NORMAL RAT PITUITARY CELLS: STUDY OF PRIMARY CULTURE, SECRETION OF PROLACTIN, AND BINDING OF PROLACTIN-^{125}IODINE

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
PATRICIA ANNE PAYNE
1975

ABSTRACT

NORMAL RAT PITUITARY CELLS: STUDY OF PRIMARY CULTURE, SECRETION OF PROLACTIN, AND BINDING OF PROLACTIN-^{125}IODINE

By

Patricia Anne Payne

Dispersed cells of normal pituitaries from three ages of female rats: immature or 14 days old, mature or 45 days old, and retired breeders or greater than six months of age were cultured for periods extending to 14 days. These cultures were characterized for their ability to release prolactin *in vitro* relative to their protein and deoxyribonucleic acid (DNA) concentrations. A significant difference in quantities of prolactin released into the medium by the pituitary cell cultures was demonstrated by radioimmunoassay. Immature pituitary cells released the lowest levels of prolactin, mature pituicytes secreted midrange values, and retired breeder pituicytes released the highest levels of prolactin. These normal rat pituitary cells were demonstrated to specifically bind prolactin-^{125}I, thus illustrating the presence of receptors for prolactin on pituicytes. A high positive correlation was demonstrated between per cent specific binding of prolactin and prolactin released *in vitro* among cultures of pituicytes. Secretion rates of prolactin and the number of binding sites may be

related. As binding material for prolactin receptor assays, this study has demonstrated the utility of cell cultures.

NORMAL RAT PITUITARY CELLS: STUDY OF PRIMARY CULTURE, SECRETION OF PROLACTIN, AND BINDING OF PROLACTIN-^{125}IODINE

By

Patricia Anne Payne

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Physiology

ACKNOWLEDGMENTS

I extend thanks to the many people who have supported me throughout my research. My thanks for their advice, help, laughter, time and caring. These people were critical for the completion of this study and thesis.

I would especially like to thank Dr. William L. Frantz for his guidance and encouragement during my research. Appreciation is also extended to Drs. C. W. Welsch, E. M. Convey and J. B. Scott, members of my advisory committee. Thanks is expressed for the use of the facilities of Dr. Clifford Welsch. Without the use of his equipment, this research would have been impossible.

To Olan Dombroske goes special thanks for his invaluable help and understanding. Gratitude is expressed to Charles Brooks for the instruction and utilization of his radioimmunoassay procedure and to Karen Kowalski for the hours spent in preparation of this manuscript. To my parents, family and R.C.B., my thanks are dimensionless and inadequate.

I am grateful for the financial support from Dr. William Frantz through the National Science Foundation, Grant No. GB-30686 and the Department of Physiology.

TABLE OF CONTENTS

TABLE OF CONTENTS cont'd Page

TABLE OF CONTENTS cont'd Page

LIST OF TABLES

LIST OF FIGURES

Introduction

Synthesis and release of prolactin are regulated by the interplay of genetic and environmental factors. The former are slow in development and more profound in expression, as illustrated by the fluctuations in prolactin serum concentrations in sexual development. The latter may be characterized as "fine tuning" and produce transient and regulatory effects as exhibited by prolactin in the development of mammary glands prior to lactation. Releasing and inhibiting factors (hormones) are elicited by the neural or physical stimulation of other hormones. The possibility of direct "feedback control" or autoregulation of a hormone and possibly prolactin has a good theoretical basis in kinetic analysis. This system entails the specific recognition of prolactin by receptors on or in the pituitary producing and releasing the hormone.

Such a mechanism of autoregulation has been demonstrated with the secretion of thyroid hormones in rats and mice, and the regulatory factors for thyroid hormones: thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH) (Bowers *et al.*, 1967; Vale *et al.*, 1967; Averill, 1969). To date, no definitive studies have shown specific binding of prolactin to normal mammotrophic cells of the anterior pituitary gland as a mechanism for prolactin regulation upon its own synthesis and secretion. This thesis describes the development and initial findings of an *in vitro* controlled experimental system for prolactin regulation.

Pituitary cell cultures are easily manipulated and conceivable for assaying radioreceptor activity and hormonal concentrations of the incubation medium. Utilization of cultures of normal anterior pituitary cells from female rats at various stages of sexual development provides a means to compare the prolactin binding activity with synthesis and release of prolactin *in vitro*. These findings compared to the *in vivo* serum and pituitary concentrations of prolactin of female rats of the same ages would aid in supporting or negating the *in vitro* results. This research was designed to elucidate this relationship of ontogeny, prolactin secretion and specific prolactin binding to cells of the female rat anterior pituitary after cultivation. Pituitary cells from three ages of female rats: 14 days of age, 45 days of age, and greater than six months of age were cultured and assayed for their ability to secrete prolactin and to bind prolactin-^{125}I.

Literature Review

I. The Development of In Vitro Systems for Pituitary Study

A. Organ Cultures of the Rat Pituitary

Organ culture of the bisected pituitaries has been a widely used method to study the regulatory mechanisms for prolactin (PRL) synthesis, storage, and release. When isolated from the hypothalamus, the pituitary *in vitro* will continue to secrete PRL (Meites *et al.*, 1959; 1961).

Addition of crude hypothalamic extracts to the culture medium decreased PRL release (Talwalker, 1963). Kragt and Meites (1967) demonstrated a negative dose response effect of hypothalamic extract on the release of PRL into the medium *in vitro*. These findings support previous studies *in vivo*, which suggested the existence of a hypothalamic prolactin inhibiting factor (PIF) (Grosvenor *et al.*, 1964; Amenomori and Meites, 1970).

Lesions in the median eminence, the pathway for nerve tracts and blood vessels from the hypothalamus to the pituitary, increased rat serum PRL levels (Chen *et al.*, 1970; Welsch *et al.*, 1971). Anterior pituitary glands, transplanted under the renal capsule, also produced higher concentrations of serum PRL (Everett, 1954; 1956).

B. Cell Cultures of the Rat Pituitary

Use of anterior pituitary cells in long term cultures has been limited to tumor cell cultures. Tashjian (1968) cloned or isolated several pituitary cell lines from

X-ray irradiated Wistar-Furth rats. Each cell line was further characterized as to growth hormone (GH) and PRL secretion (Tashjian et al., 1970), karyotype (Sonnenschein et al., 1970) and hormone release in response to hydrocortisone (Tashjian et al., 1970). In 1973, studies by Gautvik and Tashjian demonstrated a dose dependence between Ca^{+2} ions in the extracellular medium and increases in medium hormone concentration. Magnesium ions had the reverse effect. A similar increase in hormone release was noted with high levels of K^{+} in the presence of calcium (Gautvik and Tashjian, 1973b).

A similar cell line was derived from Wistar-Furth rats with anterior pituitary tumors induced with estradiol benzoate by Sonnenschein (1973). Anterior pituitary tumor cells were dispersed with viokase[R], a pancreatic enzyme mixture and with a microliter dilution technique cloned for a specific cell type. The morphology of the individual lines of cloned cells varied in terms of the percentages of dendrite-fibroblast type cells and clusters of epitheliod cells in suspension. Morphology was not, however, indicative of function as cells of different morphology were able to secrete GH and PRL (Sonnenschein et al., 1974).

Hopkins and Farquhar (1973) utilized a variety of dissociating agents and inhibitors i.e. trypsin, collagenase, neuraminidase, and soybean trypsin inhibitor. With isolated anterior pituitary cells from Sprague-Dawley

Viokase[R], Viobin Corporation

male rats that were cultured for short periods, they were able to quantitate ^{3}H-leucine incorporation into prolactin as seen by gel electrophoresis and autoradiographic electron microscopy. Other methods of trypsin dispersions of anterior pituitary cells have been developed by Ishikawa (1969) for cell morphology and classification studies and for the effects on release of adrenocorticotropin (ACTH) by hypothalamic extracts. Portanova et al. (1970) and Sayers and Portanova (1974) isolated anterior pituitary cell types from a trypsin dispersed preparation by velocity sedimentation at unit gravity. This technique also provided a useful way to study the pituitary mammotrophs or acidophils (Hymer et al., 1973).

Recently, the collagenase-viokase dispersion method of Vale et al. (1972) has been widely used. This is an adaptation of a system for studying thyrotropin releasing factor (TRF) or thyrotropin releasing hormone (TRH) and luteinizing releasing factor (LRF) as a challenge for thyroid stimulating hormone (TSH) and luteinizing hormone (LH), respectively. Baker et al. (1974) extended the use of viokase to disperse pituitary cells for immunochemical study of monolayer cultures. Over a 32 day culture period, the cells persisted and PRL and ACTH regularly were present in the medium.

With these methods of anterior pituitary gland dispersion, it is possible to maintain viable cells which retain many in vivo characteristics. Application of these

procedures to long term culture of normal rat pituitary cells is feasible if structure and function are maintained and replication provides a sufficient number of cells for study.

II. Effects of Age and Sex on Prolactin Secretion

Serum concentrations and pituitary contents of prolactin vary significantly with age and sex of the animal. Yamamoto et al. (1970) utilized in vivo incorporation of ^{14}C-leucine into PRL for the study of female rats of 12 to 140 days of age. Prolactin synthesis increased at days 37 to 45 relative to 13 to 21 day old animals continued to increase with age. In males, a similar increase at day 45 was noted but the rate of increase with age was not as great as noted for female rats. Prolactin concentrations of serum and per 100g body weight for all age groups of rats were higher in females than in males.

Voogt et al. (1970) found similar age-related concentrations of PRL in pituitaries and serum of female rats. At ages prior to vaginal opening, serum concentrations were 13-21 ug per ml, and a three-fold increase was noted on the day of vaginal opening. From 41-45 days of age, prolactin levels were similar to those found in 3-month-old estrous rats (70-80 ug/ml). Pituitary prolactin concentration trends paralleled serum amounts except on the day of vaginal opening. Voogt also produced higher levels

of prolactin in pre-pubertal females with daily injections of 0.50 ug estradiol benzoate.

In support of a stimulatory effect of estrogen on prolactin release, Ieiri et al. (1971) demonstrated that pituitary prolactin content and release is increased at proestrus and estrus in rats. Using male rats, Negro-Vilar et al. (1973) found fluctuations in secretion rates but reported lower values for prolactin concentrations similar to those reported by Ieiri et al. (1971). They found significant elevations of serum PRL during periods that coincided with rapid growth of the testes and accessory sex organs.

In comparing 21-month-old rats in constant estrus with 3-month-old cycling rats, the older females had approximately a two-fold greater anterior pituitary prolactin content by the modified pigeon crop assay of Reece and Turner (1937) by Clemens and Meites (1971). Simultaneously, high concentrations of follicle stimulating hormone (FSH) and low pituitary luteinizing hormone (LH) were also noted. As the rat matured, the synergistic and antagonistic effects of the gonadotropins and steroids in the maturation process may stimulate prolactin synthesis and release as the requirements for prolactin in sexual development increase. Whether this age related prolactin secretion was maintained in vitro was of interest in the study undertaken. The regulation of prolactin by gonadotropins and steroids was however not investigated.

III. Control of Prolactin Synthesis and Release

A. Hypothalamic Regulation: Inhibiting and Releasing Factors and the Biogenic Amines

The presence of a hypothalamic prolactin inhibiting factor (PIF) was reported by Meites et al. (1959), Pasteels et al. (1961), Talwalker et al. (1963). Others have suggested the existence of an avian hypothalamic prolactin releasing factor (PRF) (Kragt and Meites, 1965). In mammalian species, a PRF has been termed to explain initiation of lactation following injection of crude hypothalamic extracts in estrogen primed rats (Meites et al., 1960).

In vivo, catecholamines and biogenic amine precursors have been shown to inhibit rat prolactin release by increasing release of PIF or possibly by inhibiting TRH at the pituitary, thus acting as an antagonist to the TRH effect on the release of PRL (Chen and Meites, 1975). Kamberi et al. (1971) observed no direct inhibition by the catecholamines on release of prolactin with perfused hemipituitary glands. Biogenic amines placed in the ventricular nucleus were inhibitory and were dose and class dependent. Dopamine inhibited prolactin at 2.5 to 5 ug levels but epinephrine and norepinephrine at the same dose had no effect (Shaar and Clemens, 1974). Ojeda and McCann (1974) suggested a control mechanism that depends upon a balance in development of stimulating (estrogen sensitive) and inhibiting (dopaminergic) receptors for prolactin during sexual development.

Organ culture studies of anterior pituitary glands indicate that biogenic amine inhibition of prolactin release is dose dependent. With 80 to 640 ng per ml doses of dopamine, prolactin inhibition is significant, 200 to 1000 ng per ml doses of norepinephrine or epinephrine are required for similar inhibition (Koch et al., 1970). These findings report the need for pharmacological doses of catecholamines for inhibition of the release of prolactin from pituitary glands in vitro. Implants of acetylcholine into the third ventricle have also been shown to inhibit PRL release as has pilocarpine, a stimulant of the cholinergic receptor (Grandison et al., 1974).

B. Steroids

Increase in serum prolactin concentrations at puberty has been attributed to antecedent increases in estrogen at puberty (Minaguchi et al., 1968; Voogt, 1971). Prolactin secretion in vitro was increased by estrogen and progesterone but to a greater degree with estrogen. Estrogen and testosterone, but not 17 α-hydroxyprogesterone injections stimulated ^{3}H-leucine incorporation into prolactin in castrated and intact female rats, MacLeod et al. (1969).

Sar and Meites (1968) reported that progesterone increased pituitary prolactin content in vivo. In contrast Jones et al. (1965) and MacLeod et al. (1969), found no corresponding PRL increases with progesterone treatment.

These discrepancies however appear to be dose and system dependent (Blake et al., 1972; Kalra et al., 1973).

C. Stimulating and Inhibiting Compounds, Thyroid Hormones and Gonadotropins

Much of the work on stimulation and inhibition of prolactin secretion by other hormones has concerned the effects of thyroid hormones and TRF. Evidence for a two-to-five-fold increase in PRL secretion by pituitary cells treated with TRH in culture was noted by Tashjian et al. (1971). Triiodothyronine (T_3) inhibited the stimulatory effects of TRH on PRL secretion but did not increase the cell replication rate (Tsai and Samuels, 1974). Nicoll and Meites (1963) found that thyroid hormones directly increased prolactin release. In vivo, Rivier and Vale (1974) have reported increased PRL secretion with TRF and its analogs. Using a bovine pituitary cell culture, Machlin et al. (1974) noted a release of PRL, GH and TSH by TRH in a bovine pituitary cell incubation.

The precursor of serotonin, 5-hydroxytryptophan (5-HTP), stimulated release of PRL and TSH. Alone and in combination with the biogenic amine inhibitors, reserpine and α-methyl-metatyrosine (αMMT), serotonin had similar PRL and TSH effects. Simultaneous administration of dopamine and TRH to male rats elicited only TSH release without a PRL elevation (Chen and Meites, 1975).

Ergot drug derivatives have also been reported to suppress serum prolactin and block the stimulatory effects of estradiol benzoate (Lu et al., 1972; Brooks and Welsch, 1974). Welsch et al. (1971) linked the increased incidence of rat mammary tumors to elevated serum prolactin concentrations and increased estrogen serum concentrations. Reduction of mammary tumor incidence was possible with the inhibition of PRL release by 2-bromo-α-ergocryptine (Welsch and Gribler, 1973).

Prolactin implanted in the median eminence terminated rat pregnancies in early gestation (Clemens and Meites, 1968) and caused resumption of cycling in postpartum diestrous rats. Voogt et al. (1969) showed prolactin implants to stimulate FSH release. Increased FSH and LH concentrations were also noted when pseudopregnancy was terminated with prolactin implants (Voogt and Meites, 1971). In ovariectomized virgin rats, LH and FSH concentrations are increased while prolactin quantities were depressed. Perphenazine, a PRL releaser, with methallibure, a gonadotropin suppressor, also served to decrease PRL secretion (Ben-David et al., 1971). From this data, one might propose the existence of an antagonism between PRL and gonadotropin secretions that is related to the stage of sexual development of the rat.

D. Prolactin and Growth Hormone

The role of prolactin on its own regulation is unclear. In female rats, implants of 250 ug of PRL into

the median eminence significantly increased LH and FSH but failed to elevate serum PRL. No change in levels of PIF or PRF was noted (Voogt and Meites, 1971). MacLeod and Abad (1968) showed a direct inhibition of prolactin synthesis, as measured in the pituitaries of females with multiple pituitary tumor explants, by an *in vitro* assay of ^{3}H-leucine incorporation.

There appears to be no effect of GH on PRL secretion. Both hormones are produced by the acidophilic cells and are similar in chemical structure (li *et al.*, 1969). These common factors may explain the similarities in their functional responses. McGarry *et al.* (1968) have noted increased glucose utilization, greater nitrogen retention, increase in the calcium levels of the urine and stimulation of body growth by both GH and PRL.

It is evident with the effects of hormones and related compounds upon prolactin synthesis and release that a more defined and controllable system for the study of PRL would provide greater insight into PRL regulation. Pituitary cells *in vitro* would provide such a system for study of this mechanism on a cellular level, especially, in the areas of dose-dependent and direct effects of hormones on the pituitary.

IV. Correlation of Cell Number, Protein, and Deoxyribonucleic Acid

A standardized method of cell counting using a Neubauer hemocytometer and the vital stain crystal violet

has proven to be one of the most useful procedures. Absher (1974) has reported a 10% counting error with multiple samplings for counting and a standardized counting method. In evaluation of the dye exclusion technique for viability with trypan blue, Tennant (1964) found 85% of the counted cells were capable of replication under optimal conditions.

Lowry et al. (1951) determinations for protein have been used in conjunction with cell number to study cellular growth phases and hormone regulation by a specific pituitary cell population (Tashjian et al., 1968, 1970, 1971). A more sensitive and accurate assay for determining cell numbers with respect to hormone synthesis and release involves DNA analysis. If a random method of cell sampling is maintained, all phases of mitosis will be represented with equal probability.

The diphenylamine DNA assay of Burton (1959) has been further modified by Giles and Myers (1965) to increase sensitivity to a DNA concentration of 5 ug and convenience of assay. The methods for extractions and determinations of Schmidt and Thannhause (1945) and Schneiter (1945) are more time consuming.

In 1955, Leslie found the DNA concentrations in male rat liver to increase with age (0.59 pg per cell at 10 days to 1.14 pg per cell at day 182). Values were approximately 25% higher in females of corresponding ages. Hepatectomy initially decreased DNA content per cell but average values increased during regeneration supporting

hyperplasia. Hepatomas exhibited a DNA content per cell greater than control (1.0 pg per cell versus 0.913 pg per cell). Cunningham et al. (1950) reported DNA content for normal liver cells to be 6.1 pg per nucleus and 8 pg per nucleus in hepatomas. The studies of Thomson et al. (1953) indicated the DNA content of kidney, heart, bone, and spleen consistently within a range of 6.46 pg per nucleus to 6.90 pg per nucleus. Studies of Leavitt et al. (1973) with isolated pituitary cells from ovariectomized rats reported a higher DNA content per cell (10.82 pg) than the 6.6 pg per normal pituitary cell reported by Vendrely (1955).

V. Radioimmunoassay

An excellent review of the aspects of protein hormone receptor bindings for the radioimmunoassay (RIA) may be found in Principles of Competitive Binding Assays (1971) edited by Odell and Daughaday. The initial binding studies of labeled protein hormone were developed for insulin by Berson (1953). In 1959, the first studies of competitive binding inhibition of antibody and labeled insulin (antigen) competing with human plasma insulin were performed by Yalow and Berson. The binding or complexing of antigen to antibody assumes that labeled and unlabeled hormones are identical in their binding capabilities, and that antigen-antibody recognition is specific. The specific antigen-antibody recognition model of immunology has been applied to other protein including prolactin (antigen) binding to specific gamma globulins (antibodies).

Concentrations of serum rat prolactin have been assayed by the biological pigeon crop proliferation method (Lyons, 1937; Reece and Turner, 1937), the densitometric analysis (Nicoll et al., 1969) and the double antibody radioimmunoassay of Niswender et al. (1969). The latter method utilizes rabbit antisera for rat prolactin and a second ovine gamma globulin for precipitation of the antigen-antibody complex. Neill and Reichert (1971) have compared the values of the rat prolactin influence of the NIAMD Rat Prolactin Radioimmunoassay kit of Parlow with values obtained using pituitary extracted and purified rat prolactin. All measured levels of PRL were virtually identical except in serum from hypophysectomized rats. In 1972, Gala and Kuo compared serum prolactin levels measured by pigeon crop assay, electrophoretic microdensitometry (ED) and RIA in rat anterior pituitary organ culture medium. Measurement by bioassay yielded greater PRL values than those demonstrated by ED or RIA. Densitometer and RIA measurements corresponded closely in value.

The conversion of per cent labeled hormone binding to concentrations of PRL for unknowns is calculated by comparison to a standard curve of per cent specific PRL binding plotted against known concentrations of a reference prolactin. In application of the basic assumption, caution must be exercised with interpretation of the standard curve reliability, the sensitivity or range of hormone detection, the specificity of the antigen-antibody

reaction, the precision of standard curve values and the accuracy of the calculated mean values (Midgley et al., 1969).

Feldman and Rodbard (1971) have developed a mathematical model on the basic RIA assumptions: 1) antigen and antibody are separate homogeneous chemical forms; 2) a one to one complex of antigen and antibody without cooperativity or allosteric factors exists; 3) labeled and unlabeled hormone react identically; 4) antigen-antibody reaction will continue until a steady state or equilibrium is attained; 5) bound (B) and free (F) antigen may be separated without equilibrium disruption and the B/F ratio may be measured. With the curves generated by the ratio of B/F plotted against B, Feldman and Rodbard have been able to detect nonequilibrium and cross reactivity in RIA systems. With analysis, radioimmunoassay provides a specific rapid means for determination of PRL levels in serum and culture medium.

VI. Radioreceptor Assay

The basic assumption which apply to the radioimmunoassay also apply to the radioreceptor assay (RRA) for membrane binding sites. The major consideration in the binding of a hormone to its binding site on a membrane is the biological activity or binding ability of the hormone. Immunoreactivity cannot always be equated with biological activity. The reverse is also true. To be considered a true "receptor" the binding site-hormone complex must initiate or elicit a specific function or reaction (Cuatrecasas, 1974).

In the development of the binding assay of ovine prolactin, a lactoperoxidase iodination produced a biologically active hormone (Frantz and Turkington, 1972; Posner, 1974). Ovine prolactin was found to bind specifically to mouse mammary where its function in lobular-alveolar development and lactation were documented (Frantz and Turkington, 1972). Specific binding has also been demonstrated in mouse liver, kidney, midbrain, and primary sex organs and characterized for time and temperature dependent equilibrium conditions and competitive hormone binding (Frantz et al., 1974). From data obtained in prolactin binding to mammary carcinoma cells, Turkington (1974) has proposed that the number of receptors is directly related to the prolactin dependency of the carcinoma. Shiu and Friesen (1974a) have reported specific prolactin binding in rabbit mammary gland, liver, ovary, and kidney.

Kelly et al. (1974) have demonstrated an ontogenic, sex and physiological state interaction of prolactin binding in the livers of rats, rabbits, and guinea pigs. Binding increased as the age of the animal and its requirement and levels of PRL increased. This correlation may suggest that prolactin induced its own receptors.

Recently Frantz et al. (1975) have shown a high degree of specific binding of prolactin to the cells of cultured tumor and normal rat pituitary glands. Shiu and Friesen (1974b) have also been able to solubilize and purify prolactin receptors from rabbit mammary epithelial

cells. Development of this method of solubilization of receptors will enable further study on the mechanisms of the receptor for prolactin. Study of the specific binding values of prolactin to levels of prolactin secretion may also aid in knowledge of the prolactin receptor and a possible autoregulation for prolactin.

Materials and Methods

I. Animals

Female Sprague-Dawley rats of the desired age groups: 13 day old or immature, 45 day old or mature, and 180 day old or retired breeders were obtained from Spartan Research Animals, Inc. (Haslett, MI). To decrease effects of transportation stress, all rats were caged and maintained for at least 24 hr after arrival under $25^{o} \pm 1^{o}$ and 15 hr artificial illumination on a standard Purina Rat Chow diet (Ralston Purina Co., St. Louis, MO) and tap water ad libitum.

II. Method of Pituitary Gland Cell Culture

A. Growth Medium Preparation

Pituicyte Dulbecco's Modified Eagle Medium (PDME) for cell culture maintenance was prepared using a modification of the medium of Sonnenschein (1974). The constituents of PDME are: 13.47 g per L Dulbecco's Modified Eagle Medium Powder (Appendix I) (GIBOC, Grand Island, NY), 15% Horse Serum (Difco, Detroit, MI), 2.5% Fetal Calf Serum (Difco), 812 ml triple distilled water, 5 mM N'-2-Hydroxyethyl-piperazine-N'-Ethanesulfonic Acid (HEPES) buffer (GIBCO). Antibiotic-Antimycotic mixture (GIBCO) is equivalent to 100 units per ml pencillin-G, 100 ug per ml streptomycin and 0.25 ug per ml fungizone^{R}, a mycostatin, and 72 ug Anti-PPLO-agent (GIBCO).

^{R} Fungizone, E. R. Squibb & Sons

The medium is adjusted to pH 7.2 with 7.5% sodium bicarbonate (GIBCO). Vacuum filtration through a 47mm, 0.45 μ pore filter (Gelman Instrument Co., Ann Arbor, MI) and a sterile Pyrex Millipore-filtering apparatus (Millipore Corp., Bedford, MA) produced a medium suitable for cell culture. The medium is stored at 4^{o} until used.

B. Method of Cell Dispersion and Culture

Groups of 10-12 rats of a specified age class were guillotined within 20 sec after removal from their cages. Heads were washed with 95% ethanol under a laminar flow hood (Type WS Series 300, Westinghouse, Grand Rapids, MI) before the anterior pituitary glands were removed aseptically.

A medial cephalic incision was made through the skin from the region of the foramen magnum ventrally to the orbitals with small scissors and the skin retracted. With the aid of bone cutters or scissors, any remaining spinal cord was removed. Lateral incisions through the skull along parietal temporal sutures were made and the bone flap was reflected anteriorly. The cerebral hemispheres were reflected dorsally and the optic chiasma severed. In 45 day old or mature females and retired breeder females, anterior pituitaries were teased from the neurohypophysis and the surrounding connective tissue in situ with small surgical forceps. In the case of immature females, the entire pituitary gland was removed.

In all cases, pituitary fragments were placed in a sterile 100 X 15 mm Falcon petri dish (Bioquest, Cockeysville, MD) containing 10 ml sterile Puck's Saline A solution and isolation of the adenohypophysis completed. The pituitary tissue was minced (1 mm^3 blocks), and washed twice with 10 ml sterile Puck's Saline A, then a final saline solution containing 0.25% trypsin (Difco Laboratories, Detroit, MI) was added. This tissue suspension was transferred via a 10 ml sterile siliconized pipette to a 125 ml screw capped Pyrex Erlenmeyer flask containing a 5 cm Teflon stirring bar. Ten ml 1:5000 Ethylenediaminetetraacetic acid or versene (GIBCO, Grand Island, NY) were added to the flask.

Two 20 min incubations were performed at 37^{o} in a Model 805 incubator (Precision Scientific Co., Chicago, IL). The tissue fragments were dispersed during incubation by the agitation provided by a magnetic stirrer (Fischer Scientific, USA) set below the foaming point of the dispersing mixture. After 20 min of incubation, 10.0 ml of the trypsinized cell suspension were removed and 5 ml placed in each of two 13 X 100 mm screw capped Pyrex test tubes. The suspensions were diluted 1:1 (v/v) with Pituicyte Dulbecco's Modified Eagle Medium (PDME) and centrifuged at 500 X g for 10 min at room temperature ($25 \pm 1^{o}C$) in a clinical chemical centrifuge (International Equipment Co., Needham Heights, MA). The enzyme-medium supernatant was decanted, and the cell pellet was

resuspended in PDME. Viable cell number was determined by staining with 0.1% trypan blue or 0.02% crystal violet and counting by the method of Absher (1974) (see Materials and Methods, Section III below). The final suspension of cells was diluted to a concentration of 2.0 X 10^5 cells per ml. One ml fractions were transferred to 30 ml Falcon tissue culture flasks (Bioquest) and an additional 2 ml of PDME was added. Eight to ten flasks were prepared from a single dispersion of pituitary glands.

Flasks were incubated at 37° in sealed plexiglass chambers under constant aeration in a 95% O_2:5% CO_2 humidified atmosphere for maintenance of a pH of 7.4. Cells were observed on day of culture for morphology and density and each subsequent day. Medium was replaced every 48 hr with fresh PDME medium at 37°.

III. Methods of Cell Number, Protein and Deoxyribonucleic Acid: Assay and Correlation

A. Measurement of Cell Number and Viability

A fraction of the cell suspension obtained from cells of a specific culture and flask was assayed for cellular population. Cells contained in the medium and adhering to the flask were counted. A 0.01% trypan blue solution dissolved in 0.02 M citric acid is excluded by viable cells. This technique of dye-exclusion required incubation for 20-30 min at 37°. A 0.02% crystal violet or vital dye solution dissolved in 0.02 M citric acid stains only

the viable cells. The latter stain permitted rapid counting without incubation. Cell number was determined by the method of Absher (1974) with a Neubauer hemocytometer. The number of observed viable cells was corrected for factors of dilution and volume to obtain the total number of cells in suspension.

B. Measurement of Protein and Deoxyribonucleic Acid (DNA)

Protein concentrations were measured by light absorption by the standard method of Lowry et al. (1951) and quantitated using a standard bovine serum albumin curve with a concentration range of 5-500 ug. These assays were performed in duplicate with samples of each cell culture (varying cell numbers).

The concentrations of DNA were measured by the diphenylamine modification of Giles and Myers (1965). Cells of different populations and cultures were dissolved in hot 10% perchloric acid prior to assay. Absorbance readings at 595 mμ were compared with a calf thymus DNA standard curve of 1-50 ug. Values for DNA, protein, and cell number were also applied to prolactin ^{125}I binding values, expressing final counts in dpm per ug protein or DNA and dpm per cell number.

IV. Method of Radioimmunoassay

A. Sample Collection

At each 48 hour medium change, two medium samples, each from the decanted pool of medium of three randomly chosen 30 ml flasks of a specified culture, were collected. The pooled medium samples were centrifuged at 500 X g for 20 min at 4^{o} in a Sorvall RC2-B (Sorvall, Newtown, CT) to remove cells and debris. The medium was decanted and stored at -20^{o} until prolactin concentration was determined by the double antibody radioimmunoassay (Niswender et al., 1969).

B. Prolactin Radioimmunoassay

Prolactin concentrations in the culture medium were determined by a modification of the technique of Niswender et al. (1969). This double antibody radioimmunoassay was established by Charles L. Brooks (Department of Anatomy, Michigan State University, East Lansing, MI 48824) from a standard Radioimmunoassay Rat Prolactin Kit distributed by the National Institute for Arthritis and Metabolic Diseases (NIAMD).

All samples were assayed at three different concentrations in a final volume of 800 ul. For each sample 480, 460, and 420 ul of phosphate buffer saline-1% bovine serum albumen (PBS-BSA) were pipetted into three 12 X 75 mm glass tubes followed by the addition of 20, 40, and 80 ul of the sample to each tube respectively. Two hundred ul

of rabbit anti-rat prolactin (AB) diluted 1:25,000 were added per tube and the mixture incubated at 4^{o} for 24 hr. One hundred ul of the I^{125} chloramine-T labeled NIH R-P-1 rat prolactin I^{125} (*H), approximating 30,000 cpm, were added to each tube and the samples were agitated and stored at 4^{o} for 24 hr. Thereafter, 200 ul of a second antibody ovine anti-rabbit gamma globulin (1:100), were added and incubation at 4^{o} continued for 72 hr. The double antibody prolactin complex was precipitated on day five by combining 3.0 ml of phosphate buffered saline (PBS) per culture tube and was centrifuged at 1000 X g for 30 min at room temperature. The tubes were decanted of supernatant, inverted for 30 min, and permitted to air dry prior to gamma counting on a Nuclear Chicago Autogamma Counter (Model No. 1185, Nuclear Chicago/Searle, Chicago, IL) with a 50% counting efficiency.

In addition to triplicate samples of medium containing unknown amounts of prolactin, five tubes containing only 100 ul of labeled hormone were prepared. Five samples with normal rabbit serum (NRS) were made with 500 ul PBS-BSA and 200 ul 0.3% normal rabbit serum (NIAMD Anti-Rat Prolactin Serum-2) to account for the antiserum binding. The determinations of unknown prolactin concentrations were calculated by comparing triplicate samples of medium to a standard curve of per cent specific binding of prolactin plotted as a function of rat prolactin reference concentrations (NIAMD-RP-1) ranging from 0.2-20

ng which were assayed for *H binding. The degree of total antibody binding is also calculated by excluding prolactin or unknowns from a series of nine assay tubes.

The sigmoid curve generated from the plot of per cent prolactin binding against prolactin concentration (ng) was used for determining assay sensitivity and the prolactin content of the unknowns. All sample PRL concentrations were expressed as ng PRL per ml medium.

V. Prolactin Radioreceptor Assay

A. Ovine Prolactin Iodination

In a 2.0 ml polyethylene serum vial, 5 ug of ovine prolactin (NIH-S-11; 25.6 IU/mg) were iodinated using the modified lactoperoxidase method of Frantz et al. (1974). To 100 ul of 40 mM diethylbarbituric acid buffer (Mallinckrodt Chemical Works, St. Louis, MO) pH 7.0, was added 0.5 mCi of carrier free Na ^{125}I (New England Nuclear, Boston, MA or Amersham/Searle, Chicago, IL). In immediate succession were added the PRL (5 ug in 25 ul of HOH) 10 ug lactoperoxidase (Calbiochem, La Jolla, CA) in 25 ul of 40 mM diethylbarbituric acid and 250 ug of hydrogen peroxide (Mallinckrodt Chemical Works) diluted 1:30,000 with distilled water. Reaction time was 5 min at 25^{o} followed by the addition of 0.5 ml of chilled 16% sucrose solution. The mixture was immediately transferred to a 0.9 X 14 cm Sephadex G-75 column (Pharmacia Fine Chemicals, Piscataway, NJ) in a 4^{o} cold room. The column had been

previously washed with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and equilibrated with 20 ml 40 mM diethylbarbituric acid solution (barbital buffer). Elution of the prolactin-125 iodine was performed at 4° with mM barbital buffer and one ml fractions were collected. Radioactivity of the fraction was determined by counting 1 ul volumes spotted on filter paper. The first peak of radioactivity was transferred to a 1.4 X 15 cm DEAE-cellulose (Diethylaminoethyl cellulose, Sigma Chemical Co.) column equilibrated with 1% bovine serum albumin and 5 mM potassium phosphate buffer (Mallinckrodt Chemical Works), 5-500 mM gradient of potassium phosphate buffer pH 7.0. Fractions of one ml were collected and the first major peak of radioactivity was tested for biological activity with mouse mammary homogenates (Frantz et al., 1974) or cultured tumor pituitary cells (Frantz et al., 1975).

In later prolactin iodinations used for prolactin binding studies, the following procedural changes were made. Prolactin was dissolved in 0.1M ammonium bicarbonate, pH 8.1, and diluted with 4 ml glass distilled water to give a final 0.01M ammonium bicarbonate concentration (Vanderlaan, personal communication). Lactoperoxidase was dissolved with 0.4M sodium acetate (J. Baker Chemical Co., Phillipsburg, NJ) at a pH of 5.6. The Sephadex G-75 column was washed and the iodinated

preparation was eluted with 0.1M sodium acetate buffer. All other buffers, concentrations, volumes and procedures were the same.

B. Radioreceptor Cell Preparation

When a specific cell culture had reached approximately 2.0 X 10^6 cells per 30 ml flask, cultures were terminated and the cells collected for prolactin radioreceptor assay (PRRA). The medium was stored for RIA as previously described (see Materials and Methods; Section IV, Method of Radioimmunoassay). The cell monolayer was washed three times with Puck's Saline A solution, pH 7.4 and then scraped into 3.0 ml of Puck's Saline A solution with the aid of a rubber policeman. A 0.1% trypan blue vital dye exclusion cell count was performed on the dispersed cells. The dispersed cells and medium were then centrifuged separately at 480 X g for 20 min at 4^o in a Sorvall "Superspeed" (Sorvall Co., Newtown, CT). The supernatant was decanted and the cells resuspended and pooled in Puck's Saline A solution to the desired cell concentration for individual binding experiments. If not used immediately, cells were stored at -20^o (until used for binding).

C. Method of Prolactin Receptor Assay

In the radioreceptor assay of Frantz et al. (1974) total binding (TB) and non-specific binding (NSB) tubes were determined by the direct competition of ^{125}I PRL with

o-LH and o-PRL respectively. Each sample to test for non-specific binding sample [in a 500 ul polyethylene micro-tube (Beckman, USA)] consisted of: (1) 200 ul of incubation medium (1% bovine serum albumin, 0.4 mM NaCl and 2 mM tris (hydroxymethyl) aminoethane pH 7.2); (2) 2 ug cold prolactin (PRL); (3) radiolabeled prolactin-^{125}I in the range of 10,000 1000,000 cpm; and (4) 100 ul of cell suspension (approximately 1 X 10^6 cells).

The total binding samples contained the above quantities of incubation medium and prolactin-^{125}I, but had 2 ug of ovine luteinizing hormone (o-LH) instead of o-PRL. Cell samples were counted for two min prior to binding in a gamma counter to determine total available, iodinated prolactin as disintegrations per min (dpm). After this precount, the desired number of cells (1.0 X 10^4 - 1.0 X 10^6 cells) was added in 100 ul volumes to each tube. After agitating samples by vortex and incubation at 4^o for 30 min, the incubation was terminated by centrifugation at 10,000 X g in a Beckman Model 152 Microfuge (Beckman Corp.) for 5 min also at 4^o. The supernatant was aspirated and the cell pellet was washed with 100 ul of cold glass distilled water which was quickly aspirated. The incubation tubes were cut at the level of the cell pellet and resuspended in Lowry C. After the final gamma count for 20 min the protein content of the reacted cells was assayed in a standard protein determination (Lowry et al., 1951).

VI. Prolactin Binding Analysis

A. Calculation of Per Cent Total, Non-Specific and Specific Binding

From precount, final count and ug protein per sample, it is possible to calculate per cent total, non-specific, and specific binding of ^{125}I PRL for anterior pituitary cells. Per cent total binding (TB) per sample is defined as the final count of the total binding LH tubes in dpm per 100 ug protein divided by the mean of the TB precount of the sample series X 100. Non-specific percentages are calculated as the quotient of final dpm of PRL binding (NSB) tubes per 100 ug protein divided by the average NSB precount sample series X 100. The per cent of specific binding (SB) is calculated as the difference between the averages of TB final counts (dpm per 100 ug protein) and NSB final counts (dpm per 100 ug protein) divided by the average TB final count (dpm per 100 ug protein) multiplied by 100.

B. Scatchard Analysis

The binding of a protein hormone to a binding site assumes many features of the antigen-antibody binding complex theory: (1) homogeneous form of the hormone; (2) achievement of chemical equilibrium without disruption after reaction; (3) labeled and unlabeled hormone react with the same affinity; (4) little or no binding site cooperativity; and (5) a one to one correlation of hormone

molecule and binding site (Kahn et al,, 1974).

The latter three assumptions apply to the first law of mass action. In the steady state of the hormone (H)-receptor (R) binding complex (HR) the equation:

$$H + R \underset{K_2}{\overset{K_1}{\rightleftharpoons}} HR$$

describes the equilibrium and the K values are the reaction rate constants. The affinity or equilibrium constant for dissociation (Kd) may be defined in terms of the second order chemical kinetic reaction:

$$\frac{K_2}{K_1} = \frac{H\ (R\text{-}HR)}{HR} = K_d$$

Expanding and isolating the hormone-receptor term the concentration of HR equals:

$$\frac{HR - H\ (HR)}{K_d} \quad \text{or} \quad \frac{H\text{-}R}{H} = \frac{1}{K_d}\ (R\text{-}HR)$$

K_d may also be defined as the inverse the association constant, K_a

$$K_d = \frac{1}{K_a} = \frac{K_2}{K_1}$$

from which the Scatchard (1949) expression of bound hormone (B) to free hormone (F) is derived:

$$\frac{HR}{R} = K_a \ (R\text{-}HR)$$

assuming one free binding site per one free binding site molecule. According to the terminology of Scatchard, this expression extends to number of binding sites (q) and bound hormone (B) and

$$\frac{B}{F} = K_a \ (q\text{-}B)$$

By plotting the concentration of bound, labeled hormone against free labeled hormone a steady state reaction curve is generated. By standard Scatchard notation, the plot of the concentration ratio of bound labeled hormone to the free labeled hormone (B/F) in cpm against bound hormone (B) in cpm produces a straight line for the idealized system with an abscissa intercept equivalent to the number of binding sites (q) and the slope interpreted as the negative K_a ($-K_a$), equivalent to the K_d.

Experimental

I. Correlation of Deoxyribonucleic Acid and Protein Content to Cell Number

A. Objective

In order to better quantify per cent specific binding and cell number, an assay more sensitive to cell number and indicative of a random cell population was chosen. A study of the correlation of cell number with the concentration of deoxyribonucleic acid (DNA) and protein was performed on two rat pituitary cell cultures.

B. Procedure

Triplicate samples of the different cell concentrations utilized for prolactin binding studies were performed on two readily available rat pituitary cell cultures: $C_8$11RAP (Sonnenschein, 1974) and a normal retired breeder rat pituitary cell culture. Both cell types were collected several weeks prior to use and were stored at -20° in 10% glycerol-PDME, a cryoprotective agent. As previously described (see Materials and Methods, Section III), cell number, protein and DNA were determined.

DNA concentrations of unknowns were compared with a standard curve of triplicate samples of calf thymus DNA (Sigma, St. Louis, MO) at the concentrations: 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 ug per sample. Protein concentrations were determined by the method of Lowry *et al.* (1951) on aliquots of carefully counted prolactin binding

cells from $C_8$11RAP and retired breeder cultures. A standard protein curve was constructed for absorbance plotted against 10, 40, 50, 80, 100, 120, 150, 180, 200 ug per sample of bovine serum albumin (Sigma Chemical Co.).

C. Results

The relationships of DNA and protein concentrations to cell number for $C_8$11RAP and retired breeder cells are illustrated in Figure 1 with the ratios of DNA (pg) per cell: protein (ng) per cell. Protein and DNA content increased with increasing cell number as expected. The mean values and the standard error of the mean (SEM) as illustrated in Figure 1 are given in Tables I and II.

D. Discussion

With both cell cultures, protein and DNA concentrations increased with increasing cell number. The $C_8$11RAP tumor cells had significantly greater DNA concentrations as indicated by the Student's t test at the 0.95 confidence interval in comparison to the values of DNA for the cells of immature and retired breeder rats (Table I). Protein contents, however, were not significant by the Student's t test at the 0.95 confidence interval greater for the normal retired breeder cultures vs $C_8$11RAP cells (Table II). These relationships were also illustrated in the ratios of DNA: protein for both cell types (Figure 1). The tumor derived cell had a ratio of 0.2643 compared

Table I.

DNA Content of Tumor Rat Pituitary Cells ($C_8$11RAP) and Normal Rat Retired Breeder Pituitary Cells

Type	Cell Number (per ml)	Average DNA ug	±SEM	Sample Size (n)
$C_8$11RAP	1.0×10^5	1.33	0.17	6
	2.0×10^5	6.13	0.07	6
	5.0×10^5	10.23	0.63	6
	1.0×10^6	13.0	1.34	6
** Average DNA content per cell ± SEM = 19.27 pg ± 4.1674				
Retired Breeder	3.4×10^4	1.17	0.19	6
	1.75×10^5	4.87	0.13	6
	3.4×10^5	5.16	0.12	5
	5.0×10^5	8.17	1.74	6
** Average DNA content per cell ± SEM = 9.79 pg ± 1.54				

* Immature

Average DNA content per cell ± SEM = 14.97 pg ± 4.418

* Insufficient number of cells to assay a range of cell concentrations. Above value obtained for a concentration of 1.0×10^5 cells.

** Significant difference in DNA per cell between $C_8$11RAP and Retired Breeder cells at 0.05 level.

Table II.

Protein Content of Tumor Rat Pituitary Cells ($C_8$11RAP) and Normal Rat Pituitary Retired Breeder Cells

Cell Type	Cell Number (per ml)	Mean ± (mg)	SEM	Sample Size (n)
$C_8$11RAP	2.2×10^5	29.50	5.06	6
	3.6×10^5	35.60	3.08	11
	1.0×10^6	49.0	2.91	10
	1.5×10^6	141.0	4.86	7
** Average protein per cell = 72.90 ng ± 18.79				
Retired Breeder	1.0×10^5	24.67	1.52	6
	1.6×10^5	28.71	2.23	5
	2.2×10^5	36.38	3.02	8
	1.6×10^6	58.60	7.58	5
** Average protein per cell = 122.83 ng ± 35.77				

** Non-significant difference in protein per cell between Retired Breeder and $C_8$11RAP cells at 0.05 level.

Figure 1.

Mean concentrations + standard errors of the mean for DNA (ug) and log protein mean concentrations (mg) of $C_8$11RAP and Retired Breeder cells.

Ratio DNA per cell: Protein per cell:

$C_8$11RAP = 0.2643

Retired Breeder = 0.0797

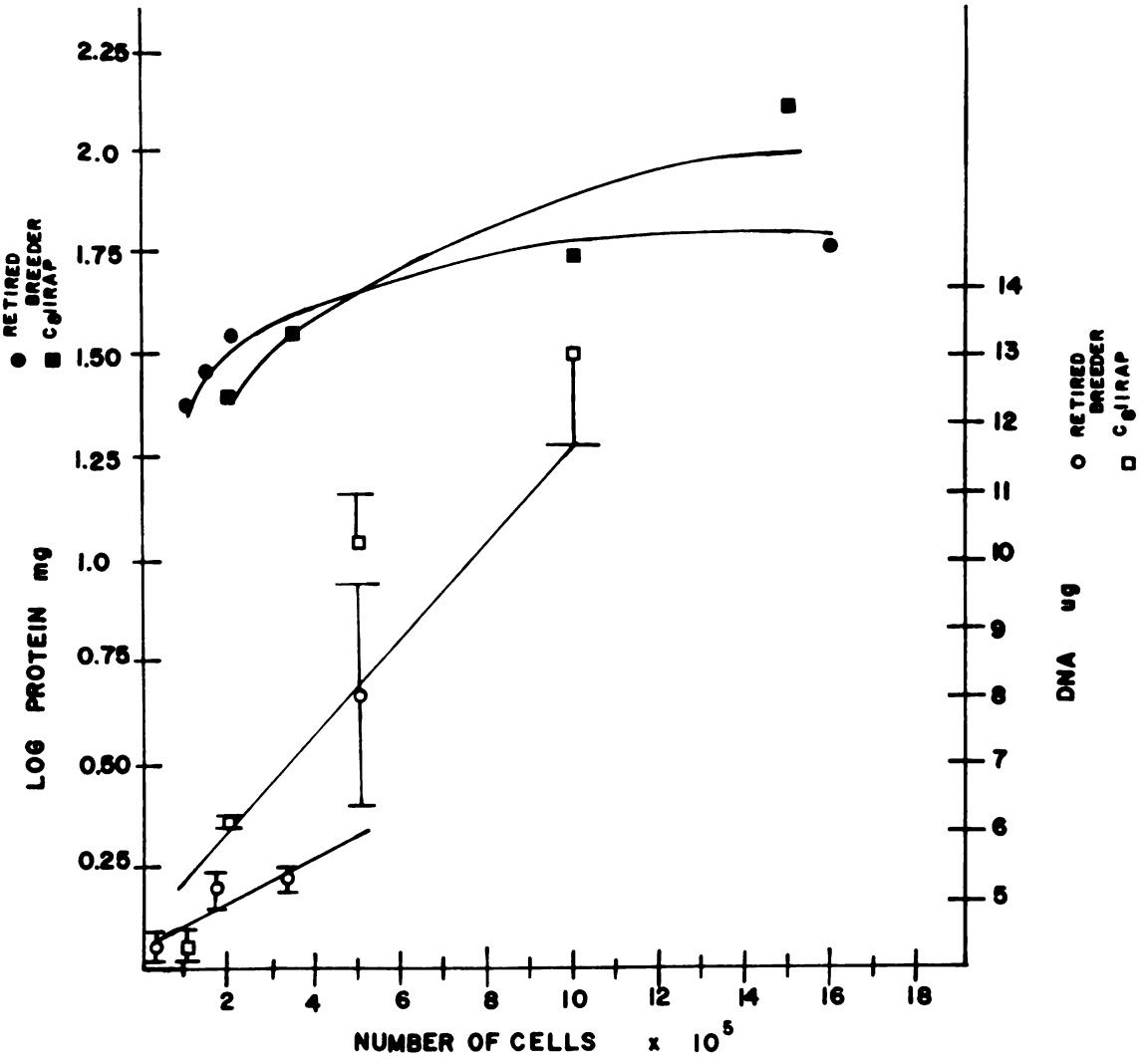
RETIRED BREEDER
C3H/IRAP
LOG PROTEIN mg
2.25
2.0
1.75
1.50
1.25
1.0
0.75
0.50
0.25
DNA ug
14
13
12
11
10
9
8
7
6
5
2
4
6
8
10
12
14
16
18
NUMBER OF CELLS x 10^5

FIGURE 1

with a ratio of 0.0797 for the normal cell. These values were indicative of the activity of the individual cell types.

The neoplastic $C_8$11RAP cell replicated at a faster rate than the normal pituitary culture. The higher DNA values for $C_8$11RAP were indicative of this rate. A heteroploid or aneuploid state existing in the tumor cell was possible. In 1970, Sonnenschein et al. demonstrated karotype differences in a rat pituitary tumor derived cell culture. The average protein concentration of normal pituitary cell was greater than that of tumor cells. These results may be correlated to the high prolactin levels released into the medium by normal cells. With a greater prolactin synthesis and release than $C_8$11RAP (Payne, unpublished data), retired breeder cells might be expected to have a higher protein content (see Experimental, Section II). The high standard errors of the mean for protein may partially be attributed to the different days of cell harvest or collection and the stage of prolactin synthesis and release. As previously shown, prolactin concentrations of the medium varied during the period of collection.

The DNA concentrations per cell (Table I) do not correspond to the normal diploid chromosome content of 6.6 pg per cell as determined by Vendrely et al. (1955). These values more closely correspond to the 10.82 pg per cell levels obtained by Leavitt et al. (1973) for isolated

rat anterior pituitary cells. This higher value was attributed to cellular aneuploidy or polyploidy. The greater DNA content of the immature rat (14.97 pg ± 4.418) in comparison with the retired breeder cell (9.79 pg ± 1.54) followed the trend observed in other ontogenic studies (Leslie, 1955) and is indicative of the faster replication and developmental rates of the younger animal. The average DNA contents per cell for normal and neoplastic pituitary cells were also greater than the levels obtained for other tissues of the normal rat. Thomson et al. (1953) indicated a DNA range of 6.46 pg per nucleus to 6.90 pg per nucleus.

The greater DNA content for normal rat pituitary cells may be the result of a greater susceptibility of chromosomes to damage during trypsinization or culture i.e. translocation of chromatin during mitosis producing aneuploidy and/or polyploidy. It would be of interest to extend this study to all age groups at various days of culture and to compare protein, DNA, and cell number to prolactin secretion in vitro.

II. Concentrations of Prolactin of the Culture Medium as a Function of Age of Animal and Days in Culture

A. Objectives

In female rat serum and pituitary glands, concentrations of prolactin remain low until day 37, when a sharp increase in pituitary content is noted with a doubling in rate on approximately day 45 of vaginal opening and again between 46-78 days with a sustained rate

until day 400 (Yamamoto et al., 1970; Voogt et al., 1970). Since concentrations of prolactin in both the serum and the anterior pituitaries differ according to the stage of sexual development, the study to measure the release of prolactin by cells cultured from anterior pituitary glands of sexually immature, mature and old (retired breeder) female rats was undertaken. Whether the in vitro situation increased, maintained or decreased the amount of prolactin release without chronic hypothalamic inhibition or stimulation was also studied. The concentrations of prolactin of specific cultures were then correlated with the degree of specific binding of these cells.

B. Procedures

1. Culture

Cell cultures of pituitary glands for each age were maintained in a humidified 95% O_2:5% CO_2 atmosphere at 37°. The incubation was changed at 48 hr intervals (see Materials and Methods, Section II).

2. Assay Procedure

For each culture derived from a specific age group, samples of medium from two pools of medium of four flasks per pool for each 48 hr period were collected and frozen at -20° until radioimmunoassays were performed within one month of collection. Culture medium (PDME) samples not used in culture were also assayed. The period of cultivation

for cells for a specific age of animal was dependent upon the individual culture dispersion or pituitary gland cell separation. Cultivation ended with cell death or termination of the culture for prolactin binding studies. The average length of the incubation period was ten days with two day intervals of medium collection.

C. Results

The concentrations of prolactin found in the culture medium of each age for 10-14 day periods of culture are shown in Table III. Mean concentrations of prolactin $\pm$ standard error of the mean (SEM) for individual cultures are also listed.

A three-factor nested analysis of variance for unequal sample sizes for the factors contributing to prolactin secretion concentrations. Each of the following factors were accounted for in the analysis: (1) the fixed effect of age for individual cultures; (2) the random effect of culture units or flasks as samples within a culture; (3) the fixed effect of day in culture; and (4) the interaction of culture units and day in culture which contribute to the differences in levels of prolactin and inseparable from experimental error (Appendix II). The significance of the contribution of each factor was tested by the F-distribution. If calculated F values proved significant (Table II) and the tested hypothesis of "no factor effect" (H:E=0) was rejected, further

analysis was performed on the interaction of age and day in culture. Individual levels of prolactin for specific days in culture were compared for single cultures by Scheffé's analysis of contrasts (Appendix V).

D. Discussion

Analysis of variance indicated a significant contribution to prolactin concentrations of the medium by the different ages of animals used for culturing, the day in culture, and the interaction of the age of the animal and the day in culture at the 0.90 confidence interval (Table IV). With respect to the age-dependence of cultures, variations in concentrations of prolactin were greatest for cells derived from female retired breeder rats when compared with immature females, followed by mature vs immature, and the least age-dependent difference with retired breeder vs mature females (Appendix IV and Table IV). Significant differences in the variances from the means for prolactin concentrations for specific days in culture were found for Day 4 vs the first day and all subsequent days in culture and the first two collections (Days 2 and 4) and the latter periods of Days 8 and 10 (Appendix VI).

Scheffé's analysis of contrasts (Appendix VI) indicated Day 10 as significantly different when compared with the prolactin values of other days in culture. Day 4, however, exhibited the most significant difference in prolactin concentration from the mean during the ten day period of culture.

Table III. Medium Prolactin Concentrations of Immature, Mature, and Retired Breeder Female Rat Pituitary Gland Cell Cultures

Age of Rat Used for Cell Dispersion (Three Dispersions Per Age)	Day in Culture 2	4	6	8	10	12	14	Mean (N) ± SEM PRL Concentration of the Designated Culture Dispersion	
	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	
Immature	101.30	35.83	42.66	18.88	6.25	22.83	27.92	36.52 (7)	11.67
14 Days	10.63	7.42	4.73	12.25	14.92	15.75		10.95 (6)	1.75
	68.83	67.42	32.92	54.50	5.06			45.75 (5)	12.04
Mature	485.0	305.0	443.30	370.0	383.30	465.0	211.66	380.47 (7)	36.62
45 Days	161.66	178.50	52.08	38.99	19.87	25.0		71.37 (6)	29.14
	200.0	50.50	21.50	375.0	96.0			148.77 (5)	64.17
Retired	215.16	319.60	192.50	183.30	253.30	82.93	319.16	223.71 (7)	31.50
Breeder	150.0	600.0	184.37	223.83	297.50	13.42		244.85 (6)	80.74
6 Months	392.50	890.0	577.50	253.30	123.0			447.26 (5)	133.93
Mean (N) ± SEM for individual Days in Culture	297.50	609.04	256.92	255.0	200.0	591.30	186.24		

Table IV.

Analysis of Factor Significance: Age, Day in Culture, and the Interaction of Age and Day in Culture

Comparison	Calculated F-Value	*Critical F-Value (v_1,v_2)	Level of Significance
Age	3.66	3.46 (2,6)	0.10
Day in Culture	2.76	2.41 (5,54)	0.001
Interaction Age and Day in Culture	16.17	3.70 (10,54)	0.001

* Rohl and Sokal (1969). Table S. Critical values of the F-distribution pp.168-197.

The variations in levels of prolactin released into the medium for the different classes of female rats in sexual development qualitatively corresponded to the trend of those for the *in vivo* female rat sexual development. Quantitatively, *in vitro* concentrations of PRL exceeded *in vivo* serum quantities of the same age. Concentrations of prolactin were impossible to compare on a single pituitary gland or weight of pituitary gland basis. The sensitivity of the primary cell culture, its metabolic rate, and the heterogeneous cell population of epithelial cells and fibroblasts must be considered in the evaluation of prolactin in the medium. The time required for recovery after trypsinization and establishment of cells in culture were additional factors contributing to the variations in prolactin concentrations.

III. The Binding of Prolactin-^{125} Iodine to Normal Rat Pituicytes and Its Correlation to Secretion of Prolactin *In Vitro*

A Objective

The ability of prolactin to inhibit its own synthesis and release has been suggested by MacLeod *et al*. (1970) from prolactin secretion studies both *in vivo* and *in vitro*. Posner *et al*. (1974) has suggested prolactin may regulate its own receptors either by inducing or activating them. The degree of specific binding of prolactin to pituicytes correlated with levels of prolactin in the culture medium may

support this hypothesis. This system may provide a model for the study of the direct action or feedback of prolactin on the pituitary cells which synthesize and release it and also for its effect on the regulation of other hormones of the pituitary.

B. Procedures

1. Culture

Cells of pituitary glands were cultured according to the ages of female rat: immature, mature, and retired breeder. At a period of sufficient growth (10 to 14 days after initial dispersion of the pituitary glands), cells were collected as previously described. The media of each group of cultured cells after collection were stored at -20^{o} until assayed for its concentrations of prolactin (see Materials and Methods, Section II, IV, and V).

2. Binding Assay

The methods of preparation and binding of iodinated prolactin to those cells is cited in the Materials and Methods (Section V). To all samples the previously described binding components were added. Assays varied in the addition of: (1) the type of cells: immature, mature, or retired breeder normal rat pituitary cells; (2) the cell number: $1x10^{4}$ to $1x10^{6}$ cells per sample; and (3) initial counts of prolactin-^{125}I per sample: 10,000 dpm

to 100,000 dpm. In each assay, separate cell cultures of each age group and different preparations of iodinated prolactin were used. All binding assays were performed at 4^{o} for 30 min.

C. Results

The percentages of total, non-specific and specific binding were calculated for each binding assay of prolactin-^{125}I with pituicytes of immature, mature and retired breeder rats (see Materials and Methods, Section VI). The significance of the difference of means of the final counts for total versus non-specific bindings was analyzed by the one-tailed Student's t test to ascertain if the mean of total binding counts was significantly less than the mean of non-specific binding counts (Table V).

The relationship of the per cent of specific binding and the concentrations of released prolactin (ng/ml) on the day of collection of cells (Table VI) was also analyzed by the method of correlation (Appendix VII). The line generated by the method of linear regression (Appendix VIII) of per cent specific binding of prolactin plotted against prolactin levels of the medium (Figure 2) illustrated the positive correlation of the two variables.

D. Discussion

The values of per cent specific binding of prolactin presented in Table V support the theory that receptors

Table V. The Binding of Prolactin-^{125}I to Cultured Normal Pituitary Cells of Immature, Mature, and Retired Breeder Female Rats

Type of Pituicyte	Pre Count (dpm)	Total Counts Bound DPM/100 ug protein ± SEM (n)	Non-Specific Counts Bound DPM/100 ug protein ± SEM (n)	Per Cent Specific Binding	T Value ($T_{\alpha,d}$)
A Immature	10,000	1,832±142 (3)	1,382±337 (2)	30	1.23 $T_{.4,4}$ = 0.941 2.1412
B Mature	60,000	1,138±132 (3)	788±99 (3)	31	$T_{0.1,4}$ = 2.132 1.7185
C Mature	27,000	2,299±804 (5)	767±383 (5)	78	$T_{0.1,8}$ = 1.397 1.446
D Mature	80,000	51,623±3,890 (4)	36,089±10,011 (6)	21	$T_{.1,8}$ = 1.397 3.713
E Mature	27,000	18,878±3,750 (3)	4,430±1,037 (3)	77	$T_{.01,4}$ = 3.495 3.238
F* Mature	40,000	37,570±9,339 (4)	7,283±533 (4)	81	$T_{.01,6}$ = 3.143 3.025
G Mature	100,000	15,973±1560 (4)	10,201±1,100 (6)	48	$T_{.01,8}$ = 2.896
H Retired Breeder	35,000	8,529±646 (5)	2,299±804 (5)	50	2.073 $T_{.1,8}$ = 1.86
I Retired Breeder	60,000	597±264 (3)	248±22 (3)	56	1.3174 $T_{0.4,4}$ = 1.533

* Fresh preparation of cells

for prolactin exist on the cells of the pituitary gland. Calculated t values for the means of total and non-specifically bound counts (Table V) further illustrated the significance of these findings. Scatchard plot analysis of bound and free iodinated preparations, receptor-saturation, and competitive inhibition analyses was impossible due to the limited availability of binding material and the variability in the capabilities of receptor-binding (biological activity) of the iodinated preparations.

The analysis for correlation between per cent specific binding and levels of prolactin released indicated a high positive correlation ($r=0.8147\pm0.2366$) for the different ages (Table VI and Appendix VII) as depicted in Figure 2. The data obtained from binding assays indicated that prolactin may induce (synthesize or activate) receptors for prolactin. The cellular mechanism controlled by the specific binding of prolactin to pituicytes was not elucidated and requires further study.

Table VI. The Correlation of Per Cent Specific Binding and Prolactin Secreted by Three Ages of Rat Pituicytes

Cell Type	% Specific Binding (y_1)	ng PRL Secreted (y_2) ng/ml
Immature	30	11.0
Mature	48	165.4
Mature	28	127.4
Mature	21	50.5
Mature	29	28.2
Retired Breeder	56	288.0
Retired Breeder	13	63.6

0.8147 ± 0.2366 = Correlation Coefficient (r) $\pm$ Standard Error of the Correlation Coefficient (S_r)

Figure 2.

Correlation of per cent specific binding of prolactin-^{125}I and prolactin secreted (ng per ml) for immature, mature and retired breeder pituitary cells.

Correlation Coefficient = 0.8147±0.2366 significant at 0.01 level.

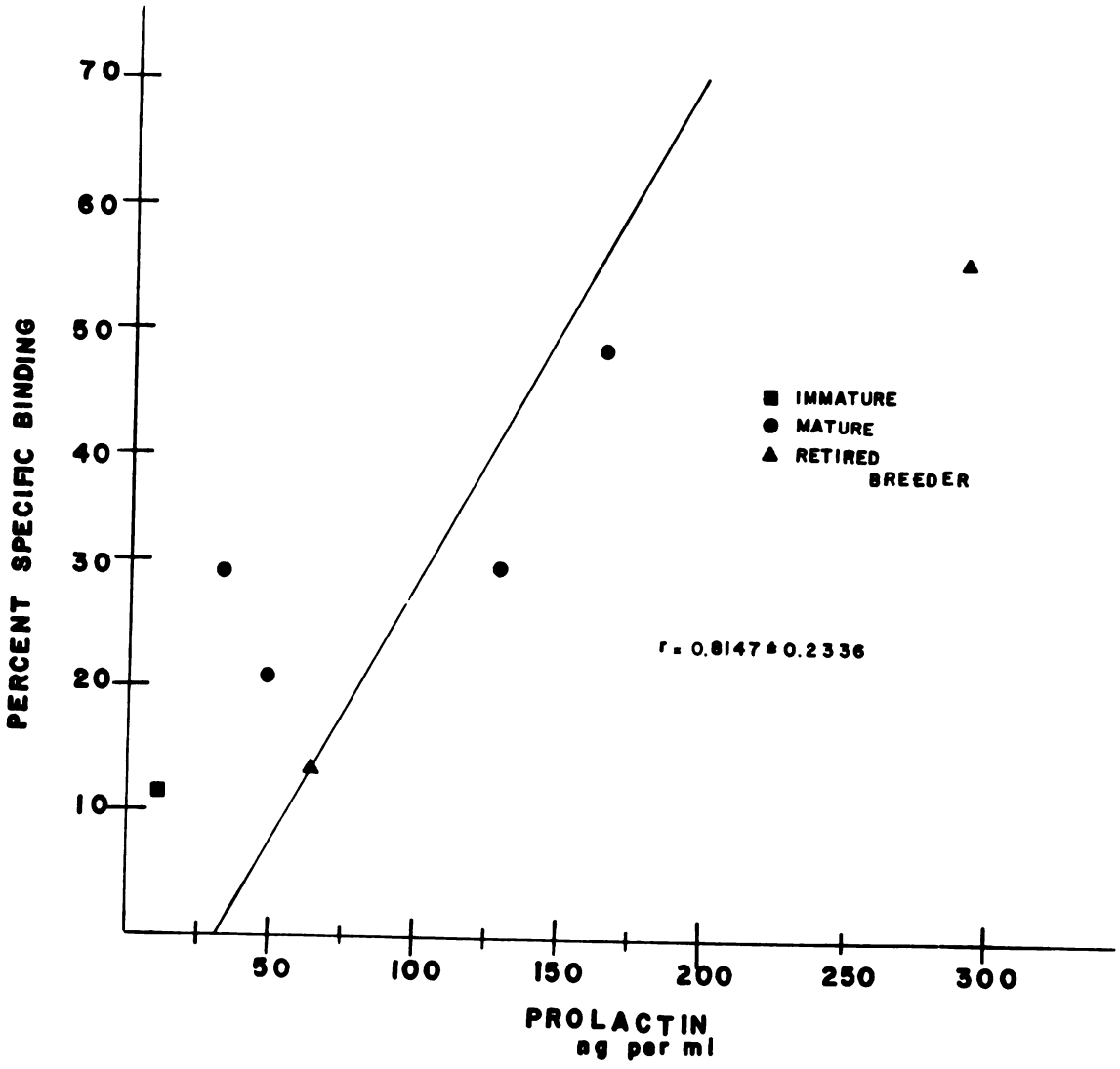
PERCENT SPECIFIC BINDING
70
60
50
40
30
20
10
IMMATURE
MATURE
RETIRED BREEDER
r = 0.8147 ± 0.2336
50
100
150
200
250
300
PROLACTIN
ng per ml

FIGURE 2

General Discussion

In the mixed cell cultures of the rat pituitary, the age of the animal, the number of days in culture and the interaction of age and day in culture significantly influenced the levels of prolactin released as determined by radioimmunoassay. Protein values per cell for retired breeder cells were found not to be significantly greater than those for the tumorous pituitary cells of the $C_8$11RAP line. The DNA values were greater in both cell types than those reported by Vendrely (1955), Leslie (1955) and Cunningham et al. (1950). The concentration of DNA per cell of the $C_8$11RAP culture was, however, significantly greater than those for the retired breeder and the immature cultures. These levels of DNA suggested chromosomal abnormalities (polyploidy or aneuploidy) which may have influenced the levels of prolactin secreted and specific binding of prolactin. From the study of the release of prolactin in vitro by normal immature, mature, and retired breeder female rats and the binding of their respective cells with iodinated prolactin, cell cultures have demonstrated their ability.

Cell cultures are advantageous in binding studies of prolactin because of the ease of collection without enzymatic or physical damages. Decreased damage to binding sites and maximized exposure for interaction with surface area at the membrane were also advantages that other preparations do not have. Iodinated prolactin bound

specifically to normal cultured rat pituitary cells. A positive correlation (0.8147) of per cent specific binding and prolactin released into the medium was demonstrated. Whether this mechanism has an autoregulatory effect in the pituitary is unclear. The present variability of individual iodinated preparations in their biological binding activity and the cultures with varying percentages of the cell types of the pituitary and different stages of metabolism make such a conclusion difficult to support quantitatively. Levels of endogenous prolactin from cultured pituitary cells may bind and saturate binding sites during assays competing with both added prolactin and prolactin-^{125}I for binding sites. However, if the rate of binding is rapid (Frantz *et al.*, 1974) and an equilibrium state is attained (Kahn *et al.*, 1974), this factor may be minimized.

The possibility also exists that binding sites located on pituitary cells may change with aging of the rat. Older rats may have a greater number of binding sites per cell or higher binding affinities for prolactin. Baker *et al.* (1974) have reported the greater persistence of the acidophil vs the basophil and chromophobe cell population of the rat pituitary *in vitro*. Histological studies of the pituitary have demonstrated a greater number of acidophils per pituitary in mature female and lactating rats (Meites *et al.*, 1961). If these conditions existed in cell culture, then the high per cent specific binding

and concentration of prolactin would be expected.

It may also be postulated that prolactin may induce its own receptors. The function of such a mechanism in the pituitary is obscure. However, a higher degree of specific binding of prolactin may be indicative of a control mechanism for prolactin release by exocytosis (Hopkins and Farquhar, 1973) rather than a dual synthesis and release system. If this theory is true, the exhaustion of prolactin stores by otherwise viable cells would be expected. Such a condition was not exhibited as decline in cell number and poor morphology were associated with low prolactin concentrations.

The presence of a quantal response or attainment of a threshold level of prolactin binding for release of prolactin would produce a sigmoid curve for per cent specific binding plotted against prolactin concentrations. This quantal response might involve the adjustment of new threshold points. Therefore, higher levels of specific binding would release greater amounts of prolactin concentration or per cent specific binding plotted against age of the rat. In respect to normal physiological states, a positive feedback alone for increase in release of prolactin with binding of prolactin to the pituitary would be indicative of a pathological rather than a normal regulatory mechanism.

Recent assays in our lab have indicated an increase in non-specific binding values for iodinated prolactin.

The factor(s) which contributed to increased non-specific binding and variability of iodinated preparations is (are) unknown. During the procedure of enzymatic iodination, the labeling of prolactin with ^{125}iodine may disrupt the stearic configuration necessary for its biological activity. The o-prolactin provided by NIH may consist of a mixture of monomers and polymers of prolactin as suggested by current research in our laboratory. These variable forms may decrease the number of biologically active molecules of prolactin.

Study of the biochemistry of the prolactin molecule, the characterization of the method and degree of the iodination of prolactin by the lactoperoxidase-hydrogen peroxide oxidation, and the conditions of ionic concentration and pH are required for production of a standardized iodinated prolactin. The kinetics of the binding reaction: its dependence on time and temperature of incubation must also be investigated.

From the study of primary cultures of the pituitary of the female rat, the heterogeneous cell population of the primary cultures demonstrated the necessity of an established, cloned or single cell derived acidophilic culture for more definitive studies of the binding and secretion of prolactin. This approach appeared most feasible with dispersion of retired breeder pituitary glands. These glands had the greatest number of cells per pituitary and the best growth characteristics in

culture. If the addition of a factor for prolonged cellular growth and maintenance would not interfere with osmotic or ionic regulation or experimental study such a substance should be investigated.

If a stable and easily standardized radiolabelled hormone can be developed, a rapid and efficient binding assay for clinical application may be achieved. Hormonal dependence of various pathologies and their response to treatment may be elucidated with binding assays. Cultured cells would greatly aid in this development because of the ease of maintenance and use, the availability of cells, and the relative homogeneity of an established culture.

APPENDICES

APPENDIX I: COMPOSITION OF DULBECCO'S MODIFIED EAGLE (DME) MEDIUM POWDER*

Component	mg per L
L-Arginine · HCl	84.00
L-Cystine · 2HCl	62.57
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCl · H_2O	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophane	16.00
L-Tyrosine (Disodium salt)	104.20
L-Valine	94.00
$CaCl_2$ (anhydrous)	200.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.10
KCl	400.00
$MgSO_4$ (anhydrous)	97.72
NaCl	6400.00
$NaH_2PO_4 \cdot H_2O$	124.00
Glucose	1000.00

Component	mg per L
Phenol Red (a)	15.00
Sodium Pyruvate	1100.00
D-Ca Pantothenate (b)	4.00
Choline Chloride	4.00
Folic Acid	4.00
i-Inositol	7.20
Nicotinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00

* GIBCO, Grand Island, NY

APPENDIX II:

Concentrations of Prolactin: Three Way Nested Analysis of Variance of the Means for Unequal Sample Sizes[1] r.f. Table I

$$Y = \mu + \alpha_i + B_{(i)j} + \gamma_k + (\alpha\gamma)_{ik} + (B\gamma)_{(i)jk} + E(ijk)$$

Y = prolactin concentration

μ = prolactin mean for all samples

α = fixed effect of the age of the i^{th} group of rats (culture)

$B_{(i)j}$ = random effect of j^{th} culture flasks or units in i^{th} culture

α_k = fixed effect of k^{th} time or day in culture

$(\alpha\gamma)_{ik}$ = interaction of age and day in culture

$(B\gamma)_{(i)jk}$ = interaction of culture flasks and day in culture, inseparable from experimental error

$E_{(ijk)}$ = experimental error

[1] Sokal and Rohlf (1969), pp. 274-286.

APPENDIX III: ANALYSIS OF VARIANCE FOR PROLACTIN RELEASE AND AGE OF ANIMAL

Table VII.

Source of Variation[2]	Degrees of Freedom (d.f.)	Sum of Squares	Mean Squares	Expected Mean Squares
Age = A	2	654,202.27	327,101.14	$\sigma^2 + 6\sigma^2_B + 18\sigma^3_{i=1} (\alpha_i)^2$
Culture Flasks = B	6	536,125.74	89,354.29	$\sigma^2 + 6\sigma^2_B$
Days of Collection = C	5	116,376.48	23,275.30	$\sigma^2 + 3\sigma^2_{BC} + 9\Sigma^{rk}_{k=1}{}^2/_5$
Interaction of Age and Day in Culture = AC	10	1,363,328.31	136,332.83	$\sigma^2 + 3\sigma^2_{BC} + \frac{3\sigma^3\sigma^k(\alpha\gamma)^2 ik}{i=ij=1}{10}$
Experimental Error = BC + E	54	455,172.58	8,429.12	$\sigma^2 + 3\sigma^2_{BC}$

[2] ibid. pp. 344-353

APPENDIX IV:

Tukey's T-Test for Analysis of Differences in Medium Prolactin Concentrations for Immature, Mature and Retired Breeder Female Rats[3]

$$t_T = \frac{(\bar{y}_{1.} - \bar{y}_{2.})}{\sqrt{MS_E \left(\frac{1}{r_1} + \frac{1}{r_2}\right)}}$$

t_T = calculated t value

$\bar{y}_{1.}$ = mean prolactin level of age group 1

$\bar{y}_{2.}$ = mean prolactin level of age group 2

MS_E = mean square error of culture flasks = B

r_1 = number of samples in age group 1

r_2 = number of samples in age group 2

Comparison	Retired Breeder vs Neonate	Mature vs Neonate	Retired Breeder vs Mature
T Value	*22.34	*15.77	*6.57

---

[3] Gill, J.L. (1976) Design and Analysis of Experiments in the Animal and Medical Sciences, to be published, Iowa State University Press, Ames, Iowa.

* Significant at a critical T value (t,v) of 4.325 (3,49) and 0.01 level of significance from "Upper Percentage Points Tukey's T ($q\alpha$,t,v) of Studentized Range", 1969. Order Statistics and Their Use in Testing and Estimation. Vol. I. Aerospace Research Lab. US Government Printing Office, Washington.

APPENDIX V:

Scheffé's Paired Contrasts for Analysis of Effect of Day in Culture on Concentrations of Prolactin Released into the Medium

---

Scheffé's Mean $\pm$ Confidence Interval[4]

$$\bar{q}_k \pm \sqrt{(t-1) f_{\alpha, t-1, n-t} \; v(\bar{q}_k)}$$

where $v(\bar{q}_k) = \sum_{i=1}^{t} (C^2_{ik}/r_i) \; MS_E$[4]

$\bar{q}_k$ = mean prolactin concentration of the k^{th} contrast

t = number of groups (ages)

n = total number of samples

$v(\bar{q}_k)$ = variance of the mean $\bar{q}_k$

r_i = replication or number of samples per day

C_{ik} = constant ($\sum C_{ik} = 0$)

MS_E = mean square error

$f_{\alpha, t-1, n-t}$ = F-distribution value for a level of significance = α and $t-1$, $n-t$ degrees of freedom

[4] Gill, (1976), to be published.

APPENDIX VI: SCHEFFÉ'S ANALYSIS OF PROLACTIN RELEASE VS DAY IN CULTURE

TABLE VIII.

Contrasts of Days in Culture	Prolactin Mean ($\bar{q}_k$) ± Confidence Interval	Contrasts of Days in Culture	Prolactin Mean ($\bar{q}_k$) ± Confidence Interval
Day 2 vs Day 4	*311.54 ± 185.25	Day 4 vs Day 6	*352.12 ±185.25
Day 2 vs Day 6	40.0 ± 185.20	Day 4 vs Day 8	*354.04 ± 185.25
Day 2 vs Day 8	42.5 ± 185.25	Day 4 vs Day 10	*409.04 ± 185.25
Day 2 vs Day 10	97.5 ± 185.25	Day 6 vs Day 8	1.92 ± 185.25
Day 2 and 4 vs Days 8 and 10	*451.54 ± 265.98	Day 8 vs Day 10	55.0 ± 185.25

* Significant at the 0.05 level

APPENDIX VII:

Product-Moment Correlation Coefficient[5] r.f. Figure 2

$$r_{12} = \frac{\Sigma y_1 y_2}{\sqrt{\Sigma {y_1}^2}\,\Sigma {y_2}^2}$$

r_{12} = correlation coefficient of per cent specific binding (y_1) and levels of prolactin (y_2)

$\Sigma y_1 y_2$ = summation of the products of values for each y_1 and its corresponding y_2

$\Sigma {y_1}^2$ = summation of the squared values of each y_1

$\Sigma {y_2}^2$ = summation of the squared values of each y_2

Standard error of the correlation coefficient

$$S_r = \sqrt{(1-r^2)/n-2}$$

S_r = standard error of correlation coefficient

r = correlation coefficient

n = number of samples considered in the correlation

[5] Sokal and Rohlf (1969), pp. 494-548.

APPENDIX VIII:

Linear Regression Analysis[6] r.f. Figures 1 and 2

Linear Regression Equation

$$\hat{Y} = a + b_{xy}X$$

$\hat{Y}$ = estimated value of Y for a given x value

a = Y intercept of the regression line

b_{xy} = slope of the regression line or regression coefficient

X = given x value

$$b_{y \cdot x} = \frac{\Sigma xy}{\Sigma x^2}$$

$x = X - \bar{X}$ $\bar{X}$ = mean of X values

$y = Y - \bar{Y}$ $\bar{Y}$ = mean of Y values

$a = \bar{Y} - b_{y \cdot x}\bar{X}$

Logarithmic Transformation of a Dependent Variable[7]

$$\log \hat{Y} = \log a + b \ (\log e) \ X$$

Curvilinear Regression by Orthogonal Polynomial[8]

$$\hat{Y} = A + B\xi_1 + C\xi_2 + D\xi_3$$

$\hat{Y}$ = estimate of Y for a given x

ξ_i = coefficient of orthogonal polynomial

A,B,C,B= regression coefficients corresponding to given x values

[6] Sokal and Rohlf (1969), pp. 404-420.

[7] ibid. p. 477.

[8] ibid. p. 470.

REFERENCES

References

Absher, M., 1973. Hemocytometer Counting. In: Tissue Culture Methods and Applications, 395-397. Academic Press, New York.

Amenomori, Y. and J. Meites, 1970. Effects of a hypothalamic extract on serum prolactin levels during the estrous cycle and lactation. P Soc Exp M 134: 492-495.

Averill, R.L.W., 1969. Depression of thyrotropin releasing factor induction of thyrotropin release by thyroxine in small doses. Endocrinol 85:67-71.

Baker, B.L., J.R. Reel, S.D. Van Dewark, Y.Y. Yu, 1974. Persistence of cell types in monolayer cultures of dispersed cells from the pituitary pars distalis revealed by immunohistochemistry. Anat Rec 179: 93-106.

Ben-David, M., A. Danon, F.G. Sulman, 1971. Evidence of antagonism between prolactin and gonadotropin secretion: effect of methallibure on perphenazine-induced PRL secretion in ovariectomized rats. J Endocr 51: 719-725.

Blake, C., R. Norman and C.H. Sawyer, 1972. Effects of estrogen and/or progesterone on serum and pituitary gonadotropin levels in ovariectomized rats. P Soc Exp M 141:1100-1103.

Bowers, C.Y., A.V. Schally, G.A. Reynolds, and W.D. Hawley, 1967. Interactions of L-thyroxine or L-triiodothyronine and thyrotropin-releasing factor on the release and synthesis of thyrotropin from the anterior pituitary gland of mice. Endocrinol 81:741-747.

Brooks, C.L., and C.W. Welsch, 1974. Reduction of serum prolactin in rats by 2 ergot alkaloids and 2 ergoline derivatives: a comparison. P Soc Exp M 146: 863-867.

Burton, K., 1956. A study of the conditions and mechanisms of the dephenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62: 315-323.

Chen, C.L., Y. Amenomori, K.H. Lu, J.L. Voogt and J. Meites, 1970. Serum prolactin levels in rats with pituitary transplants or hypothalamic lesions. Neuroendocr 6:220-227.

Chen, H.J. and J. Meites, 1975. Effects of biogenic amines and TRH on release of prolactin and TSH in the rat. Endocrinol 96:10-14.

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

Clemens, J.A., and J. Meites, 1971. Neuroendocrine status of old constant-estrous rats. Neuroendocr 7:249-256.

Cuatrecasas, P., 1974. Membrane receptors. Ann R Bioch 43:169-214.

Cunningham, L., A.C. Griffin, and J.M. Luck, 1950. Polyploidy and cancer. The desoxypentosenucleic acid content of nuclei of normal, precancerous, and neoplastic rat tissues. J Gen Physl 34:59-63.

Everett, J.W., 1954. Luteotrophic function of autografts of the rat hypophysis. Endocrinol 54:685-690.

Everett, J.W., 1956. Functional corpora lutea maintained for months by autografts of the rat hypophysis. Endocrinol 58:786-796.

Feldman, H., and D. Rodbard, 1971. Mathematical theory of radioimmunoassay. In: Principles of Competitive Binding Assay, edited by W. O'Dell and W. Daughaday, 158-203. J.B. Lippincott Co., Philadelphia.

Frantz, W.L., J. Roger W. Turkington, 1972. Formation of biologically active ^{125}I PRL by enzymatic radioiodination. Endocrinol 91:1545-1548.

Frantz, W.F., J.H. MacIndoe, and R.W. Turkington, 1974. Prolactin receptors. Characteristics of the particulate fraction binding activity. J Endocr 60: 485-497.

Frantz, W.L., C. Sonnenschein, P.A. Payne and O.C. Dombroske, 1975. The binding of o-prolactin ^{125}I to cultured anterior pituitary tumor cells and normal cells. Nature 255:636-638.

Gala, R.R., and E.Y. Kuo, 1972. Prolactin production by rat anterior pituitaries cultured in vitro as estimated by crop gland assay, densitometric analysis and radioimmunoassay. P Soc Exp M 139:1349-1352.

Gautvik, K.M., and A.H. Tashjian, Jr., 1973a. Effects of Ca^{++} and Mg^{++} on secretion and synthesis of growth hormone and prolactin by clonal strains of pituitary cells in culture. Endocrinol 92:573-583.

Gautvik, K.M., and A.H. Tashjian, Jr., 1973b. Effects of cations and colchicine on the release of prolactin and growth hormone by functional pituitary tumor cells in culture. Endocrinol 93:793-799.

Giles, K.W., and A. Myers, 1965. An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature 206:93.

Grandison, L., M. Gelato, and J. Meites, 1974. Inhibition of PRL secretion by cholinergic drugs. P Soc Exp M 145:1236-1239.

Grosvenor, C.E., S. M. McCann, and M.D. Nallar, 1964. Inhibition of suckling-induced release of prolactin in rats injected with acid extract of bovine hypothalamus. Program 46th Meeting Endocrine Society, San Francisco, p. 96.

Hopkins, C.R., and M.G. Farquhar, 1973. Hormone secretion by cells dissociated from rat anterior pituitaries. J Cell Biol 59:276-303.

Hymer, W.C., W.H. Evans, J. Kraicer, A. Mastro, J. Davis, and E. Griswolid, 1973. Enrichment of cell types from the rat adenohypophysis in sedimentation at unit gravity. Endocrinol 92:275-287.

Ieiri, T., Y. Akikusa, and K. Yamamoto, 1971. Synthesis and release of prolactin and growth hormone in vitro during the estrous cycle of the rat. Endocrinol 89:1533-1537.

Ishikawa, H., 1969. Isolation of different types of anterior pituitary cells in rats. Endocrinol Japon 16:517-529.

Jones, A.E., J.N. Fisher, U.J. Lewis, and W.P. Vanderlaan, 1965. Electrophoretic comparison of pituitary glands from male and female rats. Endocrinol 76:578-583.

Kahn, C.R., P. Freychet and J. Roth, 1974. Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J Biol Chem 249:2249-2257.

Kalra, P.S., C.P. Fawcett, L. Krulich and S.M. McCann, 1973. The effects of gonadal steroids on plasma gonadotropins and prolactin in the rat. Endocrinol 92:1256-1268.

Kamberi, I.A., R.S. Mical and J.C. Porter, 1971. Effect of anterior pituitary perfusion and intraventricular injection of catecholamines on PRL release. Endocrinol 88:1012-1020.

Kelly, P.A., B.I. Posner, T. Tsushima, and H.G. Friesen, 1974. Studies of insulin, growth hormone and prolactin binding: ontogenesis, effects of sex and pregnancy. Endocrinol 95:532-539.

Koch, T., K.H. Lu, and J. Meites, 1970. Biphasic effects of catecholamines on pituitary prolactin release in vitro. Endocrinol 87:673-675.

Kragt, C.L., and J. Meites, 1965. Stimulation of pigeon pituitary prolactin release by pigeon hypothalamic extract in vitro. Endocrinol 76:1169-1176.

Kragt, C.L., and J. Meites, 1967. Dose-response relationships between hypothalamic PIF and prolactin release by rat pituitary tissue in vitro. Endocrinol 80: 1170-1173.

Leavitt, W.W., G.L. Kimmel and J.P. Friend, 1973. Steroid hormone uptake by anterior pituitary cell suspensions. Endocrinol 92:94-103.

Leslie, I., 1955. The nucleic acid content of tissues and cells. In: The Nucleic Acids, Vol. II, edited by E. Chargaff and J.N. Davidson, 1-50. Academic Press, New York.

Li, C.H., J.S. Dixon, T-B. Lo, Y.A. Padkov, and K.D. Schmidt, 1969. Amino-acid sequence of ovine lactogenic hormone. Nature (lond) 224:695-696.

Lowry, O.H., N.J. Rosenbrough, A.L. Facci and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275.

Lu, K.H., and J. Meites, 1972. Effects of L-dopa on serum prolactin and PIF in intact and hypophysectomized, pituitary-grafted rats. Endocrinol 91:868-872.

Lyons, W.R., 1937. The preparation and assay of mamotropin. Cold Spring Harbor Symposium on Quantitative Biology 5:198-209.

Machlin, L.J., L.S. Jacobs, R. Miller, N. Cirulis, and R. Kimes, 1974. An assay for growth hormone and prolactin-releasing activities using a bovine pituitary cell culture system. Endocrinol 95:1350-1358.

MacLeod, R.M., and A. Abad, 1968. The control of prolactin and growth hormone synthesis in rat pituitary glands. Endocrinol 83:799-806.

MacLeod, R.M., A. Abad, and L.L. Eidson, 1969. In vivo effect of sex hormones on the in vitro synthesis of prolactin and growth hormone in normal and pituitary tumor-bearing rats. Endocrinol 84:1475-1483.

McGarry, E.E., D. Rubinstein and J.C. Beck, 1968. Growth hormones and prolactins: biochemical, immunological and physiological similarities and differences. Ann N Y Acad Sci 148:559-571.

Meites, J., 1959. Mammary growth and lactation. In: Reproduction in domestic animals, edited by H.H. Cole and P.T. Cupps, Vol. I., 539-593. Academic Press, New York.

Meites, J., R. Kahn, and C.S. Nicoll, 1961. Prolactin production by rat pituitary in vitro. P Soc Exp M 108:440-443.

Midgley, A.R., Jr., G.D. Niswender, and R.W. Rebar, 1969. Principles for the assessment of the releability of radioimmunoassay methods. In: Karolinska Symposia Research Methods in Reproductive Endocrin., edited by E. Diczfalusay and A. Diczfalusay, 163-184. Stockholm.

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

Negro-Vilar, A., L. Krulich, and S.M. McCann, 1973. Changes in serum prolactin and gonadotropins during sexual development of the male rat. Endocrinol 93:660-664.

Neill, J.D., and L.E. Reichert, Jr., 1971. Development of a radioimmunoassay for rat prolactin and evaluation of NIAMD rat prolactin radioimmunoassay. Endocrinol 88:548-555.

Nicoll, C.S., J.A. Parsons, R.P. Fiorindo, and C.W. Nicols, Jr., 1969. Estimation of prolactin and growth hormone levels by polyacrylamide disc electrophoresis. J Endocrinol 45:183-196.

Nicoll, C.S., and J. Meites, 1963. Prolactin secretion in vitro: effects of thyroid hormones and insulin. Endocrinol 72:544-551.

Niswender, G.D., A.R. Midgley, S.E. Monroe and L.E. Reichert, 1969. Radioimmunoassay for rat prolactin. P Soc Exp M 130:793-797.

Ojeda, S.R., and S.M. McCann, 1974. Development of dopaminergic and estrogenic control of PRL release in the female rat. Endocrinol 95:1499-1505.

Pasteels, J.L., 1961. Secretion de prolactin par l'hypophyse in culture de tissues. C R Ac Sci D 253:2140-2142.

Portanova, R., D.K. Smith and G. Sayers, 1970. A trypsin technic for the preparation of isolate rat ant pituitary. P Soc Exp M 133:573-576.

Posner, B.I., P.A. Kelly, R.R. C. Shiu, and H.G. Friesen, 1974. Studies of insulin, growth hormone and prolactin binding: tissue distribution, species variation and characterization. Endocrinol 95:521-531.

Reece, R.D., and Turner, C.W., 1937. The lactogenic and thyrotropic hormone content of the anterior lobe of the pituitary gland. Missouri Agr Exp Sta Bull, No. 226.

Rivier, C. and W. Vale, 1974. In vivo stimulation of prolactin secretion in the rat by thyrotropin releasing factor, related peptides and hypothalamic extracts. Endocrinol 95:978-985.

Rohlf, F.J., and R.R. Sokal, 1969. Statistical Tables. W.H. Freeman and Company, San Francisco.

Sar, M., and J. Meites, 1968. Effects of progesterone, testosterone, and cortisol on hypothalamic prolactin inhibiting factor and pituitary PRL content. P Soc Exp M 127:426-429.

Sayers, G. and R. Portanova, 1974. Secretion of ACTH by isolated ant pit cells: kinetics of stimulation by corticotropin-releasing factor and inhibition by corticosterone. Endocrinol 94:1723-1730.

Schmidt, G. and S.J. Thannhauser, 1945. A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J Biol Chem 161:83-89.

Schneider, W.C., 1945. Phosphorus compounds in animal tissues I: extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J Biol Chem 161:293-303.

Shaar, C.J., and J.A. Clemens, 1974. The role of catecholamines in the release of anterior pituitary prolactin in vitro. Endocrinol 95:1202-1212.

Shiu, R.P.C., and H.G. Friesen, 1974a. Properties of a prolactin receptor from the rabbit mammary gland. Biochem J 140:301-311.

Shiu, R.P.C., and H.G. Friesen, 1974b. Solubilization and purification of a prolactin receptor from the rabbit mammary gland. J Biol Chem 24:7902-7911.

Sokal, R.R., and F.J. Rohlf, 1969. Biometry, W.H. Freeman and Company, San Francisco.

Sonnenschein, C., U.I. Richardson, and A.H. Tashjian, Jr., 1970. Chromosomal analysis, organ specific function and appearance of sex clonal strains of rat pit tumor cells. Exp Cell Res 61:121-128.

Sonnenschein, C., M. Posner, S. Saududdin and M. Krasnay, 1973. Estrogen sensitive cell lines uptake of labeled sex steroids and growth properties. Exp Cell Res 78:41-46.

Sonnenschein, C., M. Posner, K. Sahr, R. Farook, and R. Brunnelle, 1974. Estrogen sensitive cell lines. Establishment and characterization of new cell lines from estrogen induced rat pituitary tumors. Exp Cell Res 84:399-411.

Talwalker, P.K., A. Ratner and J. Meites, 1963. In vitro inhibition of pituitary prolactin synthesis and release by hypothalamic extract. Am J Phys 205:213-218.

Tashjian, A.H., Y. Yasumura, L. Levine, G.H. Saao, and M.L. Parker, 1968. Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. Endocrinol 82:342-352.

Tashjian, A.H., Jr., F.C. Bancroft, L. Levine, 1970. Production of both prolactin and growth hormone by clonal strains of rat pituitary tumor cells. J Cell Biol 47:61-70.

Tashjian, Jr. A.H., N.J. Barowsky, and D.K. Jensen, 1971. Thyrotropin releasing hormone: direct evidence for stimulation of prolactin production by pituitary cells in culture. Bioc Biop R 43:516-523.

Tennant, J.R., 1964. Evaluation of the trypan blue technique for determination of cell viability. Transplant 2:685-694.

Thomson, R.Y., W.C. Hutchinson, F.C. Heapy, and J.N. Davidson, 1953. The deoxyribonucleic acid content of the rat cell nucleus and its use in expressing the results of tissue analysis, with particular reference to the composition of liver tissue. Biochem J 53:460-474.

Turkington, R.W., 1974. Prolactin receptors in mammary carcinoma cells. Cancer Res 34:758-763.

Tsai, J.S., and H. Samuels, 1974. Thyroid hormone action: stimulation of growth hormone and inhibition of PRL secretion in cultured GH_1 cells. Bioc Biop R 59: 420-428.

Vale, W., R. Burgus, and R. Guillemin, 1967. Competition between thyroxine and TRF at the pituitary level in the release of TSH. P Soc Exp M 125:210-213.

Vale, W., G. Grant, M. Amoss, R. Blackwell, and R. Guillemin, 1972. Culture of enzymatically dispersed anterior pituitary cells: functional validation of a method. Endocrinol 91:562-571.

Vendrely, R., 1955. The deoxyribonucleic acid content of the nucleus. In: The Nucleic Acid, edited by E. Chargaff and J.N. Davidson, 155-180. Academic Press, New York.

Voogt, J.L., J.A. Clemens and J. Meites, 1969. Stimulation of pituitary FSH release in immature female rats by prolactin implant in median eminence. Neuroendocr 4:157-163.

Voogt, J.L., C.L. Chen, and J. Meites, 1970. Serum and pituitary prolactin levels before, during and after puberty in female rats. Am J Phys 218:396-399.

Voogt, J.L. and J. Meites, 1971. Effects of an implant of prolactin in the median eminence of pseudopregnant rats on serum and pituitary LH, FSH and prolactin. Endocrinol 88:286-292.

Welsch, C.W., T.W. Jenkins, and J. Meites, 1970. Increased incidence of mammary tumors in the female rat grafted with multiple pits. Cancer Res 30:1024-1029.

Welsch, C.W., M.D. Squiers, E. Cassell, C.L. Chen and J. Meites, 1971. Median eminence, lesions and serum prolactin influence of ovariectomy and ergocornine. Am J Phys 221:1714-1717.

Welsch, C.W., and C. Gribler, 1973. Prophylaxis of spontaneously developing mammary carcinoma in C3H/HeJ female mice by suppression of prolactin. Cancer Res 33:2939-2946.

Welsch, C.W., T. Jenkins, Y. Amenomori, and J. Meites, 1971. Tumorous development of in situ and grafted anterior pituitaries in female rats treated with diethylstilbesterol. Experientia 27:1350-1352.

Yalow, R.S., and S.A. Berson, 1959. Assay of plasma insulin in human subjects by immunological methods. Nature (Lond) 184:1648-1649.

Yamamoto, K., L.M. Taylor, and F.E. Cole, 1970. Synthesis and release of GH and prolactin from the rat anterior pituitary in vitro as functions of age and sex. Endocrinol 87:21-26.