STUDIES ON THE HYPOTHALAMIC REGULATION OF FOLLICLE-STIMULATING HORMONE RELEASE

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ABSTRACT

STUDIES ON THE HYPOTHALAMIC REGULATION OF FOLLICLE-STIMULATING HORMONE RELEASE

by James C. Mittler

The regulation of adenohypophysial function by the central nervous system has been shown to be by neurohumoral rather than by direct nervous pathways. Neurohumoral cells in the hypothalamus liberate chemical mediators which are conveyed by the hypothalamo-hypophysial portal blood to the anterior pituitary, where they influence secretion of anterior pituitary hormones. In early 1963, when the work reported in this thesis was begun, some evidence had already been reported that hypothalamic extracts contained substances which stimulate synthesis or release of pituitary ACTH, TSH, LH, and STH, and also inhibited synthesis and release of prolactin. Direct evidence for a substance with stimulatory action on secretion of follicle-stimulating hormone (FSH) however, seemed scanty and unconvincing, although there was abundant circumstantial evidence for the existence of such a substance in the hypothalamus. The search for an FSH-releasing factor (FSH-RF) seemed of interest and of practical importance.

FSH secretion is known to be regulated by feedback mechanisms involving gonadal steroid hormones, possibly acting through the hypothalamus, or directly on the pituitary, or at both sites. The site(s) of action of steroid hormones is currently the subject of much controversy. Study of pituitary tissue *in vitro* seemed to be the ideal method for demonstrating the existence of an FSH-RF in the hypothalamus and for localizing the site(s) of action(s) of steroid hormones.

Explants of rat anterior pituitary tissue were found to release significant amounts of FSH only during the first 3 days of a 9-day culture period. Cultures were performed at 37°C in tissue culture medium 199 containing 10% calf serum and under an atmosphere of 95% O_2 - 5% CO_2. Hypothalamic stimulation of FSH release was demonstrated by means of paired cultures from opposite halves of 72 adult female anterior pituitaries. At the end of the 3rd and 6th days of culture, the medium was removed. Fresh medium containing neutralized 0.1N. HCl acid extract of rat hypothalamus (including pituitary stalk and median eminence) was added to one-half of the culture dishes and medium containing similarly prepared cerebral cortical extract was added to the remainder. Each pituitary equivalent was cultured with an equivalent of 4 hypothalami from ovariectomized female rats. Brain extracts were not added during the first 3 days of culture. After termination of culture, the medium was assayed by the method of Steelman and Pohley using medium

equivalent to 2 pituitaries and 50 I.U. of HCG per assay rat. Medium from the first 3 days of culture was assayed separately. Pituitary tissue cultured with cerebral cortical extract (control) did not release significant amounts of FSH, whereas pituitary cultured with hypothalamic extract (experimental) released highly significant amounts of FSH ($p < .001$).

During a 6-hour incubation period, neutralized acidic extracts of rat hypothalamic tissue stimulated release of FSH from adult male rat pituitary tissue. A graded response was observed in release of FSH by pituitary tissue in response to doses of extract ranging from 1/32 to 1/4 hypothalamic equivalent per incubated pituitary.

Castration was found to increase and testosterone proponate injections (2 mg/day) to reduce the FSH-releasing activity of hypothalamic extracts.

Brain extracts had no effect upon the activity of purified FSH in vitro in tissue culture or 6-hour incubation, nor when injected together with FSH into assay rats.

Testosterone (1 μgm/ml), estradiol (0.25 μgm/ml), progesterone (1 μgm/ml) and the last two in combination failed to inhibit FSH release in tissue culture. Estradiol failed to antagonize the stimulatory effects of hypothalamic extract in both tissue culture and in incubation experiments. Testosterone failed to antagonize hypothalamic extract during 6-hour incubation experiments. Steroids were added to media dissolved in a minute volume of absolute ethanol.

An appendix describes studies on the hypothalamic content of prolactin inhibiting factor (PIF). Epinephrine in oil (0.25 mg) and acetylcholine bromide (25 mg per kilogram body weight) were found to decrease the amount of PIF in the hypothalamus. Both treatments were administered twice daily and one hour before removing the hypothalami. Hypophysectomy had no effect on PIF content.

STUDIES ON THE HYPOTHALAMIC REGULATION OF FOLLICLE-STIMULATING HORMONE RELEASE

By

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I. INTRODUCTION

Much research in the past few decades has been devoted to elucidating the function of the anterior pituitary gland. It has recently become apparent that the functions of the anterior pituitary are regulated to a major extent by the central nervous system. The portion of the central nervous system most closely associated with control of anterior pituitary function is the hypothalamus.

The control of the adenohypophysis by the central nervous system has been shown to be by neurohumoral rather than by direct nervous means. Neurohumoral cells in the hypothalamus liberate chemical mediators which are conveyed by the hypothalamo-hypophysial portal blood to the anterior pituitary, where they influence secretion of anterior pituitary hormones. In early 1963, when the work reported in this thesis was begun, some evidence had already been reported that hypothalamic extracts contained substances which stimulate synthesis and release of pituitary ACTH, TSH, LH, and STH and also inhibit synthesis and release of prolactin. Direct evidence for a substance with stimulatory action on secretion of follicle-stimulating hormone (FSH) however, seemed scanty and unconvincing, although there was abundant circumstantial evidence for the existence of such a substance in the hypothalmus. The search for an

FSH-releasing factor (FSH-RF) seemed of theoretical interest and also of practical importance.

FSH secretion is known to be regulated by feedback mechanisms involving gonadal steroid hormones, possibly acting through the hypothalamus, or directly on the pituitary, or at both sites. The site(s) of this action of steroid hormones is currently the subject of much controversy. Study of pituitary tissue *in vitro* seemed to be the ideal method for demonstrating the existence of an FSH-RF in the hypothalamus and for localizing the site(s) of action(s) of steroid hormones.

II. REVIEW OF LITERATURE

Neural control of the secretions of the anterior pituitary, first proposed by Hohlweg and Junkmann (1932), is now well established. Harris (1948, 1955, 1960) reviewed evidence that release of hormones is influenced by the hypothalamus and that the mechanism of control is neurohumoral by way of the hypothalamo-hypophysial portal system. Szentagothai et al. (1962) and Nalbandov, ed. (1963) have reviewed more recent literature.

Benoit and Assenmacher (1953, 1955) have published extensive reviews of the enormous clinical and experimental literature on neural regulation of reproductive functions of the anterior pituitary. More recent, specialized reviews are those of Everett (1964) and Bogdanove (1964). Meites, et al. (1963, 1966) reviewed the work on neural regulation of prolactin secretion. Greep (1963) reviewed anatomical relationships of the brain and pituitary and the complex vascular connections between them. Some of the more important and more recent findings will now be discussed.

1. Evidence from Effects of Brain Lesions

Atrophy of the gonads and different parts of the genital tract after electrolytic lesions involving the median eminence and adjacent parts of the basal tuberal region of

the hypothalamus has been reported in both sexes in the rat (Bogdanove and Halmi, 1953; Bogdanove et al., 1957; Bogdanove, 1957; Slusher, 1958; D'Angelo, 1959, and Taleisnik and McCann, 1961). The basal tuberal region of the hypothalamus may be defined as including the pituitary stalk, median eminence, arcuate nucleus, and ventromedial nucleus. D'Angelo (1959), Bogdanove and Schoen (1959), and Lisk (1960) found that lesions which completely destroy the arcuate nucleus result in gonadal atrophy in male and female rats. Corbin (1963) found that bilateral lesions of the ventromedial nucleus-mammilary body area result in atrophy of testes, seminal vesicles, and ventral prostates in male rats. Similar findings have been reported for the female guinea pig (Dey, 1941, 1943), female cat (Laqueur et al., 1955), male dog (Daily and Ganong, 1958), Davidson and Ganong, 1960; Davidson et al. 1960b); and ewe (Clegg and Ganong, 1960).

Dávid et al. (1965) presented convincing evidence that the effects of lesions and stalk section cannot be explained as the result of impaired blood supply to the anterior pituitary. Studies on partially hypophysectomized rats showed that 70% of the adeno hypophysis may be removed without impairment of function. Stalk section causes much less loss than this. Furthermore, Haun and Sawyer (1960); McCann and Friedman (1960), Nikitovitch-Winer (1965) and others found that prolactin secretion actually rises after such lesions.

An impressive body of evidence, accumulated in recent years, indicates that neural elements involved in the control of LH as well as FSH secretion must be located outside the basal tuberal region of the hypothalamus. Constant estrous rats with ovaries containing developing and sometimes cystic follicles, but no corpora lutea, have been observed by several authors following anterior hypothalamic lesions (for review, see Flerko, 1961). Similarly, Clegg and Ganong (1960) produced constant estrus in the ewe with anterior hypothalamic lesions.

Donovan and van der Werff ten Bosch (1959a) found that anterior hypothalamic lesions could hasten onset of puberty in the female rat, and Donovan and van der Werff ten Bosch (1959b) obtained early onset of heat in the ferret after anterior hypothalamic lesions were placed during the winter anestrous period.

Bogdanove and Schoen (1959) obtained precocious puberty in female rats with anterior hypothalamic lesions; the animals became estrous. In those animals in which the arcuate nucleus was damaged, luteinization occurred also; but complete destruction of the arcuate nucleus resulted in atrophy of the gonads. Curiously, male rats were not affected by these lesions. However, Bogdanove et al. (1964) later observed hypertrophy of prostate and seminal vesicles (but not testes) after large periventricular lesions which had little apparent effect in females.

D'Angelo and Kravatz (1960) and Flerko and Bardos (1961) observed that female rats made "constant estrus" by anterior hypothalamic lesions failed to show compensatory hypertrophy after unilateral ovariectomy. These writers suggest that receptors sensitive to "estrogen deficiency" are located in the anterior hypothalamus. However, these findings conflict with those of Desclin et al. (1962).

2. Evidence from Behavior of Pituitary Tissue Implanted in the Hypothalamus

Flerko (1963) described and diagramed the region of the hypothalamus in which anterior pituitary implants maintain normal function and cytology. This region is designated the "hypophysiotrophic" area. Implants into the "hypophysiotrophic area" maintained target organs, especially the gonads, in hypophysectomized rats. Pituitary tissue implanted outside this area rapidly lost its normal cytology and function.

The "hypophysiotrophic area" is of considerable length orocaudally. It extends from the anterior and paraventricular nuclei ventrally to the region of the basal portions of the ventromedial nuclei and extends posteriorly to the mammilary bodies. It includes the whole of the arcuate nuclei and is very thin in the dorso-ventral direction.

The most complete studies of pituitary implants into the hypothalamic hypophysiotrophic area were done by Halasz et al. (1962, 1965). Also, Knigge (1962) obtained castration

cells and testicular maintenance with such implants in hypophysectomized male rats.

Flament-Durand (1964, 1965) obtained particularly impressive results with pituitary implants into the region of the arcuate nucleus in female rats. Castration cells resulted in these implants when the animals were ovariectomized. She had poor results with male rats, and did not confirm Knigge's (1962) results.

3. Evidence from Use of Hypothalamic Extracts

Campbell et al. (1961, 1964) demonstrated that direct infusion of median eminence extracts into the pituitaries of rabbits was effective in inducing ovulation, and Nikitovitch-Winer (1962) similarly induced ovulation by infusion of median eminence extract into the pituitaries of pentobarbital-blocked proestrus rats. Nikitovitch-Winer and Everett (1958, 1959) found that rat pituitary tissue which had lost its normal functions and characteristic cytological appearance after being transplanted under the kidney capsule, could be restored to normal appearance and functions by retransplantation under the median eminence. Similarly, Evans and Nikitovitch-Winer (1965) found that pituitary grafts under the kidney capsule could be restored to normal cytology and function by continuous infusion with median eminence extracts. Some of their animals went into constant estrus.

McCann et al. (1960) and McCann (1962a) first demonstrated the presence of a luteinizing hormone-releasing factor (LH-RF) in acid extracts of stalk-median eminence tissue of rats using the ovarian ascorbic acid depletion method of Parlow (1958). These results have been confirmed by Courrier et al. (1961) and Guillemin et al. (1963). Courrier et al. (1961) and Johnson (1963) observed also that hypothalamic extracts could induce ovulation in androgen-sterilized female rats. Schiavi et al. (1963) used acid extracts of sheep hypothalami to produce ovulation in rats rendered anovulatory by hypothalamic lesions. Ramirez and McCann (1963) devised a sensitive test for luteinizing hormone-releasing activity. Rats which had been ovariectomized for 6 weeks were treated with a single injection of estrogen (50 μgm of estradiol benzoate) and progesterone (25 mg). This treatment blocked LH release and plasma LH levels fell markedly within 3 days. Hypothalamic extracts were then injected and caused a marked rise in plasma LH. Johnson (1964) found that hypothalamic extract caused release of hypophysial LH in immature male rats. Schally and Bowers (1964) demonstrated stimulation of LH release <u>in vitro</u>. LH-RF has now been purified (Guillemin et al., 1963; Schally and Bowers, 1964; Ramirez et al., 1964, and Nikitovitch-Winer, 1965).

Igarashi and McCann (1964b) and Igarashi et al. (1964a) presented evidence for the presence of a follicle-stimulating hormone-releasing factor (FSH-RF) in rat

hypothalamus using *in vivo* methods, e.g. the estrogen-progesterone blocked ovariectomized rat described above and also used rats with hypothalamic lesions. These results were confirmed by Kuroshima et al. (1965). These workers employed an HCG augmented mouse uterine weight assay for FSH devised by Igarashi and McCann (1964a) but not properly validated. Later, de Rivieres and Mauléon (1965) demonstrated that this method was sensitive to LH, growth hormone, and prolactin, as well as FSH.

Gellert et al. (1964) have also presented evidence for the existence of FSH-RF by producing precocious puberty in female rats with injections of hypothalamic extracts. Independently of the work presented in this thesis, Kuroshima et al. (1965) demonstrated stimulation of release of FSH by a hypothalamic extract *in vitro*.

Kobayashi et al. (1963a) reported that crude saline extracts of hypothalamus induced an increase in "total gonadotropin" release from monolayer cultures of rat anterior pituitary tissue. Gonadotropin content of cells was increased also. Cerebral cortical extract, vasopressin, and oxytocin were without effect. These workers used the immature mouse uterine weight assay method, which measures the combined effect of FSH and LH. While FSH alone may cause some growth of ovarian follicles, Fevold (1941) and Greep et al. (1942) demonstrated that LH is necessary for estrogen stimulation and consequent uterine growth. The results of Kobayashi et al. (1963a) may be considered of doubtful significance

since they never injected hypothalamic extract alone into mice as a control. Furthermore, Courrier et al. (1963c) reported that saline extracts of sheep hypothalamus contain substantial quantities of FSH activity, whereas FSH activity is absent in acid extracts of hypothalamus.

Kobayashi et al. (1963) demonstrated that hypothalamic extracts stimulated the uptake of tritiated leucine by PAS positive cells of the anterior pituitary in vitro. Hypothalamic extracts seem therefore to stimulate the synthesis of FSH, LH, and/or thyrotropic hormone in vitro.

Moszkowska (1959) and Moszkowska and Kordon (1961) reported that more gonadotropin was released when pituitaries and hypothalami were grafted together on ovaries than when pituitaries were grafted alone. Similarly, Moszkowska (1959) and Moszkowska and Scemama (1964) reported that incubation media contained more FSH and LH when pituitaries and hypothalami were incubated together than when pituitary tissue was incubated alone. These authors did not do control experiments to test the possibility that pars tuberalis tissue adherent to the hypothalami might have contributed to gonadotropin release.

Suzuki et al. (1961) claimed that slices of male rat hypothalamic tissue stimulated gonadotropin synthesis by slices of male rat pituitary during a 3-hour incubation. Castration of the donor increased the potency of the hypothalamic slices.

In an excellent study, Talwalker et al. (1963) demonstrated that the hypothalamus contains a substance which inhibits prolactin secretion in vitro. This finding was confirmed by Schally et al. (1965).

4. Steroid Feedback

A classical review on the steroid control of anterior pituitary gonadotropic functions by feedback mechanisms was that of Greep and Chester Jones (1950). The gonadal steroids are conceived as regulating pituitary FSH and LH secretion by acting on the pituitary and/or hypothalamus to inhibit gonadotropin secretion. Reciprocal relationships between rates of sex steroid and pituitary gonadotropin secretions are required for certain phases of normal gonadal function.

In the extensive review by Greep (1961) the conclusion is drawn that it is now firmly established that gonadectomy results in a marked increase in gonadotropin secretion and in increased storage of gonadotropic hormones in the hypophysis. Among the more recent literature, Couzens and Nelson (1961) found a 3 to 4-fold increase in pituitary FSH content as well as an approximately 4-fold increase in pituitary LH concentration within 7 days after ovariectomy in female rats. Paesi et al. (1955) reported that ovariectomy causes a 5-fold increase in pituitary FSH content in the female rat within 3 months. Male rat pituitaries contained more than 5 times as much FSH as females and did not increase significantly in content after

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castration. In contrast, Hellbaum et al. (1961) found that gonadectomy caused a rise in pituitary FSH and in LH in both sexes in the rat.

Gans (1959a, b) and Gans and van Rees (1962) were able to detect FSH and LH in serum in gonadectomized male and female rats but not in intact rats. Gans and de Jongh (1963) found that serum FSH was much higher in gonadectomized male and female rats than in intact animals, and that serum of castrated male rats contained more FSH than that of ovariectomized female rats. Intact male rats were found to have much larger amounts of FSH in their pituitaries than intact female rats.

Parlow (1964a) found a 5-fold increase in FSH content in the pituitary of the mouse after ovariectomy. Serum FSH rose from undetectable to very substantial levels, 9 to 12 μgm NIH-FSH-S1 equivalents per ml. LH content did not rise. Davidson et al. (1960a) found that the FSH content of the male dog pituitary rises as much as 16-fold after castration, and LH content doubles.

Parlow (1964c) used much improved assay techniques, e.g., the OAAD assay for LH and a greatly improved version of the Steelman-Pohley assay for FSH. He found that both FSH and LH levels rose in the pituitary and blood of the female rat after ovariectomy; 0.1 to 0.4 μgm estradiol injected each day inhibited the pituitary and serum response to gonadectomy; 2.0 μgm estradiol per day were required to prevent the FSH response to castration. The duration of

this experiment was 16 days. Beyler and Potts (1962) found that estradiol treatment (4 to 400 μgm/kg body wt/day for 14 days) caused decreased pituitary FSH content in the male rat. Testosterone treatment (0.2 to 20 mg/kg/day) antagonized this effect of estradiol and by itself did not decrease pituitary FSH potency. Gans (1959a, b) found that estradiol benzoate (2 μgm/day) or testosterone propionate (100 μgm/day) inhibited the post-castration rise in serum FSH and LH in male and female rats. Paesi et al. (1955) and Hellbaum et al. (1961) obtained similar results. The parabiotic technique was used by Miyake (1961) to demonstrate inhibition of gonadotropin secretion by estrogens or androgens.

Parlow (1964c) also found that treatment of male rats with testosterone propionate (1 mg/day for 3 weeks) starting immediately after castration prevented the post-castration rise in serum FSH and serum and pituitary LH. Pituitary FSH content rose with testosterone treatment. Hellbaum et al. (1961) treated gonadectomized male and female rats with testosterone propionate (3 mg/day). Pituitary LH content (but not FSH content) decreased. Hoogstra and Paesi (1957) found that testosterone propionate treatment (2 mg/day) increased the pituitary FSH content in intact and gonadectomized male and female rats. Estradiol benzoate (2 μmg/day) did not modify this response.

Ramirez and McCann (1965) found that only 1/3 or 1/4 as much testosterone propionate was required to inhibit the post-castration rise in plasma LH in the immature male

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rats as compared with the post-puberal male rats. These authors claimed that 75 to 100 μgm testosterone propionate per 100 gm body weight per day was a "physiological" dose for an adult male rat.

Van Rees (1961) studied release of FSH by rat pituitaries *in vitro* after treatment *in vivo*. Ovariectomy caused enormously increased FSH release during a 2-hour incubation. Intact male rat pituitaries contained much more FSH than those from intact females, but released much less in vitro. Treatment of intact and gonadectomized rats with testosterone propionate *in vivo* caused decreased release *in vitro*.

McCann (1962b) concluded that, in the ovariectomized female rat, progesterone alone has only a feeble inhibitory effect on secretion of LH, but estradiol benzoate treatment sensitizes the animal to this inhibitory action. Similarly, Sager et al. (1966) found that estrone and progesterone synergize in inhibiting the rise in plasma FSH after ovariectomy. Ramirez and McCann (1963a) found that treatment with a single injection of estradiol benzoate (50 μgm) and progesterone (25 mg) caused a marked fall in plasma LH activity in 3 days in female rats which had been ovariectomized for 6 weeks. The pituitary of such an animal seems, however, to be sensitized to the effects of substances which stimulate release of LH. Igarashi and McCann (1964b) similarly obtained inhibition of FSH release and sensitization to FSH-RF.

Testosterone alone apparently cannot account for the testicular feedback mechanism. Bogdanove (manuscript in

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preparation) studied pituitary cytology and pituitary FSH content (using an improved version of the Steelman-Pohley assay) in male rats after castration and testosterone propionate treatment. This excellent work demonstrated increased FSH stores in the pituitary and increased FSH in the serum 2 weeks after castration. With testosterone propionate treatment (3.0 mg each 2 days), the FSH stores in the pituitary increased still further while FSH content of the serum decreased markedly. Pituitary cytology changed with castration and became still more abnormal with testosterone propionate treatment, indicating that some testicular factor other than androgen participates in regulation of FSH secretion in the male rat. In addition, Johnsen (1964) concluded from a study of human clinical material that a testicular-hypophysial feedback mechanism occurs separately from testosterone.

5. Evidence from Implantation of Steroid Depots

Another approach to localizing the control of gonadotropic function in the brain has been to implant crystalline steroids into various regions. Such steroids are slowly absorbed; thus they provide a means of localizing sites of negative feedback action.

The region most often identified with steroid sensitivity is the basal-tuberal portion of the hypothalamus, particularly the median eminence. Davidson and Sawyer (1961a) implanted estradiol benzoate into various regions

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of the brain in the female rabbit. Failure of copulation-induced ovulation, and eventual ovarian atrophy, followed implants into the basal tuberal region, especially the posterior median eminence. Negative results were reported for implants in the mammillary bodies, anterior pituitary, etc. These findings were extended by Kanematsu and Sawyer (1963d). Kanematsu and Sawyer (1963b, c) found that such brain implants caused cytological changes indicating decreased gonadotropic stimulation in the pituitaries of ovariectomized rabbits. Kanematsu and Sawyer (1964) found also that these implants caused decreased amounts of LH in the plasma of ovariectomized rabbits. Similarly, Ramirez et al. (1964) found that implants of estradiol in the median eminence prevented the rise in plasma LH after ovariectomy in rats.

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

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

An estrogen-sensitive structure in the anterior hypothalamus was reported by Flerko and Szentagothai (1957). Small fragments of ovarian tissue were implanted into the region of the paraventricular nuclei and caused decreased uterine size. Liver tissue was used as a control.

The question of steroid effects directly upon anterior pituitary gonadotropic function remains controversial. Lisk (1962) implanted testosterone into the pituitaries and brains of male and female rats and obtained gonadal atrophy only from implants in certain regions of the hypothalamus. Davidson and Sawyer (1961b), however, obtained some evidence of inhibition of gonadotropin secretion from effects of intrapituitary implants of testosterone propionate in male dogs.

Flerko and Szentagothai (1957) found that implantation of small fragments of ovarian tissue into the pituitary had no effect on gonadotropin secretion whereas similar

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implants in certain regions of the hypothalamus were highly effective. Davidson and Sawyer (1961a) and Kanematsu and Sawyer (1963b) reported similar results using estradiol benzoate implants.

These findings conflict sharply with those of Bogdanove (1963b) who found that ovarian or estradiol dipropionate implants were effective directly on the pituitary. Such implants produced localized regression of castration cells in ovariectomized female rats. In addition, Ramirez et al. (1964) found that estradiol implants in the anterior pituitaries of female rats prevented the rise in plasma LH after ovariectomy, and Rose and Nelson (1957) obtained inhibition of the castration response from estradiol injected into the hypophysial fossa. Kanematsu and Sawyer (1964), in contrast to some of their earlier work, reported an increase in plasma LH following estradiol benzoate implants in the anterior pituitaries of ovariectomized rabbits.

Evidence for a direct stimulatory action of estrogen on prolactin secretion by the anterior pituitary was presented by Kanematsu and Sawyer (1963d), by Ramirez and McCann (1964b), and by Flament-Durand (1965). Nicoll and Meites (1964) obtained <u>in vitro</u> evidence for a direct effect.

Attramadal (1964) reported localization of tritium labeled estradiol injected systemically into gonadectomized rats of both sexes. Estradiol was taken up most rapidly by pituitary, uterus, and vagina. In the pituitary, estradiol was found in the cytoplasm of the basophils. In the

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hypothalamus, estradiol was found in the nuclei of cells in the supraoptic and paraventricular nuclei. Eisenfeld and Axelrod (1966) reported that tritiated estradiol is selectively accumulated by the hypothalamus as well as by anterior pituitary, uterus, and vagina. These findings of selective affinities by both pituitary and hypothalamus for estradiol are considered evidence for direct action on both organs.

6. Neural Control of Ovulation

In the female rat, the immediate cause of ovulation appears to be a marked rise in plasma LH on the afternoon of proestrus, and it clearly is closely controlled by the central nervous system (Ramirez and McCann, 1964a), as are other phases of the ovarian cycle. The hypothalamus-pituitary system of the male rat, unlike the female, does not secrete gonadotropins in a cyclic fashion. Ovaries transplanted to castrated male rats develop large follicles, but these do not ovulate. Harris (1964) and Gorski and Wagner (1965) reviewed literature demonstrating that this difference exists in the hypothalamus, not in the pituitary. Several excellent and thorough reviews on the extensive literature concerned with the nervous control of ovulation have recently appeared; those of Sawyer (1959), Villee (1961), and Everett (1961a, 1964) are particularly distinguished.

Sawyer (1959) found that ovulation results from electrical stimulation of the hypothalamus, but not of the

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pituitary in the rabbit. In the rat, Barraclough and Gorski (1961) localized two regions concerned with stimulation of LH secretion by means of electrical stimulation experiments. An anterior (suprachiasmatic) region appeared to be concerned with the ovulatory burst of LH, and a posterior (arcuate-ventromedial) region appeared to be concerned with basal, estrogen-regulated secretion of LH.

Sawyer et al. (1947) and Markee et al. (1948) found that the neural mechanism involved in the ovulation response to copulation in the rabbit has adrenergic links. Evidence for cholinergic links also was presented by Sawyer et al. (1949, 1950, 1951).

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III. TISSUE CULTURE EXPERIMENTS ON FSH RELEASE

A. Materials and Methods

Preparation of Brain Extracts: Five month old rats of the Wistar strain (Wilson & Son, Acton, Indiana) which had been ovariectomized for approximately 6 weeks, were used as donors for hypothalamic tissue. Preliminary trials indicated that ovariectomy resulted in increased FSH-RF activity by the hypothalamus. Each hypothalamus, including pituitary stalk and median eminence, was removed rapidly and placed on dry ice. The hypothalamic tissue was homogenized in 0.1 N HCl (0.2 ml/hypothalamus) and centrifuged at 12,000 x g for 45 minutes at 4°C. The supernatant was neutralized with 1 N NaOH and incorporated directly into tissue culture medium 199 (Difco Laboratories, Detroit, Michigan) containing 10% calf serum, 25 U/ml penicillin, and 50 μgm/ml streptomycin. Medium containing similarly prepared extracts of rat cerebral cortical tissue served as a control in each experiment in which hypothalamic extracts were used. Each 3 ml of culture medium contained extract equivalent to 2 hypothalami (about 20 mg of brain tissue).

Culture Procedure: The procedure outlined by Nicoll and Meites (1963) was adapted for these experiments. Mature virgin female rats of the Wistar strain were used as donors

for cultured pituitary. The rats were stunned and decapitated, and the posterior pituitary was removed and discarded. The anterior pituitary was removed and cut into 8 explants, of approximately equal size, with a single-edge razor blade which had been soaked in ether and absolute alcohol.

The cultures were performed in 3.5 cm x 1 cm sterile disposable plastic Petri dishes (Falcon Plastics, Inc.), each containing 3 ml of culture medium 199. Medium 199 is the formulation of Morgan, Morton, and Parker (1950) and was purchased from Difco Laboratories, Detroit, Michigan. Stock solutions of antibiotics were sterilized by filtration through Millipore filters with pore size of 0.45 μ and were stored at -20°C. In each dish, 8 explants were placed upon a strip of washed (95% alcohol overnight, 100% alcohol overnight, then anhydrous ether overnight) lens paper, which was supported on a 1 cm x 2 cm x 0.4 cm platform of #46 grid stainless steel mesh (United Surgical Supply Co.). The cleaning procedure for the stainless steel platforms included soaking in 1/2 conc. HCl for 15 min., then immersing in distilled water, absolute alcohol and anhydrous ether, and then drying in air.

Explants from opposite halves of the same anterior pituitary were placed in a control and an experimental dish, so that approximately equal cell populations were obtained. The cultures were maintained in an air-tight Plexiglass chamber for 9 days at 36°C under continuous gassing with 95% O_2 - 5% CO_2 at a flow rate of about 200 ml/minute.

The gas was humidified by passing through a sintered glass filter which was immersed in a cylinder of distilled water.

At the end of the 3rd and 6th days of culture, the medium was removed and fresh medium was added. The 1st, 2nd, and 3rd 3-day medium samples were collected separately and stored at -20°C until assayed. Upon termination of culture the explants were weighed, fixed, and studied histologically. Aldehyde-fuchsin (Gomori, 1950, as modified by Elftman, 1959b) and periodic acid - Schiff (Elftman, 1959a) staining methods were used. Standard recommendations were followed in washing and sterilizing all glassware and instruments. Merchant et al. (1960) and Paul (1960) were used as references.

Assay Procedure: FSH activity was measured by the human chorionic gonadotropin (HCG) - augmentation method of Steelman and Pohley (1953). Weanling female rats (Holtzman Co., Madison, Wisconsin), 25 days old, were injected subcutaneously with the test materials to which had been added a total dose of 50 IU HCG (Nutritional Biochemicals Corporation, Cleveland, Ohio). The animals received 3 injections per day for 3 days. The material for each group of animals was divided into 3rds and frozen until day of use. Injection solutions were kept at 4°C between injections. Approximately 72 hours after the 1st injection, the ovaries were removed and weighed. All ovaries (each pair were weighed together) were recorded as mg/100 gm body weight.

The standard error of the mean was calculated for each combined group using the following equation:

$$S_{\bar{x}} = \sqrt{\frac{N\ (\Sigma\ X^2) - (\Sigma\ X)^2}{N^2\ (N-1)}}$$

where N is the number of observations (X).

Students "t" test was used to determine the significance of the differences between groups as described by Snedecor (1956).

B. Results on Release of FSH *In Vitro*

Twenty male rat pituitaries were cultured for 9 days. Medium was collected each 3 days. At the end of the 9-day period, half of the explants were fixed in Bouin's solution and studied histologically; the remainder were frozen at -20°C for assay. Survival was good.

The level of FSH activity released in each 3-days of culture is indicated by the results presented in Figure 1. Each assay rat received tissue culture medium from the equivalent of 0.4 cultured male rat pituitaries. There were 6 assay animals per group. Data are presented as bar graphs; standard errors of the mean for each group are indicated also. Animals receiving HCG only had ovaries weighing 94 $\pm$ 6 mg/100 gm body weight.

Significant FSH activity was present only in the culture medium from the first 3 days of culture. FSH activity was not detected in medium from the 2nd or 3rd 3-day period or in the explants at the end of 9 days.

Fall Release -- Mean Ovarian Weights (mg per 100 gm body weight)

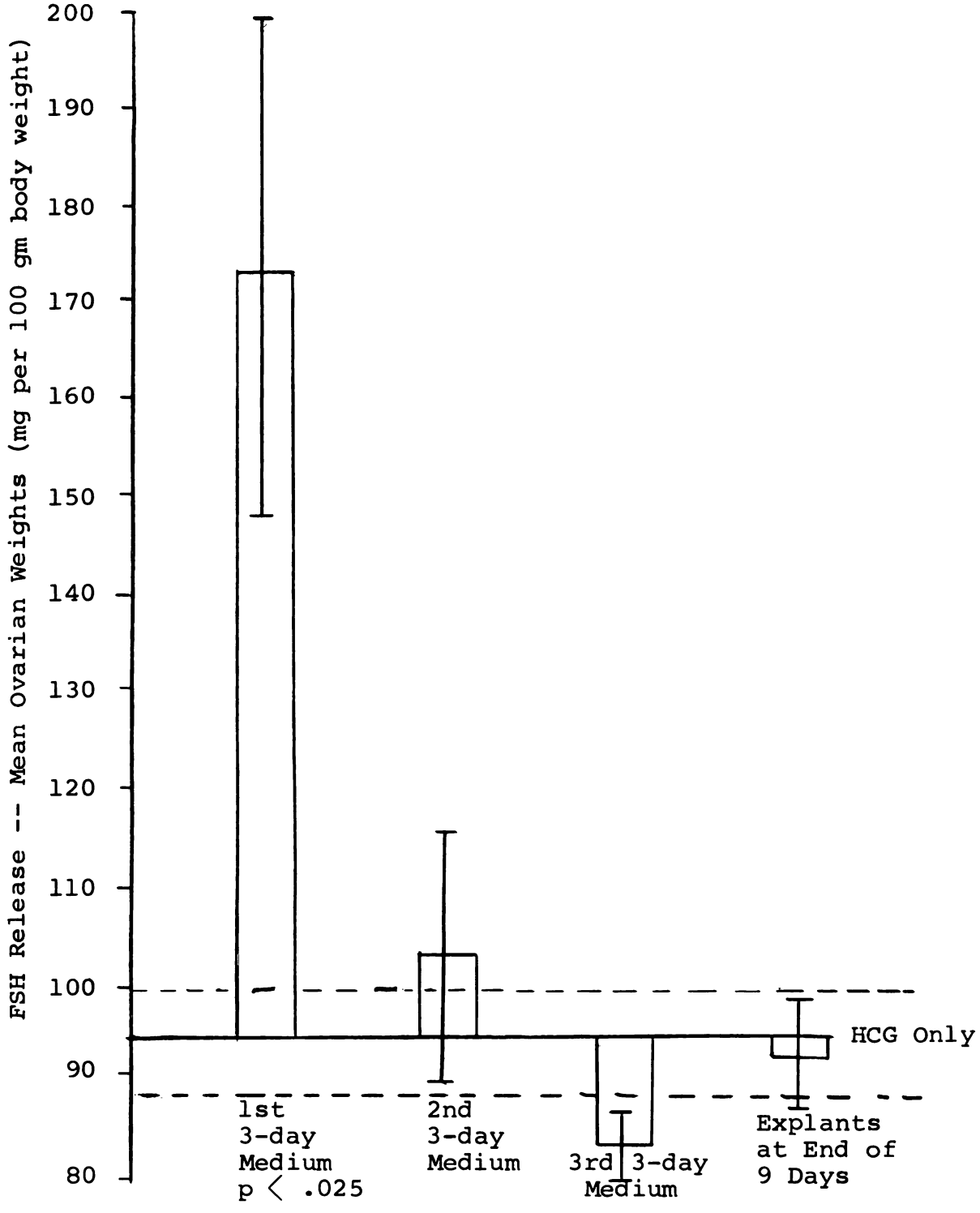

Fig. 1. Release of FSH by Pituitary Tissue *in vitro* in Relation to Time. Lines on bars refer to standard error of the means, as do dotted lines.

These results indicate that pituitary tissue releases FSH *in vitro*, but in the absence of stimulation it does not synthesize or release appreciable quantities of FSH for longer than 3 days.

Dose-response curves of tissue culture medium and NIH-FSH-S1 are shown in Figure 2. Pooled tissue culture medium samples, representing tissue cultured for 9 days, were administered at 2 dose levels (0.2 or 0.4 pituitary equivalents per assay rat) to groups of 6 assay animals. Purified sheep FSH was similarly administered at dose levels of 75 μgm or 150 μgm. The curves are plotted with a common intercept (threshold dose).

The virtually identical dose-response curves obtained indicate that living rat pituitary tissue releases *in vitro* a substance which is biologically identical with the highly purified FSH extracted from sheep pituitaries collected at slaughterhouses, and that, therefore, it is proper to make quantitative comparisons between the two.

C. Stimulation of FSH Release by Hypothalamic Extract *In Vitro*

1. *Effects of Hypothalamic and Cerebral Cortical Extracts on FSH Release*

In this experiment, brain extracts were added to the media only during the last 6 days of a 9-day culture. The medium from each group of 4 culture dishes, representing the hormone released by the equivalent of 4 anterior pituitaries,

Mean Ovarian Weights (mg per 100 gm body weight)

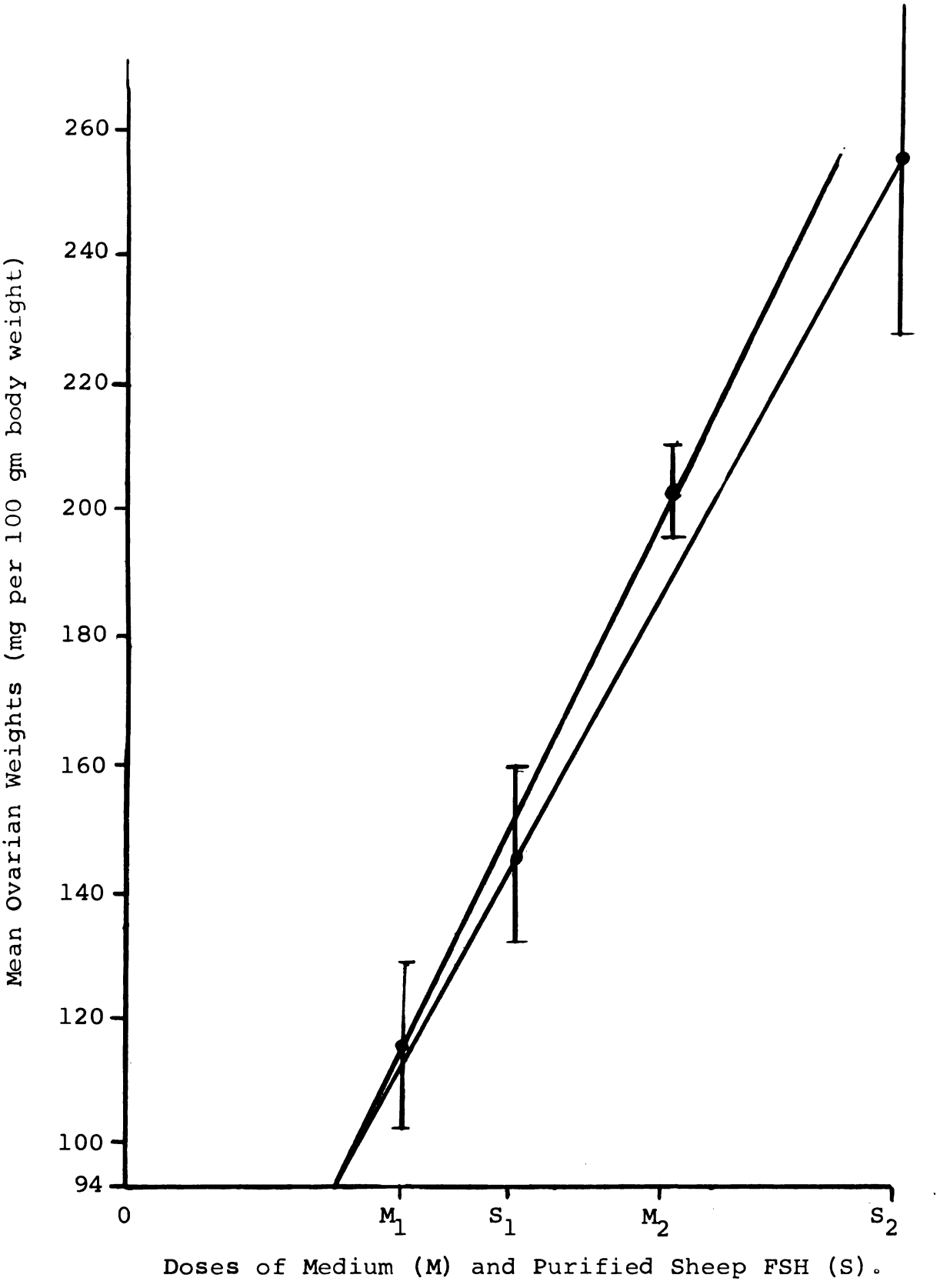

Fig. 2. Dose-Response Curves: Tissue Culture Medium FSH and NIH-FSH-S1.

was injected into 2 assay animals after a portion (1/16th) was removed for assay of luteinizing hormone. A total of 9 paired groups of assay animals was used; hence there were 18 rats given medium from pituitary explants cultured with hypothalamic extract and 18 rats given medium from pituitary cultured with cerebral cortical extract. A total of 72 dishes were used.

The data for the 9 paired groups are presented in Table 1. In every group the medium from pituitary exposed to hypothalamic extract produced a greater ovarian response than the medium from tissue exposed to cerebral cortical extract. Twenty assay rats treated with the augmentation dose of 50 IU HCG alone had ovarian weights of 100 $\pm$ 5 mg/100 g body weight, while 9 animals treated with 50 IU HCG and neutralized acid extract of hypothalamus (7.5 hypothalami per assay rat) had ovarian weights of 93 $\pm$ 7 mg/100 g body weight. Thus, the mean responses to the augmentation dose alone, to hypothalamic extract, and to the medium from explants cultured with cortical extract were virtually identical. This indicates that no detectible amount of FSH was present in the control medium during the last 6 days of culture. Only the culture medium from explants cultured with hypothalamic extract contained significant FSH activity ($p < .001$).

The tissue culture medium from the first 3 days of culture was assayed subsequently. The anterior pituitary explants were not cultured with either hypothalamic or

Table 1. Effects of acid extracts of rat cerebral cortex and hypothalamus on FSH release by rat pituitary *in vitro*.

	Mean Ovarian Weights of Assay Rats (mg/100 g body wt.)	
Group	Medium from anterior pituitary culture with cortical extract*	Medium from anterior pituitary culture with hypothalamic extract*
1	76	166
2	78	125
3	89	94
4	94	125
5	89	150
6	130	142
7	122	145
8	98	225
9	114	173
	Average 99 ± 7†	Average 149 ± 10†

*Medium from 4 anterior pituitary equivalents injected into 2 assay rats.

†Standard errors were calculated on the basis of 18 assay rats. Cortical vs. Hypothalamic Groups: $t = 4.1$ $p < .001$.

cerebral cortical extracts during this period. Medium equivalent to 1.87 cultured anterior pituitaries was injected into each assay rat. Medium from the control and experimental culture dishes produced average ovarian weights of 130 ± 11 and 149 ± 8 mg/100 g body weight, respectively. This indicates that significant amounts of FSH were released by the anterior pituitary explants during the first 3 days

of culture, and these amounts did not differ significantly between the control and experimental culture dishes. These ovarian weights cannot be strictly compared with those of the assay rats given medium from the last 6 days of culture, since there is some variation in response among different shipments of assay rats.

The pituitary explants at the end of 9 days of culture consisted of a wide rim of viable tissue and a necrotic core. Acidophils and a small number of periodic acid-Schiff positive cells were clearly visible. Photomicrographs are presented in Figures 3 and 4 of sections of fresh tissue and 9-day explants, respectively, stained with aldehyde-fuchsin. No obvious differences were seen in the explants cultured with hypothalamic extract as compared to those cultured with cerebral cortical extract.

According to Purves and Griesbach (1955), there are two types of gonadotropic cells in the anterior pituitary. These are termed central gonadotrophs which produce luteinizing hormone and peripheral gonadotrophs which are scattered close to the surface of the pituitary and produce FSH. If so, there was virtually complete survival and healthy appearance of FSH-secreting cells in the tissue culture and incubation systems described in this thesis.

2. Effects of Incubating a Purified FSH Preparation With and Without Brain Extracts on FSH Activity

This experiment was performed to determine whether or not

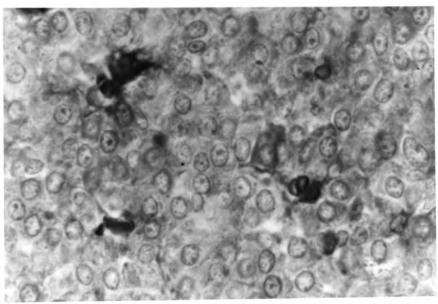

Fig. 3. Photomicrograph of Section of Uncultured Female Rat Pars Anterior Tissue. Aldehyde-Fuchsin X630.

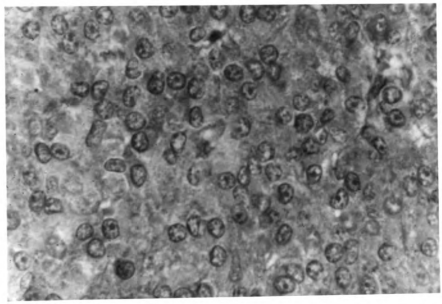

Fig. 4. Photomicrograph of Section of Female Rat Pars Anterior Tissue Cultured for 9 Days. Aldehyde-Fuchsin X630.

FSH activity could be increased or decreased as a result of incubation alone or incubation with brain extracts. Ovine FSH (NIH-S1) was incubated alone or with neutralized acid extracts of rat hypothalamus or cerebral cortex for 3 days in culture medium and injected into assay animals. The dose in each case was 150 μg, and all substances were incorporated in culture medium 199. The results are presented in Table 2. Eight animals (group 4) received FSH which had been incubated with cerebral cortical extract from ovariectomized rats in doses equivalent to 75 mg of fresh tissue per assay rat. The rats in group 5 each received FSH which had been incubated with extract equivalent to 75 mg of fresh hypothalamus from ovariectomized rats. The animals in group 3 received FSH which had been incubated alone. Eight animals (group 2) received non-incubated FSH. These results indicate that cerebral cortical and hypothalamic extracts had no significant effect on FSH activity in vitro.

3. Effects of Injections of Hypothalamic And Cerebral Cortical Extracts on Response of Assay Animals to FSH

Each of the 6 assay animals received 150 μg of ovine FSH mixed with a neutralized acid extract of 75 mg of rat cerebral cortical tissue. Six other rats received FSH together with extract equivalent to 7.5 rat hypothalami (about 75 mg of brain tissue). Six animals received FSH alone. The results are presented in Table 3. Neither

Table 2. Effects of incubating FSH with and without brain extracts.

Group and Treatment	No. of Assay Rats	Mean Ovarian Wt. (mg/100 g body wt.)*
1. Untreated Assay Rats	10	24 ± 1
2. HCG only	10	87 ± 4
3. Non-incubated FSH	8	186 ± 14
4. Incubated FSH	6	171 ± 18
5. FSH incubated with cerebral cortical extract	8	150 ± 6
6. FSH incubated with hypothalamic extract	8	156 ± 6

*Mean and standard errors.

Table 3. Effects of injections of hypothalamic and cerebral cortical extracts on response of assay animals to FSH. (50 IU HCG given to all groups.)

Group and Treatment	No. of Assay Rats	Mean Ovarian Wt. (mg/100 g body wt.)*
1. HCG only	5	91 ± 9
2. FSH	6	215 ± 28
3. FSH and Hypothalamic extract	6	188 ± 13
4. FSH and Cerebral cortical extract	6	184 ± 10

*Mean and standard errors.

hypothalamic nor cerebral cortical extracts influenced the ovarian response of the assay rats to FSH.

4. Discussion

These results demonstrate the existence of a factor(s) in crude acid extract of hypothalamus from ovariectomized rats which acts directly on the anterior pituitary to stimulate release of FSH. Stimulation cannot be attributed to any effects of hypothalamic extracts directly on FSH in the medium, since such extracts did not significantly activate or inactivate FSH *in vitro*. The hypothalamic extract also did not significantly influence the response of the assay animals to FSH injections. The first 3 days of culture medium from the control and experimental groups were assayed and found not to differ significantly in the amounts of FSH released, thus confirming the equivalence of the control and experimental pituitary explants. The presence of FSH activity in the control medium from the first 3 days of pituitary culture and its absence, under the conditions of our assay, in the medium from the last 6 days culture, indicate that FSH is released in detectible amounts only for a few days in the absence of hypothalamic stimulation. Incorporation of a hypothalamic extract into the culture medium at the end of the third and sixth days of culture apparently can maintain FSH release by the pituitary explants.

Our in vitro results appear to be in agreement with the report of Igarashi and McCann (1964b), in which rat hypothalamic extracts were used to stimulate FSH release in vivo. The specificity of their FSH assay method in the mouse requires further study and evaluation, whereas the Steelman-Pohley assay method has been shown to be highly specific for FSH. Our results are also in apparent agreement with an independent study by Kuroshima et al. (1965) in which stimulation of FSH release was obtained by incubating sheep and beef hypothalamic extracts with rat pituitaries. Igarashi et al. (1964b) have reported partial purification of the FSH-releasing factor and obtained evidence that it is a small polypeptide different from vasopressin and corticotropin-releasing factor. Kobayashi et al. (1963a) found that cerebral cortical extracts, purified vasopressin and oxytocin had no effects on gonadotropin release in vitro. Igarashi and McCann (1964b) and Kuroshima et al. (1965) also reported that cerebral cortical extract had no effect on FSH-release. Lack of FSH activity in acid extracts of hypothalamus was reported also by Igarashi and McCann (1964b), Kuroshima et al. (1965) and Courrier et al. (1963).

The question has been asked whether these results represent stimulation of release or release secondary to stimulation of synthesis or both. Since it was decided to do a thorough histological study on the explants in expectation of finding cytological changes caused by hypothalamic

factors and since this work was handicapped by a relatively insensitive assay, there was insufficient explant tissue left over to bioassay and therefore to prove a net increase in activity in the system. Such results would not be conclusive, however; no one has yet determined whether or not intracellular FSH has the same specific activity as FSH that has passed through the cell membrane. The possibility remains, therefore, that an activation process could take place without protein synthesis. Kobayashi et al. (1965) have demonstrated that hypothalamic extract stimulates uptake of tritiated leucine by pituitary basophil cells *in vitro*; therefore, it appears that protein synthesis is being stimulated.

D. Effects of Steroid Hormones on FSH Release *In Vitro*

In the first five of the following experiments, a total of 174 pituitaries from ovariectomized rats were cultured for 6 days in medium 199 without calf serum. Each culture dish contained explants derived from 4 pituitary halves. Half of each pituitary was cultured in plain medium; the opposite half was exposed to steroid(s). The experimental medium in experiment 2 contained estradiol (0.25 μgm/ml). The experimental medium in experiment 3 contained progesterone (1.0 μgm/ml). Experimental medium number 4 contained the above doses of estradiol and progesterone in combination. Experimental medium number 5

contained testosterone propionate (1.0 μgm/ml). Each assay rat received medium from the equivalent of 1/2 ovariectomized rat pituitary cultured for 6 days.

In the last experiment, 24 intact female rat pituitaries were cultured for 6 days in 199 medium without calf serum. Explants from 2 pituitary halves were in each dish. During the first 3 days, the pituitaries were cultured in plain medium. During the last 3 days, the control medium contained hypothalamic extract representing 3 adult male rats per pituitary equivalent. The experimental group received hypothalamic extract and estradiol (0.25 μgm/ml).

Culture media and injection solutions in each experiment were kept in independent groups and injections were arranged so that data on independent groups of 3 control assay rats, with a matching group of 3 experimental assay rats were obtained. Combined data are presented in Table 4.

The steroids used came from stock solutions prepared in absolute ethanol. The progesterone (Smith, Kline, and French, Philadelphia, Pa.) and testosterone propionate (Nutritional Biochemicals Corp., Cleveland, Ohio) were each prepared in solutions to contain 400 μgm/ml. Those of estradiol (American Steroids Co., Hato Rey, Puerto Rico) were made up to contain 100 μgm/ml. Control amounts of ethanol were added to control medium.

Results: Data obtained in cultures with steroids are presented in Table 4. Estradiol (No. 2), progesterone (No. 3), estradiol and progesterone in combination (No. 4)

Table 4. Effects of steroids added directly to the medium on FSH release *in vitro*.

Expt. No.	Treatment of assay rats	No. of Paired Groups	Total No. of Assay Rats	Mean Ovarian Weight Increase*	
				Control	Exptl.
1.	Fresh tissue homogenate (1/2 pit. per assay rat)	--	8	214 ± 15	--
2.	Control: Medium from pituitary tissue cultured with plain medium Exptl.: Medium from pituitary tissue cultured with estradiol (0.25 μgm/ml)	7	42	178 ± 9	173 ± 17
3.	Control: as above Exptl.: Medium from pituitary tissue cultured with progesterone (1.0 μgm/ml)	6	36	148 ± 9	143 ± 9
4.	Control: as above Exptl.: Medium from pituitary tissue cultured with estradiol and progesterone	4	24	203 ± 13	200 ± 19
5.	Control: as above Exptl.: Medium from pituitary tissue cultured with testosterone propionate (1.0 μgm/ml)	6	36	175 ± 9	225 ± 15
6.	Control: Medium from pituitary tissue cultured with hypothalamic extract Exptl.: Medium from pituitary tissue cultured with hypothalamic extract and estradiol (0.25 μgm/ml)	2	12	39 ± 1	37 ± 2

*Data are presented as mean ovarian weight increase over the group receiving HCG only. (Means and standard errors.)

and testosterone propionate (No. 5) failed to inhibit FSH release from ovariectomized rat pituitary tissue *in vitro*. Ovarian weights from a group (No. 1) of assay rats receiving injections of a fresh tissue homogenate are included also.

Explants were stained with aldehyde-fuchsin and studied histologically. Survival was excellent. No obvious differences between control and experimental pituitary halves were obtained with any treatment. Signet cells were maintained 6 days *in vitro*.

In an experiment with somewhat different design (No. 6), estradiol failed to antagonize the stimulatory effect of hypothalamic extract on intact rat pituitary tissue. In Section IVD of this thesis, experiments were done in which estradiol and testosterone failed to antagonize hypothalamic extract during a 6-hour incubation period.

Discussion: The doses of steroids added to the media were rather high. Actual content of steroid in the medium was not measured. Uptake of steroids by pituitary tissue (see Attramadal, 1964, and Eisenfeld and Axelrod, 1966), binding to proteins in solution, binding to plastic in the Petri dishes, etc., probably caused substantial reduction in the actual amounts present.

The maximum possible total doses of steroids which the assay animals could have received were 1.5 μgm of testosterone propionate, 1.5 μgm of progesterone, 0.375 μgm estradiol, or the combination of the last two. These levels are generally considered negligible.

These results seem to corroborate the findings of Flerko and Szentagothai (1957) Davidson and Sawyer (1961c) Lisk (1962) and Kanematsu and Sawyer (1963b) whose results indicate that steroid hormones do not exert a direct negative feedback effect on the pituitary. However, these results seem incompatible with the findings of Rose and Nelson (1957), Davidson and Sawyer (1961b) and Bogdanove (1963b), who found evidence for a direct negative feedback effect.

The possibility remains, however, that changes in experimental design might produce different results. For example, data indicating whether or not a long preculture with steroids would inhibit the effect of later addition of hypothalamic extract were not obtained, but it is noteworthy that Igarashi and McCann (1964b) and Kuroshima et al. (1965) found that a single injection of large doses of estrogen and progesterone in vivo actually seemed to sensitize the pituitary to FSH-RF three days later. In addition, unpublished work from two laboratories indicates that testosterone treatment sensitizes the pituitaries of castrated male rats to FSH-RF.

A number of findings, which can be interpreted as indicating a direct action of steroid(s) on FSH release, require comment. Hoogstra and Paesi (1957) reported that testosterone treatment causes increased pituitary FSH content in intact and castrated male and female rats in doses which are known to cause decreased release. Parlow (1964c) reported that testosterone treatment (1 mg/day)

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caused increased pituitary FSH and decreased serum FSH in castrated male rats. Bogdanove (manuscript in preparation) and Beyler and Potts (1962) obtained similar results. On the other hand, Paesi et al. (1955) found that estradiol treatment causes decreased pituitary FSH content in intact and castrated male and ovariectomized female rats, and Parlow (1964c) found that estradiol treatment lowered FSH activity in both pituitary and serum. Van Rees (1961) found that treatment with testosterone *in vivo* caused increased pituitary FSH content but decreased release *in vitro* during a 2-hour incubation period whereas estrogen treatment caused decreased pituitary FSH content but actually seemed to increase the rate of release *in vitro*. It is noteworthy that these workers all injected steroids for 2 or more weeks.

The above findings can be reconciled with the concept that there is no steroid feedback on pituitary FSH release if we assume that FSH-releasing factor(s) stimulates both synthesis and release of FSH, and therefore can produce two dose-response curves. Testosterone is known to be less effective than estradiol as an inhibitor of FSH secretion (see Miyake, 1961) and may not completely inhibit it. In the work quoted above, testosterone may be presumed to reduce FSH-RF secretion until FSH release is almost completely suppressed while FSH synthesis occurs to a small degree; therefore, pituitary content rises. The findings of Kobayashi et al. (1963a, 1964, 1965) in which hypothalamic

extract caused increased gonadotropic activity in cultured cells, and the tissue culture findings in this thesis support the idea of stimulation of FSH synthesis. Evidence for a distinct stimulation of release arises from the depletion of pituitary FSH content observed after intracarotid injection of hypothalamic extract by Dávid et al. (1965) and Schally et al. (manuscript in preparation).

The possibility of direct feedback on the pituitary cannot be excluded on the basis of evidence presently available. More work is needed.

IV. INCUBATION EXPERIMENTS ON FSH RELEASE

A. Materials and Methods

Experimental Animals: Rats of the Sprague-Dawley strain (Spartan Research, Haslett, Michigan) were used in all experiments. Donors of hypothalami and incubated pituitaries were sexually mature male rats weighing 250-350 grams. Unless otherwise noted, female rats, 22 days old, were used for the FSH bioassays. The diet consisted of Wayne Lab-Blox (Allied Mills, Inc., Chicago, Illinois). Assay rats received bread and milk as a supplement. The animals were maintained in a temperature controlled ($75 \pm 1^{\circ}F$) and artificially illuminated room.

Preparation of Acid Extracts: Animals were killed by guillotine. The hypothalami (including pituitary stalk and median eminence) were removed rapidly and placed in ice-cold 0.1 N. HCl (0.2 ml per hypothalamus). Hypothalami and acid were later homogenized and centrifuged at 12,000 x g for 40 minutes at $4^{\circ}C$. Supernatents (acid extracts) were stored at $-20^{\circ}C$.

Incubations: A short term incubation method derived from the method of Saffran and Schally (1955) was used. Rats were killed by decapitation and the heads were rapidly opened. Pituitaries were placed on filter paper soaked in

incubation medium and dissected. Anterior pituitary tissue was separated from posterior pituitary, sliced longitudinally and placed into 25 ml flasks, each containing 2 ml of medium 199 (Difco Laboratories, Detroit, Michigan). Opposite halves from each pituitary were placed in flasks for different experimental groups. Each flask contained 8 pituitary halves. Incubations were carried out in a Dubnoff metabolic shaker (60 cycles per minute) under constant gassing with 95% O_2 - 5% CO_2 at 37°C.

Thirty minutes after the last pituitary was dissected out, the medium in the flasks was replaced with fresh medium. Next, the acid extracts were neutralized to approximately pH 7.0 with 1. molar NaOH and added to the flasks as rapidly as possible. Six hours later, the incubation was terminated; the incubated pituitary tissue was weighed, and the media were frozen for storage.

Assay: FSH activity was measured by the method of Steelman and Pohley (1953) as modified by Parlow and Reichert (1963). The specificity and reliability of this method seemed well established (Parlow, 1964b). Weanling female assay animals received test materials in a total volume of 3.0 ml containing 50 IU of Human Chorionic Gonadotropin (Nutritional Biochemicals Corporation, Cleveland, Ohio). Animals received injections of 0.5 ml twice daily, 8 to 10 hours apart, for 3 days. All treatments were administered in medium 199, and solutions were kept at 4°C between injections. The animals were ovariectomized or killed on

the fourth day and the ovaries were freed from adherent fat and connective tissue and weighed on a torsion balance.

Most of the materials were tested at two dose levels since it was realized that demonstration of a dose-response curve with each preparation provides strong evidence for the validity of the results. Each assay rat received medium from the equivalent of $\frac{4}{3}$ or $\frac{8}{3}$ pituitaries. Purified ovine FSH (NIH-FSH-S2) at two dose levels (100 μgm or 200 μgm) was used as a standard preparation. These doses represent the lower portion of the dose-response curve. Assay animals were randomly distributed among experimental groups. Neutralized acidic extracts of cerebral cortical and hypothalamic tissue have been shown to be without significant effect on the assay when administered subcutaneously (Kuroshima et al., 1965; Courrier et al., 1963; also see Sections IIIC and IVD of this thesis).

All data obtained were plotted on graph paper and regression lines were plotted for preliminary analysis. The standard errors of the mean were calculated for each point and t-tests were carried out. Estimation of relative potency by graphical means and of statistical significance by t-tests gave good agreement with potencies and confidence limits computed by the following method of statistical analysis derived from Bliss (1956).

When, as in the Steelman-Pohley assay, the response y can be plotted as a straight line against arithmetic dosage units, and when the plotted lines for the standard

(subscript 1) and for each of the m-1 unknowns (subscripts 2 to m) meet at zero dose within the sampling error, the relative potency is given by the ratio of their slopes. The doses of each preparation are spaced at equal arithmetic intervals and coded: x = 0, 1, or 2. The slopes are computed with a common intercept a' at x = 0, so that $Y_1 = a' + b_1 x_1$, $Y_2 = a' + b_2 x_2 \ldots Y_m = a' + b_m x_m$. In coded units the relative potency P'*, for unknown 2, for example is the ratio:

$$P'* = \frac{b_2}{b_1}$$

To recover the potency P* in original units requires only simple arithmetic.

When the assay is fully balanced with the same number of assay animals at each of 2 dose levels, and when a zero dose is not included the common intercept is determined as:

$$a' = \frac{5 \Sigma y - 3 T'_b}{f m}$$

Where the total number of doses is 2m, each treatment has f replicate responses, y, which total T_t, and $T'_b = \Sigma (x T_t)$. The slope for each preparation is then computed as

$$b_i = \frac{1}{5} \left\{ \frac{T'_b}{f} - 3 a' \right\}$$

The error variance about the m lines is determined as

$$s^2 = \frac{\Sigma y^2 - a' \Sigma y - \Sigma(b_i T'_b)}{n}$$

where the number of degrees of freedom n = 2fm - m - 1.

The index of precision, λ, is computed as

$$\lambda = \frac{ms}{\Sigma b_i}$$

The 95% confidence limits of P'* are next computed as

$$X_{p'*} = CP'_{*i} - K \pm \sqrt{(C-1)\ (CP'^{2}_{*i} + 1) + K\ (K - 2\ CP'_{*i})}$$

where $K = (C - 1)\ (\frac{9}{9 + m})$

$$C = \frac{b_i^2}{b_i^2 - c_{11}\ s^2\ t^2}$$

$$c_{11} = \frac{1}{5f} + \frac{9}{5fm}$$

t = tabular value of student's t at p = 0.05 for n degrees of freedom.

B. Assay of FSH-RF Activity by 6-Hour Incubation Technique

In these experiments FSH-release was studied in a 6-hour incubation system. Attempts were made to quantitate the amount of FSH released by tissue in contact with hypothalamic extract or cerebral cortical extract. A study was done also on response of anterior pituitary tissue to different doses of hypothalamic extract.

1. Stimulation of FSH Release by Male Rat Hypothalamic Extracts

The results of three similar experiments are shown in Table 5. Each pituitary equivalent was incubated in the presence of hypothalamic extract equivalent to 2.0 adult male rat hypothalami. Similarly prepared cerebral cortical extracts from equivalent amounts of tissue were used as osmotic controls since previous work had indicated that these had no effect on FSH release.

In experiments 1, 2, and 3, 40, 24, and 32 pituitary halves respectively released readily measurable amounts of FSH during incubation. Their opposite halves, in the presence of hypothalamic extract, released significantly greater amounts of hormone.

2. Effect of Graded Doses of Hypothalamic Extract on FSH Release by Male Rat Pituitary In Vitro

The effect of varying doses of hypothalamic extract were evaluated in two separate experiments whose combined data are shown in Figure 5. In these experiments, each assay animal received medium from the equivalent of 2 adult male rat anterior pituitaries. Doses of hypothalamic extract, drawn from pooled groups of 24 animals, were 0, 1/32, 1/16, 1/8, 1/4 and 1/2 of a hypothalamus per incubated pituitary equivalent. The data are presented as the mean ovarian weights. Hypothalamus donors, pituitary donors,

Table 5. Effects of acid extracts of rat cerebral cortex and hypothalamus on FSH release by adult male rat pituitary *in vitro*.

Experiment Number	Number of Assay Animals per Group per Dose Level	Treatment	FSH Release as μgm/NIH-FSH-S2 per mg Pituitary Tissue per Hour*	Relative Release: Cortex vs. Hypothalamus*	λ
1	5	Cortical Extract	3.13 (2.57-4.08)	2.12 (1.90-2.49)	.278
		Hypothalamic Extract	6.62 (5.82-7.82)		
2	3	Cortical Extract	2.21 (1.67-3.59)	2.66 (2.34-3.10)	.395
		Hypothalamic Extract	5.87 (5.09-7.00)		
3	4	Cortical Extract	0.72 (0.25-1.06)	4.24 (3.48-5.62)	.260
		Hypothalamic Extract	3.16 (2.42-3.89)		

*Mean and 95% Confidence Limits.

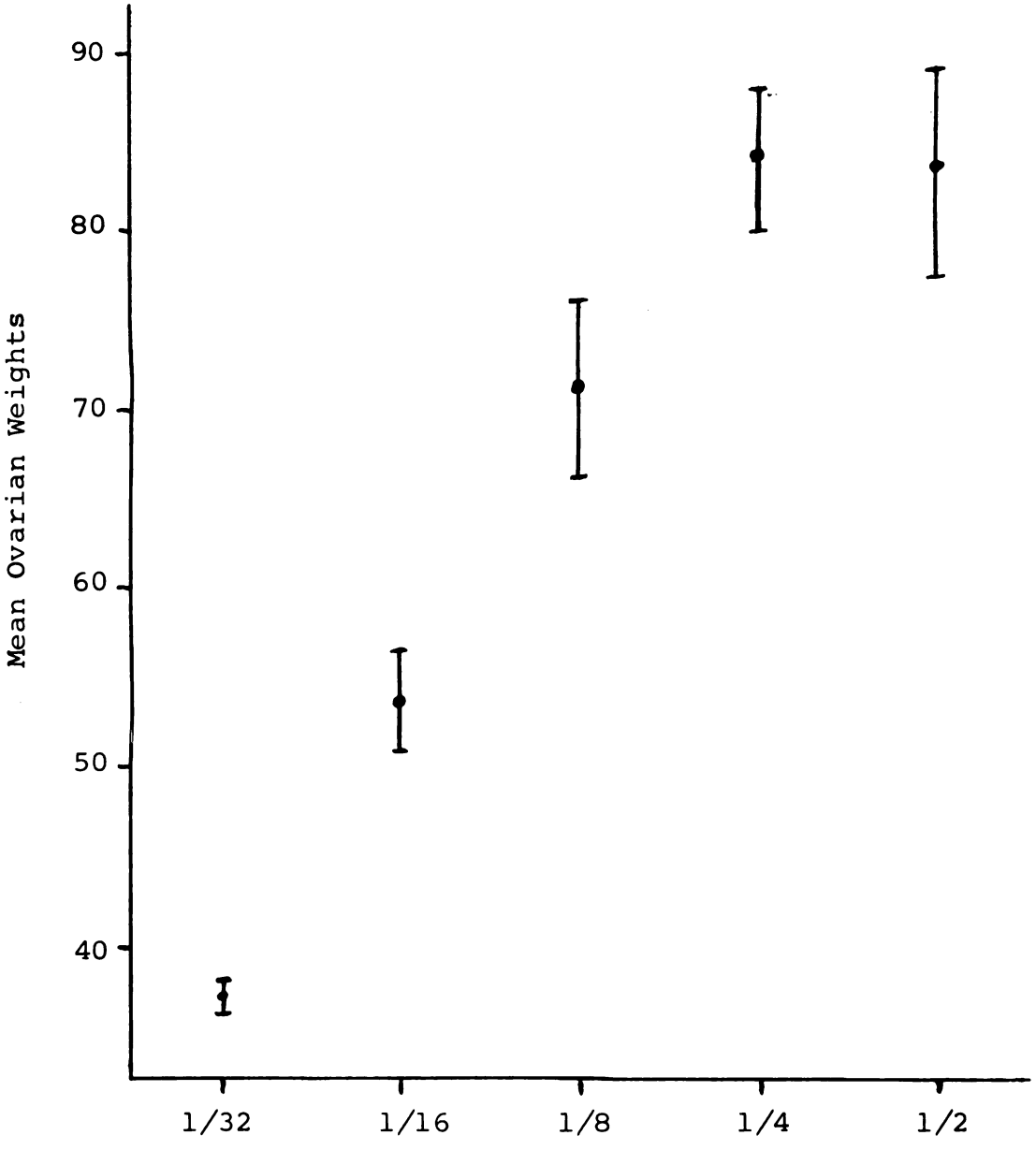

Fig. 5. Effects of Graded Doses of Hypothalamic Extract on FSH Release by Pituitary Tissue *in vitro*.

and assay animals were carefully chosen for uniform body weight. Vertical bars refer to standard errors of the mean, and the student's "t" test was used to compare groups.

The dose of 1/32 hypothalamus per incubated pituitary produced no significant stimulation of FSH release. With 6 assay animals per group, the dose of 1/8 hypothalamus produced a significant ($p < .001$) increase in ovarian weight when compared with the dose of 1/32. With 4 assay rats per group, the dose of 1/16 hypothalamus produced a significant ($p < .005$) stimulation when compared with 1/32; and the dose of 1/4 produced a significantly ($p < .001$) greater response than the dose of 1/16. Doses of 1/2 and 2 hypothalami per incubated pituitary were used also and were not associated with further increases in ovarian weights.

Discussion: Previous work on FSH-RF used the long-term ovariectomized female rat pretreated with 50 μgm estradiol benzoate and 25 mg progesterone 3 days before use *in vivo* (Igarashi and McCann, 1964b; Igarashi et al. 1964a; Kuroshima et al. 1965) or *in vitro* (Kuroshima et al., 1965) systems. Also used previously were ovariectomized rats with median eminence lesions (Igarashi and McCann, 1964b) and long term tissue cultures of pituitary tissue from normal female rats (Kobayashi et al., 1963a, 1965). The present study indicates that male rat pituitaries provide a satisfactory means of demonstrating FSH-RF activity. The graded response observed in the second part of this work indicates a potential usefulness of this method in quantitatively assaying FSH-RF.

In the 3rd experiment in Table 5, pituitaries from old rats (body weights, 400-450 grams) with atrophic testes released significantly less FSH in incubation than did those from younger rats (experiments 1 and 2). The relative release, however, was significantly greater in the old rats. The low absolute release perhaps reflects the influence of low endogenous FSH-RF activity before the pituitaries were removed. The high relative release indicates that the pituitaries were still highly responsive. These observations are consistent with the idea that reproductive failure with age in the male rat is the result of hypothalamic rather than pituitary deterioration.

C. Effects of Castration and Androgen Treatment On Hypothalamic FSH-RF Content

Data on the effects of testosterone injections and castration on FSH-RF activity in rat hypothalamus are tabulated in Table 6. Data are presented as the ratios of FSH activity in the control and experimental media.

In the first two experiments, hypothalami from groups of animals which had been injected with 2 mg of testosterone propionate (Nutritional Biochemicals Corporation, Cleveland, Ohio) in corn oil each day for two weeks were compared with those receiving control oil injections. Each incubated pituitary equivalent was incubated with the equivalent of 0.2 hypothalami from adult male rats weighing 275-300 grams. Pituitary tissue incubated with extracts from the steroid treated rats released significantly less FSH than did the controls.

Table 6. Effects of treating hypothalamus donors on FSH-RF activity.

Treatment	Experiment Number	Hypothalamic equivalents per Incubated Pituitary	Number of Assay Animals per Group per Dose Level	Relative Potency: Media with Experimental Hypothalamic Ext./Media with Control Extract*	λ
Testosterone Propionate (2 mg per day for 2 weeks)	1	0.2 ♂	3	0.52 (0.00-0.82)	.235
	2	0.2 ♂	5	0.71 (0.49-0.89)	.231
Castration (2 weeks)	3	0.05 ♂	4	1.29 (1.07-1.57)	.224
	4	0.05 ♂	4	1.47 (1.22-1.90)	.324
Ovariectomy (4 weeks)	5	2.0 ♀	2	1.58 (1.17-2.89)	.339

*Mean and 95% Confidence Limits.

In the second two experiments, pituitary tissue incubated with hypothalamic extracts from male rats which had been castrated for two weeks released significantly more FSH than tissue incubated with control extracts. Each pituitary equivalent was incubated with the equivalent of 0.05 hypothalami.

One experiment (No. 5) is shown comparing hypothalami from ovariectomized rats with those from intact female rats. Gonadectomy appears to cause increased hypothalamic FSH-RF content in the female also. 2.0 hypothalami per incubated pituitary were used, a dose which causes maximal stimulation when male hypothalami are used. This experiment suggests, therefore, that intact female hypothalami contain much less FSH-RF than intact male hypothalami. This result may not be reproductible, however. FSH-RF content seemed to vary widely among different shipments of female rats but not of male rats. With female hypothalami the ovarian weights frequently indicated that there was no significant FSH-RF activity in control or experimental groups, or that both caused maximal stimulation.

Discussion: The finding that a steroid (testosterone propionate) can cause reduced FSH-RF content in the hypothalamus of the male rat is consistent with the observation of Dávid et al. (1965) that estradiol treatment causes decreased hypothalamic FSH-RF content in the hypothalamus of the ovariectomized female rat.

A disturbing point arises however, since it is known that testosterone not only participates in negative feedback relationships with the hypothalamus-pituitary axis but also has gonadotropic properties. Greep (1961) reviews the extensive literature on this topic. Although FSH plays an essential role in the growth of spermatogenic tissue (see Greep and Fevold, 1937; Simpson, Li, and Evans, 1951; and Woods and Simpson, 1961), Albert (1961) reviews work in which it was demonstrated that testosterone alone can maintain testicular weight. More recently, Nelson and Merckel (1938) and Randolph et al. (1959) found that testosterone and testosterone propionate maintained complete spermatogenesis in the hypophysectomized mouse. Ludwig (1950) concluded that doses of testosterone and testosterone propionate of about 0.1 mg/day caused decreased weight and histological evidence of degeneration in the testes of the male rat through effects on pituitary function, whereas implants into the testes and doses of 1.0 to 3.0 mg per day maintained testicular weights and normal histology by direct action.

Greep and Fevold (1937) and Randolph et al. (1959) found that LH alone could maintain spermatogenesis, presumably by stimulating the Leydig cells to secrete testosterone which in turn acted on the seminiferous tubules. In the normal animal, growth and maintenance of spermatogenic tissue appears to result from the combined and synergistic effects of FSH and LH (Simpson, Li, and Evans, 1951).

As was discussed in the literature review, testosterone alone cannot account for the negative gonadotropic feedback system in the male. Therefore, it is possible that the observed decrease in hypothalamic FSH-RF content resulted both from direct action on the hypothalamus and from release of some unidentified substance from the testes.

The finding of increased FSH-RF content in the hypothalamus of male and female rats after castration corroborates the work of Dávid et al. (1965) who found increased FSH-RF activity in the hypothalamus of the ovariectomized female. The increases in total-gonadotropin stimulating activity after gonadectomy reported by Kobayashi et al. (1963a) in the female rat and by Suzuki et al. (1961) in the male rat are also compatible with these results.

Variations in light intensity during the moving of our laboratory in the fall of 1964 are a possible source of the apparent sex difference in hypothalamic FSH-RF content. Fiske (1941) reported that constant light hastened the advent of puberty in female rats. She also used the HCG augmentation phenomenon to demonstrate that this resulted specifically from an increase in FSH secretion. Male rats were less affected. Dempsey and Searles (1943) reported that constant illumination produced constant estrus without ovulation in rats. Dempsey and Searles (1943), Desclin (1961a, 1961b), and Ifft (1962) reported that constant illumination caused increases in size of nucleoli in many of the nuclei of the female rat.

Variations in diet are a conceivable source of variations in hypothalamic function. It is well known that nutritional deficiency causes regression of gonadal function. Srebnik et al. (1961), Srebnik and Nelson (1962) and Srebnik (1964) studied this phenomenon in rats and found a marked sex difference. Gonadal function in female rats was much more severely affected than in males.

D. Additional Control Experiments

1. Effects of Steroid Hormones on the Hypothalamic Stimulation of FSH Release In Vitro

Adult male rat pituitary tissue was incubated with hypothalamic extract. In all groups, the dose of hypothalamic extract was 0.2 hypothalamic equivalent per pituitary equivalent. In the experimental groups, steroids were dissolved in a minute volume of absolute ethanol and were added to the medium together with the hypothalamic extract. Steroids used were testosterone (0.5 or 1 μgm/ml) or estradiol (0.25 μgm/ml). These steroids were obtained from American Steroid Co., Hato Rey, Puerto Rico. All media were assayed at 2 dose levels for FSH.

The results are presented in Table 7. No evidence for an effect of steroids directly on pituitary tissue was noted. These results indicate that steroids carried over in hypothalamic extract did not influence the results obtained in the hypothalamic FSH-RF content studies in the previous section. This evidence is particularly important

Table 7. Effects of adding steroids to media together with hypothalamic extract on FSH release *in vitro*.

Treatment	Total Assay Animals per Group per Dose Level	Experimental / Control	95% Confidence Limits	λ
Testosterone (1 μgm/ml)	4	0.84*	0.58-1.36*	.428
Testosterone (0.5 μgm/ml)	2	1.14	0.48-1.80	.258
Estradiol (0.25 μgm/ml)	4	0.79*	0.54-1.34*	.428

0.2 Hypothalamic Equivalents per Incubated Pituitary.

Total No. of Assay Animals - 32

*Combined data from 2 experiments.

since Eisenfeld and Axelrod (1966) reported that the hypothalamus selectively accumulates tritiated estradiol.

2. Effects of Incubating a Purified FSH Preparation With and Without Brain Extract on FSH Activity

Ovine FSH (NIH-S2) was incubated for 6 hours. Each incubation flask contained 300 μgm of FSH. Assay animals received 100 μgm of FSH. Assay animals received 100 μgm or 200 μgm of FSH in each treatment group. Unincubated FSH served as the control. There were 3 experimental groups: FSH incubated alone, FSH incubated with neutralized cerebral cortical extract equivalent to 8 mg of fresh tissue per flask, and FSH incubated with extract equivalent to 0.8 hypothalami per flask.

The data are presented in Table 8. FSH activity was not increased or decreased as a result of incubation alone or incubation with brain extracts. These findings corroborate results presented above in this thesis.

3. Effects of Injections of Steroids or Brain Extracts on Response of Assay Animals to FSH

Ovine FSH (NIH-S2) was mixed with estradiol, testosterone, cerebral cortical extract, or hypothalamic extract in doses designed to serve as controls for previous experiments. The doses were as follows:

Testosterone: Assay animals received 100 μgm FSH and 2/3 μgm

Table 8. Effects of incubating NIH-FSH-S2 with and without brain extracts.

Treatment	Assay Animals per Group per Dose Level	Experimental / Control	95% Confidence Limits
FSH Incubated Alone	2	1.07	0.82-1.38
FSH Incubated With Cortical Extract	2	1.16	0.78-1.57
FSH Incubated With Hypothalamic Extract	2	1.08	0.88-1.34

Total No. of Assay Animals - 16

λ = .540

testosterone or 200 μgm FSH and 4/3 μgm testosterone.
Estradiol: Assay animals received 100 μgm FSH and 1/6 μgm estradiol or 200 μgm FSH and 1/3 μgm estradiol.
Cerebral cortical extract: Assay animals received 100 μgm FSH and neutralized acid extract equivalent to 8/3 mg. of cerebral cortical tissue or twice these doses.
Hypothalamic extract: Assay animals received 100 μgm FSH and neutralized acid extract equivalent to 0.267 rat hypothalami or twice these doses.
Controls: Assay animals received 100 or 200 μgm NIH-FSH-S2.
All treatments were administered in tissue culture medium 199.

The data are presented in Table 9. No indication was observed that steroids or brain extracts could influence the assay animals. The data on brain extracts corroborate results presented earlier in this thesis as well as those of Courrier et al. (1963), Igarashi and McCann (1964b) and Kuroshima et al. (1965), and indicate that results obtained in this work cannot be attributed to effects of hypothalamic extracts directly on assay animals.

The negative findings with steroids corroborate the results of Schuetz et al. (1964a, b) who found that steroids failed to inhibit the HCG augmentation of FSH effects. Payne and Runser (1958) obtained some FSH-augmenting activity with doses of estrogen far higher than those used here, but no effect at all with testosterone propionate. Smith and Bradbury (1963) obtained effects on the ovary, but only with very high doses of diethylstilbestrol.

Table 9. Effects of injections of steroids and brain extracts on the response of assay animals to FSH.

Treatment	Assay Animals per Group per Dose Level	Experimental / Control	95% Confidence Limits	λ
Testosterone	4	0.92	0.40-1.72	.581 (Combined Data from Replicate Experiments)
Estradiol	4	0.97	0.53-1.75	
Cortical Extract	2	0.92	0.50-1.49	.394
Hypothalamic Extract	2	0.77	0.40-1.22	

All doses were designed as controls for previous experiments.

Total No. of Assay Animals = 32.

V. GENERAL DISCUSSION

Work reported in this study clearly demonstrates that rat hypothalamus contains FSH-RF activity. Pituitary tissue exhibited a graded response to different doses of FSH-RF. Gonadectomy raised hypothalamic FSH-RF content and testosterone treatment lowered it. Steroid hormones did not inhibit FSH release or antagonize the stimulatory action of hypothalamic extract *in vitro*.

Comparison With Other Work

The results obtained in the 9-day tissue culture part of this work are similar to the results of Kobayashi et al. (1963a) in which hypothalamic extract caused increased gonadotropin both in the tissue culture medium and in the cells. However, the use of a nonspecific assay for gonadotropin makes it difficult to assess the significance of these results. LH and/or FSH may have been stimulated. In addition, their work lacked proper controls. Kobayashi et al. (1965) reported that hypothalamic extract stimulated uptake of radioactive leucine by pituitary basophils, but again this may be related to one or a combination of three hormones.

The hypothalamic stimulation of FSH release obtained in the 6-hour release obtained in the 6-hour incubation experiments (Chapter VII) is similar to an independent study

by Kuroshima et al. (1965) in which pituitary tissue was incubated 1 hour. These workers did not present evidence that their method was suitable for quantitative measurements of FSH-RF. The work presented in this thesis corroborates the findings of Igarashi and McCann (1964b), Igarashi et al. (1964a), Gellert et al. (1964) and Evans and Nikitovitch-Winer (1965) who found evidence for FSH-RF activity in hypothalamic acid extracts by means of in vivo systems of questionable specificity.

Content studies of hypothalamic releasing factors remain few in number. Ratner and Meites (1964) demonstrated that suckling and estrogen treatment could reduce the hypothalamic content of prolactin inhibiting factor (PIF). Vernikos-Danellis (1964, 1965) demonstrated that adrenalectomy and stress caused increases in hypothalamic content of corticotropin-releasing factor (CRF), whereas treatment with cortisol causes a reduction. Sinha and Meites (1965) found that thyroidectomy causes an increase in hypothalamic content of thyrotropin-releasing factor (TRF). Fiel and Meites (1965) reported that nutritional deficiency can cause a reduction in hypothalamic somatotropin-releasing activity (SRF) and Pecile et al. (1965) found a reduction in SRF with age. Ramirez and Sawyer (1965) and Chowers and McCann (1965) reported fluctuations in hypothalamic luteinizing hormone-releasing activity (LRF) during the estrus cycle in the rat. Piacsek and Meites (in press) also reported that gonadectomy and injections of gonadal steroids altered LRF content.

Recently, Dávid et al. (1965) reported that FSH-RF activity in hypothalamic extracts, as measured by a carotid injection system, was approximately doubled when the hypothalamus donors were ovariectomized. If the ovariectomized donors were then treated with estradiol benzoate, FSH-RF content declined to less than 1/4 of the control value. Unfortunately, their results were presented simply as bar graphs, without statistical analysis.

Significance of This Work

The work presented here provides strong evidence for the existence of a substance(s) in hypothalamic extracts which stimulate the release of FSH from the pituitary. This work is the first, so far as I know, to demonstrate stimulation of FSH release (specifically) from male rat pituitary tissue. The dose-response curves obtained here demonstrates a potentiality for quantitative assay of FSH-RF. Since all or almost all established hormones exhibit graded dose-response relationships, whereas nonspecific agents usually do not, demonstration of a dose-response curve for FSH-RF is circumstantial evidence for its physiological significance. Both Dr. G. Duncan (The Upjohn Co., Kalamazoo, Mich.) and Dr. S. M. McCann (Dept. of Physiology, Southwestern Medical School, Dallas, Texas) have successfully used my *in vitro* FSH-RF assay (private communication to J. Meites).

The demonstrated ability of castration and testosterone propionate treatment to influence the hypothalamus is evidence that the site of negative feedback action by steroids is the hypothalamus. Failure of steroids to directly inhibit FSH release *in vitro* or to antagonize the stimulatory effect of hypothalamic extract is inconsistent with the concept of negative feedback directly on the pituitary. Ability to alter the hypothalamic content of FSH-RF by treatment of the donor is also further evidence that this substance has physiological significance and is not merely an artifact of chemical and physical extraction of the hypothalamus.

Suggestions For Further Work

There is a large practical need for the purification, structural determination, and synthesis of FSH-RF for use in medicine and agriculture. Once this is done, and pure FSH-RF is available, it will be practical to demonstrate unequivocally that FSH-RF stimulates synthesis, as well as release, of FSH. It also will be possible to do satisfactory studies on the effects of FSH-RF on pituitary cytology and to study effects on cell metabolism, including oxygen consumption. In addition, pure FSH-RF could be used to firmly establish the shape of the dose-response curve.

Since my results do not completely rule out the possibilities of steroid effects directly on the pituitary, more experiments are needed. For example, a long pre-culture

with steroids might influence the response to hypothalamic extract added later.

Much work remains to be done on hypothalamic FSH-RF content in female rats. The effects of ovariectomy and treatment with steroids should be studied more completely. Effects of nutritional deficiency, melatonin, and changes in light intensity are of interest also. Differences in hypothalamic FSH-RF content between male and female rats are suggested by my data and should be investigated thoroughly.

Developmental and comparative aspects are of interest. The hypothalamus should be studied from fetal stages to senility, and from fish to reptiles and birds.

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APPENDIX I

STUDIES ON PROLACTIN INHIBITING FACTOR (PIF)

Meites (1959) found that virgin female rats injected subcutaneously for 10 days with 10 μgm estradiol daily followed by twice daily injections of 25 mg acetylcholine iodide/kg body weight or 0.25 mg epinephrine in oil for 5 days, showed extensive lobulo-alveclar mammary growth and lactation. Meites, Talwalker, and Nicoll (1960) obtained similar results with epinephrine, acetylcholine iodide, and serotonin in estrogen-primed female rabbits. Desclin (1960) confirmed the finding of Meites (1959) that epinephrine could initiate mammary secretion in estrogen-primed rats. Meites, Nicoll, and Talwalker (1963) reviewed work demonstrating that epinephrine, acetylcholine, and serotonin can initiate lactation, maintain mammary secretion and produce pseudopregnancy in rats under certain experimental conditions. These results are interpreted as indicating that these neurohumors stimulate, directly or indirectly, the release of prolactin from the pituitary.

Talwalker, Ratner and Meites (1963) demonstrated the presence of a prolactin-inhibiting factor (PIF) in acid extracts of rat hypothalami. The method developed by these authors was applied by Ratner and Meites (1964) as an <u>in vitro</u> assay method for PIF and used to obtain evidence

indicating that certain stimuli, including the suckling stimulus, act indirectly on the pituitary by depleting the hypothalamus of its PIF content.

Since Talwalker, Ratner and Meites (1963) found that epinephrine and acetylcholine had no effect on the pituitary *in vitro*, it seemed of interest to attempt to explain the lactational effects of these neurohumors by demonstrating effects on the PIF content of the hypothalamus. Effects of graded doses of hypothalamic extract on pituitary prolactin release and of hypophysectomy on hypothalamic PIF content were considered worth studying also.

Materials and Methods

The work reported here follows in detail the methods used in the paper by Ratner and Meites (1964) except where otherwise noted. For each of 2 experiments designed to investigate the effects of epinephrine, experimental hypothalamus donors were 12 Carworth CFN female rats treated with epinephrine in oil (0.25 mg per injection) twice daily for five days and killed 1 hour after the last injection. The Parke-Davis Co. supplied most of the epinephrine used. Twelve CFN female control animals received injections of 0.1 ml of corn oil twice daily.

For each of 2 experiments designed to study the effects of acetylcholine, 12 CFN female rats comprising the experimental group received 25 mg acetylcholine bromide (Eastman Co., Rochester) per kilogram body weight twice daily. Control animals received saline injections.

Twelve intact female Sprague-Dawley rats were used as control hypothalamus donors in one experiment. Twelve hypophysectomized rats of the same age and strain also served as controls.

The incubation procedures involved use of neutralized acid extract equivalent to 2 hypothalami for each pituitary equivalent incubated. In each incubation, 12 adult Spartan rat pituitaries were divided among three flask pairs, i.e., there were 2 pituitary equivalents per flask. The medium from each incubation was injected into 12 pigeons. In an experiment in which graded doses of hypothalamic extract were used, 6 incubated pituitaries and 6 pigeons were used for each dose level. Dose levels of hypothalamic extract ranged from 1/2 to 4 hypothalami per incubated pituitary. Control pituitary halves were incubated with equivalent amounts of cerebral cortical extract.

Results: The results from the content study experiments are presented in Table 1A. Effects of graded doses of hypothalamic extract are presented in Figure 1A as differences between control and experimental ratings for each bird; vertical bars represent standard errors of the mean.

Epinephrine treatment produced a definite reduction in hypothalamic PIF content. Both experiments are significant at the 0.5 level. Acetylcholine bromide produced results which achieved acceptable levels of statistical significance only when the 2 experiments were combined.

Table 1A. Effects of treatments on hypothalamic PIF content.

Epinephrine:

Exp.	No. of Pigeons	Mean Responses (RTU)	P.
1	11	C = 0.500 E = 0.955	< .05
2	12	C = 1.000 E = 1.708	< .05
Combined Data	23	C = 0.761 E = 1.308	< .0025

Acetylcholine Bromide:

Exp.	No. of Pigeons	Mean Responses (RTU)	P.
1	12	C = 0.480 E = 0.750	< .06
2	12	C = 0.833 E = 1.083	< .10
Combined Data	24	C = 0.656 E = 0.917	< .006

Hypophysectomy:

No. of Pigeons	Mean Responses (RTU)	P.
10	C = 0.950 (intact) E = 0.925 (hypophysectomized)	Not significant

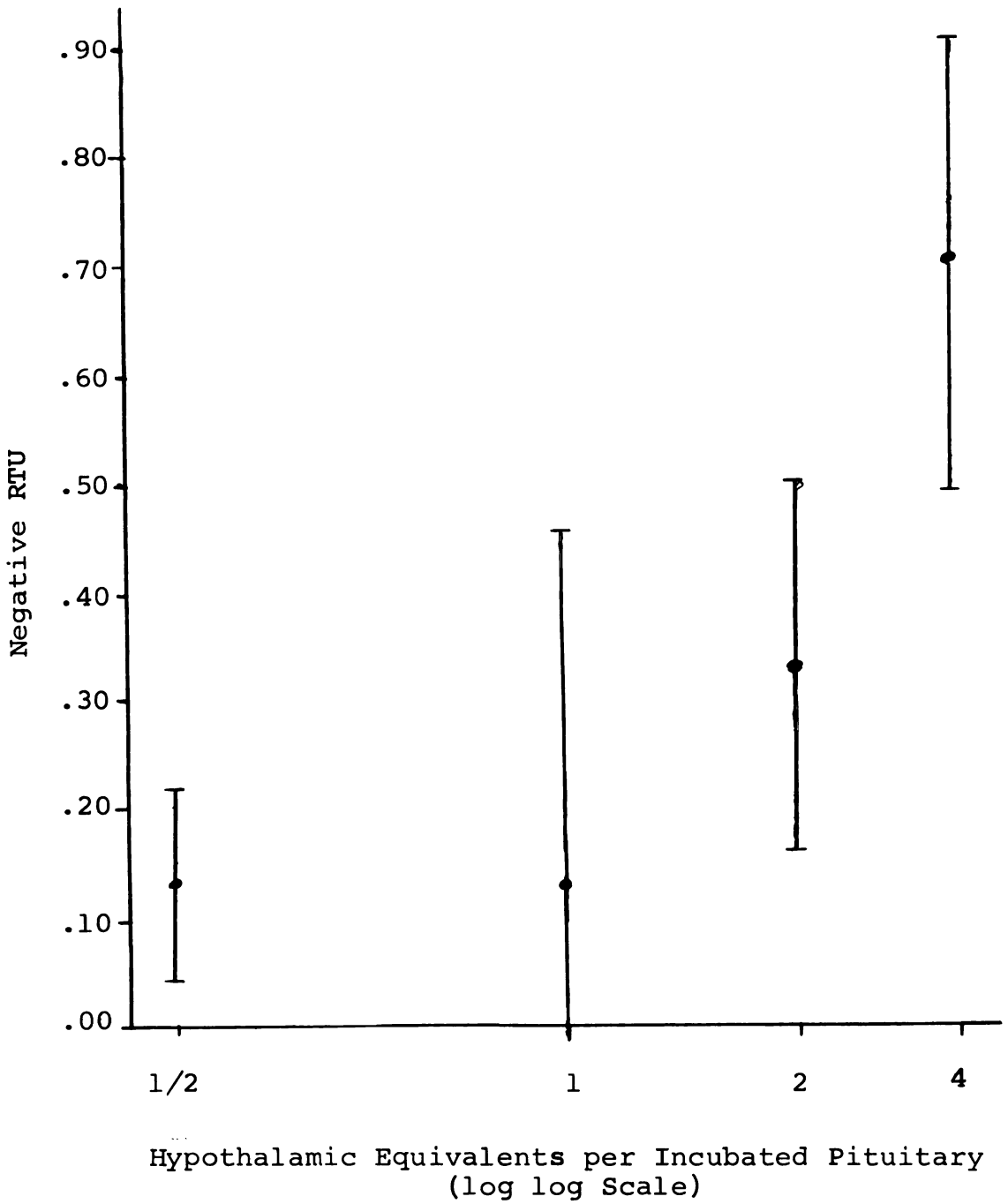

Fig. 1A. Effects of Graded Doses of Hypothalamic Extract on Pituitary Prolactin Release.

Hypophysectomy had no effect on hypothalamic PIF content.

Treatment of data from the PIF dose-response curve poses certain problems. It is known that the Reece-Turner unit (RTU) is linearly related to the logarithm of the dose of prolactin in μgm or International Units (I.U.) within a certain dose range, as are all but a few hormonal dose-response relationships. It is postulated that the log dose of PIF is inversely related to the amount of prolactin released in μgm or I.U. within a certain range of dose. Therefore, the RTU should be related inversely to the log log PIF within a certain dose range, i.e.,

$$RTU = -K_1 \log \log PIF + K_2$$

where K_1 and K_2 are constants related to threshold effects. For the purposes of this study, it is convenient to analyze these negative RTU's by first subtracting each experimental crop rating from the matched control, then graphing results as means and standard errors of the mean, as is done in Fig. 1A.

Because of small numbers and birds differing considerably in age and physical condition, the attempt to demonstrate a graded response (dose-response curve) was not conclusive. However, the results do suggest a relationship between amount of hypothalamic extract and degree of inhibition.

Discussion

Results obtained in this work indicate that epinephrine and acetylcholine can reduce the hypothalamic PIF content and presumably, its rate of release. It appears, therefore, that the lactogenic effects of these neurohormones are indirect and mediated through the hypothalamus. The failure of hypophysectomy to modify PIF content may be considered as preliminary evidence that there is no feedback of prolactin on the hypothalamus. However, this work is by no means conclusive.

Because of the small numbers and lack of uniformity in the birds, the graded response experiment could not be conclusive. It is, however, encouraging. Since almost all hormones exhibit a log-dose response relationship, whereas non-specific agents rarely do, demonstration of a logarithmic relationship for PIF on prolactin release would be further evidence that PIF is of physiological significance and is not merely an artifact of extraction of the hypothalamus. More recent work by Kragt (Ph.D. thesis, Michigan State University, Dept. of Physiology, 1966) definitely establishes that PIF shows a definite dose-response relationship to pituitary prolactin release in vitro.

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APPENDIX II

LIST OF PUBLICATIONS BASED ON WORK PRESENTED IN THIS THESIS

1. Mittler, J. C. and J. Meites, Stimulation of Pituitary Release by Hypothalamic Extract *in vitro*, in *Program, 46th Annual Meeting, The Endocrine Society*, 1964. (Abstract No. 8).

2. Mittler, J. C. and J. Meites, *In Vitro* Stimulation of Pituitary Follicle-Stimulating Hormone Release by Hypothalamic Extract, *Proc. Soc. Exptl. Biol. Med.*, *117*: 309-313, 1964.

3. Mittler, J. C. and J. Meites, Effects of Castration and Androgen on FSH-releasing Activity of Hypothalamic Extracts in Rats, *Endocrinology*, *78*: 500-504, 1966.