcellular fractions from rat liver. Exp. Cell. Res. 18: 187.

Riddle, O. and Bates, R. W. 1939. The preparation, assay and actions of lactogenic hormone. In Sex and Internal Secretions. 2nd ed. E. Allen (Ed.). Williams and Wilkins, Baltimore.

Riddle, O. and Braucher, P. F. 1931. Studies on the physiology of reproduction in birds. XXX. Control of the special secretion of the crop-gland in pigeons by an anterior pituitary hormone. Am. J. Physiol. 97: 617.

Rivera, E. M. and Bern, H. A. 1961. Influence of insulin on maintenance and secretory stimulation of mouse mammary tissue by hormones in organ-culture. Endocrinology. 69: 340.

Roberts, S. and Keller, M. A. 1955. Influence of epinephrine and cortisone on the metabolism of the hypophysis and hypothalamus of the rat. Endocrinology. 57: 64.

Rockefort, G. J., Rosenberger, J. and Saffran, M. 1959. Depletion of pituitary corticotrophin by various stresses and by neurohypophysial preparations. J. Physiol. 146: 105.

Rose, S. and Nelson, J. 1956. Hydrocortisone and ACTH release. Aust. J. Exp. Biol. and Med. Sci. 34: 77.

Rose, S. and Nelson, J. F. 1957. The direct effect of oestradiol on the pars distalis. Aust. J. Exp. Biol. and Med. Sci. 35: 605.

Rothchild, I. 1960a. The corpus luteum-pituitary relationship: the association between the cause of luteotropin secretion and the cause of follicular quiescence during lactation; the basis of a tentative theory of the corpus luteum-pituitary relationship in the rat. Endocrinology. 67: 9.

Rothchild, I. 1960b. The corpus luteum-pituitary relationship: the lack of an inhibitory effect of progesterone on the secretion of pituitary luteotrophin. Endocrinology. 67: 54.

Rothchild, I. and Quilligan, E. J. 1960. The corpus liteum-pituitary relationship: on the reports that oxytocin stimulates the secretion of luteotropin. Endocrinology. 67: 122.

Royce, P. C. and Sayers, G. 1958. Biological characterization of a corticotropin-releasing factor. Fed. Proc. 18: 132.

Ryan, K. J. and Smith, O. W. 1961. Biogenesis of estrogens by the human ovary. II. Conversion of progesterone-4-C^{14} to estrone and estradiol. J. Biol. Chem. 236: 710.

Sanders, A. and Rennels, E. G. 1957. The cytology and functional capacity of the rat pituitary gland after autotransplantation. Anat. Rec. 127: 362.

Sawyer, C. H. 1960. Reproductive behavior. In Handbook of Physiology, Sect. 1: Neurophysiology, Vol. II. J. Field (Ed.). American Physiological Society, Washington, D. C.

Sawyer, C. H., Markel, J. E. and Townsend, B. F. 1949. Cholinergic and adrenergic components in the neurohumoral control of the release of LH in the rabbit. Endocrinology. 44: 18.

Sayers, G. and Sayers, M. A. 1948. The pituitary-adrenal system. Recent Progress in Hormone Research. 11: 81. Academic Press, New York.

Schaberg, A. and De Groot, C. A. 1958. The influence of the anterior hypophysis on the morphology and function of the adrenal cortex in vitro. Exp. Cell. Res. 15: 475.

Selye, H. 1934. On the nervous control of lactation. Am. J. Physiol. 107: 535.

Selye, H. 1954. The effect of cortisol upon the mammary gland. Acta Endocrinologica. 17: 394.

Selye, H. and McKeown, T. 1934. The effect of mechanical stimulation of the nipples on the ovary and the sexual cycle. Surg. Gynec. and Obst. 59: 886.

Selye, H., Collip, J. B. and Thomson, D. L. 1934. Nervous and hormonal factors in lactation. Endocrinology. 18: 237.

Selye, H., McKeown, C. S. and Collip, J. B. 1936. Effect of testosterone on the mammary gland. Proc. Soc. Exp. Biol. and Med. 34: 201.

Setlow, R. B. and Pollard, E. C. 1962. Molecular Biophysics. Chapt. 15, p. 489. Addison-Wesley Publishing Co., Inc., Reading, Mass. and London, England.

Sgouris, J. T. and Meites, J. 1952. Inactivation of prolactin by body tissues in vitro. Am. J. Physiol. 169: 301.

Snedecor, G. W. 1950. Statistical Methods. Iowa State College Press, Ames, Iowa.

Soliman, F. A. and Reineke, E. P. 1955. Influence of estrogen and progesterone on radioactive iodine uptake by rat thyroid. Am. J. Physiol. 183: 63.

Solomom, J. and Greep, R. O. 1959. The effect of alterations in thyroid function on the pituitary growth hormone content and acidophil cytology. Endocrinology. 65: 158.

Soulairac, A. and Desclaux, P. 1947. Modifications hypophysaires apres lesions hypothalamiques. Action de la phloridzine. Ann. d'Endocrinol. 8: 343.

Stricker, P. and Grueter, F. 1928. Action du lobe anterieur de l'hypophyse sur la montie laiteuse. Compt. Rend. Soc. Biol., Paris. 99: 1978.

Struempler, A. W. and Burroughs, W. 1959. Stilbesterol feeding and growth hormone stimulation in immature ruminants. J. Anim. Sci. 18: 427.

Stutinski, F. 1957. Action de l'ocytocine exogene sur la production d'un deciduome traumatique chez la ratte allaitante. Compt. Rend. Acad. Sci. 244: 1537.

Sulman, F. G. and Winnik, H. Z. 1956. Hormonal effects of chlorpromazine. Lancet. 1: 161.

Swingle, W. W., Fedor, E. J., Barlow, G. Jr., Collins, E. J. and Perlmut, J. 1951a. Induction of pseudopregnancy in the rat following adrenal removal. Am. J. Physiol. 167: 593.

Swingle, W. W., Seay, P., Perlmutt, J., Collins, E. J., Barlow, G. Jr., and Fedor, E. J. 1951b. An experimental study of pseudopregnancy in the rat. Am. J. Physiol. 167: 586.

Swingle, W. W., Seay, P., Perlmutt, J., Collins, E. J., Fedor, E. J. and Barlow, G. Jr. 1951c. Effect of electrical stimulation of uterine cervix upon sexual development of prepuberal rats. Am. J. Physiol. 167: 599.

Sydnor, K. L. and Sayers, G. 1954. Blood and pituitary ACTH in intact and adrenalectomized rats after stress. Endocrinology. 55: 621.

Tabachnick, I. A. and Trentin, I. J. 1951. Effect of suckling on adrenal ascorbic acid level of mice. Fed. Proc. 10: 339.

Talwalker, P. K. and Meites, J. 1961. Mammary lobulo-alveolar growth induced by anterior pituitary hormones in adreno-ovariectomized and adreno-ovariectomized-hypophysectomized rats. Proc. Soc. Exp. Biol. and Med. 107: 880.

Teubenhaus, M. and Soskin, S. 1942. Release of luteinizing hormone from anterior pituitary by an acetylcholine like substance from the hypothalamic region. Endocrinology. 29: 958.

Thomas, T. B. and Emerson, G. A. 1945. Cytological findings in alloxan diabetes in the rabbit. Texas Repts. Biol. and Med. 3: 142.

Thompson, K. W., Vincent, M. M., Jensen, F. L., Price, R. T. and Shapiro, E. 1959. Production of hormones by anterior pituitary cells in serial culture. Proc. Soc. Exp. Biol. and Med. 102: 403.

Trowell, O. A. 1958. The culture of mature organs in synthetic medium. Exp. Cell. Res. 16: 118.

Victor, J. and Anderson, D. H. 1937. Stimulation of anterior hypophysis metabolism by theelin or dihydrotheelin. Am. J. Physiol. 120: 154.

Villee, C. A. 1961. Some problems of the metabolism and mechanism of action of steroid sex hormones. Chapt. 11, p. 643. In Sex and Internal Secretions. Vol. I. W. C. Young (Ed.). The Williams and Wilkins Co., Baltimore.

Wallach, D. P. and Reineke, E. P. 1949. The effect of varying levels of thyroidal stimulation on the ascorbic acid content of the adrenal cortex. Endocrinology. 45: 75.

Warren, S. and Le Compte, P. M. 1952. The Pathology of Diabetes Mellitus. 3rd ed. Lea and Fabiger Co., Philadelphia.

Weichert, C. K. and Boyd, R. W. 1933-34. Induction of typical pseudopregnancy in the albino rat by means of experimental hyperthyroidism. Anat. Rec. 58: 55.

West, C. D., Damast, B. L., Sarro, S. D., and Pearson, O. H. 1956. Conversion of testosterone to estrogens in castrated, adrenalectomized human females. J. Biol. Chem. 218: 409.

Winnik, H. Z. and Tennenbaum, L. 1955. Apparition de galactorrhee au cours du traitement de largactil. Pr. Med. 63: 1092.

Wolf, J. M. 1935. Reaction of ovaries of mature female rats to injection of oestrin. Proc. Soc. Exp. Biol. and Med. 32: 757.

Yamada, T. 1959. Studies on the mechanism of hypothalamic control of thyrotropin secretion: comparison of the sensitivity of the hypothalamus and of the pituitary to local changes of thyroid hormone concentration. Endocrinology. 65: 920.

Yokoyama, A. and Ota, K. 1959a. The effects of hypothalamic lesions on litter growth in rats. Endocrinol. Japon. 6: 14.

Yokoyama, A. and Ota, K. 1959b. Effects of oxytocin replacement on lactation in rats bearing hypothalamic lesions. Endocrinol. Japon. 6: 268.

Yokoyama, A. and Ota, K. 1960. Studies on the mammary glands of lactating rats bearing hypothalamic lesions. Endocrinol. Japon. 7: 1.

Zizine, L. 1956. Inhibition par la testosterone de 1' hypertrophie surrenalienne compensatrice. Compt. Rend. Soc. Biol., Paris. 150: 1333.

Zeckwer, I. T., Davison, L. W., Keller, T. B. and Livengood, S. C. 1935. The pituitary in experimental cretinism: structural changes in the pituitaries of thyroidectomized rats. Am. J. Med. Sci. 190: 145.