INFLUENCE OF DIFFERENT PHYSIOLOGICAL PARAMETERS ON PROLACTIN BINDING ACTIVITY IN PROLACTIN TARGET TISSUES

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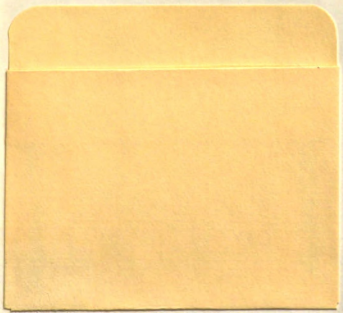

ABSTRACT

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and was highest on the days of diestrus during the estrous cycle. It is concluded that the increase in prolactin binding activity in the ovaries of immature rats is associated with the appearance of corpora lutea and that prolactin has a role in ovarian function during the estrous cycle of the rat.

3. Prolactin binding activity was measured in ovarian and mammary gland microsomal membrane preparations during pregnancy and lactation. The binding activity of prolactin in the ovaries was significantly increased on days 3 and 6 of gestation and on days 4 and 10 of lactation. Prolactin binding activity in the mammary tissue remained unchanged throughout pregnancy and increased by 2-fold on days 4 and 10 of lactation. These data suggest that the binding activity of prolactin is correlated with physiological requirements for the hormone during pregnancy and lactation.

4. Prolactin binding activity was measured in liver, kidney, and adrenal microsomal membrane preparations of 15, 23, 28, 33, 38, 43 and 75 day-old female rats. The binding activity in the liver increased and reached a peak at 43 days of age whereas the prolactin binding activity in the kidney and adrenal tissue steadily decreased until 43 days of age in these rats. Estradiol benzoate injected at 1 μg for 5 days in immature female rats significantly increased prolactin binding activity by 4-5 fold in the liver tissue. It is concluded that estrogen secretion near the time of puberty may stimulate prolactin binding activity in the liver, and that the functions of prolactin may be more important in the kidney and adrenals of the immature rat than in the adult rat.

5. Prolactin binding activity was measured in microsomal membranes of liver tissue from intact, ovariectomized, ovariectomized-thyroidectomized, and ovariectomized-thyroidectomized rats injected with thyroxine (T_4) or estradiol benzoate (EB). Thyroidectomy and ovariectomy each reduced prolactin binding activity in liver tissue significantly. The combination of ovariectomy and thyroidectomy decreased prolactin binding activity more than thyroidectomy or ovariectomy alone. Doses of 2.5 μg or 10 μg T_4/100 g BW daily returned prolactin binding activity in the thyroidectomized rats to intact control values, and in the ovariectomized-thyroidectomized rats to the ovariectomized values. A dose of 2 μg EB/rat increased prolactin binding activity above that of intact controls.

Scatchard analysis showed that ovariectomy and thyroidectomy decreased the number of prolactin binding sites in the liver as compared to those in intact controls or in ovariectomized-thyroidectomized rats treated with EB and T_4. It is concluded that the thyroid and ovaries are important regulators of prolactin binding activity in the liver of the rat.

6. Prolactin binding activity was measured in liver and mammary gland microsomal membranes of ovariectomized rats treated with estradiol benzoate (EB) (5 and 20 μg) or estradiol benzoate (5 μg) and progesterone (4 mg) for 10 days. Estradiol benzoate or the combination of EB and progesterone significantly increased prolactin binding activity in the liver tissue approximately 4-fold as compared to controls. The prolactin binding activity in the mammary tissue was significantly decreased by EB and the combination

of EB and progesterone. One week after the treatment was terminated the effects of EB and EB and progesterone were still present in the liver and mammary tissue when assayed in a second group of ovariectomized rats. It is concluded that EB (5 or 20 μg) or a combination of EB and progesterone are able to stimulate prolactin binding activity in the liver and depress binding of prolactin in the mammary tissue.

7. The effects of adrenalectomy and hydrocortisone acetate treatment on prolactin binding activity in liver microsomal membranes was measured in ovariectomized rats. Adrenalectomy for either 6 or 24 days slightly lowered prolactin binding activity in the liver. Hydrocortisone acetate treatment, 1 mg daily for 10 days, produced a slight depression in prolactin binding activity; however, 100 μg hydrocortisone acetate/100 g BW daily to adrenalectomized rats significantly decreased the binding of prolactin in the liver. These results suggest that adrenalectomy had a tendency to lower prolactin binding activity in the liver of female rats, whereas treatment with hydrocortisone acetate produced a more marked reduction in prolactin binding activity.

INFLUENCE OF DIFFERENT PHYSIOLOGICAL PARAMETERS ON PROLACTIN BINDING ACTIVITY IN PROLACTIN TARGET TISSUES

By

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INTRODUCTION

Part of the definition of the term hormone states that hormones are substances carried by the circulating blood to another part of the body where they evoke systemic functions by acting on specific tissues and organs. In order for this hormone system to be specific there would have to be "recognition" by the target tissue for the particular hormone. Substantial evidence now exists that the initial interaction of polypeptide hormones with their target cells is with a hormone specific receptor site on the target cell. The receptor molecule has been localized on the plasma membrane of the cell. Recently membrane receptors have been isolated for prolactin, luteinizing hormone, follicle stimulating hormone, insulin and others. The study of hormone receptors has provided another tool for further characterization of hormone action.

In the last year the development of a specific radioreceptor assay for prolactin (Shiu et al., 1973) has made it possible to measure the binding activity of prolactin in target tissues, and with the use of Scatchard analysis to estimate the number of receptors per mg of tissue and determine the binding affinity of prolactin for these receptors. The major emphasis of this thesis is on the physiological characterization of prolactin receptor binding activity in several target organs, mainly the liver and ovaries.

The studies presented follow three lines of investigation: (1) establishment of specific binding for prolactin in liver, ovarian and mammary gland tissues and demonstration of no cross reactivity with other hormones (2) the development of prolactin receptor binding activity and possible correlation with prolactin levels in the plasma (3) measurement of prolactin receptor binding activity in target tissues during different physiological conditions, such as pregnancy, lactation, hyperthyroidism, estrogen deficiency, etc.

LITERATURE REVIEW

I. General Hypothalamic Control of Anterior Pituitary Function

A. Anatomy of the Hypothalamus

The physiological relationship between the hypothalamus and the anterior pituitary is made clearer by an understanding of their anatomy. The role of the hypothalamus in pituitary function is depicted in the colored illustrations of the nervous system by Netter (1968).

The hypothalamus is described as the most ventral portion of the diencephalon. It is bordered anteriorly by the lamina terminalis, posteriorly by the interpeduncular fossa, dorsally by the hypothalamic sulcus in the third ventricle and ventrally by the tuber cinereum. There are three distinct hypothalamic regions. In a cephalocaudal direction they are: anterior or supraoptic area, middle or tuberal area and a caudal or mammillary area. The supraoptic area contains two sharply defined hypothalamic nuclei, the paraventricular nucleus and supraoptic nucleus. In the tuberal area the hypothalamus reaches its widest extent and the medial portion forms the central gray substance of the ventricular wall. At the lower end the periventricular arcuate complex form the base of the third ventricle. Also located in the tuberal region are the

dorsomedial, ventromedial, and lateral hypothalamic nuclei. The mammillary portion consists of the mammillary bodies and the dorsally located cells of the posterior hypothalamic nucleus.

B. Anatomy of the Hypothalamo-Pituitary Connections

The pituitary gland is attached to the brain by the infundibulum or tuber cinereum, an extension of the third ventricle which is prolonged downward as the pituitary stalk. Part of the tuber cinereum and the uppermost part of the neurohypophysis is called the median eminence. It is in this region, the infundibulum and median eminence that the hypophysial portal blood vessels originate. There are no direct neural connections between the hypothalamus and anterior pituitary; rather, the connection is by way of the hypophysial portal blood vessels.

Popa and Fielding (1930) first described the pituitary portal circulation and erroneously indicated that the flow of blood was from the anterior pituitary to the hypothalamus. Subsequent studies suggested that the flow was from the hypothalamus to the anterior pituitary (Wislocki and King, 1936; Houssay et al., 1935). Green and Harris (1949) directly observed the pituitary portal circulation in living rats under the microscope. They indicated that the portal vessels originated in the median eminence and infundibular stem, and stated that the blood flowed caudally toward the anterior pituitary. These observations were confirmed in the dog by Torok (1954) and in the mouse by Worthington (1955). More detail on the anatomy of the pituitary portal system was given by Daniel (1966).

C. Hypothalamic Hypophysiotropic Hormones

Several books and reviews have been written on hypothalamic control of anterior pituitary function and the hypothalamic hypophysiotropic hormones (Meites, 1970a; Szentagothai et al., 1972; Burgus and Guillemin, 1970; McCann, 1971; Reichlin and Mitnick, 1973; Vale et al., 1973; Schally et al., 1973). Only some of the most important and classical references on this topic will be reported.

The hypothalamus regulates secretion of the anterior pituitary hormones by producing specific hypophysiotropic hormones. Taubenhaus and Soskin (1941) suggested that the rat hypothalamus secretes an acetylcholine-like substance into the portal vessels to elicit pituitary LH release. Later, Markee et al., (1948) and McDermott et al. (1951) proposed that epinephrine, acetylcholine, and histamine might be involved in the release of gonadotropins, ACTH and other pituitary hormones. The direct influence of the hypothalamus on anterior pituitary function was first demonstrated by Harris and Jacobson (1952). They observed the return of estrous cycles in hypophysectomized rats when the anterior pituitary was removed and transplanted under the median eminence. However, when the anterior pituitary was transplanted under the temporal lobe of the brain in hypophysectomized rats, normal estrous cycles were not resumed. Harris (1955) proposed the "chemotransmitter hypothesis", according to which neurohormones released from the hypothalamus were responsible for regulating pituitary function. These neurohormones of which Harris (1955) wrote were later revealed to be a special

class of polypeptides. Saffran and Schally (1955) and Guillemin and Rosenberg (1955) were among the first to report that crude acidic hypothalamic extracts caused release of ACTH in vitro and in vivo. Saffran and Schally (1955) called the active substance "corticotropin releasing factor" (CRF). McCann et al. (1960) demonstrated the presence of LRF (luteinizing hormone releasing factor) in acid extracts of rat hypothalami. Talwalker, Ratner and Meites (1963) and Pasteels (1961-63) provided evidence for PIF (prolactin inhibiting factor) activity. GHRF activity was first demonstrated by Deuben and Meites (1963). Evidence for the existence of other hypophysiotropic hormones followed, such as TRH (Shibusawa et al., 1959; Schreiber, 1963; Guillemin et al., 1962), FSH-RF (Igarashi and McCann, 1964; Mittler and Meites, 1962), MIF and MRF (Kastin and Schally, 1966; Taleisnik and Orias, 1964), GIF (Krulich et al., 1968) and PRF in birds (Kragt and Meites, 1965; Meites and Nicoll, 1966).

Very recently the structures for several of the hypophysiotropic hormones have been elucidated. Boler et al. (1969) and Burgus et al. (1969) first reported the structure of thyrotropic releasing factor (TRH or TRF), and subsequently the amino acid sequence of LRF (Matsuo et al., 1971), MIF (melanocyte stimulating hormone inhibiting factor) (Nair et al., 1971) and GIF (somatostatin) (Brazeau et al., 1973) were reported.

II. Hypothalamic Control of Prolactin Secretion

A. Hypothalamic Prolactin Release-Inhibiting Factor (PIF)

The regulation of prolactin secretion is unique in that it is the only anterior pituitary hormone that is chronically inhibited by the mammalian hypothalamus under most conditions. Any disturbance in the connection of the hypothalamus with the anterior pituitary can result in increased release of prolactin. Everett (1954; 1956) demonstrated that transplantation of the pituitary underneath the kidney capsule resulted in sustained release of prolactin, whereas release of all other anterior pituitary hormones was sharply reduced. This work was later confirmed by Nikitovitch-Winer and Everett (1958) and Chen et al. (1970). Various other experimental techniques have been employed to demonstrate hypothalamic inhibition of prolactin secretion, such as placement of lesions in the median eminence or "hypophysiotropic area" of the hypothalamus (Chen et al., 1970; Welsch et al., 1971), culture or incubation of the anterior pituitary in vitro (Meites et al., 1961) and administration of appropriate drugs (Meites, 1962; Meites et al., 1963).

Inhibition of pituitary prolactin release by the mammalian hypothalamus is exerted via the action of a PIF. Culture of anterior pituitary tissue in vitro has demonstrated that the anterior pituitary can synthesize and release prolactin autonomously when removed from the hypothalamus and other body influences (Meites, 1959a). Addition of crude acid extracts of rat hypothalamic tissue resulted in decreased release of prolactin (Talwalker et al., 1963; Pasteels et al.,

1963). Kragt and Meites (1967) demonstrated a negative dose-response relationship between the quantity of hypothalamic extract added and the amount of prolactin released in vitro. This work was later confirmed by Chen (1969) with the use of a specific radioimmunoassay for rat prolactin.

There have been several in vivo demonstrations of PIF activity. Grosvenor et al. (1964) injected hypothalamic extracts into postpartum lactating rats and reported that they inhibited prolactin release following suckling. Kurashima et al (1966) reported prevention of pituitary prolactin depletion in response to cervical stimulation during estrus by hypothalamic extracts. Injection of crude extract of rat hypothalamus reduced serum prolactin in cycling and lactating rats (Amenomori and Meites, 1970) and decreased serum prolactin in normal and orchidectomized male rats (Watson et al., 1971).

PIF activity in the hypothalamus can be altered by several means including use of central acting drugs, hormones, and physiological stimuli. Perphenazine (Danon et al., 1963), reserpine (Ratner et al., 1965), haloperidol (Dickerman et al., 1972a), Na pentobarbital (Wuttke et al., 1971a), epinephrine and acetylcholine (Mittler and Meites, 1967), estrogen (Ratner and Meites, 1964), progesterone, testosterone and cortisol (Sar and Meites, 1968), a norethynodrel-menstranol combination (enovid) (Minaguchi and Meites, 1967a) and the suckling stimulus (Ratner and Meites, 1964; Minaguchi and Meites, 1967b) were found to decrease hypothalamic PIF activity in rats. Ergocornine (Wuttke et al., 1971b), L-Dopa and monoamine oxidase inhibitors (Pargyline, iproniazid, and Lilly compound-15641)(Lu and

Meites, 1971) and prolactin itself (Chen et al., 1967; Clemens and Meites, 1968; Voogt and Meites, 1971) were shown to increase PIF activity in the hypothalamus. Work by Lu and Meites (1972) demonstrated that L-Dopa, the immediate precursor of dopamine, increased PIF activity in the hypothalamus and elicited the presence of PIF activity in the systemic blood of rats. These results together with numerous other studies confirmed the view that drugs which increased hypothalamic catecholamines also stimulated synthesis and release of PIF, whereas drugs that reduced catecholamines in the brain depressed hypothalamic PIF activity (Meites et al., 1972).

B. Hypothalamic Prolactin-Releasing Factors (PRF)

Unlike the mammalian hypothalamus which inhibits prolactin secretion, the avian hypothalamus appears to exert a stimulatory influence on prolactin secretion and apparently contains a prolactin-releasing factor (PRF). Kragt and Meites (1965) demonstrated that an extract of pigeon hypothalamus stimulated prolactin release by the pigeon pituitary in vitro. Hypothalamic extracts from the chicken, quail, tricolored blackbird, duck and turkey (Meites, 1967; Nicoll, 1965; Gourdji and Tixier-Vidal, 1966; Chen et al., 1968) also induced release of prolactin when incubated with pituitaries from these species. The existence of a PRF in the mammalian hypothalamus is probable but less clear. Meites et al. (1960a) reported initiation of lactation by injection of crude hypothalamic extracts into estrogen-primed rats. Injections of crude cerebral extracts also initiated lactation in some rats. This work was later confirmed by Mishkinsky et al. (1968).

Nicoll et al. (1970) observed both prolactin inhibiting and prolactin releasing activity in rat hypothalamic extract when incubated with rat pituitary. PIF activity was reported to be present predominantly in the dorsolateral part of the preoptic area, and PRF activity mainly in the median eminence and a narrow basal portion of the preoptic area (Krulich et al., 1971). Valverde and Chieffo (1971) reported only PRF activity in an extract of porcine hypothalamus. However, others have failed to show PRF activity in comparable systems (Meites et al., 1972).

Synthetic pyro-glutamyl-histidyl-proline amine (TRH or TRF) has been observed by several laboratories to induce prolactin release. TRH has been shown to increase release of prolactin in the cow (Convey et al., 1972; Kelly et al., 1973a), rat (Tashjian et al., 1971), human (Hwang et al., 1971; Bowers et al., 1971; Jacobs et al., 1971) and monkey (Josimovich et al., 1974). Recently our laboratory reported that in the rat TRH was able to induce prolactin release in vivo (Mueller, Chen and Meites, 1973) and in vitro (Dibbet et al., 1972). This work raises the possibility that TRH and PRF in mammals are the same. However, under most physiological conditions, TSH and prolactin are not released together (Meites, 1973), i.e. hot and cold temperatures (Mueller et al., 1974), during suckling, administration of L-Dopa (Chen et al., in press), etc. It is possible that TRH is similar to PRF structurally.

C. Role of Catecholamines, Serotonin and Acetylcholine

In the last few years much attention has been given to the role of neurotransmitters on prolactin and gonadotropic secretion. The

substances which serve as neurotransmitters in the brain are acetylcholine and three monoamines, i.e. dopamine, norepinephrine and serotonin. A more thorough review of the localization of these transmitters in the brain is presented by Cooper et al., (1970), Anton-Tay and Wurtman (1971), and Fuxe and Hokfelt (1969). With the development of histochemical fluorescence techniques for identifying biogenic amines in the brain, it has been demonstrated that norepinephrine and serotonin are highly concentrated in the hypothalamus and midbrain (Vogt, 1954; Brodie et al., 1959). Anden et al. (1964) showed that the median eminence is rich in dopaminergic nerve terminals and Bjorklund et al. (1970) demonstrated noradrenergic terminals in the median eminence. Chemical assays showed relatively large amounts of norepinephrine and dopamine in the median eminence (Rinne and Sonninen, 1968). More recently, Piezzi et al. (1970) have observed that the bovine median eminence also contains high concentrations of serotonin. Since the techniques for detection of cholinergic neurons are not as sensitive or as specific as histochemical fluorescence, much less is known about the distribution of these fibers in the brain. However, Shute and Lewis (1969) have demonstrated cholinergic fibers in the lateral preoptic and mammillary regions of the hypothalamus.

These amines may be released into the hypothalamo-pituitary portal system from neurons whose cell bodies lie in the medial hypothalamus and whose nerve endings are terminated in the median eminence and the infundibulum near the primary capillary loops of the portal system (Fuxe and Hokfelt, 1969). Halasz (1969) has shown that the medial basal area of the hypothalamus, the so-called hypophysiotropic area, is essential for tonic release of anterior pituitary hormones.

Coppola et al. (1966) and others postulated that a sympathetic tonus, originating in the hypothalamus, normally acted to stimulate the release of FSH and LH while restraining the secretion of prolactin. In the absence of this tonus, FSH and LH secretion were suppressed while prolactin release was stimulated. Evidence for this postulate came from pharmacological studies in which certain drugs which interferred with or enhanced catecholamine activity were used, and gonadotropin secretion was monitored. Drugs such as reserpine, a catecholamine depletor, and chlorpromazine, a potent adrenergic blocker, were shown to stimulate deciduoma formation (Barraclough and Sawyer, 1959), block ovulation (Coppola et al., 1966), induce pseudopregnancy (Coppola et al., 1965), and induce lactation in rabbits (Meites, 1957; Kanematsu et al., 1963). These reports suggest an increase in prolactin secretion and a decrease in gonadotropin secretion.

Early work from our laboratory indicated that injections of epinephrine and norepinephrine induced lactation in estrogen-primed rats and rabbits (Meites, 1959a; 1962; Meites et al., 1963). The observations that such drugs as reserpine and chlorpromazine were able to stimulate pseudopregnancy and lactation (Meites, 1959a) suggested that they induced prolactin release. Ratner et al. (1965) further demonstrated that reserpine decreased hypothalamic production of PIF, providing an explanation of how reserpine evoked increased prolactin secretion. Mizuno et al. (1964) further demonstrated that injections of iproniazid, a monoamine oxidase inhibitor and therefore depressor of catecholamine metabolism, inhibited postpartum lactation in rats, suggesting decreased secretion of prolactin. This provided the first evidence that brain catecholamines are

inhibitory to anterior pituitary secretion of prolactin. Further work by Lu et al. (1970) showed that reserpine, chlorpromazine, α-methyl-meta-tyrosine and α-methyl-para-tyrosine, which are all catecholamine depressants, produced increases in serum prolactin levels indicating that they decreased hypothalamic PIF activity. On the other hand drugs as L-dopa (the immediate precursor of dopamine) or monoamine oxidase inhibitors (pargyline, iproniazid, or Lilly compound-15641), each known to enhance hypothalamic catecholamine activity, significantly decreased serum prolactin values (Lu and Meites, 1971). Lu and Meites (1972) later showed that L-Dopa increased PIF activity in the hypothalamus. Another neuroleptic drug, haloperidol, reduced brain catecholamines and also markedly elevated serum prolactin by decreasing PIF in the hypothalamus (Dickerman et al., 1974). These reports led to the concept that hypothalamic catecholamines, including dopamine and norepinephrine, act as neurotransmitters to increase the release of PIF, which in turn enters the pituitary portal vessels to inhibit pituitary prolactin release (Meites et al., 1972).

There is evidence to suggest that the catecholamines have a direct effect on the anterior pituitary to alter prolactin release. Gala and Reece (1965) observed that some doses of epinephrine increased prolactin release by rat pituitary. However, Jacobs et al. (1968), MacLeod (1969), and Birge et al. (1970) reported that catecholamines including dopamine, norepinephrine and epinephrine inhibited prolactin release by rat pituitary tissue in vitro and concluded that catecholamines may represent the undefined hypothalamic PIF. Koch et al. (1970) demonstrated a biphasic effect of catecholamines

on prolactin release. High doses of catecholamines produced inhibition, intermediate doses no effect and low doses caused stimulation of prolactin release. Recent reports suggest that catecholamines, particularly dopamine, may be a PIF. Shaar et al. (1973) demonstrated that dopamine when incubated with pituitary halves inhibited prolactin release as compared to controls without dopamine. This work has been confirmed by Samli and MacLeod (1974), Ojeda et al. (1974) and Dibbet et al. (1974). Subsequently, Shaar and Clemens (1974) have shown that hypothalamic extracts subjected to catecholamine absorption on alumina gel lost their ability to inhibit prolactin release. Their results indicated that the prolactin inhibiting activity of hypothalamic extracts can be totally accounted for by the endogenous catecholamines normally present in the hypothalamus and further indicate that a catecholamine may be a PIF. These same workers presented evidence that dopamine is present in the portal blood system (Shaar, personal communication). However, Takahara et al. (1974) have evidence that a non-catecholamine PIF is present in the hypothalamus.

Indoleamines and catecholamines appear to work in opposition on control of pituitary prolactin release (Meites et al., 1972). When serotonin is given centrally, it increases prolactin (Kamberi et al., 1970; 1971). Systemic injection of 5-hydroxytryptophan, a precursor of serotonin, also increases serum prolactin (Lu and Meites, 1973). It is interesting to note that indoleamines exert an inhibitory effect on pituitary secretion of gonadotropins (Kamberi et al., 1970; 1971; Fraschini, 1970), in opposition to the effects of the catecholamines (Kamberi et al., 1970; Schneider and McCann, 1970).

Early investigations with cholinergic drugs indicated that atropine, a cholinergic blocking agent, could inhibit ovulation in the rat (Everett et al., 1949) and in the rabbit (Sawyer et al., 1949). Other drugs which act like atropine also blocked ovulation (Sawyer, 1963). Kamberi and Bacleon (1973) reported that injections of stropine into the third ventricle inhibited the proestrous surge of gonadotropins and prolactin.

Cholinergic drugs have been shown to influence lactation and pseudopregnancy. Atropine in high doses given systemically induced lactation (Meites et al., 1960b) and pseudopregnancy (Gitsch and Everett, 1958), whereas low doses inhibited lactation (Jacobson et al., 1950) and pseudopregnancy (Grosvenor and Turner, 1958). Pilocarpine, a cholinomimetic agent, or physostigmine, an acetylcholine esterase inhibitor, induced lactation in rats (Meites et al., 1960). However lactation is not a specific indicator of prolactin secretion. Recent work from our laboratory (Grandison et al., 1974) has shown that injections of acetylcholine into the lateral ventricle of female rats significantly decreased serum prolactin. Systemic injections of either pilocarpine or physostigmine also decreased serum prolactin levels in female and male rats. The mechanisms by which acetylcholine inhibits prolactin release is not clear at present. Previous work has shown that acetylcholine has no direct effect on pituitary prolactin release (Talwalker et al., 1963). There is the possibility that the effects of acetylcholine are mediated through the hypothalamus and affect the PIF/PRF system.

III. Functions of Prolactin

A. Mammary Gland

Eighty-two different actions have been reported for prolactin. Historically the best-known category of actions consists of effects related to reproduction (Nicoll and Bern, 1972). The most widely known of these in mammals is stimulation of mammary gland development and lactation, and in avian species stimulation of pigeon crop milk production.

Classical experiments by Lyons and co-workers (1958) used hypophysectomized, ovariectomized, and adrenalectomized rats as a model, and showed that injections of estrogen and GH produced ductal growth whereas injections of estrogen, progesterone, GH and prolactin elicited lobulo-alveolar growth. These studies were confirmed by the in vitro experiments of Elias (1957) and Rivera (1964). Undeveloped mammary glands from mice and guinea pigs were cultured for 5-7 days with combinations of insulin, estrogen, progesterone, GH and prolactin, and these showed lobulo-alveolar growth. Prolactin and GH can induce mammary lobulo-alveolar growth equivalent to that seen in pregnancy in the absence of ovarian hormones. Thus transplantation of a pituitary mammotropic tumor that secreted high amounts of prolactin, GH and ACTH into adreno-orchidectomized rats of the inbred Fischer strain resulted in mammary lobulo-alveolar growth equivalent to that seen during pregnancy (Clifton and Furth, 1960). Related experiments by Talwalker and Meites (1961) showed that prolactin and GH injections into rats

which were adrenalectomized and ovariectomized or adrenalectomized, ovariectomized and hypophysectomized were able to stimulate lobulo-alveolar growth. Turner (1939), Lyons et al. (1958) and Meites and Hopkins (1961) demonstrated that ovarian hormones have no ability to stimulate mammary gland development in the absence of anterior pituitary hormones. It has been suggested by Meites and Nicoll (1966) that the gonadal steroids stimulate secretion of anterior pituitary hormones (prolactin and GH) and synergize with the pituitary hormones to sensitize the mammary tissue to these hormones.

After development of mammary glands, the minimal requirements for initiating or maintaining lactation appear to be prolactin and ACTH or adrenal cortical hormones (Meites, 1966). Combinations of prolactin and adrenal glucocorticoids were observed to initiate lactation in hypophysectomized rats (Folley, 1956), and in hypophysectomized, adreno-gonadectomized rats (Lyons et al., 1958). Adrenal glucocorticoid hormones alone initiated lactation in the pregnant rat (Talwalker and Meites, 1961) and cow (Tucker and Meites, 1965), and either prolactin or hydrocortisone acetate induced lactation in the pregnant rabbit (Meites, 1966). There is an increase in secretion of prolactin and adrenal cortical hormones at the end of pregnancy, permitting the onset of lactation. The milking stimulus together with milk removal from the mammary gland serves to maintain postpartum lactation (Meites et al., 1972).

A look at the cellular actions of prolactin provides some clues as to how it stimulates mammary gland development and milk production. More recently Turkington (1972a) has reviewed the molecular biology of prolactin. He reported the ability of prolactin to

stimulate RNA synthesis leading to the production of specific milk proteins. Ovine prolactin can induce synthesis of casein, α-lactalbumin and β-lactoglobulin in mammary epithelial cells formed in culture (Turkington, 1968). The induction of milk protein synthesis is prevented by actinomycin D and this suggests that prolactin may require new DNA-directed RNA synthesis for milk protein synthesis. RNA and DNA content increase between day 18 or pregnancy and day 3 of lactation and the combination of insulin, prolactin and hydrocortisone stimulate RNA synthesis in both prepartum and postpartum tissue (Mohrenweiser and Emery, 1973). When lactating rats are hypophysectomized the mammary gland undergoes several changes. Gland weight decreases and levels and synthesis of RNA and DNA are retarded (Baldwin and Martin, 1968). DNA levels and rates of synthesis were maintained by prolactin which also partially maintained gland weight. Rates of casein and cytoplasmic protein synthesis were maintained at normal levels by prolactin. For maintenance of optimal conditions of the mammary gland, both prolactin and cortisol seemed necessary. In mammary gland explants addition of prolactin to the insulin-hydrocortisone medium allows daughter cells to complete their ultrastructural differentiation; this includes appearance of secretory protein granules (Mills and Topper, 1970; Green and Topper, 1970). There is ample evidence that prolactin is a true metabolic hormone with respect to its action on the mammary gland. It was of interest therefore to measure prolactin receptor binding activity of the mammary gland of the rat throughout pregnancy and lactation as part of this thesis.

B. Pigeon Crop Sac

In a review by Riddle (1963) the pigeon crop sac was described. Only two limited lateral areas of the total crop are involved in "milk" production. Histological examination showed that only the mucosa hypertrophies and forms crop-milk. The deeper lying cells persistently divide and contribute to a thickening of the crop wall. These cells form globules of fat, begin to disintegrate, and the desquamated dead cells form the whitish masses called crop milk. Riddle and Braucher (1931) demonstrated that the crop-sac response is controlled by an anterior pituitary hormone. Riddle, Bates and Dykshorn (1932; 1933) isolated and named the hormone "prolactin" and showed that it evoked a crop-sac response as well as stimulated milk secretion in mammals. Nicoll and Sherry (1967) demonstrated that the prolactin-induced stimulation of crop-sac mucosa is mediated by RNA and protein synthesis. Riddle and coworkers described a systematic method for quantitative assay of prolactin based on weight increase of the crop sac after 4 daily intramuscular injections of prolactin. Another assay developed by Lyons and Page (1935) involved intracutaneous injection over the crop sac. Until the advent of a radioimmunoassay for prolactin (Niswender et al., 1969), the pigeon crop sac response was the standard assay procedure for prolactin and is still used as a bioassay for prolactin. As reviewed by Bern and Nicoll (1968) one of the main uses of the pigeon crop sac assay has been to distinguish between prolactins from various vertebrates such as teleosts and poikilothermic tetrapods and mammals.

C. Ovaries

Prolactin is the major luteotropin in the rat. In hypophysectomized rats with fresh corpora lutea, prolactin administration maintained functional corpora lutea and deciduoma formation (Astwood, 1941; Evans et al., 1946; Ahmad et al., 1969). Increased levels of prolactin can maintain functional corpora lutea for prolonged periods of time. Everett (1956) showed that removal of the anterior pituitary in rats from its connection with the diencephalon and placing it under the kidney capsule caused a prolonged secretion of prolactin and maintenance of luteotropic activity. Subsequent studies by Nikitovitch-Winer and Everett (1958) in which anterior pituitary grafts were placed under the kidney and in the anterior chamber of the eye, also demonstrated a prolonged luteotropic activity in rats. Chlorpromazine, reserpine (Barraclough and Sawyer, 1959) and perphenazine (Ben-David, 1968), are all stimulators of prolactin secretion (Lu et al., 1970; Danon et al., 1963), and all prolong luteotropic activity. Injections of ovine lactogen for 5-7 days from the day of estrus induced pseudopregnancy in normal or hysterectomized rats (Anderson, 1968). Saito et al. (1970) showed that prolactin administration to hypophysectomized rats, soon after ovulation had been induced by PMS and HCG, also induced pseudopregnancy.

The above studies indicate that prolactin is able to stimulate progesterone secretion from rat corpora lutea. That prolactin was able to maintain progesterone secretion was shown by MacDonald and Greep (1968). The mechanism of action of prolactin on corpora lutea

involves ovarian enzymes. Hashimota and Wiest (1969) explained the luteotrophic action of prolactin by its antagonistic action to luteal 20-α-hydroxysteroid dehydrogenase and in part by its mobilization of a progesterone precursor. The enzyme, 20-α-hydroxysteroid dehydrogenase (20αHD) converts progesterone to a 20α dihydro derivative which has no progesterone activity. A 3-4 fold increase in 20αHD activity in the ovary is induced by treatment of rats with various ergot alkaloids, and this increase is prevented by exogenous prolactin (Lamprecht et al., 1969). Prolactin administration to rats bearing three day old corpora lutea increases progesterone secretion, decreases the synthesis of 20αHD, and influences the rate of cholesterol turnover in lutein tissue (Armstrong et al., 1969). In a subsequent study by Armstrong et al. (1970) 30 day old female rats were treated with PMS and HCG to induce ovulation and then they were cervically stimulated to induce pseudopregnancy. Hypophysectomy of these rats decreased progesterone secretion and increased the level of 20α-hydroxypregn4-en-3-one, an inactive progesterone derivative. Prolactin treatment begun a few hours after hypophysectomy considerably increased progesterone and decreased the levels of the inactive progesterone derivative, and increased free and esterified cholesterol levels. The activity of two other enzymes involved in progesterone metabolism, 5α-reductase and 3β-hydroxysteroid dehydrogenase, is decreased after prolactin and administration (Zmigrod et al., 1972). In addition to influencing progesterone metabolism, prolactin also causes cholesterol accumulation in both interstitial and luteal compartments of the ovary in intact and hypophysectomized immature rats (Zarrow and Clark, 1969). Behrman et al.,

(1970) and Behrman and Greep (1972) demonstrated that prolactin was able to induce enzymes controlling luteal cholesterol ester turnover, sterol acyl transferase and sterol esterase.

In addition to being the main luteotropin in the rat, there is evidence that prolactin also may influence luteal function in the mouse, hamster, ferret, rabbit, cow, pig, and sheep. In 1935, Dresel reported that injections of prolactin into cycling mice produced persistent leucocytic vaginal smears and a suspension of estrus. Prolactin is part of the luteotropic complex in the hamster (Greenwald, 1967a). Pseudopregnancy was induced in cyclic hamsters with a combination of prolactin and FSH (Grady and Greenwald, 1968). In the ferret, isolation of the hypophysis by pituitary stalk section does not interfere with luteal function (Donovan, 1963). Stalk sectioned animals in whom ovulation is induced by sterile mating show typical pseudopregnant changes, including the ovaries which contain large secretory corpora lutea. Donovan (1967) reported that the factor secreted by the isolated hypophysis to maintain luteal function was prolactin. Prolactin was the only hormone to support luteal function in the hypophysectomized ferret. Unlike the rat, mouse and ferret, the role of prolactin in larger animals is not clear. It may act synergistically with other hormones to maintain luteal function. In the hypophysectomized rabbit, prolactin is not able to maintain corpora luteal function (Kilpatrick et al., 1964). Hilliard and Sawyer (1966) showed that although prolactin was not able to acutely stimulate steroidogenesis in the rabbit ovary, it could enhance the responsiveness of the preovulatory ovary to LH and thus increase basal progesterone secretion. They concluded

that prolactin affects steroidogenesis in the rabbit ovary by maintaining availability of steroid precursors. A later study by Hilliard et al. (1968) showed that LH accelerates the synthesis and release of 20∝ -hydroxypregn-4-en-3-one (20∝-OH) from rabbit ovarian interstitial tissue, concomitant with a loss of interstitial cholesterol. When ovarian cholesterol is depleted by LH, less steroid is released, and chronic prolactin treatment promotes cholesterol storage, restores the basal output of 20∝-OH and enhances sensitivity of the ovary to LH in the intact rabbit. In the hypophysectomized rabbit it is necessary for prolactin and estrogen or LH to be administered in order to elevate cholesterol stores and induce progestin release. Bartosik et al. (1967) and Romanoff et al. (1966), using perfused bovine ovaries in vitro, demonstrated that prolactin infusion caused an increase in progesterone secretion rate and an increase in acetate-1-^{14}C incorporation into progesterone. Prolactin infusion into ovaries in situ was seen to increase the secretion rate of progesterone in sheep (Domanski and Dobrowolski, 1966; Domanski et al., 1967). Hixon and Clegg (1969) reported that 50 mg of prolactin was able to significantly increase ovarian progesterone secretion in the hypophysectomized ewe. Cook et al. (1969) showed that prolactin was able to enhance progesterone secretion in the pig but had no effect on luteal function in the ewe.

The most dramatic effect of prolactin on corpora lutea function is seen during pregnancy. Cutuly (1941) showed that rats hypophysectomized 1 to 5 days after mating and treated with lactogenic hormone, were able to implant and maintain pregnancy for periods ranging from 6 days to term. Cutuly (1941) suggested that

lactogenic hormone was capable of stimulating corpora lutea function. Meites and Shelesnyak (1957) administered large doses of prolactin and observed a lengthening of pregnancy by extending functional activity of corpora lutea. Hypophysectomy at days 1, 4, or 8 of pregnancy in the hamster resulted in regression of corpus luteum (Greenwald, 1967b). Treatment with prolactin and FSH increased vascularity of the corpus luteum and maintained pregnancy in these hamsters. Prolactin and FSH seem to be the luteotropic complex in the mouse as well as the hamster (Choudary and Greenwald, 1969). Pregnant mice hypophysectomized on day 6 and injected with 500 ug prolactin and 200 ug FSH maintained pregnancy. In dwarf mice prolactin seems to be the sole hormone necessary to maintain pregnancy and support corpora luteal function (A. Bartke, 1966a; A. Bartke, 1971c; A. Bartke, 1973). Robson et al. (1971) reported that in mice, prolactin is the major hormone to support pregnancy during the preimplantation period and after implantation LH seems to be necessary. In rabbits hypophysectomized during the second week of gestation, severe follicular involution and degeneration of corpora lutea became apparent within seven days (Spies et al., 1968). Prolactin in combination with either FSH and more effectively with estrogen restored ovarian luteal and interstitial tissue comparable to that of intact pregnant rabbits.

In the rat, prolactin is the major if not sole stimulus for luteal function until days 7 to 8 of pregnancy, when placental prolactin and pituitary LH become essential members of the luteotropic complex. From day 12 of pregnancy until parturition, LH is no longer required and placental prolactin alone may be sufficient

by itself to maintain the corpus luteum (Neill and Smith, 1974). The following reports seem to substantiate the above statement. Clemens et al. (1969a) showed that prolactin was necessary to maintain pregnancy in the rat from days 1 to 6. The pregnant rats were given hypothalamic implants of prolactin and this appeared to induce luteal regression and cessation of progesterone secretion. Rats hypophysectomized on day 5 or 6 of pregnancy and given prolactin, were only able to maintain pregnancy until day 10 (Greenwald and Johnson, 1968; Yang et al., 1973). If prolactin and ICSH or FSH or estrogen were given, rats maintained pregnancy until term showing that prolactin by itself is essential during the first half of pregnancy.

Morishige and Rothchild (1973; 1974) have shown that blocking prolactin secretion with ergocornine caused regression of corpora lutea until days 7-8 of pregnancy; after this period inhibition of pituitary prolactin secretion did not induce luteolysis. LH antiserum did not induce luteolysis until days 7-8, when it became highly effective. They also observed that in addition to hypophyseal LH, placental prolactin is necessary for maintenance of corpora lutea at days 7-8. In the absence of hypophyseal prolactin but in the presence of hypophyseal LH, removal of the uterus and thus placental prolactin at days 7-8, caused regression of corpora lutea. The corpus lutea of the rat are maintained at day 12 of pregnancy or later without hypophyseal support (Astwood and Greep, 1938). Moreover, Madhwa Raj and Moudgal (1970) showed that LH antiserum became ineffective in inducing abortion at day 12. The maintenance of corpora lutea and progesterone secretion during the last half

of pregnancy is probably accounted for by secretion of a placental prolactin (Neill and Smith, 1974). Recent work using a radioreceptor assay has reported 2 peaks of placental prolactin during pregnancy, one at days 10-14 and another at days 17-21 (Kelly et al., 1973). It also appears that prolactin is necessary to maintain corpora luteal function during lactation, since inhibition of lactation and corpora luteal function was induced by a hypothalamic implant of prolactin in postpartum rats (Clemens et al., 1969b).

Prolactin has the ability to be luteolytic as well as luteotropic in rats (Malven and Sawyer, 1966). The luteolytic effect of prolactin in the rat seems to be exerted on corpora lutea which have lost their capacity to secrete progesterone (Lam and Rothchild, 1973). In hypophysectomized rats, administration of prolactin either 2 or 5 days after hypophysectomy enhances luteal regression (MacDonald and Greep, 1969; Saito et al., 1970), whereas if prolactin administration is started soon after hypophysectomy it maintains the corpora lutea (Malven, 1969; Saito et al., 1970). The function of prolactin in the normal cycling rat may be to induce luteolysis. Wuttke and Meites (1971) demonstrated that the rise of prolactin during the estrous cycle of the rat serves to induce luteolysis of the older crop of corpora lutea during each cycle. This work was later confirmed by Gelato et al. (1972) and by Grandison and Meites (1972) in mice. Since one of the most important functions of prolactin is to regulate corpora luteal function it was thought of interest to study the ontogeny of prolactin receptor binding activity in ovaries from pubescence through adulthood, and during pregnancy and lactation.

D. Male Reproductive System

The role of prolactin in the male has not been clearly defined. In a recent report by Negro-Vilar et al. (1973), changes in serum prolactin as well as the gonadotropins were monitored during sexual development of the male rat. The serum hormone levels were correlated with organ growth rates and they found that the initial increase in testicular growth was preceded by a sharp rise in FSH and prolactin at 25 days of age. In immature hypophysectomized rats, pituitary transplants induced a significant increase in testicular growth (Negro-Vilar and Saad, 1972). They assumed prolactin was responsible for the testicular growth by activation of steroidogenic pathways leading to androgen biosynthesis. Hafiez et al. (1971) and Musto et al. (1972) showed that prolactin administration can increase 3-B-hydroxysteroid dehydrogenase and 17-B-hydroxysteroid dehydrogenase activity in the testis of dwarf mice. Injections of prolactin to hypophysectomized rats raised testosterone to detectable levels in half of the rats tested (Hafiez et al., 1972). In the same study LH increased the levels of testosterone in all rats, and when a combination of prolactin and LH were given there was a greater increase in testosterone than when LH alone was given. Prolactin has also been shown to promote accumulation of cholesterol esters in the mouse testis (Bartke, 1971a). Prolactin may influence testicular function as there is evidence reported that it stimulates testosterone production and synergizes with LH. Bartke (1971b) reported that LH produces more androgen in hypophysectomized rats when prolactin is present. Several reports have shown some effects of prolactin on

spermatogenesis in rats and mice (A. Bartke, 1965; A. Bartke, 1966a; A. Bartke and C.W. Lloyd, 1970a; A. Bartke, 1971b). However, these effects may be secondary to the ability of prolactin to stimulate androgen synthesis.

In addition to influencing testicular function, prolactin can influence the male accessory organs. Prolactin stimulated growth of seminal vesicles in tissue culture when added to medium (Bengmark and Hesselsjo, 1964). In castrate male mice prolactin caused an increase in seminal vesicle weight (Bartke and Lloyd, 1970b), and when testosterone was given in combination with prolactin the weight of these organs was increased further (Bartke, 1967). Castrate guinea pigs given testosterone and prolactin had seminal vesicles twice the size of the castrates receiving only testosterone (Antliff et al., 1960). Prolactin also synergizes with testosterone to increase the size of the prostate. Gonadectomized-hypophysectomized rats treated with either LH or STH alone produced no response in prostate weight but testosterone caused a doubling in ventral and anterior prostate weight (Chase et al., 1957). In these same animals prolactin plus testosterone produced a significant increase over the effect of testosterone alone. A similar report was presented by Grayhack (1963) who also showed that prolactin and testosterone synergize to increase prostate weight. In the absence of testosterone, prolactin can synergize with other hormones to stimulate prostatic growth. In immature castrate rats prolactin and ACTH synergize to increase ventral prostate weight (Tullner, 1963). If these immature rats are hypophysectomized as well as castrated, prolactin, ACTH and thyroxine can act to restore the weight of the ventral prostate.

Prolactin has also been shown to increase citric acid content of the lateral prostate in hypophysectomized-castrate rats in combination with testosterone (Grayhack, 1963; Grayhack and Lebowitz, 1967). More recently Moger and Geschwind (1972) reported that prolactin synergized with testosterone to increase fructose and citric acid content of the dorsolateral prostate, seminal vesicles and coagulating glands. They also showed that prolactin was able to increase zinc uptake in the absence of testosterone. In prostatic tissue homogenates of fourteen-week-old rats, prolactin stimulated adenyl cyclase activity whereas testosterone had no effect (Golder et al., 1972). This may be a possible mechanism of action for prolactin. It seems apparent that prolactin may indeed be an important "co-factor" in the male reproductive system.

E. Metabolic Function

Prolactin has been reported to be a somatic as well as a metabolic hormone (Riddle, 1963; Bern and Nicoll, 1968; Nicoll and Bern, 1972). Licht and Jones (1967) showed that prolactin increased food consumption and increased lean body weight in adult male lizards. These same effects of prolactin were seen in juvenile lizards along with a decrease in hepatic lipid content (Licht and Hoyer, 1968). Licht (1967) and Tassava (1969) demonstrated that injections of prolactin or pituitary grafts were able to stimulate tail regeneration in the lizard. A more recent report by Callard and Chan (1972) indicated a synergistic effect of prolactin and corticosterone to restore liver weight and glycogen content in the hypophysectomized lizard.

A somatic role of prolactin in tadpoles has been well established. Berman et al. (1964) and later Enemar et al. (1968) reported that mammalian prolactin caused increased body weight and increased tail length in tadpoles. It seems that prolactin is antagonistic to thyroxine in tadpoles and favors the retention of larval structures (Bern and Nicoll, 1968). Bern et al. (1967) countered the tail-resorbing influence of thyroxine added to aquarium water by injections of prolactin. Later Brown and Frye (1969a) showed that prolactin inhibited metamorphosis and promoted further growth in thyroidectomized tadpoles. In subsequent work, Brown and Frye (1969b) showed that prolactin was ineffective in stimulating growth in post-metamorphic frogs. Vellano et al. (1970) were able to show that in castrate or castrate-thyroidectomized newts, prolactin administration increased tail height. They concluded that height of tail in the newt is prolactin dependent. As well as stimulating growth in amphibians, prolactin has also been reported to increase carbohydrate content of the liver (Brown and Brown, 1971) and induce arginase activity in larval liver (Guardabassi et al., 1970).

Early work by Bates et al. (1937) and Schooley et al. (1941) demonstrated that prolactin had the ability to maintain body weight in hypophysectomized pigeons and in normal young pigeons, and was most effective in stimulating appetite and increasing intestinal, pancreatic, and liver tissue weight. Prolactin also synergized with other hormones to promote organ and body growth. Miller and Riddle (1943) reported that prolactin, desoxycorticosterone and thyroxine in combination had a greater effect than any of these hormones given

alone. One unit of prolactin daily reduced the amount of weight lost to less than half in untreated hypophysectomized pigeons, whereas when prolactin, desoxycorticosterone and thyroxine were given the hypophysectomized pigeons regained all their lost weight. In a later study Bates et al. (1962) reported that adequate doses of either prolactin or GH alone or together increased organ weight and body weight in hypophysectomized pigeons. An even greater increase was observed when thyroxine and prednisone were given together with GH and prolactin. They noted synergisms in the order of 50 fold when all 4 hormones were given. Prolactin not only increased liver size but stimulated liver function in the pigeon. Goodridge and Ball (1967) treated pigeons with prolactin and found an increased rate of hepatic fatty acid synthesis and increased rate in conversion of glucose to fatty acids. They also observed an increased utilization of glucose by tissues of prolactin-treated birds, and an elevated level of enzymes involved in lipogenesis. Prolactin is somatotrophic in other birds as well as the pigeon. In the Spotted Munia prolactin enhances body growth and influences spleen and liver weight (Chandala and Thapliyal, 1968; Chandola and Thapliyal, 1973). Increases in body fat were the result of prolactin administration to the white-throated Sparrow (Meier and Martin, 1971).

In mammals there is strong evidence indicating that prolactin is a metabolic hormone capable of stimulating growth. Bates et al. (1935) were able to stimulate growth in dwarf mice with a crude prolactin preparation. In a subsequent study Bates and co-workers (1942) demonstrated that pituitary extracts containing prolactin

caused a 30% increase in body weight of dwarf mice. Knobil (1959) observed striking stimulation of growth in young normal rats which was comparable to that seen in response to growth hormone. In male rats hypophysectomized for 7 days, prolactin caused an increase in body weight and cartilage width as compared to saline injected hypophysectomized rats (Cargill Thompson and Crean, 1963). As reported in the pigeons, prolactin also synergizes with other hormones in the rat (Bates et al., 1964; Milkovic et al., 1964). Prolactin alone caused an increase in nose to tip of tail length in normal rats, and when prolactin, GH and ACTH were given in combination a rapid increase in body weight of nearly 4 g per day was seen. Rats with transplantable mammotropic tumors, which secrete large amounts of GH, prolactin and ACTH, developed diabetes as well as enlarged livers (Bates et al., 1966; Wilson, 1969).

Prolactin appears to affect the metabolism of carbohydrate and fat in both liver and adipose tissue. Winegrad et al. (1959) removed epididymal fat pads from male rats and incubated with ovine prolactin. Prolactin in this preparation increased the production of CO_2 from glucose and increased the incorporation of glucose carbon into long chain fatty acids. It's interesting to note that prolactin and GH worked differently. GH also increased glucose oxidation to CO_2 but this increase was not accompanied by an increase in fatty acid synthesis from glucose. They felt that the data indicated prolactin stimulated phosphogluconate oxidative pathway in glucose utilization. Similar results were reported on the effect of prolactin on adipose tissue by Moore and Ball (1962) and Beck et al. (1964). They saw the same effect of prolactin and also noted the difference

between GH and prolactin, that is GH did not increase the incorporation of glucose carbons into long chain fatty acids in adipose tissue as did prolactin. Nejad et al. (1962) studied the conversion of glucose carbon to fatty acids and CO_2 in liver slices of hypophysectomized rats. In hypophysectomized rats the conversion of glucose to fatty acids and CO_2 was impaired. Administration of 300 ug GH for 14 days was not able to repair this defect but when 100 ug prolactin with 3 ug l-thyroxine (which was ineffective alone) were given, the lipogenesis was repaired. It is of interest to point out here that prolactin affects the metabolism of the mammary gland in the same way it does adipose tissue and liver, as cited above (Heitzman, 1968; Wang et al., 1971; Strong et al., 1971).

In mice prolactin has been reported to induce liver glycogenolysis (Elghamry et al., 1966), increase xanthine oxidase activity in liver and mammary gland (Chandra and Cole, 1961) and stimulate hepatic RNA synthesis as well as increase body weight by 16% (Chen et al., 1972). Rats bearing mammotropic tumors which secrete large amounts of prolactin, GH and ACTH, showed increased incorporation of acetate into long chain fatty acids, increased protein content and increased acetyl-CoA carboxylase activity which is the rate limiting enzyme in fatty acid biosynthesis (MacLeod et al., 1968). Placental lactogen stimulated the incorporation of glycine-2-C^{14} into liver protein of rats (Burt et al., 1969). The authors discussed the point that pregnant animals have higher incorporation of glycine-1-C^{14} than non-pregnant animals, and liver in pregnancy has higher nucleic acid content which may be related to the action of human placental lactogen. Turkington (1972b) also observed large increases in rates of RNA formation in liver during pregnancy and lactation.

Houssay and Penhas (1956) demonstrated a diabetogenic effect of prolactin in dogs that were hypophysectomized-adrenalectomized and had 15-18% of their pancreas removed. In a later study, DeBodo and Altszuler (1958) reported that prolactin seemed to improve the insulin hypersensitivity of hypophysectomized dogs. Continued administration of prolactin in hypophysectomized dogs caused elevation of post-absorptive blood sugar levels toward normal and decreased the responsiveness to insulin. These authors point out that prolactin differs from GH in that it does not produce severe hypoglycemia after first treatment. More recent work by Rothgeb et al. (1971) showed again that prolactin administration in the dog increased the concentration of glucose in the post-absorptive state but produced no change in insulin. Prolactin also increased the rate of glucose production and uptake in the post-absorptive state. Again mention is made of the difference between prolactin and GH. In the GH treated dog insulin was very high and this was not so with prolactin treatment. However, prolactin like GH in the dog increased glucose turnover, liver glycogen and plasma free fatty acids (Winkler et al., 1971). This effect of prolactin is not due to GH contamination since the amount of GH believed to be in the prolactin preparation was injected without any effect. In the cow prolactin administration caused an increase in non-esterified fatty acids (Williams et al., 1966), and feeding and arginine infusion in cows resulted in a significant increase in plasma prolactin (McAtee and Trenkle, 1971).

Human studies also point to the possibility of prolactin being a metabolic hormone. In 1958, Bergenstal and Lipsett observed that administration of ovine prolactin to human subjects who had been

hypophysectomized caused a retention of nitrogen and an increase in urinary amino acid nitrogen excretion, indicating these patients were in a positive nitrogen balance. In humans both placental lactogen and pituitary prolactin seem to induce carbohydrate intolerance and are thought to be physiological antagonists of insulin (Beck and Daughaday, 1967; McGarry et al., 1968). An intravenous injection of insulin into men and women caused a significant increase in plasma prolactin and rather low levels of glucose (10 mg/100 ml) also stimulated prolactin release (Wilson et al., 1972). The metabolic effects of human prolactin were assessed in a recent study in women. Berle (1973) reported that administration of human prolactin resulted in an increase in plasma B-hydroxybutyrate, a product of fatty acid degradation, and free fatty acids and a decrease in pyruvate and lactate indicating an effect on glucose metabolism and utilization.

There seem to be some common threads in the action of prolactin in these different species. Prolactin is able to stimulate growth in amphibians, birds, rats and mice, and can stimulate liver protein synthesis in rats and mice and influence carbohydrate and lipid metabolism in pigeons, rats, dogs, cows and man. It is quite possible that in avian and mammalian species there exists a "Metabolic Complex" of hormones consisting of GH, prolactin, adrenal corticoids and thyroid hormones. This is suggestive in the work of Bates et al. (1974). Part of the data to be presented in this thesis deals with studies measuring prolactin binding to liver membrane preparations.

F. Salt and Water Balance

Osmoregulation in lower vertebrates and mammals has been reported to involve prolactin. Burden (1956) showed that killfish were unable to survive in fresh water without the pituitary. Pickford and Phillip (1959) demonstrated that the pituitary factor able to overcome the effects of hypophysectomy in euryhaline fish was prolactin. According to Ball (1969) euryhaline teleosts are unable to survive in fresh water after hypophysectomy for more than a limited period, but they can live for much longer in sea water or in a fish Ringer solution. Prolactin seems to be specific since injections of ADH, oxytocin, GH, TSH or ACTH are without effect in hypophysectomized teleosts (Schreibman and Kallman, 1966). Blood osmotic pressure and sodium concentration fall after hypophysectomy in teleost. When the hypophysectomized fish are given injections of prolactin, they are able to maintain sodium levels near normal (Ball and Ensor, 1965; Ball and Ensor, 1967; Dharmamba, 1970). Prolactin has been reported to have the same sodium retaining effect in intact fish (Utida et al., 1971). Intact seawater adapted fish were given prolactin injections and showed significantly high plasma sodium concentrations. In some cases the sodium levels rose so high it became toxic to the fish.

In the teleost pituitary there is a specific region which is a source of prolactin-like hormone (Bern and Nicoll, 1968). The erythrosinophilic or eta cells are organized into a distinct rostral lobe. Ectopic transplants of rostral lobe in hypophysectomized fish permit survival in fresh water (Ball, 1965). The rostral lobe

comprises 8% of the total gland and increases to 42% in specimens held for long periods in fresh water (Blanc-Livini and Abraham, 1970). Prolactin in the rostral lobe also increases in fish held in freshwater for extended periods of time. This evidence indicates that prolactin is an important hormone in teleosts for sodium regulation. It's interesting to note that Hirano et al. (1973) reported an increase in labelled thymidine incorporation into DNA of the urinary bladder of the flounder after prolactin administration. The increase in sodium absorption after prolactin treatment followed closely the time course of thymidine incorporation. It's possible therefore, that prolactin action (sodium retention) in teleosts may be mediated through RNA and protein synthesis.

In the domestic duck prolactin has been postulated to aid in adaptation from freshwater to an estuarine environment. Prolactin infused intravenously in domestic duck caused an increase in nasal fluid output (Peaker et al., 1970). Therefore it may stimulate the inactive salt gland to start secreting at a high rate. Further work by Ensor and Phillips (1970) showed that the pituitary prolactin levels in the domestic duck increased after 2-3 days of salt loading. These workers concluded that prolactin may have a direct effect on salt gland of these birds and cause it to excrete sodium. These reports indicate a species difference in prolactin's osmoregulatory action.

The overall effect of prolactin in mammals seems to be sodium retention and promotion of antidiuresis, which is similar to its action in the teleosts. Rats given a single injection of either bovine or ovine lactogenic hormone show a decrease in the rate of

urinary excretion of sodium, which was interpreted to be due to a direct effect of these hormones on the renal tubules (Lockett and Nail, 1965). Lockett (1965; 1967) reported a direct effect of lactogenic hormones on the renal tubules in a perfused kidney preparation in cats. The perfusion of the cat kidneys with lactogenic hormone resulted in an increased renal blood flow and glomerular filtration rate followed by a retention of sodium. It's interesting to note that in Lockett's preparation, GH was also antidiuretic and caused the retention of sodium but did not alter renal blood flow or glomerular filtration rate. Prolactin in rats has been reported also to influence clearance of insulin and para-amino hippuric acid (Matthews, 1963). Ensor et al., (1972) demonstrated that dehydration in non-lactating rats was correlated with a 25% decrease in pituitary prolactin. In lactating female rats this fall was increased to 50%. Further experiments injecting ovine prolactin into intact female rats showed prolactin to be antidiuretic. Relkin and Adachi (1973) and Relkin (1973) reported that rats maintained on a low sodium diet had increased plasma and pituitary prolactin, whereas no change was seen in GH or TSH. They proposed that prolactin may enhance the aldosterone secretory rate in sodium deprived rats since aldosterone secretion was increased in these rats. Pallmore et al. (1970) reported that the pituitary is necessary to maintain aldosterone secretion in rats. The important hormone could possibly be prolactin. Salt loading (400 meq NaCl) in the ewe appears to negate the sodium retaining action of aldosterone. If these animals were injected with sheep pituitary prolactin the sodium retaining action of aldosterone was restored in spite of the high

salt (Burstyn et al., 1972). Further work by the same group of investigators (Horrobin et al., 1973) showed that ewes given 80 meq of NaCl, aldosterone promotes sodium retention. If cortisol is given along with the aldosterone it antagonizes the action of aldosterone and the animals lose sodium. Prolactin is able to override the effects of cortisol and aldosterone, again causing sodium retention. Therefore it's possible that prolactin may sensitize the renal tubules to the action of aldosterone. Rats treated with 2-bromo-α-ergokryptine had an increased sodium excretion as well as an increased fluid volume (Richardson, 1973). The actions of 2-bromo-α-ergokryptine on the kidney were attributed to the ability of the drug to decrease prolactin secretion.

In the human, Horrobin et al. (1971) reported that a single injection of prolactin produced a significant decrease in water excretion, sodium excretion, and increased plasma sodium levels. They suggested that prolactin may act on the proximal tubule. More recent studies on humans (Buckman and Peake, 1973a; Buckman and Peake, 1973b) showed that plasma prolactin was decreased after administration of hypotonic fluid and increased after administration of hypertonic fluid. The reports in humans seem somewhat conflicting. It is hard to fit the prolactin responses to hypo- and hyper-tonic fluids into the proposed action of prolactin on the kidney tubules to enhance sodium retention. Relkin (1974) reported this same phenomenon in rats. He saw a large increase in prolactin when he infused a 3% NaCl solution into rats and a decrease in prolactin with infusion of hypotonic NaCl solution. It's quite possible that the mechanisms of prolactin action as an osmoregulator are many sided, that is,

prolactin may respond to both a change in sodium concentration and a change in osmolality. In light of the present work, it was felt of interest to determine whether or not prolactin binds specifically to rat kidney homogenates.

G. Adrenal Function

Tullner (1963) and Bates et al. (1964) observed that prolactin was able to augment the adrenal weight response to ACTH. This appeared to indicate that prolactin may influence adrenal function. A clinical report by Ingvarsson (1969) showed that prolactin may cause sensitization of an ACTH refractory adrenal cortex. In a female rheumatoid arthritic patient, dependent on steroids, prolactin administration lessened the dosage of steroids required for treatment. After prolactin administration, a low excretion of 17-ketogenic steroids was found and a considerable increase in excretion of 17-ketogenic steroids in response to administration of ACTH after prolactin treatment. Witorsch and Kitay (1972) demonstrated that prolactin decreased adrenal 5α-reductase activity in hypophysectomized rats. Adrenal 5α-reductase converts corticosterone to reduced metabolites. The data suggest that prolactin plays an alternate physiological role in regulating hormone secretion by preventing the intra-adrenal conversion of corticosterone to reduced metabolites. More recently Lis et al. (1973) reported that prolactin was able to stimulate corticosterone synthesis. Using isolated rat adrenal cells in vitro they observed that prolactin administered in vivo partially restored the corticosterone biosynthesis in

adrenals from hypophysectomized rats. Further the combined treatment to hypophysectomized rats of ACTH and prolactin gave higher maximal response than treatment with ACTH alone. In addition to affecting adrenal corticoids, Piva et al. (1973) showed that prolactin also influences adrenal progesterone. Dexamethasone treated castrated female rats given an intravenous injection of ovine prolactin exhibited a marked increase in adrenal progesterone as measured by progesterone plasma levels. Prolactin was almost three times as effective as either human LH or ovine GH. With these reports on the ability of prolactin to influence adrenal steroid production, it was thought of interest to measure specific binding of prolactin to adrenal membrane preparations.

IV. Prolactin Interactions with Other Hormones

The endocrine system is a highly integrated affair, and excesses or deficiencies in one gland may alter the rate of production of hormones by others (Turner and Bagnara, 1971). Almost every physiologic adjustment within the endocrine system is effected by a balance between hormones acting together or in sequence. For example, complete and normal functioning of mammary glands requires estrogens, progesterone, insulin, prolactin, oxytocin, adrenal steroids, thyroid hormones and possibly others. The estrous and menstrual cycles require a multitude of hormones acting in concert to produce the observed changes (Schwartz and McCormack, 1972). Hormones in the body fluids never act alone, and one may be modified (potentiated, limited or secretion pattern altered) by others that

are present. There is evidence that progesterone, emanating from the placenta, prevents premature expulsion of the fetus by blocking the response of the uterine musculature to other hormones (Turner and Bagnara, 1971).

Synergisms are an important phenomenon in endocrinology. Some hormones increase the effectiveness of others that are present with them in low concentrations. Bates et al. (1964) and Milkovic et al. (1964) reported that for normal growth in rats several hormones are needed, such as growth hormone, prolactin, ACTH, and thyroxine. They also observed that the combination of hormones in physiological doses was much more effective in stimulating growth than any of the hormones administered alone in large quantities.

A. Effects of Estrogen, Testosterone and Progesterone on Prolactin Secretion

Estradiol can directly stimulate rat pituitary prolactin release when added to pituitary culture in vitro (Meites, 1966). Injections of estrogen in vivo depressed hypothalamic PIF activity in the rat (Ratner and Meites, 1964). This work indicated that estrogen acts to promote prolactin release both via the hypothalamus and by a direct action on the anterior pituitary. There are several reports which show that estrogen administration in vivo in rats increases serum and pituitary prolactin levels. Implants of minute quantities of estrogen in the median eminence of rats with DMBA-induced mammary tumors caused an increase in pituitary and serum prolactin levels as compared to rats implanted with cholesterol

(Nagasawa et al., 1969). In ovariectomized rats estrogen also stimulated prolactin secretion and it appears that small doses of estrogen are more effective than large doses (Chen and Meites, 1970). The ability of estrogen to stimulate prolactin secretion in ovariectomized rats was confirmed by Blake et al. (1972) and Kalra et al. (1973). Kalra et al. (1973) also observed that estrogen administration was able to stimulate the release of prolactin when injected on the morning of estrus into intact female rats and enhance prolactin release in castrate males. Data to be presented in this thesis will demonstrate that estrogen can also influence prolactin receptor binding activity in liver and mammary tissue.

In culture of rat pituitaries progesterone failed to stimulate prolactin release (Meites, 1966) whereas Meites (1959) reported that in vivo administration of large doses of progesterone increased pituitary prolactin content and elicited mammary growth and secretion in rats. More recent work on the progesterone effect on prolactin release indicated that in ovariectomized rats it can partially counteract the stimulatory action on prolactin release (Chen and Meites, 1970). Blake et al. (1972) observed that progesterone did not alter prolactin secretion in castrate female rats. However, Kalra et al. (1973) reported that 5, 10, and 25 mg of progesterone administered to ovariectomized rats stimulated prolactin release and a lower dose of 1.5 mg progesterone had no effect on prolactin. This may account for the discrepancy between the work of Kalra et al. (1973) and Blake et al. (1972). Certainly there is no doubt that prolactin enhances progesterone synthesis in several species as has been reviewed elsewhere in this literature survey (see Section II.B. Ovaries).

Testosterone appears to have no direct action on the pituitary to enhance prolactin secretion (Meites, 1966), whereas in vivo administration of testosterone has been observed to increase prolactin content slightly in the pituitary and stimulate mammary growth and secretion in rats (Meites, 1959). Kalra et al. (1973) reported that testosterone propionate in doses of 0.5 mg to 2 mg per rat significantly increased serum prolactin levels in castrate male and female rats. Testosterone propionate was more effective in male rats at 0.5 mg and 1 mg doses. As reviewed (see II. Functions of Prolactin C. Males) in this thesis, prolactin is able to stimulate testosterone synthesis in male rats and synergize with testosterone to stimulate growth of male accessory sex organs.

B. Thyroid Hormones

Early work suggested that thyroidectomy (McQueen-Williams, 1935) or thiouracil feeding (Meites and Turner, 1947) diminished pituitary prolactin content in rats. Administration of thyroid hormones in vivo on the other hand has been reported to stimulate pituitary prolactin secretion and milk production (Meites, 1966). Lu et al. (1972) recently confirmed that thyroidectomy decreased serum and pituitary prolactin levels. The in vitro data on thyroid hormones and prolactin release support the in vivo results. Incorporation of small amounts of thyroxine and triiodothyronine into a culture system significantly increased prolactin release over that of pituitary tissue not cultured with those hormones (Nicoll and Meites, 1963). Chen and Meites (1969) reported that in vivo

injection of thyroxine did not alter hypothalamic PIF activity in the rat suggesting that thyroxine acts directly on the pituitary to stimulate prolactin release. Subsequent work by Dibbet et al. (1973) showed that pituitaries from male thyroidectomized rats released significantly less prolactin into medium 199 than intact controls, whereas thyroxine treated male rats released slightly more prolactin into the medium than controls. In addition they again showed that triiodothyronine directly stimulates prolactin release when added to incubation medium containing pituitary halves.

In addition to influencing prolactin release by thyroid hormones, there is evidence to show that prolactin and thyroxine may act synergistically on some organ systems. Bates et al. (1962) and Miller and Riddle (1943) demonstrated that prolactin and thyroxine among other hormones are able to act synergistically in the pigeon to stimulate body and visceral growth. In the rat prolactin and thyroxine in combination restored lipogenesis in the liver after hypophysectomy (Nejad et al., 1962). Addition of thyroxine to culture medium of rat mammary glands containing suboptimal amounts of prolactin resulted in improved development of mammary glands (Singh and Bern, 1969). This same work was confirmed in vivo using rabbits by Nilsson et al. (1970). They observed that development of mammary gland was incomplete in thyroidectomized rabbits injected with prolactin. In light of the present work on prolactin-thyroid interaction it was thought of interest to determine the effects of hypo- and hyperthyroidism on prolactin receptor binding activity in female rats.

C. Glucocorticoids

In the rat, Meites (1966) reported that cortisol administered *in vivo* was able to increase prolactin content in the pituitary and stimulate mammary growth and secretion. Morishige and Leathem (1973) observed that in protein deficient pregnant and non-pregnant rats, adrenalectomy decreased serum prolactin. Exogenous corticosterone treatment restored serum prolactin levels to normal. These authors suggested that corticosterone may restore hypophysial prolactin secretion to normal, although Nicoll and Meites (1964) were not able to demonstrate a direct effect of either cortisol or corticosterone on pituitary prolactin release.

At parturition, in rats, prolactin and corticoids synergize to initiate lactation and promote milk secretion both *in vivo* (Meites, 1966) and *in vitro* (Turkington, 1972). In avian species corticosterone and prolactin act synergistically to stimulate gonadal growth (Meier *et al.*, 1971) and fat storage (Meier and Martin, 1971). Evidence will be reported in this thesis to show that adrenal corticoids influence prolactin receptor binding activity in target tissues.

D. Growth Hormone

There are no data available as of yet that show that growth hormone can alter the secretion of prolactin. However, there are many conditions during which both hormones are released or depressed or act synergistically to produce specific effects.

A classical example of synergism between the two hormones is in stimulating development of mammary glands. In ovariectomized-hypophysectomized rats given injections of GH and prolactin, considerable lobulo-alveolar growth was observed (Talwalker and Meites, 1961). For a more detailed discussion of this topic the reader is referred to Lyons et al. (1958) and Meites (1966). Growth hormone and prolactin also synergize with other hormones such as ACTH and thyroxine to stimulate body growth (Bates et al., 1964; Milkovic et al., 1964) and lipogenesis from glucose in the liver (Nejad et al., 1962) of rats as well as avian species (Bates et al., 1962).

There are many similarities between the two hormones. They are both produced by the acidophils of the anterior pituitary, and there are several mammotropic pituitary tumors which secrete large amounts of both prolactin and growth hormone (MacLeod et al., 1968; Meites, 1972). A review by McGarry et al. (1968) described the similarities in function of the two hormones. Both prolactin and growth hormone are able to stimulate free fatty acid synthesis, increase urinary calcium, induce carbohydrate intolerance, increase glucose utilization, cause nitrogen retention, stimulate body growth and were renotropic in rats or humans.

Prolactin and growth hormone not only resemble each other in some of their functional aspects, but also respond similarly to certain treatments. In female rats both growth hormone (Dickerman et al., 1972b) and prolactin (Voogt et al., 1970) increase at vaginal opening. During the estrous cycle of rats prolactin peaks late in the afternoon of proestrus (Wuttke and Meites, 1970) and growth hormone peaks on the day of estrus (Dickerman et al., 1972b).

Surgical procedures such as ovariectomy decrease serum prolactin (Chen and Meites, 1970) and decrease plasma growth hormone (Dickerman, 1971) in rats, whereas estrogen treatment increases both hormones (Chen and Meites, 1970; Dickerman, 1971). Thyroidectomy in rats also decreases prolactin (Lu et al., 1972) and growth hormone (Dickerman et al., 1972b).

The mechanisms underlying the similarities between these two hormones have yet to be unraveled. There is evidence that hormones such as human growth hormone, ovine prolactin, and human chorionic somatomammotropin, which are active as growth promoting and lactogenic hormones, have similarities in their amino acid sequences (Li, 1972).

E. Gonadotropins

In many physiological states there seems to be a divergence between prolactin and gonadotrophin secretion by the pituitary, including the puberal state (Kragt and Maskin, 1972; Voogt et al., 1970), suckling (Amenomori et al., 1970; Diebel and Bogdanove, 1970), and after castration or estrogen administration (Chen and Meites, 1970; Kalra et al., 1973). When prolactin secretion is low, gonadotrophin secretion is high, and vice versa. Clemens et al. (1969b) reported that implantation of prolactin into the median eminence of postpartum lactating rats permitted cycling to resume in otherwise diestrous rats. Earlier work by the same investigators (Clemens and Meites, 1968) showed that implantation of prolactin but not of cocoa butter in the median eminence of rats in early pregnancy

resulted in termination of pregnancy and resumption of estrous cycles. The above results indicate that prolactin may influence secretion of the gonadotropins by the pituitary. Subsequent work by Voogt et al. (1969) demonstrated that prolactin implanted into the median eminence of immature female rats stimulated pituitary FSH release. In pseudopregnant rats a prolactin implant increased serum LH and FSH and caused termination of pseudopregnancy (Voogt and Meites, 1971). This antagonism between prolactin and gonadotrophin secretion was also reported by Ben-David et al. (1971a). They showed that when the pituitary is secreting high amounts of gonadotrophin, prolactin secretion is suppressed.

V. Protein Hormone Receptors

A. ACTH and Angiotensin

For a hormone to activate a target tissue, it must first bind to some constituent of the cell. This first step in polypeptide hormone action had been studied indirectly for many years by measuring this effect of the hormone. Early work showed reversible binding of tritiated vasopressin to bladder tissue, localization of ^{131}I-insulin in sarcolemma and soluble fractions of muscle cells, and that the first step in TSH action on thyroid and of insulin on rat diaphragm was rapid, firm reversible binding to a superficial site, presumably receptors (Lefkowitz et al., 1971). This conclusion for TSH was based on the demonstration that thyroid slices incubated with TSH at 1°C then washed and incubated at 37°C without hormone,

showed persistent effects attributable to TSH stimulation. It was not until 1969 that two groups of researchers using ^{125}I-ACTH and ^{125}I-angiotensin, respectively, that methods applicable for direct study of the interaction of peptide hormones and their specific receptors on target cells were demonstrated.

Lefkowitz et al. (1969; 1970a) reported specific binding of ^{125}I-ACTH to adrenal tissue extracts. This binding was proportional to the amount of extract added, was not altered by insulin, and could be correlated to ACTH's ability to stimulate adenyl cyclase activity. Subsequent work by this group (Lefkowitz et al., 1970b) led to the development of a radioreceptor assay for ACTH utilizing the hormone-receptor complex. This assay was reported to have an absolute sensitivity of 1 pg of ACTH. The further characterization of ACTH receptors revealed that the membrane fraction of the adrenal extract displayed the greatest ability to bind ^{125}I-ACTH; only labeled ACTH bound to the fraction, FSH, HPL, and insulin did not; the use of Scatchard analysis showed two sets of receptors for ACTH, high affinity, low capacity and low affinity, high capacity; proteolytic enzymes, phospholipase, and sulfhydryl reagents all decreased ACTH binding whereas RNAase and DNAase were without an effect which suggested these receptors may be partially protein and/or lipid (Lefkowitz et al., 1971).

At about the same time Goodfriend and Lin (1969) demonstrated specific binding of labeled angiotensin to tissue fragments of rat uterus, rabbit aorta, bovine adrenal cortex, and a cell free particulate preparation from bovine adrenal cortex. Similar physical-chemical characterization work was done on angiotensin receptors as

had been reported for ACTH. Lin and Goodfriend (1970) reported that the binding of angiotensin was not altered by oxytocin, serotonin, acetylcholine, vasopressin or bradykinin; binding of angiotensin to uterus was maximum in 20 minutes at 10°C; binding was temperature and pH dependent; and heating and freeze-thawing decreased the specific binding of angiotensin to uterine tissue, although freeze-thawing had less of an effect on adrenal particles. Further work by Goodfriend and Lin (1970) showed binding for angiotensin II and angiotensin I and this binding did not correlate with ATPase or adenyl cyclase activity unlike the binding of ACTH.

These reports on the binding of ACTH and angiotensin were the beginnings of an approach to hormone research that has been extended by dozens of laboratories to many other peptide hormones. The general approach to the study of hormone-receptor interaction has been physical-chemical characterization and somewhat less of an approach has been physiological characterization.

B. Growth Hormone and Other Hormones

Analogous to the system described by Lefkowitz et al. (1970) for a radioreceptor assay for ACTH, Lesinak et al. (1973) used cultured human lymphocytes to establish a radioreceptor assay for human growth hormone (HGH). They demonstrated that ^{125}I-HGH binds specifically to cultured human lymphocytes since other species of growth hormone, as well as bovine TSH and pork insulin did not alter binding. The authors proposed this system as a quantitative biological assay. More recently Tsushima et al. (1974) reported use

of rabbit liver membrane preparations for a radioreceptor assay to measure GH. The use of this type of assay system has revealed that plasma as well as pituitary immuno-reactive HGH comprises at least two discrete components, "big" HGH and "little" HGH (Gorden et al., 1973). Apparently big HGH component has much less activity in the radioreceptor assay than in radioimmunoassay, whereas little HGH component has similar activity in both assays. It is possible that this tool can have important clinical applications.

Human growth hormone also has been reported to bind specifically to rat and rabbit liver microsomal membrane preparations (Posner et al., 1974) and to membrane fractions of 7,12-dimethylbenzanthracene (DMBA) induced rat mammary tumors (Kelly et al., 1974a). The ontogeny of growth hormone receptor binding activity in the liver of female rats revealed that specific binding increased as the animals reached sexual maturation, and during late pregnancy the binding for GH was 222% of control levels (Kelly et al., 1974b).

Binding and receptor interaction have been reported for several other peptide hormones, such as glucagon binding to rat liver plasma membranes (Robdell et al., 1971), ADH binding to porcine renal membranes (Campbell et al., 1972), calcitonin binding to purified renal plasma membranes of rat (Marx et al., 1972), parathyroid hormone interaction with rat renal plasma membranes (Malbon and Zull, 1974), oxytocin receptors in uterus of rat and sow (Soloff and Swartz, 1974), TRH binding to plasma membranes of bovine anterior pituitary (Borden and Labrie, 1973), and binding of somatomedin to skeletal, liver and placental membranes (Hintz et al., 1974). All hormone-receptor binding reactions were shown to be specific and in the case

of TRH, ADH, glucagon and parathyroid hormone binding was correlated with stimulation of adenyl cyclase activity.

C. Insulin

Insulin-receptor interaction has been described for liver cell membranes, fat cell membranes and human lymphocytes. House and Weideman (1970) demonstrated binding of ^{125}I-insulin to membranes from hepatic parenchymal cells. Maximum binding was observed to occur in less than 2.5 minutes at 0°C. Cuatrecasas et al. (1971) further described the binding of insulin to liver cell membranes as a saturable process which is a time dependent reaction following second order kinetics. Radiolabeled insulin was only displaced from the membranes by unlabeled insulin. Hormones such as glucagon, GH, albumin or biologically inactive, reduced or oxidized chains of insulin had no ability to alter the binding of ^{125}I-insulin. A more quantitative study on the interaction of insulin and its receptor in liver plasma membranes revealed two classes of receptors, a high affinity, low capacity site and a low affinity, high capacity site (Kahn et al., 1974). At 30° the binding of insulin to liver membranes was a rapid reaction and by 15 minutes binding had reached almost 90% of its maximum value. The amount of binding was increased at 4°, although it took longer to reach a steady state. This indicates that the binding reaction is temperature dependent.

Insulin binding to fat cell membranes and human lymphocytes is very similar to binding in liver cell membranes. There also seems to be two types of receptor sites in fat cells and lymphocytes that

is high affinity, low capacity and low affinity, high capacity (Gavin et al., 1973; Hammond et al., 1972). Binding is also temperature dependent and is increased at lower temperatures in fat cells and lymphocytes.

Insulin receptors in liver, fat cells and lymphocytes respond similarly to treatment with various enzymes. Trypsin destroys binding of insulin to its receptors in its target tissues, whereas treatment of these receptors with phospholipase A and C enhances insulin binding (Cuatrecasas et al., 1971; Cuatrecasas, 1971; Krug et al., 1972). Apparently perturbation of phospholipids of liver and fat cell membranes by digestion with phospholipase C or A results in the appearance of new binding sites for insulin (Krug et al., 1972).

There are two other substances, wheat germ agglutinin and concanavalin A, both plant lecithins, which interact with insulin receptors. The wheat germ agglutinin enhances the specific binding of insulin to fat cells and liver membranes, whereas higher concentrations inhibit binding of insulin (Cuatrecasas and Tell, 1973). The enhancement is not due to unmasking of receptors and the inhibition occurs because the wheat germ agglutinin in high concentrations binds to a site on the insulin macromolecule (Cuatrecasas, 1973). Concanavalin A only displaces insulin from its receptor on fat cells and liver membranes. Both plant proteins have insulin-like activity (Cuatrecasas and Tell, 1973).

Some physiological characterization of insulin-receptor interaction has been done. Kahn et al. (1973) reported that obese-hyperglycemic mice bind less insulin to liver membranes than their

thin litter mates. They showed that this was due to a decrease in the number of receptors and correlated well with the insulin resistance these obese mice exhibit. There is no change in insulin binding in the liver during development in the rat but receptor binding does increase significantly during pregnancy (Kelly et al., 1972). These same workers (Kelly et al., 1974b) also demonstrated insulin binding to kidney membranes as well as DMBA induced mammary tumors (Kelly et al., 1974a). Whether binding in the kidney and these DMBA tumors is related to function has yet to be determined.

D. Gonadotropins, LH and FSH

Hormone-receptor interactions have been studied in the testis for human luteinizing hormone (HLH), human chorionic gonadotropin (HCG) and human follicle stimulating hormone (HFSH) and in the ovary for HLH and HCG. The receptor preparations used for the testis vary from crude homogenates to purified plasma membrane preparations (Catt and Dufau, 1973). In these receptor preparations, interstitial cells, intact cells and homogenized cell fractions, HLH and HCG, show the same amount of binding (Cat and Dufau, 1973). Human FSH on the other hand binds more appreciably to the seminiferous tubule homogenate (Bhalla and Reichert, 1974). Means (1973) reported that HFSH bound membrane fraction of tubular testicular cells, indicating that the tubular binding sites for HFSH are located on the surface of the cell. Autoradiographic studies have shown this to be true for the binding sites of HLH and HCG on the interstitial cells. In the case of all three hormones, HLH, HCG and

HFSH, the binding reaction to their respective receptors is temperature dependent with higher initial association at 37°C, the optimum pH is in the range of 7.5, and is not significantly altered by calcium (Catt and Dufau, 1973; Means, 1973; Bhalla and Reichert, 1974).

Altering the structure of HCG by removing the sialic acid or galactase residues and removing sialic acid from FSH does not affect the binding of these hormones to their receptors (Catt and Dufau, 1973; Means, 1973). However, Dufau et al. (1974) have reported that the disulfide bonds form an important component of the receptor for HCG in the testis and appear to be essential for hormone-receptor interaction. Solubilization of the gonadotropin receptor in the testis showed similar binding as the homogenate of receptors but the affinity of the receptors for ^{125}I-HCG was reduced by 50% (Dufau and Catt, 1973).

The binding of HCG and HFSH has been correlated with stimulation of adenyl-cyclase system. The synthesis and release of cyclic AMP into incubation medium of rat testis was detectable after addition of HCG, this same response was seen for HCG and testosterone synthesis (Catt and Dufau, 1973). An early response to FSH was observed to be accumulation of cAMP and an increase in protein kinase activity (Means, 1973). The stimulation of adenyl cyclase and protein kinase activity seems to be a common result in the interaction of protein hormones and their receptors.

The binding of HLH and HFSH in the testis appears to be age dependent. HFSH binds more to testis homogenates of immature rats than adult rats (Means and Vaitukaitis, 1972) whereas HLH has

higher specific binding in testis homogenates of 130 day old rats than either 5 day or 22 day old rats (Sharpe et al., 1973). The binding of HLH correlates well with increase in Leydig cells seen at around 50 days in the rat.

The receptors for HLH and HCG in the corpus luteum of the human, bovine and rat respond similarly as the gonadotropin receptors in the testis. The binding reaction for the gonadotropins in the corpus luteum is temperature dependent with equilibrium being reached within 30 minutes at 37°C, the optimum pH is 7.5, and calcium or magnesium don't appreciably alter the binding (Lee and Ryan, 1972; Cole et al., 1973; Haour and Saxena, 1974). In the rat corpus luteum, trypsin, α-chymotrypsin, and phospholipase C and D inhibited binding of HCG and HLH (Lee and Ryan, 1972), whereas in the bovine corpus luteum only pepsin and phospholipase A were able to inhibit the binding of HCG. The use of these enzymes indicates that the receptors in the corpus luteum are lipoproteins and possibly covered by glycoproteins.

The binding of radiolabeled HCG and HLH were considered to be specific since both unlabeled HCG and HLH administered in vivo and in vitro inhibited the binding of HLH and HCG by the corpus luteum (Lee and Ryan, 1972; Braendle et al., 1972). The subunits of LH (ovine and human) and HCG are considerably less potent than the native hormones in inhibiting the binding of ^{125}I-HLH by corpus luteum (Lee and Ryan, 1973). This is also true for gonadotropin (LH, HCG, and FSH) receptors in the testis (Catt and Dufau, 1973; Means, 1973).

Initial radioautography studies revealed that binding of ^{125}I-HLH and HCG was predominantly localized to the surface corpus luteum (Midgley, 1973). Subsequent work with electron microscopic radioautographs showed that the majority of the silver grains (from ^{125}I-HCG) in luteal and thecal cells was located at the plasma membranes of these cells (Han et al., 1974). These studies were confirmed with localization of LH (HCG) binding sites by fractionation of subcellular organelles (Rajaniemi et al., 1974a). This analysis demonstrated that specific radioactivity (^{125}I-HCG) was predominantly with fractions representing mostly plasma membrane particles.

Luteal and testis binding sites have been applied to the development of radioligand-receptor assays for HCG, LH and FSH (Saxena et al., 1974; Catt et al., 1971; Reichert and Bhalla, 1974). These receptor assays are not as sensitive as radioimmunoassays for LH but they provide a powerful tool for measuring biological activity of these hormones (Catt and Dufau, 1973). Reichert and Bhalla (1974) used a rat testis tubule receptor assay to compare the properties of FSH from several species. A more practical application of this type of receptor assay used plasma membranes of bovine corpora lutea of early pregnancy to measure HCG and in this way, detect pregnancy within 6 to 8 days after conception (Saxena et al., 1974).

E. Prolactin

Radioautographic studies have shown that an intravenous injection of ^{125}I- or ^{131}I-prolactin becomes distributed throughout the body (Birkinshaw and Falconer, 1972; Rajaniemi et al., 1974b). In

both males and females uptake was shown by liver and kidney. Female rats and mice showed weak labeling of prolactin in the ovaries and mammary tissue and male rats displayed some uptake by the testis, prostate and seminal vesicles (Rajaniemi et al., 1974b). Midgley (1973) using autoradiographic analysis demonstrated specific binding of ^{125}I-ovine prolactin to the corpora lutea of rats. He also reported that the functional state of the corpora affected the binding of prolactin, that is, new corpora bound more prolactin than old corpora. These radioautographic studies demonstrated that prolactin bound to the surface of the cell in the tissues, and indicated that the receptors for prolactin on its target tissues are located on the membrane.

With these radioautographic reports providing a basis, the direct study of prolactin and its receptor interaction was started. Turkington and Frantz (1972) using tissue homogenates reported specific binding for prolactin in mammary glands, liver, kidney, and seminal vesicles. The ^{125}I-ovine prolactin binding by these tissues was not altered by either LH or TSH. Subsequent work by Turkington et al. (1973) and Friesen et al. (1973) demonstrated the specific binding of radiolabeled prolactin to purified plasma membranes of adrenals and ovaries in addition to liver, kidney and lactating mammary glands. The pigeon crop sac also binds prolactin and this binding induces proliferation (Mishkinsky et al., 1972).

The binding of ^{125}I-labeled prolactin to mammary gland particles was complete by 20 minutes at either 4°C or 37°C, although at 37°C more prolactin was bound (Frantz et al., 1974). Scatchard analysis of prolactin binding to mammary gland particles revealed a Kd of

9.1×10^{-9} mol/l and the number of binding sites were estimated to be 15.5×10^{-13} mol/mg protein (Frantz et al., 1974). These same workers reported that treatment of mammary gland particles with trypsin decreased the specific binding of prolactin whereas neuroaminidase, RNAase and DNAase had no effect. This suggests that peptide bonds are required for prolactin-binding activity. Heating of the binding particles at 70°C for 10 minutes completely removed hormone-displaceable binding activity further suggesting its macromolecular nature (Frantz et al., 1974). Solubilization of prolactin receptors from mammary gland increased the affinity of the receptor five-fold over the particulate receptor (Shiu et al., 1974).

An application of prolactin binding sites in mammary gland has been the development of a radioreceptor assay for prolactin (Shiu et al., 1973). The assay is able to measure prolactin in several species as well as distinguish between prolactin preparations of varying potencies. Probably the most important clinical application of this system is the determination of prolactin receptor binding activity in mammary carcinomas. Kelly et al. (1974a) demonstrated that DMBA induced rat mammary tumors specifically bound ^{125}I-ovine prolactin and this binding was correlated to the growth response of these tumors to prolactin, that is, prolactin dependent tumors displayed the greatest amount of binding. Turkington (1974), using three different types of experimental mammary carcinomas, showed the same phenomenon. The DMBA-induced carcinomas which are prolactin dependent displayed greatest binding; the R3230AC carcinoma, which is responsive to prolactin for milk protein synthesis but not growth, showed some binding; and the C3HBA carcinoma, a relatively

autonomous tumor, had no detectable binding. Prolactin receptors have also been demonstrated in an estrogen-receptor deficient rat mammary carcinoma and these receptors are similar to those found in lactating rat mammary tissue (Costlow et al., 1974). These results provide evidence for prolactin receptors in mammary carcinomas and suggest that the degree of prolactin dependence of such carcinomas may be characterized by the relative number of prolactin receptors present. This is a powerful tool for the clinician which may provide him with an easy test of responsiveness of human breast cancers to hormones and evaluation of specific treatments.

The topic of this thesis is the study of prolactin receptors in mammary glands, ovaries and liver tissue with the major emphasis on physiological characterization. Recently one such report has appeared on prolactin receptors in the liver (Kelly et al., 1974b). They found that receptors increase with age, during pregnancy and lactation and females have more receptors for prolactin than males.

There are certain properties that all protein hormone receptors have in common, as the foregoing discussion has revealed. These properties are: 1) high affinity for binding a specific hormone; 2) a requirement for a high degree of structural specificity in the hormone which is bound; 3) the hormone binds rapidly and the population of receptors is easily saturable with hormone, but binding is reversible; 4) a restricted distribution among various cell types of the animal, being present in highest concentration in the hormonal target organ; 5) the receptor is macromolecular in nature, with protein and lipid moieties; and 6) formation of the hormone-receptor complex serves to activate hormone-dependent processes in the target

cell (Turkington et al., 1973). Hormone-receptor interaction can be summarized by the following schematic representation using prolactin binding to mammary alveolar cells as a model:

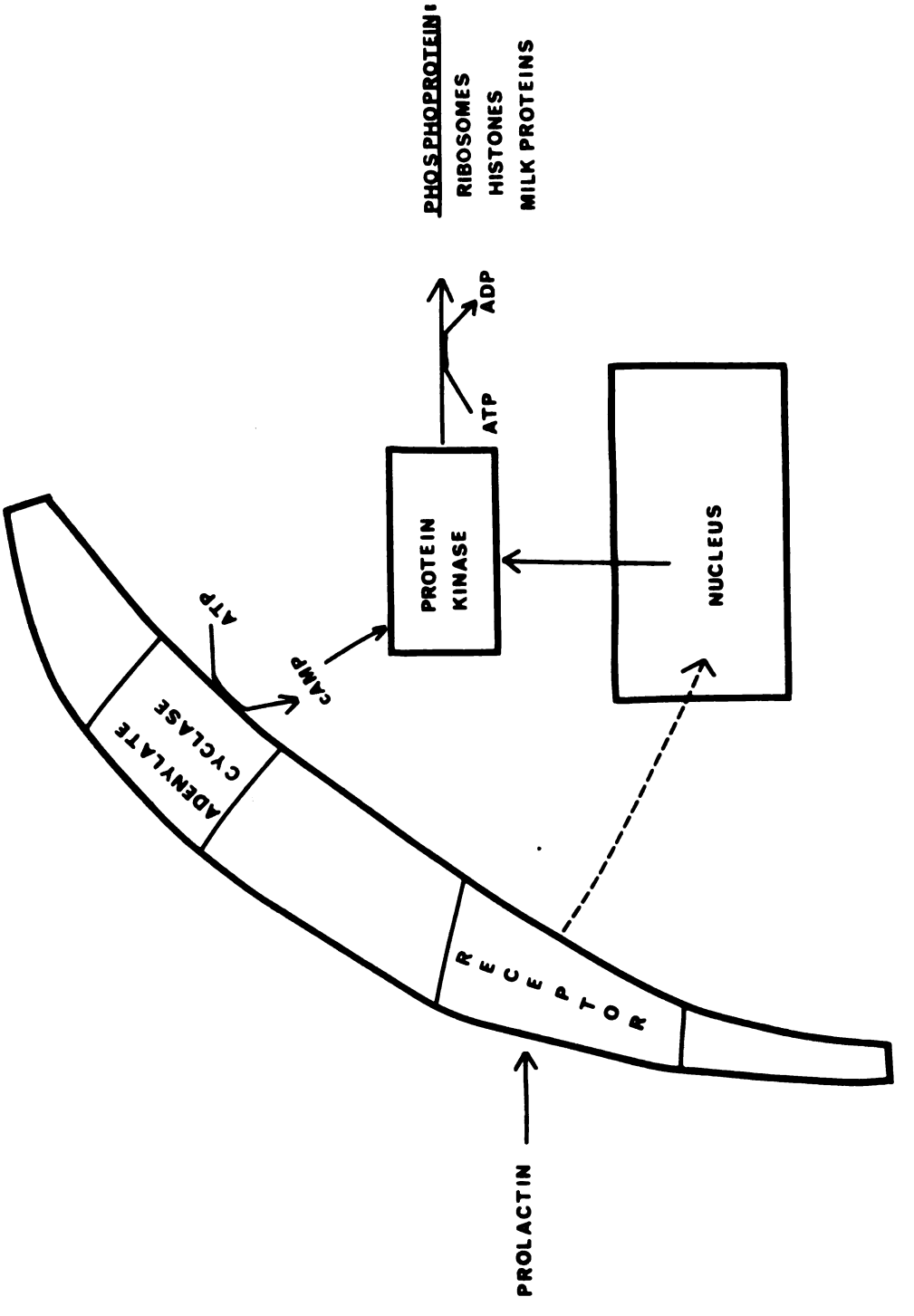

Figure 1. Prolactin - Receptor Interaction (Turkington et al., 1973)

MATERIALS AND METHODS

I. Animals

Immature and mature female rats of the Sprague-Dawley strain were purchased from Spartan Research Animals, Inc. (Haslett, Michigan). Mature female rats of the Long-Evans strain were obtained from Blue Spruce Farms, Inc. (Altamont, New York). All rats were housed in metal wire cages in temperature-controlled (25°C±1°C) and artificially illuminated (lights on from 5:00 AM until 7:00 PM daily) rooms, and were maintained on a standard diet of Purina Rat Chow (Ralston Purina Co., St. Louis, Missouri) and tap water ad libitum.

All surgically treated animals were given a postoperative intramuscular injection of 0.2 ml Longicil S (Fort Dodge Laboratories, Fort Dodge, Iowa) immediately following surgery. Thyroparathyroidectomized animals were maintained on .1% calcium lactate solution ad libitum for eleven days post-operatively, followed by tap water for the remainder of the experimental period. Adrenalectomized animals were maintained on .9% sodium chloride solution ad libitum for the entire experimental period.

Estrous cycles were determined by examining daily vaginal smears. Only females showing at least 2 regular 4 or 5 day cycles were used in the ovarian cycle experiments. Pregnancy was

designated by examining for sperm in vaginal lavage. The day sperm were found was considered day 1 of pregnancy.

II. Surgical Procedures

All surgery was performed under ether anesthesia with clean, non-sterile technique.

A. Thyro-parathyroidectomy

A midline incision was made about one-half an inch in length on the ventral side of the rat approximately half way between the sternum and the first cervical vertebrae. The submaxillary salivary glands, subcutaneous facia and the skin were retracted, exposing the sternoid muscle. The sternoid muscle was also divided in the midline parallel to the striations of the muscle, exposing the trachea, the thyroid and parathyroid glands. All tissue from the incision was held with small retractors throughout the operation. First the isthmus of the thyroid was severed using a small pair of scissors. Next the thyroid gland was dissected away from the trachea using fine curved forceps with great care to avoid injury to the carotid artery and the recurrent laryngeal nerves which lies deep near the thyroid on either side. Finally the subcutaneous facia was sutured with silk and the skin closure was completed by means of autowound clips.

B. Ovariectomy

Bilateral incisions a half inch in length were made, approximately one inch caudal to the last rib and approximately three-quarter inches ventral to the spinal column, through the skin and lateral abdominal muscles. The ovary together with the uterus and adipose tissue were withdrawn. A pair of forceps was inserted through the mesentery near the oviducts, a ligature was placed around the fat to avoid damage to the uterus. The ovary was removed and the uterus and adipose tissue returned to the body cavity. The muscle was closed with silk, and the skin with auto-wound clips.

C. Adrenalectomy

Bilateral incisions one-half inch in length were made, approximately one-quarter inch rostral to the last rib and about three-quarter inches ventral to the spinal column, through the skin and lateral abdominal muscles. Fine curved forceps were used to locate the fat pad situated atop the kidney which contains the adrenal gland. The adrenal gland was teased apart from the fat pad with forceps and care was taken to avoid damaging the kidney. The muscle was closed with silk, and the skin with auto-wound clips.

III. Preparation of Hormones

A. Hormones for Injection

Sodium-L-Thyroxine Penthydrate (Nutritional Biochemicals Co., Cleveland, Ohio) solutions were prepared in 0.87% saline by first adjusting the pH to 10 with .1 N NaOH so that the thyroxine would dissolve. The thyroxine (T_4) solution was then titrated to pH 7.9 by using a pH meter and 0.1 N HCL. Finally the volume was brought to 10 ml by addition of .87% saline, pH 7.9 to yield a concentration of 100 μg/ml or 10 μg/.1 ml. The T_4 solution of 25 μg/ml was prepared by taking 2.5 ml of the 100 μg/ml solution and diluting to 10 ml with .87% saline, pH 7.9, yielding a solution of 2.5 μg/.1 ml.

Estradiol benzoate (Nutritional Biochemicals Co., Cleveland, Ohio) was prepared from a stock solution of 100 μg estradiol benzoate per ml, by taking a certain amount of the stock solution and diluting it with Mazola corn oil to yield a volume to appropriate concentration for injection in the various experiments.

Hydrocortone Acetate (Merck Sharp & Dohme, West Point, Pa.) in saline suspension was used. The stock solution of 50 mg/cc was diluted using 0.87% saline solution to a final concentration of 100 μg/0.1 cc. Progesterone (Searle Chemicals, Inc.) was prepared in corn oil. The concentration of the final solution was 20 mg/cc.

Ovine prolactin (NIH-S10) and ovine growth hormone (NIH-S11) were prepared by addition of the hormone to 0.87% NaCl made alkaline by 0.1 N NaOH until the hormone dissolved, and then titrated to

pH 7.9 by addition of 0.1 N HCl. Each hormone was prepared every other day for the course of the experiment. Both ovine prolactin and growth hormone were injected in a concentration of 1 mg per .2 cc.

B. Hormones for Cross-reactivity Studies

All hormones used for cross-reactivity studies were dissolved in alkaline distilled water and then diluted to final concentration using 0.25 M TRIS-HCL containing 10mM $CaCl_2$ adjusted to pH 7.8. The following hormones were used: ovine prolactin (NIH-S10; 25.6 units/mg); ovine growth hormone (NIH-S11; 0.56 IU/mg); ovine luteinizing hormone (NIH-S15; 0.99 USP units/mg); ovine thyroid stimulating hormone (NIH-S6; 2.47 USP units/mg); ovine follicle stimulating hormone (NIH-S7; 30.6 IU/mg).

IV. Radioreceptor Assay for Prolactin

A. Preparation of Tissue Samples

At the termination of each experiment tissue samples were immediately removed from the animals and placed on dry ice and stored frozen. At the time of assay the tissue samples were thawed and homogenized in 3 to 5 volumes 0.3 M sucrose. All homogenizations were carried out at 4°C or in ice baths. Liver tissue was homogenized using a Virtis tissue homogenizer (Virtis Co., Gardener, N.Y.)

at medium speed for three minutes; kidney, adrenal, and ovarian tissue were homogenized using a pyrex brand tissue grinder (A.H. Thomas Co., Philadelphia, Pa.); mammary tissue was homogenized using a Waring blender adapted with a mini-cup (A.H. Thomas Co., Philadelphia, Pa.) and subjected to two 15 second pulses of homogenization. After homogenization the samples were centrifuged at 750 g for 20 minutes at 4°C and the supernatant collected. The supernatant was centrifuged at 15,000 g for 20 minutes at 4°C and the supernatant was again collected. This second supernatant fraction was centrifuged at 100,000 g for 90 minutes at 4°C and at the end of this centrifugation the pellet was collected. The pellet contains microsomal membranes. The pellet was resuspended in 0.25 M TRIS-HCL containing 10mM $CaCl_2$ adjusted to pH 7.8. In order to resuspend the pellets they were rehomogenized using a pyrex brand tissue grinder. The final product was a fine suspension of microsomal membranes. This microsomal membrane preparation was assayed for protein content by Lowry method (Lowry et al., 1951). The preparation was diluted to a final concentration of 300 µg protein/100 µl for liver tissue, 1000 µg protein/100 µl for kidney tissue and 200 µg protein/100 µl for adrenal, ovarian and mammary tissue.

B. Iodination of Ovine Prolactin

Ovine prolactin (NIH-S10; 25.6 IU/mg) was iodinated using a lactoperoxidase method developed in our laboratory. Ovine prolactin (5 ug in 20 ul distilled water), 1.0 mCi carrier free Na ^{125}I (Amersham/Searle, Chicago, Illinois), 5 ug lactoperoxidase in 10 µl

of distilled water, and 20 µl of 30% hydrogen peroxide solution (Mallinckrodt Chemical Works, St. Louis, Mo.) diluted 1:30,000 in distilled water were added to the reaction vial in the order stated. The reaction was carried out for two and one-half minutes at which time 200 µl of 16% sucrose solution was added to the vial. The entire contents of the reaction vial were layered on a Sephadex G50 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) column coated with 1% Egg albumin in 0.25 M TRIS-HCL containing 10mM $CaCl_2$ at pH 7.8. The column was eluted with 0.25 M TRIS-HCL containing 10mM $CaCl_2$ at pH 7.8. The major peak from the Sephadex G50 filtration was repurified on a Sephadex G100 column. The major peaks from the Sephadex G100 filtration were then tested for their ability to bind specifically to microsomal membrane preparations and then used in the radioreceptor assay. The labeled o-prolactin was diluted to approximately 50,000-70,000 cpm/100 µl for the assay.

C. Assay Procedure

The procedure used is a modification of the method developed by Shiu et al. (1973). Two series of tubes were assayed for each tissue sample (Microsomal membrane preparation). The first is total binding tubes (TB) and they contain the following: (1) microsomal membrane sample in 100 µl; (2) diluent which is 0.25 M TRIS-HCL and 10 mM $CaCl_2$ with 0.1% BSA (Bovine serum albumin) at pH 7.8 - 300 µl; (3) labeled ^{125}I-ovine prolactin in 100 µl. The second is non-specific binding tubes (NSB) and they contain: (1) microsomal membrane sample in 100 µl; (2) diluent same as in TB tubes - 200 µl;

(3) labeled ^{125}I-ovine prolactin in 100 µl; and (4) unlabeled ovine prolactin 1 µg/100 µl. The final volume of each set of tubes (TB and NSB) is 500 µl. Both TB and NSB tubes are allowed to incubate at 4°C for 24 hours in the case of liver membranes and for 48 hours for all other membrane preparations. At the end of the incubation period three millileters of cold diluent are added to the TB and NSB tubes and they are centrifuged at 750 g for 30 minutes at room temperature. This centrifugation allows a pellet to form which is the aggregation of labeled hormone bound to the receptors on the membranes. At the end of this centrifugation the tubes are decanted and left inverted for at least 10 minutes. They are then counted in a gamma counter (Nuclear Chicago, Searle Inc.) for 60 seconds. TB and NSB tubes are assayed in quadruplicate for each sample.

Specific Binding is then calculated for each sample based on total binding and non-specific binding. Specific binding equals cpm ^{125}I-o-prolactin bound to receptor in absence of unlabeled o-prolactin (total binding) minus cpm in presence of unlabeled o-prolactin (non-specific binding). Percent specific binding is then the cpm (specific binding) divided by the total cpm of ^{125}I-ovine prolactin added to the assay tubes. All data are expressed in terms of percent specific binding.

V. Scatchard Analysis

There are several mathematical procedures for treatment of data obtained in competitive protein binding assays, radioimmunoassays,

and other types of binding of small molecules by macromolecules (Rodbard, 1973). These methods are based on an idealized model for the dynamics of the reaction between the radioligand (antigen) and the binding material (antibody). They entail transformations of the response variable in accord with the model, often to linearize the response and also to provide constants which are useful in describing the displacement curves observed. These constants represent quantitative estimates for the parameters of the model. Since the underlying model is an idealization of the analytical curves which are generated for extraction of the constants, it does not fit the data perfectly. The idealized model can be summarized in part by the following important assumptions (Kahn et al., 1974): (1) the hormone is present in a homogeneous form (prolactin in this case); (2) no appreciable cooperativity exists between binding sites; (3) one hormone molecule can react with only one binding site (even though the site can occur in groups). In addition, in order to secure valid data using labeled hormone tracer, it is generally regarded to be essential that: (4) labeled and unlabeled hormone behaved identically; (5) full chemical equilibrium was achieved by the end of the experiment; (6) bound and free hormone were perfectly separated without perturbing the equilibrium.

If assumptions 1-3 are made for the interaction between a hormone and a single class of receptor sites then by invoking the first order law of mass action, the hormone-receptor interaction at steady state can be described as:

$$H + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HR \qquad (1)$$

where H is the concentration of free hormone; R the concentration of receptor sites; HR, the concentration of hormone-receptor complex; k's are the constants of proportionality and they are also rate constants. This reaction system can be seen to react with second order chemical kinetics. The affinity constant k_d (equilibrium constant for dissociation) can now be defined as follows. Let the rate of appearance of complexed hormone and receptor be described as

$$\frac{dHR}{dt} = k_1(R)x(H) \quad \text{or} = k_1(R\text{-}HR)xH \tag{2}$$

and let the rate of loss of complex through dissociation be

$$\frac{-dHR}{dt} = k_{-1}(HR) \tag{3}$$

then

$$\frac{dHR}{dt} = \frac{-dHR}{dt} \tag{4}$$

and so,

$$k_1(R\text{-}HR)H = k_{-1}(HR) \tag{5}$$

Since the k_d (or K_m) has been defined as, k_{-1}/k_1, then,

$$K_d = K_m = \frac{k_{-1}}{k_1} = \frac{H(R\text{-}HR)}{HR} \tag{6}$$

and expanding the above equation

$$K_d = \frac{HR\text{-}H(HR)}{HR} \tag{7}$$

rearranging

$$HR = \frac{HR - H(HR)}{K_d} \tag{8}$$

and

$$\frac{H-R}{H} = \frac{1}{K_d}(R-HR) \tag{9}$$

which is the Scatchard relation.

Alternatively, since $K_d = 1/K_a = \frac{k_1}{k_{-1}}$, the Scatchard expression may be written

$$\frac{HR}{R} = K_a(R-HR) \tag{10}$$

If we assume that 1 mole of hormone is bound to 1 mole of receptor (assumption 3), then HR represents the concentration of bound H and substituting q for R according to the notation which has been developed for the RIA, we get

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

This formulation generates a straight line when the ratio bound/free hormone is plotted as a function of bound hormone, with a slope of $-K_a$ and the intercept on the bound axis equal to q. If the model chosen above to describe the system is correct the data should fit this relationship.

Data for Scatchard analysis was generated using the standard inhibition curve where the amount of labeled hormone is constant and the dosage of cold hormone is varied. The data were handled as follows: 1) counts of ^{125}I-ovine prolactin that were bound at

each dose level were noted; non-specific binding of labeled prolactin was determined using an excess amount of unlabeled prolactin; this value was subtracted from the counts bound at each dose level in order to obtain specific binding. 2) The counts for specific binding at each dose level were divided by the value for the total amount of labeled prolactin added (cpm); this value represents the ratio of labeled prolactin bound to the total amount of labeled prolactin (B/T). 3) The total amount of prolactin (labeled + unlabeled) was estimated for each tube from the amount of unlabeled prolactin administered at each dose level and the amount of labeled prolactin present as determined from the specific activity. 4) The total amount of hormone present was multiplied by the value for B/T to give an estimate of the total amount of prolactin bound at each dose level of unlabeled prolactin. 5) The bound value obtained was subtracted from the total amount of prolactin present to give an estimate of the amount of free prolactin present at each dose level. 6) Finally, the value for the total amount of hormone bound was divided by the value for total amount of free hormone. 7) The B/F ratio thus calculated was then plotted against the B for each dose level of unlabeled prolactin. From this plot as previously mentioned we obtained a fairly straight line, indicating a reasonable fit for the model. An unweighted linear regression was therefore made on the data and the slope was interpreted as $-K_a$ and the x intercept as the maximum bindable prolactin.

On the assumption that 1 mole of prolactin binds to 1 mole of receptor (assumption 3 above), the moles of maximum bindable

prolactin can be translated into moles of receptor or q. In order to convert from nanograms to moles, the values for the slope is multiplied by the nanogram molecular weight for prolactin (-2.3×10^{13}) to give K_a (affinity constant) and the value for the X-intercept is divided by 2.3×10^{13} to give moles of receptor.

VI. Statistical Analysis

Sample means and standard errors were calculated. The level of significance of difference in specific binding of prolactin for various treatment groups were determined using an analysis of variance (ANOVA) for unequal group numbers (Sokal and Rohlf, 1969). If the result of the ANOVA was significant, Duncan's new multiple range test (Duncan, 1955) was used to evaluate the significance of differences between groups. The data for ontogenesis of prolactin binding activity were analyzed by using an unweighted linear regression to establish the dependence of binding activity and time during development (Sokal and Rohlf, 1969).

EXPERIMENTAL

I. Demonstration of Specific Binding of Prolactin to Liver, Ovarian and Mammary Gland Membrane Preparations

A. Objectives

Specific binding for prolactin has been reported for lactating mammary tissue, liver, kidney, adrenal, ovaries and seminal vesicles (Friesen et al., 1973; Turkington et al., 1973). Inasmuch as we were interested in working with prolactin receptor binding activity, it was necessary to establish specificity of binding for the tissues we selected as model systems. The present study was undertaken to assure that no cross reactivity of the labeled prolactin and other protein hormones exists at the prolactin binding sites, and to determine the doses of unlabeled prolactin that would displace labeled prolactin in liver, ovarian, and mammary tissues.

B. Procedures

1. Membrane Preparations

Microsomal membrane preparations were made for liver, ovarian and mammary gland tissue by the methods previously described (see

Materials and Methods, IV. Radioreceptor Assay). All membrane preparations in this study were aliquoted at 300 μg protein/100 μl.

2. Animals

Liver membranes were prepared from intact female Sprague-Dawley rats. Long Evans female rats, which had shown two consecutive estrous cycles were used for the donors of ovaries for membrane preparations. The ovaries from different stages of the cycle were pooled. Mammary gland microsomal membranes were obtained from the glands of lactating rats.

3. Hormone Preparations

Hormones used were NIH ovine preparations of TSH, LH, GH, FSH and prolactin. In the cross reactivity studies dosages of 10, 100, and 1000 ng of TSH, LH and GH were used. For the competitive inhibition curves between ^{125}I-labeled prolactin and unlabeled prolactin, using the different membrane preparations, unlabeled prolactin was added in a dose range of 0.5 ng to 1000 ng.

4. Assay Procedures

Total binding of ^{125}I-prolactin was determined for each of the membrane preparations (see Materials and Methods, section IV. Radioreceptor Assay). To assay tubes which contained a membrane preparation diluted to 300 μg protein/100 μl diluent and approximately 50,000

to 70,000 cpm of ^{125}I-prolactin, were added the different unlabeled hormones such as GH, LH, prolactin, etc. This was done in order to determine the amount of ^{125}I-prolactin added to the tubes that could be displaced by these unlabeled hormones.

In this experiment binding of ^{125}I-prolactin was expressed as a percent of the total hormone bound in the absence of any unlabeled hormones.

C. Results

Figures 2, 3 and 4 show that LH, TSH or GH were not able to displace the labeled prolactin from either liver, ovarian or mammary gland microsomal membrane preparations during the incubation period. Ovine GH showed a slight cross reactivity at the 1000 ng level, but this could be accounted for by the stated contamination of the NIH preparation with prolactin. The amount of prolactin stated to be present in the ovine GH-NIH preparation was less than 0.5 IU/mg. On the other hand, non-labeled prolactin readily displaced the labeled prolactin in all three microsomal membrane preparations tested (Figures 2, 3 and 4).

D. Conclusions

These results demonstrate that only unlabeled prolactin can displace the labeled prolactin in liver, ovarian and mammary tissue microsomal membrane preparations. These data also show that prolactin is able to compete with the radiolabeled hormone at physiological

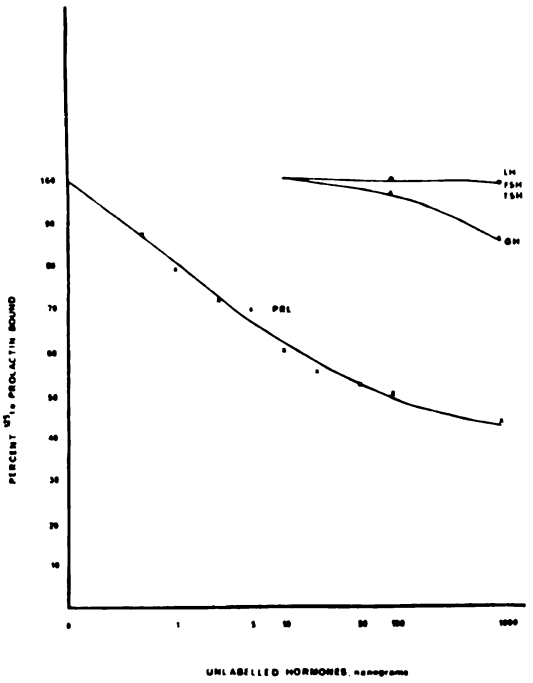

Figure 2. Competitive Displacement of ^{125}I-Radiolabelled Ovine Prolactin from Mammary Gland Membranes of Female Rats. Rat mammary gland membranes were incubated with 77,000 cpm of ^{125}I-o-Prolactin. Total bound cpm were approximately 16,000. Non-specific binding was 10% of the total radioactivity added.

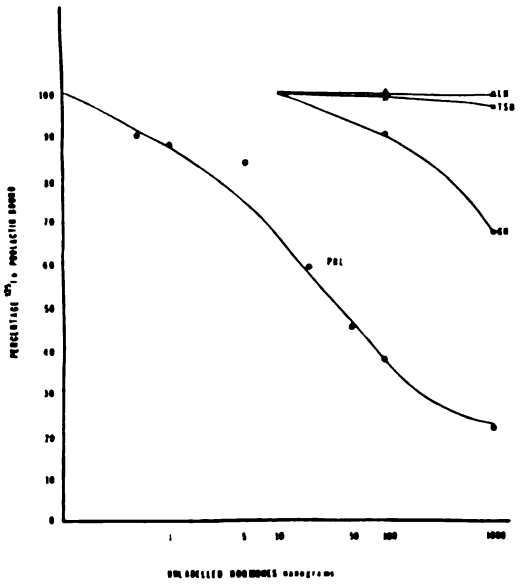

Figure 3. Competitive displacement of ^{125}I-radiolabelled ovine prolactin from ovarian membranes of rats. Rat ovarian membranes were incubated with 100,000 cpm of ^{125}I-o-Prolactin. Total bound cpm were approximately 14,500. Non-specific binding was 6% of total radioactivity added.

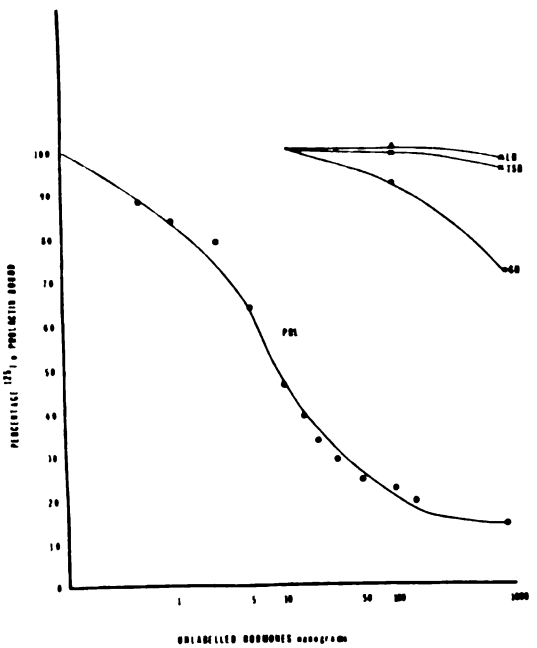

Figure 4. Competitive displacement of ^{125}I-radiolabelled ovine prolactin from liver membranes of female rats. Rat liver membranes were incubated with 70,000 cpm. Total bound cpm were approximately 7000. Non-specific binding was 2% of the total radioactivity added.

levels since inhibition was observed with as little as 0.5 ng of prolactin in liver and ovarian tissue and 5 ng in the mammary tissue. Since the shapes of the curves were different, (Figure 5) this could represent a difference in the number of receptor sites and/or affinities of the preparations for prolactin. These results are in agreement with the reports for lactating mammary glands (Shiu et al., 1973) and liver tissue (Kelly et al., 1974b).

II. Prolactin Binding Activity in Ovarian Tissue During Prepuberal Development and the Estrous Cycle in the Rat

A. Objective

Prolactin has been reported to stimulate ovarian progesterone secretion and be the major luteotropic hormone in the rat (Armstrong et al., 1970; Neill and Smith, 1974). In the immature rat the ovaries appear to show growth and atresia of follicles, but unlike the adult rat no mature follicles (Schwartz et al., 1974) or corpora lutea are present. The adult rat which ovulates every four or five days has both large and mature follicles as well as corpora lutea. Since as the rat matures the ovaries tend to increase their sensitivity to gonadotropins (Schwartz et al., 1974) and possibly prolactin, a partial explanation for this could be an increase in receptors. It was therefore of interest to measure prolactin binding activity in the ovaries of immature and adult cycling female rats.

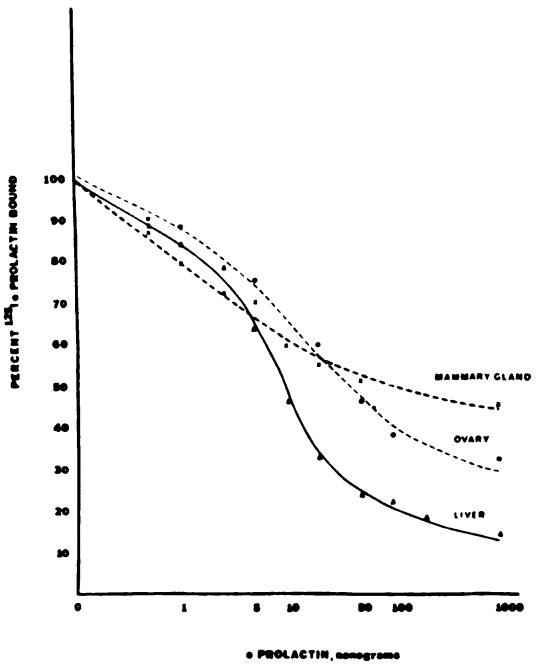

Figure 5. Competitive displacement of ^{125}I-radiolabelled ovine prolactin from mammary gland, ovarian, and liver membranes of female rats.

B. Procedure

1. Animals

Sprague-Dawley female rats were killed by decapitation at 21 and 30 days of age and a third group were killed on the day of vaginal opening.

In the first cycle study (Table II) Long Evans female rats weighing 250-300 grams were followed for 2 consecutive estrous cycles and killed on the different days of the cycle, and in the second study (Table III) Sprague-Dawley female rats weighing 225-250 grams were used and treated in the same manner as the animals of study 1. At the termination of the experiments the ovaries were removed and stored frozen until assayed. Both 4-and 5-day cycling females were used as ovarian donors. The 5-day cycling rats had 3 days of diestrus whereas the 4-day cycling rats had only 2 days of diestrus. The ovaries from diestrus day 1 and diestrus day 2 of the 5-day cycling females were pooled and considered to be the same as diestrus day 1 of a 4-day cycling female since preliminary results showed that prolactin binding activity in the ovaries on these two days of the 5-day cycle was not statistically different from prolactin binding on diestrus day 1 of a 4-day cycling female rat. Ovaries from diestrus day 3 of 5-day cycling females were pooled with ovaries from diestrus day 2 of 4 day cycling females for the same reason mentioned above.

2. Tissue preparation and assay procedure

In the prepuberal study ovaries from 15 animals were pooled for each determination and in the estrous cycle studies ovaries from 5 animals were pooled for each determination. The ovaries were cleaned and homogenized in 0.3 M sucrose and microsomal membrane preparations were made. The ovaries were diluted to 200 ug protein/ 100 ul except in the first study on the estrous cycle (Table II) where they were diluted to 300 µg protein/100 µl and specific binding of ^{125}I-radiolabelled prolactin was measured as previously described.

3. Statistical Analysis

An analysis of variance was performed and followed by Duncan's Multiple Range Test for a comparison among means.

C. Results

During prepuberal development in the rat, prolactin binding to the ovaries was low (Table I) and on the day of first estrus or vaginal opening (Table I, group 3) prolactin binding activity significantly increased. The level of prolactin binding activity on first estrous day was similar to that seen in adult cycling female rats on the day of estrus (Table II).

During the estrous cycle there were fluctuations in the binding activity of prolactin (Table II and Table III). Prolactin binding was significantly higher during diestrus or the luteal phase than during proestrus or estrus.

D. Conclusions

Prolactin binding activity increased in the ovaries as the rat matured, suggesting increased prolactin action on the ovaries with maturation. During the estrous cycle of the adult rat, it reached the highest level during diestrus, when corpora lutea were mainly present and when prolactin is most capable of influencing luteal function.

In the immature animals there is no maturation of the follicles and no luteinization (Schwartz et al., 1974) until the first ovulation or sexual maturation. At this time the corpora lutea appear and there also is an increase in prolactin binding activity. Since prolactin is the major luteotropic hormone in the rat, this pattern appears to be dependent on an increase in binding sites for prolactin in the corpora lutea.

The function of prolactin during the estrous cycle has not yet been clearly defined although recent evidence suggests that prolactin causes luteolysis of the old crop of corpora lutea (Wuttke and Meites, 1971; Grandison and Meites, 1972). Prolactin binding activity was high throughout the cycle in the rat and may be correlated with both the luteotropic and luteolytic actions of prolactin on the ovaries.

Table I. Prolactin Binding Activity in Ovarian Membranes During Prepuberal Development in the Rat

Days of Age	% Specific Binding** of ^{125}I-oPRL
1. 21 (3)+	3.4 ± 0.6
2. 30 (3)	4.5 ± 0.3
3. 36 - 39 (3)	7.3 ± 0.3*

+No. of determinations for each mean.

*P < 0.01 for Day 36-39 vs. Day 21; Day 36-39 vs Day 30

**In all studies presented (Tables I-XVII) NSB was 2-5% of total radioactivity added to each tube.

Table II. Prolactin Binding Activity in Ovarian Membranes During the Estrous Cycle in the Rat

Day of Cycle	% Specific Binding of ^{125}I-oPRL
Proestrus (2)[+]	9.0 ± 0.1
Estrus (2)	7.8 ± 0.04
Diestrus I (2)	10.0 ± 0.9
Diestrus II (2)	14.0 ± 2.0

[+]No. of determinations for each mean.

Table III. Prolactin Binding Activity in Ovarian Membranes During the Estrous Cycle in the Rat

Day of Cycle	% Specific Binding of ^{125}I-oPRL
Proestrus (6)[+]	8.7 ± 0.3*
Estrus (6)	7.0 ± 0.3
Diestrus I (9)	10.0 ± 0.3**
Diestrus II (5)	11.0 ± 0.7**

[+]No. of determinations for each mean.

**P∠0.01 for D II vs. Pro.; D II vs Estrus; D I vs. Estrus

* P∠0.05 for Pro. vs. Estrus; Pro. vs. D I

III. Prolactin Binding Activity in Ovaries and Mammary Tissue During Pregnancy and Lactation in the Rat

A. Objectives

Prolactin is an essential hormone during pregnancy (Neill and Smith, 1974) and lactation (Meites, 1966). It is necessary for maintenance of the corpora lutea during the first six days of gestation (Clemens et al., 1969a), and together with the adrenal glucocorticoids initiates and maintains lactation (Meites, 1966). Since prolactin is important for both the ovaries and mammary tissue, it was of interest to measure prolactin binding activity in these tissues during pregnancy and lactation.

B. Procedures

1. Animals

Sprague-Dawley female rats weighing 225-250 grams were housed 4 to a cage together with 1 male rat. The day sperm were found in the vaginal lavage was considered day 1 of pregnancy. Control females were followed for 2 consecutive estrous cycles. Rats were killed on the day of estrus and on 1, 3, 6, 12, 16 and 20 days of pregnancy. Two additional groups of pregnant rats were maintained for the full gestation period, and their litter size was adjusted to six pups on day 1 of parturition and suckled for either 4 or 10

days. At the end of the experiment both the ovaries and mammary glands were removed and stored frozen until assayed.

2. Tissue preparation and assay procedures

Ovaries from 4 animals were pooled and homogenized in 0.2 M sucrose solution and microsomal membranes were prepared. The mammary glands from individual rats also were homogenized and microsomal membranes were collected. Both ovaries and mammary tissue were diluted to 200 μg/100 μl and specific binding of ^{125}I-radiolabelled prolactin was measured.

3. Statistical Analysis

An analysis of variance was followed by Duncan's Multiple Range Test for a comparison among the means.

C. Results

From day 1 of pregnancy (Table IV, group 2) there was an increase in the binding of prolactin to the ovaries, reaching a peak on days 3 and 6 of pregnancy (Table IV, groups 3 and 4). By day 12 of pregnancy (Table IV, group 5) the prolactin binding in the ovaries has significantly decreased reaching its lowest level on day 20 of pregnancy (Table IV, group 7). As the animals suckled, the binding of prolactin again significantly increased (Table IV, groups 8

Table IV. Prolactin Binding Activity in Ovarian Membranes During Pregnancy and Lactation in the Rat

Days of Pregnancy and Lactation			% Specific Binding of ^{125}I-oPRL
1.	Estrus	(5)+	6.0 ± 0.4
2.	1	(4)	7.5 ± 0.2**
3.	3	(4)	9.1 ± 0.1*
4.	6	(4)	9.6 ± 0.1*
5.	12	(4)	6.9 ± 0.6
6.	16	(4)	6.3 ± 0.6
7.	20	(5)	4.5 ± 0.5
8.	4	(4)	7.3 ± 0.4**
9.	10	(5)	8.3 ± 0.2*

+ No. of determinations for each mean
* $P < 0.01$ for Days 3 and 6 vs. Estrus; Days 3 and 6 vs. Day 12, 16 and 20; Day 10 Lactation vs. Estrus
** $P < 0.05$ for Day 4 Lactation vs. Estrus; Day 1 vs. Estrus

and 9) although the values did not reach the levels of binding seen in the first half of gestation.

The prolactin binding activity in the mammary tissue (Table V) during pregnancy was relatively low and showed little change during the entire period. On days 4 and 10 of lactation (Table V, groups 8 and 9) prolactin binding activity increased by about 100%.

D. Conclusions

These results show that prolactin binding activity in the ovaries is the highest during the first half of gestation in the rat, and that prolactin binding is significantly increased in the mammary tissue during lactation.

Pituitary prolactin has been demonstrated to be essential for maintaining luteal function in the rat during the first 6 days of gestation (Clemens et al., 1969a; Morishige and Rothchild, 1974) and also during lactation (Clemens et al., 1969b). The binding of prolactin in the ovaries follows this same pattern, i.e., the binding is highest during the first half of gestation and lactation. Shiu et al. (1973) reported that rat placental lactogen increases during the second half of gestation and Neill and Smith (1974) proposed that placental lactogen supports progesterone secretion during the second half of gestation. It is possible that placental lactogen binds to the ovaries during the second half of gestation and therefore pituitary prolactin binding is decreased.

Table V. Prolactin Binding Activity in Mammary Gland Membranes During Pregnancy and Lactation in the Rat

Days of Pregnancy and Lactation (no. of rats/group)	% Specific Binding of ^{125}I-oPRL
1. Estrus	1.0 ± 0.1
2. 1	1.0 ± 0.1
3. 3	1.0 ± 0.1
4. 6	0.9 ± 0.1
5. 12	0.8 ± 0.1
6. 16	1.0 ± 0.1
7. 20	1.0 ± 0.05
8. 4	2.6 ± 0.03*
9. 10	2.2 ± 0.2 *

*$P < 0.01$ as compared to all other groups

The mammary tissue, unlike the ovaries, shows very little binding of prolactin during pregnancy but the binding significantly increased at lactation. Placental lactogen is similar to pituitary prolactin in that it maintains corpora lutea and stimulates growth and milk secretion by rat mammary gland (Neill and Smith, 1974). Shiu et al. (1973) demonstrated that human placental lactogen also binds to lactating rabbit mammary glands. So the low pituitary prolactin binding in the mammary gland during the later half of pregnancy when the glands are very well developed could be due to the saturation of these sites by placental lactogen.

The binding for prolactin in the lactating mammary glands was low even though significantly increased over the levels observed during pregnancy. One explanation for this is that as the rats suckle, the levels of endogenous prolactin are highly elevated and the binding sites for prolactin become partially saturated, resulting in low binding activity. The effects of high endogenous levels of prolactin on binding in different target organs has yet to be determined.

IV. Normal Development and Effects of Estrogen on Prolactin Binding Activity in Liver, Adrenals and Kidneys of Immature Female Rats

A. Normal Development and Effects of Estrogen on Prolactin Binding Activity in Liver of Immature Female Rats

1. Objectives

Prolactin binding activity in the liver had been reported to increase with age and reach a peak shortly after puberty in the female rat (Kelly et al., 1974b). Our laboratory had preliminary indications that estrogen could stimulate prolactin binding activity in the liver. Since binding was low in the immature animal, it was thought of interest to determine whether immature rats were responsive to estrogen stimulation in terms of changes in prolactin binding activity in the liver.

2. Procedures

a. Animals

Immature female Sprague-Dawley rats 10, 18, 23, 28, 33, 38 and 70 days of age were treated with either 1 µg estradiol benzoate in 0.1 cc corn oil or with the vehicle alone. The rats were injected for 5 days at the end of which time they were killed and their livers were removed and stored frozen.

b. Tissue preparation and assay procedure

Liver microsomal membrane preparations were made and diluted to 300 μg protein per 100 μl diluent, as described previously. Specific binding of ^{125}I-radiolabeled prolactin was determined for each sample.

c. Statistical Analysis

The data were analyzed using an analysis of variance for unequal sample size, followed by Duncan's Multiple Range test for comparison among means. The data for the controls were also subjected to an unweighted least squares fit for an exponential function, whereas the data for the treated animals were analyzed by an unweighted linear regression (Sokal and Rohlf, 1969).

3. Results

These data demonstrate that prolactin binding activity increases with age (Figure 6) and this developmental pattern follows an exponential growth curve (correlation coefficient 0.98). The adult level emerges about the time of sexual maturation, since at the time of vaginal opening (38-40 days, Figure 6) the sharpest increase was seen and thereafter the curve reached a plateau.

The estrogen treated rats (Figure 6) showed a marked increase in binding as compared to the control rats of corresponding age

(Table VI). However, those rats treated at 10 days of age and killed at 15 days of age did not respond to estrogen treatment, as can be seen in Table VI. Their binding was at approximately the same level as the untreated controls. It is interesting to note that the prolactin binding activity of the estrogen treated rats displayed a linear pattern (correlation coefficient of 0.97) of development, indicating that estrogen modified the pattern observed in the untreated rats.

B. Ontogeny of Prolactin Binding Activity in the Adrenal Glands and Kidneys

1. Objectives

We had observed that the adult pattern of prolactin binding activity in the liver emerged at the time of puberty in the female rat. Since both the adrenal glands and the kidneys had been shown to specifically bind ^{125}I-radiolabeled prolactin, the question arose as to whether their development of prolactin binding activity would follow the same pattern as in the liver. The present study was undertaken to answer this question.

2. Procedures

a. Animals

The adrenal glands and kidneys were removed from the 23, 28, 33, 38, 43 and 75 day old control rats of the previous study (see A. 2. Procedures a. Animals).

b. Tissue preparation and assay procedure

Adrenal glands from 4 animals were pooled and a membrane fraction was prepared. A total of 4 kidneys were pooled for each membrane preparation. This was done in order to have enough protein to assay. The adrenal membranes were diluted to 100 µg protein/100 µl and the kidney membranes to 1000 µg protein/100 µl. Specific binding of ^{125}I-prolactin was measured and each sample was assayed in quadruplicate (Materials and Methods, section IV).

c. Statistical analysis

The data were analyzed by using an unweighted least squares fit for an exponential function (Sokal and Rohlf, 1969), and the difference between means was tested by analysis of variance followed by Duncan's Multiple Range test.

Table VI. Prolactin Binding Activity in Liver Homogenates of Untreated and Estradiol Benzoate Treated Female Rats

Age in Days (no. of rats/group)	% Specific Binding of ^{125}I-oPRL	
	Controls	EB-treated
15 (5)	2.2 ± 0.1	2.4 ± 0.3
23 (11)	2.5 ± 0.2	10.5 ± 1.0
28 (12)	2.8 ± 0.2	13.5 ± 1.0
33 (12)	4.8 ± 0.6	18.5 ± 0.7
38 (12)	5.9 ± 0.5	19.6 ± 2.0
43 (12)	9.8 ± 0.8	20.6 ± 0.9
75 (7)	12.0 ± 0.9	17.0 ± 1.2

Table VII. Prolactin Binding Activity in Adrenal and Kidney Homogenates of Growing Female Rats

Age in Days	% Specific Binding of ^{125}I-oPRL	
	Adrenals	Kidneys
23	12 ± 2 (3)*	2.4 ± 0.5 (6)*
28	11 ± 0.7 (3)	5.4 ± 0.2 (6)
33	7.4 ± 0.9 (3)	4.3 ± 0.2 (6)
38	4.6 ± 1 (3)	1.2 ± 0.2 (7)
43	3.1 ± 0.5 (3)	0.7 ± 0.1 (6)
75	1.8 ± 0.3 (3)	0.7 ± 0.1 (8)

*Number of samples used for determining standard error per mean.

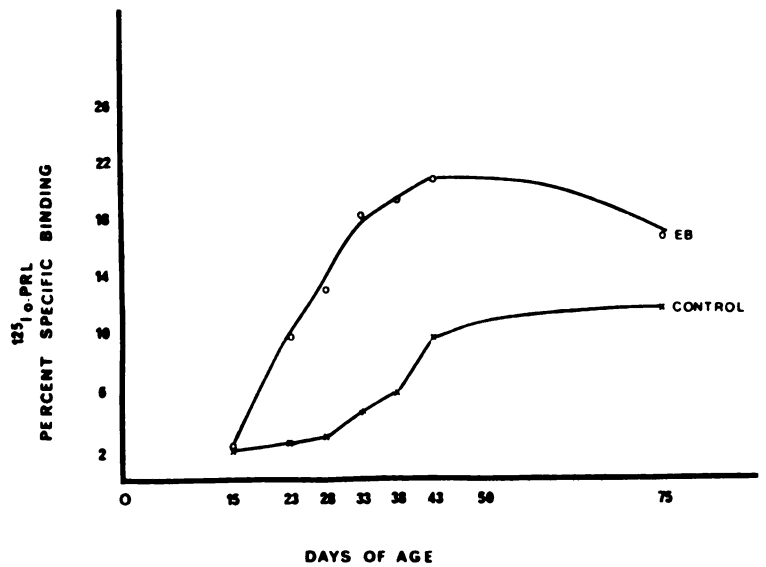

Figure 6. Normal development and effects of estrogen on prolactin binding activity in liver tissue of immature female rats.

3. Results

Prolactin binding activity (Figure 7) in the adrenal glands and kidneys decreased exponentially (correlation coefficients -0.98 and -0.97, respectively) with age. This pattern was the reverse of what was seen in the liver (Figure 6). The decline continued until puberty and then prolactin plateaued to adult levels. The binding in the adult rat adrenal gland was approximately 1/6 the binding seen in the immature animal at 23 days of age (Table VI), and this binding at 23 days was significantly different (P 0.01) from the binding observed at either 43 or 75 days of age. The kidneys of 28 day old rats (Table VII) displayed binding of radiolabeled prolactin about 7-fold greater than in the adult female rat 75 days old (P 0.01 for 28 days vs. 75 days).

The binding of prolactin in the adrenal glands of immature rats was much greater than observed in either the liver (Table VI) or kidneys (Table VII) of immature female rats. It should be pointed out that adrenal binding is expressed as per 100 μg protein whereas liver binding is expressed as per 300 μg protein and kidney binding as per 1000 μg protein. Thus the adrenal gland binds by far more radiolabeled prolactin per ug of protein than either liver or kidney tissue.

C. Conclusions

These data show that prolactin binding activity in the liver increases with age and reaches a plateau at puberty, whereas the binding in the adrenal glands and kidneys decreases with age and also plateaus at puberty. The binding activity in the liver correlates well with the pattern of prolactin secretion in immature rats since the highest levels of serum prolactin were seen at puberty and thereafter (Voogt et al., 1970). It is difficult to interpret the high binding in the immature adrenals and kidneys as compared to the low binding in the adult. One can speculate that the function of prolactin may be more important in these tissues during prepuberal development than in the adult.

In the liver, estrogen was able to increase prolactin binding activity in the immature animal, although the animals treated at 10 days of age did not respond to the estrogen treatment. Ten days of age could possibly be a period when the mechanism(s) involved in prolactin binding activity in the liver may be refractory to the effects of estrogen. The mechanism(s) of estrogen stimulation are not yet known. Estrogen increases prolactin levels in 21 day old female rats (Voogt et al., 1970) as well as in adult female rats (Chen et al., 1970), and it also influences liver size (Leathem, 1961). It is possible that at puberty estrogen stimulates not only prolactin secretion but also prolactin receptor binding activity in the liver.

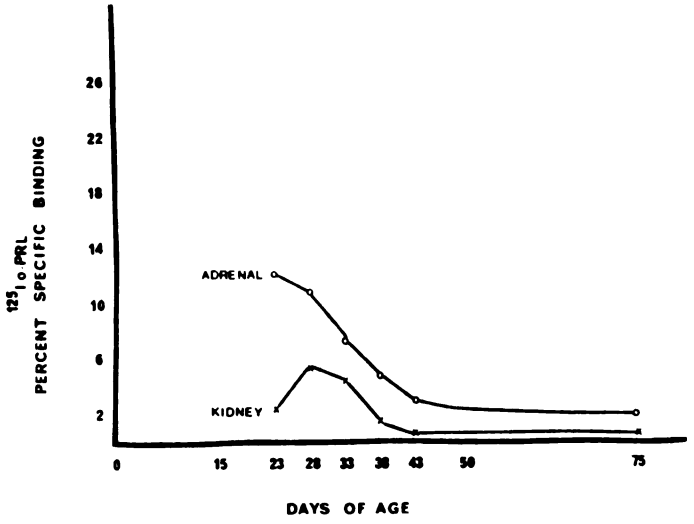

Figure 7. Ontogeny of prolactin binding activity in kidney and adrenal tissues of immature female rats.

V. Effects of the Thyroid and Ovaries on Prolactin Binding Activity in Rat Liver

A. Objectives

Recent reports by several laboratories (Turkington and Frantz, 1972; Friesen et al., 1973) and our own laboratory have demonstrated that prolactin binds specifically to liver tissue, indicating the presence of prolactin receptors in this tissue. The nature and functions of prolactin receptors in the liver, as well as the mechanisms regulating their induction and maintenance have not yet been determined.

Among the endocrine glands that may influence liver functions are the thyroid and ovaries. Thyroid hormones are known to be protein anabolic (Friesen and Lipner, 1971), and can stimulate GH secretion (Dickerman et al., 1972b). Liver size and protein content are reduced after thyroidectomy (Turner and Bagnara, 1971). Estrogen also has been reported to influence liver size and function (Leathem, 1961), and can increase prolactin (Meites et al., 1972) and GH secretion (Dickerman et al., 1972b). It was of interest therefore, to determine the effects of thyroidectomy, ovariectomy, thyroxine and estrogen on prolactin receptor binding activity in the liver of female rats.

B. Procedures

1. Animals

Mature, virgin female Sprague-Dawley rats weighing 200-225 grams each were used.

Three separate experiments were performed. In experiment 1, intact controls and thyroidectomized rats were injected sc daily with 0.2 ml of 0.85% NaCl for 30 days. Two other groups of thyroidectomized rats were injected sc with 2.5 or 10 μg L-thyroxine (T_4) per 100 gm body weight for 30 days. In the second experiment, ovariectomized and ovariectomized-thyroidectomized rats were injected sc with 2.5 or 10 μg T_4 per 100 gm body weight. Treatment was begun 31 days after surgery and continued for 15 days. In the third experiment, the animals were injected sc daily as follows: (1) intact female rats, 0.85% NaCl; (2) ovariectomized-thyroidectomized rats, 0.85% NaCl; (3) ovariectomized-thyroidectomized rats, 2.5 μg T_4 per 100 gm body weight (4) ovariectomized-thyroidectomized rats, 2 μg estradiol benzoate (EB) in corn oil and (5) ovariectomized-thyroidectomized rats, T_4 and EB.

2. Tissue preparation and assay procedure for prolactin binding activity.

The liver tissue from each rat was homogenized in 0.3 M sucrose and microsomal membranes were collected according to the methods previously described (see Materials and Methods, IV.

Radioreceptor Assay). Each liver membrane sample used for the assay contained 300 μg of protein as determined by Lowry protein method (1955).

The specific binding of ^{125}I-labeled ovine prolactin to these membranes was determined by the methods outlined in section IV, Radioreceptor Assay, Materials and Methods. Each sample was assayed in quadruplicate.

3. Statistical analysis of data

The data were first analyzed by an analysis of variance for unequal sample size (Sokal and Rohlf, 1969), followed by Duncan's Multiple range test for comparison of means among all groups (Duncan, 1955).

4. Scatchard Analysis

This analysis was carried out according to the procedures outlined in section V. Scatchard Analysis (Materials and Methods).

C. Results

In experiment 1 (Table VIII), thyroidectomy (group 2) reduced prolactin binding activity to less than a third of that present in intact controls (group 1). Doses of 2.5 and 10 μg T_4/100 gm body weight (groups 3 and 4) restored prolactin binding activity to the

intact control level. The effects of the two doses of T_4 on specific prolactin binding activity in the liver were not statistically different from each other.

Rats both ovariectomized and thyroidectomized (group 2, Table IX) showed significantly less prolactin binding activity than rats only ovariectomized (group 1). Treatment with either dose of T_4 (groups 3 and 4) restored liver prolactin binding activity to levels present in the ovariectomized rats (group 1).

In the third experiment (Table X) thyroidectomy and ovariectomy (group 2) again significantly decreased prolactin binding activity in the liver as compared to intact controls (group 1). Replacement therapy with either 2.5 μg T_4/100 gm body weight (group 3) or 2 μg EB (group 4) partially restored prolactin binding activity. However, both the T_4 (group 3) and the EB (group 4) treated rats had significantly less binding than the intact control rats (group 1). When ovariectomized-thyroidectomized rats were treated with both T_4 and EB (group 5), prolactin binding activity was increased above that of the intact controls. The prolactin binding activity in intact controls in this experiment was about twice that seen in intact controls in experiment 1 (Table VIII). The iodinated preparations of ovine prolactin used for the assays in experiments 1, 2, and 3 were not the same, and as mentioned previously (see Materials and Methods, section IV. Radioreceptor Assay), the iodinated ovine prolactin used in experiment 3 was repurified. This latter is believed to be mainly responsible for the higher specific binding seen in the third experiment.

Data were obtained for the competitive inhibition of labeled hormone by different amounts of unlabeled prolactin with receptor preparations from intact control and experimental animals (Fig. 8). A Scatchard analysis is shown in Figure 9 on the data from intact control animals and reveals the presence of a low concentration (2.113×10^{-14}M/300 μg protein) of binding sites for prolactin of very high affinity (Ka = $5.03 \times 10^{12} M^{-1}$). The combination of ovariectomy and thyroidectomy resulted in approximately a 9-fold reduction in the number of these binding sites. Replacement with T_4 and EB in the ovariectomized-thyroidectomized animals restored the number of binding sites to levels slightly higher than found in controls (3.65×10^{-14}M/300 μg protein). The affinity constants of the sites in the control and experimental groups were not substantially different. These data from Scatchard analysis are summarized in Table XI.

D. Conclusions

These results indicate that either thyroidectomy or ovariectomy decrease prolactin binding activity in the liver. Thyroidectomy was more effective in this respect than ovariectomy. Injections of T_4 returned prolactin binding activity in the thyroidectomized rats to the level of the intact controls, and in the ovariectomized-thyroidectomized rats to the level of ovariectomized rats. Estrogen replacement together with T_4 increased prolactin binding activity above that of intact control levels. Scatchard analysis revealed

Table VIII. PRL Binding Activity in Liver Homogenates of Thyroidectomized (Tx) and Thyroxine (T_4) Treated Female Rats

Treatment and no. of rats/group	% Specific Binding of I^{125}-oPRL
1. Intact Females (5)	6.4 ± 0.9
2. Tx (5)	1.6 ± 0.3*
3. Tx + 2.5 μg T_4/100 gm BW (8)	6.5 ± 0.8
4. Tx + 10 μg T_4/100 gm BW (7)	5.3 ± 0.4

*$P < 0.01$ as compared to all other groups

Table IX. PRL Binding Activity in Liver Homogenates of Ovariectomized (Ovx)-Thyroidectomized (Tx) Rats Given or Not Given Thyroxine (T_4)

Treatment + no. of rats/group	% Specific Binding* of I^{125}-ovine PRL
1. Ovx + saline (7)	3.9 ± 0.2
2. Ovx + Tx (5)	1.0 ± 0.1*
3. Ovx + Tx + 2.5 μg T_4/100 gm BW (8)	3.8 ± 0.7
4. Ovx + Tx + 10 μg T_4/100 gm BW (8)	3.9 ± 0.2

* $P < 0.01$ as compared to all other groups

Table X. Prolactin Binding Activity in Liver Homogenates of Ovariectomized (Ovx)-Thyroidectomized (Tx) Rats Given Thyroxine (T_4) and/or Estradiol Benzoate (EB)

Treatment and no. of rats/group	% Specific Binding of I^{125}-oPRL
1. Intact females (11)	13.3 ± 1.2
2. Ovx + Tx (8)	1.8 ± 0.3*
3. Ovx + Tx + 2.5 μg T_4/100 gm Bd wt (8)	9.5 ± 0.7*
4. Ovx + Tx + 2 μg EB/rat (8)	9.9 ± 1.0*
5. Ovx + Tx + 2.5 μg T_4 and 2 μg EB (8)	20.2 ± 1.7*

*$P < 0.01$ as compared to intact females

Table XI. Scatchard Analysis

Treatment Group	Maximum Bindable Prolactin (ng)	Maximum Bindable Prolactin (moles)	Ka ($Moles^{-1}$)
Intact controls (IC)	0.486	2.113×10^{-14}	5.03×10^{12}
Tx, ovx	0.061	2.660×10^{-15}	5.61×10^{12}
T_4, EB	0.841	3.655×10^{-14}	5.14×10^{12}

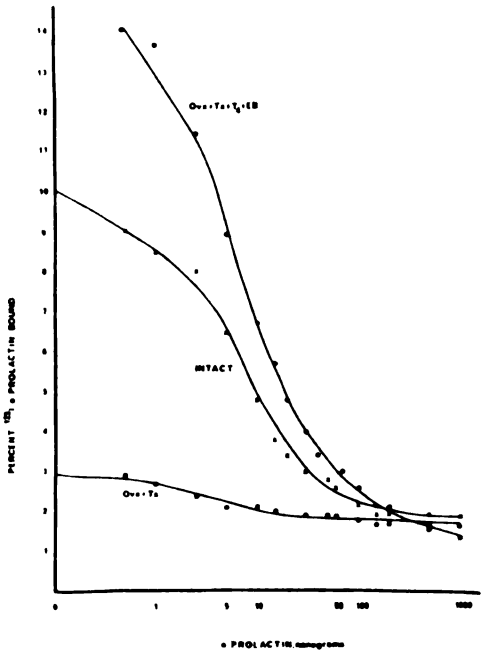

Figure 8. Binding of ^{125}I-ovine prolactin to liver membranes of ovariectomized-thyroidectomized, intact controls and ovariectomized-thyriodectomized rats injected with T_4 and EB as a function of unlabelled o-prolactin (noted on abscissa). The ordinate represents the binding of ^{125}I-o-prolactin as a percent of the total ^{125}I-o-prolactin in the incubation. Each tube contained 300 ug protein and 70,000 cpm ^{125}I-o-prolactin. Non-specific binding was 2% of the total radioactivity added.

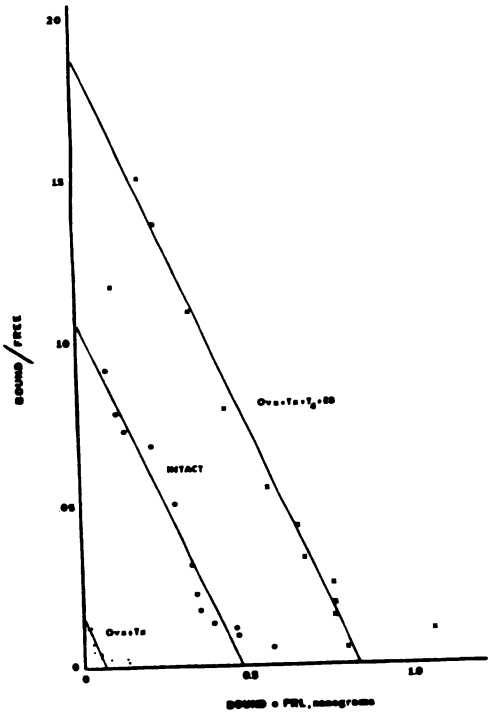

Figure 9. Scatchard plot of data for competitive displacement of ^{125}I-ovine prolactin with unlabelled o prolactin from liver membranes of ovariectomized-thyroidectomized, intact controls, and ovariectomized-thyroidectomized rats injected with T_4 and EB. The ordinate represents ratio of nanograms bound/free hormone, and abscissa the nanograms of o-prolactin bound to liver membranes. The affinity constant (Ka) is the negative slope of the line. The intercept on the abscissa is the total amount of o-prolactin bound to the receptors.

that the differences in prolactin binding activity were due to an alteration in the number of "receptor" binding sites in the liver. Since thyroidectomy can decrease liver proteins generally (Turner and Bagnara, 1971), this decrease also may have included receptor proteins for prolactin. The mechanism(s) by which the ovaries influence prolactin receptors in the liver is unknown, although estrogens can increase pituitary prolactin release (Meites et al., 1972) and can alter liver function (Leathem, 1961).

VI. Effects of Ovarian Hormones on Prolactin Binding Activity in Liver and Mammary Tissues

A. Effects of Estrogen or Estrogen and Progesterone on Prolactin Binding Activity in Liver

1. Objectives

Estrogen and progesterone have been reported to stimulate prolactin secretion (Chen and Meites, 1970; Kalra et al., 1973). Since estrogen greatly increases prolactin secretion, it was thought of interest to determine if estrogen and progesterone could also stimulate prolactin binding activity in the liver.

2. Procedures

a. Animals

Approximately 45 Sprague-Dawley female rats weighing 200-225 grams were used in these experiments. The rats were ovariectomized and treatment was begun one week after surgery. The rats were assigned to the following groups: (1) controls, corn oil, 0.2 ml, (2) estradiol benzoate (EB), 5 μg/0.2 ml, (3) EB, 20 μg/0.2 ml, (4) EB, 5 μg/0.1 ml and progesterone, 4 mg/0.1 ml. The treatments were given sc daily for 10 days. At the end of the treatment period, half of the rats were killed 24 hours after the last injection and the other half were killed 7 days after the last injection.

b. Tissue preparation and assay procedure

Liver microsomal membrane preparations were made and all tissue was aliquoted at 300 μg protein/100 μl. The specific binding of ^{125}I-radiolabeled prolactin was measured for each rat. The samples were assayed in triplicate.

c. Statistical analysis

The data were analyzed by analysis of variance followed by Duncan's Multiple Range Test for comparisons among the means.

3. Results

Table XII shows that estrogen in both dose levels (groups 2 and 3) as well as the combination of EB and Progesterone (group 4) significantly increased prolactin binding activity as compared to the ovariectomized controls (group 1). The effects of the two doses of EB and the combination of EB and Progesterone on the specific prolactin binding activity in the liver were not statistically different from one another.

In the second half of this experiment (Table XIII), in which the rats were killed 7 days after the last treatment, the specific prolactin binding in the liver of ovariectomized rats treated with EB (groups 2 and 3) or EB + prog (group 4) was still significantly higher than the controls. Although the binding was slightly less than that seen 24 hrs after the last injection (Table XII), the stimulatory effects of estrogen were still present.

B. Effects of Estrogen or Estrogen and Progesterone on Prolactin Binding Activity in Mammary Tissue

1. Objectives

Estrogen and progesterone stimulate the development of mammary glands (Meites, 1966) as well as influence prolactin secretion (Chen et al., 1970; Kalra et al., 1973) in the rat. It was therefore

Table XII. Prolactin Binding Activity in Liver Homogenates of Ovariectomized (Ovx) Rats Treated with Estrogen (EB) or Estrogen and Progesterone (Prog), and Killed 24 hours After Last Injection

Treatment (no. of rats/group)	% Specific Binding of ^{125}I-oPRL
1. ovx + corn oil (5)	7.9 ± 0.9
2. ovx + EB, 5 µg (5)	31 ± 1.4*
3. ovx + EB, 20 µg (5)	29 ± 2 *
4. ovx + EB, 5 µg + Prog, 4 mg (5)	29 ± 2 *

*$P < 0.01$ as compared to ovx + corn oil

Table XIII. Prolactin Binding Activity in Liver Homogenates of Ovariectomized (Ovx) Rats Treated with Estrogen (EB) or Estrogen and Progesterone, (Prog) and Killed 7 Days after Last Injection

Treatment (no. of rats/group)	% Specific Binding of ^{125}I-oPRL
1. ovx + corn oil (5)	7 ± 1.9
2. ovx + EB, 5 µg (5)	29 ± 2 *
3. ovx + EB, 5 µg (5)	25 ± 2 *
4. ovx + EB, 5 µg + Prog 4 ng (5)	23 ± 3 *

*$P < 0.01$ as compared to ovx + corn oil

of interest to observe the effects of estrogen and progesterone on prolactin binding activity in the mammary tissue.

2. Procedures

a. Animals

The mammary tissue was removed from the same ovariectomized rats as in the previous study (VI. A. 1. Procedures a. Animals) treated with varying doses of estrogen or the combination of estrogen and progesterone.

b. Tissue preparation and assay procedure

Microsomal membrane preparations were made from mammary tissue and diluted to 200 μg protein/100 μl. Specific binding of ^{125}I-radiolabeled prolactin was measured and each sample was assayed in quadruplicate.

c. Statistical analysis

The data were analyzed using analysis of variance followed by Duncan's Multiple Range Test for comparisons among the means.

3. Results

Treatment of ovariectomized rats with either 5 or 20 μg EB (Table XIV, groups 2 and 3) or a combination of EB + Prog (group 4) significantly reduced prolactin binding activity by approximately 1/2 in mammary tissue. The effects of the two doses of EB and the combination of EB and Prog on specific prolactin binding activity in the mammary tissue were not statistically different from one another.

Table XV shows that when the other half of the rats were killed one week after the last injection, EB (groups 2 and 3) and the combination of EB and Prog (group 4) also significantly reduced prolactin binding activity in the mammary tissue. However, prolactin binding in the controls (group 1, Table XV) was higher than in the controls (group 1) in Table XIV, when killed 24 hours after the last injection. It is possible that the longer period of ovariectomy (Table XII) increased the prolactin binding activity in this mammary tissue.

C. Conclusions

These data demonstrate that estrogen (5 or 20 μg) or a combination of estrogen and progesterone are able to stimulate prolactin binding activity in the liver and decrease binding of prolactin in the mammary tissue. As mentioned before, the mechanism(s)

Table XIV. Prolactin Binding Activity in Mammary Tissue Homogenates of Ovariectomized (Ovx) Rats Treated with Estrogen (EB) or Estrogen and Progesterone (Prog), and Killed 24 Hours After Last Injection

Treatment (no. of rats/group)	% Specific Binding of ^{125}I-oPRL
1. ovx + corn oil (5)	1.4 ± 0.2
2. ovx + EB, 5 μg (5)	0.7 ± 0.1*
3. ovx + EB, 20 μg (5)	0.5 ± 0.04*
4. ovx + EB, 5 μg + Prog, 4 mg (5)	0.8 ± 0.06*

*P∠0.01 as compared to ovx + corn oil

Table XV. Prolactin Binding Activity in Mammary Tissue Homogenates of Ovariectomized (Ovx) Rats Treated with Estrogen (EB) or Estrogen and Progesterone (Prog), and Killed 7 Days After Last Injection

Treatment (no. of rats/group)	% Specific Binding of ^{125}I-oPRL
1. ovx + corn oil (5)	3.8 ± 0.5
2. ovx + EB, 5 μg (5)	0.8 ± 0.08*
3. ovx + EB, 20 μg (5)	0.7 ± 0.1*
4. ovx + EB, 5 μg + Prog, 4 mg (5)	0.5 ± 0.1*

*$P < 0.01$ as compared to ovx + corn oil

involved in the effect of estrogen on prolactin binding activity remains to be defined. Evidence from our previous work indicates that estrogen increases the number of binding sites in the liver (see Experimental section V. 3. Results). Estrogen has also been shown to alter liver size and function (Leathem, 1961), as well as to stimulate prolactin release (Chen and Meites, 1970).

The effects of estrogen and estrogen and progesterone on mammary tissue seem to fall in line with early work reported by Meites and Sgouris (1953). They suggested that although large doses of estrogen increase pituitary prolactin secretion, they render the mammary glands less sensitive to the lactational action of prolactin. Large doses of estrogen, or combinations of estrogen and progesterone, stimulated mammary growth but prevented prolactin from initiating lactation in castrated rabbits. When estrogen administration was terminated, prolactin initiated copious lactation. Further work by Meites (1970b) demonstrated that large doses of estrogen were able to inhibit growth of DMBA-induced rat mammary tumors even though serum prolactin levels were high. Since these tumors are responsive to prolactin, the author concluded that estrogen may possibly inhibit the action of prolactin on the mammary tumor. The evidence we have reported here suggests that large doses of estrogen may inhibit the action of prolactin on the mammary gland by decreasing prolactin binding activity. Another possibility is that the high serum prolactin produced by estrogen treatment may result in greater saturation of prolactin binding sites in the mammary gland, and hence less measurable prolactin binding.

In a recent article, Kelly et al. (1974a) reported prolactin binding activity in DMBA-induced rat mammary tumors. The data showed that prolactin dependent tumors had the highest prolactin binding, and a negative relationship was observed with prolactin binding activity in the liver, i.e., the liver prolactin binding was lowest in those animals bearing mammary tumors with highest prolactin binding. We also have observed a reciprocal relationship between liver and mammary tissue binding in response to estrogen and progesterone treatment, i.e., when the binding in the liver was high, that in the mammary tissue was low.

VII. Effects of Adrenals on Prolactin Binding Activity in Liver of Female Rats

A. Effects of Adrenalectomy and Hydrocortisone Treatment on Prolactin Binding Activity in Liver

1. Objectives

The adrenal glucocorticoids influence protein synthesis and carbohydrate metabolism of the liver (Litwack and Singer, 1972) as well as the general body metabolism of rats (Frieden and Lipner, 1971). There is also evidence that glucocorticoids may reduce prolactin secretion in rats whereas adrenalectomy may increase prolactin secretion (Ben David et al., 1971b; also Mueller, unpublished). Since we had observed such a profound effect of thyroid

and ovarian hormones on prolactin binding activity in the liver, it was of interest to investigate the effects of another class of metabolic hormones, this is, the glucocorticoids, on prolactin binding activity in the liver.

2. Procedures

a. Animals

Virgin, female Sprague-Dawley rats weighing 200-225 gms were used. The animals were ovariectomized or ovariectomized-adrenalectomized for 14 days at which time treatment was begun. The ovariectomized rats were treated sc with either 0.85% saline or 1 mg hydrocortisone acetate and the ovariectomized-adrenalectomized animals were injected sc with 0.85% NaCl. The treatment was continued for 10 days. The ovariectomized-adrenalectomized animals were maintained on 0.9% saline drinking water for the duration of the experiment.

b. Tissue preparation and assay procedure

Liver tissue was homogenized in 0.3 M sucrose and microsomal membranes were prepared and diluted to 300 μg protein/100 μl, as described previously. Specific binding of ^{125}I-radiolabeled prolactin was measured. Each sample was assayed in quadruplicate.

c. Statistical analysis

The data were analyzed using analysis of variance for unequal sample size (Sokal and Rohlf, 1969).

3. Results

Adrenalectomy (Table XVI, group 2) and hydrocortisone acetate treatment (group 3) each lowered prolactin binding activity in the liver. Analysis of variance showed marginal significance ($P < 0.05$) since the effect of either treatment was not very pronounced.

B. Effects of Adrenalectomy and Hydrocortisone Acetate Replacement Therapy on Prolactin Binding Activity in the Liver

1. Objectives

The previous experiment had shown a tendency for adrenalectomy and a large dose of hydrocortisone acetate to decrease prolactin binding activity. The present study was designed to investigate whether a replacement dose of hydrocortisone would restore prolactin binding to control level.

2. Procedures

a. Animals

Sprague-Dawley virgin female rats weighing approximately 300 gms were used. The rats were ovariectomized for 40 days and then adrenalectomized on day 41. Rats ovariectomized or ovariectomized-adrenalectomized received a sc injection of 0.85% saline and another group of ovariectomized-adrenalectomized rats was given 100 μg hydrocortisone/100 gm body weight sc daily. Treatment was begun on the same day the adrenalectomies were performed and continued for 6 days.

b. Tissue preparation and assay procedure

Liver microsomal membrane preparations were made and specific binding of ^{125}I-radiolabeled prolactin was measured as described previously. Each sample was assayed in triplicate.

c. Statistical analysis

The data were analyzed using an analysis of variance for unequal sample size followed by Duncan's Multiple Range Test for comparison among the means.

3. Results

In this second experiment, adrenalectomy (Table XVII, group 2) again lowered prolactin binding activity, although this was not significant as compared to the controls. Hydrocortisone treatment (group 3), however, produced a significant decrease in prolactin binding activity.

C. Conclusions

These results show that adrenalectomy had a tendency to lower prolactin binding activity in liver of female rats, whereas treatment with hydrocortisone acetate produced a more marked reduction in prolactin binding activity than was observed by adrenalectomy alone. Generally, the glucocorticoids are considered to be protein catabolic hormones and therefore decrease protein synthesis (Frieden and Lipner, 1971), although they stimulate gluconeogenesis in the liver (Litwack and Singer, 1972). This may be one explanation for the effects of a glucocorticoid hormone on prolactin binding activity, that is, it possibly decreases the receptor proteins for prolactin.

Table XVI. Prolactin Binding Activity in Liver Homogenates of Ovariectomized (Ovx) Rats Adrenalectomized (Adx) or Hydrocortisone Acetate Treated Rats

Treatment and no. of rats/group	% Specific Binding of I^{125}-oPRL
1. Ovx (5)	6.4 ± 0.6
2. Ovx-Adx (6)	4.8 ± 0.7
3. Ovx + hydrocortisone acetate (8)	4.8 ± 0.6

Table XVII. Prolactin Binding Activity in Liver Homogenates of Ovariectomized (Ovx)-Adrenalectomized (Adx) Rats Given or Not Given Hydrocortisone Acetate (HC)

Treatment and no. of rats/group	% Specific Binding of I^{125}-oPRL
1. Ovx + saline (9)	6.0 ± 0.5
2. Ovx + Adx (7)	5.0 ± 0.6
3. Ovx + Adx + 100 μg HC/100 gm BW (10)	3.7 ± 0.3*

P < 0.01 as compared to ovx + saline controls

GENERAL DISCUSSION

The data presented in this thesis demonstrate that ^{125}I-radiolabeled prolactin binds specifically to ovarian, mammary and liver microsomal membranes, and that prolactin binding activity changes under different physiological conditions in all tissues studied. This suggests that binding sites for prolactin vary with the physiological requirements for the hormone, such as an increase in prolactin binding activity during lactation in the mammary gland.

The ontogeny of prolactin binding activity does not follow the same pattern in all tissues that specifically bind prolactin. In liver and ovarian tissues the binding for prolactin increased as the animal continued to develop, and reached adult levels at or about the time of vaginal opening in the rat. On the other hand, kidney and adrenal tissues showed significantly higher binding for prolactin in the immature rat, followed by a steady decrease in binding activity as the rat continued to mature. The levels of prolactin binding activity in the kidney and adrenals also reach adult levels at about the time of vaginal opening. Kelly et al. (1974b) reported similar findings for prolactin binding activity in the liver of rats, but no reports have as yet appeared on prolactin binding activity in the ovaries, kidneys or adrenal glands of immature rats.

The increase in binding for prolactin in the ovaries of developing rats correlates well with the appearance of corpora lutea in the ovaries at vaginal opening or at first ovulation. According to Schwartz et al. (1974), until the first ovulation there are only follicles present in the developing ovaries. As the rat approaches sexual maturation, there is follicular maturation and ovulation. These authors also suggested that there is an increase in the number of receptor sites for the gonadotropins in the ovaries as the rat matures, resulting in increased sensitivity of the ovaries to these hormones. The binding data presented on prolactin in the ovaries tends to support this statement.

Vaginal opening appears to be an important time for prolactin binding activity. This is also the period when prolactin serum levels rise and begin to show cyclic fluctuations (Voogt et al., 1970). Estrogen levels in the serum increase preceding vaginal opening as well (Ramirez, 1973). In immature rats, estrogen stimulated prolactin binding activity in the liver, and preliminary observations indicated that estrogen decreased prolactin binding activity in the kidney and adrenals (Marshall, Gelato and Meites, unpublished data). It is possible therefore that the pattern of prolactin binding activity in these tissues of the immature rat is related to the increase in estrogen at about the time of vaginal opening. However, the relatively high amount of prolactin binding activity in the adrenals and kidneys of 20-30 day old female rats suggests that prolactin has some functions on these organs at this time. Prolactin has been shown to influence both kidney and adrenal function in

the adult rat. It has been reported that a decrease in prolactin caused an increase in sodium and potassium excretion by the kidney (Richardson, 1973) and Lis et al. (1973) observed that prolactin was able to partially restore corticosterone biosynthesis in the adrenals of hypophysectomized rats. The immature animal may be more sensitive to these actions of prolactin.

The most pronounced effect of prolactin as a luteotropic agent is seen during early pregnancy or pseudopregnancy. Data presented in this thesis show that prolactin binding activity is the highest during the first six days of gestation when pituitary prolactin is known to be essential for the support of progesterone secretion from the corpus luteum (Clemens et al., 1969a). During the second half of gestation binding of prolactin in the ovaries goes down and reaches a low level just before parturition. At this time there is another prolactin-like hormone to be considered. Placental lactogen in the rat shows two peaks during gestation, one at days 10-12 and another at days 17-21 (Shiu et al., 1973). Neill and Smith (1974) reported that a placental prolactin is probably responsible for support of the corpus luteum during the second half of gestation. It is possible that the high levels of placental lactogen during the second half of gestation as measured by Shiu et al. (1973) compete with pituitary prolactin for binding sites on the corpus luteum and therefore binding for pituitary prolactin is decreased. Competition between radiolabeled ovine prolactin and human placental lactogen has been demonstrated by Shiu et al. (1973) for binding sites on lactating mammary gland membranes. The

ovarian preparation for binding studies used in the present study were membrane fractions of whole ovaries, and the corpora lutea were not separated. Therefore the binding activity of prolactin may have been different if only the corpora lutea had been measured during pregnancy.

Prolactin is the major luteotropic hormone in the rat, but it has also been shown to be luteolytic (Malven and Sawyer, 1966). These authors reported that prolactin was luteolytic or luteotropic depending on the time prolactin was administered after hypophysectomy. Prolactin given two or more days after hypophysectomy had a luteolytic action whereas when given soon after hypophysectomy it was luteotropic. Although these authors demonstrated a luteolytic action for prolactin, it's physiological significance was not known. In lactating rats two generations of corpora lutea are present, one resulting from pregnancy and another from parturition-induced ovulation (Discussion in Malven and Sawyer, 1966). Normally the corpora lutea of pregnancy regress after parturition. If lactation is prevented, the corpora lutea of pregnancy regress more slowly. Presumably the suckling induced prolactin surges cause the regression of the non-functional corpora lutea of pregnancy. Recent work indicated that the role of prolactin during the estrous cycle in the rat may be luteolysis. Wuttke and Meites (1971) demonstrated that blockade of the proestrous surge of prolactin by ergocornine resulted in an accumulation of old corpora lutea in the ovaries of rats. This work has been confirmed in the rat (Gelato et al., 1972) and mouse

(Grandison et al., 1972). The binding for prolactin during the cycle is relatively high and suggests a function for prolactin during this time.

An interesting question is why is prolactin luteotropic at one time and luteolytic at another? The answer apparently lies in the ovary and how it responds to prolactin. Prolactin inhibits enzymes such as 20α-hydroxysteroid dehydrogenase (Hashimoto and Wiest, 1969), 5α-reductase and 3β-hydroxysteroid dehydrogenase (Zmigrod et al., 1972) which metabolize progesterone to an inactive dihydro compound. In this way prolactin is a luteotropic agent. The mechanism(s) for the action of prolactin as a luteolytic agent are not clearly defined. Malven and Sawyer (1966) proposed that there is a decreased responsiveness of the corpora lutea to prolactin with time. Hashimoto and Wiest (1969) reported that the ratio of prolactin to LH may dictate either support or luteolysis of corpora lutea. In subsequent work, Malven (1969) suggested that the luteolytic action of prolactin may involve some undefined control mechanism over stromal cell development, since the distinctive histological characteristic of a luteolytic response to prolactin is the relative increase in the stromal cell population. The ovarian binding studies reported here were done on whole ovaries. Corpora lutea were not separated from other ovarian tissue. The data show that prolactin binds to ovaries of immature animals which have no corpora lutea. Midgley (1973) demonstrated that prolactin binds to interstitial ovarian tissue as well as luteal tissue. Therefore it is possible that prolactin may be influencing other structures

of the ovary other than the corpora lutea. Binding studies separating corpora lutea from other ovarian tissues may provide some interesting clues on the actions of prolactin on the ovaries during the cycle.

The actions of prolactin on the mammary gland have been well defined. Prolactin becomes attached to its receptor located on the mammary cell membrane (Turkington, 1972a; Shiu et al., 1973) and initiates a series of cellular events involving DNA and RNA directed synthesis of protein kinase, leading to mammary growth and the production of milk proteins. The cellular events following the action of prolactin on the corpora lutea have not been studied as extensively as the mammary gland, but it is known that prolactin stimulates progesterone secretion and this effect is mediated by depression of enzymes involved in the metabolism of progesterone (Hashimoto and Wiest, 1969; Zmigrod et al., 1972). Data presented in this thesis indicate that as the physiological requirement by the ovaries and the mammary gland for the action of prolactin are increased, the binding activity of prolactin is increased. Clemens et al. (1969a) reported that pituitary prolactin is essential during the first six days of gestation to support progesterone secretion from the corpus luteum. The binding activity for pituitary prolactin throughout pregnancy was found to be highest on days 3 and 6 of gestation.

At parturition prolactin serum levels increase and lactation begins. Prolactin is necessary for milk secretion in the rat, and as the need for prolactin is increased, the number of receptor sites for this hormone in the mammary gland are also increased. Frantz

et al. (1974) showed that lactating mouse mammary glands bind more prolactin than non-lactating glands. Recent work by Kelly et al. (1974a) demonstrated that DMBA induced rat mammary tumors which are dependent on prolactin for growth have more binding sites for prolactin than DMBA tumors which are not dependent on prolactin. Similar work by Turkington (1974) also showed that tumors in rats and mice which were most responsive to prolactin for growth had the greatest number of receptors for prolactin.

Another tissue that shows increased prolactin binding activity as its functions increase is the liver. Turkington (1972b) observed an increase in liver function during gestation as evidenced by stimulation of RNA and protein synthesis. Kelly et al. (1974b) found that prolactin binding activity in the liver of pregnant rats was 3-fold higher than in non-pregnant rats. This elevation in prolactin binding activity could possibly reflect stimulation of liver function by prolactin. There are indications that prolactin stimulates protein synthesis in the liver (Burt et al., 1969; Chen et al., 1972). These studies, together with the data presented in this thesis, provide evidence that the number of binding sites for prolactin in a tissue are indicative of physiological functions for this hormone on these tissues.

Some hormonal treatments can alter prolactin binding activity in tissues. Thyroidectomy significantly reduced prolactin binding activity in the liver and replacement therapy with thyroxine returned binding to intact levels. Hydrocortisone acetate treatment significantly decreased prolactin binding activity in the liver.

Unpublished observations (Marshall, Gelato and Meites) in our laboratory indicate that thyroxine and hydrocortisone acetate produce similar effects on prolactin binding activity in the kidney, adrenals and liver.

Thyroidectomy decreases the amount of protein in the liver (Turner and Bagnara, 1971) and thus may reduce the binding sites for prolactin since they are considered to be protein in nature (Frantz et al., 1974). This is supported by Scatchard analysis which showed that the actual number of binding sites for prolactin in the livers of thyroidectomized rats decreased as compared to intact controls, and there was little change in the affinity constant of these sites. Thyroxine is an anabolic hormone whereas hydrocortisone acetate is a catabolic hormone (Frieden and Lipner, 1971). The effects of thyroxine and hydrocortisone acetate on prolactin binding activity in the liver, kidneys and adrenal glands could be related to their function as regulators of protein synthesis. Thus, in order for a certain level of prolactin binding activity to be maintained in these tissues, both thyroid and adrenal hormones may be necessary, and an imbalance in these hormones could result in a change in the numbers of binding sites.

Estrogen has been considered to be one of the most important regulators of prolactin secretion (Meites et al., 1972). A recent report by Leung and Sasaki (1973) implicated prolactin as a possible regulator of estrogen receptors in mammary tissue. Data presented in this thesis show that estrogen may be important in regulating prolactin binding activity in the liver and mammary gland. In the

liver, prolactin binding activity was increased 4-5 fold after administration of estrogen to adult ovariectomized and immature female rats. Estrogen has been shown to influence liver function and is implicated in liver protein synthesis (Leathem, 1961). Thus estrogen may increase prolactin binding activity in the liver as a result of increasing general protein synthesis. On the other hand, mammary tissue as well as kidney and adrenal tissues did not show stimulation but inhibition of prolactin binding activity after administration of estrogen. The reason for the decrease in prolactin binding activity in the mammary gland after estrogen administration is not clear, but may be due to the relatively high doses of estrogen employed. High doses of estrogen have been shown to inhibit the action of prolactin on the mammary gland (Meites et al., 1972), reducing lactation and mammary tumor growth. Lower doses of estrogen may increase prolactin binding sites in the mammary gland although this remains to be demonstrated. In DMBA induced rat mammary tumors, low levels of estrogen stimulate growth of the tumors and high doses inhibit growth (Meites, 1970b). Further work needs to be done in order to elucidate the actions of different doses of estrogen on prolactin binding activity.

The relatively high amounts of prolactin binding activity in tissues such as the liver of adult and immature rats and the adrenals and kidneys of immature rats indicates that these tissues are target organs for prolactin. Prolactin has been shown to be a somatic as well as metabolic hormone in mammals, and is generally considered to be a "growth hormone" in avian species (Nicoll and

Bern, 1972). There is a substantial amount of data linking prolactin to metabolic function such as stimulation of hepatic RNA synthesis (Chen et al., 1972), stimulation of glucose utilization (Berle, 1973), stimulation of growth in rats (Knobil, 1959) and many other functions reviewed elsewhere in this thesis. Another function that has been attributed to prolactin is that of an osmoregulator, and this has been recognized as an important role of prolactin in teleosts. Work by Lockett (1965; 1967), Horrobin et al. (1971), Buckman and Peake (1973a; 1973b), and Relkin (1973) indicate an osmoregulatory role for prolactin in mammals as well. At least 82 separate functions have been ascribed to prolactin (Nicoll and Bern, 1972) and the advent of receptor physiology may be the key to separating "function" from "fiction".

Research on prolactin receptors is a new area and much work needs to be done in order to determine their physiological significance and their role in target tissues. Receptors are believed to be the intermediates between the presence of a hormone at a target tissue site and initiation of cellular events in the tissue. The studies presented in this thesis suggest a relation between prolactin binding activity and physiological functions in several tissues.

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APPENDIX

CURRICULUM VITAE AND LIST OF PUBLICATIONS

CURRICULUM VITAE

NAME: GELATO, Marie C.

DATE OF BIRTH: July 7, 1947

PLACE OF BIRTH: New York City, New York

PRESENT ADDRESS: Department of Physiology
Michigan State University
East Lansing, Michigan 48824

FUTURE ADDRESS: Max Planck Institute for
Biophysical Chemistry
Department of Neurobiology
34 Gottingen-Nikolausberg
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EDUCATION:

Degree	Year	Institution	Major Field of Study
B.A.	1965-1969	Hunter College	Biol. Sciences
M.S.	1969-1971	Michigan State Univ.	Physiology
Ph.D.	1972-1975	Michigan State Univ.	Neuroendocrinology

HONORS:

(1) Elected associate member of Sigma Xi, Spring, 1973.
(2) Elected full member of Sigma Xi, Spring, 1974.

POSITIONS HELD:

(a) Post-doctoral Fellow, Max Planck Institute, January, 1975.
(b) Teaching Assistant in Physiology, Michigan State University, 1970-present.
(c) Instructor, Department of Physiology, Michigan State University, Summer term, 1972.
(d) Research Assistant in Physiology, Michigan State University, 1970.

TALKS PRESENTED AT SCIENTIFIC MEETINGS:

Meetings	Year	Topic
5th Annual Meeting of Society for the Study of Reproduction	1972	Inhibition of Luteolysis by Iproniazid during the Estrous Cycle in Rats

RESEARCH PUBLICATIONS

1. W. Wuttke, M. Gelato, and J. Meites. Mechanisms of Pentobarbital Actions on Prolactin Release. Endocrinology 89:1191, 1971.

2. W. Wuttke, M. Gelato, and J. Meites. Effects of Na-Pentobarbital on Hypothalamic PIF, LRF, and FSH, RF and on Serum Prolactin, LH and FSH. Brain-Endocrine Interaction. Median Eminence: Structure and Function. Int. Symp. Munich 1971, p. 267-79 (Karger, Basel, 1972).

3. M. Gelato, S.K. Quadri, and J. Meites. Inhibition of Prolactin Release by a Thalidomide-Related Compound (CG 603). Proc. Soc. Exp. Biol. Med. 140:167, 1972.

4. S. Dickerman, G. Kledzik, M. Gelato, J. Chen and J. Meites. Effects of Haloperidol on Serum and Pituitary Prolactin, LH and FSH, on Hypothalamic PIF and LRF, and on Ovulation in Rats. Neuroendocrinology 15:10-20

5. L. Grandison, M. Gelato and J. Meites. Inhibition of Prolactin Secretion by Cholinergic Drugs. Proc. Soc. Exp. Biol. Med. 145: 1236-1239

6. M. Gelato, S. Marshall, M. Boudreau, J. Bruni, G.A. Campbell and J. Meites. Effects of Thyroid and Ovaries on Prolactin Binding Activity in Rat Liver. Endocrinology (in press).

7. M. Gelato, S. Marshall, M. Boudreau and J. Meites. Estrogen Stimulation of Prolactin Binding Activity in the Liver of Immature and Mature Female Rats. (in preparation).

8. M. Gelato, G. Kledzik, S. Marshall, G.D. Riegle and J. Meites. Prolactin Binding Activity in the Ovaries during the Estrous Cycle, Pregnancy and Lactation. (in preparation).