ABSTRACT

STUDIES ON THE NEUROENDOCRINE CONTROL OF PROLACTIN RELEASE IN MAMMALS AND BIRDS

by Clifford Lee Kragt

Several studies related to the physiology of prolactin are presented in this dissertation. Most of the emphasis was placed on comparing the neuroendocrine mechanisms regulating prolactin release from the pituitaries of rats and pigeons. In vitro and in vivo techniques were employed, involving incubation of the pituitary or transplantation of the pituitary. In addition, the physiological effects of "mammotropic" pituitary tumors were studied in male and female rats. Polyacrylamide gel electrophoresis was also tested for its potential usefulness as a semi-quantitative assay for prolactin. The following results were obtained:

1. An improved method for assaying PIF was developed. Male rat pituitaries were incubated in a Dubnoff shaker for 4 hours in flasks containing 2 ml of Medium 199 and sodium bicarbonate buffer. They were constantly gassed with 95% $O_2$-5% $CO_2$. The prolactin released by the equivalent of 2 male anterior pituitaries into 2 ml of medium was assayed in 5 pigeons, the control medium being injected on one side of the crop sac and the experimental medium on the other side.

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Little variation was observed in prolactin release by male pituitaries from experiment to experiment. The assay readily detected the smallest amount of hypothalamic extract tested (1 hypothalamus/ml) in this experiment. This assay for PIF may be sensitive enough to detect as little as one-quarter of a hypothalamus per ml of solution.

2. Hypothalamic extract containing PIF was tested at several levels for its effect on prolactin release by the above in vitro method. It was found that the amount of prolactin released into the medium was inversely related to the logarithm of the amount of hypothalamic extract present in the incubation medium over the range tested (1-4 hypothalami/ml). Hypothalamic extract in the same amounts also inhibited the release of prolactin from female rat pituitaries when incubated in vitro in a similar manner.

3. Earlier evidence indicated that the avian hypothalamus does not inhibit but may actually stimulate the release of prolactin from the avian pituitary. Hypothalamic extract equivalent to one hypothalamus from a parent pigeon increased the release of prolactin by the pigeon pituitary twofold when incubated in vitro for two hours. Extracts of pigeon hypothalamus and rat hypothalamus exerted no effect on the release of prolactin by the pituitary of the opposite species in vitro. Extracts of hypothalami from male and female chickens, and quail, also significantly stimulated the

release of prolactin by the pigeon pituitary *in vitro*. It was concluded that the hypothalamus of birds contains a "prolactin releasing factor" (PRF) in contrast to the hypothalamus of mammals which contains a "prolactin inhibiting factor" (PIF).

4. Transplantation of the pituitary of the rat to a site remote from the hypothalamus causes it to secrete increased amounts of prolactin and some growth hormone. Pigeon pituitary tissue was transplanted subcutaneously directly over the crop sac of an intact pigeon. It was found that tissue which had not vascularized disappeared 10 days after transplantation. Viable pituitary tissue did not release detectable amounts of prolactin 10 days after transplantation as indicated by the complete lack of crop sac stimulation. These results provide additional evidence that the pituitary in birds has to be stimulated by the hypothalamus in order to release prolactin.

5. The effects of a subcutaneous pituitary transplant and thyroxine injections on body and mammary growth were investigated in immature hypophysectomized rats. A single transplanted pituitary in a hypophysectomized rat maintained body growth at 47% of the intact control during a 30 day period. Mammary gland development was also stimulated by the transplant. Thyroxine increased body growth significantly in hypophysectomized animals with a pituitary transplant.

However, thyroxine inhibited mammary development in rats with a pituitary transplant. It was concluded that a pituitary transplant releases detectable amounts of prolactin and growth hormone.

6. Subcutaneous transplantation into female rats of a $MtT_{F4}$ pituitary tumor which secretes prolactin, growth hormone and ACTH had been reported to induce ovarian atrophy and luteal regression despite the high circulating levels of prolactin. The effect of adrenalectomy in tumor-bearing animals was studied in order to determine whether the high ACTH release by the tumor was responsible for the failure of prolactin to maintain luteal function in these rats. It was observed that removal of the adrenals prevented the decrease in ovarian weight and prevented luteal regression in tumor-bearing animals. It was concluded that adrenal corticoids reduce the sensitivity of the ovaries to prolactin and thereby cause luteolysis. Atrophy of the interstitial tissue and the decrease in ovarian weight were possibly due to inhibition by the tumor of LH release by the host animal's own pituitary.

7. Transplantation of the $MtT_{W15}$ pituitary tumor to intact and castrated male Wistar rats was also studied in order to determine the actions of high levels of prolactin and growth hormone in the male. The pituitary tumor produced gigantism in both intact and castrated animals. Tumor and body weights were greater in the intact than in the castrated

tumor-bearing animals. Upon sacrifice, it was observed that the adrenals were greatly enlarged which indicated that ACTH was also being secreted by the tumor. Atrophy of the testes and accessory glands was also observed in tumor-bearing males. This was analogous to the effects of the $MtT_{F4}$ tumor transplants in female rats. It was concluded that the pituitary tumor could be readily transplanted to males, and that testicular atrophy may have been due to excessive adrenal steroid release, as had been previously demonstrated in female rats transplanted with a similar tumor.

8. Anterior pituitary hormones of the rat were separated by gel electrophoresis. It was found that in this system, prolactin was the fastest migrating major component and had a greater mobility than albumin. Growth hormone, LH, and TSH all migrated much slower and could not be separated from one another in the region nearest the origin. It was concluded that prolactin could be readily separated from the other rat pituitary hormones by this technique, but that accurate quantitation could not be accomplished.

# STUDIES ON THE NEUROENDOCRINE CONTROL OF PROLACTIN RELEASE IN MAMMALS AND BIRDS

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# INTRODUCTION

Recent evidence indicates that the release of 5 of 6 hormones (FSH, LH, TSH, STH, and ACTH) from the anterior pituitary of mammals is stimulated by factors from the hypothalamus which reach the pituitary through the hypothalamo-hypophysial portal system. Prolactin release, on the other hand, is inhibited by the hypothalamus. Placement of lesions in the hypothalamus, pituitary transplantation, incubation of pituitary tissue and other experimental techniques have indicated that a prolactin inhibiting factor (PIF) in the hypothalamus suppresses the synthesis and release of prolactin in mammalian species under most conditions. Since an important requirement for the establishment of a specific hormone is the demonstration of a dose-response effect on its target tissue, an attempt was made to determine whether a negative dose-response relationship exists between the amount of rat hypothalamic extract added to an incubation medium and the amount of prolactin released by incubated rat pituitary.

In contrast to the rat and other mammalian species, several observations have suggested that the release of prolactin from the avian pituitary may not be inhibited but may be stimulated by the hypothalamus. It was of interest,

therefore, to test the effects of hypothalamic extracts from birds on release of prolactin by the pigeon pituitary to determine whether a stimulating or an inhibiting factor for prolactin release was present. It was also of interest to observe whether mammalian hypothalamic extracts had any effect on prolactin release by the avian pituitary, and whether avian hypothalamic extracts had any effect on prolactin release by the mammalian pituitary.

Studies were also carried out to determine the amounts of prolactin and growth hormone released by the transplanted rat pituitary, as indicated by mammary gland development and body growth in hypophysectomized immature rats. The effects of "mammotropic" pituitary tumor transplants, which secrete large quantities of prolactin, growth hormone, and ACTH, were also studied with particular reference to their luteotropic action on the ovaries and their effects on the testes. Finally, the polyacrylamide gel electrophoresis was evaluated for its possible use as a means of measuring the effects of hypothalamic extracts on anterior pituitary function in the rat.

# REVIEW OF LITERATURE

## I. GENERAL INTRODUCTION

It has been known for many years that the central nervous system exerts an important regulatory influence on the secretion of hormones by the anterior pituitary. Environmental factors such as light, temperature, and rainfall can alter gonadal development in birds (Rowan, 1925) and mammals (Marshall, 1942). Ovulation in the rabbit, normally induced in response to copulation, was also observed following electrical stimulation of the head (Marshall and Verney, 1936; Harris, 1937), which further indicated that neural elements were involved in the release of anterior pituitary hormones. Histological examination of the anterior pituitary revealed a lack of direct innervation from any source, and early workers (Rasmussen, 1938) were unable to determine the mechanism of integration between the nervous and endocrine systems.

The observation that a portal circulatory system was present between the median eminence and anterior pituitary was first reported by Popa and Fielding (1930). Since these workers incorrectly concluded that the direction of blood flow was from the pituitary upward to the hypothalamus, the possibility that this vascular route could be the link between the two systems was temporarily excluded. Subsequent direct

observation of blood flow in these vessels by Green and Harris (1949) in the rat and by Benoit and Assenmacher (1952) in the duck firmly established that blood flow, as Wislocki and King (1936) had suggested, was from the hypothalamus downward to the anterior pituitary. Harris (1955), combining his findings with those of others, formulated the theory of neurohumoral regulation of anterior pituitary secretion in mammals. Concurrently, Benoit and Assenmacher (1955) supplied abundant experimental evidence to indicate that this theory was also applicable to the avian class.

## II. COMPARISON OF MAMMALIAN AND AVIAN HYPOTHALAMO-HYPOPHYSIAL SYSTEMS

### A. Anatomical Relationships

Classically the dual embryonic origin of the pituitary tissue has led anatomists to divide it structurally into two parts, the neurohypophysis and the adenohypophysis. According to Rioch, Wislocki and O'Leary (1940), the neurohypophysis includes not only the posterior lobe and the infundibular system but also the median eminence. The tuber cinereum is a centrally located mound on the central surface of the diencephalon and is an extension of the floor of the third ventricle. From the surface of the tuber cinereum a medial process extends ventrally toward the posterior lobe of the pituitary; that portion of the process nearest the tuber cinereum is referred to as the median eminence (Baker, 1962). The adenohypophysis includes the pars distalis, pars

intermedia and the pars tuberalis (a thin sheet of tissue of oral ectodermal origin partially enveloping the infundibular stem). Several distinct differences exist between the avian and mammalian pituitary. In the avian pituitary, there is complete separation of posterior and anterior pituitary lobes by a connective tissue septum. A second difference is the complete absence of an intermediate lobe in birds (Wingstrand, 1951). In addition, the anterior lobe of the avian pituitary has also been divided into cephalic and caudal portions, poorly delineated by a slight anatomical constriction.

### B. Hypothalamo-hypophysial Vasculature in Mammals and Birds

In mammals (Fig. 1), the two anterior cerebral arteries, the two internal carotid arteries, the two posterior communicating arteries and the connection with the basilar artery together constitute a complete anastomatic ring on the base of the brain, the Circle of Willis. The internal carotid artery reaches the base of the brain lateral to the tuber cinereum, continues cephalad and gives off small branches to the base of the brain. Two recent reviews of pituitary-hypothalamic vasculature (Landsmeer, 1963; Greep, 1963) indicate that in most mammals there is a dual blood supply to the anterior pituitary. Blood courses via the hypothalamic artery to the floor of the third ventricle and then percolates to the anterior pituitary via the portal

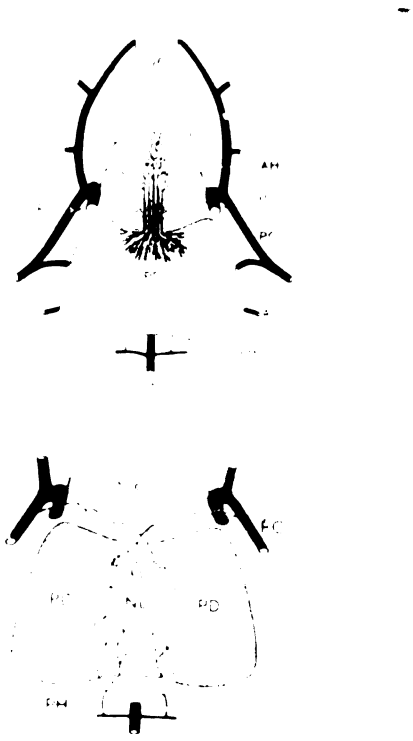

Fig. 1. (Upper) Vasculature of ventral aspect of rat pituitary. Abbreviations: OC=optic chiasma; AH=anterior hypophysial artery; IC=internal carotid artery; P=peduncular artery; PC=posterior communicating artery; PH=posterior hypophysial arteries; PD=pars distalis; AC=anterior communicating artery; B=basilar artery. (Lower) Dorsal aspect of rat pituitary. S=infundibular stalk; NL=neural lobe (from: Daniel and Prichard, 1956).

system. In rats this is the only blood supply to the anterior pituitary. Other mammalian species may possess an anterior hypophysial artery (Zeman and Innes, 1963).

The posterior lobe of most species is supplied by the inferior hypophysial artery. The rat is again unusual in that the neural lobe has dual arterial supply and venous drainage. Anteriorly a branch of the peduncular and posteriorly bilateral branches of the basilar artery supply the lobe. Some vascular connections between the posterior and anterior lobes of the pituitary have also been reported (Greep, 1963). Drainage of blood from the entire hypophysis occurs by way of small veins which pass to neighboring sinuses of the dura.

In certain avian species (Fig. 2), such as the white-crowned sparrow (passerine), the right common carotid artery is absent (Vitums et al., 1964). The arterial blood supply to the brain is supplied only by the left common carotid artery leading to asymmetry in brain vascularity. However, in the pigeon two common carotids are present (Wingstrand, 1951). The common carotids divide into external and internal branches just as in the mammal. The two internal carotids join in the region of the sella turcica. An inferior hypophyseal artery is also present. After the carotids have separated again, they pass rostrally along the sides of the pituitary and make a sharp bend before they reach the rostral end of the organ and pass through the diaphragma sella into the brain cavity. The Circle of Willis in birds

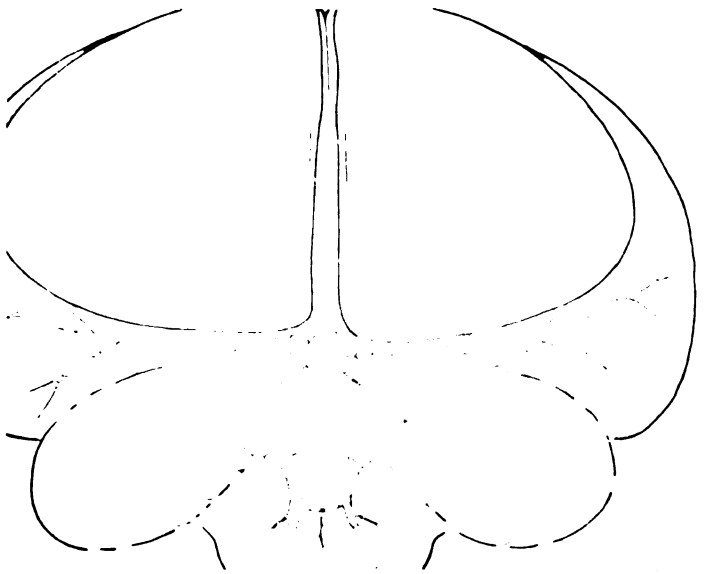

Fig. 2. Diagram of the arteries of the preoptic area of the brain of a white-crowned sparrow. Antero-ventral aspect. a. optic chiasma; b. hypophysis; c. optic lobe; d. medulla oblongata; e. hemisphere; 1. internal carotid artery; 2. intercarotid anastomosis; 3. basilar artery; 4. anterior ramus; 5. infundibular artery; 6. ventral optic lobe-artery; 7. posterior cerebral artery; 8. middle cerebral artery; 9. anterior cerebral artery; 10. preoptic arteries; 11. ethmoidal artery (from: Vitums et al., 1964).

is incomplete caudally, since the posterior rami of the carotids usually do not fuse. Again the pigeon is an exception since there is fusion. In the pigeon as well as nearly all other birds the circle is usually incomplete anteriorly, since the anterior cerebral arteries do not communicate as in mammals. Those vessels which do anastomose have capillary dimensions.

When the carotid touches the diencephalic wall it divides into two main trunks. Inside the brain the carotid emits a few vessels on each side which are the main arteries of the hypophysial region. The dense capillary network of the median eminence is supplied by the infundibular arteries. It is drained by the portal vessels which pass along the portal zone of the pars tuberalis to the secondary plexus in the pars distalis. In most investigated birds the portal vessels are the only afferent vessels to the pars distalis. The portal vessels enter the pars distalis in the transitional zone between the cephalic and caudal lobes and spread to all parts of the gland.

The neural lobe is supplied by arteries which, in the pigeon, are true inferior hypophyseal arteries coming from the intercarotid anastomosis. Blood from the neural lobe drains directly into the sinus cavernosus. Connections of the anterior lobe with the capillary bed of the neural lobe are absent in birds. Blood is drained from the pars distalis by three larger and several smaller outlets in the periglandular capsule. These drain into the posterior and

anterior intercavernous sinuses. The "venae carotides" are the most important outlets for the sinus cavernosus in birds. These carry the blood to the occipital region where it is finally transported to the internal jugular vein (Wingstrand, 1951).

## III. NEURAL REGULATION OF RELEASE OF ANTERIOR PITUITARY HORMONES OTHER THAN PROLACTIN IN MAMMALS AND BIRDS

Classical methods used to study neuroendocrine mechanisms include placement of hypothalamic lesions, stalk section, pituitary transplantation and *in vitro* culture and incubation systems. Central nervous system depressor or stimulatory drugs as well as hormones known to affect the nervous system have also been extensively employed by many researchers. The literature containing data obtained using these and other basic techniques to study each of the anterior pituitary hormones has increased greatly. Instead of presenting an extensive review of all of these papers in this dissertation, a calendar of events listing selected major contributions and reviews for each of the anterior pituitary hormones has been compiled (Tables 1-4) by the author. Reports for each of the anterior pituitary hormones have been categorized on the basis of techniques employed and within each of these categories listed chronologically.

A. Regulation in Mammals

On the basis of results obtained by placement of lesions, stalk section and pituitary tranplantation experiments (Tables 1-4), the existence of hypothalamic factors regulating anterior pituitary hormone secretion was postulated. In general, in mammalian species, it is now believed that the synthesis and release of anterior pituitary hormones are stimulated by small molecular weight polypeptides which can be extracted from hypothalamic tissue.

Requisites proposed by Guillemin and Schally (1963) which they feel must be met in order to definitely demonstrate the existence of a neurohumor have been partially accomplished for each of the pituitary hormone releasing factors. Corticotropin releasing factor (CRF), luteinizing hormone-releasing factor (LRF), follicle stimulating hormone-releasing factor (FSH-RF), growth hormone-releasing factor (SRF), and thyrotropin-releasing factor (TRF) have all been partially purified from crude extracts of hypothalamic tissue (Tables 1-4). An international standard has been proposed by Guillemin and Sakiz (1965) for TRF. Schally and Bowers (1964) have published the relative proportions of amino acids present in LRF but the sequence has not been determined. It is of considerable interest that many peptides of known amino acid composition and sequence, including $\alpha$ and $\beta$ CRF, appear to possess the ability to release ACTH from the pituitary (Guillemin and Schally, 1963). Progress on the purification

Table 1. Evidence for Neural Control of LH and FSH

| WORKER/S | DATE | AREA OF CONTRIBUTION |
|---|---|---|
| Harris | 1955 | Review |
| van der Werff ten Bosch | 1959 | " |
| Everett | 1961 | " |
| Assenmacher | 1963 | " |
| Bogdanove | 1964 | " |
| McCann and Ramirez | 1964 | " |
| Guillemin | 1964 | " |
| Rothchild | 1965 | " |
| Dey | 1941 | Hypothalamic lesions |
| Benoit and Assenmacher | 1952 | or stalk section |
| Bogdanove and Halmi | 1953 | " |
| Benoit and Assenmacher | 1955 | " |
| Shirley and Nalbandov | 1956 | " |
| Daily and Ganong | 1958 | " |
| D'Angelo | 1959 | " |
| Ralph | 1959 | " |
| Davidson and Ganong | 1960 | " |
| Benoit | 1962 | " |
| Flerko | 1962 | " |
| Egge | 1964 | " |
| Everett | 1954 | Pituitary transplant |
| Desclin | 1956 | " |
| Everett | 1956 | " |
| Everett and Nikitovitch-Winer | 1963 | " |
| Ma and Nalbandov | 1963 | " |
| Assenmacher and Bayle | 1964 | " |
| Kobayashi *et al.* | 1963 | *In vitro* |
| Mittler and Meites | 1964 | " |
| Schally and Bowers | 1964 | " |
| Mittler and Meites | 1966 | " |
| Piacsek | 1966 | " |
| Van Tienhoven *et al.* | 1954 | Drugs and Hormones |
| Everett | 1961 | " |
| Fraps | 1961 | " |
| Barraclough | 1963 | " |
| Bogdanove | 1964 | " |
| Rothchild | 1965 | " |
| Piacsek | 1966 | " |

Continued

Table 1 - Continued

| WORKER/S | DATE | AREA OF CONTRIBUTION |
|---|---|---|
| Martini | 1965 | Intra-carotid |
| McCann and Taleishnik | 1960 | Releasing factor |
| Guillemin *et al.* | 1963 | purification |
| Schally and Bowers | 1964 | " |
| Igarashi and McCann | 1964 | " |

Table 2. Evidence for Neural Control of ACTH

| WORKER/S | DATE | AREA OF CONTRIBUTION |
|---|---|---|
| Harris | 1955 | Review |
| Long | 1956 | " |
| Sayers *et al.* | 1958 | " |
| Saffran and Saffran | 1959 | " |
| Ganong and Forsham | 1960 | " |
| Flink | 1961 | " |
| Fortier | 1962 | " |
| Yates and Urquhart | 1962 | " |
| Ganong | 1963 | " |
| Vernikos-Danellis | 1965 | " |
| McCann and Brobeck | 1954 | Hypothalamic lesion |
| Shirley and Nalbandov | 1956 | or stalk section |
| Royce and Sayers | 1958 | " |
| De Wied | 1961 | " |
| Egge | 1964 | " |
| Fortier *et al.* | 1957 | Pituitary transplant |
| Critchlow *et al.* | 1963 | " |
| Ma and Nalbandov | 1963 | " |
| Matsuda *et al.* | 1964 | " |
| Assenmacher and Bayle | 1964 | " |
| Saffran *et al.* | 1955 | *In vitro* |
| Guillemin | 1955 | " |
| Guillemin *et al.* | 1959 | " |
| Munson and Briggs | 1955 | Drugs and Hormones |
| Martini *et al.* | 1960 | " |
| Leeman *et al.* | 1962 | " |
| Guillemin | 1962 | " |
| Doephfner | 1963 | " |
| Vernikos-Danellis | 1965 | " |
| Vernikos-Danellis | 1964 | Intra-carotid |
| Guillemin and Schally | 1963 | Releasing factor |
| Dhariwal *et al.* | 1966 | purification |

Table 3. Evidence for Neural Control of TSH

| WORKER/S | DATE | AREA OF CONTRIBUTION |
|---|---|---|
| Greer | 1957 | Review |
| D'Angelo and Traum | 1958 | " |
| Schreiber | 1963 | " |
| D'Angelo | 1963 | " |
| Schreiber | 1964 | " |
| Uotila | 1939 | Hypothalamic lesions |
| Shirley and Nalbandov | 1956 | or stalk section |
| D'Angelo | 1958 | " |
| Reichlin | 1960a | " |
| Bogdanove | 1962 | " |
| Assenmacher and Tixier-Vidal | 1962 | " |
| Egge and Chiasson | 1963 | " |
| Everett | 1954 | Pituitary transplant |
| Everett | 1956 | " |
| D'Angelo | 1963 | " |
| Ma and Nalbandov | 1963 | " |
| Assenmacher and Bayle | 1964 | " |
| Schreiber *et al.* | 1961 | *In vitro* |
| Guillemin *et al.* | 1963 | " |
| Bowers *et al.* | 1965 | " |
| Sinha and Meites | 1965 | " |
| von Euler and Holmgren | 1956 | Drugs and Hormones |
| Bogdanove and Crabill | 1961 | " |
| Guillemin *et al.* | 1965 | Releasing factor |
| Guillemin and Sakiz | 1965 | purification |

Table 4. Evidence for Neural Control of STH

| WORKER/S | DATE | AREA OF CONTRIBUTION |
|---|---|---|
| Meites | 1964 | Review |
| Cahane and Cahane | 1938 | Stalk section or |
| Bogdanove and Lipner | 1952 | lesions |
| Shirley and Nalbandov | 1956 | " |
| Hinton and Stevenson | 1959 | " |
| Reichlin | 1960 | " |
| Reichlin | 1960b | " |
| Reichlin | 1961 | " |
| Goldberg and Knobil | 1957 | Pituitary transplant |
| Martini *et al.* | 1959 | " |
| Hertz | 1959 | " |
| Swelheim and Wolthuis | 1962 | " |
| Dao and Gawlah | 1963 | " |
| Halasz *et al.* | 1963 | " |
| Ma and Nalbandov | 1963 | " |
| Meites and Kragt | 1964 | " |
| Assenmacher and Bayle | 1964 | " |
| Meites *et al.* | 1962 | *In vitro* |
| Franz *et al.* | 1962 | " |
| Deuben and Meites | 1964 | " |
| Deuben and Meites | 1965 | " |
| Schally *et al.* | 1964 | " |
| Pecile *et al.* | 1965 | Intra-carotid |
| Meites and Fiel | 1965 | " |
| Muller *et al.* | 1965 | Releasing factor |
| Pecile *et al.* | 1965 | purification |
| Ishida *et al.* | 1965 | " |
| Dhariwal *et al.* | 1965 | " |

of the other factors has not proceeded much beyond preliminary stages. Sephadex and CMC column chromatography and fractionation have been widely employed (Igarashi and McCann, 1964; Schally and Bowers, 1964; Dhariwal et al., 1965).

All of the releasing factors, either crude extracts or relatively purified preparations, have been shown to stimulate release of anterior pituitary hormones in vitro (Saffran et al., 1955; Schreiber et al., 1961; Deuben and Meites, 1964; Mittler and Meites, 1964; Piacsek, 1966). In vivo experiments have provided evidence for normal physiological functions by the releasing factors. Ovarian ascorbic acid depletion and ovulation has been repeatedly induced in primed-premature rats by treatment with hypothalamic extracts (Campbell et al., 1961; McCann, 1962). Treatment of normal animals with TRF preparations has been shown to increase circulation levels of TSH (Ducommun et al., 1965). This is one of the few reports of a correlation between plasma levels of tropic hormone and administered releasing factor. Vernikos-Danellis (1964) demonstrated such a relationship between injections of median eminence extracts and ACTH release. Pituitary depletion of FSH, ACTH, and growth hormone following intra-carotid injections of hypothalamic extracts has been used as an end point for demonstrating the other respective releasing factors in vivo (Martini, 1965; Vernikos-Danellis, 1965; Meites and Fiel, 1965).

Definite fluctuations in hypothalamic content of all releasing factors has been observed to correlate well with observed physiological changes. Castration and steroid treatments have been reported to alter the hypothalamic contents of LRF and FSH-RF (Mittler and Meites, 1966; Piacsek, 1966). Hypothalamic content of LRF has been reported to parallel the pituitary LH content during the cycle of the female rat (Ramirez and McCann, 1965). A decrease in the hypothalamic content of CRF has been detected following stress in rats (Vernikos-Danellis, 1964). It has been observed that exposure of rats to cold (temperatures below 23°C) increased the TRF content of the hypothalamus in an inverse proportion to decreasing temperature (Sinha and Meites, unpublished observations). Starvation has been shown to cause a decrease in both hypothalamic SRF and pituitary growth hormone content (Meites and Fiel, 1965). Suckling, estrogen, reserpine, epinephrine and acetycholine have also been reported to alter hypothalamic content of PIF (Ratner and Meites, 1964; Ratner et al., 1965; Mittler and Meites, unpublished observations).

The many different interrelationships involved in the environment-brain-pituitary sequence have definitely not been elucidated. However, Halász and Pupp (1965) have suggested on the basis of recent experiments with the "deafferentiated hypothalamic island" that environmental factors, both internal and external, may act directly at brain levels

higher than the hypothalamus. Alteration of hypothalamic releasing factor content may then be the result of afferent impulses coursing to the "hypophysiotrophic" area.

A log-dose response relationship has been determined for each of the releasing factors except PIF. McCann (1962) demonstrated this relationship for LRF in vivo using the primed Parlow rat. Piacsek (1966) has shown that this relationship also holds for in vitro stimulation of LH release. Mittler and Meites (1966) using a six hour incubation system found that the quantity of FSH released into the medium by rat pituitaries was also a function of the log of the dose of hypothalamic extract added. This relationship has not yet been demonstrated in vivo. Release of ACTH from the pituitary is related to the log of the dose of hypothalamic extract injected into the carotid artery (Vernikos-Danellis, 1964). Using the same in vivo system a similar relationship was also demonstrated to exist by Meites and Fiel (1965) for SRF. Deuben and Meites (1964) had previously reported that the release of growth hormone in vitro by a cultured pituitary was also related to the log of the amount of hypothalamic extract added to the culture.

Currently, no evidence has been reported on the detection of neurohumors in portal blood. However, Nallar and McCann (1965) have reported plasma LRF was detectable in hypophysectomized rats. The observation that increased release of gonadotropic hormones occurred when hypophysectomized

rats bearing subcutaneous pituitary transplants were exposed to constant light, very strongly suggests that releasing factors may also be present in systemic circulation in this animal preparation (Piacsek, 1966).

Additional data must be obtained to determine the precise mechanism(s) of actions for these neurohormones. The possibility that multiple peptides may stimulate the release of a single hormone has been exemplified by the observation that α CRF, β CRF, α MSH and β MSH all stimulate release of ACTH from the pituitary (Guillemin and Schally, 1963). The amino acid analysis of the α and β CRF indicates that they may be precursors for α and β MSH. This logically leads to the possibility that neurohumors may be precursors for anterior pituitary hormones, since it has been demonstrated that α MSH makes up a portion of both the ACTH and lipotropic hormone (Li, 1962, 1965) molecule. Only further research will answer these exciting questions.

B. Regulation in Birds

Study of the neuroendocrine mechanisms in birds has at certain times paralleled but more commonly lagged considerably behind that in mammals. Hypothalamic lesions or pituitary stalk section have been reported to cause gonadal atrophy in the domestic duck (Benoit and Assenmacher, 1952) and also prevent light induced gonadal development (Assenmacher and Benoit, 1953). Stalk section in laying hens has an effect similar to that of hypophysectomy on the gonads, producing

a loss in body weight, but has little or no effect on thyroid or adrenal weight (Shirley and Nalbandov, 1956). Ralph (1959) found that localized hypothalamic lesions in the medial region of the ventral portion of the paraventricular nucleus were most effective in preventing ovulation in the hen. Assenmacher and Tixier-Vidal (1962) also reported that stalk section has little effect on thyroid function in the duck. These observations do not agree with those of Egge and Chiasson (1963) who reported that thyroid weight and epithelial cell activity were reduced with lesions localized in the preoptic region of the supraoptic nucleus of the hen. The reasons for these differences are not apparent at this time.

Transplantation of the pituitary to the kidney capsule in chickens (Ma and Nalvandov, 1963) has little effect on the secretion or release of TSH and apparently leads to an increased rate of adrenal function. Assenmacher and Bayle (1964) also noted a decrease in gonadotropin secretion by the transplanted duck pituitary; however, they unlike Ma and Nalbandov (1963) did observe considerable atrophy of the thyroids and adrenals. These marked differences in results await explanation.

Assessment of pituitary function of birds by <u>in vitro</u> techniques has not been applied to the study of hormone release except as related to prolactin. In addition, no attempts have been made to purify the releasing factors

from avian hypothalamic sources. In brief, research techniques (Tables 1-4) such as hypothalamic lesioning, stalk sectioning and pituitary transplantation provide strong evidence for hypothalamic regulation of pituitary secretion in birds. Differences between hypothalamic regulatory mechanisms in mammals and birds are readily apparent. Little data has yet been accumulated on the detection, isolation, and characterization of avian neurohumors as compared to that available on mammalian neurohumors.

## IV. NEURAL REGULATION OF PROLACTIN RELEASE FROM THE ANTERIOR PITUITARY OF MAMMALS AND BIRDS

### A. Physiological Indications of Neural Control

#### 1. Experiments in mammalian species

Seyle (1934) demonstrated that the stimulus of suckling caused milk secretion, and was the first to suggest that it was due to prolactin release. He supported his hypothesis with the observation that when the nipples of the nursing female rat were removed, thus preventing suckling, the mammary glands involuted. Also, he reported that suckling of virgin, cycling, female rats by foster litters resulted in the induction of pseudopregnancy and lactation (Seyle and McKeown, 1934). These physiological responses have definitely been shown to be dependent on prolactin (Riddle and Bates, 1939). Non-lactating women, when suckled by infants, also exhibit mammary gland development and lactation (Gellhorn, 1908).

Mammary gland regression following removal of the pups has been experimentally retarded by irritative stimuli such as topical application of turpentine to the nipples (Mixner and Turner, 1941). Ota and Yokoyama (1965) have recently found that lactation can be reinitiated in female rats from which young have been removed for several days by adding foster litters. Maqsood and Meites (1961) reported that electrical stimulation of the nipples also induced mammary secretion in estrogen-primed female rats. Initiation and reinitiation of lactation and retardation of mammary gland involution by neural stimuli eminating from the nipples was most probably due to prolactin release from the pituitary. Reece and Turner (1936) and Grosvenor and Turner (1957) reported that suckling of lactating female rats by young caused a depletion of pituitary prolactin content. However, other workers have been unable to demonstrate pituitary prolactin depletion following suckling in rats (Meites and Nicoll, 1966).

Pseudopregnancy has been induced in the female rat by a variety of means among which are sterile copulation, mechanical stimulation of the cervix with a glass rod, and electrical stimulation of the cervix during proestrus (Long and Evans, 1922; Slonaker, 1929; Shelesnyak, 1931). Lactogenic hormone or prolactin has long been known to be the anterior pituitary hormone which causes enlargement of the corpora lutea and progesterone secretion characteristic of

pseudopregnancy (Evans et al., 1941). Depletion of pituitary prolactin has also been reported to occur after mechanical stimulation of the cervix (Herlyn et al., 1965), although confirmation of these results would be desirable.

2. Experiments in avian species

Prolactin has been shown to initiate several physiological processes in birds. In addition to its more commonly known stimulatory action on pigeon crop "milk" formation (Riddle and Braucher, 1931), prolactin injections into hens or roosters induced broodiness (Nalbandov, 1953). Pituitary prolactin content was also reported to be highest during brooding periods of the pigeon (Schooley and Riddle, 1938), hen (Saeki and Tanabe, 1955), pheasant (Breitenbach and Meyer, 1959) and turkey (Cherms et al., 1962). However, the question of whether this broodiness is a direct or indirect effect of prolactin has been debated by Riddle (1963) and Lehrman (1963). Nevertheless, it has been suggested that visual and tactile stimuli such as seeing or touching eggs or young in the nest leads to prolactin release and subsequent incubation, crop development and broody behavior in the domestic pigeon (Medway, 1961) and ring dove (Lehrman, 1964).

Electrical stimulation of the head of chickens terminates broodiness, presumably by interfering with prolactin secretion (Nakajo, 1952). It should be recalled that such stimulation caused prolactin and LH release, and pseudopregnancy to occur in rabbits and rats. Stimulation of

tactile receptors of the breast by topical application of turpentine to the apterium (Medway, 1961) of the pigeon was extremely toxic but nevertheless did not stimulate crop gland development.

Prolactin release in response to seasonal environmental changes has also been suggested (Meier and Farner, 1964; King and Farner, 1965) to be responsible for premigratory deposition of fat in passerine species. Bates et al. (1962) reported that prolactin administration to the hypophysectomized pigeon also produced increased fat storage. The seasonal cessation of reproduction in non-domestic avian species has also been suggested to be the result of the antigonadal action of prolactin released as a result of the change in photoperiod (Riddle and Bates, 1939). Induction of prolactin release in ducks and pigeons by increasing photoperiodicity or continuous light has been claimed by Assenmacher et al. (1962). However, the evidence presented was questionable since quantification of prolactin content of the pituitary was made to histological measurement of the crop sac epithelium and not by biological assay. The authors admit that crop gland stimulation in pigeons exposed to constant illumination was not visually apparent. Only a few microns difference in thickness of the crop was detected microscopically, and since one microgram of prolactin injected intradermally leads to crop changes visible to the naked eye, this reported thickening appears to be non-physiological and not related to prolactin.

In conclusion, it can be stated that abundant evidence exists which indicates that environmental stimuli, detected and transmitted by the nervous system, lead to prolactin release in mammals and birds.

## B. Effects of Hypothalamic Lesions and Stalk Section

### 1. Experiments in mammalian species

Initial experiments with hypothalamic lesions and pituitary stalk section were plagued by conflicting and inconsistent results (Desclin, 1940; Uotila, 1940; Dempsey and Uotila, 1940). Most discrepancies were later found to be due to rapid regeneration of the portal system (Greep and Barrnett, 1951) following transection. Originally, when studying the effects of stalk section on prolactin secretion, the gain in weight of the pups after suckling lesioned and non-lesioned mothers was used as an end-point (Jacobsohn and Westman, 1945). All pups died shortly after section and it was therefore postulated that prolactin secretion decreased and mammary glands involuted. This end-point of course is the result of multiple changes resulting from stalk section. Subsequent experiments (Jacobsohn, 1949; Donovan and van der Werff ten Bosch, 1957; Yokoyama and Ota, 1959), using supplementary oxytocin injections, demonstrated that the principle reason for death of the pups after placing lesions in the mother was starvation because of abolition of the oxytocic reflex and cessation of ACTH release and that

in fact mammary gland development persisted after stalk section (Gale et al., 1961). Stalk section has now been shown to increase prolactin release from the pituitary in the female rat (McCann and Friedman, 1960), the male rat (De Voe et al., 1966), the rabbit (Haun and Sawyer, 1960), the cat (Grosz and Rathballer, 1961), and women (Eckles et al., 1958).

Sawyer and co-workers (Haun and Sawyer, 1961; Sawyer et al., 1963; Kanematsu et al., 1962) presented evidence supporting the proposal made earlier by Everett (1964) that the center responsible for control of LH release and prolactin release may be identical. Lesions of the same basal tuberal area which prevented ovulation also induced mammary development in estrogen-primed rabbits. More recent physiological evidence has made this hypothesis less and less tenable (Rothchild, 1965). Everett and Quinn (1966) have currently provided definite anatomical proof for the existence of separate hypothalamic regulatory centers for LH and prolactin secretion. In addition, Schally et al. (1964) have published evidence that purified LH releasing factor (LRF) is free of prolactin inhibiting factor (PIF).

2. Experiments in avian species

Avian neuroendocrinologists have emphasized the importance of the neurosecretory stainable material in the hypothalamus and infundibular stalk in the control of anterior pituitary secretory processes. Several workers have

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correlated the quantity of aldehyde fuchsin positive neurosecretory material with the seasonal status of gonadal development (Assenmacher, 1957; Farner and Oksche, 1962; Oksche et al., 1964). The chemical significance of this neurosecretory material is uncertain and recently it has been suggested that such material may be more important relative to posterior lobe function than anterior lobe secretion of hormones (Graber and Nalbandov, 1965). Avian neuroendocrinologists may therefore be over emphasizing its importance. With this reservation in mind, it should be noted that variations in the diameter of hypothalamic nuclei and neurosecretory material have also been reported to occur during incubation in the chicken (Legait, 1955). This may be associated with high prolactin secretion.

Many studies have been reported on the effects of placement of lesions in the hypothalamus and pituitary stalk section as related to gonadal development in avian species (Benoit and Assenmacher, 1952). Very few have considered the effect of such treatments on prolactin release from the pituitary. Stalk section in the hen (Shirley and Nalbandov, 1956) and duck (Assenmacher and Tixier-Vidal, 1960) do not appear to cause increased prolactin secretion as is true in mammals. These later workers reported that the pituitary cells which were least sensitive to stalk section were the prolactin cells. The evidence, however, is too limited to draw definite conclusions.

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## C. Pituitary Transplantation

### 1. Experiments in mammalian species

The full importance of the observation made by Desclin (1950) that the transplanted anterior pituitary of the rat induced pseudopregnancy was not fully appreciated until studied extensively by Everett (1954). Transplantation of the pituitary to the kidney capsule of the hypophysectomized rat resulted in a decrease in release of all pituitary hormones except prolactin (Everett, 1956). These results have been repeatedly confirmed (Alloiteau, 1958; Quilligan and Rothchild, 1960; Montemurro and Gardner, 1961, 1963; Wolthuis and de Jongh, 1963; Nikitovitch-Winer, 1965). Cytologically the pituitary grafts contained only prolactin cells (Saunders and Rennels, 1957). Re-transplantation of the pituitary from the kidney capsule back to the sella turcica led to re-differentiation of the cells and near normal tropic hormone secretion (Nikitovitch-Winer and Everett, 1958; Everett and Nikitovitch-Winer, 1963). Gonadotropic function was sufficient to sustain pregnancy. It has been suggested that even though the transplanted rat pituitary continuously secretes prolactin at a level higher than during the estrous cycle, it may not be secreting prolactin at maximum capacity (Meites et al., 1963; Mayer, 1963; Everett, 1956), since estrogen can further stimulate release.

Mammary growth has been induced by pituitary grafts in intact and hypophysectomized rats (Meites and Hopkins, 1960; Cowie et al., 1960; Dao and Gowlak, 1963; Meites and Kragt, 1964). Subcutaneous isografts also interrupted the estrous cycle of the mouse (Montemurro and Gardner, 1961) and produced mammary development in intact and hypophysectomized mice (Bardin et al., 1964). However, it appears that the guinea pig may be unusual in that intraocular pituitary transplants in hypophysectomized guinea pigs can maintain the reproductive tract (Schweizer et al., 1940), and mammary glands regress after stalk section (Aron and Marescaux, 1962). Intramammary implants of anterior pituitary fragments in hypophysectomized guinea pigs also failed to maintain mammary gland integrity (Russel, 1962). However, hormones other than prolactin may also be necessary for development in the guinea pig.

2. Experiments in avian species

Transplantation of the avian pituitary has been performed in the hen (Ma and Nalbandov, 1963), the male duck (Assenmacher and Bayle, 1964) and pigeon (Bayle and Assenmacher, 1965). In both species gonadal development was extremely depressed. According to Ma and Nalbandov, "there was no apparent increase in prolactin secretion."
A physiological end-point for prolactin in the chicken and duck is however difficult to specify. Cytological evidence reported by Tixier-Vidal et al. (1965) indicated that there

were abundant prolactin and LH cells present in ectopic pituitary transplants. Conclusions on hormonal content based on histological observations may be misleading. Of much greater significance is the observation reported by Bayle and Assenmacher (1965) that there was no difference between crop weights and epithelial mucosal thicknesses of hypophysectomized pigeons with pituitary autotransplant when compared with those from hypophysectomized pigeons without transplants. If the transplanted pituitary had been secreting abundant amounts of prolactin, as is true of mammalian pituitary transplants, the crop sac would have been extensively stimulated. Since this was not observed and because the crop sac is a definite target organ for prolactin, it can be concluded that the transplanted pigeon pituitary does not release appreciable amounts of prolactin in contrast to the mammalian pituitary.

### D. Drugs and Hormones

#### 1. Experiments in mammalian species

Numerous drugs and hormones induce release of prolactin from the anterior pituitary of the rat (Meites, 1962). Steroids, especially estrogens increased the prolactin content of the pituitary (Reece and Turner, 1936a) and induced mammary gland development in female rats. The rise in circulating estrogen levels prior to parturition has also been indicated to be one of the main mechanisms responsible for

initiation of post-partum lactation (Meites and Turner, 1942). Kanematsu and Sawyer (1961, 1963) have suggested that estrogens may act both at the pituitary and hypothalamic levels. In rabbits, estradiol implants in the posterior median eminence elevated the pituitary prolactin content but elicited no mammary gland stimulation; only implants in the anterior pituitary induced mammary gland development and lactation. These results are in agreement with those of Nicoll and Meites (1963, 1964), who found that estradiol benzoate increased prolactin release from the rat pituitary when cultured in vitro. Ratner and Meites (1964) found that estradiol benzoate injections for 10 days into cycling rats also depleted the hypothalamic PIF content. This observation further supports the concept of dual mechanisms of action for estrogen. Injections of testosterone prcpionate increased pituitary prolactin content of spayed female rats about 40% and induced extensive mammary development (Reece and Mixner, 1939). Progesterone has been reported to have only a slight stimulatory effect on prolactin content of the anterior pituitary of spayed rats, but synergized with estrogen to give a pronounced increase in prolactin content (Reece and Bivins, 1942).

Tranquilizers such as reserpine and chloropromazine, administered chronically to human females, resulted in galactorrhea (Sulman and Winnik, 1956). Pseudopregnancy (Barraclough and Sawyer, 1959) and mammary development

(Meites et al., 1959) occurred in female rats when the above drugs were administered. These observations definitely indicate that an increase in prolactin release from the pituitary occurred and suggested that some neural inhibitory mechanism had been suppressed. Reserpine treatment has also been reported to increase the incidence of mammary cancer in certain strains of mice receiving concurrent estrogen treatment (Lacassagne, 1961).

In the course of development of the field of neuroendocrinology, the posterior pituitary hormones have repeatedly been considered as potential releasers for the anterior pituitary hormones. Prolactin has been no exception. Benson and Folley (1956) suggested that oxytocin may be a stimulator of prolactin release in the rat. Subsequent reports have proven this hypothesis to be incorrect. The initial observation of these workers, that oxytocin retarded mammary gland involution was valid; however, the mode of action suggested was incorrect. Oxytocin has now been shown to exert its effect by evacuating the glandular contents and thereby prevents involution due to congestion (Meites et al., 1960). It is now generally held that the neural lobe hormones are not of primary importance in release of anterior pituitary hormones. However, chemical structural similarities have been reported to exist between the posterior lobe hormones and the hypothalamic neurohumors, and the possibility exists that similar biological activities may be possessed by related chemical structures.

## 2. Experiments in avian species

No drug or hormone when administered to pigeons has been conclusively demonstrated to release prolactin from the birds pituitary. An early paper (Riddle and Lahr, 1944) indicated that doves treated with progesterone pellet implants and housed in nests containing "dummy" eggs had extensive crop development. Subsequent reports (Meites and Turner, 1947; Medway, 1961) indicated that the egg itself was responsible for the effect and that progesterone administration alone was ineffective. Diethylstilbestrol, testosterone propionate, progesterone and estrone were all found to have no effect on pigeon pituitary prolactin content and crop gland development (Meites and Turner, 1947).

Reserpine has also been claimed to stimulate prolactin release and crop gland development when injected into pigeons (Lefranc, 1958; Tixier-Vidal and Assenmacher, 1962). In this laboratory we have not been able to duplicate these results and question the validity of the methods used for assessment of crop gland stimulation by the French workers. Histological differences in gland thickness after reserpine treatment were reported by the above authors, but no visual proliferation was detected.

When oxytocin was considered to induce prolactin release in mammals (Benson and Folley, 1956), it was also tested in pigeons (Chaudhury and Chaudhury, 1962). These workers found that intradermal and systemic administration

of oxytocin both produced increases in crop weight, and suggested that prolactin release had occurred. Mizuno and Meites (1963), using the same oxytocin doses as the above workers, could find neither a change in pigeon crop weight nor any visual proliferation. Hohn (1963) also reported no effect of oxytocin on the pigeon crop gland. Another substance which has been suggested to affect avian prolactin and growth hormone acidophils is intermedin or MSH (Legait and Legait, 1955). However, this requires confirmation.

## E. In Vitro Studies

### 1. Experiments in mammalian species

Previous to Everett's (1954) pituitary transplantation experiments, numerous workers had cultured pituitary tissue *in vitro* (Anderson and Haymaker, 1935; Cutting and Lewis, 1938) but with limited success. Since the transplanted pituitary secreted considerable prolactin *in vivo*, it became of interest to see whether the same was true *in vitro*. Using organ culture techniques, Meites *et al*. (1961) and Pasteels (1961) demonstrated that abundant prolactin was released by cultured pituitaries. In view of the previous work (Benson and Folley, 1956) which suggested that oxytocin may stimulate prolactin release, the direct effect of this and other known hypothalamic constituents were tested *in vitro*. Oxytocin, vasopressin, epinephrine and acetylcholine were all found to have no effect on prolactin

release (Nicoll and Meites, 1962). In vivo administration of estrogen (Ratner et al., 1963) and estrogen incorporated into culture medium both augmented the amount of prolactin released into the medium (Meites and Nicoll, 1965). Pituitaries of all mammalian species tested synthesized more prolactin than was present in fresh pituitary tissue (Nicoll and Meites, 1962). Gala and Reece (1965) recently reported stimulation of prolactin release in vitro cultures by epinephrine, but their results do not appear to be convincing and are clearly in conflict with all other recent work indicating that epinephrine does not directly stimulate pituitary function.

Co-cultures of hypothalamic pieces (Pasteels, 1961a; Danon et al., 1963) and pituitaries, or addition of hypothalamic extracts (Talwalker et al., 1963) to pituitaries incubated in vitro, inhibited the release of prolactin from the pituitaries. This substance present in the extracts has been named prolactin inhibiting factor (PIF). Suckling, estrogen administration or reserpine injections, all known to induce prolactin release in vivo, have been shown to deplete the PIF content of the rat hypothalamus (Ratner and Meites, 1964; Ratner et al., 1965).

Grosvenor (1965) has reported that hypothalamic extracts show no dose-response curve and act in an 'all-or-none' fashion when given in vivo to rats. The assay procedure he used is highly questionable. Circulating levels of estrogens

and progesterone are known to influence the pituitary content of prolactin (Reece and Turner, 1937; Reece and Bivins, 1942). Crude hypothalamic extracts contain FSH-RF and LRF which could directly augment secretion of pituitary FSH and LH, which in turn stimulate estrogen and possibly progesterone secretion, and thus indirectly increase pituitary prolactin. Such hypothalamic extracts also contain TRF and CRF, which indirectly could result in increased thyroxine and adrenal steroids, both of which can stimulate prolactin secretion (Meites et al., 1953). Such crude extracts also contain epinephrine, norepinephrine, acetylcholine and serotonin, all of which have been shown to increase prolactin secretion in vivo (Meites et al., 1963). The lack of a log dose-response relationship then may be due to the influences of other neurohumors and hormones. The author believes that the use of an in vitro system provided results with greater validity, since the target organs are not present and no factor except PIF has been shown to influence the pituitary directly (Talwalker et al., 1963).

2. Experiments in avian species

In contrast to mammalian pituitaries, pigeon pituitaries when cultured in vitro did not release very much prolactin into the medium (Nicoll and Meites, 1962). The rat pituitary actually synthesizes from 10-15 times as much prolactin as is present in fresh tissue, whereas the pigeon pituitary releases an amount very near to the original gland

content during 6 days of culture. Tixier-Vidal and Goudji (1965) have also observed that prolactin was released when cultured in vitro for up to 14 days. Comparison of their results with those of Pasteels (1961) for rat pituitary tissue in culture also led them to the conclusion that the avian pituitary released much less prolactin than mammalian pituitary. The inability of Gala and Reece (1965a) to demonstrate release of prolactin when culturing pigeon pituitary can be attributed to poor culture techniques. The data previously published (Kragt and Meites, 1965) and presented in this dissertation and an additional report by Nicoll (1965), definitely indicate that pituitaries from pigeons and tri-colored blackbirds release detectable amounts of prolactin in incubation or culture systems.

### F. Hypothalamic Factors

#### 1. Experiments in mammalian species

Only limited attempts have been made to chemically identify and purify hypothalamic PIF after the initial demonstration of its existence (Talwalker et al., 1963). Purified LRF preparations (Schally et al., 1964) have been found to be free of PIF activity. Using the questionable in vivo assay procedure described previously, Grosvenor has found PIF activity to be present in the polypeptide fraction of rat and bovine stalk median eminence extracts prepared by McCanns' laboratory (Grosvenor et al., 1965). However,

McCanns' laboratory has not been able to separate LRF and PIF activities (Dhariwal et al., 1965). Recently, Kuroshima et al. (1966) have reported that porcine hypothalamic extracts also contain PIF. Injection of one hypothalamic equivalent intraperitoneally 15 minutes prior to cervical stimulation prevented the depletion of pituitary prolactin previously shown to occur by Herlyn et al. (1965) one-half hour after stimulation. This in vivo method for testing PIF is open to the same criticisms as that of Grosvenor (1965).

2. Experiments in avian species

The data presented in this dissertation represent the first attempt to test avian hypothalamic material for its effect on prolactin release. An interesting report has recently appeared indicating that in plasma of hypophysectomized chickens a substance is present which depletes ovarian ascorbic acid in the Parlow rat (Nalbandov, personal communication). Since the gonads of the bird are atrophic it is exciting to postulate that this substance may be a hypothalamic releasing factor for LH. Thus some evidence has been reported for the existence of at least two hypothalamic factors in birds which influence anterior pituitary hormone secretion.

## EXPERIMENTAL

### I. DOSE-RESPONSE RELATIONSHIPS BETWEEN THE AMOUNTS OF HYPOTHALAMIC EXTRACT ADDED TO A MEDIUM AND RELEASE OF PROLACTIN BY THE RAT PITUITARY IN VITRO

#### Objectives

Dose-response relationships have been determined for all hypothalamic factors regulating anterior pituitary function with the exception of PIF. The purpose of this experiment was to determine whether a dose-response relationship existed between the amounts of hypothalamic extract added to a medium and release of prolactin by the rat pituitary. Cerebral cortical extract was also tested at several levels for its effect on prolactin release by the pituitary *in vitro*.

Large variations have been observed in the amount of prolactin released by female pituitaries from experiment to experiment, even when incubated under similar conditions (Nicoll and Meites, 1962, 1963). These fluctuations may be due to the alteration of steroid titers in the blood during the estrous cycle. To avoid these fluctuations, male pituitaries were incubated and the amount of prolactin released in 4 separate experiments was compared. The effects of several doses of male hypothalamic extract on release of prolactin by the male pituitary were also observed.

## Materials and Methods

Mature male and female Sprague-Dawley rats (Spartan Research Farms) were maintained in a temperature controlled (75±1°F) and artifically illuminated (14 hr. light - 10 hr. dark) room. These rats were used as hypothalamic and pituitary donors in these experiments. They were supplied with water and Wayne Lab Blox ad libitum. Rats were sacrificed by decapitation, and anterior pituitaries and/or hypothalami were removed. The hypothalami and an equal volume of cerebral cortical tissue were individually homogenized in chilled .1 N HCl and centrifuged (12,000 g) for 30 minutes at 4°C. The supernatant was decanted, neutralized with 1.0 N NaOH, and stored on ice until used within less than 1 hour. Each anterior pituitary was separated from the posterior lobe, quartered and each quarter placed in one of four flasks containing medium 199 (Difco Laboratories, Detroit, Michigan) maintained at pH 7.4 with bicarbonate buffer. Each flask contained 8 pituitary quarters or the equivalent of 2 pituitaries. Final concentrations of hypothalamic extract present in the 2 ml volume of medium were 0, 1, 2, and 4 hypothalamic equivalents per ml of medium. All incubations were carried out in a Dubnoff metabolic shaker (60 cycles/min) under constant gassing with humidified 95% $O_2$-5% $CO_2$ at 37.5±.5°C for periods of 2 or 4 hours. At the end of incubation the medium was separated from the anterior pituitary quarters, frozen and stored at -20°C until assayed. Pituitary tissue

was weighed so that prolactin release could be expressed on the basis of per unit pituitary weight.

Prolactin was assayed by the intradermal pigeon crop technique (Reece and Turner, 1937). One crop side was injected with either 1 $\gamma$ or 5 $\gamma$ of NIH-B-1 prolactin standard. A total of 10 birds were injected with each standard dose in each of two experiments. The opposite side of the crop sac was injected with medium from flasks in which rat pituitaries had been incubated. Each unknown was assayed at one dosage level in a total of 5 assay pigeons. By this design 4 unknown preparations could be assayed for prolactin in the same 20 pigeons and expressed in terms of NIH-B-1 prolactin standard by use of Bliss (1952) statistics. The statistics used were those designed for three point assays. Since the slopes of the individual assays of standard NIH prolactin did not differ significantly, the data of two separate assays were pooled and the combined slope used in the calculations of potencies for the unknown samples. Statistical procedures are presented in Table 5.

## Results

### 1. Standard-NIH-B-1 prolactin assay data (Fig. 3)

The mean crop responses of 20 pigeons given 1 $\gamma$ and 5 $\gamma$ NIH-B-1 were 0.45 and 1.38 RTU, respectively. The slope of the curve was 1.32 and was statistically greater than zero ($P < 0.01$). The index of precision ($\lambda$) was .190. This curve

Table 5. Statistical formulae used for prolactin and PIF assays

1. Slope = b

$$b = \frac{\Sigma xy - \frac{(\Sigma x)\ (\Sigma y)}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

2. Analysis of Variance (ANOV)

| Item | d.f. | s.s. | m.s. |
|---|---|---|---|
| A. Deviations from mean | n-1 | $\Sigma y^2 - \frac{(\Sigma y)^2}{n}$ | |
| B. Deviations due to regression | 1 | $\frac{(\Sigma xy - \frac{(\Sigma x)\ (\Sigma y)}{n})^2}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$ | |
| C. Deviations from regression | n-2 | (A - B) = $d^2_{y.x}$ | $d^2_{y.x}/n-2 = s^2_{y.x}$ |

3. Standard Deviation of the Slope = s.d. (b)

$$\text{s.d. (b)} = \frac{s_{y.x}}{\sqrt{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}}$$

4. Index of Precision $\lambda = \frac{\text{s.d. (b)}}{b}$

5. Potency = m' = $\frac{\bar{Y}u - \bar{Y}s}{bc}$

6. Confidence Limits of Potency

$$X_1 = C^2M \pm tCs_m$$

where: $C^2 = \frac{B^2}{B^2 - s^2 t^2}$

$B^2$ = s.s. of deviation due to regression

$s^2$ = m.s. deviation of error

$t$ = tabular t value

$$s_m = \sqrt{\frac{1}{N_u} + \frac{1}{N_s} + \frac{(\bar{Y}u - \bar{Y}s)^2}{B^2 - s^2 t^2}} \cdot \lambda$$

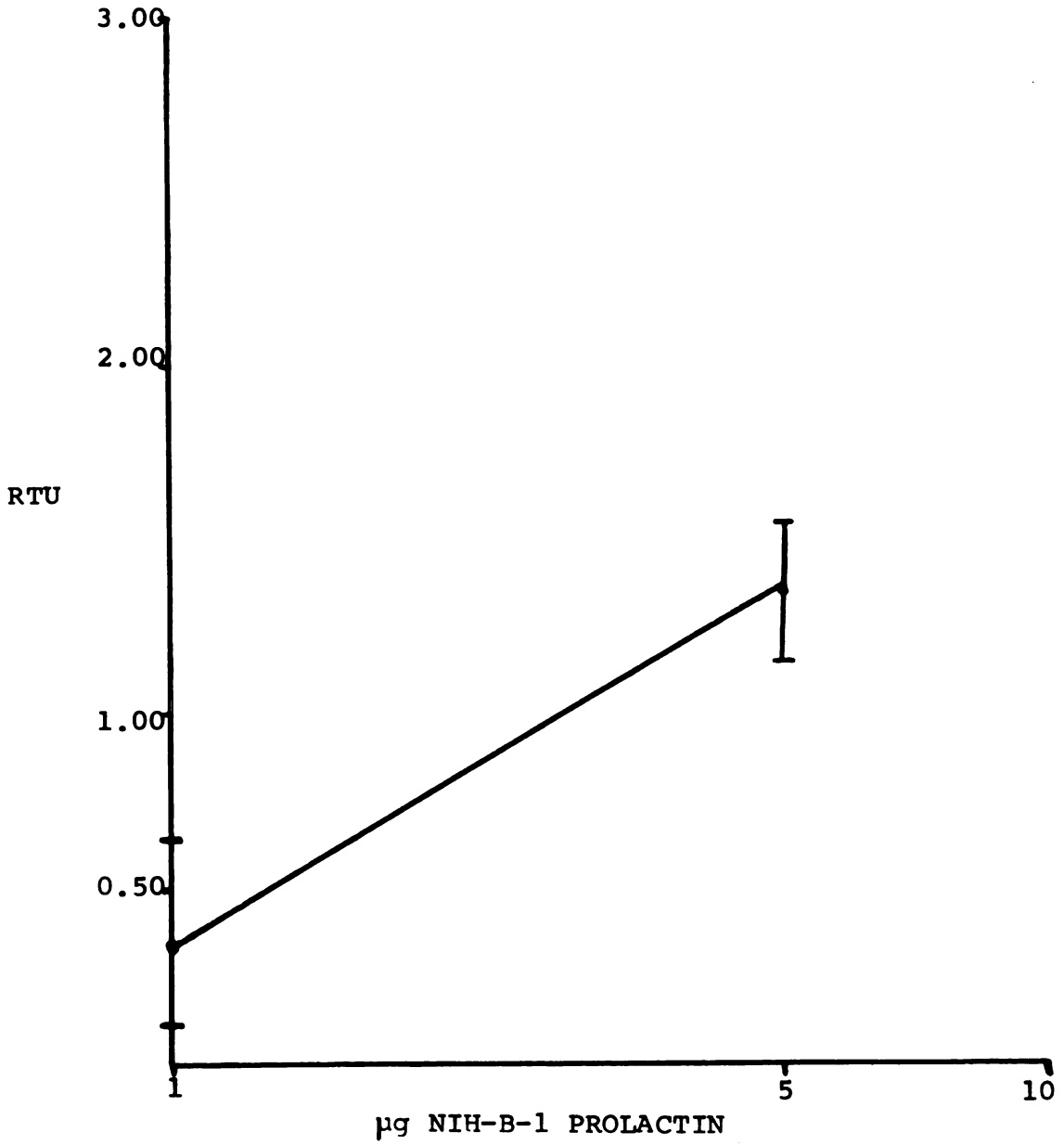


Fig. 3. Dose-response relationship for NIH-B-1 prolactin standard; data pooled from 2 assays.

was used to calculate the potencies of the unknown quantities of prolactin released into the medium by rat pituitaries.

2. Dose-response relationships between hypothalamic extract from female rats and prolactin release by female rat pituitaries in vitro (Table 6)

The data indicate that when progressively greater amounts of hypothalamic extract were incubated for 2 hours with female rat pituitaries, less prolactin was released into the medium (Table 6). This relationship was evident when prolactin release was expressed as RTU/pituitary, RTU/10 mg pituitary or as ug NIH-B-1 equivalent/10 mg pituitary. When the μg of prolactin released/10 mg pituitary tissue was plotted against the logarithm of the dose of hypothalamic extract added, a linear curve with a negative slope was obtained. Data from Experiment 2 (Table 6) are plotted in Figure 4. The large difference in amount of prolactin released by pituitaries from random cycling females in these 2 experiments can probably be attributed mainly to fluctuations in secretory activity of the pituitary during the estrous cycle, and perhaps also to differences in age of the donor rats. There was about a 50 gram difference in weight between the donor rats used in the two experiments. In this system, 1 hypothalamic equivalent had no effect on the release of prolactin, whereas 2 and 4 hypothalamic equivalents significantly inhibited release.

Table 6. Dose-response relationship between hypothalamic extracts from female rats and quantity of prolactin released by female rat pituitaries *in vitro*

| Exper. no. | Hypothal. equiv. added | Prolactin released into medium | | | 95% Confidence limits | $\lambda^*$ |
|---|---|---|---|---|---|---|
| | | RTU/2 AP | RTU/10 mg AP | µg NIH-B-2 Equiv./10 mg AP | | |
| 1 | 0 | 9.60 | 3.84 | 57.92 | 37.61-74.54 | .250 |
| | 1 | 9.75 | 3.05 | 63.40 | 41.17-97.64 | |
| | 2 | 6.50 | 2.32 | 15.70 | 10.40-23.72 | |
| | 4 | 2.75 | 0.92 | 2.88 | 1.91- 4.35 | |
| 2 | 0 | 2.25 | 1.18 | 1.20 | 0.79- 1.81 | .290 |
| | 1 | 2.50 | 1.39 | 1.52 | 1.00- 2.30 | |
| | 2 | 1.50 | 0.08 | 0.90 | 0.60- 1.36 | |
| | 4 | 0.75 | 0.03 | 0.56 | 0.37- 0.85 | |

*$\lambda$ = Index of precision of standard curve

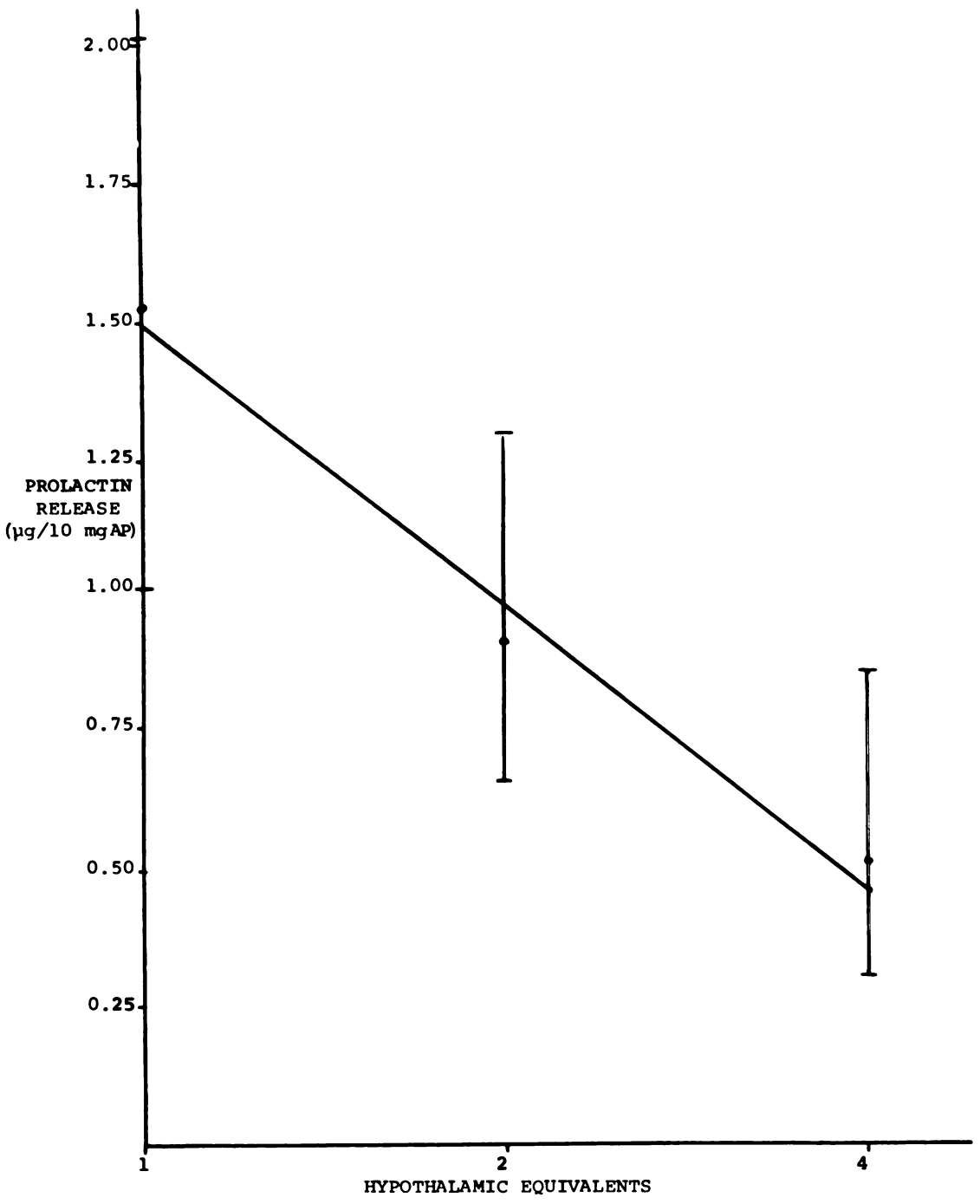


Fig. 4. Dose-response relationship between hypothalamic extract from female rats and prolactin release by female rat pituitaries *in vitro*.

3. Dose-response relationships between cerebral cortical extract from female rats and prolactin release by female rat pituitaries *in vitro* (Table 7)

None of the various doses of cerebral cortical extracts incubated for 2 hours with pituitaries had any significant effect on the release of prolactin. All of the 95% confidence limits over-lapped, and therefore there were no statistically significant differences ($P > 0.05$) between any of these values (Table 7).

4. Dose-response relationships between hypothalamic extract from male rats and prolactin release by male rat pituitaries *in vitro* (Table 8)

Previous work had indicated that hypothalamic extracts from male rats were equi-potent to those of female rats in their ability to inhibit prolactin release. However, male rat pituitaries released about one-half as much prolactin as female pituitaries when incubated *in vitro* (Ratner, 1965). For this reason the duration of incubation was increased to 4 hours. The results indicate that the amounts of prolactin released by male pituitaries *in vitro* were considerably less variable than those released by female pituitaries. In four separate assays of medium in which 8 male pituitary quarters from 4 different male donors were incubated, the total amount of prolactin released was 10.50, 9.75, 9.25, and 8.75 RTU, respectively. It also appears that the 4 hour incubation period employing male pituitaries provides a more sensitive assay for PIF than the previously used 2 hour incubation period of female pituitaries. In both experiments (Table 8)

Table 7. Dose-response relationships between cerebral cortical extract from female rats and prolactin release by female rat pituitaries *in vitro*

| Exper. no. | Cerebral cortical extract added (ml extract/flask) | Prolactin released into medium | | | 95% Confidence limits | $\lambda^*$ |
|---|---|---|---|---|---|---|
| | | RTU/2 AP | RTU/10 mg AP | µg NIH-B-1 equiv./10 mg AP | | |
| 1 | 0.0 | 4.00 | 1.54 | 5.60 | 3.70- 8.46 | .226 |
| | 0.2 | 4.75 | 1.98 | 7.70 | 5.10-11.63 | |
| | 0.4 | 4.00 | 1.60 | 5.82 | 3.85- 8.79 | |
| | 0.8 | 3.25 | 1.16 | 3.84 | 2.54- 5.80 | |

*$\lambda$ = Index of precision of standard curve

Table 8. Dose-response relationships between hypothalamic extract from male rats and prolactin release by male rat pituitaries *in vitro*

| Exper. no. | Hypothal. equiv. added | Prolactin released into medium | | | 95% Confidence limits | $\lambda^*$ |
|---|---|---|---|---|---|---|
| | | RTU/2 AP | RTU/10 mg AP | µg NIH-B-1 equiv./14 10 mg AP | | |
| 1 | 0 | 9.25 | 5.00 | 31.20 | 20.26-48.05 | .195 |
| | 1 | 5.00 | 4.55 | 8.55 | 5.66-12.91 | |
| | 2 | 4.00 | 2.57 | 5.37 | 3.56- 8.11 | |
| | 4 | 2.25 | 1.73 | 3.84 | 2.54- 5.80 | |
| 2 | 0 | 8.75 | 4.73 | 26.20 | 17.01-40.34 | .185 |
| | 1 | 5.50 | 3.14 | 8.88 | 5.80-13.59 | |
| | 2 | 3.25 | 1.76 | 4.11 | 2.70- 6.25 | |
| | 4 | 2.75 | 1.67 | 3.64 | 2.41- 5.49 | |

*$\lambda$ = Index of precision of standard curve

it was observed that as the amount of hypothalamic extract added to a flask was increased, there was a corresponding decrease in the amount of prolactin released into the medium. Data from Experiment 1 are plotted in Figure 5. An inverse linear relationship exists between the logarithm of the amount of hypothalamic extract added and the amount of prolactin released by the pituitary.

## Discussion

The amount of prolactin released by female and male rat pituitaries when incubated *in vitro* was observed to be inversely related to the logarithm of the dose of hypothalamic extract added to the medium. A minimum of 2 hypothalamic equivalents was necessary to produce a statistically significant inhibition of prolactin release by female pituitaries incubated for 2 hours. The dose-response curve in this system was linear between the dosages of 1 and 4 hypothalamic equivalents. A total of four hypothalamic equivalents produced an average inhibition of about 80% when incubated for 2 hours. Therefore, higher dosages of extract could also have been used before maximum inhibition would have occurred. It is interesting that only one hypothalamic equivalent was necessary to significantly inhibit prolactin release by the male pituitary during a 4 hour incubation. It appears that PIF in as little as 0.25 hypothalamic equivalent may be detected by the 4 hour assay system. The dose-response

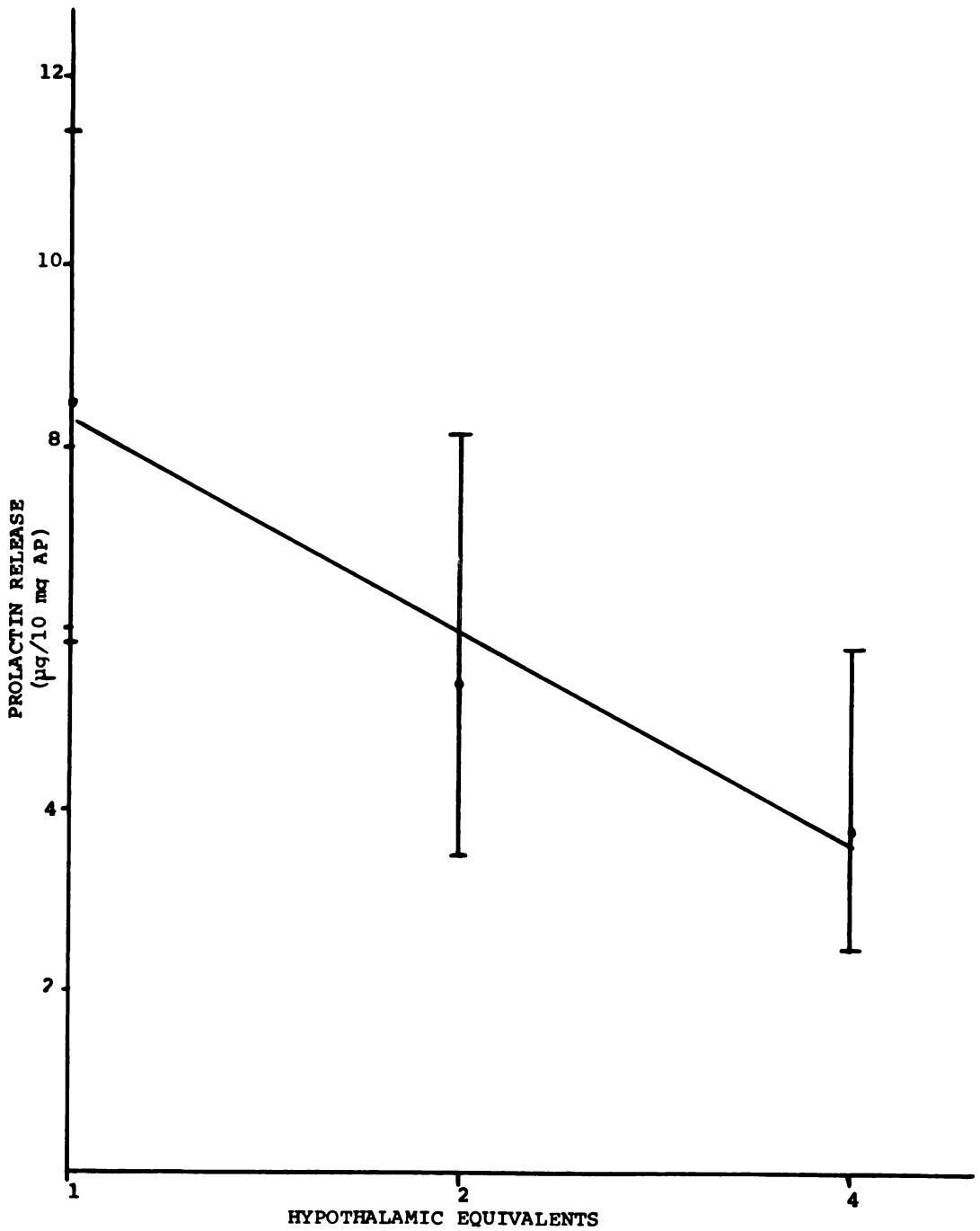


Fig. 5. Dose-response relationship between hypothalamic extract from male rats and prolactin release by male rat pituitaries in vitro.

relationship was linear between the dosage range of 1 to 4 hypothalamic equivalents, but appeared to approach maximum inhibition at the higher dosage. Use of male pituitaries for assaying PIF makes comparison of results of one experiment with those of others more valid. Only small variations in the amount of prolactin released by the male pituitaries were observed from one experiment to another.

The greater sensitivity of the 4 hour assay system with male pituitaries for detection of PIF may be due to the longer exposure of the pituitary to the hypothalamic factor, resulting in a greater difference in prolactin release between control and experimental flasks. PIF may also be long acting once it has affected the pituitary. This possibility is supported by the observation that as little as 1/16 of a hypothalamus was sufficient to significantly increase release of FSH from male rat pituitaries when they were incubated for 6 hours (Mittler and Meites, 1966). However, it is realized that PIF and FSH-RF may not necessarily parallel each other in action, _in vitro_.

The data indicate that PIF exists as a specific factor in the hypothalamus for the regulation of prolactin secretion in male and female rats, since the pituitary release of prolactin _in vitro_ responded as a logarithmic function of the dose of hypothalamic extract added to the incubation flask. This is contrary to the observation made by Grosvenor (1966), who reported that injections of acid hypothalamic

extracts acted in an "all or none" manner and failed to show a dose-response curve *in vivo*. However, his *in vivo* system has not been shown to be specific for PIF. Crude hypothalamic extracts contain at least 8 factors which can indirectly increase prolactin release *in vivo*, i.e., epinephrine, norepinephrine, acetylcholine, serotonin, FSH-RF, LRF, TRF and CRF. The former 4 substances have been shown to initiate mammary secretion and promote prolactin secretion in rats (Meites *et al.*, 1963). The latter 4 substances, by acting on the anterior pituitary, can induce release by the target organs of estrogen, progesterone, thyroid hormones and adrenal cortical hormones, all of which have also been shown to stimulate prolactin release *in vivo* (Meites *et al.*, 1959, 1963). Thus, crude hypothalamic extracts contain at least 8 agents which can increase prolactin secretion *in vivo* and only one factor (PIF) which inhibits it. This is believed to explain why crude extracts of hypothalamus were found to initiate mammary secretion in rats (Meites, *et al.*, 1960). It is doubtful that a specific *in vivo* assay for PIF will be possible until it has been purified and freed of the above substances. None of these 8 substances have been shown to influence prolactin release by the anterior pituitary *in vitro*.

## II. CONTROL OF PROLACTIN RELEASE IN AVIAN SPECIES

### A. Effect of Hypothalamic Extracts on Pigeon Pituitary Prolactin Release in vitro

#### Objectives

Several observations suggest that the control of prolactin release by the avian pituitary differs from that of mammals. These differences are summarized in Table 9. The purpose of these experiments was to determine the effects of pigeon hypothalamic extracts on prolactin release by the pigeon pituitary, and the effects of pigeon or rat hypothalamic extracts on prolactin release by the pituitary of the opposite species.

#### Materials and Methods

Male and female White King squabs, 4-6 weeks old, were used as donors of pituitary and hypothalamic tissues, and also served as assay animals. They were maintained under seasonal lighting conditions during the summer of 1964 in southern Michigan, and were fed water and Ryde's pigeon mix ad libitum. Parent pairs of mature White King pigeons were also sacrificed on the day young were hatched, and served as donors for pituitary and hypothalamic tissue for certain experiments. The crops of the parent pigeons are normally filled with crop milk at this time, indicating that they are releasing large amounts of prolactin. Rats used in this study were virgin females (150-200g) of the Sprague-Dawley strain (Spartan Research Labs., Haslett, Michigan). They were

Table 9. Differences in control of prolactin release in mammals and birds

| Treatment | Prolactin release by AP | |
|---|---|---|
| | Mammals | Birds |
| 1. Postpartum lactation | + | |
| Post-hatching crop milk secretion | | + |
| 2. Drugs and hormones | | |
| Estrogens | + | - |
| Testosterone | + | - |
| Progesterone | + | - |
| Reserpine | + | - |
| Epinephrine | + | - |
| Acetylcholine | + | - |
| 3. Transplantation of AP | Increase | No increase |
| 4. Culture of AP *in vitro* | Large | Small |
| 5. Effect of hypothalamic extract on AP | Inhibition | Stimulation |

maintained under artificial illumination (14 hr. light/day) in a temperature-controlled (75±1°F) room, and fed water and Wayne Lab Blox ad libitum.

The rats and pigeons were sacrificed by decapitation and the anterior pituitaries and hypothalami were quickly removed. The hypothalami and an equal quantity of pigeon cerebral cortical tissue were individually homogenized in chilled .1 N HCl and centrifuged (12,000g) for 30 min. at 4°C. The supernatant was frozen at -20°C for subsequent use the following day. Each anterior pituitary was separated from the posterior lobe in both pigeons and rats, halved, weighed and incubated for 2 or 4 hours in 25 ml Erlenmeyer flasks containing 2 ml of medium 199 (Difco Laboratories, Detroit, Michigan) maintained at pH 7.4 with bicarbonate buffer. The number of anterior pituitary and hypothalamic equivalents incubated per flask are indicated in each experiment. All incubations were carried out in a Dubnoff metabolic shaker (60 cycles/min) under constant gassing with humidified 95% $O_2$-5% $CO_2$ at 37.5±.5°C. At the end of incubation, the medium was separated from the anterior pituitary halves, lyophilized and stored as a dry powder at -20°C until ready for assay.

The samples were assayed for prolactin by the intradermal pigeon-crop technique of Lyons (1937) as modified by Reece and Turner (1937). Each injection was in a .2 ml volume, and the responses were rated in Reece-Turner units and converted to IU by the method previously described by Nicoll and Meites (1963). For this study a linear log-dose response

curve was obtained by injecting 5 doses of NIH prolactin standard into groups of 10 pigeons each, with the following units: $Y = 2.79 + 1.22 \log x$, where Y is the Reece-Turner unit of response and x is the IU of prolactin activity. The index of precision was .39, which agrees with results of previous assays (Nicoll and Meites, 1963; Mizuno et al., 1964). The slope was significant ($P < 0.01$). Significance of differences between groups was analyzed by using the t-test for paired observations (Snedecor, 1956). The differences in the amounts of prolactin released by the incubated pituitary in different experiments can be accounted for by changes in duration of incubation, variations in prolactin secretion by pituitaries from different individual animals, etc. Valid comparisons are considered to be those made in the same experiment, between prolactin released by pituitary halves from the same pituitary, incubated in separate flasks at the same period of time. Under similar treatments, the trends of the results in each experiment were always the same.

## Results

### 1. Prolactin Content of Fresh Anterior Pituitary and of Medium after 4-Hour Pituitary Incubation (Table 10)

Anterior pituitaries from 15 male and female pigeons, each 4-6 weeks old, were homogenized in physiological saline (pH 8.0-8.4) and assayed at three dose levels (1, 4, and 8 mg per assay bird). Anterior pituitaries from 3 pairs of

Table 10. Prolactin content of fresh pigeon anterior pituitary and of medium after four-hour pituitary incubation

| Assay material | No. of donor birds | AP wt (mg) | No. of assay birds | Dose per bird | Total prolactin ($IUx10^{-3}$) | ($IUx10^{-3}$) prolactin per mg AP | ($IUx10^{-3}$) prolactin per AP |
|---|---|---|---|---|---|---|---|
| Fresh AP of 4- to 6-week-old pigeons | 15 | 4.2 | 5 | 1 mg | 28.1± 9.8† | 28.1 | 118.0 |
| | | | 5 | 4 mg | 50.2±16.1 | | |
| | | | 5 | 8 mg | 68.9± 4.9 | | |
| Fresh AP of parent pigeons | 6 | 7.0 | 5 | 2 mg | 59.9± 7.4 | 37.9* | 263.2 |
| | | | 5 | 4 mg | 99.9±34.0 | | |
| Medium from incubated 4- to 6-week-old pigeons | 10 | 4.8 | 5 | 2 pit. equiv. | 13.0± 5.0 | 1.4 | 6.7 |
| Medium from incubated AP of parent pigeons | 10 | 7.8 | 5 | 2 pit. equiv. | 78.8± 5.4 | 5.1 | 39.8 |

AP = anterior pituitary.
* Extimated value obtained by extrapolation from original Reece-Turner ratings.
† = ± values in this and all following tables are standard errors of the mean.

parent pigeons obtained on the day young were hatched were similarly homogenized, and assayed at 2 dose levels (2 and 4 mg per assay bird). Five assay birds were used for each dose level. The average pituitary weights of the 4-6 week old and parent pigeons were 4.2 and 7.0 mg, respectively. One mg of fresh pituitary tissue from 4-6 week old pigeons produced a response equivalent to $28.1 \times 10^{-3}$ IU prolactin. The responses at the three dose levels yielded a straight line when plotted against the log of the dose (Fig. 6). Comparison of the prolactin in 4 mg of pituitary tissue from 4-6 week old pigeons ($50.2 \times 10^{-3}$ IU) with that in the same quantity of pituitary tissue from parent pigeons ($99.4 \times 10^{-3}$ IU), indicated that the parent pigeon pituitary contained approximately twice as much prolactin as the 4-6 week old pigeon pituitary. When expressed as total IU prolactin per pituitary, the pituitary of the parent pigeon contained an average of $263.2 \times 10^{-3}$ IU and the pituitary of the 4-6 week old pigeon contained $118 \times 10^{-3}$ IU.

In order to determine the quantity of prolactin released by pituitary tissue <u>in vitro</u>, four anterior pituitary halves from 4-6 week old pigeons were placed in each of 5 flasks and incubated for 4 hours. Four anterior pituitary halves from parent pigeons were similarly incubated in each of 5 additional flasks. Following incubation, the medium from each flask was frozen in an acetone-dry ice bath and lyophilized to dryness. Prior to assay, the material was

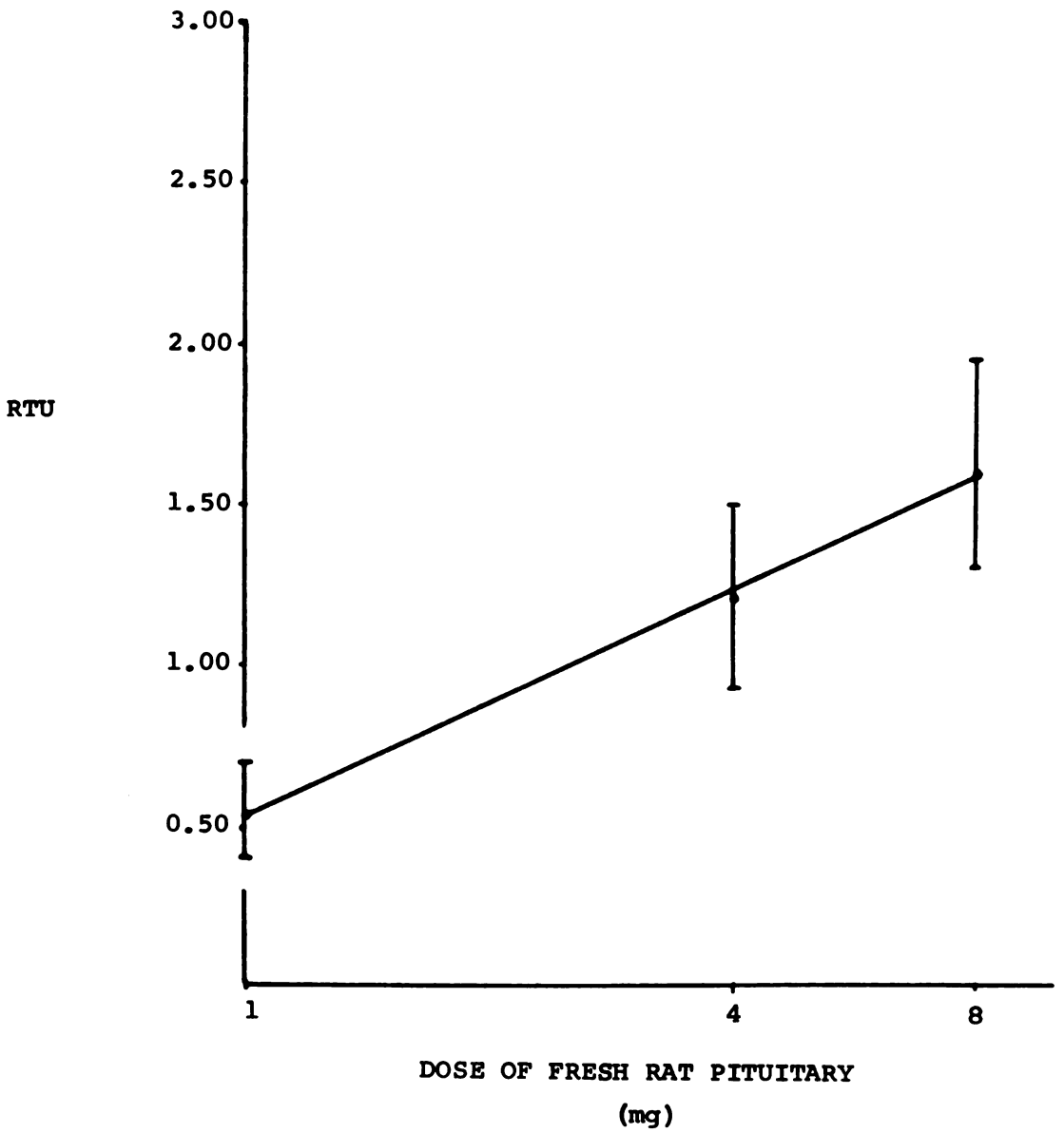


Fig. 6. Dose-response relationship between fresh rat pituitary and pigeon crop stimulation.

dissolved in saline (pH 8.0-8.4) and all the medium of each flask was assayed in one bird. The average pituitary weights from 4-6 week old and parent pigeons were 4.8 and 7.8 mg, respectively. The anterior pituitaries from parent pigeons released about 6 times as much prolactin into the medium as pituitaries from 4-6 week old pigeons. When expressed on the basis of IU of prolactin per mg of anterior pituitary tissue the release of the former group was approximately 4 times as great as by the latter group. Data from additional incubations (Expts. 3 and 4, Table 8) indicated that the amount of prolactin released by the parent pigeon pituitary was 2-5 times greater than that released by pituitaries from 4-6 week old pigeons. Comparisons of prolactin release from pituitaries of parent pigeons on the day young were hatched with pituitaries from non-hatching adult pigeons, also showed significantly greater release by the former (unpublished observations).

2. Effects of Pigeon Hypothalamic and Cerebral Cortical Extracts on Release of Prolactin *in vitro* by the Anterior Pituitary of 4-6 Week Old Pigeons (Table 11)

Paired-flask experiments were performed as follows: anterior pituitaries were hemisected and each half was placed in a separate flask; therefore the pituitary tissue placed in each of the two flasks was approximately homogeneous in composition and weight. Hypothalamic extract from parent or 4-6 week old pigeons was added to each experimental flask and an equivalent amount of cerebral cortical extract was

Table 11. Effect of pigeon hypothalamic and cerebral cortical extracts on release of prolactin *in vitro* by the anterior pituitary of 4- to 6-week-old pigeons

| Expt. no. | Assay medium | No. of donor birds | No. of assay birds | Dose per bird in pit. equiv. | Prolactin in medium ($IUx10^{-3}$) | Sig. diff. between CCE and HE |
|---|---|---|---|---|---|---|
| 1 | AP+CCE* | 20 | 5 | 1 | 21.6±12.8 | > .10 |
| | AP+HE | | | | 32.9±17.9 | |
| 2 | AP+CCE* | 24 | 6 | 1 | 15.1± 6.4 | > .10 |
| | AP+HE | | | | 27.0± 8.2 | |
| 3 | AP+CCE** | 16 | 8 | 1 | 9.5± 4.8 | < .05 |
| | AP+HE | | | | 20.2± 4.9 | |
| 4 | AP+CCE** | 20 | 5 | 2 | 29.4±11.8 | < .05 |
| | AP+HE | | | | 66.9±16.2 | |
| 5 | AP+CCE** | 9 | 3 | 1 | 10.9± 7.4 | < .05 |
| | AP+HE | | | | 63.6±23.6 | |
| 6 | AP+CCE*** | 12 | 3 | 1 | 10.9± 7.5 | < .05 |
| | AP+HE | | | | 84.4±27.8 | |

AP = anterior pituitary; CCE = cerebral cortical extract; HE = hypothalamic extract.
* = CCE and HE from 4- to 6-week-old pigeons; 2 hypothalami/pituitary.
** = CCE and HE from parent pigeons; 1 hypothalamus/pituitary.
*** = CCE and HE from parent pigeons; 2 hypothalami/pituitary.

always added to the control flask. The medium from each flask pair was assayed in the same pigeons, by injecting medium from the control flask into one crop side and medium from the experimental flask into the opposite crop side. This experimental design lessens the variation in results due to differing prolactin contents of individual pituitaries as well as that due to differing sensitivities of individual assay birds.

The effects of hypothalamic extracts prepared from 4-6 week old pigeons on release of prolactin by the pigeon pituitary were tested as follows: a total of 2 anterior pituitary halves were incubated for 4 hours in each of 5 flask pairs. To each experimental flask 1 ml of hypothalamic extract (2 hypothalamic equivalents) was added, and an equal amount of cerebral cortical extract was added to the control flask. The medium from each flask pair was assayed in one pigeon. The results (Expts. 1 and 2) show that although the media from the pituitary halves incubated with hypothalamic extract contained somewhat more prolactin than the media from the pituitary halves incubated with cerebral cortical extract, this difference was not statistically significant.

The effects of extracts of hypothalami removed from parent pigeons on the day young were hatched on release of prolactin from the anterior pituitaries of 4-6 week old pigeons were also tested. A total of 4 anterior pituitary halves were incubated in each of 4 flask pairs. One ml of

hypothalamic extract (2 hypothalamic equivalents/ml) was added to each experimental flask and an equal volume of cerebral cortical extract was added to each control flask. The medium from each flask pair was assayed in two pigeons; therefore, each assay bird received medium from the equivalent of one incubated pituitary (Expt. 3). A significantly greater quantity of prolactin was present in the medium from flasks to which hypothalamic extracts had been added ($20.2 \times 10^{-3}$IU) than in medium from flasks to which cerebral cortical extract had been added ($9.5 \times 10^{-3}$IU). This experiment was repeated (Expt. 4), employing 5 flask pairs, and the medium from each pair was assayed in one pigeon. In this assay, each bird received medium equivalent to two anterior pituitaries. Again, medium from flasks to which hypothalamic extracts had been added contained significantly more prolactin ($66.9 \times 10^{-3}$IU) than medium from control flasks ($29.4 \times 10^{-3}$IU).

In Experiment 5, incubation of 6 anterior pituitary halves per flask in a single flask pair, and assay of the medium in 3 birds, yielded a significantly greater amount of prolactin in the flasks to which hypothalamic extract (1 hypothalamic equivalent/pituitary) had been added ($63.6 \times 10^{-3}$IU) than in the control flasks ($10.9 \times 10^{-3}$IU). Experiment 6 shows that addition of a greater amount of hypothalamic extract (2 hypothalamic equivalents/pituitary) to a flask containing 6 anterior pituitary halves also elicited release of statistically greater amounts of prolactin ($84.4 \times 10^{-3}$IU) than

released by 6 halves in a flask to which cerebral cortical extract had been added ($10.9x10^{-3}$IU). Although more prolactin was released into the medium when extract equivalent to two hypothalami per pituitary were incubated than when only one hypothalami per pituitary were incubated, this difference was not statistically significant.

A dose-response relationship was also determined for the effect of four different doses of hypothalamic extract from parent pigeons on the amount of prolactin released per pituitary equivalent during 4 hours of incubation. Pigeon pituitaries were quartered to provide 4 homogeneous tissue samples for incubation. One sample was incubated with each of 4 dosages of hypothalamic extract (0.1, 0.5, 1.0, and 2.0 hypothalamic equivalents per pituitary). Medium from 2 flasks in which a total of 20 pituitary halves had been incubated with the same amount of hypothalamic extract were pooled for assay in 5 pigeons. The average amount of prolactin released by one pituitary equivalent incubated for 4 hours with cerebral cortex was $12.67\pm1.55x10^{-3}$IU. This value was obtained by averaging values obtained in 7 separate experiments using a total of 43 assay pigeons. A linear log-dose response relationship was obtained when hypothalamic extract was incubated with the pituitary tissue (Fig. 7). The one point which fell below the line was due to a group of insensitive assay birds. One-tenth hypothalamic equivalent had no significant effect on prolactin release. It appears

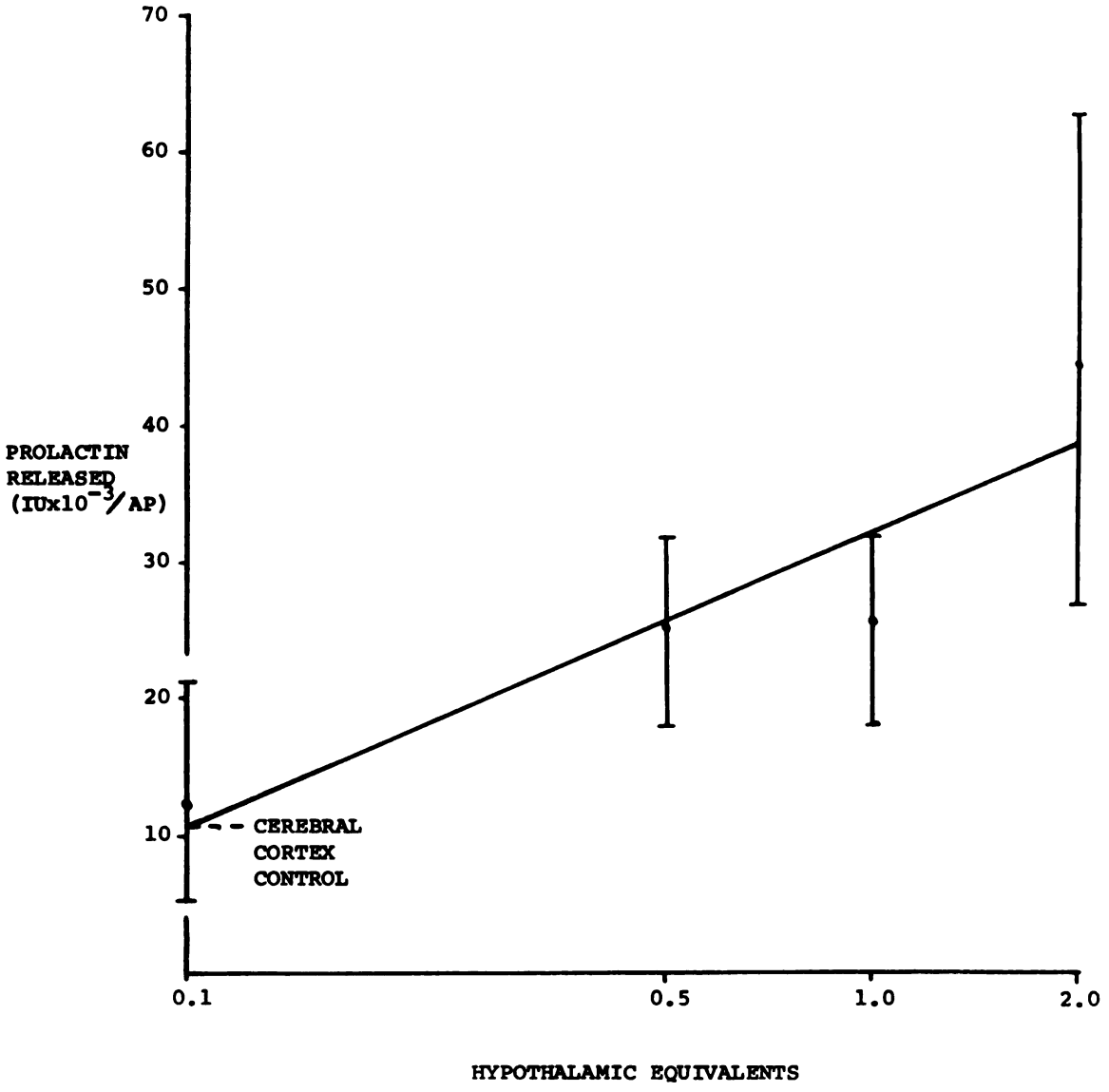


Fig. 7. Dose-response relationship between hypothalamic extract from parent pigeons and release of prolactin by the pigeon pituitary *in vitro*.

that 1 hypothalamic equivalent increased the amount of prolactin released about 2-fold and that 2 hypothalamic equivalents increased release about 4-fold above the control incubated with cerebral cortex.

### 3. Assay of Hypothalamic and Cerebral Cortical Extracts for Prolactin Activity

Hypothalamic and cerebral cortical extracts from pigeons were found not to contain crop stimulating activity. Four non-incubated hypothalamic and cerebral cortical equivalents were assayed in each of 5 pigeons. In addition, medium containing 2 hypothalamic equivalents which had been incubated for 4 hours was also tested in each of 5 pigeons. Neither incubated nor non-incubated neutralized hypothalamic extracts produced any response when assayed by the intradermal pigeon crop technique. Similarly, hypothalamic extracts from rats were also found to be free of prolactin when assayed by the pigeon crop test (Talwalker et al., 1963).

The effects of incubating pituitary with and without cerebral cortical extract on release of prolactin were also tested. Medium from 6 anterior pituitary halves incubated with cerebral cortical extract and medium from similar control halves incubated without extract were injected into a total of 3 assay birds. The quantity of prolactin released in 4 hours of incubation was 10.9±7.4 and 10.9±7.4 IU per pituitary, respectively, indicating that cerebral cortical extract had no effect on prolactin release.

4. Comparative Effects of Hypothalamic Extracts from Pigeons and Rats on Release of Prolactin by the Anterior Pituitary of the Opposite Species (Table 12)

Four anterior pituitary halves from 4-6 week old pigeons were incubated (Expt. 1) in each of 4 flask pairs. One ml of hypothalamic extract (4 hypothalami/ml) from random cycling female rats was added to each of the experimental flasks and an equal amount of cerebral cortical extract was added to the control flask. Medium from each flask was frozen and lyophilized. The contents of each flask were assayed in 2 pigeons; each bird, therefore, received one pituitary equivalent. There was no significant difference ($P > .20$) between the prolactin content of the experimental and control flasks. The same experiment was repeated (Expt. 2) using 4 flask pairs. Each flask pair was assayed in one pigeon; therefore, each bird received medium from the equivalent of two incubated pituitaries. Again, no significant difference in prolactin content was noted between flasks to which cerebral cortical extract had been added and flasks to which rat hypothalamic extract had been added.

To further test the effects of rat hypothalamic extracts on the release of prolactin from pigeon anterior pituitaries, Experiments 3 and 4 were performed. Four anterior pituitary halves from parent pigeons, sacrificed on the day the eggs were hatched, were incubated in each of 3 flask pairs for 4 hours. One ml of rat hypothalamic extract

Table 12. Comparative effects of hypothalamic extracts from pigeons and rats on *in vitro* release of prolactin by the anterior pituitary of the opposite species

| Expt. no. | Assay medium | No. of assay birds | Dose per bird in pit. equiv. | Prolactin in medium (IU x $10^{-3}$) AP + CCE | AP + HE | Sig. diff. between CCE and HE |
|---|---|---|---|---|---|---|
| 1 | 4- to 6-wk-old pigeon AP and rat CCE or HE* | 8 | 1 | 10.3± 4.4 | 8.5± 4.9 | > .20 |
| 2 | 4- to 6-wk-old pigeon AP and rat CCE or HE* | 8 | 2 | 12.3± 6.2 | 9.1± 3.7 | > .20 |
| 3 | Parent pigeon AP and rat CCE or HE* | 6 | 1 | 17.7± 7.0 | 19.9±10.9 | > .20 |
| 4 | Parent pigeon AP and rat CCE or HE* | 4 | 1 | 23.1±10.7 | 16.9± 8.0 | > .20 |
| 5 | Rat AP and 4- to 6-wk-old pigeon CCE or HE* | 10 | 0.3 | 91.5±40.9 | 79.7±24.5 | > .20 |
| 6 | Rat AP and 4- to 6-wk-old pigeon CCE or HE* | 10 | 0.6 | 272.4±77.5 | 286.9±75.0 | > .20 |
| 7 | Rat AP and parent pigeon CCE or HE** | 10 | 0.6 | 37.7± 9.6 | 39.5±13.2 | > .20 |

AP = anterior pituitary; CCE = cerebral cortical extract; HE = hypothalamic extract.
* = 4-hr incubation period.
** = 2-hr incubation period.

(4 hypothalami/ml) was added to each of the experimental flasks and an equivalent amount of cerebral cortical extract was added to each control flask. The medium from each flask pair was treated as before and assayed in two pigeons. The prolactin content of the control medium was $17.1 \times 10^{-3}$IU per pituitary equivalent, and the experimental medium contained $19.1 \times 10^{-3}$IU per pituitary equivalent. This experiment was repeated using 2 flask pairs and a total of 4 assay birds. The quantity of prolactin released in the control flask was $23.1 \times 10^{-3}$IU per pituitary equivalent and that released in the experimental flask was $16.9 \times 10^{-3}$IU per pituitary equivalent. No statistically significant differences ($P > .20$) were noted between the prolactin contents of control and experimental flasks in either of the above two experiments. These results indicate that rat hypothalamic extract, which inhibits release of prolactin from rat anterior pituitary (Talwalker et al., 1963) at this dose level (2 hypothalami/pituitary), had no effect on the release of prolactin by the anterior pituitaries from either 4-6 week old or parent pigeons.

The effect of pigeon hypothalamic extracts on release of prolactin by the rat pituitary in vitro was studied in the next series of experiments. Six anterior pituitary halves from 3-4 months old random cycling virgin female rats of the Sprague-Dawley strain were incubated in each of 2 flask pairs (Expt. 5). One ml of hypothalamic extract (6 hypothalamic equivalents/ml) from 4-6 week old pigeons was added to each experimental flask and an equivalent amount of

cerebral cortical extract was added to each control flask. After incubation for 4 hours, the medium was assayed in a total of 10 pigeons. Medium equivalent to .3 of a rat pituitary was injected into each pigeon crop half. The media from the control and experimental flasks contained $91.5 \times 10^{-3}$IU and $79.7 \times 10^{-3}$IU prolactin per pituitary equivalent, respectively. This experiment was repeated a second time (Expt. 6). In this trial each crop half of 10 assay birds was injected with medium equivalent to .6 of a pituitary. Control medium contained $272.4 \times 10^{-3}$IU prolactin per .6 pituitary equivalent and experimental medium contained $286.9 \times 10^{-3}$IU prolactin per .6 pituitary equivalent. No statistically significant differences existed between the prolactin content of experimental and control flasks in either of the above two experiments.

The effect of extract of hypothalami from parent pigeons on release of prolactin by rat pituitary was also determined. Six pituitary halves from virgin cycling female rats were incubated in each of 2 flask pairs (Expt. 7). Two ml of hypothalamic extract (3 hypothalami/ml) from parent pigeons were added to each experimental flask and equal amounts of cerebral cortical extracts were added to the control flasks. The flasks were incubated for 2 hours and the medium from each flask pair was injected into each side of the crop sac. The medium from the experimental and control flasks contained $39.5 \times 10^{-3}$IU and $37.7 \times 10^{-3}$IU prolactin per .6 pituitary equivalent, respectively. The above results indicate that extracts

of hypothalami from both 4-6 week old and parent pigeons had no effect on release of prolactin by the rat anterior pituitary.

5. Effects of Some Known Hypothalamic Constituents on Release of Prolactin *in vitro* by Pigeon Anterior Pituitary (Table 13)

Preliminary screening of known hypothalamic constituents, such as epinephrine, oxytocin, lysine-vasopressin and arginine-vasopressin was also undertaken. Four pituitary halves were incubated in each flask (3 flask pairs). In the three experimental flasks, single dosages of epinephrine bromide (40 μg/ml), oxytocin (1 U/ml), lysine-vasopressin (1 U/ml), and arginine-vasopressin (1 U/ml) were assayed for prolactin releasing activity. The media from each flask pair was assayed in 2 pigeons; therefore, each pigeon was injected with medium equivalent to one pituitary.

No significant effect was noted on the amount of prolactin released when the pituitaries were incubated with these agents. Other factors should also be tested at multiple dose levels including the avian antidiuretic hormone, arginine-vasotocin, to exclude them as possible stimulators of avian prolactin release.

## B. Stimulation of Pigeon Pituitary Prolactin Release by Other Avian Hypothalamic Extracts *in vitro*

### Objectives

In order to determine whether the stimulatory mechanism found to exist in the pigeon hypothalamus was characteristic

Table 13. Effect of known hypothalamic constituents on *in vitro* release of prolactin by pigeon anterior pituitary

| Treatment | Dose | No. of assay pigeons | Prolactin ($IU \times 10^{-3}$) Control | Prolactin ($IU \times 10^{-3}$) Experimental | Sig. diff. between control & exper. |
|---|---|---|---|---|---|
| Epinephrine | 40 µg/ml | 6 | 9.55± 4.60 | 14.13± 6.25 | > .3 |
| Oxytocin (pitocin) | 1U | 6 | 11.30± 6.70 | 12.15± 4.17 | > .3 |
| Lysine-vasopressin | 1U | 6 | 24.38±12.47 | 24.38±12.47 | > .3 |
| Arginine-vasopressin | 1U | 4 | 20.68±10.01 | 19.80±14.32 | > .3 |

of other avian species, hypothalamic extracts from male and female chickens and quail were tested *in vitro*. White Leghorn chickens and *Coturnix Coturnix Japonica* quail were obtained from the Michigan State University Department of Poultry Science for these experiments. Hypothalamic extracts were prepared and incubations were carried out as described in the pigeon experiments.

## Results

### 1. Effect of Hypothalamic and Cerebral Cortical Extracts from Chickens on Release of Prolactin *in vitro* by the Pigeon Anterior Pituitary (Table 14)

Four anterior pituitary halves from 4-6 week old pigeons were incubated (Expts. 1 and 2) in each of 3 flask pairs. One ml of hypothalamic extract (6 hypothalami/ml) from either 1-1 1/2 year old White Leghorn roosters (Expt. 1) or laying hens (Expt. 2) was added to each of the experimental flasks and an equal amount of cerebral cortical extract was added to each control flask. Medium from each flask was frozen and lyophilized. The contents of each flask were assayed in 2 pigeons; each bird, therefore, received one pituitary equivalent. A third experiment was performed, using hypothalamic extract from laying hens but at a dosage (8 hypothalami/ml) greater than in Experiment 2. Four flask pairs were used in this experiment.

In all three experiments greater amounts ($P < .05$) of prolactin were released by the incubated pigeon pituitary

Table 14. Effect of chicken hypothalamic (HE) and cerebral cortical extract (CE) on release of prolactin *in vitro* by the pigeon anterior pituitary (AP)

| Expt. no. | Assay medium | No. of donor chickens | No. of assay pigeons | Prolactin in medium (IU x $10^{-3}$) | Sig. diff. between CE and HE |
|---|---|---|---|---|---|
| 1 | AP + CE<br>AP + HE (male)<br>3 hypothal/AP | 12 | 6 | 12.37± 7.17<br>40.94±10.50 | < .05 |
| 2 | AP + CE<br>AP + HE (female)<br>3 hypothal/AP | 12 | 6 | 22.60± 5.07<br>40.26± 7.34 | < .05 |
| 3 | AP + CE<br>AP + HE (female)<br>4 hypothal/AP | 32 | 8 | 25.00± 5.32<br>54.86±12.80 | < .05 |

hypothal = hypothalamus

when either male or female chicken hypothalamic extracts were added to the medium. It appears, therefore, that hypothalamic extracts from male and female chickens contain prolactin stimulating activity.

2. Effect of Quail Hypothalamic and Cerebral Cortical Extracts on Release of Prolactin in vitro by Pigeon Anterior Pituitary (Table 15)

Fifty male and 50 female quail (Coturnix Coturnix Japonica), 20-24 months of age, were sacrificed by decapitation and the pituitaries and hypothalami were collected as described previously. Six anterior pituitary halves from 4-6 week old pigeons were incubated in each of 5 flask pairs. One ml of hypothalamic extract (9 hypothalami/ml) from either male (Expt. 1) or female (Expt. 2) quail was incubated in each of the experimental flasks and an equivalent amount of cerebral cortical extract was added to each control. Medium from each flask was frozen and lyophilized. The contents of 5 experimental and 5 control flasks were pooled and assayed in a total of 7 birds by the paired assay technique. Each assay pigeon was injected on each crop side with medium removed after incubation with the equivalent of 2 pigeon pituitaries.

In both experiments, greater amounts ($P < .01$) of prolactin were released when either male or female quail hypothalamic extracts were added to the incubated pituitaries than when cerebral cortical extract was used. The data suggest that quail hypothalamic extracts also contain prolactin stimulating activity.

Table 15. Effect of quail hypothalamic (HE) and cerebral cortical extract (CE) on release of prolactin *in vitro* by pigeon anterior pituitary (AP)

| Expt. no. | Assay medium | No. of donors | No. of assay birds | Prolactin in medium (IU x $10^{-3}$) | Sig. diff. between CE and HE |
|---|---|---|---|---|---|
| 1 | AP + CE<br>AP + HE (male)<br>3 hypothal/AP | 50 | 7 | 20.04± 9.00<br>48.78± 8.99 | < .01 |
| 2 | AP + CE<br>AP + HE (female)<br>3 hypothal/AP | 50 | 7 | 22.62± 6.14<br>45.76±14.7 | < .01 |

hypothal = hypothalamus

## Discussion

The results of these experiments provide direct evidence that a different hypothalamic mechanism controls the release of prolactin by the pigeon pituitary than controls release of prolactin by the mammalian pituitary. In contrast to the mammalian hypothalamus, which contains a "prolactin inhibiting factor" (Talwalker *et al.*, 1963), the hypothalamus of parent pigeons on the day young were hatched was found to contain prolactin stimulating activity, and presumably a "prolactin stimulating factor." The equivalent of only one hypothalamus from a parent pigeon was sufficient to increase release of prolactin 2-4 fold by the incubating pigeon pituitary. A recent observation indicates that an extract of hypothalamus from the tri-colored blackbird may also promote prolactin release by the pituitary of this bird when cultured *in vitro* (Nicoll, 1965). The present experiments show that a prolactin stimulating factor is also present in the hypothalamus of male and female chickens and quail. Epinephrine, oxytocin, lysine vasopressin and arginine vasopressin had no effect on prolactin release *in vitro*, indicating that these are not the agents responsible for prolactin release. Prolactin *per se* was not found in either hypothalamus or cerebral cortical extracts from pigeons.

On the day of hatching, the parent pigeons used in these experiments simultaneously exhibited extensive crop "milk" production, a high pituitary prolactin content and increased

prolactin stimulating activity in the hypothalamus. On the other hand, the 4-6 week old pigeon showed no significant crop development, a relatively low pituitary prolactin content and a small but statistically non-significant amount of prolactin stimulating activity in the hypothalamus. The data suggests that prolactin stimulating activity may be present in the 4-6 week old pigeon hypothalamus, but at the dose level used (2 hypothalamic equivalents/pituitary), this stimulation was insufficient to elicit a statistically significant increase in prolactin release.

An analogy can be made between the postpartum lactating rat and the parent pigeon after hatching its young. Following parturition in the rat, pituitary prolactin content increases 2-3 times over that present during pregnancy (Meites, 1959) and stimulates lactogenesis. Similarly in the pigeon, pituitary prolactin content at the time the eggs are hatched increases at least 2-fold over that of non-brooding pigeons (Schooley and Riddle, 1938) and stimulates crop "milk" secretion. When cultured in vitro, anterior pituitaries of lactating rats release 2-4 times as much prolactin per day as do anterior pituitaries from mature cycling females (Meites et al., 1963). The present results indicate that the pituitary of the parent pigeon on the day young are hatched similarly released 2-5 times as much prolactin as the pituitary from 4-6 week old pigeons when incubated for 4 hours in vitro. However, the mechanisms by which these two species meet the

physiological demand for prolactin differ. Ratner and Meites (1964) found that the suckling stimulus depletes the "prolactin inhibiting factor" of the rat hypothalamus, and thereby increases release of prolactin by the pituitary. In the parent pigeon, tactile and visual stimuli received from the eggs or by viewing the mate or young may be responsible for stimulating the hypothalamus (Lehrman, 1964) and thus promote pituitary prolactin release and crop "milk" secretion. The precise efferent pathways to the pigeon hypothalamus at the time of brooding or hatching of eggs are as yet unknown. Whether different amounts of prolactin stimulating activity are present in the hypothalamus of male and female pigeons, both of which secrete crop milk, remains to be determined.

The mammalian and pigeon hypothalami do not appear to exert their respective activities when incubated with pituitaries of the opposite species. The failure of rat hypothalamus to inhibit prolactin release by the pigeon pituitary, and the inability of pigeon hypothalamus to stimulate release of prolactin by the rat pituitary, indicate that the hypothalamic mechanism for releasing prolactin is different for each of the two classes. These results also suggest that the "prolactin inhibiting factor" of the rat hypothalamus and the "prolactin stimulating factor" of the pigeon hypothalamus differ chemically.

## C. EFFECT OF SUBCUTANEOUS TRANSPLANTATION OF THE PIGEON PITUITARY OVER THE PIGEON CROP SAC

### Objectives

Transplantation of the mammalian pituitary to a site remote from the hypothalamus results in secretion of abundant amounts of prolactin. The lack of a definite end-organ response for prolactin in chickens and ducks makes interpretation of data on prolactin release by transplants in these species uncertain. The purpose of these experiments was to determine the effects of subcutaneous transplantation of the pigeon pituitary directly over the crop sac. This method should serve as a very sensitive indicator of whether or not prolactin can be released by the avian pituitary when transplanted.

### Materials and Methods

Forty immature 4-6 week old White King pigeons (Cascade Squab Farms, Grand Rapids, Michigan) maintained under natural illumination and temperature during the months of February and March (East Lansing, Michigan) were used in these experiments. Pigeons used as pituitary donors were sacrificed by decapitation and the anterior pituitary was rapidly removed. In those experiments in which living tissue was to be transplanted, pituitaries were transferred to a Petri dish containing medium 199 (Difco Laboratories, Detroit, Michigan) until transplanted 3-4 minutes later. In other experiments in which

dead tissue was to be transplanted, the pituitaries were placed on foil and frozen in a refrigerator at -20°C for 15 minutes, removed, thawed and again refrozen. Prior to transplanting the tissues, each pituitary was halved and one-half of the same pituitary was transplanted on each side of the crop sac. Transplantation was accomplished by making a quarter inch midline incision through the integument with a scissors without puncturing the crop sac. A forceps or iris scissors was then inserted between the muscle layer of the crop sac and that of the integument and the connective tissue gently forced apart for a distance of about 1 or 1 1/2 inches on both sides of the midline to form a channel about one-fourth inch wide. A pituitary half was then placed between the integument and crop sac at a point in this channel furthest from the midline with a fine forceps. This was repeated on the other crop side and the incision closed with a wound clip. Pigeons transplanted with dead pituitary tissue were sacrificed 2 or 5 days after the initial surgery. Pigeons transplanted with living pituitary tissue were sacrificed at 5 or 10 days after surgery. The crop sacs and integument were removed *in toto* over the area of the transplant. In all instances these two tissues were then gently separated and examined for the presence of pituitary tissue. If tissue was present, it was removed and fixed in Bouin's for histological study after routine hemotoxylin and eosin staining. The degree of crop sac stimulation was rated in Reece-Turner units

(RTU), similar to those used for the assay of prolactin by the intra-dermal technique (Reece and Turner, 1937).

## Results

When dead pigeon anterior pituitary tissue was transplanted over the crop sac it rapidly became necrotic and released the prolactin contained in the tissue (Table 16). Two days post-transplantation, 7 out of 10 crop sacs were stimulated (group 1). Visually the degree of stimulation was rated at 4.75 RTU for the 10 transplants. No pituitary tissue was recovered; however, necrotic nodules of tissue were present on the crop sac. By 5 days after transplantation of dead tissue, the necrotic pituitary tissue had completely disappeared (group 2). Only 1 pigeon crop had any indication of stimulation which indicates that any previous stimulation had already regressed.

All 10 pituitaries were recovered from group 3 which had been transplanted with living tissue. The condition of the transplant and its location in respect to the crop sac are shown in Figure 8. Histological examination indicated that all transplants were well vascularized and that only the centers had become necrotic. This has also been reported to occur in rat pituitary transplants (Everett and Nikitovitch-Winer, 1963). Three of the 10 crop sacs showed minimal stimulation by prolactin. These responses were most probably due to the slow leakage of prolactin from the necrotic tissues

Table 16. Effect of subcutaneous pigeon pituitary transplants directly over the crop sac

| Group | Treatment | Day post-transplant. sacrificed | No. of transplant. made | No. of transplants surviving | No. of crop sacs stimulated | Degree of crop stimulation RTU/10 transplants |
|---|---|---|---|---|---|---|
| 1 | Dead AP tissue | 2 | 10 | 0 | 7 | 4.75 |
| 2 | Dead AP tissue | 5 | 10 | 0 | 1 | 0.25 |
| 3 | Living AP tissue | 5 | 10 | 10 | 3 | 0.75 |
| 4 | Living AP tissue | 10 | 10 | 8 | 0 | 0.00 |

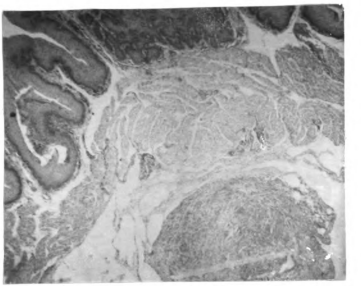

Fig. 8. Photomicrograph of subcutaneous pigeon pituitary transplant and adjacent crop sac mucosa (35x).

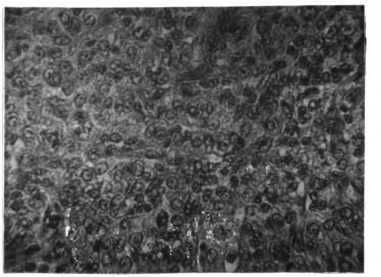

Fig. 9. Photomicrograph of subcutaneous pigeon pituitary transplant (94x).

in the center of the transplants. None of the crop sacs of pigeons sacrificed 10 days after living pituitary halves had been transplanted exhibited any stimulation in response to the transplant (group 4). Eight of these 10 transplants were recovered. Histological examination of these pituitary tissues (Fig. 9) indicated that they were well vascularized and very viable. It appears that if transplants do not become vascularized soon enough after transplantation they become necrotic and disappear within 10 days. However, this was not the case for most living pituitaries in these experiments, since the percent take was from 80-100 percent.

## Discussion

The pigeon pituitary does not secrete detectable amounts of prolactin when transplanted subcutaneously over the crop sac. This is contrary to the mammalian pituitary which has been demonstrated to release abundant prolactin when transplanted to a site remote from the hypothalamus (Everett and Nikitovitch-Winer, 1963; Meites and Hopkins, 1960). These data agree with the *in vitro* results published previously by this laboratory (Kragt and Meites, 1965) and elsewhere (Nicoll, 1965), which indicated that hypothalamic extracts from avian sources stimulate the release of prolactin from the pituitaries of pigeons or tri-colored blackbirds when incubated or cultured. Bayle and Assenmacher (1965) have also demonstrated that the transplanted pigeon pituitary does not secrete prolactin by a system employing transplants in the kidney capsule.

This is a less sensitive system than transplantations over the crop sac. Their transplantations were carried out in hypophysectomized pigeons as recipient animals, which has the advantage of excluding any possible influence from the host pituitary. Both the present experiments and those of Bayle and Assenmacher (1965) show that transplantation of living pigeon anterior pituitary does not result in prolactin release.

It appears that in order for the pigeon pituitary to actively release prolactin during brooding and rearing of the young a stimulatory substance must be liberated from the hypothalamus. The existence of such a releasing factor has already been demonstrated (Kragt and Meites, 1965). The next logical experiment that should be preformed is to transplant pituitary halves over the crop sacs, and 10 days later, inject neutralized avian hypothalamic extracts intra-dermally near the viable transplant and note the effects on the crop sac. This should result in crop sac stimulation.

## III. EFFECTS OF A PITUITARY HOMOTRANSPLANT AND THYROXINE ON BODY AND MAMMARY GROWTH IN IMMATURE HYPOPHYSECTOMIZED RATS

### Objectives

Studies on growth hormone release by pituitary transplants in the anterior chamber of the eye, kidney capsule and peritoneal cavity have yielded contradictory results (Martini and de Poli, 1956; Greer, 1957; Goldberg and Knobil, 1957;

Martini et al., 1959; Khazin and Reichlin, 1961; Ahren and Rubinstein, 1963). No appreciable body growth was detected in hypophysectomized rats given ocular transplants of pituitary. Hypophysectomized rats which received a subcutaneous pituitary transplant also failed to show any body weight gains after 2 to 3 months (Dao and Gawlak, 1963). However, all the rats given ocular transplants weighed more than 150 grams at the time of hypophysectomy and transplantation, and the Sprague-Dawley rats used by Dao and Gawlak (1963) initially weighed 94-135 grams. These rats may not have been as sensitive to growth hormone as younger hypophysectomized rats.

Hertz (1959) reported that transplantation of 4 pituitaries under the kidney capsule of immature hypophysectomized male and female rats resulted in body growth gains equal to 2/3 of that of intact control rats, and suggested that, in the rat, growth hormone production and release are not completely dependent upon hypothalamic stimulation. Swelheim and Wolthuis (1962) observed slight stimulation of body and tail growth after transplantation of a single pituitary underneath the kidney capsule of young hypophysectomized rats. Relocation of the pituitary from the kidney capsule of the area of the median eminence was reported to restore growth to nearly normal in hypophysectomized female rats (Nikitovitch-Winer and Everett, 1958; Smith, 1963). The essential role of the thyroid for the maintenance of normal body growth is well established (Asling et al., 1949; Ray et al., 1950). Thyroidectomy has

been reported to result in rapid disappearance of the acidophil cells of the anterior pituitary, which are associated with growth hormone secretion, while administration of thyroid preparations apparently increases the activity of these cells (Koneff et al., 1949; Knigge, 1958). Reappearance of acidophils in pituitary transplants following thyroxine treatment was observed by Halasz et al., 1963). In addition, Contopoulos et al. (1958) reported that thyroidectomy decreased the growth hormone content of the rat pituitary. It was the purpose of the present study to determine the effects of a subcutaneous pituitary transplant and thyroxine injections on body growth mammary development and several organ weights in young hypophysectomized rats.

## Materials and Methods

Hypophysectomized female rats (Sprague-Dawley strain, Hormone Assay Labs., Chicago, Illinois), 36-38 days old and weighing 60-70 g each, were fed a diet consisting of Wayne Lab Blox, oranges, bread and milk. Rats which gained more than 7 grams 10 days after hypophysectomy were not used in this experiment. Ten days after hypophysectomy the rats were divided into uniform groups of 10 each and treated as follows: (a) daily subcutaneous injection of .2 ml saline; (b) transplantation of an anterior pituitary and daily injection of .2 ml saline; (c) transplantation of an anterior pituitary and daily subcutaneous injection of 5 μg thyroxine (Smith, Kline

and French Labs., Philadelphia, Pennsylvania) per 100 g body weight; (d) daily injection of 5 μg thyroxine per 100 g body weight, and (e) daily injections of .2 ml saline to intact controls of the same age as the hypophysectomized animals.

The transplanted anterior pituitaries came from 3- to 4-month-old Sprague-Dawley females, weighing 175-225 g each. After decapitation, the posterior pituitary was removed and discarded, and the whole anterior pituitary transplanted subcutaneously in the area of the right inguinal mammary pad of the hypophysectomized host rat. All injections were made subcutaneously beginning on the day after transplantation and continuing for 30 days. Body weight was recorded daily for each rat, and tail length was measured at the beginning and end of the experiment.

Thirty days after transplantation, the animals were sacrificed, and the right inguinal mammary pad was removed and fixed in Bouin's fluid. After staining by a standard procedure (Nandi, 1959), the mammary glands were rated for degree of development, from 1-5, by a method described previously (Talwalker and Meites, 1961). Ratings from 1-3 indicate progressive degrees of duct development only, whereas ratings of 4-5 indicate lobulo-alveolar as well as extensive duct growth. The tibias from the right rear leg were removed and fixed in buffered formalin for subsequent staining with silver nitrate (Papkoff and Li, 1962). The weights of the pituitary transplant, liver, kidneys, spleen, adrenals, thymus, ovaries

and uterus were recorded, and the pituitary transplant, adrenals and ovaries were fixed in Bouin's fluid for histological examination. The sella turcica of all hypophysectomized rats was examined under magnification for pituitary fragments.

## Results

The data on body growth and organ weights are summarized in Table 17. Rats with a single subcutaneous pituitary transplant (group 2) exhibited significantly greater body growth than the hypophysectomized controls (group 1). Body weight, tail growth, tibial epiphysial cartilage width, and spleen and thymus weight were significantly increased in group 2. Hypophysectomized rats injected with thyroxine (group 3) showed a slight but insignificant body weight gain over the hypophysectomized controls (group 1), and tail length and thymus weight were significantly increased. Thyroxine injections into hypophysectomized rats with a subcutaneous pituitary transplant (group 4) significantly increased body weight, tail growth, and kidney, spleen and thymus weight over those of the animals with a pituitary transplant only (group 2). The final body weight gains of the group 4 rats averaged 75.5% of that of the intact controls (group 5). A small decrease in cartilage width was observed in the pituitary-transplanted rats given thyroxine as compared to the rats with a pituitary transplant only. Figure 10 shows the average daily body growth curves for each of the five groups during the experiment.

Table 17. Effects of a pituitary transplant and thyroxine on body and tail growth, tibial cartilage width and organ weights of hypophysectomized rats.

| Group and treatment | No. of rats | Body wt. orig. final gain g. | Tail growth cm. | Tibial width μ | Liver g/100g |
|---|---|---|---|---|---|
| 1. Hypox. controls | 10 | 66.5<br>75.0±1.0<br>9.5 | 0.91 | 138.2±2.5 | 3.89±0.08<br>(2.92)[2] |
| 2. Hypox. + A.P. | 9 | 65.2<br>101.7±2.9<br>36.5 | 2.20 | 194.8±9.0 | 4.05±0.02<br>(4.12) |
| 3. Hypox. + thyroxine | 9 | 64.1<br>83.3±1.9<br>19.2 | 2.44 | 131.0±1.6 | 5.00±0.14<br>(4.17) |
| 4. Hypox. + A.P. + thyroxine | 8 | 66.7<br>125.9±5.4<br>59.2 | 4.88 | 171.5±8.2 | 4.35±0.14<br>(5.19) |
| 5. Intact controls | 7 | 78.9<br>157.3±10.7<br>78.4 | 5.07 | - - - | 4.01±0.11<br>(6.28) |

[1]Hypox. - hypophysectomized rats

[2]Actual organ weights

| Kidneys mg/100g | Spleen mg/100g | Thymus mg/100g | Adrenals mg/100g | Ovaries mg/100g | Uterus mg/100g |
|---|---|---|---|---|---|
| 734.0±2.0 (551.0)[2] | 209.1±1.3 (157.0)[2] | 277.0±14.1 (208.5)[2] | 20.1±1.3 (15.0)[2] | 9.8±0.41 (7.4)[2] | 45.9±2.2 (34.4)[2] |
| 737.2±17.4 (751.1) | 313.6±16.6 (321.1) | 342.5±15.0 (349.4) | 13.3±1.4 (13.6) | 6.6±0.4 (6.8) | 35.3±2.2 (35.5) |
| 997.0±10.0 (832.0* | 278.5±52.7 (261.1) | 339.4±13.8 (281.7) | 19.5±1.7 (16.2) | 7.4±0.7 (6.2) | 36.1±2.0 (30.1) |
| 842.0±3.0 (1020.0) | 349.7±4.1 (401.5) | 427.6±21.9 (543.1) | 14.9±1.1 (14.8) | 6.7±0.3 (8.4) | 32.6±2.2 (40.8) |
| 919.0±25.0 (1440.0) | 374.6±9.4 (580.7) | 244.7±14.1 (283.9) | 46.5±1.9 (62.7) | 57.4±4.1 (88.9) | 209.4±16.0 (337.4) |

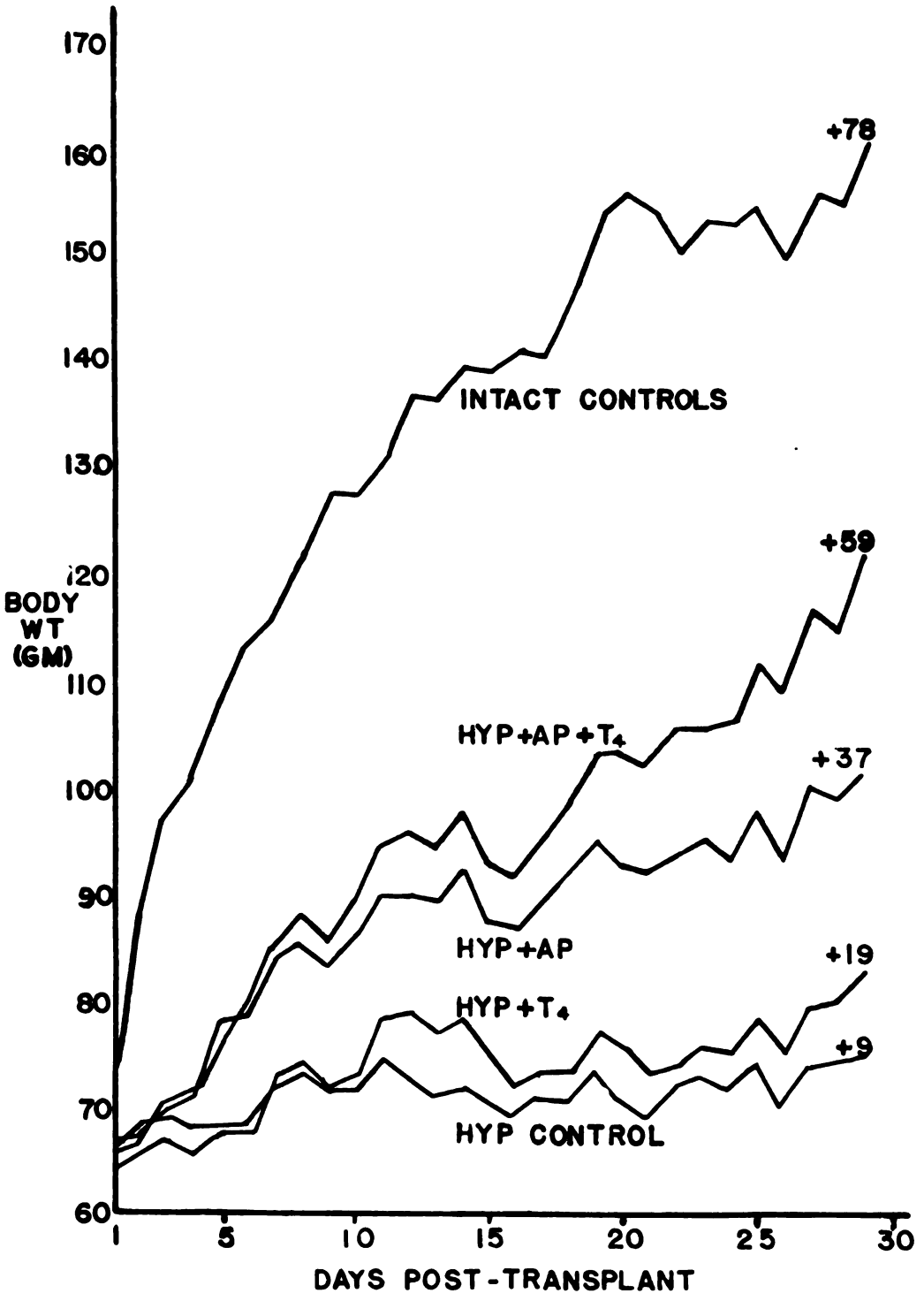


Fig. 10. Body growth curves of normal rats and hypophysectomized rats with and without pituitary transplants and similar rats injected with thyroxine.

The mammary glands of the hypophysectomized control rats consisted of only a bare duct system (Fig. 11, A), while those of the rats treated with thyroxine showed even a lesser degree of development (Fig. 11, B). Extensive duct growth and moderate lobuloalveolar development were observed in the inguinal mammary glands located near the transplanted anterior pituitary (Fig. 11, C), while thyroxine treatment of pituitary-transplanted animals resulted in reduced mammary growth (Fig. 11, D). The ratings for these mammary glands are given in Table 18.

Histological examinations of the adrenals and ovaries revealed no noticeable differences between the four hypophysectomized groups. Only small follicles and atrophied interstitial tissue were observed in the ovaries. The anterior pituitary transplants averaged 6.8±1.2 mg in weight in the untreated hypophysectomized rats (group 2), and weighed 7.56±1.1 mg in the rats given thyroxine (group 3). Most pituitary transplants had a rich vascular supply, and histological examination indicated the presence of more normal-appearing cells in the transplants of the rats given thyroxine than in rats not given thyroxine. Viability of these pituitary transplants was further indicated by the progressive increase in body weight of these rats, and the localized mammary growth.

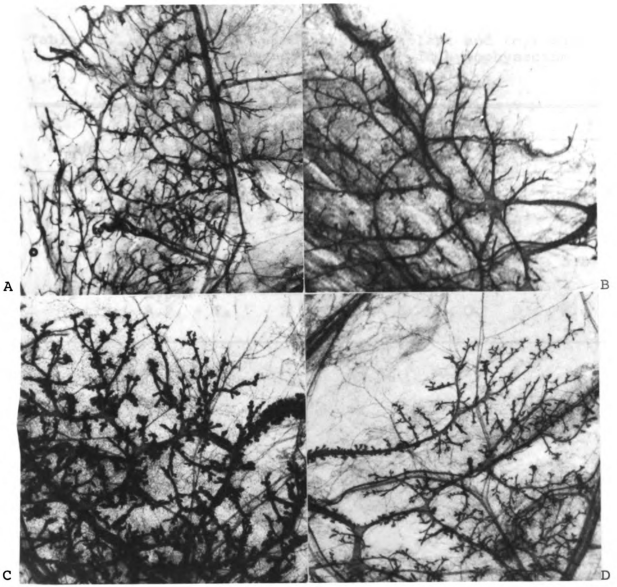


Fig. 11. Representative inguinal mammary glands from the 4 hypophysectomized groups of rats at end of 30-day experiment. (A) control, atropic duct system; (B) thyroxine injection only, greater atrophy of ducts; (C) anterior pituitary transplant only, extensive development of ducts and end buds with limited alveolar growth; (D) anterior pituitary transplant and daily thyroxine injections, marked inhibition of mammary development as compared to C. (3X).

Table 18. Effects of a pituitary transplant and thyroxine on mammary gland development in hypophysectomized rats

| Group | Treatment | Rating | | | | | |
|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | Av. |
| 1 | Hyped | 6 | 4 | 0 | 0 | 0 | 1.4 |
| 2 | Hyped + $T_4$ | 7 | 1 | 0 | 0 | 0 | 1.0 |
| 3 | Hyped + A.P. | 0 | 2 | 3 | 5 | 0 | 3.3 |
| 4 | Hyped + A.P. + $T_4$ | 2 | 1 | 6 | 0 | 0 | 2.4 |

## Discussion

In the present study, transplantation of a single pituitary from 3- to 4-month-old female donor rats into young hypophysectomized immature rats induced a significant increase in body weight, tail length, tibial cartilage width, and in weight of the spleen and thymus. This indicates that the transplanted pituitary synthesized and released substantial amounts of growth hormone without any apparent hypothalamic stimulation. The continuous increase in body growth over a period of 30 days suggests persistent synthesis and release of growth hormone rather than a mere initial leaching of hormone from the transplant. These results are in substantial agreement with those of Hertz (1959), but are in disagreement with the reports of other workers (Martini and de Poli, 1956; Greer, 1957; Martini *et al.*, 1959; Goldberg and Knobil, 1957; Khazin and Reichlin, 1961; Ahren, 1963; Dao and Gawlak, 1963) who failed to note any significant gain in body weight in older hypophysectomized rats given pituitary transplants.

This laboratory has recently reported that an extract of rat hypothalamus can stimulate growth hormone release from the rat pituitary *in vitro* and *in vivo* (Deuben and Meites, 1964). The hypothalamic growth hormone releasing factor is believed to be essential for normal growth hormone release by the pituitary *in situ*. Transplantation of the pituitary to a location remote from the hypothalamus apparently results in a

reduced but nonetheless substantial secretion of growth hormone. Prolactin may also have contributed to a slight degree to the growth effects observed, since prolactin secretion by the transplant is increased after removal of hypothalamic inhibition (Meites et al., 1963).

Thyroxine significantly augmented body growth in the hypophysectomized rats with a pituitary transplant. It also increased body growth of the tail, liver and kidney above that of the rats with a transplant only. Tibial epiphysial width was significantly reduced in these animals. These results add support to the postulated role of thyroxine in directly stimulating hormone production by the pituitary (Knigge, 1958). This laboratory has previously reported that thyroxine or triiodothyronine can directly stimulate the rat pituitary in vitro to increase the release of prolactin (Nicoll and Meites, 1963). A direct synergism may also have occurred between the growth hormone released by the pituitary transplant and the thyroxine injected, since such an effect is well established (Asling et al., 1949; Ray et al., 1950). It is probable, therefore, that in the present experiment thyroxine increased body weight in the hypophysectomized rats by a direct action on the transplanted anterior pituitary, resulting in increased growth hormone release, and by synergizing with the released growth hormone.

Local mammary growth was stimulated by the pituitary transplant in the hypophysectomized rats. This is in agreement

with another report in which an implant of pituitary adjacent to mammary gland tissue induced localized mammary growth in hypophysectomized rats (Dao and Gawlak, 1963). Since the transplanted rat pituitary can secrete greater amounts of prolactin than the pituitary *in situ* (Meites *et al.*, 1963), and can also release growth hormone, as indicated in the present study, it is believed that these two hormones account for the mammary growth observed. Ovarian hormones are not believed to have participated in this mammary growth, since the ovaries and uteri were atrophic and there was no histological evidence of ovarian activity. Previously it was reported that injections of prolactin and growth hormone together could induce mammary lobulo-alveolar development in adreno-ovariectomized or hypophysectomized, adreno-ovariectomized rats (Talwalker and Meites, 1961). These and other related observations are believed to necessitate some revision of the widely held concept (Lyons, 1958) that ovarian hormones are *essential* for lobulo-alveolar mammary development in the rat. The mechanism by which thyroxine inhibited mammary development in these rats is not clear, but other workers have also observed a depression of mammary growth in rats given thyroid preparations (Leonard and Reece, 1941; Trentin *et al.*, 1948).

## IV. EFFECTS ON GONADAL FUNCTION OF PITUITARY "MAMMOTROPIC" TUMORS TRANSPLANTED INTO MALE AND FEMALE RATS

### A. Effect of Adrenalectomy on Ovarian Function in Female Fischer Rats Bearing Anterior Pituitary Tumor ($MtT_{F4}$) Transplants

#### Introduction

In the course of numerous experiments involving transplantation of pituitary tumors (Furth - $MtT_{F4}$) into host female rats of the Fischer (CDF) inbred strain, several workers (Mizuno et al., 1964; Milkovic et al., 1964) reported suppression of ovarian weight and corpus luteum regression. This particular tumor has been shown to secrete growth hormone, prolactin and corticotropin in super-abundant amounts. It was originally induced by chronic estrogen stimulation and was readily transplantable to other host female rats of this highly inbred strain. During initial passages the tumor tissue was estrogen dependent, but the tumor used in these experiments (passages 47, 51, and 52) were autonomous. Unpublished observations have indicated that the ovaries of rats bearing the "mammotropic" $MtT_{W15}$ tumor-bearing were not atrophied and contained many well developed corpora lutea. The only difference between the $MtT_{F4}$ and the $MtT_{W15}$ tumors is that the latter secretes STH and prolactin but presumably does not secrete appreciable amounts of corticotropin.

Normal female rats after adrenalectomy have 2-3 normal cycles followed in turn by persistent corpora lutea and recurrent pseudopregnancies (Swingle et al., 1951). This

suggests that adrenalectomy can result in increased prolactin secretion. Bates et al. (1964) reported that administration of high dosages of ACTH, but not prolactin or growth hormone into intact female rats suppressed ovarian weight. Vaginal estrus was inhibited and a reduction in ovarian weight also occurred in mice treated with ACTH daily for 2-6 weeks (Jarrett, 1965). Milkovic et al. (1964) found that adrenalectomy prevented the suppression of ovarian weight that occurs in $MtT_{F4}$ tumor-bearing animals. These observations suggested that the ACTH-adrenal axis must be partially responsible for ovarian atrophy in $MtT_{F4}$ tumor-bearing females and may also be a factor in luteolysis. The purpose of this series of experiments was to determine the cause of luteal failure in the $MtT_{F4}$ transplanted rat despite the high circulating levels of prolactin, the luteotropic hormone of the rat. The temperal changes in ovarian function were observed after tumor transplantation. Adrenalectomy of tumor-bearing females was also performed in order to observe any changes in ovarian histology which might occur after removal of adrenal steroids.

## Materials and Methods

Mature virgin female Fischer rats (CDF) 3-4 months old were used for these studies. They were maintained in a temperature controlled ($75\pm1^{o}F$) and artificially illuminated (14 hrs. light-10 hrs. dark) room. They were fed Wayne Lab Blox ad libitum supplemented with Dash dog food.

Tumor-bearing animals were initially received from Dr. Jacob Furth at Columbia University. In an initial experiment daily vaginal smears were taken for two weeks prior to transplantation of tumor tissue into 25 rats, and continued thereafter for three months in order to determine the effect of the transplant on the estrous cycle. Serial transplantation of the tumor tissue was made by preparing a mince of pooled tumor tissue fragments in medium 199 from several animals carrying a previous passage of the tumor. The mince was drawn into a tuberculin syringe and each animal was injected in the nape of the neck with 0.1 ml of the tissue suspension (passage 47). Vaginal smears were also followed in 25 animals bearing a subsequent passage (passage 51) of the tumor, in order to test the validity of the observations made previously. Concurrently 2-3 representative animals were sacrificed at each of the following time periods: (1) the day of tumor transplantation, (2) 10 days after the last estrous smear, (3) 20 days after, (4) 30 days after, and (5) 50 days after exhibition of the last estrous vaginal smear. Body, adrenal and ovarian weights were recorded and the ovaries and adrenals fixed for histological study by staining with hemotoxylin-eosin. Correlation of vaginal smears changes with temporal changes in ovarian histology was therefore possible.

A group of 30 female rats was divided equally into two groups: fifteen were transplanted with tumor tissue and the others remained untreated. Thirty days following pituitary tumor transplantation, eight animals with transplants and

eight non-transplanted controls were adrenalectomized. At this time the tumor tissue was just palpable (1-2 mm in diameter). The remaining seven in each group served as non-adrenalectomized, tumor-bearing and non-tumor-bearing controls. All four groups were sacrificed 60 days after the initial transplantation of tumor tissue and, therefore, 30 days after adrenalectomy. Ovaries were fixed and stained for histological study. This experimental design was choosen since preliminary experiments indicated that adrenalectomy of host animals already possessing very large tumors (40-50 g) resulted in death of the majority of the animals.

## Results and Discussion

Tumors became palpable on the average of 25 days post-transplantation as previously reported by other workers (Mizuno et al., 1964). After transplantation the animals exhibited an average of about 5 complete estrous cycles or about 25 days of normal cycling. At this time the majority of the animals (22/25) stopped cycling and became continuously anestrous. The other animals (3/25) exhibited a single pseudo-pregnancy (13-18 days) before becoming anestrous. It is interesting to note that when the tumor is just becoming palpable, enough prolactin is being released to induce pseudo-pregnancy in some instances, and in most enough for even longer prolongation of luteal life. The histological appearance of the ovaries indicated that corpora lutea are very large at 20

days after (Fig. 12) tumor palpation and absent at 30 (Fig. 13) and 50 days (Fig. 14) after tumor palpation as compared to those in a normal cycling female (Fig. 15). This observation definitely indicated that luteolysis occurred sometime between 20 and 30 days after the last estrous smear or after a life span approximating that of normal pregnancy. The histological study, therefore, indicated temporal ovarian changes which were not detected by vaginal smears. It is evident that ovulation did not occur at this time. Large tertiary follicles were present, the interstitial tissue was completely atrophic and luteal tissue was absent. This suggests a lack of luteinizing hormone secretion by the host pituitary.

Data on body, organ, and tumor weights of intact (group 1), adrenalectomized (group 2), intact tumor-bearing (group 3) and adrenalectomized tumor-bearing (group 4) female Fischer rats are presented in Table 19. All animals initially present in groups 1 and 2 survived. One animal died in group 3 and three died in group 4 due to stress from the growing tumor. Final tumor weights in the intact and adrenalectomized animals were similar. The nearly 10-fold increase in adrenal weight in tumor-bearing rats is indicative of the great quantity of ACTH being secreted by the tumor.

A point to be emphasized is the observation that adrenalectomy in tumor-bearing animals prevented the decrease in ovarian weight (Table 19) and luteolysis (Fig. 13), both of

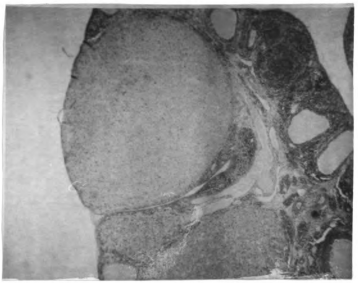

**Fig. 12.** Photomicrograph of ovary from female rat 20 days after palpation of $MtT_{F4}$ tumor (35x).

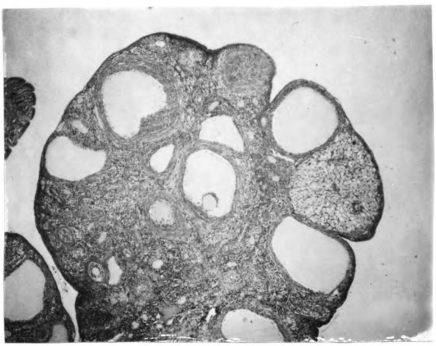

**Fig. 13.** Photomicrograph of ovary from a female rat 30 days after palpation of $MtT_{F4}$ tumor (35x).

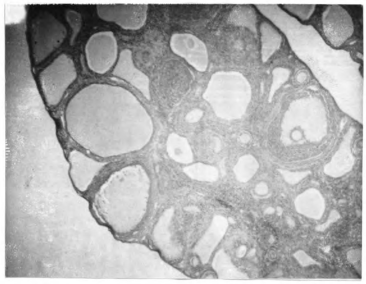

Fig. 14. Photomicrograph of ovary from a female rat 50 days after palpation of $MtT_{F4}$ tumor (35x).

Fig. 15. Photomicrograph of ovary from a normal cycling female (35x).

Table 19. Effect of adrenalectomy on organ weights of female Fischer rats with and without pituitary tumor ($MtT_{F4}$) transplants

| Group and treatment | No. of animals Initial | Final | Final body wt (g) | Tumor wt (g) | Adrenal wt (mg) | Ovarian wt (mg) | Uterine wt (mg) | Pituitary wt (mg) |
|---|---|---|---|---|---|---|---|---|
| 1. Intact | 7 | 7 | 171.7±4.3* | --- | 42.1±2.3 | 50.0±2.8 | 341.8±29.6 | 10.6±0.6 |
| 2. Adrenal-eetomy | 8 | 8 | 182.1±3.5 | --- | --- | 54.9±2.6 | 336.0±27.4 | 14.5±0.4 |
| 3. Intact + $MtT_{F4}$ | 7 | 6 | 166.0±6.6 | 12.6±2.64 | 407.0±51.6 | 30.7±2.3 | 211.7±18.1 | 7.8±0.1 |
| 4. Adrenal-ectomy + $MtT_{F4}$ | 8 | 5 | 222.8±11.1 | 12.1±3.32 | --- | 63.0±4.4 | 263.2±20.8 | 14.0±0.5 |

*Standard error

which occur in intact tumor-bearing animals. Enlarged corpora lutea are present in both adrenalectomized non-tumor-bearing animals (Fig. 16) and also those with tumors (Fig. 17). This, therefore, indicates that adrenal steroids secreted in response to the super-abundant ACTH released by the tumor were responsible for ovarian atrophy and luteal regression. An increase in uterine weight in adrenalectomized tumor-bearing animals (group 4) was observed when compared to their respective control (group 3), again indicating resumption of ovarian function.

Pituitaries of adrenalectomized females with and without tumors were significantly heavier than those of normal cycling females. This observation does not agree with the results of Milkovic et al. (1964) who reported that adrenalectomy had no effect on pituitary weight suppression by the $MtT_{F4}$ tumor. At 30 days post-transplantation, adrenalectomy definitely prevented pituitary weight suppression in this experiment. This difference may be due to the fact that Milkovic et al. (1964) recorded total pituitary weights whereas the weights presented in this thesis are only for anterior pituitaries.

Several mechanisms by which adrenal steroids can exert an ovarian suppressive action can be postulated. It is possible that the steroids of the adrenals inhibit ovarian sensitivity to gonadotropins. ACTH administration to mature mice have been shown to decrease the ovarian response to administered PMS (Jarrett, 1965). Steroid action on the pituitary may also be responsible for ovarian atrophy.

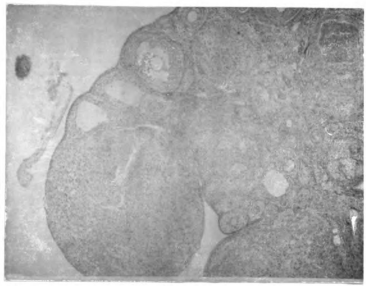

Fig. 16. Photomicrograph of ovary from a rat adrenalectomized for 30 days (35x).

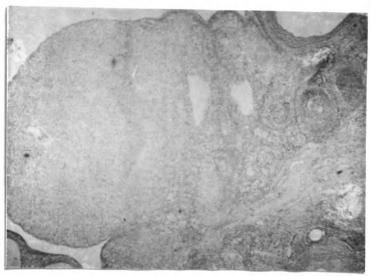

Fig. 17. Photomicrograph of ovary from $MtT_{E4}$ tumor-bearing rat adrenalectomized at the time the tumor became palpable and sacrificed 30 days later (35x).

Corticoids have been reported to decrease prolactin release *in vitro* (Nicoll and Meites, 1964) by rat anterior pituitary. However, the authors concluded that this decrease could be attributed to pharmacological toxicity. Estrogens and androgens of the adrenals may also be circulating in sufficient quantities in tumor-bearing animals to suppress host pituitary gonadotropin secretion. Shipley and Meyer (1965) have reported that large doses of adrenal cortical hormones can depress gonadal function. The possibility exists that luteal failure occurred in the absence of ovulation and LH secretion, as indicated by the lack of vaginal cornification and complete atrophy of interstitial tissue. Rothchild (1965) postulated that normal luteal regression in the rat is due to the luteolytic action of LH. Why then did the corpora lutea in these tumor-bearing animals disappear when LH was not being secreted? The most plausible explanations that can be offered at this time are that the high corticoid levels in the blood decreased the sensitivity of the ovaries to the luteotropic action of the high levels of circulating prolactin. Additional experiments would more fully answer this question.

## B. Effects of an Anterior Pituitary Tumor ($MtT_{W15}$) Transplant on Body Growth, Organ Weights and Organ Histology in Castrated and Intact Male Wistar Rats

### Introduction

Very little work has been reported on the effects of a mammotropic tumor transplant in male rats. Clifton and Furth

(1960) reported that when the $MtT_{F4}$ tumor was transplanted to male Fischer rats, this induced extensive mammary development and secretion. Wistar female rats, 3-4 months after pituitary tumor ($MtT_{W15}$) transplantation, developed mammary tumors (Sinha and Meites, unpublished observations). The growth rate of these mammary tumors when transplanted to other host females appeared to be stimulated by the two hormones released by the pituitary tumor, prolactin and growth hormone. The combination of purified prolactin and growth hormone were shown to maintain mammary lobulo-alveolar development in male rats in the absence of gonadal or adrenal steroids (Meites, 1965).

Numerous workers have also reported that prolactin and growth hormone have a direct effect on the accessory sex organs of the male rat (Grayhack, 1963; Gunn et al., 1965) by synergizing with androgen. The dorsolateral prostate of the rat has been reported to be the most prolactin-sensitive accessory gland in male rats (Grayhack, 1963). Anatomically, this portion is homologous to human prostatic tissue (Price and Williams-Ashman, 1961). Haran-Ghera (1966) observed that mammotropic pituitary tumors in the male mouse stimulated extensive growth of the seminal vesicles. The purpose of this experiment was to test whether the $MtT_{W15}$ tumor, previously reported to secrete great amounts of prolactin and growth hormone, could be transplanted to the male Wistar rat. The effects of chronic hormone stimulation on the mammary glands

and accessory sex glands of the castrated and intact male rats were also observed.

## Materials and Methods

Forty male Wistar rats (85-95 g each) were used for this experiment. They were housed in a temperature controlled ($75\pm1^{\circ}F$) and artificially illuminated (14 hrs. light, 10 hrs. dark) room. They were fed Wayne Lab Blox ad libitum. The animals were divided equally into two groups of 20 animals each; one group was castrated and the other remained unoperated. The following day 10 animals of the intact group and 10 animals of the castrate group were transplanted with pituitary tumor tissue ($MtT_{W15}$) as described previously. Body weights were recorded weekly for the 6 months duration of the experiment. Those animals surviving and an equal number of control castrate and intact males were sacrificed 6 months after the tumor tissue was implanted.

Body, tumor, adrenal, testicular, prostate, seminal vesicle and host pituitary weights were recorded. The testis, host pituitaries, accessory sex glands and mammary glands were fixed in Bouin's fluid for subsequent study after routine hemotoxylin and eosin staining. Tumor size was also approximated throughout the experiment by frequent palpation.

## Results and Discussion

Body growth curves for the 4 groups of animals are shown in Fig. 18. Castrate-non-tumor-bearing and castrate-tumor-

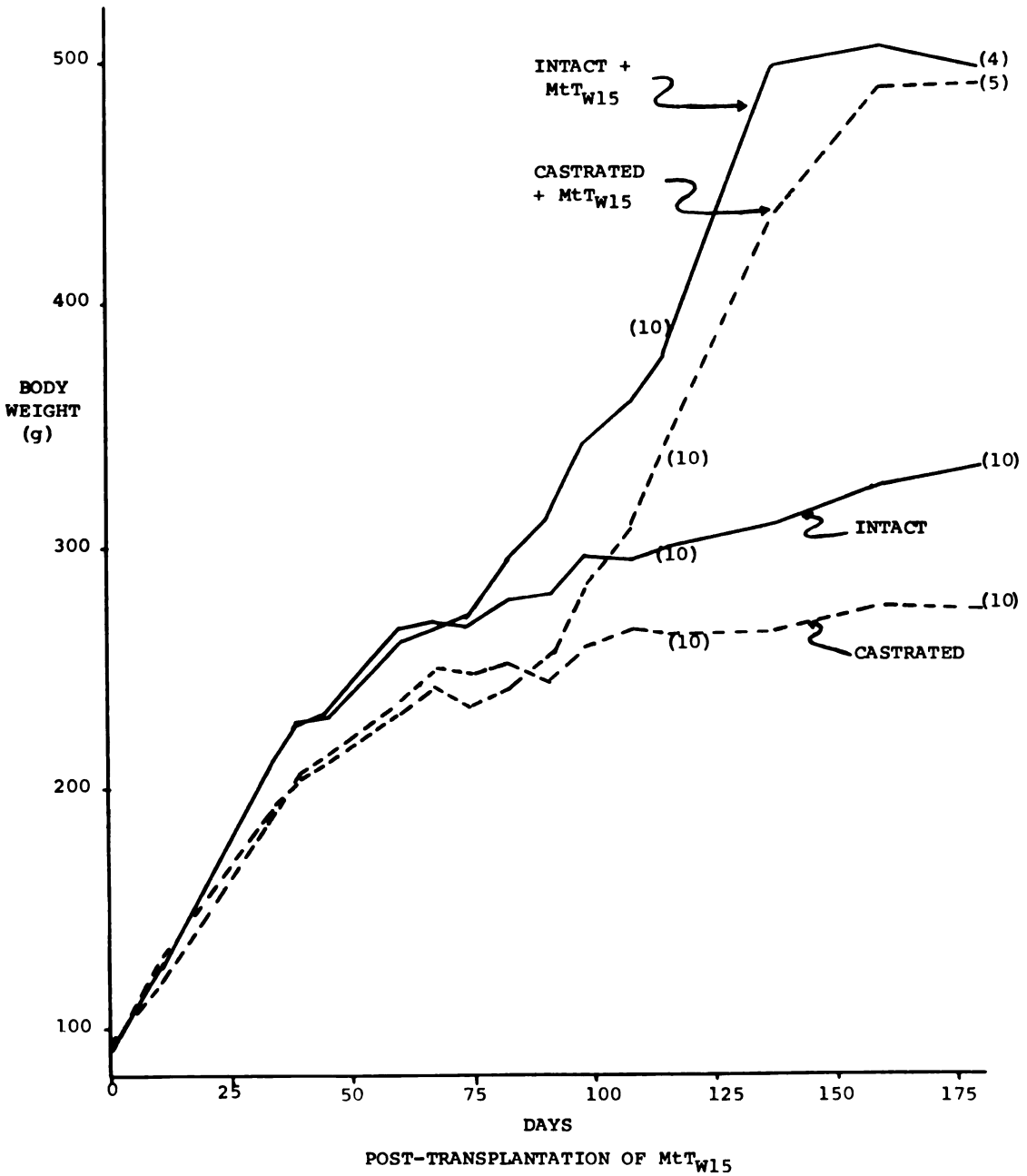


Fig. 18. Body growth curves for normal intact, intact tumor-bearing, castrated, and castrated tumor-bearing male rats.

bearing animals always exhibited body weights less than that of their respective intact controls. Tumor-bearing rats continued to gain weight at a linear rate long after the body weight of the two control groups had reached a plateau. Body and organ weights of the animals at the time of sacrifice are presented in Table 20. All 10 animals survived in both the intact and castrated groups. However, the tumors were so large that only 4 non-castrated and 5 castrated males survived in the other two groups. Therefore, to obtain a non-biased statistical treatment of the data, only 5 animals from each of the previous two groups were sacrificed. Final body weights of tumor-bearing rats were significantly greater than non-tumor bearing animals. The reason that the final body weights in intact and castrated-tumor-bearing animals are approximately the same is presumably due to the fact that the largest intact rats had the largest tumors, and therefore were subjected to the most stress and died prior to termination of the experiment. The tumors in intact males became palpable about one month after transplantation, similar to tumor tissue transplanted to females. However, the tissue in the castrated males was palpable a week later than that in intact males and continued to grow at a slower rate. Final tumor weight in the intact males was about 1 1/2 times that of the castrated male.

A surprising observation was the greater than 6 fold increase in adrenal weight in the tumor-bearing animals.

Table 20. Body and organ weights of castrated and intact male Wistar rats with pituitary tumor transplants

| Group | Final body wt (g) | Tumor wt (g) | Adrenal wt (mg) | Testis wt (g) | Seminal vesicle (mg) | Prostate Dors-lat (mg) | Prostate Ventral (mg) | Pituitary wt (mg) |
|---|---|---|---|---|---|---|---|---|
| 1. Intact | 331.4±6.3* | --- | 28.6± 2.2 | 2.69±.17 | 508.8±58. | 376.0±45. | 366.2±69 | 8.6±0.2 |
| 2. Castrated | 276.6±6.8 | --- | 31.2± 2.2 | --- | 9.4±.7 | 5.0± 1. | 5.0±1. | 15.2±1.0 |
| 3. Intact + tumor | 485.0±12.6 | 61.5±6.1 | 197.0±14.3 | 0.85±.10 | 221.0±87. | 81.3±13. | 81.3±13. | 7.7±0.5 |
| 4. Castrated + tumor | 481.6±25.0 | 37.0±3.2 | 200.2± 8.5 | --- | 43.2±2.0 | 9.8±.4 | 9.8±.4 | 13.0±0.0 |

*Standard error

In females, this tumor ($MtT_{W15}$) secreted only prolactin and growth hormone. From the adrenal weights presented it is obvious that tremendous amounts of ACTH must also have been secreted. This increase is too great to be attributed solely to stress of the tumor on the output of ACTH by the host pituitary. A difference in function of the pituitary tumor tissue in female and male animals has not been reported. It is probable that the tumor transplanted in this experiment possessed ACTH secreting cells. We have previously observed in this laboratory that repeated transplantation of the mammotropic $MtT_{F4}$ tumor resulted in greater secretion of ACTH and in less secretion of growth hormone and prolactin (unpublished observation). This tumor, therefore, was similar to the $MtT_{F4}$ in hormonal secretory activity. A marked reduction in testicular weight (Table 20) and disappearance of testicular interstitial tissue (Figs. 19 and 20) and cessation of spermatogenesis was observed. This was analogous to the observation previously reported in this dissertation on the effect of ACTH secreting tumors on ovarian function in the female. It appears, therefore, that LH secretion was inhibited, and possibly FSH as well, in the male by ACTH secretion.

Castration of course reduced ventral and dorsolateral prostate weights and seminal vesicle weights. An unexpected decrease in these organ weights also occurred in intact tumor-bearing rats as an indirect effect of suppression of testicular function. The greater weight of these accessory organs in

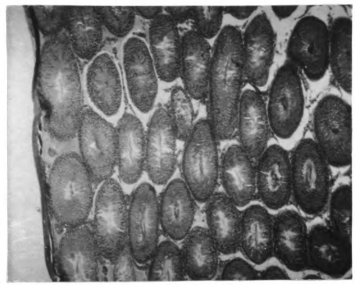

Fig. 19. Photomicrograph of testis from a normal male rat (35x).

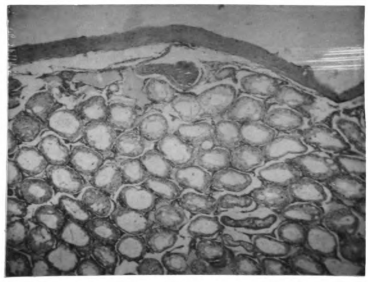

Fig. 20. Photomicrograph of testis from a $MtT_{W15}$ tumor-bearing male (35x).

castrated-tumor-bearing animals than castrated males can be attributed only in part to the direct effects of prolactin and growth hormone. Increased adrenal androgens may also have influenced these weights to an appreciable extent. An experiment using adrenalectomized-castrated males should be designed and performed to test this possibility.

Tremendous mammary development and secretion were observed in all tumor-bearing animals (Figs. 21-23). One of the five castrated-tumor-bearing males sacrificed had a palpable mass in the inguinal region. Histological examination (Fig. 24) indicated that it possibly was an epithelial mammary gland tumor.

## V. APPLICATION OF ELECTROPHORETIC TECHNIQUES TO SEPARATE ANTERIOR PITUITARY HORMONES

Anterior pituitary homogenates have been separated into several protein components by paper and starch gel electrophoretic techniques. Levey and Roberts (1958) separated pituitary proteins of normal and thyroidectomized rats using paper electrophoresis. The paper strips were divided into two portions and assayed for growth hormone. Of the three major components that were observed, the slowest moving component was growth hormone. This protein band was "thyroid labile" and disappeared after thyroidectomy. It was also of interest that thyrotropin was also recoverable from this same region.

Smithies (1959) developed a starch gel medium which provided greater resolution of proteins than that provided by

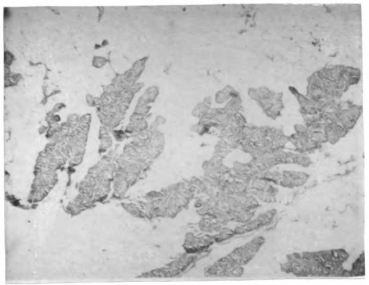

Fig. 21. Photomicrograph of mammary gland of normal male rat (420).

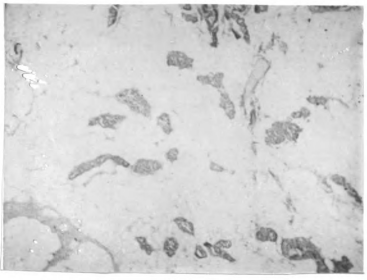

Fig. 22. Photomicrograph of mammary gland of castrated male rat (420x).

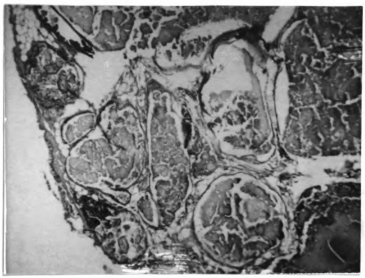

Fig. 23. Photomicrograph of mammary gland representative of normal and castrated male rats with transplanted $MtT_{W15}$ tumor (420x).

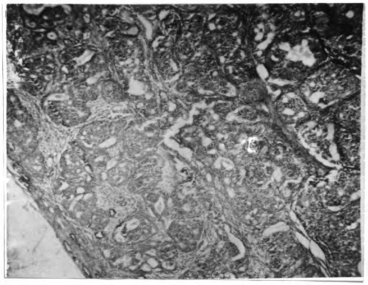

Fig. 24. Photomicrograph of mammary tumor in castrated tumor-bearing male rat (35x).

paper electrophoresis. Ferguson and Wallace (1960, 1963) separated crude ovine pituitary extracts into 50 components using starch gel. NIH standard ovine prolactin was separated into 17 components by the same system. This great heterogeneity of purified prolactin has not been confirmed, and the greatest number of bands reported by other workers (Barrett et al., 1962) was only four. Baker et al. (1963) separated rat pituitary homogenates using starch gel and assayed eluates from various gel segments for prolactin activity. They concluded that most of the prolactin activity was present in the fastest migrating, most acidic protein component. Some activity was also detected in the nearest adjacent more basic component. Human anterior pituitary homogenates were separated on starch gel by Lloyd and Meares (1962). Using the mouse uterine assay they found that the gonadotropic activity was among the slowly migrating components and could not be correlated with any individual protein zone stained with nigrosine. Gonadotropic hormones were also found to migrate slowly in starch gel by Catt and Moffat (1965). It is interesting that when electrophoresis was carried out at pH 8.5, protein migration was observed to occur similarly in both the starch and polyacrylamide gel media (Catt and Moffat, 1965; Jones et al., 1965). The prolactin band was composed of two closely adjacent single bands in some instances.

Development of polyacrylamide gel electrophoresis by Ornstein and Davis (1962) has provided a method for the greatest resolution of proteins yet known. Electrophoretic comparison of pituitary glands from male and female rats was accomplished using polyacrylamide gel by Jones et al. (1965). They were able to isolate the prolactin band by correlating known physiological differences in prolactin content of male and female pituitaries with quantitative differences in particular stained bands and then assaying the corresponding non-stained bands. In this way certain portions of the gel were not assayed for hormonal activity and may have escaped detection. The prolactin band was reduced in pituitaries from ovariectomized female rats and increased in males treated with estradiol. Growth hormone migrated at a considerable distance behind albumin and prolactin. Thyroidectomy reduced the thickness of the growth hormone band and replacement thyroxine increased the thickness to near normal (Lewis et al., 1965). Pituitary glands from dwarf mice were observed to be lacking a growth hormone band (Lewis et al., 1965a). The administration of thyroxine did not produce any change in pituitary growth hormone content.

The polyacrylamide gel electrophoresis technique, therefore, has been repeatedly demonstrated to resolve microgram quantities of protein mixtures into individual protein components. It became apparent that this technique might prove helpful in the separation and semi-quantitation of microgram

quantities of pituitary hormones released into incubation medium by pituitaries *in vitro*. It may also be possible to detect alterations in hormonal release after addition of hypothalamic extracts to this system. The purpose of this study was to separate the hormones in fresh rat pituitary homogenates using this technique. Proteins eluted from segments of the gels were assayed for prolactin growth hormone, TSH, LH, ACTH and FSH activities. The data presented should serve as a guide for subsequent study of hormones released into an incubation medium.

## Materials and Methods

Adult CFN female rats (Carworth Farms, Inc.), each weighing 175-200 g, were housed under controlled illumination (14 hrs. light-10 hrs. dark) and temperature ($75\pm1^{\circ}F$), and were fed Wayne Lab Blox pellets *ad libitum*.

Disc electrophoresis was performed by the technique of Ornstein and Davis (1962). Tetramethylethylenediamine was added to the large pore gel to facilitate polymerization as suggested by Lewis (1963). The 7.5% standard gel preparation was used at pH 9.5. Fresh anterior pituitaries were homogenized with an agate mortor and pestle and suspended in phosphate buffer (pH 7.4) at a concentration of 2 mg fresh tissue per .1 ml of buffer. Twelve glass tubes (62 x 5 [I.D.] mm] containing gel were used for each electrophoretic test. Buffer containing homogenized pituitary was mixed with an

equal volume of upper gel solution and will be referred to as the sample gel solution. Sample gel (.3 ml) containing 3 mg of homogenized pituitary tissue was added to each column and a current of 2.0-2.5 milliamperes applied per tube. The duration of each electrophoresis was judged by watching the bromophenol-buffer front, and varied from 1-1.5 hours in length. At the termination of electrophoresis the 12 gel columns were removed from the glass tubes. Previous work had indicated that the polyacrylamide gel cylinders contracted upon freezing on dry ice. Therefore, in order to establish points of reference for subsequent cutting, the distance from origin to bromophenol-buffer front was measured for each of the twelve cylinders, and at each 1 cm interval a hole was punched through the gel with a sharp dissecting needle.

Two of the cylinders were stained for one hour with Amido Swartz dye in 6% acetic acid solution. The remaining ten were frozen immediately in test tubes on a slab of dry ice. The electrophoresis, staining and destaining were completed on the same day. The stained protein bands were then used together with the perforated reference points to locate the corresponding protein bands in the 10 unstained cylinders. Each cylinder was allowed to thaw prior to cutting the five segments as indicated (Fig. 25). Similar segments from the ten tubes were pooled and expressed through the orifice of a 2 ml syringe into conical centrifuge tubes, each containing 2 ml of distilled water. The hormones were extracted from

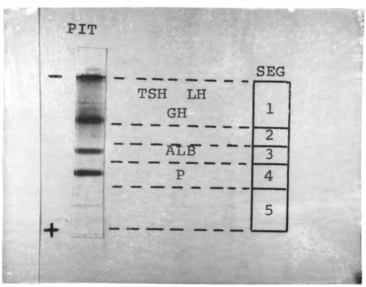


Fig. 25. Photograph of a stained polyacrylamide electrophoresis gel column on which hormones in homogenized rat pituitary had been separated.

the homogenized gels by allowing the tubes to stand overnight at 4°C. The following morning they were centrifuged and the supernatant was collected and stored at -20°C. Supernatants of similar gel fractions were collected from several electrophoretic determinations and pooled.

Pooled eluates from each group of segments were assayed for prolactin activity by the intradermal pigeon crop technique (Reece and Turner, 1937). GH, TSH, LH, FSH and ACTH were assayed by the multiple assay technique described by Jensen et al. (1939). A total of 50 immature female rats of the Sprague-Dawley strain (Hormone Assay Lab., Chicago, Illinois), hypophysectomized at 26-28 days of age, were used in this experiment. Tibial epiphysial cartilage width, thyroid cell height, ovarian interstitial tissue repair, follicle development and adrenal lipid dispersion served as histological endpoints for each of the hormones mentioned above. Tibias and adrenals were fixed in 10% neutral formalin solution. Tibial epiphysial cartilage width was measured with an ocular micrometer after the halved tibia had been stained with silver nitrate. Adrenals were sectioned while frozen and stained with Sudan black. All other tissues used for histological study were fixed in Bouin's solution, dehydrated, cut at 10 μ, and stained with hemotoxylin and eosin. Thyroid cell height was measured by taking the average of 40 readings with an ocular micrometer. A total of 6 assay animals were used in two experiments for each pooled segment

eluate. Each animal was injected with the supernatant recovered from the equivalent of 6 mg fresh anterior pituitary tissue initially present in the sample gel.

## Results and Discussion

Biological assay results are presented in Table 21. Buffer alone (group 1) or a buffer extract of polyacrylamide gel segments not containing protein (group 2), were injected into assay animals as experimental controls. Neither of these solutions stimulated the pigeon crop sac nor the endocrine organs of the hypophysectomized rats. In two experiments prolactin activity was localized in gel segments 3 and 4. The results, therefore, disagree with those of Lewis et al. (1965) who reported that crop stimulating activity was found only in the major band of segment 4. Supporting the findings are those of Baker et al. (1963) who reported that crop stimulating activity was present in several bands when anterior pituitary tissue was separated by starch electrophoresis. In addition, purified NIH ovine prolactin was placed on polyacrylamide gel under conditions similar to those used for fresh tissue in the experiments, and also was resolved into at least three distinct components. It is possible that polymers are formed when prolactin is subjected to some chemical treatments, and when placed in gel electrophoresis, multiple bands are resolved. In these experiments, the majority of crop stimulating activity was present in the

Table 21. Hormonal activities present in various segments of a polyacrylamide column

| Seg. no. | Treatment | Total no. of assay animals | Prolactin (IU) expr. 1 | Prolactin (IU) expr. 2 | Growth hormone tibial width (μ) expr. 1 | Growth hormone tibial width (μ) expr. 2 | TSH-thyroid cell height (μ) | LH |
|---|---|---|---|---|---|---|---|---|
| 1. | Buffer | 6 | - | - | 135±10* | 132±8 | 3.8±.6 | - |
| 2. | Gel eluate | 6 | - | - | 133±12 | 130±5 | 4.0±.8 | - |
| 3. | Segment 1 | 6 | - | - | 195±13 | 174±10 | 6.6±.8 | + |
| 4. | Segment 2 | 6 | - | - | 139±6 | 127±5 | 3.8±.5 | - |
| 5. | Segment 3 | 6 | .066 | .035 | 159±8 | 147±9 | 3.8±.7 | - |
| 6. | Segment 4 | 6 | .144 | .192 | 134±7 | 132±5 | 3.5±.6 | - |
| 7. | Segment 5 | 6 | - | - | 129±6 | 128±5 | 3.7±.7 | - |
| 8. | Homogenized ant. pit. | 6 | + | + | 276±12 | 207±12 | 8.1±.9 | + |

*Standard error

fastest migrating major band, and in addition, appreciable activity was also detected in segment 3 in a minor band associated with albumin.

In two experiments GH was localized in segment 1 (group 3) and these results are in agreement with those of Lewis et al. (1965). A slight amount of GH activity was also present in segment 3. This increase in width was less than 40 μ and, therefore, can not be definitely attributed to GH. It may have been due to prolactin which has been shown to possess some tibial stimulating activity (Reisfeld et al., 1961; Popkoff and Li, 1962). Another possible explanation for finding similar GH activity associated with proteins migrating at different rates is the observation that GH may bind to plasma protein (Hadden and Prout, 1964).

TSH activity was found in segment 1. The average thyroid cell height was 6.6 μ (group 3) as compared to 4.0 μ or less in all other groups. LH activity was also present in segment 1, as can be seen by the degree of ovarian interstitial tissue repair present in the photomicrographs (Figs. 26, 27, 28). Nearly complete repair was present in assay animals injected with extract from segment 1 (Fig. 28), while all ovaries from other groups were similar to those of hypophysectomized immature animals (Fig. 26). These results definitely indicate that hormones of glycoprotein composition migrate very slowly in this system. This possibility was suggested by Lewis et al. (1965) who observed PAS positive staining material near the origin of each gel.

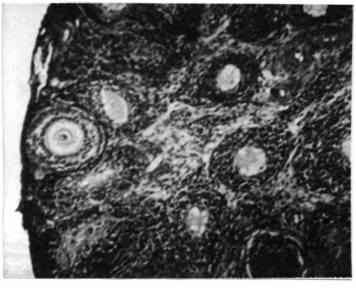

Fig. 26. Photomicrograph of ovary from hypophysectomized immature rat (100x).

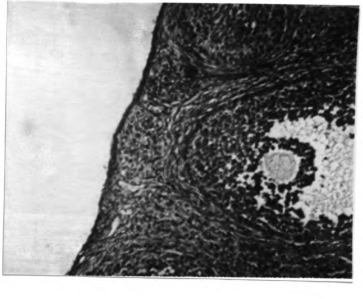

Fig. 27. Photomicrograph of ovary from hypophysectomized immature rat treated with homogenized rat pituitary (100x).

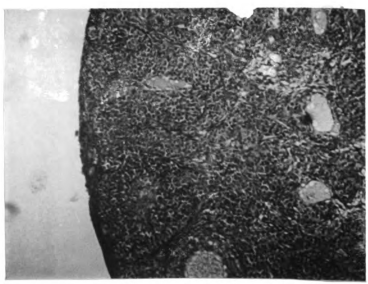

Fig. 28. Photomicrograph of ovary from hypophysectomized immature rat treated with eluate from gel segment 1 (100x).

Numerous tertiary follicles were present in the ovaries of assay animals injected with pituitary homogenate (Fig. 27). No follicular development was observed in any of the assay animals given eluates of gel segments. The quantity of extract injected may have been below the minimal effective dosage for detection of FSH. Likewise, no ACTH activity was detected, as indicated by the lack of lipid dispersion in histological sections of the adrenal. Since ACTH is a low molecular weight polypeptide, its rate of migration may have been so rapid that it would have migrated into the lower buffer.

## GENERAL DISCUSSION

In this thesis several allied studies related to the physiology of prolactin were undertaken. The significance of each portion of these data has been discussed following each experiment in the preceding section of this thesis. Therefore, major emphasis will be placed on the possible indications for further research.

I believe that the best method for assaying PIF on the basis of the data obtained is to incubate male rat pituitary quarters, for 4 hours, with dosages of hypothalamic extract in the range of .25-2.0 hypothalami per ml of culture medium. Male pituitaries release prolactin with much less variation than female pituitaries. Prolongation of incubation to 6 hours may increase the sensitivity of the assay still further. For routine screening of many samples containing possible PIF, as would occur during chemical fractionation of hypothalamic extracts, I would suggest incubating 8 male pituitary halves divided homogeneously between two flasks in a single flask-pair design. After incubation for at least 4 hours, the 2 ml volumn of medium from each flask could be assayed in 5 pigeons. Use of a standard curve with each prolactin assay permits conversion of Reece-Turner units of prolactin to ug of NIH-standard equivalents. This design is ideal when numerous

samples are to be compared with one another. However, when one solution is to be compared with only one other, it is best to take advantage of the two crop sides and assay each solution in the same bird. The results can then be analyzed statistically using students t-test for paired observations (Snedecor, 1956).

Several observations on prolactin release by the transplanted rat pituitary are interesting. Prolactin release by the transplant is definitely greater than that of the pituitary in the intact female during the cycle, since luteal function and mammary growth are observed. However, prolactin secretion still is not maximal. Since the pigeon appears to have a stimulatory mechanism to meet the physiological need for prolactin during crop "milk" formation, it is possible that maximum secretion of prolactin requires stimulation such as occurs during lactation in the rat. In this respect the data presented by Ratner (1965) indicated that PIF was depressed in the hypothalamus of lactating female rats and that hypothalamic extracts from these rats did not inhibit prolactin release <u>in vitro</u>. It is possible that the mammalian species evolved this system for regulation of prolactin secretion to provide maximum efficiency for milk secretion. Investigation of the effects of hypothalamic extracts from lactating female rats on prolactin release by the pigeon pituitary would be of interest.

The study of neuroendocrinology in avian species is just beginning. Some evidence has been provided indicating that there may be an LRF in birds (Nalbandov, personal communication). The existence of other neurohormones for release of other avian anterior pituitary hormones has not been tested in vitro. Transplantation of the pituitary of the chicken suggests that TSH secretion by the transplanted pituitary may be very similar to that of the intact pituitary. Also, the adrenals of the chicken do not permanently regress when the bird is hypophysectomized, and it has been suggested that adrenals may be maintained by an extrahypophyseal ACTH (Ma and Nalbandov, 1963). Does the hypothalamus of the bird produce ACTH? Placement of hypothalamic lesions in the pigeon also suggests that ACTH, may actually be inhibited (Miller, 1961). It would be of interest to test avian hypothalamic extracts in vitro for possible neurohumors which regulate release of FSH, LH, TSH, ACTH, and growth hormone, and see whether the mechanisms for control differ from mammals. Thus far, only a "prolactin releasing factor" has been demonstrated.

Numerous experiments of biochemical nature can also be suggested for the study of the control of pituitary hormone secretion. The indication that the isolated anterior pituitary actually synthesizes appreciable amounts of prolactin can be more fully answered by combining several techniques. It has been well demonstrated that prolactin activity released into the medium is increased 10-15 times above that of fresh

rat pituitary tissue in organ culture (Nicoll and Meites, 1962). Prolactin synthesis by a cell-free system of anterior pituitary may also prove feasible. Simple determinations of total proteins in medium and homogenized pituitary after incubation could be compared with that of fresh pituitary homogenized in medium without incubation, as a preliminary indication of protein synthesis. Detection of the incorporation of a labeled amino acid into a hormone during incubation and its possible alteration by addition of hypothalamic extract is already being performed by certain laboratories and should prove useful as related to prolactin. Fractionation of hypothalamic extracts for PIF and PRF should also be performed. However, methods for purification of rat prolactin from a mixed protein solution remain to be worked out. Once the labelled prolactin has been synthesized and purified, the hormone could be administered to animals to study the mode of action of the molecule.

Transplantation of the rat pituitary subcutaneously has proven to be a simple and very effective technique for studying pituitary hormone release. From the data presented in this thesis, it is apparent that the transplanted pituitary releases growth hormone in small but detectable amounts. This technique was also employed to determine whether secretion of growth hormone by the pituitary could be altered by administration of thyroxine. The synergistic actions of growth hormone and thyroxine on growth make interpretation of

these results difficult. Pituitary cytology would help answer this question.

It is apparent from data presented in this thesis that adrenal secretions are the cause for ovarian atrophy in $MtT_{F4}$ tumor-bearing female rats. Pituitary function is definitely inhibited, as indicated by the significant decrease in pituitary weight in tumor-bearing animals. LH release was definitely inhibited as indicated by the complete atrophy of the interstital tissue of the ovary. Since the uterine weights of tumor-bearing rats are lower than the controls, the suppression of LH cannot be attributed to increased estrogen from the adrenal. On the basis of other reported data it can be proposed that adrenal corticoids suppressed pituitary LH release (Shipley and Meyers, 1965). Additional studies of the role of the adrenal in luteolysis in the intact female rat during the cycle and pseudopregnancy would be of great interest. Treatment of normal cycling rats with prolactin and cortisol concurrently would determine whether corticoids reduce the sensitivity of the ovary to prolactin. It is very interesting to note that a tumor secreting the same hormones as the $MtT_{F4}$ when transplanted to the male also caused testicular atrophy. Whether this effect was also mediated via the adrenal remains to be determined. Spermatogenisis (primarily controlled by FSH) was completely inhibited in the male, whereas follicular development (also primarily controlled by FSH) was not affected in the female. Chronic administration of high dosages of

corticoids to male rats does not suppress testicular seminiferous tubule function (Albert, 1961), but chronic administration of androgen has a great depressing effect. Androgen administration into intact females causes complete interstitial tissue atrophy while follicular development continues normally. It is possible, therefore, that ovarian and testicular atrophy seen in tumor-bearing animals may be due to adrenal androgen secretion which in turn acts on the host's pituitary to suppress gonadotropin secretion.

Study of homogenates of fresh rat pituitary by polyacrylamide gel electrophoresis indicated that pituitary hormones could be separated by this technique. Recent results reported by Robboy and Kahn (1966) also indicated that this technique is useful for the study of hormone release into a culture medium. These workers were able to detect differences in the mobilities of proteins from pituitary tissue and proteins released into the medium by this method. This technique has also previously been demonstrated to be useful for demonstrating differences in pituitary content of hormones for animals under different experimental conditions (Lewis et al., 1965). Comparison of the mobilities of prolactin in pituitary homogenates in several species would also be of great interest. Fractionation of hypothalamic extracts by this technique, using a smaller lower gel pore size, may also be of value. Preliminary experiments using oxytocin and vasopressin could be undertaken to determine methods for staining and detection

of peptides. Differences in hypothalamic contents of various factors that have been reported could then be used to locate these factors on the column.

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