CENTRAL NERVOUS SYSTEM INHIBITION OF PROLACTIN SECRETION AND EFFECT OF ANTERIOR PITUITARY HORMONES ON INDUCTION OF NORMAL AND NEOPLASTIC MAMMARY GROWTH IN THE RAT

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ABSTRACT

CENTRAL NERVOUS SYSTEM INHIBITION OF PROLACTIN SECRETION

AND

EFFECT OF ANTERIOR PITUITARY HORMONES ON NORMAL AND NEOPLASTIC MAMMARY GROWTH

by Purnachandra Keshavrao Talwalker

1. The effects of chlorpromazine, a central nervous system (CNS) depressant, on mammary growth and secretion was investigated in the rat. Chlorpromazine stimulated mammary lobulo-alveolar (L-A) growth, and initiated mammary secretion in estrogen-primed rats or maintained mammary secretion in post-partum rats after litter withdrawal. In the estrogen-primed rat, initiation of mammary secretion normally requires prolactin and ACTH. These results indicate therefore, that chlorpromazine promotes secretion of both prolactin and ACTH by the anterior pituitary (AP).

2. The ability of the rat hypothalamus to inhibit prolactin secretion by AP in vitro was determined by a 2-hour incubation of rat AP. Homogenates or acid extracts of rat hypothalamus inhibited prolactin synthesis and release by AP, whereas acid extracts of cerebral cortex had no effect, indicating the presence of a prolactin inhibiting factor (PIF) in rat hypothalamus. Acetylcholine, epinephrine, norepinephrine, serotonin, histamine, substance P, oxytocin, arginine or lysine vasopressin and bradykinin had no effect on pituitary

prolactin release, indicating that PIF is different from any of these substances normally present in the hypothalamus. PIF in the rat hypothalamus was dialyzable, showing that it is a small molecule and perhaps a peptide similar to other neurohumoral releasing factors. Acid extracts of hypothalami from cattle, sheep, and swine origin also showed PIF activity.

3. The *in vivo* and *in vitro* capacity of the rat "mammotropic" pituitary tumor (Furth, MtT.F_4) to secrete prolactin was determined. The prolactin contents of MtT.F_4 and normal AP-transplants were very low as compared with normal AP *in situ*. Considerable amounts of prolactin activity could be detected in the blood plasma of rats bearing MtT.F_4, but not in the plasma of cycling, estrogen primed, pregnant or lactating rats, or in rats bearing a single AP-transplant. MtT.F_4 released considerable amounts of prolactin into the medium during 3 or 6 days of culture, although the amounts of hormone released were small than by normal AP in culture. It is concluded that the MtT.F_4, like the normal AP removed from hypothalamic inhibition, synthesizes and releases prolactin at a very rapid rate, thereby retaining very little prolactin in the tissue.

4. MtT.F_4 was successfully transplanted into Sprague-Dawley rats from the inbred Fischer strain of rat. However, tumor growth and percentage "take" in the Sprague-Dawley strain were lower than in the Fischer strain.

The tumor in the Sprague-Dawley rats apparently secreted prolactin, STH and ACTH as it does in the Fischer rat, and thus remained functionally similar to the original tumor.

5. A MtT.F_4 transplant stimulated mammary L-A growth in adreno-ovariectomized Fischer rats. Also, injections of combination of prolactin and STH stimulated mammary L-A growth in adreno-ovariectomized-hypophysectomized Carworth CFN rats. This demonstrates that the AP hormones, prolactin and STH, can promote mammary L-A growth in the apparent absence of ovarian and adrenal cortical hormones.

6. Mammary tumors were induced in ovariectomized Sprague-Dawley rats, with limited treatment with estradiol or prolactin and STH, following a single DMBA (7, 12-dimethyl-1, 2-benzanthracene) feeding. No mammary tumors developed in untreated ovariectomized rats fed carcinogen. These results indicate that prolactin and STH can produce mammary tumors in carcinogen treated rats in the apparent absence of ovarian hormones. They also indicate that the pituitary hormones, prolactin and STH, are essential during the initiating phase of mammary carcinogenesis in the rat.

7. Differences in the susceptibility of the mammary gland to chemical carcinogens were investigated by using 4 different strains of rats and 3 different carcinogens. The mammary glands of Sprague-Dawley strain were much more susceptible to production of tumors (33%) than the other strains studied (0-7%, Carworth CFN, MSU Chemistry Department,

and Hunt-Hoppert inbred strain). 3-methylcholanthrene and 9, 10-dimethyl-1, 2-benzanthracene were more effective in inducing mammary tumors in Sprague-Dawley strain than, 3, 4-benzpyrene.

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Dedicated

to my parents

Shri Keshavrao

and

Sou. Kamalabai

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INTRODUCTION

One of the characteristics of humans is the desire to simplify the complexities of nature by generalized explanations. Primitive man invoked spirits and demons to explain natural phenomena. With the advent of science, such explanations have become unsatisfactory and we now insist on more mechanistic hypotheses that can be experimentally verified. The value of a theory, hypothesis or a concept must be judged either in terms of variety and type of observations that can be accounted for, or by its effectiveness in stimulating research and influencing the design of experiments.

Recently in endocrinology, as a result of studies involving central and peripheral nerve lesions, pituitary stalk section, hypophyseal transplants, electrical stimulation, neurohumoral stimulants, pharmacological agents and *in vitro* cultures of pituitary tissue, the concept has developed that the central nervous system (CNS), and particularly hypothalamus is essential for normal secretion of 5 of the anterior pituitary hormones, but it inhibits prolactin secretion by way of its neurovascular linkage to the pituitary (1).

Psychotropic agents such as chlorpromazine, which suppresses the CNS, evoke galautorrhea in women and induce pseudopregnancy in the rat, indicating release of pituitary prolactin (2, 3). This suggests that chlorpromozine overcomes CNS

inhibition to pituitary prolactin secretion, and thereby fosters its release. The effect of chlorpromozine on mammary growth and secretion in the rat was therefore investigated.

Hypothalamic inhibition of pituitary prolactin release is probably mediated through release of a hypothalamic factor(s) which is transported through the hypothalamo-hypophyseal portal system to the anterior-pituitary. Such a concept requires the demonstration of the existence of a prolactin inhibiting factor (PIF) in the hypothalamic tissue. Therefore, it was decided to determine whether homogenates or acid extracts of rat hypothalamus could inhibit pituitary prolactin secretion, and whether such inhibition could be due to one of the known neuropharmacological agents present in the hypothalamus.

Removal of hypothalamic inhibition to the pituitary, by transplanting it from its normal cranial site to other parts of the body, or by culturing it <u>in vitro</u>, fosters prolactin release (1). In some strains of rats and mice, the pituitary transplant may grow into a tumor and secrete large amounts of prolactin as indicated by mammary gland stimulation and a pseudopregnancy response in the rat (4-6). The rat "mammotropic" pituitary tumor developed by Furth, similarly induces mammary gland stimulation in the host rat (7). The functional behaviour of this pituitary tumor appears to be analogous to a normal pituitary removal from its hypothalamic connections, insofar as prolactin secretion is concerned.

Therefore, the capacity of this pituitary tumor to elaborate prolactin *in vivo* and *in vitro* was investigated, and this was compared with prolactin secretion by normal rat pituitary *in situ*.

In the second part of this thesis, effect of anterior pituitary hormones on normal and neoplastic mammary growth were studied. The importance of estrogen for the development of the mammary lobulo-alveolar (L-A) system is well established. Estrogen stimulate mammary L-A growth in intact rats and mice, but is ineffective in the absence of the anterior pituitary. Since estrogen can increase pituitary prolactin and probably STH secretion, this suggests that estrogenic stimulation of mammary L-A growth is probably mediated in part through increasing prolactin and STH secretion (1).

Clifton and Furth (8) observed that in adreno-gonadectomized rats, mammary L-A growth was induced by transplanting a pituitary tumor which produces large amounts of prolactin STH and ACTH. Such a tumor provided a continuous source of these hormones in increasing concentrations as the tumor grew in the hosts. These workers did not check for completeness of adreno-gonadectomy, and histological examination of the mammary glands was not reported. It was thought of interest therefore, to re-examine and extend this study in adreno-ovariectomized rats, and examine the evidence for estrogenic activity and completeness of adrenalectomy.

The results showed that in the apparent absence of estrogens, the hormones (prolactin, STH and ACTH) secreted by the pituitary tumor stimulated mammary L-A growth in adreno-ovariectomized rats. This emphasized the importance of prolactin and STH in mammary growth in the rat. It was decided therefore, to determine whether frequent injections of large doses of pituitary hormones (thus mimicking the effects of the pituitary tumor transplant) could stimulate mammary L-A growth in adreno-ovariectomized-hypophysectomized rats.

Studies involving mammary carcinogenesis in the rat suggested that mammary tumorogenesis consists of a two-phase mechanism: (a) an initiating phase in which a carcinogen produces an irreversible alteration in the cells of the mammary gland, and (b) a promoting phase in which hormones stimulate growth of these altered cells (7). Although pituitary hormones maintain or stimulate growth of already established mammary tumors in rats, it is not clear whether hormones participate in the initiating phase of mammary carcinogenesis by a chemical carcinogen. Therefore, the role of estrogen and of prolactin and STH during the initiating phase of mammary tumorogenesis by a chemical carcinogen was investigated.

Experiments I, II and V in this thesis have already been published, while all other experiments reported here are as yet unpublished.

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Section One

CENTRAL NERVOUS SYSTEM INHIBITION OF PROLACTIN SECRETION

I. EFFECTS OF CHLORPROMAZINE ON MAMMARY GLANDS OF RATS

ABSTRACT

The effects of chlorpromazine were determined on mammary growth and initiation of milk secretion in virgin rats, and on maintenance of mammary structure and secretion in postpartum rats after litter removal. When chlorpromazine was administered in doses of 5 or 15 mg/kg body weight for 5 days, only the higher dose was effective in inducing lobulo-alveolar growth and initiating milk secretion in rats initially primed with 10 µg estradiol daily for 10 days. The higher dose of chlorpromazine also maintained mammary lobulo-alveolar structure and secretion in postpartum rats for 10 days after litter removal. Chlorpromazine produced a significant increase in adrenal weight and decrease in thymus weight, indicating adrenal stimulation. In estrogen-primed rats neither prolactin nor ACTH alone could initiate mammary secretion; however they were effective when given in combination. The present data indicate that chlorpromazine promotes the secretion of both prolactin and ACTH. Hypophysectomy following estradiol treatment prevented chlorpromazine from initiating mammary secretion, showing that its effects are mediated through the anterior pituitary and not directly on the mammary gland.

Recently attention has been focused on the influence of several tranquilizing drugs on anterior pituitary function. Chlorpromazine may inhibit TSH (1) and FSH-LH (2,4) secretion, and stimulate secretion of ACTH in several species (3). Chlorpromazine apparently promotes prolactin secretion as indicated by induction of pseudopregnancy, maintenance of corpora lutea beyond the normal period of pseudopregnancy in the rat (4) and occasional galactorrhea in women (5,6). It became of interest to determine whether this drug could induce mammary growth and secretion in the rat, since these processes are dependent upon anterior pituitary secretion of prolactin and ACTH.

METHODS

Virgin female rats of the Carworth strain, weighing 200-250 gm each, were maintained in a temperature controlled ($74 \pm 2°F$) and artificially illumined (14 hours per day) room. They were fed a normal laboratory diet, and food and water were available ad libitum. Three different experiments were performed as follows:

Experiment 1: The effects of chlorpromazine on mammary growth were investigated in virgin rats. Thirty-two rats were divided into three groups of 12, 10 and 10 each, and were injected subcutaneously with saline or chlorpromazine in doses of 5 or 15 mg/kg body weight for 15 days.

Experiment 2: The ability of chlorpromazine to induce mammary secretion was determined in estrogen-primed virgin

rats. Fifty-five rats were divided into 9 groups and injected subcutaneously with 10 μg estradiol daily in 0.1 ml corn oil for 10 days. This treatment induces lobulo-alveolar (L-A) development in the rat and is necessary to render the mammary tissues responsive to hormones which stimulate milk secretion (7). For the subsequent 5 days the groups received subcutaneous injections as follows: (1) controls, saline 0.1 ml once daily; (2) and (3), chlorpromazine, 5 and 15 mg/kg body weight, respectively, once daily; (4) and (5), prolactin (Ovine, 20 I.U./mg), 1.0 and 2.0 mg, respectively, twice daily; (6) and (7) ACTH, 1.0 and 2.0 I.U., respectively, twice daily; (8) prolactin, 1.0 mg and ACTH 1.0 I.U., each twice daily. In the last group (9) rats were hypophysectomized by the parapharyageal approach after 10 days treatment with estradiol and then injected with chlorpromazine, 15 mg/kg body weight, for 5 days once daily. Chlorpromazine and prolactin were dissolved in physiological saline, and ACTH was injected in a gel-preparation.

Experiment 3: The effects of chlorpromazine on the maintenance of L-A structure and secretion in postpartum rats were studied. Thirty rats were bred and placed in individual cages. On the first day following parturition the litters were reduced to six young each. During the following three days the litters were weighed daily to check lactational performance of the mother, and on the 4th day the litters were withdrawn. The lactating rats were then divided into three

groups of 10 each and injected subcutaneously with saline or chlorpromazine in doses of 5 or 15 mg/kg body weight for the following 10 days.

The day after the last day of treatment the rats were sacrificed and the right inguinal mammary glands were removed and prepared for histological examination by standard techniques (7). The ovaries, uterus, adrenals and thymus were excised from the animals of Experiment 1, and weighed on a Roller-Smith balance.

RESULTS

Experiment 1: (Table I). The mammary glands of the saline-treated controls (gr. 1) consisted essentially of a few ducts and end buds (Fig. 1. For figures see appendix for published article). The mammary glands from the rats treated with the lower dose of chlorpromazine (gr. 2) did not differ from the controls (gr. 1). At the higher dose level (gr. 3), chlorpromazine induced considerable L-A development (Fig. 2). The weights of the ovaries and uterus from gr. 2 and 3 were not significantly different from the controls (gr. 1). However, the average adrenal weights were significantly higher ($P < .05$) and average thymus weights were significantly lower ($P < .01$) in gr. 3 than in the controls (gr. 1).

Experiment 2: (Table II). Estradiol treatment for 10 days induced L-A development but no secretion in virgin female rats (7). Subsequent treatment with saline for 5 days

(gr. 1) resulted in mammary regression to almost a bare duct system in all rats and no secretion was observed (Fig. 3). Treatment with the lower dose of chlorpromazine (gr. 2) had no effect, since the mammary glands regressed to the same extent as in the controls (gr. 1). However, treatment with the higher dose of chlorpromazine (gr. 3) induced maintenance of L-A structure and induction of secretion in all rats of this group (Fig. 4).

Experiment 3: (Table III). In postpartum rats after removal of litters and treatment with saline for 10 days (gr. 1), the mammary glands regressed from a secretory L-A structure to ducts with a few small closed alveoli and no secretion (Fig. 8). Mammary glands from rats treated with the lower dose of chlorpromazine (gr. 2) did not differ histologically from the controls (gr. 1). Mammary involution was markedly inhibited in the rats treated with the higher dose of chlorpromazine (gr. 3). L-A structure and some secretion were maintained in 9 out of 10 rats (Fig. 9).

In order to determine whether the effects of chlorpromazine were mediated through prolactin alone or also required ACTH, the two hormones were administered individually and in combination to estradiol-primed rats. When prolactin was administered at dose levels of 1 or 2 mg twice daily it produced maintenance of L-A structure but no secretion (Fig. 5). When ACTH was injected at dose levels of 1 or 2 I.U. twice daily, there was no secretion and the mammary glands regressed

(Fig. 6) as in the controls. However, when both prolactin and ACTH were administered, 4 out of 5 rats showed mammary secretion and maintenance of L-A structure (Fig. 7). Hypophysectomy following estradiol treatment, and subsequent treatment with chlorpromazine at the higher dose level, resulted in complete regression of L-A structure. This indicates that the effects of chlorpromazine are mediated through the pituitary.

DISCUSSION

The results of the present study show that an appropriate dose of chlorpromazine in the rat can (a) induce L-A growth (b) initiate mammary secretion after estrogen-priming and (c) retard mammary involution following litter removal in postpartum rats. L-A development is believed to be induced in virgin rats by increased secretion of prolactin and stimulation of ovarian luteal function. Hypophysectomy following ostradiol treatment prevented chlorpromazine from initiating mammary secretion, showing that its action is mediated through the anterior pituitary and not directly on the mammary gland. Both prolactin and ACTH were necessary to initiate mammary secretion in catrogen-primed rats. Neither hormone was effective alone. The rat is apparently exceptional in this respect, since prolactin alone can initiate mammary secretion in intact animals of other species with developed mammary glands (8).

In postpartum rats, maintenance of mammary structure and secretion is partially dependent upon the suckling stimulus which has been shown to induce release of both prolactin and ACTH from the anterior pituitary (8). Mammary involution which normally follows litter removal or weaning can be partially inhibited by administration of prolactin or cortisone (9). In hypophysectomized rats, milk secretion can also be partially maintained by injecting prolactin and ACTH (10). Maintenance of L-A structure and secretion in postpartum rats with chlorpromazine in the present experiment is therefore believed to be due to increased secretion of both prolactin and ACTH.

Chlorpromazine has been reported to induce ACTH release in several species (3). In the virgin female rats treated for 15 days with chlorpromazine, there was a significant increase in adrenal weight and decrease in thymus weight. Multiple injections of chlorpromazine may induce maximum secretion of ACTH in rats, as has been reported for reserpine (11). Stimulation of prolactin secretion by chlorpromazine is indicated by its ability to induce pseudopregnancy, maintain corpora lutea beyond the normal period of pscudopregnancy in rats (4), evoke galactorrhea in women (5,6) and in the present study, initiate mammary secretion in estrogen-primed rats. Preliminary assays in our laboratory of pituitaries from postpartum rats following chlorpromazine administration suggests that prolactin is released.

Recently, it has been proposed that factors which suppress FSH-LH secretion promote the secretion of prolactin (12). Chlorpromazine has been reported to inhibit ovulation in the rat in response to mechanical stimulation of the uterine cervix or injection of estradiol when administered during estrus (2). There is also evidence that factors which promote the secretion of ACTH suppress LH secretion (13). It has been observed that non-specific stresses, drugs and emotional disturbances inhibit ovulation and at the same time promote ACTH secretion (14, 15). Harris (16) concluded that most conditions which promote ACTH secretion also inhibit TSH secretion. Chlorpromazine and reserpine have been reported to inhibit TSH secretion (1,17). It is possible therefore, that some of the factors which promote the secretion of ACTH and prolactin also suppress FSH, LH and TSH secretion by the anterior pituitary.

Recent studies have shown that transplantation of the anterior pituitary from its normal cranial site to other parts of the body favors secretion of prolactin (18). In our laboratory we have observed that pituitary implants underneath the kidney capsule secrete sufficient prolactin to initiate mammary secretion in estrogen-primed rats (19). It has been suggested that the hypothalamus may normally inhibit prolactin secretion by way of its neurovascular linkage to the anterior pituitary (18). Barraclough and Sawyer (4) suggested that reserpine and chlorpromazine

depress the hypothalamus and thus remove inhibition to prolactin secretion. Studies of the effects of chlorpromazine on ACTH secretion indicate that its action is at the prepituitary and probably at the hypothalamic level (3,20). The ability of chlorpromazine and various other factors such as the suckling stimulus (8), adrenalectomy (21), numerous drugs, electrical stimulation, and nonspecific stresses (22-25) to induce secretion of prolactin and ACTH suggests the existence of a common mechanism(s) regulating the secretion of these two hormones.

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FOOTNOTES

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TABLE 1. Effects of Chlorpromazine on Mammary Growth in the Rat

Group No.	Treatment 15 days 1 x daily	No. rats with L-A* Growth	Organ weight in mg per 100 gm Body Weight			
			Adrenal	Thymus	Ovary	Uterus
1 (12)**	Controls, Saline, 0.85%	0	25.8±1.5 ***	99.9±8.8	26.1±1.8	207.1±19.0
2 (10)	Chlorpromazine 5 mg/kg BW	0	--	--	--	--
3 (10)	Chlorpromazine 15 mg/kg BW	7	30.2±2.0	57.1±6.7	25.3±2.4	191.8±11.6

*L-A = Lobulo-alveolar **No. of rats in the group *** S.E. $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

TABLE 2. Effects of Chlorpromazine on Initiation of Lactation in Estrogen-Primed* Rats

Group No.	No. Rats Treated	Treatment (5 days)	Dose	No. Rats with Secretion
1	10	Saline 0.85%	0.1 ml 1xd**	0
2	5	Chlorpromazine	5 mg/kg BW 1xd	0
3	10	Chlorpromazine	15 mg/kg BW 1xd	10
4	5	Prolactin	1 mg/kg BW 2xd	0
5	5	Prolactin	2 mg/kg BW 2xd	0
6	5	ACTH	1 I.U. 2xd	0
7	5	ACTH	2 I.U. 2xd	0
8	5	Prolactin + ACTH	1 mg + 1 I.U. 2xd	4
9	5	Hypophysectomy*** +Chlorpromazine	15 mg/kg BW 1xd	0

*Estradiol, 10 ug, 1x daily, injected for 10 days

** d = daily

*** Hypophysectomy was carried out at end of 10 days of estradiol treatment

TABLE 3. Effects of Chlorpromazine on Maintenance of Mammary Structure and Secretion in Postpartum Rats after Litter Removal

Group No.	No. Rats Treated	Treatment (10 days)	Dose 1xdaily	No. Rats with Maintenance of L-A* and Secretion
1	10	Controls, 0.85% Saline	0.1 ml	0
2	10	Chlorpromazine	5 mg/kg BW	0
3	10	Chlorpromazine	15 mg/kg BW	9

*L-A = lobulo-alveolar structure

II. *IN VITRO* INHIBITION OF PITUITARY PROLACTIN SYNTHESIS AND RELEASE BY HYPOTHALAMIC EXTRACT

ABSTRACT

Anterior pituitaries from rats were incubated for 2 hours at 37.5°C in a Dubnoff metabolic shaker in an atmosphere of 95% O_2 - 5% CO_2. At the end of the indubation period, 169% more prolactin was found in the medium and anterior pituitaries than in fresh non-incubated anterior pituitary, demonstrating that net synthesis and release of prolactin had occurred. When anterior pituitaries were incubated together with homogenate or acid extract of rat hypothalamus, prolactin levels in the medium and anterior pituitaries were markedly decreased, indicating inhibition of synthesis and release. Acid extract of rat cerebral cortex had no effect on pituitary prolactin synthesis or release. Incubation of purified ovine prolactin or rat prolactin, with or without homogenate or acid extract of rat hypothalamus, did not alter prolactin activity, demonstrating that the inhibitory action of homogenate or acid extract of the hypothalamus was not due to inactivation of prolactin. Acetylcholine iodide, epinephrine, l-norepinephrine bitartrate, serotonin creatinine sulfate, histamine acid phosphate, oxytocin (Pitocin), synthetic arginine and lysine vasopressin, and substance P had no effect on pituitary prolactin release *in vitro*. The results indicate that the hypothalamus contains a factor(s) which inhibits synthesis and release of prolactin by the anterior pituitary

in vitro, and this factor(s) does not appear to be any of the known neurohumoral agents in the hypothalamus.

Recent studies have emphasized that the CNS and particularly the hypothalamus participate in the control of anterior pituitary function. It has been observed that placement of hypothalamic leisons, transection of the pituitary stalk, transplantation of the anterior pituitary to non-cranial sites, use of certain CNS depressant drugs, and organ cultures of the anterior pituitary are accompanied by a marked decrease in secretion of all anterior pituitary hormones except prolactin. Under these conditions, prolactin secretion is actually increased, as indicated by prolonged maintenance of the corpus luteum in the rat, induction of mammary growth and lactation, and increased secretion of prolactin by the pituitary in vitro (1,2). These observations suggested that the hypothalamus may contain a factor(s) which normally inhibits prolactin secretion. Presumably this factor is transported through the hypothalamico-hypophyseal portal system to the anterior pituitary, where it acts to depress prolactin synthesis and/or release.

This investigation was undertaken to determine whether homogenates or acid extracts of rats hypothalamus could inhibit prolactin secretion, and whether such inhibition could be due to one of the neuropharmacological agents known to be present in the hypothalamus. Short term incubation of the anterior pituitary was adopted as a test method (3), since under these conditions the anterior pituitary is isolated from its normal neurovascular linkage and is removed from all body

influences. A preliminary report of the present work has previously been published in abstract form (4).

METHODS AND RESULTS

Animals. Mature virgin female Carworth rats of the CFN strain, 3-5 months old, were used as pituitary donors in all experiments. Rats were maintained in a temperature controlled (75 ± 1°F) and artificially illuminated (14 hrs/day) room. They were fed ad libitum on Wayne Lab Blox pellets. White King squabs, 5-8 weeks old, were used for prolactin assays. A total of 820 rats and 805 pigeons were used in this study.

Incubations. The rats were first stunned and then decapitated. The anterior pituitary was separated from the posterior lobe and placed in a Petri dish over filter paper moistened with synthetic protein-free medium "199"[3] (5). Each anterior pituitary was cut into half, weighed, and transferred to a 25 ml pyrex flask containing medium "199" (pH 7.4). A total of 6 or 12 anterior pituitary halves were placed in each flask. All incubations were carried out in a Dubnoff metabolic shaker (60 cycles per minute) under constant gassing with humidified 95% O_2 - 5% CO_2, at 37.5 ± 0.5°C.

Prolactin Assays. Prolactin activity of the samples was determined by the intradermal pigeon crop assay method using a paired assay procedure. The control sample was

injected over one side of the crop sac and the experimental sample over the other side in the same pigeons. This provided a direct comparison between the two samples. Anterior pituitaries released considerable quantities of prolactin into the medium during incubations. Therefore, the medium was injected without any further treatment, while the anterior pituitaries were homogenized with medium "199" and injected as such. Prolactin activity was expressed as IU per 100 mg of anterior pituitary incubated. The details of the assay procedure and statistical treatment have been described elsewhere (6).

Experiment 1. Synthesis and Release of Prolactin In Vitro

Anterior pituitaries from groups of 3 rats each were divided into halves, weighed, transferred to a flask containing 2 ml of medium "199", and incubated for 2 hours. At the end of this period, the medium and the incubated anterior pituitaries were separated and assayed independently for prolactin activity. In order to determine initial prolactin content, anterior pituitaries from either 5 or 6 rats of the same age group were removed at the same period, pooled, weighed and assayed for prolactin activity.

The results are summarized in Table 1. An average of 0.8 IU of prolactin was released into the medium per 100 mg of anterior pituitary (AP) incubated. Anterior pituitaries at the end of indubation contained an average of 2.7 IU

prolactin per 100 mg AP. The prolactin content of the medium and the incubated AP together was 3.5 IU per 100 mg AP as compared with 1.3 IU per 100 mg AP present at the beginning of the experiment. This represents a 169% increase in prolactin over that contained in freshly excised AP. The results of this experiment show that net synthesis and release of prolactin can occur during 2 hours of incubation. We have also observed a gradual increase in the total amount of prolactin released into the medium during a 4 hour period of incubation (unpublished).

Experiment 2. Effect of Homogenate of Hypothalamus on Prolactin Secretion In Vitro

Anterior pituitaries from 6 rats were cut into halves, weighed and placed into 2 flasks, each containing 2 ml of medium "199". Each flask contained 6 halves from the same 6 pituitaries. Preliminary studies showed that under these conditions, the incubated anterior pituitaries released the same amounts of prolactin in both flasks. The hypothalamus (including the median eminence) from the same 6 rats was homogenized and added to the experimental flask, and an equivalent volume of medium "199" was added to the control flask. At the end of 2 hours incubation, the medium and the incubated pituitary halves were removed from the control and experimental flasks, and assayed against each other in the same pigeons for prolactin activity.

The results are summarized in Table 2. When anterior pituitaries were incubated with rat hypothalamic homogenate,

significantly less prolactin was released into the medium in all 14 flasks than in the corresponding control flasks not containing hypothalamic homogenate. An average inhibition of prolactin release of 75.4% was observed. Similarly, the prolactin content of the anterior pituitary halves incubated together with hypothalamic homogenate was significantly less (51.8%) than in the corresponding control anterior pituitary halves. These results show that hypothalamic homogenate can inhibit prolactin synthesis and release *in vitro*.

Experiment 3. Effect of Acid Extracts of Hypothalamus and Cerebral Cortex on Prolactin Secretion In Vitro

The hypothalami from 36 rats were removed, pooled, weighed, homogenized with 2.4 ml 0.1N HCl, and centrifuged (12000 x G) for 30 minutes at 4°C. The supernatant was added to medium "199" and the pH was adjusted to 7.4 by adding $NaHCO_3$ solution. The final volume was made up so that each ml of medium contained the acid extract from 3 rat hypothalami. Equivalent amounts of acid extract of cerebral cortex were similarly prepared. To each of the experimental flasks, 2 ml of the medium containing acid extract of hypothalamus or cerebral cortex was added, and to each of the corresponding control flasks, 2 ml of medium "199" was similarly prepared but without any tissue extract. At the end of the incubation period, the medium and the incubated anterior pituitary halves were removed and assayed separately for prolactin activity.

The results are summarized in Table 3. When 3 rat anterior pituitaries were incubated with acid extract from 4 rat hypothalami, an average of 54% less prolactin was found in the medium and 36% less in the incubated anterior pituitaries respectively than in the corresponding controls. Greater inhibition (72% and 61%) of prolactin release into the medium and of anterior pituitary prolactin content (68% and 56%) was observed when anterior pituitaries were incubated with acid extract from 6 rat hypothalami. Acid extract of cerebral cortex had no effect on either prolactin release into the medium or anterior pituitary prolactin content. These results demonstrate that hypothalamic acid extract can inhibit synthesis and release of prolactin by the anterior pituitary *in vitro*.

Experiment 4. Effect of Incubation per se on Prolactin Activity

A. A standard NIH ovine prolactin (15 IU/mg) preparation was added to 2 ml of medium "199" in each of 6 flasks. Three flasks were incubated for 2 hours, and 3 other flasks served as controls and were not incubated. At the end of 2 hours, pooled medium from the control flasks was assayed against pooled medium from the experimental flasks.

B. Anterior pituitaries from 6 rats were divided into halves, transferred to a flask containing 4 ml of medium "199", and incubated for 2 hours. At the end of incubation, the anterior pituitary halves were removed and the medium

was equally divided and placed into 2 flasks. The medium in each control flask was not incubated, while the medium in each experimental flask was incubated again for 2 hours. Media from 3 control and 3 experimental flasks were each pooled and assayed for prolactin activity.

The results are summarized in Table 4. No significant difference was observed in prolactin content between incubated and non-incubated medium containing NIH ovine prolactin. Similarly, incubation had no effect on rat pituitary prolactin. This shows that under the above experimental conditions, incubation *per se* neither activates nor inactivates NIH ovine or rat prolactin.

Experiment 5. Effect of Homogenate or Acid Extract of Hypothalamus on Activity of Prolactin Preparations

A. Standard NIH ovine prolactin preparation was added to flasks containing 2 ml of medium "199" containing acid extract from 6 rat hypothalami. At the end of 2 hours incubation, the medium from the 3 control flasks and the 3 experimental flasks were pooled and assayed for prolactin activity.

B. Anterior pituitaries from 6 rats were divided into halves, transferred to a flask containing 4 ml of medium "199", and incubated for 2 hours. At the end of incubation, the medium was separated from the pituitary halves, and was divided equally and placed into 2 flasks. Homogenate or acid extract from 6 rat hypothalami was added to an experimental

flask, and equivalent amounts of medium "199" was added to the control flask. Each pair of flasks was further incubated for 2 hours, and the medium was assayed separately, or by combining media from either 3 control or the corresponding 3 experimental flasks.

The results in Table 5 indicate that (a) incubation of NIH ovine prolactin with rat hypothalamic acid extract does not activate or decrease prolactin activity (b) the activity of rat pituitary prolactin released into the medium is not increased or diminished when incubated with hypothalamic homogenate or acid extract of hypothalamus and (c) homogenate or acid extract of hypothalamus does not modify the pigeon crop response to prolactin.

Experiment 6. Effects of Neuropharmacological Agents on Prolactin Release In Vitro

Anterior pituitaries from 6 rats were cut into halves, weighed and placed into 2 flasks, each containing 2 ml of medium "199". Each of the two flasks contained 6 halves from the same 6 pituitaries. Acetylcholine iodide,[4] epinephrine,[4] l-norepinephrine-bitartrate,[5] serotonin creatinine sulfate,[6] histamine acid phosphate,[7] substance P (9U/mg), oxytocin (Pitocin[8]), synthetic lysine vasopressin,[9] and synthetic arginine vasopressin[9] were added to experimental flasks, while the corresponding control flasks contained an equivalent volume of medium "199". Solutions of these agents were brought to pH 7.4 before placing them into the flasks. The

dosages of these agents are given in Table 6. Each agent was added once at the beginning and again one hour after incubation was begun.

At the end of 2 hours incubation, the media from the control and the experimental flasks were removed and assayed against each other for prolactin activity. In most cases, media from 3 control flasks and the corresponding 3 experimental flasks were pooled separately and assayed, with the exception of the incubations with acetylcholine iodide, epinephrine and serotonin creatinine sulfate. In these, the medium from each of the flasks was assayed separately. The commercial preparation of oxytocin (Pitocin) solution contained 0.5% chlorobutanol as a preservative. Therefore, an equivalent amount of chlorobutanol was added to the control flask.

The results (Table 6) show that incubation of anterior pituitary with all the neuropharmacological agents employed, at the dose levels used, had no significant effect on prolactin release into the medium. This indicates that the hypothalamic inhibition of anterior pituitary prolactin release *in vitro* is not due to any of the above neuropharmacological agents.

Experiment 7. Effects of Neuropharmacological Agents on Pigeon Crop Response to NIH Ovine Prolactin

This experiment was carried out to determine whether the neuropharmacological agents employed above could modify

the pigeon crop response to NIH ovine prolactin by acting at the crop level. Standard NIH ovine prolactin was added to medium "199". The medium was divided into 2 equal halves, and acetylcholine iodide, epinephrine, serotonin creatinine sulfate, histamine acid phosphate, oxytocin (Pitocin) or Pitressin[8] was added to each half in the amounts shown in Table 7. The corresponding control halves were mixed with an equivalent volume of medium "199". Both the control and the experimental media were assayed in the same pigeons for prolactin activity. The results (Table 7) show that these neuropharmacological agents, at the dose levels used, did not modify the pigeon crop response to NIH ovine prolactin.

Experiment 8. Tests for Prolactin Activity in Homogenates and Acid Extracts of Hypothalamus and Cerebral Cortex

The hypothalamus from 6 rats were homogenized with 2 ml of medium "199". The homogenate was injected into 5 pigeons (dose 0.1 ml of homogenate/pigeon/day for 4 days). Media "199" containing acid extracts of hypothalamus or cerebral cortex were prepared as described in Experiment 3 and assayed separately for prolactin activity in 10 pigeons in doses of 0.1 ml of medium/pigeon/day for 4 days. At the dose levels used, no prolactin activity was detected in homogenate or acid extracts of hypothalamus or cerebral cortex.

DISCUSSION

The present study shows that synthesis and release of prolactin by the isolated anterior pituitary can occur during a 2 hour period of incubation, and that these processes are inhibited by an homogenate or acid extract of rat hypothalamus. An acid extract of cerebral cortex had no effect on prolactin synthesis or release, suggesting that this portion of the brain does not contain the active factor. Inhibition of prolactin secretion cannot be attributed to proteolytic descruction of prolactin, since incubation of ovine or rat hypothalamus did not decrease the biological activity of these preparations. Also, homogenate or acid extract of rat hypothalamus did not influence the pigeon crop response to prolactin.

The prolactin inhibiting factor(s) does not appear to be any of the known neuropharmacological substances present in the hypothalamus (7, 8), since incubation of the anterior pituitary with acetylcholine iodide, epinephrine, l-norepinephrine bitartrate, serotonin creatinine sulfate, substance P, oxytocin (Pitocin) or synthetic lysine and arginine vasopressin at the dose levels used, had no effect on prolactin release. Previously, we reported that injections of acetylcholine, epinephrine, norepinephrine or serotonin into estrogen-primed rats or rabbits initiated mammary secretion (9-11). The present study shows that the action of these drugs in stimulating prolactin secretaion is not by way of a

direct action on the anterior pituitary but through other mechanisms. It is possible that they act by inhibiting synthesis or release of the prolactin inhibiting factor(s) of the hypothalamus. It has been suggested that oxytocin may be responsible for inducing prolactin release from the anterior pituitary (12), but most evidence from *in vivo* and *in vitro* studies (13, 14) does not support this view.

Acid extracts of the hypothalamus apparently contain the FSH, LH, TSH, and ACTH releasing factors, as well as prolactin inhibiting factor(s). Intrapituitary infusions of acid extracts of median eminence tissue have been reported to induce release of gonadotropin as determined by ovulation in the rat or the rabbit (15, 16). Gonadotropic activity of the medium from anterior pituitary cultures was markedly increased following addition of acid extract of the hypothalamus to a culture medium (17). An acid extract of rat stalk median eminence tissue elicited LH release in rats as measured by ovarian ascorbic acid depletion (18). An acid extract of bovine median eminence tissue and a polypeptide extracted from dog hypothalamus stimulated TSH release (19, 20). Similarly, injections of acid extracts of hypothalamus stimulated ACTH release from the pituitary in the rat (21). In all these studies, acid extracts of cerebral cortical tissue were ineffective. The chemical nature of these hypothalmaic factors is not presently well defined, although the corticotropin and TSH releasing factors apparently are polypeptides (22-24).

Short term incubation of the anterior pituitary, as originally described by Saffran and Schally (3), has been used by many workers for studying release or synthesis of anterior pituitary hormones such as ACTH (25, 26), TSH (23, 24) and FSH (27). Thus incubation of beef anterior pituitary slices with S^{35} labelled methionine resulted in incorporation of the labelled amino acid into prolactin (28). When rat pituitaries were incubated with C^{14}-phenylalanine, the rate of incorporation into ACTH was found to be linear for 4 hours (29). After incubation of female rat anterior pituitaries for 2 hours, the total quantity of FSH present in medium and incubated pituitary together was found to be significantly more than in the control anterior pituitaries which were not incubated (27). It is possible that under our experimental conditions, incubation of anterior pituitary might have resulted in activation of prolactin in the pituitary from an inactive form, or that activation and synthesis might have occurred together. Arguments in favor of such activation have been presented for prolactin (30) and for ACTH (31), but no direct proof is presently available to support this view.

Certain bovine STH and ovine prolactin preparations have been reported to undergo degradation upon standing in alkaline buffers (32). However, incubation _per se_ of NIH ovine prolactin or of rat pituitary prolactin in the present study did not result in any detectable loss of activity.

Similarly, no evidence for destruction of rat ACTH was observed during a one hour period of incubation nor when ACTH was incubated together with anterior pituitary tissue (25). Neither activation nor inactivation of rat pituitary FSH released into the medium was observed during 2 hours of incubation (27). The presence of proteinase in a purified preparation of sheep ICSH (LH) was demonstrated only after prolonged incubation for 6 to 24 hours (33). In our studies homogenate or acid extract of hypothalamus did not activate or inactivate NIH ovine prolactin or rat prolactin. It has previously been reported that incubation of bovine prolactin with a variety of rat tissues reduced the biological activity of the prolactin; however, brain tissue had no effect (34). It has been reported that proteolytic digestion of bovine prolactin (35) and STH (36, 37) can be carried out to the extent of 15 - 29% and 11 - 25%, respectively, without loss of detectable biological activity. It is possible that under our experimental conditions limited degradation of prolactin may have occurred without appreciable loss of biological activity.

Earlier we reported that in estrogen-primed rats, injections of hypothalamic homogenate from rats, which presumably contains all the factors which stimulate secretion of the anterior pituitary hormones as well as the prolactin inhibiting factor(s), initiated mammary secretion (38). It should be recognized that initiation of lactation is not a

specific reaction to prolactin alone, and is influenced by other hormones, particularly ACTH and adrenal cortical steroids. ACTH, cortisone or cortisol have been reported to initiate mammary secretion in virgin rats, estrogen-primed or pregnant rats, and rabbits (See 39). The lactation-inducing effect of hypothalamic homogenate *in vivo* may therefore be due to the presence of corticotropin-releasing factor (CRF), since CRF has been shown to be a potent stimulator of mammary secretion in estrogen-primed rats (2). In addition the hypothalamus contains factors which stimulate TSH (19, 20, 23, 24) and STH (40) secretion by the anterior pituitary, both of which may exert favorable effects on mammary secretion (2). The possibility should also be considered that *in vivo* induction of mammary secretion by hypothalamic homogenate may be nonspecific in nature, since many nonspecific agents induce pseudogregnancy (2) or initiate mammary secretion (41).

Several procedures have demonstrated that removal of hypothalamic regulation of the anterior pituitary results in increased prolactin secretion, including placement of certain hypothalamic lesions, pituitary transplantation, administration of certain CNS depressant drugs, and *in vitro* cultures of anterior pituitaries (2). The concept has developed that the hypothalamus chronically inhibits prolactin secretion by way of its neurovascular linkage to the anterior pituitary (42). The results of the present *in vitro* experiments lend

support to this concept and indicate that the rat hypothalamus contains a factor(s) which inhibits prolactin synthesis and release.

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FOOTNOTES

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3. Difco Laboratories, Detroit, Michigan.
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TABLE 1. Prolactin Content of Fresh AP*, and of Medium and AP After 2 Hours Incubation

Assay Material	Total No. of APs	No. of Assays	Total No. of Pigeons	Prolactin IU/100 mg AP
Freshly Excised AP	61	11	55	1.3 ± 0.1**
Medium	69	23	92	0.8 ± 0.1
Incubated AP		23	92	2.7 ± 0.4
Medium + Indubated AP	69	--	--	3.5*** ± 0.3

* AP = Anterior Pituitary

** Mean ± standard error of mean

*** Prolactin content of combined medium and incubated AP is about 169% greater than in freshly excised AP.

TABLE 2. Effect of Homogenates of Hypothalamus on Prolactin Secretion *In Vitro*

		Medium				Incubated AP*		
		Prolactin: IU/100 mg AP**				Prolactin: IU/100 mg AP**		
Flask Pair No.	No. of Pigeons	Control (C)	Experimental≠ (E)	% Inhibition	No. of Pigeons	Control (C)	Experimental≠ (E)	% Inhibition
1	4	1.52	0.43	71.7	4	4.52	1.15	74.6
2	4	1.25	0.38	69.6	4	3.95	2.10	46.8
3	4	0.46	0.02	95.7	4	1.19	0.83	30.3
4	4	1.56	0.10	93.6	4	2.86	0.75	73.8
5	4	0.34	0.13	61.8	4	1.69	1.00	40.9
6	4	0.66	0.02	97.0	4	1.52	1.15	24.4
7	4	0.35	0.02	94.3	4	4.47	1.30	70.9
8	4	1.63	0.44	73.0	4	2.40	1.69	29.6
9	4	2.01	1.00	50.3	4	1.96	0.65	66.8
10	4	1.19	0.52	56.3	4	1.93	1.25	35.2
11	4	1.84	0.36	80.3	4	2.83	1.15	59.4
12	4	2.86	0.93	67.5	4	2.66	1.29	51.5
13	4	1.63	0.37	77.3	4	3.03	1.39	54.1
14	4	1.25	0.41	67.2	4	1.73	0.58	66.5
				75.4≠≠ ±4.1				51.8≠≠ ±4.7

* AP = Anterior Pituitary
** C *vs* E: $P < 0.01$

≠ Incubated with homogenates from 6 rat hypothalami
≠≠ Mean ± standard error of mean

TABLE 3. Effect of Acid Extract of Hypothalamus and Cerebral Cortex on Prolactin Secretion *In Vitro*

No. Pairs of Flasks	Medium: No. of Pigeons	Medium: Prolactin IU/100 mg AP: Control (C)	Medium: Prolactin IU/100 mg AP: Experimental (E)	Medium: % Inhibition	Medium: P CvsE	Incubated AP/: No. of Pigeons	Incubated AP/: Prolactin IU/100 mg AP: Control (C)	Incubated AP/: Prolactin IU/100 mg AP: Experimental (E)	Incubated AP/: % Inhibition	Incubated AP/: P CvsE
				Acid Extract of Hypothalamus						
3	8	5.9 ±0.4	2.7* ±0.3	54	< 0.01	8	5.0 ±0.3	3.2// ±0.4	36	< 0.05
3	8	8.1 ±0.5	2.3** ±0.3	72	< 0.01	8	7.8 ±0.4	2.5 ±0.3	68	< 0.01
3	8	4.7 ±0.4	1.8** ±0.3	61	< 0.01	8	5.6 ±0.6	2.5 ±0.3	56	< 0.01
				Acid Extract of Cerebral Cortex						
3	8	3.3 ±0.2	3.1 ±0.4	--	> 0.30	8	4.0 ±0.9	5.1 ±0.7	--	> 0.20
3	8	5.8 ±0.3	5.9 ±0.2	--	> 0.40	9	4.9 ±0.5	4.2 ±0.8	--	> 0.20

* Each experimental flask contained acid extract from 4 rat hypothalami

** Each experimental fask contained acid extract from 6 rat hypothalami

/ AP = Anterior Pituitary

// Mean ± standard error of mean

TABLE 4. Effect of 2 Hours Incubation on Prolactin Activity

No. of Flasks	Treatment	No. of Pigeons	Prolactin IU/ml Medium	P
		NIH Ovine Prolactin		
3	Not Incubated	8	0.35±0.08*	>0.20
3	Incubated	8	0.33±0.06	
		Rat Pituitary Prolactin (Released into Medium)		
3	Not incubated	8	0.09±0.02	>0.30
3	Incubated	8	0.10±0.02	

*Mean ± standard error of mean

TABLE 5. Effect of Homogenate or Acid Extract of Hypothalamus on Activity of Prolactin Preparations

No. Pairs of Flasks	No. of Assays	Total No. of Pigeons	Treatment	Prolactin: IU/ml Medium		P
				Control (C)	Experimental (E)	C vs E
			NIH Ovine Prolactin			
6	2	16	Hypothalamic Acid Extract	0.36 ±0.03	0.33 ±0.04	>0.40
			Rat Pituitary Prolactin (Released into Medium)			
6	6	24	Hypothalamic Homogenate	0.18 ±0.02	0.17 ±0.03	≃0.50
6	2	16	Hypothalamic Acid Extract	0.27 ±0.05	0.30 ±0.06	>0.30

TABLE 6. Effects of Neuropharmacological Agents of Prolactin Release *In Vitro*

Treatment	Dose/ml Medium (2x)	No. Pairs of Flasks	No. of Assays	Total No. of Pigeons	Prolactin: IU/100 mg AP* Control (C)	Prolactin: IU/100 mg AP* Experimental (E)	P C vs E
Acetylcholine Iodide	100 µg	6	6	24	1.2 ±0.2	1.1** ±0.3	>0.30
Epinephrine	40 µg	6	6	24	0.9 ±0.1	0.8 ±0.2	>0.20
L-Norepinephrine -Bitartrate	160 µg	6	2	16	1.4 ±0.3	1.5 ±0.1	>0.30
Serotonin Creatinine Sulfate	30 µg	5	5	26	1.9 ±0.4	2.2 ±0.2	>0.20
Histamine Acid Phosphate	500 µg	6	2	16	4.2 ±0.4	3.7 ±0.6	>0.20
Substance P	50 µg	6	2	16	1.1 ±0.3	1.4 ±0.4	>0.30
Oxytocin (Pitocin)	1 IU	6	2	16	1.5 ±0.3	1.8 ±0.4	>0.20
Lysine Vasopressin	1 IU	6	2	16	3.4 ±0.6	3.8 ±0.7	>0.20
Arginine Vasopressin	1 IU	6	2	16	2.9 ±0.6	2.4 ±0.5	>0.30

* AP = Anterior Pituitary
** Mean ± Standard error of mean

TABLE 7. Effects of Neuropharmacological Agents on Pigeon Crop Response to NIH Ovine Prolactin

Treatment	Dose/ml Medium	No. of Pigeons	Prolactin: IU/ml Medium		P
			Control (C)	Experimental (E)	C vs E
Acetylcholine iodide	200 µg	6	0.38 ±0.04	0.35 ±0.03	>0.20
Epinephrine	80 µg	6	0.42 ±0.05	0.47 ±0.06	>0.20
Serotonin Creatinine Sulfate	60 µg	6	0.36 ±0.07	0.43 ±0.05	>0.30
Histamine Acid Phosphate	1 mg	6	0.43 ±0.06	0.38 ±0.05	>0.20
Oxytocin (Pitocin)	2 IU	6	0.40 ±0.06	0.44 ±0.07	>0.40
Pitressin	2 IU	6	0.39 ±0.02	0.42 ±0.06	>0.20

II. ADDENDUM

The following additional studies were carried out after the publication of the preceeding paper.

A. Effect of Bradykinin on Pituitary Prolactin Release In Vitro

Bradykinin is present in various tissues of the body, including the brain. Since it is found in very high concentration in the hypothalamus (1, 2), it was decided to determine its effect on prolactin release *in vitro*. The method used was the same as described under Experiment 6.

The results of the present experiment (Table II-a) show that bradykinin, at the dose level used, had no significant effect on prolactin release. This would incidate that bradykinin is not the prolactin inhibiting factor in the hypothalamus.

B. Effect of Dialyzed Rat Hypothalamic Extract on Pituitary Prolactin Release In Vitro

An acid extract of rat hypothalamus inhibited pituitary prolactin release *in vitro*, as shown in the previous publication. It was decided, therefore, to determine the effect of dialysis on prolactin inhibiting activity by rat hypothalamic extract. The method used was the same as described under Experiment 3. Rat hypothalamic acid extract was neutralized with NaOH, dialyzed for 8 hours against 0.9 per cent Nacl at 4°C,and added to the experimental medium in amounts equivalent to 3 hypothalami/ml.

The results of this experiment (Table II-b) show that, under our experimental conditions, prolactin inhibiting capacity of the acid extract of the hypothalamus was lost following dialysis. This suggests that the prolactin inhibiting factor (PIF) of the hypothalamus is dialyzable, and is probably a small molecule. This is in agreement with work on the other hypothalamic factors (CRF, LRF, TRF, GRF) which indicates that they are probably all polypeptides (3).

C. Effects of Acid Extracts of Hypothalami from Different Species on Pituitary Prolactin Release In Vitro

An acid extract of rat hypothalamus inhibited synthesis and release of pituitary prolactin *in vitro*, as shown in the proceeding paper. It was therefore of interest to determine whether hypothalami from other species could similarly inhibit pituitary prolactin release *in vitro*.

Lyophilized powders of pig, sheep, and bovine hypothalamic extracts were supplied by Dr. Steelman (Merck, Sharpe, and Dohme, Rahway, N. J.). Hypothalami or cerebral cortical tissues from these species were removed as quickly as possible and immediately frozen. The tissues were homogenized with 5 ml of 0.1N Hcl/gm of brain tissue (wet weight), centrifuged (12000 x G) for 30 minutes, at 4°C. The supernatant was neutralized with NaOH and lyophilized. The lyophilized powders were tested for prolactin inhibiting activity by the method described in *Experiment 3* in the preceding paper.

The results in Table II-c show that acid extracts of pig, sheep, or bovine hypothalami inhibited pituitary prolactin release *in vitro*. An acid extract of cerebral cortical tissue was ineffective. This indicates that hypothalami from these species contain the prolactin inhibiting factor (PIF).

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TABLE II-a. Effect of Bradykinin on Pituitary Prolactin Release *In Vitro*

Dose/ml Medium (2x)	No. Pairs of Flasks	No. of Assays	Total No. of Pigeons	Prolactin: IU/100 mg AP* Control (C)	Prolactin: IU/100 mg AP* Experimental (E)	P CvsE
50 µg	4	2	16	1.4** ±0.2	1.6 ±0.1	>0.20

*AP = Anterior Pituitary

**Mean ± Standard error of mean

TABLE II-b. Effect of Dialyzed* Rat Hypothalamic Acid Extract (HAE) on Pituitary Prolactin Release *In Vitro*

Treatment (Experimental)	No.of Flask Pairs	No. of Pigeons	Medium: Prolactin: IU/100 mg AP Control (C)	Medium: Prolactin: IU/100 mg AP Experimental (E)	% Inhibition	C vs E t	C vs E p
HAE, dialyzed	2	8	1.3	1.1	--	1.2	>0.20
	2	8	1.2	1.0	--	1.7	>0.10
HAE, not dizlyzed	2	8	0.9	0.4	56	4.6	<0.01
	2	8	1.1	0.4	64	3.2	<0.01

* Rat hypothalamic acid extract was prepared as in Experiment 3, neutralized with IN HaoH, dialyzed for 8 hours against 0.9% Nacl at $4^{\circ}C$, and added to the experimental medium in amounts equivalent to 3 hypothalami/ml.

TABLE III-c. Effects of Acid Extracts of Hypothalamit from Different Species of Prolactin Release by Rat Anterior Pituitary *In Vitro*

Treatment* (Experimental)	No. Pairs of Flasks	Total No. Pigeons	Medium: Prolactin: IU/100mg AP** Control (C)	Medium: Prolactin: IU/100mg AP** Experimental (E)	Prolactin Release % Inhibition	C vs E t	C vs E p
Pig Hypoth. Ext.	4	16	1.72	0.46	72.9±4.8/	4.6	<.001
Sheep Hypoth. Ext.	4	15	0.74	0.19	74.6±7.5	4.7	<.001
Bov. Hypoth. Ext.	4	15	1.30	0.25	84.5±3.7	5.4	<.001
Bov. Cortex. Ext.	6	16	1.20	1.00	--	0.9	>0.30

*20 mg/ml, lyophilized extract

/ Mean ± Standard error mean

**AP = Anterior Pituitary

III. *IN VIVO* AND *IN VITRO* PROLACTIN SECRETION BY TRANSPLANTED RAT "MAMMOTROPIC" PITITUARY TUMORS

SUMMARY

The capacity of the functional, transplanted, autonomous, rat pituitary "mammotropic" tumor (Furth, MtT. F_4) to elaborate prolactin *in vivo* and *in vitro* was investigated. Prolactin content of transplanted anterior pituitary (AP) and MtT. F_4 were 1.6% and 4.8%, respectively, of that present in the AP *in situ*. Considerable amounts of prolactin could be detected in the blood plasma of rats bearing MtT. F_4, the levels being higher in rats with larger than with smaller tumors. Prolactin ctivity could not be detected by the assay method employed, in the blood plasma of normal cycling, estrogen-treated, pregnant or lactating rats or in rats bearing a single AP-transplant underneath the kidney capsule. The amount of prolactin released into the medium by MtT. F_4 during 3 days of culture was 8-31 times greater than that present initially, indicating active synthesis and release of prolactin by the tumor. These results show that MtT. F_4, like an AP-transplant removed from hypothalamic inhibition, synthesises prolactin and releases it rapidly, leaving little in the tissue itself.

The pioneer work of Furth has led to the identification and isolation of variety of transplantable hormone-secreting rat and mouse pituitary tumors (7). One of these is a chromophobic, autonomous rat pitituary "mammotropic" tumor, strain F_4 (MtT. F4) originally induced by estrogen (6, 9). Following transplantation in the rat this tumor appears to elaborate prolactin, STH and ACTH, but no FSH-LH or TSH (2, 3, 7, 9, 21).

Animal cell cultures rarely perform differntiated functions characteristic of the tissue of origin; this has been especially true of cultures of endocrine tissue. However, our laboratory (13, 14) as well as others (11, 15) have recently observed that cultures of rat anterior pituitary (AP) actively secrete prolactin in the medium; whereas elaboration of all other hormones is markedly diminished. The purpose of this study was to determine the capacity of MtT. F_4 to secrete prolactin *in vivo* and *in vitro* as compared to that of normal rat AP. A report of the present work has previously been published in abstract form (19).

MATERIALS AND METHODS

Animals. Mature, virgin female, highly inbred Fischer[1] rats of the CDF strain, 3-4 months old, were used in this study. The rats were maintained in a temperature controlled (75 $\pm$ 1°F) and artifically illuminated (14 hr/day) room. They were fed *ad libitum* on Wayne Lab Blox pellets supplemented

with canned (Dash) dog food. We have observed that White King Squabs 5-8 weeks old are more sensive for prolactin assays than White Carneau pigeons of the same age. Therefore, the former birds were used for all assays.

Prolactin assay. The sensitive intradermal pigeon-crop assay method was utilized. The test material was injected over one side of the crop-sac, while standard NIH prolactin was injected over the other side of the crop-sac in the same pigeons. Prolactin activity was expressed as IV/100 mg of tissue (wet weight) or IU/100 ml plasma. The details of the assay procedure has been described elsewhere (14).

Transplantation and assays of MtT. F_4 and AP. Serial transplantation of MtT. F_4 was carried out in our laboratory. The tumor transplantations were made by injecting rats subcutaneously in the back of the neck 0.1 ml of tumor mince in an equal volume of medium 199 (pH7.4) containing streptomycin (100 µg/ml) and penicillin G potassium (100 U/ml). In some rats, injections were made at two sites, resulting in the formation of two tumors. The tumors were palpable within 4-5 weeks. When the tumor reached 1-2 cm in diameter, it was removed and a portion of it was used for cultures and for histological examination; another part of it was weighed, homogenized with physiological saline and assayed for prolactin activity. A total of six tumors (passage 41) from six rats were assayed separately for prolactin activity.

Intact rats were transplanted, underneath the kidney capsule, with one AP from each of the donor rats of the same strain and approximately of the same age group. Ten days later the AP-transplants were removed, trimmed of adhering tissue under a disecting microscope, pooled from 10 rats each, weighed, homogenized with saline and assayed for prolactin activity. Some of the AP-transplants were used for histological examination. The AP from normal cycling rats was also removed, homogenized with saline and assayed for prolactin activity.

Collection of rat blood and assay for prolactin activity. Rats were anesthetized with ether and blood was collected in a heparinized syringe from the posterior abdominal artery. After centrifugation, the plasma was dialyzed, lyophilized and assayed for prolactin activity. Rats in different states were used as blood donors, as indicated in Table 2.

Cultures of MtT. F_4. A modification of the watch glass technique (5) was used, in which sterile, plastic (3.5 cm x 1 cm) Petri dishes[3] were employed. Platforms were formed from stainless steel mesh rectangles (1 cm x 2.3 cm), bent at each end to make a platform 4 mm high. These were placed in Petri dishes with 3 ml of culture medium 199[4], containing 2 U insulin,[5] 100 U penicillin G potassium and 100 µg streptomycin per ml. For some of

the cultures, calf, rabbit or horse serum[6]; or calf serum and chicken embryo extract[7] were added to medium 199, as shown in Table 3.

The MtT. F_4 was removed and placed in a Petri dish (10 cm x 1.5 cm) containing a few drops of medium. The tumor was cut into small pieces, each about 2-3 mm in diameter, and two such explants were placed on a strip of lens paper (1.5 cm x 3 cm), which then was placed on top of the platform inside the Petri dish. The tumor explants were cultured for 3 or 6 days at 35°C in 95% O_2 - 5% CO_2 atmosphere. A total 148 cultures using 6 MtT. F_4 were carried out. At the end of the culture period, medium from 8-10 culture dishes was pooled, dialyzed, lyophilized and assayed for prolactin activity. The explants were fixed in Bouins fluid, sectioned at 6 μ and stained with hematoxylin and eosin. The details of the culture procedure have been described elsewhere (14).

In order to determine whether prolactin activity could be detected in medium without MtT. F_4 cultures, medium 199 alone or medium 199 containing 20% calf, rabbit or horse serum was incubated for 3 days. At termination, the medium was dialyzed, lyophilized and assayed for prolactin activity. Also pregnant rat mammary gland or pigeon crop gland was cultured for 3 days and the medium was similarly treated and assayed for prolactin. In another experiment, standard NIH ovine prolactin was added to medium 199 or medium 199

containing 20% calf serum, and incubated for 3 days to ascertain the effects of incubation on prolactin activity.

RESULTS

The average prolactin activity (Table 1) of MtT.F_4 and AP-transplants were found to be 0.06 and < 0.02 IU/100 mg tissue (wet weight), respectively, as compared to 1.2 IU/100 mg of fresh AP. This represents 4.8 and < 1.6 per cent, respectively, of the content of prolactin present in fresh AP.

No prolactin activity was detected in the rat blood plasma (Table 2) from (a) 3-4 months old mature female cycling rats (b) female rats treated daily with 10 µg estradiol for 10 days (c) 11-12 day pregnant rats (d) 10 day postpartum, lactating rats, and (e) rats with a single AP-transplant of 10 days duration. However, considerable amounts of prolactin activity could be detected in the blood plasma of rats bearing MtT.F_4. The plasma prolactin activity was found to be higher in rats bearing larger as compared to those with smaller tumors.

Explants of MtT.F_4 actively secreted prolactin in the culture media (Table 3). The amounts of prolactin secreted during 3 days of culture (0.5-1.9 IU/100 mg MtT.F_4) was found to be 8-31 times more than the initial content of MtT.F_4 (0.06 IU/100 mg MtT.F_4). Prolactin was also detected in the medium of monolayer cultures of MtT.F_4 for 22 days (unpublished). Neither indubation of the medium alone for three days without MtT.F_4, nor medium from cultures of rat

mammary gland or pigeon crop gland showed prolactin activity. Prolactin potency was not altered when standard NIH ovine prolactin was incubated for 3 days in medium 199 with or without 20% calf serum.

Histological examination of explants at the end of the culture period showed variable degree of maintenance. The best maintenance of explants, comparable to uncultured MtT.F4, was obtained when 20% calf serum was added to medium 199 (Fig. 1 and 2). A section from an AP-transplant underneath the kidney capsule, 10 days after transplantation, is shown in Figure 3. These AP-transplants were viable and were actively secreting prolactin, as indicated by mammary growth of the hosts rats.

DISCUSSION

The prolactin content of MtT.F4 and AP-transplants is very low as compared with that in the AP *in situ*. The MtT.F4 and AP-transplant secrete considerable amounts of prolactin *in vivo*, as indicated by mammary gland stimulation or blood assays in rats with tumors. Therefore, the MtT.F4 and AP-transplant have less capacity to retain (or store) prolactin than normal AP *in situ*.

Placement of hypothalamic lesions, transection of the pituitary stalk, transplantation of the AP to noncranial sites, administrations of certain depressant drugs and cultures of AP *in vitro* have all demonstrated that the

hypothalamus exert an inhibitary effect on prolactin secretion (13). We have recently provided evidence suggesting that the hypothalamus contains a factor(s) which inhibits prolactin secretion *in vitro* (20). The decrease in the capacity of MtT.F_4 or AP-transplant to retain prolactin may be due to removal of hypothalamic inhibition. This may result in impairment of the intracellular storange mechanism, or increased permeability of the plasma membrane of the cells of the tumor and AP-transplant.

Considerable amounts of prolactin could be detected in the blood plasma of rats with a MtT.F_4. The high blood prolactin levels may be due in part to the large size of the tumor (500-1000 times larger than a normal rat AP), and it may actually secrete a smaller amount of prolactin per unit weight. The MtT.F_4 during 3 days of culture secreted 0.5-1.9 IU of prolactin per 100 mg MtT.F_4, while under similar conditions bovine and rat AP secreted 8-12 IU (Talwalker and Meites, unpublished) and 10-40 IU per 100 mg tissue respectively (13, 14). This would indicate that on a per unit weight basis, the *in vitro* capacity of MtT.F_4 to secrete prolactin is much lower than the normal AP. Nevertheless, the MtT.F_4 during 3 days of culture secreted 8-31 times more prolactin than was present initially, indicating continuous synthesis and release of prolactin. It appears that the low *in vitro* prolactin secretion by MtT.F_4 as compared with normal AP cannot be attributed to increased inactivation

of the harmone, since incubation per se of the medium containing prolactin did not alter its activity. Prolactin was not detectable by the assay procedure used, in blood plasma from normal cycling, estrogen-treated, pregnant or lactating rats, or in rats with a single AP-transplant. However, utilizing the luteotropic activity of prolactin as an assay, Wolthuis (22,23) could detect prolactin in the blood plasma of rats under similar conditions.

The MtT.F_4 was initially induced by Furth with estrogen (6, 9). It has been observed that the immediate circulatory connection with the hypothalamus is not necessary for in vivo proliferative (6), or for an in vivo and in vitro (13, 16) prolactin secretary response to estrogen. Estrogen administration in vivo has been found to deplete the rat hypothalamus of prolactin inhibiting activity (Ratner and Meites, unpublished). Thus estrogen stimulation may lead to pituitary tumor formation and increased prolactin secretion, similar to MtT.F_4, by a direct action on the AP or hypothalamus. It should be noted that transplantation alone of the AP to noncranial sites in certain strains of rats (6, 10, 12) and mice (1, 4, 8) leads to pituitary tumor formation. Thus removal of hypothalamic inhibition to the AP, by various procedures, may lead to increased prolactin secretion and formation of prolactin secreting tumors.

In conclusion, the present study shows that the MtT.F_4 synthesises and releases considerable amounts of prolactin in

vivo and in vitro although little protactin is present in the tumor tissue itself. In these aspects its behaviour is analogous to the AP when removed from hypothalamic inhibition.

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FOOTNOTES

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1. Charles River Breeding Laboratories, Brookline, Mass.
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TABLE 1. Prolactin Assays of AP, AP-Transplants, and MtT.F_4-Transplants

Tissue	No. of Donor Rats	No. of Assays	Total No. of Pigeons	Prolactin IU/100 mg. Tissue (Wet Weight)	Comparative Prolactin Content %
AP	16	4	18	1.2 $\pm$ 0.07*	100
AP-Transplant**	20	2	8	$<$ 0.02	$<$ 1.6
MtT.F_4-Transplant	6	6	30	0.06 $\pm$ 0.01	4.8

AP = Anterior Pituitary
MtT.F_4 = Mammotropic pituitary tumor, strain F_4

* Mean $\pm$ Standard error of mean

** A single anterior pituitary was transplanted underneath the kidney capsule of each rat. Ten days later the transplant was removed and assayed for prolactin activity.

TABLE 2. Prolactin Assays of Blood Plasma from Rats in Different States

Type of Donor Rat	No. of Donor Rats	No. of Assays	Total No. of Pigeons	Prolactin IU/100 ml Plasma	Remarks
Mature, female	4	4	16	0	3-4 months old
Estradiol	4	4	16	0	10 μg, 1x10 days
Pregnant	3	3	12	0	Mid-pregnancy
Lactating	5	5	20	0	10 days post-partum
AP-Transplant	8	2	10	0	10 days after single AP-transplant
MtT.F_4-Transplant	2	2	8	33	Each rat had 2 tumors (Av.diam. 2.5 cm)
MtT.F_4-Transplant	1	1	4	6.2	One tumor (Av. diam.2.4 cm)
MtT.F_4-Transplant	2	2	8	<0.4	Each rat had 2 tumor (Av.diam. about 1 cm)

AP = Anterior Pituitary

MtT.F_4 = "Mammotropic" pituitary tumor, strain F_4

TABLE 3. Prolactin Assays of Different Media from Cultures Containing MtT.F_4-Transplants

Medium	No. of Cultures	Days of Cultures	No. of Assays	Total No. of Pigeons	Prolactin IU/100 mg. MtT.F_4	Prolactin in Medium As Compared With Fresh MtT.F_4 Content
"199"	25	1-3	3	12	1.0±0.2*	x 16
"199"	10	1-3	1	4	1.9	x 31
		4-6	1	4	1.4	x 23
"199" + 20% Calf serum	47	1-3	5	20	1.0±0.20	x 16
	27	1-3	3	12	1.7±0.25	x 29
		4-6	3	12	1.2±0.22	x 20
"199" + 20% Horse serum	10	1-3	1	4	1.1	x 18
		4-6	1	4	1.0	x 16
"199" + 20% Rabbit Serum	9	1-3	1	4	0.54	x 9
		4-6	1	4	0.51	x 8
"199" + 20% Calf Serum + 5% Chicken Embryo extract	10	1-3	1	6	1.3	x 21
	10	1-3	1	6	1.0	x 16
		4-6	1	6	0.9	x 15

MtT.F_4 = Mammotropic pituitary tumor, strain F_4

* Mean ± Standard error of mean

Fig. 1. A section from transplanted "mammotropic" pituitary tumor, strain F_4 (MtT.F_4).

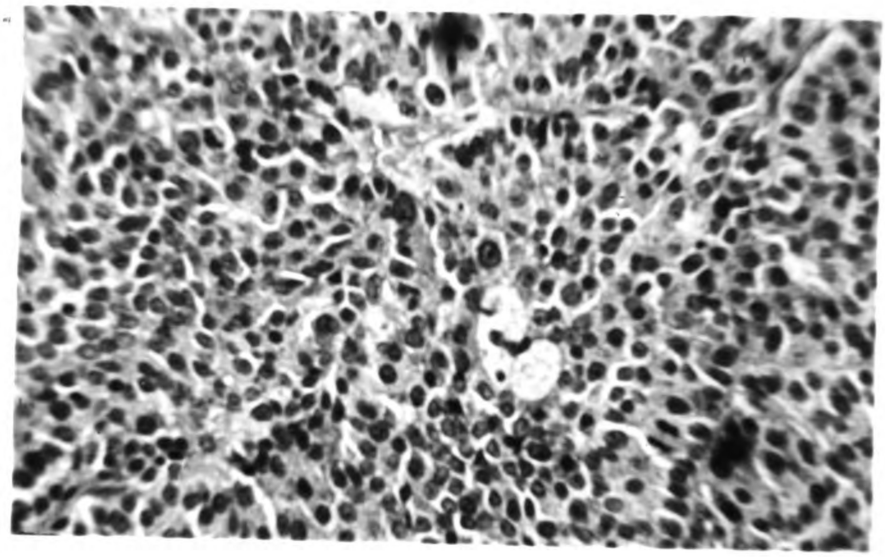


Fig. 2. A section from "mammotropic" pituitary tumor explant at the end of 3 days of culture in medium "199" containing 20% calf serum.

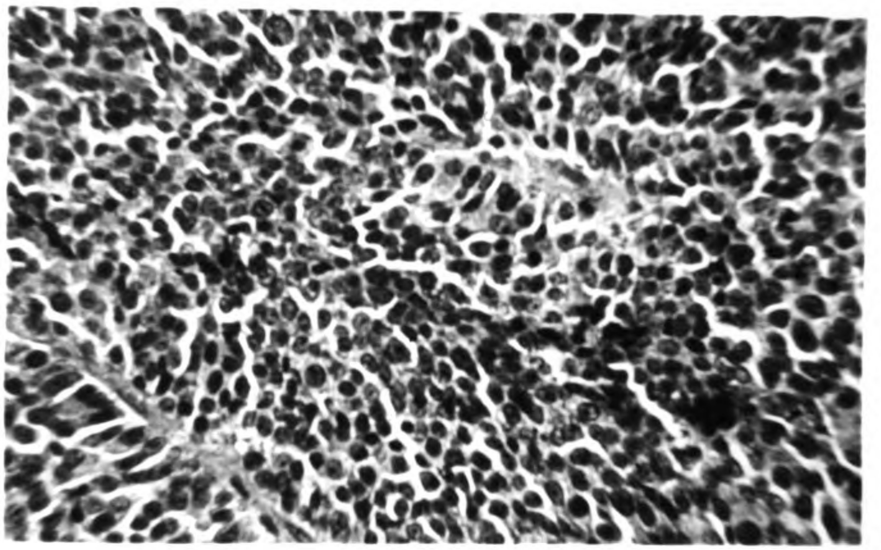


Fig. 3. A section from "mammotropic" pituitary tumor explant at the end of 3 days of culture in medium 199 without serum.

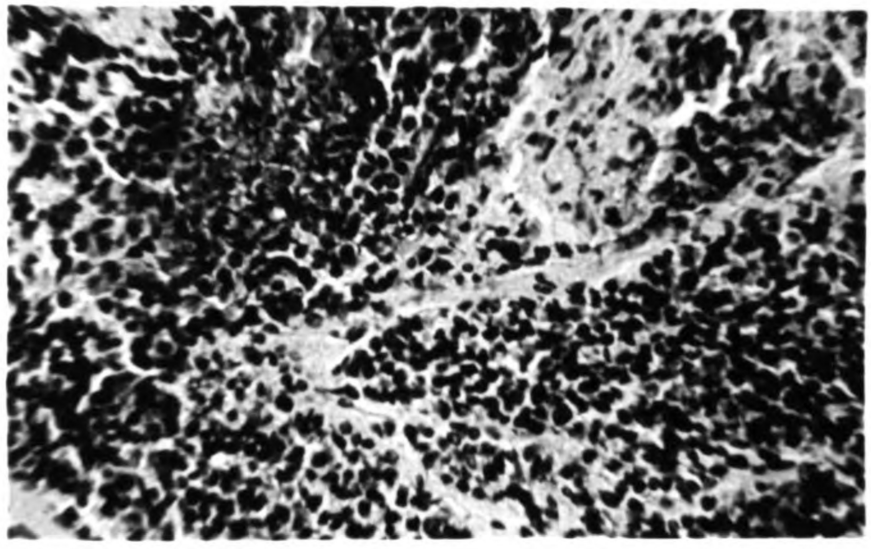
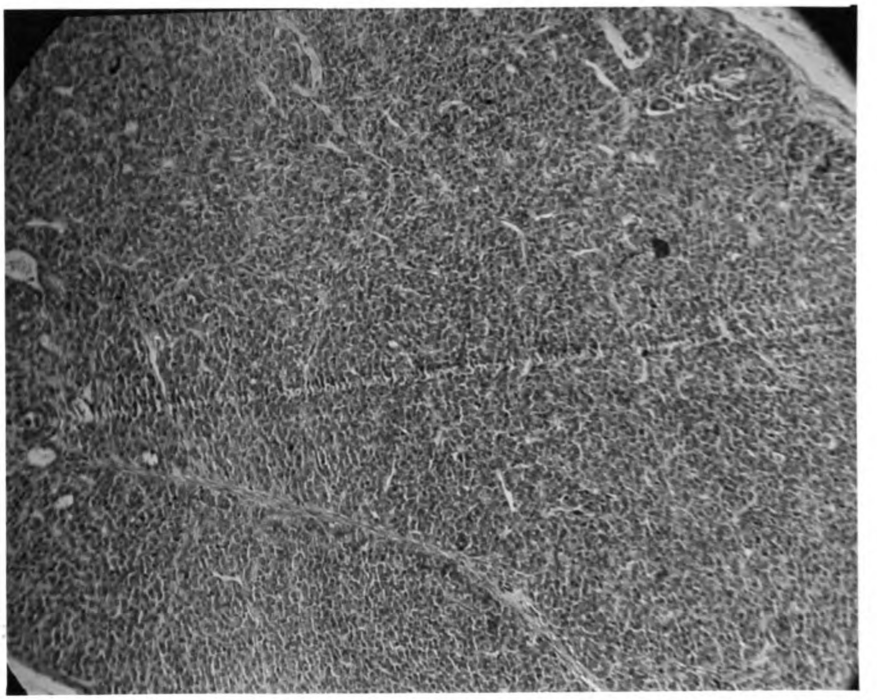


Fig. 4. An anterior pituitary transplant underneath the kidney-capsule 10 days after transplantation.



III. ADDENDUM

A. Prolactin Assays of Different Pituitary "Mammotropic" Tumor Strains

Prolactin assays of different pituitary "mammotropic" tumor (MtT.) strains of rats and mice were carried out. These tumors were supplied by Dr. Jacob Furth in the form of frozen or lyophilized material. All these tumors were transplantable autonomous and monomorphous. F_4 and F_6 were originally induced by eotrogen; W_5 by x-radiation, and MtT/50 x-radiation followed by estrogen (1). Tumors were assayed according to the method already described. The results of the assay are summarized in Table III-a. For comparison similar assays of anterior pituitaries from CFN and Fischer strains of rats are also included. These data show that prolactin content of these tumors is uniformly very low as compared to normal rat AP *in situ*, with the exception of mouse MtT/50 which has about half as much prolactin activity as normal rat AP.

B. Transplantation of Pitituary "Mammotropic"Tumor Strain F_4 (MtT.F_4) in Sprague Dawley Rat

MtT.F_4 was originally induced by diethylstilbestrol treatment in inbred Fischer rat. The tumor was initially dependent, in that following transplantation it grew only in estrogen treated rats. After 4-5 serial passages in the Fischer rat, it became autonomous, and could grow in normal hosts following transplantation. However, it still retained

its responsiveness to estrogen, as indicated by increased growth (2). Earlier attempts to transplant MtT.F_4 in other strains of rats were uniformly unsuccessful. Since the MtT.F_4 which was available to us has passed through about 40 serial passages in the Fischer rat, it was decided to determine whether by this time it could be transplanted successfully in the Sprague-Dawley strain of rats. The selection of the Sprague-Dawley strain for this study was due to our interest in mammary turmorogenesis. The mammary gland of the female Sprague-Dawley rat is more susceptible to turmorogenesis following intragastric (3, 4) or intramammary administrations (unpublished observations) of chemical carcinogens than other strains of rats. Secondly, Sprague-Dawley rats are in no way related to the Fischer strain (Dr. W. Dunning, personal communication).

Sprague-Dawley female rats, 5-6 week old, were obtained from Hormone Assay Laboratory, Chicago, Illinois. MtT.F_4 (passage 40 and 41) was transplanted subcutaneously, into animals 7-8 weeks old. The details of the transplantation procedure has been described previously. Three weeks after transplantation, rats were examined by palpation every week for tumor development. One group of rats was injected with estrodiol in oil, 10 μg, thrice weekly for six weeks, beginning one day before tumor transplantation. Another group of rats was treated with 50 IU PMS and 3 days later 25 IU HCG, followed a day later by a tumor transplant. At termination of the experiment all tumors were examined histologically.

The results are summarized in Table III-b, and show that (a) successful transplantation in Sprague-Sawley rat occurs only in 40-60% of the animals as compared with a 100% take in Fischer rats (b) estrogen or PMS and HCG treatment had no effect in altering tumor incidence and (c) mean lateney (7-8 weeks) was longer in Sprague-Dawley rats when compared with Fischer rats (3-5 weeks). It was also observed that the tumors in the Sprague-Dawley rats grew very slowly and even after five months, they never attained more than about 1/3 the size of tumors in Fischer rats. By this time most of the rats started dying of respiratory infections. An incidental observation was that most of these rats had stomach ulcers. We have not observed this latter phenomenon in Fischer rats.

Histological examination of these tumors showed that they in no way differed in appearance from original tumor transplant. Also the types of hormones secreted by this tumor appeared to be similar to that of original tumor. Enlarged adrenals and almost complete disappearance of thymus indicated ACTH release. Increased in the sizes of liver, kidney, and spleen denoted somatotropic effects, while intense mammary gland stimulation suggested prolactin secretion. Similar effects are also observed when MtT.F_4 is transplanted in the Fischer rat (1, see Figures 1-4).

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TABLE III-a. Prolactin Assays of Different Pituitary "Mammotropic" Tumor (MtT) Strains/

Species and Strain of Animal*	MtT Strain	Type of Pituitary Material	No. of Assays	No. of Assay Pigeons	Av. Prolactin Content (IU/100mg MtT or AP)**
Rat, Fischer	F_4	Fresh, frozen	1	5	0.09
Rat, Fischer	F_4	Fresh, frozen	1	5	0.07
Rat, Fischer	F_4	Fresh, frozen	4	16	0.12
Rat, Wistar	W_5	Fresh, frozen	1	6	0.03
Rat, Fischer	F_4	Lyophilized	1	5	0.06
Rat, Fischer	F_4	Lyophilized	2	10	0.05
Rat, Fischer	F_6	Lyophilized	1	5	<0.01
Rat, Wistar	W_5	Lyophilized	2	10	0.05
Mouse, LAF_1	MtT/50	Lyophilized	1	5	0.53
Rat, CFN	AP	Fresh, frozen	4	16	1.61
Rat, Fischer	AP	Fresh, frozen	4	18	1.20

/ The tumors were supplied by Dr. J. Furth.

*All rat and mouse strains were inbred, except CFN.

**AP = normal anterior pituitary in situ.

TABLE III-b. Transplantation of MtT.F_4* into Sprague-Dawley Rats

Group	Treatment**	No. of Rats	No. of Rats With Tumors	Per Cent of Rats With Tumors	Mean Latency (weeks)
1	Saline	12	5	50	7
2	Saline	12	7		8
3	Estradiol	10	6	60	8
4	PMS and HCG	12	5	41	7

* Pituitary "mammotropic" tumor, Strain F_4

** See text for details.

Fig. 1. "Mammotropic" pituitary tumor ($MtT.F_4$) in Fischer rat.

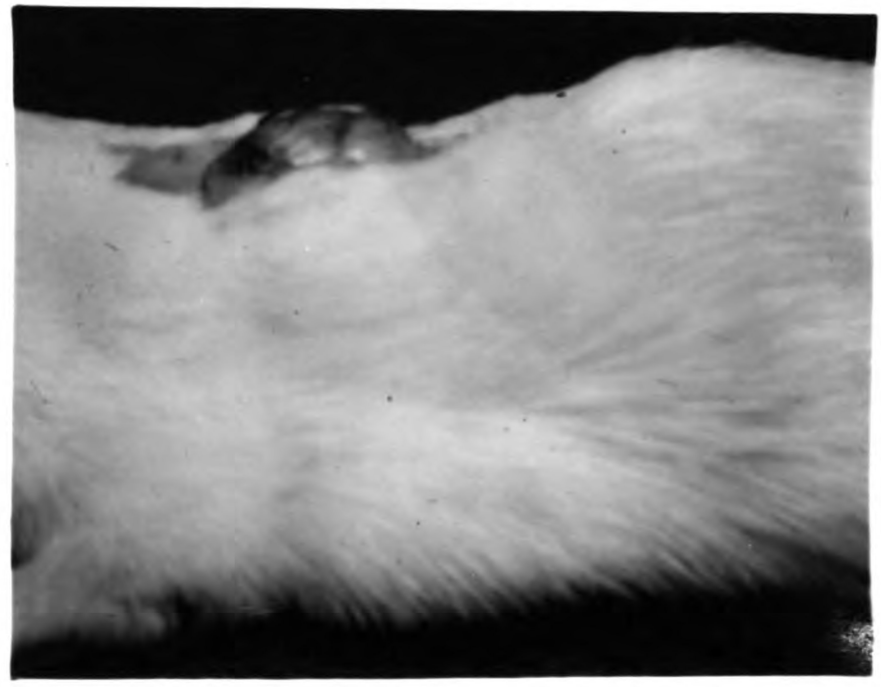

Fig. 2. "Mammotropic" pituitary tumor in Sprague-Dawley rat.

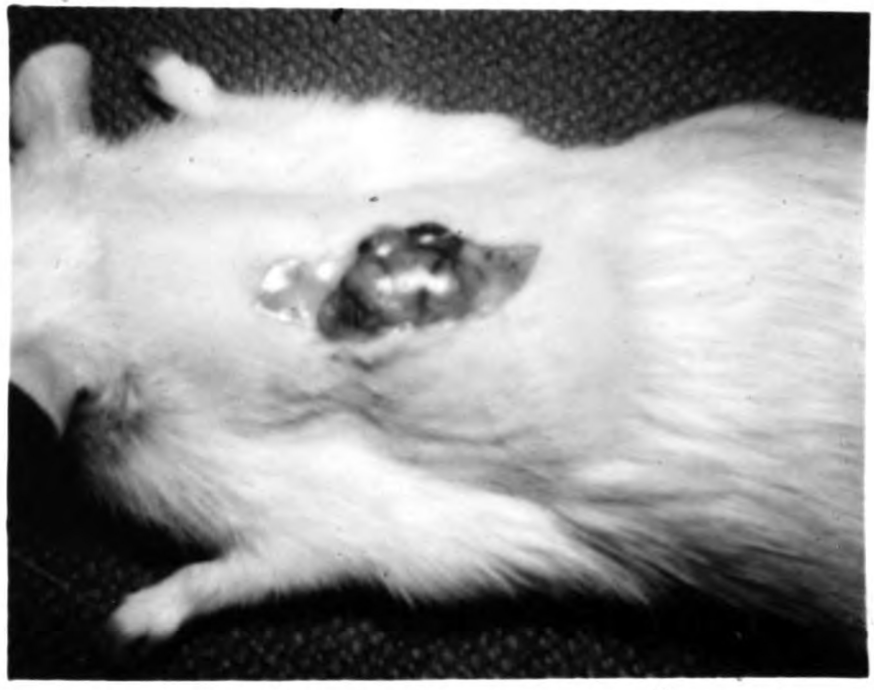

Fig. 3. Sprague-Dawley rat bearing "mammotropic" pituitary tumor ($MtT.F_4$) showing intense mammary gland stimulation.

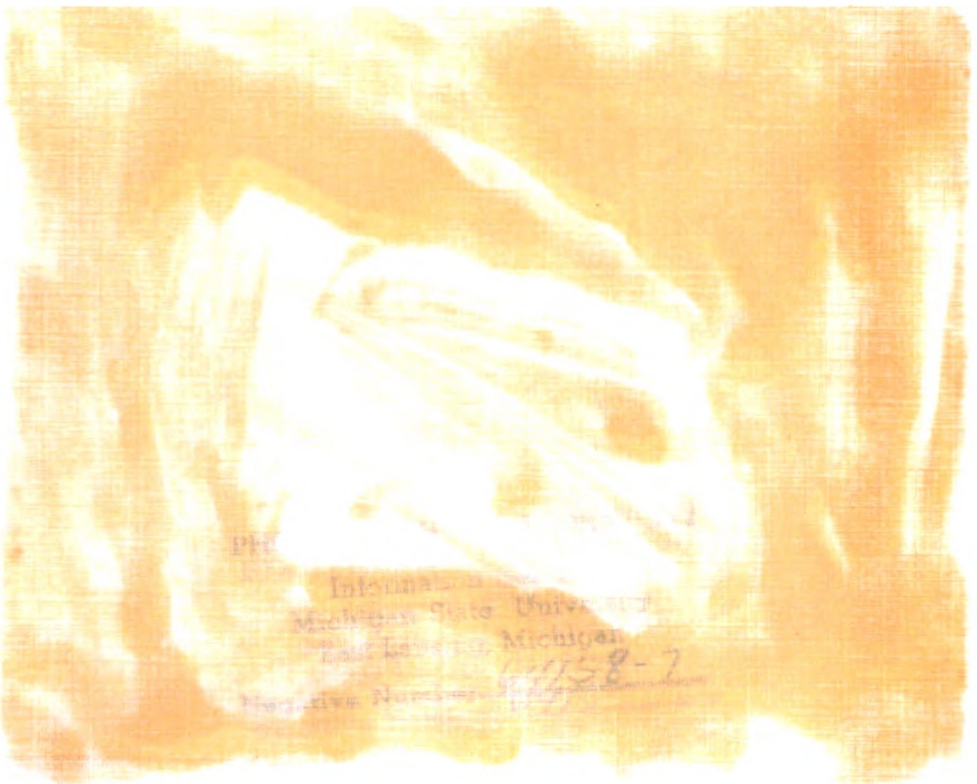

Fig. 4. Comparison of the different organs of (MtT.F_4) tumor bearing versus non tumor bearing Sprague-Dawley rats. Note enlarged liver, kidney, and adrenals of tumor bearing rat (left) as compared with non tumor bearing rat (right).

Section Two

EFFECT OF ANTERIOR PITUITARY HORMONES ON INDUCTION OF NORMAL AND NEOPLASTIC MAMMARY GROWTH IN THE RAT

I. MAMMARY LOBULO-ALVEOLAR GROWTH IN ADRENO-OVARIECTOMIZED RATS FOLLOWING TRANSPLANTATION OF "MAMMOTROPIC" PITUITORY TUMOR

The ovarian hormones estrogen and progesterone have been shown to induce mammary lobulo-alveolar (L-A) growth in intact or ovariectomized rats, and to synergize with prolactin and growth hormone (STH) in adreno-ovaricetomized-hypophysectomized rats (1). Ovarian hormones themselves little or no effect on mammary L-A development in the rat in the absence of the anterior pituitary (AP), whereas our laboratory has recently reported that AP hormones (STH and prolactin) alone induced mammary L-A growth in the absence of the ovaries, adrenals and pituitary (2). This suggested that the pituitary hormones, STH and prolactin, are of primary importance for mammary L-A growth in the rat.

Recently Furth and his co-workers (3, 4) developed many types of transplantable pituitary tumors. One of these is a monomorphous pituitary "mammotropic" tumor, strain F_4 ($MtT.F_4$), originally induced by estrogen (5). This tumor secretes prolactin, STH and corticotropin (ACTH) but no FSH-LH or TSH in the host animal (6-10). The tumor provides a continuous source of prolactin, STH and ACTH, which is secreted in increasing amounts as the tumor grows in the host. It was of interest, therefore, to determine the effect of a $MtT.F_4$ transplant on mammary growth in the rat in which the sources of estrogens have been eliminated by removal of the ovaries and adrenals.

METHODS

Highly inbred, virgin female Fischer rats of the CDF strain (Charles River Breeding Laboratories, Brookline, Mass.), 3 months old, were used in this experiment. The animals were fed ad libitum on Wayne Lab Blox pellets, supplemented with canned dog food (Dash) and slices of oranges. Rats in Groups 1 and 2 (Table 1) were sham-operated, while those in Groups 3 and 4 were adreno-ovariectomized. Following each operation, the rats were injected once subcutaneously with 30,000 units of penicillin G. The completeness of adrenalectomy was assessed in 2 ways: (a) after killing the rats at the end of experiment, they were carefully examined with a magnifying lens for adrenal remnants; and (b) 9 rats of the same strain, age, and weight were adreno-ovariectomized and given saline (1% Nacl) in their drinking water for 7 days. From the 8th day onward, the rats were given distilled water for drinking instead of saline. The day on which each rat died, after saline withdrawal, was recorded.

Seven days after the operation, rats in Groups 2 and 4 were transplanted with a Furth pituitary "mammotropic" tumor ($MtT.F_4$, passage 42) subcutaneously in the dorsal neck region. Each $MtT.F_4$ from the donor rats was removed asceptically, cut into pieces with iris scissors, and the tumor mince was suspended in equal volume of medium 199 (pH 7.4, Difco Laboratories, Detroit, Mich.). This was injected

in a 0.1 ml volume. The tumors attained palpable size within 4-5 weeks. Estrogenic activity in adreno-ovariectomized rats was determined by vaginal smears.

Eight weeks after tumor transplantation, all rats in Groups 1-4 were killed. Both inguinal mammary pads from each rat was disected free, spread flat on cork and fixed in Bouin's fluid. One mammary pad from each animal was stained by a standard procedure (2) for gross examination, and rated for development according to the following scale:

I = Few ducts; few or no end buds

II = Moderate duct growth; moderate number of end buds

III = Numerous ducts and branches; many end buds

IV = Numerous ducts and branches;moderate lobulo-alvedar growth

V = Numerous ducts with many branches;dense lobulo-alveolar growth, as in mid or lage pregnancy

The other mammary gland pad from the same animal was prepared for histological examination, as described earlier (11). Lactation ratings of 0 to 4 were assigned to each gland, depending on amount of secretion observed microscopically (11). The MtT.F_4 from each of the tumor bearing rats was also removed and weighed.

RESULTS

The data are summarized in Table I. The mammary glands from intact controls (Group 1) consisted of moderately

developed ducts with few end buds (Fig. 1) and no secretion (Fig. 2). MtT.F_4 transplantation in intact rats (Group 2) induced highly extensive mammary lobulo-alveolar growth and intense mammary secretion (Rating $>>$ 4), far greater than that observed during normal pregnancy and lactation.

Adreno-ovariectomy alone without an MtT.F_4-transplant (Group 3) resulted in marked regression of the mammary duct and end bud system (Fig. 3). Transplantation of an MtT.F_4 in adreno-ovariectomized rats (Group 4) induced extensive mammary duct growth and branching and moderate to extensive L-A development (Fig. 4). These rats showed a variable but small degree of mammary secretion (Fig. 5 and 6).

The average weight of the tumors from the adreno-ovariectomized rats (9.9 gm) was significantly greater ($P < 0.01$) than those removed from the intact rats (6.1 gm). Vaginal smears of all adreno-ovariectomized rats did not show any evidence of estrogenic activity. The 9 adreno-ovariectomized rats (separate from those used in Groups 1-4) died following withdrawal of saline and substitution with distilled water, as follows: one rat on day 5, 2 on day 7, 2 on day 8, and one each on days 11, 12, 14, and 15.

DISCUSSION

In previous *in vivo* studies, using adreno-ovariecto-mized-hypophysectomized rats and mice (1), or *in vitro* cultures of the mammary glands of mice (12), it was observed

that estrogen and progestrone were needed in addition to prolactin and STH to induce mammary L-A growth. In the present study, mammary L-A growth was induced by the hormones secreted by MtT.F$_4$. This is in agreement with the similar findings by Clifton and Furth (13).

We have reported that the MtT.F$_4$ secretes prolactin in vivo as well as in vitro cultures (6), and other in vivo studies (7-10) have demonstrated that it also secretes STH and ACTH, but no FSH-LH or TSH. This indicates that mammary L-A growth in adreno-ovariectomized rats bearing a MtT.F$_4$ was due to secretion of prolactin, STH and ACTH from the tumor. However, our previous demonstration that only prolactin and STH are needed to induce full mammary L-A development in adreno-ovariectomized rats (2), suggests that prolactin and STH were primarily responsible for the development of mammary L-A system observed in the tumor bearing rats. Lasfargues (14) also found that L-A growth can be induced in mouse mammary gland cultures by addition of STH or prolactin to the culture medium. Addition of estrogen to the culture medium was not essential.

Ovariectomy and adrenalectomy appeared to be complete in these rats. Vaginal smears of these rats did not show any evidence of estrogen activity, indicating that estrogenic was not availble to synerize with prolactin and STH to stimulate mammary L-A growth. At necrospy, gross examination did not reveal the presence of adrenal ramnants.

The 9 adreno-ovariectomized control rats died within 15 days after saline withdrawal. However, most of the rats showed a slight degree of mammary secretion, indicating the possible presence of slight amounts of glueocorticoids. The minimal requirement for initiation of mammary secretion in the rat is prolactin and gluescorticoids (1). It is also possible that the large amounts of prolactin secreted by the tumor may have decreased the amount of a glueocorticoid necessary to initiate mammary secretion.

SUMMARY

1. The influence of a hormone-secreting (prolactin, STH and ACTH), autonomous, transplanted "mammotropic" pituitary tumor (Furth, MtT.F_4) on mammary lobulo-alveolar (L-A) growth in adreno-ovariectomized(doubly operated) Fischer rats was investigated.

2. The MtT.F_4-transplant stimulated extensive mammary L-A growth in intact or doubly-operated rats. Intact tumor bearing rats showed intense mammary secretion, while doubly operated tumor bearing rats revealed only slight amounts of mammary secretion. The average weight of MtT.F_4 in doubly operated rats was higher than that in intact controls.

3. Completeness of adrenalectomy and ovariectomy was checked. Nine control doubly operated rats failed to survive more than 15 days after withdrawal of saline. Vaginal smears of these rats did not reveal any estrogenic activity.

4. It is concluded that full mammary L-A growth can be induced following adreno-ovariectomy by a MtT.F_4-transplant secreting prolactin and STH.

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FOOTNOTES

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TABLE 1. Mammary Lobulo-Alveolar Growth in Adreno-Ovariectomized Rats Following Transplantation of Pituitary "Mammotropic" Tumor Strain F_4 ($MtT.F_4$)

Group	Treatment*	No. of Rats	Av. MtF.F4 Wt. (gm)	Mammary Growth Rating**					No. of Rats With Mammary Secretion Rating**				
				I	II	III	IV	V	0	1	2	3	4
1	Intact controls	9	--	2	7	0	0	0	0	0	0	0	0
2	Intact, $MtT.F_4$-Transplant	14	6.1/ ±1.0	0	0	0	0	14	0	0	0	0	14//
3	Adrex-Ovax, No. $MtT.F_4$-transplant	13	--	12	1	0	0	0	0	0	0	0	0
4.	Adrex-Ovax with $MtT.F_4$-transplant	12	9.9 ±1.3	0	0	2	4	6	2	4	3	3	0

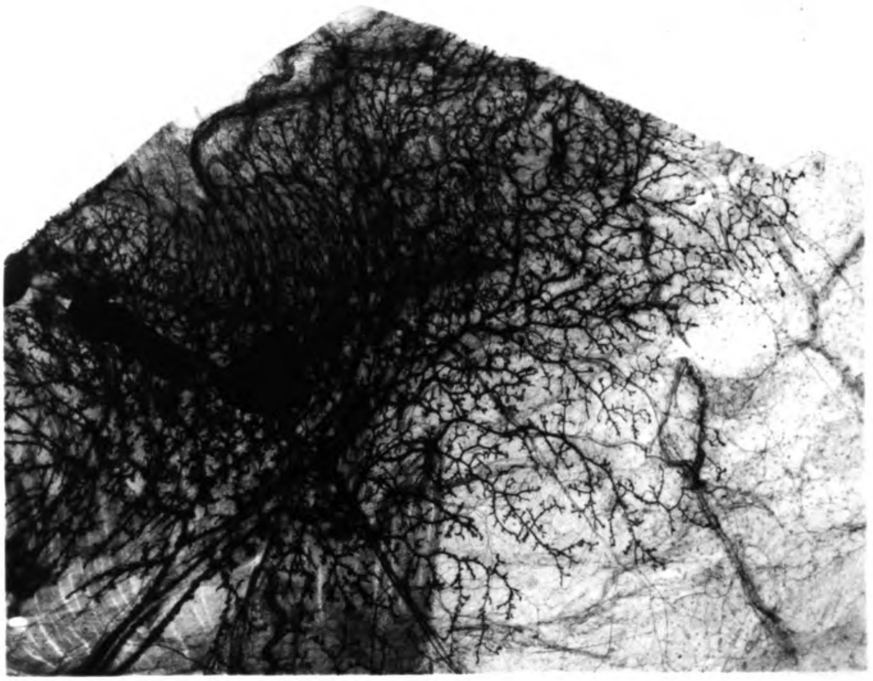
* Adrenalectomized = Adrex, Ovariectomized = Ovax

** See text for rating system

/ Mean + Standard error of mean, Group 2 vs 4: $P < 0.01$

// Mammary secretion in these rats was far greater than indicated by rating of 4.

Fig. 1. Intact controls. Moderate duct development and few end buds.



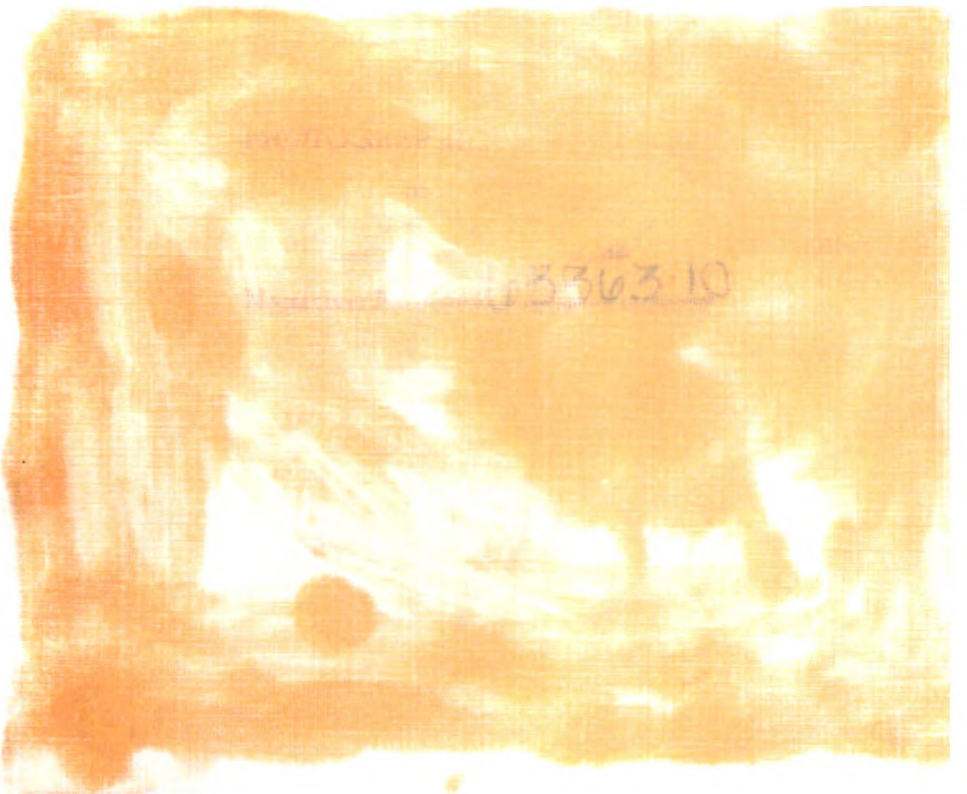


Fig. 2. Mammary gland section from the same animal as in Fig. 1. No mammary secretion present.

Fig. 3. Adreno-ovariectomized rat without MtT.F_4 transplant. Few ducts with little branching.

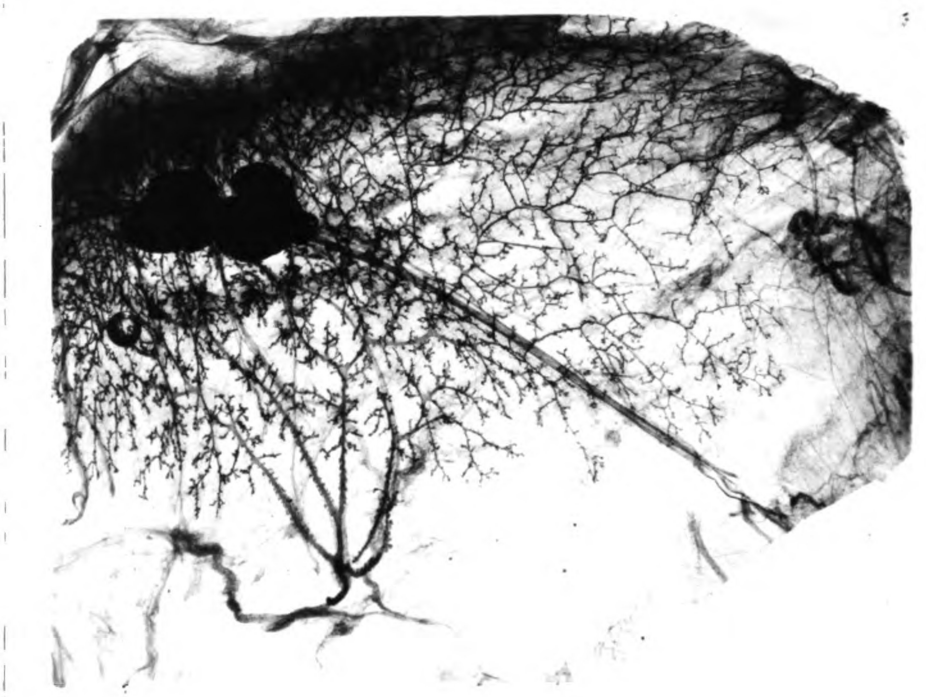


Fig. 4. Adreno-ovaricetomized rat with MtT.F_4 transplant. Extensive lobulo-alveolar growth.

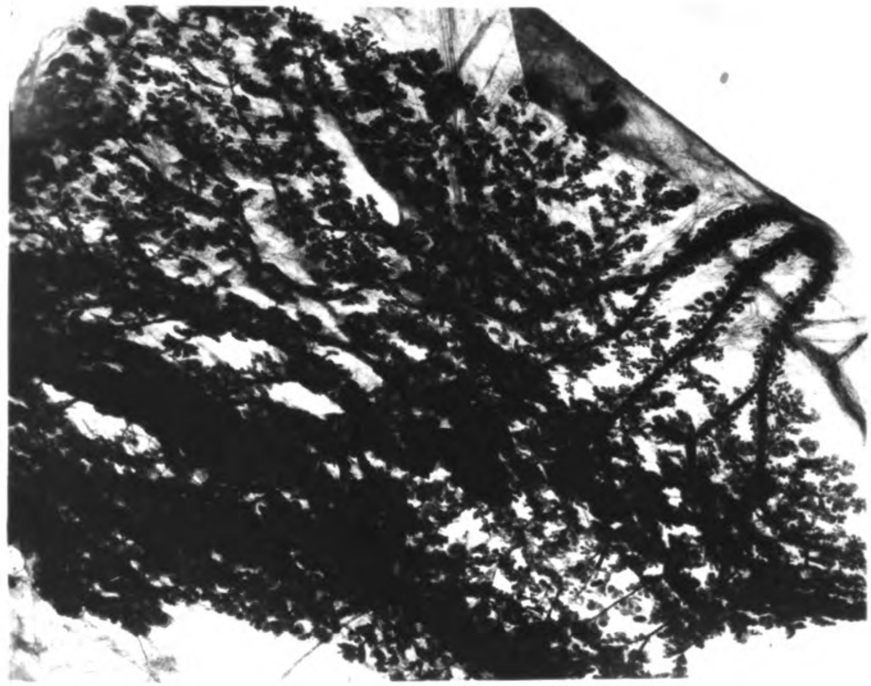
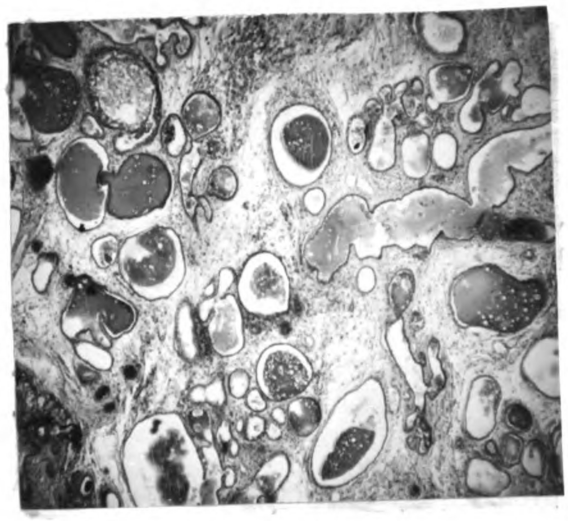


Fig. 5. Same type of rat as in Fig. 4, showing slight mammary secretion.

Fig. 6. Same type of rat as in Fig. 4, showing moderate mammary secretion.



II. INDUCTION OF MAMMARY LOBULO-ALVEOLAR GROWTH BY ANTERIOR PITUITARY HORMONES IN ADRENO-OVARIECTOMIZED AND ADRENO-OVARIECTOMIZED-HYPOPHYSECTOMIZED RATS

Studies in ovariectomized-hypophysectomized or in adreno-ovariectomized-hypophysectomized rats have indicated that hormones from the anterior pituitary and ovaries are both essential for inducing mammary lobulo-alveolar growth (-3). The adrenals are believed to be of secondary importance, although it is recognized that they secrete several steroids which can influence mammary growth (1-3). Only a small degree of lobulo-alveolar development has been produced by injecting anterior pituitary extracts into ovariectomized or adreno-ovariectomized rats (4). Recently, Clifton and Furth (5) observed complete lobulo-alveolar development in adreno-gonadectomized male rats of the Fischer strain after implanting "mammotropic" pituitary tumors intramuscularly. No data were given as to completeness of adrenalectomy in these rats. Since these pituitary tumors appear to secret both STH and prolactin (5), it was of interest to determine whether frequent injections of large doses of these two hormones or of whole anterior pituitary powder could induce lobulo-alveolar mammary growth in adreno-ovariectomized or adreno-ovariectomized-hypophysectomized rats.

METHODS

Mature virgin female Carworth rats of the CFN strain, weighing 180-240 g each, were used in this study. Rats in groups 1-5 (Table 1) were adreno-ovariectomized (doubly operated) while rats in groups 6 and 7 were hypophysectomized

by the parapharyngeal approach and 3 days later were adreno-ovariectomized (triply operated). Following each operation, the rats were injected once subcutaneously with 30,000 units of penicillin G. Completeness of adrenalectomy was assessed in two ways: (a) after killing the rats at the end of the experiment, they were carefully examined with a magnifying lens for adrenal remnants; and (b) 17 rats of the same strain, age and weight were adreno-ovariectomized and supplied with tap water instead of 1% saline. Death occurred in 16 rats on the following days: one rat on day 7, two on day 9, one on day 10, three on day 11, three on day 12, two on day 13, and one each on days 14, 15, 17 and 19. The remaining rat was killed on the 24th day, and upon examination one adrenal was found which had not been completely removed. The sella turcica of all hypophysectomized rats was examined for pituitary fragments at the end of the experiment. Only rats with complete pituitary removal are included in the results.

Twenty-four hours after adreno-ovariectomy, all groups of rats were treated for 10 days as shown in Table 1. Hormones were injected thrice daily at intervals of approximately six hours between 9 A.M. and 9 P.M. Bovine STH* and ovine prolactin* were dissolved in distilled water and anterior pituitary powder+ was suspended in physiological saline.

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*Donated by Endrocrinology Study section, NIH.

+Supplied through courtesy of Dr. J. D. Fisher of Armour Pharmaceutical Company, Kankakee, Illinois.

All rats were fed ad libitum on Wayne Lab-Blox pellets supplemented with canned Armour's Dash dog food, and were given 1% saline to drink. Slices of oranges and 5% glucose in their drinking water were made available to all hypophysectomized rats. Body weights of all rats were recorded at the beginning and end of each treatment.

The rats were killed on the day following the last injection, and both inguinal mammary pads were dissected free, spread flat on the cork and fixed in Bouin's fluid. One mammary pad from each animal was stained by a standard procedure (6), and rated for development according to the following scale:

I = Few ducts; few or no end buds

II = Moderate duct growth; moderate number of end buds

III = Numerous ducts and branches; many end buds

IV = Numerous ducts and branches; with moderate lobulo-alveolar (L-A) growth

V = Numerous ducts and branches with dense L-A growth, as in mid or late pregnancy.

RESULTS

The data are summarized in Table 1. In doubly operated rats, mammary glands from saline treated controls (Group 1) consisted of moderately developed ducts with few end buds (Fig. 1. See published article in Appendix for

figures.) Treatment with STH (Group 2) induced extensive duct growth with many branches and end buds but no L-A growth (Fig. 2). Prolactin (Group 3) also induced duct growth with many branches and end buds (Fig. 3), but 3 out of 7 rats showed limited L-A development. Combined treatment with STH and prolactin (Group 4) induced extensive duct growth and dense L-A development comparable to that seen in rats after mid or late pregnancy (Fig. 4). The results with anterior pituitary powder (Group 5) were variable and mammary growth was not equal to that produced by combined STH and prolactin treatment, but the majority of these rats showed some L-A development. In the triply operated rats, mammary glands from control animals (Group 6) consisted essentially of few small ducts with little branching and few or no end buds (Fig. 5). Combined treatment with STH and prolactin (Group 7) produced extensive duct growth with many branches and moderate L-A development (Fig.6).

Doubly or triply operated controls gained very little in average body weight during the treatment, while rats given STH gained an average of 43 g. Prolactin and anterior pituitary powder produced average body weight gains of 14 g and 20 g, respectively.

DISCUSSION

In previous studies with triply operated rats administration of estrogen and progesterone or adrenal corticoids

were needed in addition to STH and prolactin to induce lobulo-alveolar (L-A) growth (2). The results of the present experiment show that in doubly or triply operated rate combined treatment with bovine STH and ovine prolactin, or bovine anterior pituitary powder alone, can induce L-A development. Our results are therefore in agreement with those of Clifton and Furth (5) who found good L-A development in adreno-gonodectomized male rats implanted with "mammotropic" pituitary tumors. The main differences between our experiment and those reported by earlier investigators are that we injected prolactin and STH or whole anterior pituitary powder thrice instead of only once daily; injections were begun the day after surgery; and relatively large doses of thses hormones were used. Also, our rats were mature females, three to four months old, whereas Lyons et al. (2) employed mainly young rats of the Long-Evans strain.

Ovariectomy and adrenalectomy appeared to be complete in these rats. The 16 adrenalectomized rats not given saline died within 19 days. Since injections were begun on the day after removing the ovaries and adrenals, the possibility cannot be excluded that small amounts of steroid hormones were present during the first few days of treatment. It is doubtful however, that such small amounts if present would have been sufficient to synergize with the anterior pituitary hormones to induce full L-A growth. Clifton and Furth (5) implanted their pituitary tumors in rats 4 to 16 days after adreno-gonadectomy and still obtained good L-A development.

The present results cannot be interpreted to mean that ovarian and probably adrenal cortical hormones have no role in normal mammary development in rats. Estrogen and progesterone have been shown to induce full mammary growth in intact or ovariectomized rats (1) and to synergize with prolactin and STH in triply operated rats (2). However, our results suggest that the anterior pituitary is of primary importance in mammary development in the rat, since anterior pituitary hormones alone induced full mammary development in the absence of ovaries and adrenals, whereas ovarian or adrenal cortical hormones have been shown to have little or no effect on the mammary growth in the absence of the anterior pituitary (1-3).

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V. ADDENDUM

Clifton and Furth (5) reported minimal mammary secretion in adreno-gonadectomized Fischer rats bearing mammotropic pituitary tumors, suggesting that accessory adrenal cortical tissues may be present in these rats. Histological examination of stained mammary sections from our adreno-ovariectomized rats treated with prolactin and STH failed to reveal any secretory activity.

TABLE 1. Effects of Anterior Pituitary Hormones on Mammary Growth in Ovèriectomized-Adrenalectomized and Overiectomized-Adrenal-ectomized-Hypophysectomized Rats

Group and No. of Animals	Treatment* (3 x daily; 10 days)	Mammary Rating**					Av. Body Weight (g.)		
		I	II	III	IV	V	Initial	Final	Difference
	Overiectomized-Adrenalectomized Rats								
1 (9)	0.1 ml. Saline	3	6	-	-	-	222	225	+ 3
2 (7)	2 mg. STH	-	1	5	1	-	217	260	+43
3 (7)	30 IU Prol.	-	-	4	3	-	208	222	+14
4 (7)	1.33 mg. STH + 20 IU Prol.	-	-	-	1	6	220	261	+41
5 (10)	20 mg. AP	-	-	2	5	3	224	244	+20
	Hypophysectomized-Overiectomized-Adrenalectomized Rats								
6 (9)	0.1 ml. Saline	6	3	-	-	-	211	212	+ 1
7 (9)	1.33 mg. STH + 20 IU Prol.	-	-	4	5	-	182	225	+43

*STH = Growth hormone; Prol. = prolactin; AP = Anterior pituitary powder

**See text for mammary rating system.

III. MAMMARY TUMOR INDUCTION BY ESTROGEN OR ANTERIOR PITUITARY HORMONES IN OVERIECTOMIZED RATS GIVEN 7, 12-DIMEHYL-1, 2-BENZANTHRACENE

It has been well established that ovarian and pituitary hormones play a major role in the normal development of the mammary gland, and in the maintenance and growth established mammary tumors in the rat (1-8). The pituitary appears to be of primary importance in mammary growth in the rat, since a combination of growth hormone (STH) and prolactin (9) or transplantation of a pituitary "mammotropic" tumor (10, Talwalker, Meites and Dueben, unpublished), which presumably secretes prolactin and STH (5, 11, 12), induced mammary growth in adreno-gonadectomized rats. On the other hand, ovarian or adrenal steriods have little or no effect on mammary growth in the absence of the pituitary (1-3). Induction of mammary growth by estrogen in intact rats appears to be partially mediated through the stimulation of pituitary prolactin and STH secretion (3,4).

The rapid induction of mammary carcinomas by intragastric administration of 3-methylcholanthrene (3-Mc) or 7, 12-dimethyl-1, 2-benzanthracene (DMBA) has been described (13, 14). Hypophysectomy prevents mammary tumor induction by 3-Mc, whereas tumors can be induced in hypophysectomized rats provided estrogen, progesterone and STH are given together with 3-Mc(15). Dao (16) reported that ovariectomy before a single feeding of DMBA prevented mammary tumor induction in the Sprague-Dawley female rat, but tumors appeared when ovaries were transplanted concurrently or within several days after DMBA administration. This suggests

that ovarian hormones are essential for mammary tumor induction by DMBA. Since ovarian hormones influence pituitary prolactin and STH secretions, it was of interest to determine the role of estrogen, or prolactin and STH, in mammary tumor induction by single feeding of DMBA in ovariectomized Sprague-Dawley rat.

METHODS

Sprague-Dawley, virgin female rats (Hormone Assay Laboratories, Chicago, Illinois) were used in this experiment. Rats were randomly divided into 6 groups. When they reached 52 days of age, group 1 was sham-operated, while groups 2-6 were bilaterally ovariectomized. Seven days later, all rats (groups 1-6) received, once only, 20 mg of 7, 12-dimethyl-1, 2-benzanthracene (DMBA, Eastman Organic Chemicals, Rochester, N. Y.) in 1 ml corn oil, by intragastric instillation. This dose of DMBA is considered optimal, since 100 per cent of rats, so treated, develop mammary tumors (14). Groups 1-5 were treated for 7 days before and 7 days after (total 14 days) DMBA administration, as shown in Table 1. Group 6 was treated, daily, with porcine STH and ovine prolactin in increasing dosages, beginning a day after ovariectomy and continuing as follows: Days 1-15, 1 mg each; days 16-30, 1.5 mg each; days 31-45, 2 mg each; days 46-60, 2.5 mg each; and days 61-75, 3 mg each. All injections were given subcutaneously. Estrodiol was injected in 0.1 ml corn

oil; and porcine STH and ovine prolactin (15 IU/mg) were given in 0.2 ml saline.

The rats were examined for tumors by palpation twice weekly. Occurrence of the tumor was dated from the day of feeding DMBA. The number and per cent of animals developing tumors (incidence) was determined at the end of 40 weeks, and the range and mean time of appearance of first tumors (latency) were calculated. Rats were killed when they appeared ill, or when the tumors became so large that they ulcerated and bled. At necropsy, the tumors were fixed in 10% neutral formalin, sectioned at 6 μ, and stained with hematoxylin and eosin.

RESULTS

Spontaneous mammary carcinomas in Sprague-Dawley rat are of very rare occurrence (13, 14, 17). Mammary carcinomas were the only type observed in these DMBA fed rats. No fibrosarcomas were detected in the mammary region. The mammary carcinomas were grossly nodular, white and soft. Hemorrhage and central necrosis were often encountered, especially when the tumors became large. The tumors contained acini lined with many layers of epithelial cells (Fig. 1) arranged to form gland-like structures with papillary projections in the lumina. The lumina of these glandular structures were often filled with eosinophilic-staining material. Metastases of these mammary tumors were not

observed, but often infiltration of adjacent muscles and skin were found. The principal causes of death of rats appeared to be due to large size of tumors developing hemorrhage and necrosis; and increased susceptibility to respiratory infection.

The results are summarized in Table 1. When 20 mg of DMBA was fed to the intact sham-operated controls and they were thereafter given saline (group 1), 100 per cent of the animals developed mammary tumors within 52-110 (mean 68) days. Ovariectomy 7 days before DMBA administration completely prvented tumor induction (group 2). Limited treatment of ovariectomized rats with 1 and 10 µg estradiol (groups 3 and 4) increased mammary tumor incidence to 33 and 23 per cent, respectively. In the estrogen treated groups, the mean latency was slightly higher and average number of tumors per tumor bearing rat was lower than in the sham-operated intact controls. Combined daily treatment with STH and prolactin, either for 14 or 7t days (groups 5 and 6), increased mammary tumor incidence to 44 and 66 per cent, respectively. In these rats also, the mean latency was higher and the average number of tumors per tumor bearing rat was lower than in the sham-operated controls.

DISCUSSION

Recent investigations have led to the concept that mammary carcinogenesis involves two separate phases (a) initiation of carcinogenesis by a carcinogen and (b) promotion of

tumor growth by hormones. Carcingoens bring about an irreversible change of the mammary cells, whereas hormones regulate growth and function of these cells. Hormones themselves apparently are not carcinogenic (4, 16). The results of the present study show that estradiol, or porlactin and STH, administered during the initiation phase of mammary carinogenesis by DMBA, permit ovaricetomized rats to develop mammary carcinomas. Thus neoplastic transformation in the cells of the mammary gland of rats requires the participation of hormones, particularly prolactin and STH. Estrogen may participate in the initiating phase through stimulation of prolactin and STH secretion by the pituitary, and also by a direct action on the mammary gland.

It is evident from the present study that estrogen, or prolactin and STH, were only partially effective in inducing mammary tumors in ovariectomized rats fed DMBA. This may be due to the particular doses of hormones used, which may not have been optimal, and to the schedule and duration of the hormone treatments. It is also possible that other hormones may be involved, including progesterone, TSH or ACTH. However, our study clearly show that limited treatment with the pituitary hormones, prolactin and STH, in the absence of the ovaries, can induce mammary tumors with the aid of a single DMBA feeding.

Mammary tumors induced by 3-Mc or DMBA feeding in rats, are hormone dependant during the early stages of growth, and

hence they may regress when appropriate endocrine glands supplying the hormones are removed. Thus, growth of mammary tumors is inhibited by ovariectomy, ovariectomy and adrenalectomy or by hypophysectomy; and stimulated by prolactin and STH secreting pituitary "mammotropic" tumor transplant (4-8, 13, 14). Estrogen maintains or stimulates growth of these mammary tumors only in ovariectomized or ovaricetomized-adrenalectomized rats. It is ineffective in the absence of the pituitary (18). Therefore, estrogen may promote growth of these mammary tumors through stimulation of secretion by the pituitary, prolactin and STH. The present study, together with others (4-6, 15, 18) suggest that the pituitary hormones, prolactin and STH, are of primary importance both in the initiating and promoting phases of mammary carcinogenesis by 3-Mc or DMBA feeding in the rat.

SUMMARY

1. The effect of estrogen or prolactin and STH on mammary tumor induction by a single feeding of 20 mg of 7, 12-dimethyl-1, 2-benzanthracene (DMBA), in ovariectomized Sprague-Dawley rat was investigated.

2. After DMBA administration, mammary tumor incidence in sham-operated controls was 100 per cent. In ovariectomized rats, DMBA failed to induce any mammary tumor by the end of 40 weeks. However, daily treatment with 1 or 10 µg estradiol, for 7 days before and after (total 14 days) DMBA administration,

induced mammary tumors in 33 and 23 per cent of rats, respectively. Similar treatment with 1 mg STH and 1 mg prolactin, twice daily, for 14 days increased mammary tumor incidence to 44 per cent, while continuous daily treatment with increasing amounts of prolactin and STH for 75 days, starting with 0.5 mg and rising to 1, 1.5, 2, 2.5 and finally to 3 mg each per rat, at intervals of 15 days, resulted in a 66 per cent incidence of mammary tumors.

3. The average number of tumors per tumor bearing rat was lower (1.0-2.3) and mean tumor latency was longer (89-146 days) in ovariectomized rats than in sham-operated controls (3.9, and 68 days, respectively). All tumors were mammary carcinomas.

4. It is suggested that estrogen, prolactin and STH participate in the induction phase of mammary carcinogenesis by DMBA, and that the effect of estrogen on mammary tumor induction is partially mediated through stimulation of pituitary prolactin and STH secretion.

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FOOTNOTES

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+ NIH postdoctoral fellow. Present address: Department of Animal Breeding, Nihon University, Tokyo, Japan.

ACKNOWLEDGMENT

We express our appreciation to the Endocrinology Study Section, NIH, for ovine prolactin; to E. R. Squibbs, for porcine growth hormone; and to T. E. Staley for technical assistance.

TABLE 1. Effect of Hormone Administrations on Mammary Tumor Induction in Ovariectomized Rats with 7, 12-Dimethyl-1, 2-Genzathracene (DMBA)

Group and Treatment*	Duration of Treatment (Days)	Total No.of Rats	No. and Percent of Rats With Tumors	Av. No. of Tumors per Tumor Bearing Rats	Range and Mean Latency (Days)
1. Sham-operated controls, Saline	14	18	18(100)	3.9	52-110 (68)
2. Ovax-controls, Saline	14	20	0(0)	--	--
3. Ovax, 1 μg Esd. 1xdaily	14	12	4(33)	1.5	72-130(104)
4. Ovax, 10 μg Esd.1xdaily	14	13	3(23)	1.0	76-102(89)
5. Ovax, 1 mg STH + 1mg prolactin 2xdaily	14	9	4(44)	1.8	114-210(146)
6. Ovax, STH + Prolactin** 1xdaily	75	9	6(66)	2.3	94-207(122)

* Ovax = ovariectomized; STH = porcine growth hormone; Esd = Estradiol DMBA was administered 7 days after ovariectomy.

** Dosages of STH and prolactin were increased every 15 days. See text for the details.

Fig. 1. A mammary carcinoma in an ovariectomized rat induced by a single feeding of DMBA and injections of prolactin and STH. Hematoxylin and Eosin.

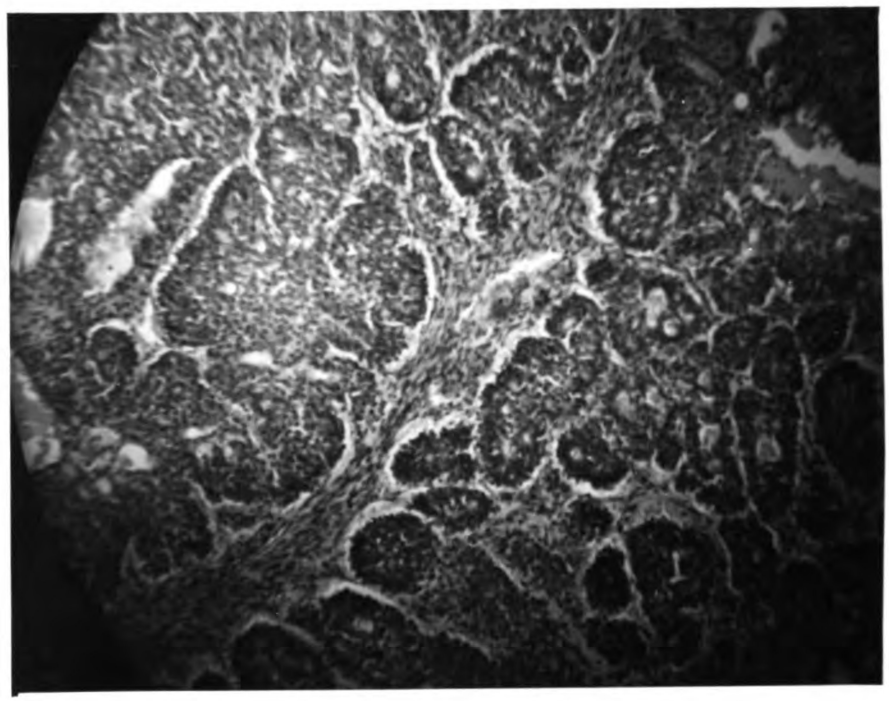
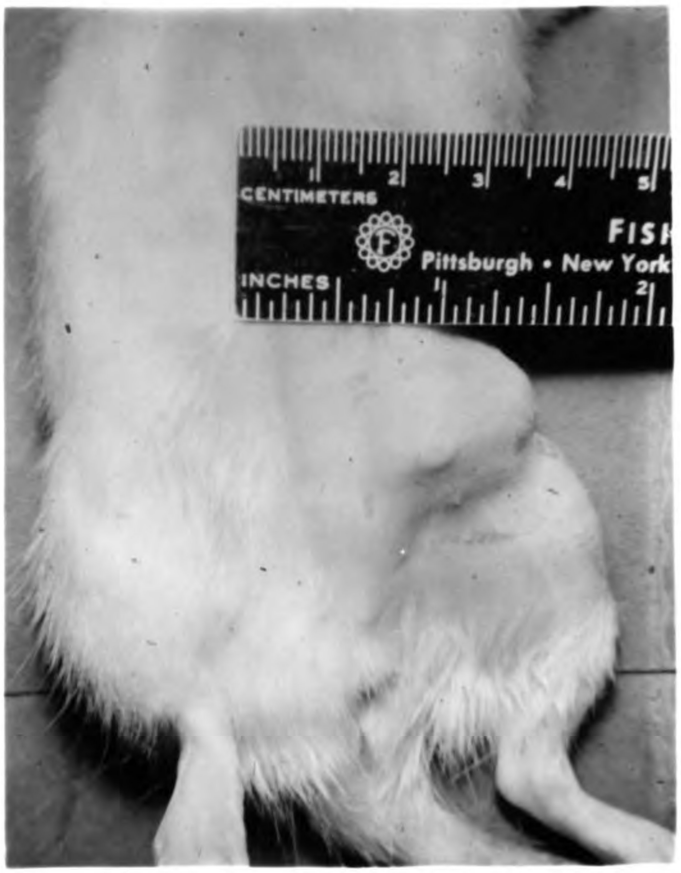


Fig. 2. Mammary tumor induced by prolactin and STH in the ovariectomized Sprague-Dawley rat fed DMBA.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III. ADDENDUM

A. Induction of Tumors in Different Strains of Rats by Intramammary Administrations of Chemical Carcinogens

Variations in the susceptibility of the rat mammary gland to oral administrations of chemical carcinogens are quite common. Thus an optimal dose of methylcholanthrene or dimethyl-benzanthracene will induce mammary tumors in 100 per cent of rats of the Sprague-Dawley strain, while Wistar and Fischer strains are quite resistant to tumor induction by this procedure. Also, the amount of carcinogen required per animal to induce such tumors is relatively high (1-5).

Application of chemical carinogens to the skin in the mammary gland region of the mouse produces skin as well as mammary tumors. However, the latent period is unusually long and the mammary tumor incidence is very low (6). Similar applications of chemical carcinogens in the rat invariably produces skin tumors (7, 8). Tumors have also been evoked in rats and mice by subcutaneous injection of chemical carcinogen or implantation of pellets containing chemical carcinogen. In these studies, the latent period for tumor induction was usually found to be considerably prolonged, depending upon potency and dosage of the chemical carcinogen used. In the albino rat the average latent period have been reported to be somewhere between 21-32 weeks (6-11). The present experiments were intended to determine the susceptibility of the mammary

glands of different strains of rats to mammary tumors following intrammary administrations of different chemical carcinogens.

METHODS

3-methylcholanthrene (Mc, 3, 4-benzpyrene or 9, 10-dimethyl-1, 2-benzanthracene (DMBA) were dissolved in corn oil and injected into the mammary gland fat pad in 1 mg doses and in a 0.1 ml volume, twice weekly for 4 weeks. All injections were made approximately at the same site. Six separate experiments on a total of 352 animals were performed in four different strains of female rats, each 45 to 50 days old at the beginning of the experiment.

Vasectomized male rats were introduced in the cages of one group of rats of the Sprague-Dawley strain treated with Mc. Vaginal smears of these rats showed pseudopregnancy-like cycles. Starting on the fifth week, each rat was palpated in the inguinal region once weekly for the presence of a tumor. When these tumors appeared, they were measured with calipers once weekly, and were removed for histological study when they reached 15-20 mm in diameter.

RESULTS AND CONCLUSION

The results of these experiments are summarized in Tables VI-a-1 to VI-a-6. It will be noted that the vast majority of the tumors produced by the three carcinogens were sarcomas classified under three different categories by Dr. Langham:

(a) Fibro (b) Rhabdomyo and (c) Leiomyo, according to the similar system suggested by Bonser (6). Of these the fibro sarcoma was the most common type.

The incidence of adenocarcinoma of the mammary gland was much higher (33%) in the Sprague-Dawley strain than in Chemistry Department (M.S.U.), Carworth CFN (originally Wistar), or in the highly inbred (32nd generation) Hunt-Hoppert strain of rat (0-7%). In the Sprague-Dawley strain, MC and DMBA were more effective in inducing mammary carcinomas than BP. Pseudopregnancy induced by vasectomized male rats increased mammary tumor incidence by 10% only (from 50 to 60%) when MC was used as a carcinogen in the Sprague-Dawley strain.

Most of the tumors in all four strains of rats developed in a comparatively short period of time (9-14 weeks) after initiating treatment with the carcinogens, and the average tumor incidence was very high (70-100%). Only one tumor was produced under each injection site.

It is concluded that among the 4 strains of rats studied, the mammary gland of Sprague-Dawley strain is the most susceptible to production of mammary tumors following intramammary injections of chemical carcinogens. Among the three carcinogens studied, MC and DMBA were more effective than BP in producing mammary tumors in the Sprague-Dawley strain. The high susceptibility of the mammary gland of the Sprague-Dawley rat to carcinogens is of considerable interest, since this strain of rats develops mammary tumors almost exclusively when given carcinogens orally (1, 2; also see the preceeding paper).

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TABLE VI-a-1. Tumor Induction with Chemical Carcinogens in MSU Chemistry Department Rats*

Treatment	No. of Rats at Beginning of Experiment	No. of Rats When First Tumor Appeared	Total No. of Rats With Tumors	Tumor Incidence %	No. Weeks When First Tumor Appeared	Latent Period (50% Incidence) Weeks	Mammary Carcinoma	Sarcoma		
								Fibro-	Rhabdomyo-	Leiomyo-
Controls	12	--	0	0	0	0	0	0	0	0
MC	24	24	23	96	8	10	0	21	2	0
BP	12	12	11	91	8	12	1	10	0	0
DMBA	13	10	7	70	7	14	1	3	2	1
						Total	2	34	4	1

*See text for abbreviations and treatments.

TABLE VI-a-2. Tumor Induction with Chemical Carcinogens in MSU Chemistry Department Rats*

Treatment	No. of Rats at Beginning of Experiment	No. of Rats When First Tumor Appeared	Total No. of Rats With Tumors	Tumor Incidence %	No. Weeks When First Tumor Appeared	Latent Period (50% Incidence) Weeks	Mammary Carcinoma	Sarcoma		
								Fibro-	Rhabdomyo-	Leiomyo
Controls	15	--	0	0	0	0	0	0	0	0
MC	15	14	14	100	7	11	0	13	1	0
BP	15	15	15	100	8	13	0	14	0	1
DMBA	15	13	10	77	8	14	1	4	4	1

*See text for abbreviations and treatments

TABLE VI-a-3. Tumor Induction with Chemical Carcinogens in Chemistry Department Rats (Est. Treated)*

Treatment	No. of Rats at Beginning of Experiment	No. of Rats When First Tumor Appeared	Total No. of Rats With Tumors	Tumor Incidence %	No. Weeks When First Tumor Appeared	Latent Period (50% Incidence) Weeks	Mammary Carcinoma	Sarcoma		
								Fibro-	Rhabdomyo-	Leiomyo-
Controls	12	--	0	0	0	0	0	0	0	0
MC	10	10	10	100	7	10	0	8	2	0
BP	10	10	8	80	8	12	0	8	0	0
DMBA	9	7	5	71	7	12	1	3	1	0
						Total	1	19	3	0

* See text for abbreviations and dosages of treatments.

TABLE VI-a-4. Tumor Induction with Chemical Carcinogens in Carworth (CFN) Rats*

Treatment	No. of Rats at Beginning of Experiment	No. of Rats When First Tumor Appeared	Total No. of Rats With Tumors	Tumor Incidence %	No. Weeks When First Tumor Appeared	Latent Period (50% Incidence) Weeks	Mammary Carcinoma	Sarcoma		
								Fibro-	Rhabdomyo-	Leiomyo-
Controls	15	--	0	0	0	0	0	0	0	0
MC	15	15	15	100	7	9	1	11	3	0
BP	15	15	15	100	7	10	1	11	3	0
DMBA	15	13	11	84	8	12	1	8	2	0
						Total	3	30	8	0

* See text for abbreviations and treatments.

TABLE VI-a-5. Tumor Induction with Chemical Carcinogens in Hunt-Hoppert Inbred Rats*

Treatment	No. of Rats at Beginning of Experiment	No. of Rats When First Tumor Appeared	Total No. of Rats With Tumors	Tumor Incidence %	No. Weeks When First Tumor Appeared	Latent Period (50% Incidence) Weeks	Mammary Carcinoma	Sarcoma		
								Fibro-	Rhabdomyo-	Leiomyo-
Controls	15	--	0	0	0	0	0	0	0	0
MC	15	14	12	85	8	14	0	10	2	0
BP	15	15	14	93	7	11	0	12	1	1
DMBA	15	--	--	--	--	--	--	--	--	--
						Total	0	22	5	1

* See text for abbreviations and treatments.

TABLE VI-a-6. Tumor Induction with Chemical Carinogens in Sprague-Dawley Rats*

Treatment	No. of Rats at Beginning of Experiment	No. of Rats When First Tumor Appeard	Total No. of Rats With Tumors	Tumor Incidence %	No. Weeks When First Tumor Appeared	Latent Period (50% Incidence) Weeks	Mammary Carcinoma	Sarcoma		
								Fibro-	Rhabdomyo-	Leiomyo-
Controls	15	--	0	0	0	0	0	0	0	0
MC	15	14	12	85	7	10	6	3	2	1
BP	15	14	13	93	8	13	1	9	2	1
DMBA	15	11	5	45	8	13	3	2	0	0
Pseudopregnancy and MC**	10	10	10	100	7	11	6	3	1	0
						Total	10	14	4	2

* See text for abbreviations and treatments.

**Vasectomized males were introduced in the cages.

Fig. 1. Mammary tumor development in the Sprague-Dawley rat following intramammary administrations of Mc.

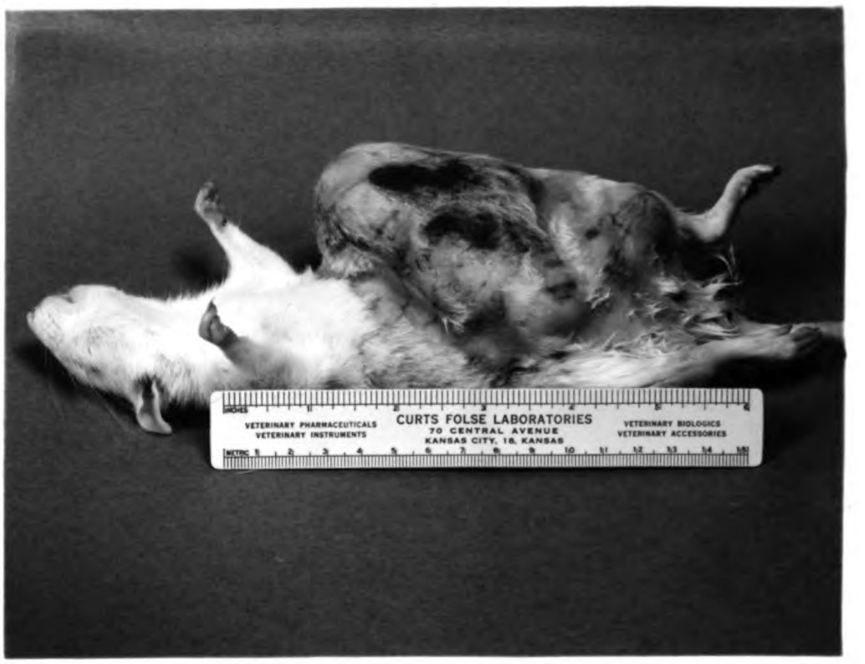
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Fig. 2. Mammary carcinoma in the Sprague-Dawley rat induced by intramammary injections of Mc.

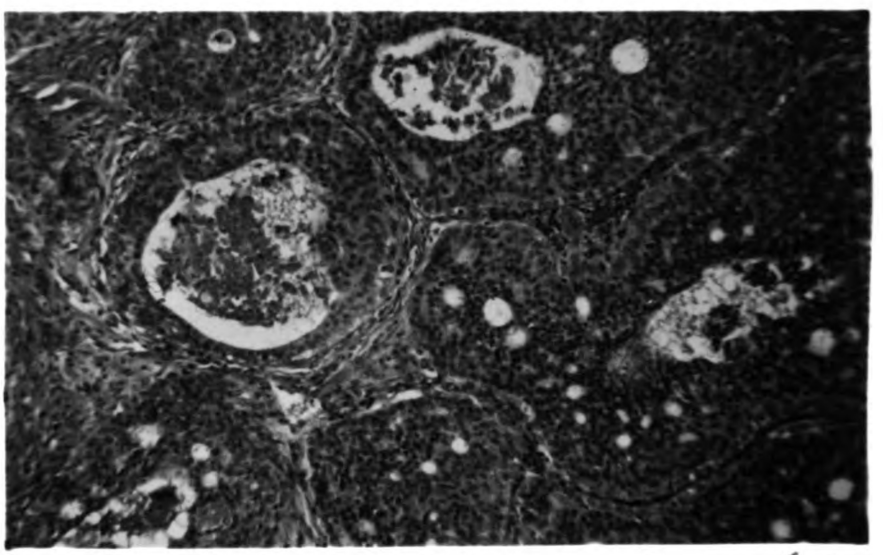


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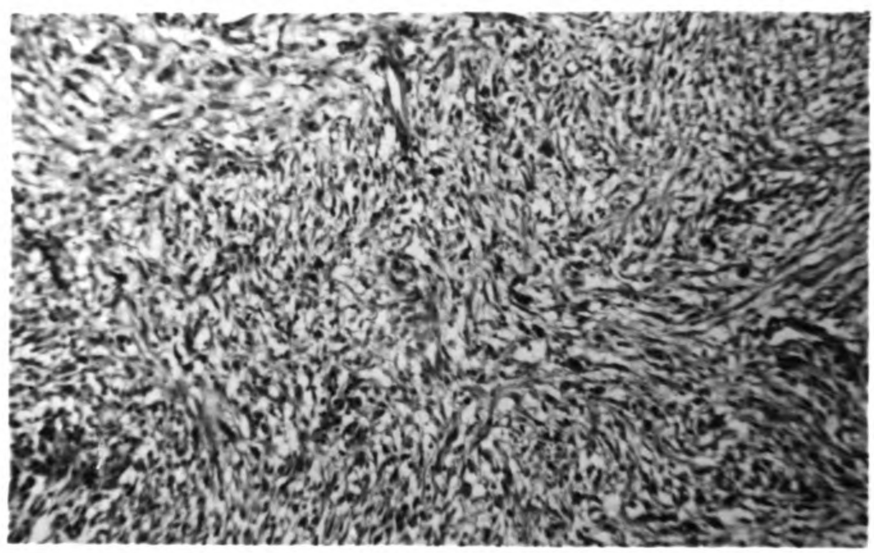
Fig. 3. Fibroadenoma induced in the Sprague-Dawley rat following intramammary administrations of Mc.



APPENDIX

Curriculum Vitae and list of papers published during graduate studies at Michigan State University in which writer was an author of co-author.

CURRICULUM VITAE

NAME: Talwalker, Purnachandra Keshavrao
(First) (Middle)

DATE OF BIRTH: May 19, 1932

PLACE OF BIRTH: Indore, Madhya Pradesh, India

NATIONALITY: Indian SEX: Male

MARITAL STATUS: Single

PRESENT ADDRESS: Department of Physiology and Pharmacology
Michigan State University
East Lansing, Michigan, U.S.A.

PERMANENT ADDRESS IN INDIA: 963 A Krishnanagar,
Bhavnagar, Gujarat, India

EDUCATION QUALIFICATIONS:

Degree	Year		Major Field of Study
B.Sc.(Honors)	1953	Gujarat University, India	Chemistry
M.Sc.	1956	Gujarat University, India	Chemistry
M.S.	1959	Michigan State Univ. East Lansing, Michigan U.S.A.	Physiology
Ph.D.	1964	Michigan State Univ.	Physiology-Biochemistry Endrocrinology as special field

HONORS:

(a) Awarded State Government of Sourashtra India, postgraduate fellowship for one year (1953-54) for obtaining highest marks at the B.Sc. examination.

(b) Elected as a full member of honorary national scientific society, Sigma Xi, U.S.A.

POSITIONS HELD:

(a) Teaching Assistant, Department of Biochemistry, M.P. Shah Medical College, India, 1955-57.

(b) Research Assistant, Department of Physiology and Pharmacology, Michigan State University, U.S.A., 1958-63.

TALKS PRESENTED AT THE SCIENTIFIC MEETINGS:

Meeting	Date	Topic
1. 44th Ann. Meeting Fed. Am. Soc. Exptl. Biol., Chicago, Illinois	April 14, 1960	Initiation and maintenance of milk secretion following chlorpromazine administration.
2. 45th Ann. Meeting Fed. Am. Soc. Exptl. Biol., Atlantic City, New Jersey	April, 1961	Initiation of mammary secretion in pregnant rats and rabbits by hydrocortisone acetate.
3. 46th Ann. Meeting Fed. Am. Soc. Exptl. Biol., Atlantic City, New Jersey	April 18, 1962	In vivo and in vitro prolactin production by rat "mammotropic" pituitary tumors.
4. North Central Conference on Comparative Endocrinology, Brooklodge, Michigan	May 6, 1962	In vitro evidence for prolactin inhibiting factor in the hypothalamus.
5. Ann. Meeting of Mich. Acad. of Sc., East Lansing, Michigan	March 26,1959	Effects of prolactin oxytocin and hydrocortisone on lactational performance of rats.
6. Ann. Meeting of Mich. Acad. of Sc., Ann Arbor, Michigan	March 27,1960	Effects of chlorpromazine on mammary glands of rats.
7. Ann. Meeting of Mich. Acad. of Sc., Detroit, Michigan	March 24,1961	Induction of lactation in pregnant animals by hydrocortisone acetate.

RESEARCH PUBLICATIONS
(During graduate studies at Michigan State University)

Co-author of a chapter in the book:

CNS and Secretion and Release of Prolactin. Chapter 8. In: Advances in Neuroendocrinology, edited by A. V. Nalbandov, University of Illinois Press, Urbana, Illinois. 1963.

Articles:

1. Talwalker, P. K., J. Meites, and C. S. Nicoll. Effects of hydrocortisone, prolactin and oxytocin on lactational performance of rats. Am. J. Physiol., 199: 1070, 1960.

2. Talwalker, P. K., J. Meites, C. S. Nicoll and T. F. Hopkins. Effects of chlorpromazine on mammary glands of rats. Am. J. Physiol., 199: 1073, 1960.

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

4. Talwalker, P. K., C. S. Nicoll and J. Meites. Induction of mammary secretion in pregnant rats and rabbits by hydrocortisone acetate. Endocrinology, 69: 802, 1961.

5. Talwalker, P. K., A Ratner and J. Meites. In vitro inhibition of pituitary prolactin synthesis and release by hypothalamic extract. Am. J. Physiol., 205: 213, 1963.

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

7. Ratner, A., P. K. Talwalker and J. Meites. Effect of estrogen administration in vivo on prolactin release in vitro. Proc. Soc. Exp. Biol. Med., 112: 12, 1963.

8. Meites, J., P. K. Talwalker and C. S. Nicoll. Failure of oxytocin to initiate mammary secretion in rabbits or rats. Proc. Soc. Exp. Biol. Med., 105: 467, 1961.

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

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

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

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

13. Meites, J., C. S. Nicoll and P. K. Talwalker. Induction and maintenance of lactation in rats by electrical stimulation of uterin cervix. Proc. Soc. Exp. Biol. Med., 102: 127, 1959.

14. Meites, J., T. F. Hopkins and P. K. Talwalker. Initiation of lactation in pregnant rabbits with prolactin, cortisol or both. Endrocinology, 73: 261, 1963.

15. Talwalker, P. K., A. Ratner and J. Meites. In vivo and in vitro prolactin production by rat "mammotropic" pituitary tumors. Federation Proc. 21: 196, 1962 (Abstract).

16. Mizuno, H., P. K. Talwalker and J. Meites. Inhibition of mammary secretion in rats by Iproniazid. Proc. Soc. Exp. Biol. Med. In press.