HORMONE CONTROL OF PROLACTIN RECEPTOR ACTIVITY IN MALE RAT ACCESSORY SEX ORGANS, CARCINOGEN-INDUCED MAMMARY TUMORS AND PIGEON CROP-SACS

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
GARY STEVEN KLEDZIK
1976

This is to certify that the

thesis entitled
Hormone Control of Prolactin Receptor Activity in Male Rat Accessory Sex Organs, Carcinogen-Induced Mammary Tumors and Pigeon Crop-Sacs
presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

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Date November 11, 1976

ABSTRACT

HORMONE CONTROL OF PROLACTIN RECEPTOR ACTIVITY IN MALE RAT ACCESSORY SEX ORGANS, CARCINOGEN-INDUCED MAMMARY TUMORS AND PIGEON CROP-SACS

By

Gary Steven Kledzik

1. Specific binding of (^{125}I) iodoprolactin was demonstrated in particulate membrane fractions of rat ventral prostates, carcinogen-induced mammary tumors, and pigeon crop-sacs. The specific binding was time and temperature dependent. Unlabeled prolactin readily displaced the binding of (^{125}I) iodoprolactin, whereas LH, FSH, TSH or GH showed no such competition. By contrast, membranes obtained from rat testis or seminal vesicles did not appreciably bind labeled prolactin.

2. Injections of testosterone propionate into intact 30 and 60 day old rats had little effect on specific prolactin binding in testes or seminal vesicles, but significantly increased prolactin binding per 200 ug of membrane protein derived from ventral prostates. There was no significant difference between the binding values of the two age groups.

3. Castration reduced the specific binding of (^{125}I) iodoprolactin in ventral prostates and injections of testosterone propionate into castrated rats increased prostatic prolactin binding. Scatchard analysis revealed that the concentration of high affinity prolactin binding sites in ventral prostates was decreased by castration and returned toward normal by testosterone injections. The dissociation constants were not significantly altered in the castrated group.

4. In vitro binding of (^{125}I) iodoprolactin was inhibited in prostatic tissue removed from intact rats 2 hours after a subcutaneous injection of unlabeled prolactin, but not in ventral prostates from rats killed 26 or 74 hours after injection. Prolactin injected together with testosterone propionate into castrated rats produced no greater increase in specific prolactin binding than testosterone alone.

5. Administration of ergocornine to intact or castrated rats did not influence the binding of labeled prolactin, and injections of 2.0 ug estradiol benzoate into castrated rats followed by 3 injections of ergocornine within 26 hours also did not significantly influence prolactin binding in the ventral prostate. However, injections of 25 ug estradiol benzoate into castrates followed by ergocornine decreased prostatic prolactin binding at a 5% level of significance.

6. In vitro binding of (^{125}I) iodoprolactin was significantly reduced in membrane preparation of ventral prostates obtained 5, 15 or 30 minutes after an intravenous injection of unlabeled prolactin. Binding of labeled prolactin to ventral prostates removed 1 hour to 8 days after an injection of unlabeled prolactin was not significantly different from control values. A single subcutaneous injection of testosterone propionate increased the binding of labeled prolactin to ventral prostates removed 3, 4 or 5 days later. The binding of labeled prolactin removed 12.5 hours to 2 days and from 6 days to 20 days after testosterone injection did not differ significantly from control values.

7. Specific prolactin binding in ventral prostates of 10 and 20 month old rats was markedly reduced as compared to 2½ month-old rats. Serum prolactin levels were significantly greater in 20 month old rats, and serum testosterone levels significantly less in 10 and 20 month-old rats, as were found in 2½ month-old rats.

8. Crop sacs from juvenile pigeons contains approximately twice as much prolactin binding activity as crop sacs from mature pigeons. Proliferation of the crop sac in response to prolactin is associated with an increase in binding of prolactin. Parent and prolactin injected pigeons, each with proliferated crop sac epithelium,

exhibited 4-5 times as much specific prolactin binding as nonproliferated crops from juvenile or mature birds.

9. A significant negative correlation was noted between administered doses of estrogen and the subsequent binding of (^{125}I) iodoprolactin to rat mammary tumor membranes. Injections of 10 or 25 ug estradiol benzoate daily for 10 days effectively inhibited mammary tumor growth and significantly reduced specific prolactin binding to mammary tumor cell membranes.

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By

Gary Steven Kledzik

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Physiology

1976

DEDICATION

Dedicated to the memory of my brother Rollie Pompili and good friend Suzy Pangborn and to my family.

ACKNOWLEDGMENTS

I wish to sincerely thank Dr. Joseph Meites for his guidance and support throughout my graduate studies. It was indeed a honor and a pleasure for me to have been his student. I also wish to thank Drs. W. D. Collings, E. Convey, G. D. Riegle, and the members of my guidance committee: Drs. C. Welsch, H. Hafs, W. Frantz, K. Moore, R. Pittman and R. Bernard for their advice and encouragement. Appreciation is expressed to all my present and former colleagues especially to Carol Bradley, Steve Marshall, Drs. K. Quadri, S. Dickerman, and M. Gelato.

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INTRODUCTION

A current view of the molecular basis of hormone action is that receptors concentrated in target cells rapidly bind a hormone and, as a consequence activates the hormone dependent cellular events (Litwack, 1972, 1975; O'Malley and Means, 1973). Although numerous reports describe macromolecules that appear to be intimately involved in selective uptake and retention of hormones in presumed target tissues, their relationship to biochemical mechanisms by which hormones influence cellular function is far from clear (Williams-Ashman and Reddi, 1971; Cuatrecasas et al., 1975). Thus, it should be realized that the term "receptor" is used for convenience and inherently implies a lack of knowledge of the precise structure and function of these substances. However, "receptor" in the context used currently in hormonal studies, does not apply to a variety of unrelated substances that bind hormones with low specificity and avidity nor does it apply to a host of enzymes catalyzing hormone metabolism. Most contemporary workers use the term "receptor" to describe those molecules in target cells that serve to recognize a specific hormone. Recognition is

demonstrated by high affinity binding of the hormone to a receptor of limited capacity (Roth et al., 1975; Cuatrecasas et al., 1975).

Receptors for steroid hormones have been localized in cytoplasmic and nuclear fractions of hormone responsive cells and have been well characterized (Gorski et al., 1968; Jensen and DeSombre, 1973; O'Malley and Means, 1973; McGuire et al., 1975). The present discussion will focus on receptors for protein hormones and, in particular those for prolactin. The experimental approach to the study of hormone-receptor interactions has been to measure the binding of a radioactively labeled hormone to intact or fractionated target cells. The fundamental principle is that the binding of radiolabeled hormone to specific receptor sites can be competitively inhibited only through native hormone or closely related analogs.

REVIEW OF LITERATURE

I. Functional Neuroanatomy of Hypothalamio-Hypophyseal System

A. General Anatomy of the Hypothalamus

The hypothalamus, though somewhat vaguely defined, comprises the most ventral portion of the diencephalon where it forms the floor and lower walls of the third ventricle. On the floor of the brain, the hypothalamus is bound cranially by the optic chiasam, laterally by the optic tracts and caudally by the mammillary bodies. Extending from the basal surface is a clear visible protuberance, the tuber cinereum prolonged as the pituitary stalk. An inconspicuous groove, the hypothalamic sulcus, separates the hypothalamus from the dorsally located thalamus (Jenkins, 1972; Haymaker et al., 1969; Martini et al., 1970).

In a craniocaudal direction, individual nuclei of the hypothalamus have been artificially divided into three groups. It is noteworthy that many hypothalamic nuclei are not as clearly defined as they appear to be in diagrams. Often they are diffuse and merge with surrounding tissue or with one another (Schreiber, 1963). The anterior

hypothalamus, a region identical with what some authors term the supraoptic region (Netter, 1968), contains the paraventricular, supraoptic and suprachiasmic nuclei. In the middle hypothalamus or tuberal area the periventricular and arcuate nuclei envelope the base of the third ventricle. Also found in the middle region are the lateral, dorsolmedial and ventromedial nuclei. The posterior hypothalamus contains three nuclei surrounding the prominent mammillary bodies; the pre mammillary, the supramammillary and the tuberomammillary nuclei.

The neural pathways entering or leaving the hypothalamus are numerous. In addition to well defined tracts, they undoubtedly include many isolated fibers connecting hypothalamic nuclei with each other and with other parts of the central nervous system. Ascending fibers from the brain stem and descending fibers from the forebrain comprise the major afferent pathways of the hypothalamus. The forebrain projections to the hypothalamus include those from olfactory and hippocampal areas. The hippocampus (and septum) probably channel sensory and neocortical input to the hypothalamus (Martini et al., 1970). Major efferent hypothalamic connections include an ascending bundle to basal forebrain areas and a descending tract to autonomic motoneurons. Most pertinent to the present discussion are efferent fibers which deliver oxytocin and antidiuretic hormone (ADH) to the posterior pituitary and efferent

fibers that terminate near the pituitary stalk. This latter group of fibers are presumed to contain hypothalamic hormones (releasing factors) that influence the synthesis and release of anterior pituitary hormones (Martini et al., 1970; Martini and Ganong, 1966; Meites, 1970a).

B. Anatomy of the Pituitary Gland

The pituitary or hypophysis is a composite gland of neural and epithelial components situated underneath the hypothalamus. In some species the pituitary lobes are encased in the sella turcica, a concavity of the sphenoid bone (Atwell, 1926). The posterior pituitary, i.e., the neurohypophysis, derived from neural tissue, consists of the pars neurosa (posterior lobe) and its upperward extension, the infundibular stem. The glial cells of the neurohypophysis are called pituicytes (Bucy, 1932). These were once thought to be the secretory cells of the posterior pituitary. However, most workers now believe that hormones of the posterior pituitary are products of hypothalamic neurosecretory cells. The neurosecretory material moves along axons of the hypothalamo-hypophyseal tract into the pars neurosa where it is stored and released as needed (Turner and Bagnara, 1971).

The anterior portion (adenohypophysis) makes up the epithelial component of the pituitary. The pars distalis (anterior lobe) forms the greater part of the adenohypophysis and is situated in the sella turcica (if

formed). The anterior pituitary also includes the pars tuberalis forming the ventral side of the pituitary stalk and the pars intermedia. The pars intermedia is regarded as a separate (middle) lobe of the pituitary in some sub-mammalian species. Histologically, the epithelial cells of the anterior pituitary can be divided into three main groups according to their staining properties: acidophils, basophils and chromophobes. The numerical relationship of the cell types varies considerably in different functional states, e.g., sex differences, lactation, castration. Prolactin and growth hormone are thought to originate in acid-stained cells; gonadotrophins and thyrothropin in basophils and ACTH in chromophobes (Ganong, 1969).

The pars tuberalis of the adenohypophysis and the infundibular stem of the neurohypophysis make up the pituitary stalk. The region corresponding to the upper-most portion of the stalk is often referred to as the median eminence (Tilney, 1936).

C. Hypothalamic Hypophyseal Vascular Connection

In contrast to the direct innervation of the neurohypophysis there is no neural pathway between the hypothalamus and the anterior pituitary. An alternate pathway by which the nervous system might influence the pituitary gland was provided by the discovery of hypophyseal portal veins (Popa and Fielding, 1930). Other studies

later established that the direction of blood flow is from the median eminence to sinusoids of the pars distalis (Houssay et al., 1935; Green and Harris, 1947). The significance of this hypothalamic-hypophyseal vascular link was unappreciated until Harris and others (Harris and Jacobson, 1952; Nikitovitch-Winer and Everett, 1958) demonstrated that the anterior pituitary loses most of its histological characteristics and secretory capacity when the portal vessels are interrupted. When anterior pituitary tissue was grafted to the hypothalamus, particularly to regions near the median eminence, it was noted that the histological appearance and functional activity of the transplants were partially maintained (Halasz et al., 1962; Knigge, 1962). In 1955 Harris proposed the "chemotransmitter hypothesis" suggesting that blood-borne substances introduced into capillaries of the hypophyseal portal system are responsible for neural regulation of anterior pituitary function.

In recent years, evidence has accumulated supporting the existence of hypothalamic hypophysiotropic hormones. Although not totally supported, it is presumed that there are chemically distinct hypothalamic hormones for each of the known adenohypophyseal hormones. Three such substances, thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH) and somatostatin (SIF) have been isolated from hypothalamic

tissue and shown to have marked influence on anterior pituitary function. However, all these hormones have been reported to influence the release of more than one pituitary hormone and to exert other neural effects (on behavior). Efforts are being made to determine the physiological significance of these observations.

II. Control of Prolactin Secretion

A. Hypothalamic Inhibition

There is substantial evidence that prolactin synthesis and release are chronically inhibited by the mammalian hypothalamus. The anatomical connections between the hypothalamus and the anterior pituitary are critical in this consideration. Thus, excised pituitaries transplanted to the kidney capsule (Everett, 1954; Chen et al., 1970) or incubated *in vitro* (Meites et al., 1961) produce a hypersecretion of prolactin. Moreover, isolation of the pituitary by stalk section (Dempsey et al., 1940), lesions in the median eminence that destroy the hypophysiotropic area (Chen et al., 1970; Welsch et al., 1971), or administration of certain CNS depressing drugs (Meites, 1962; Meites et al., 1963) enhance prolactin release.

Studies showing that extracts of hypothalamic tissue inhibited pituitary prolactin synthesis and release provided presumptive evidence for the presence of a prolactin-inhibitory-factor (PIF) in the hypothalamus.

Crude hypothalamic extracts from a variety of species (Shally et al., 1967) added to pituitary incubations (Meites et al., 1961; Talwalker et al., 1963) or cultures (Pasteels, 1961) suppressed the autonomous secretion of prolactin. Kragt and Meites (1967) were able to demonstrate a negative dose-response between the amount of hypothalamic extract added to an incubation and the subsequent release of prolactin into the medium. Recently, Nicoll (1971) reported that hypothalamic PIF activity can act to prevent Ca^{++} influx into prolactin secreting cells and thus inhibit the spontaneous release of secretory granules *in vitro*. Several other workers have demonstrated hypothalamic PIF activity *in vitro*. Grosvenor et al. (1964) reported that injected bovine hypothalamic extracts prevented a suckling-induced release of prolactin in rats and Kurashima et al. (1966) reported that porcine hypothalamic extracts inhibited prolactin release in rats following cervical stimulation. Rat hypothalamic extracts reduced serum prolactin in cycling and lactating rats (Amenomori et al., 1970) and decreased serum prolactin in intact male rats (Watson et al., 1971). Kamberi et al. (1971a) infused hypothalamic extracts into a hypophyseal portal blood vessel and noted a dose related inhibition of prolactin secretion.

Hypothalamic PIF content is readily altered by various drugs, hormones and other stimuli. Perphenazine

(Danon et al., 1963), reserpine (Ratner et al., 1965), haloperidol (Dickerman et al., 1972, 1974), sodium pentobarbital (Wuttke et al., 1971), epinephrine and acetycholine (Mittler and Meites, 1967), estrogen (Ratner and Meites, 1964), progesterone, testosterone and contisol (Sar and Meites, 1968), a norethynodrel-mentranol combination (Enovid) (Minaguchi and Meites, 1967) and the suckling stimulus (Ratner and Meites, 1964; Minaguchi and Meites, 1967) were reported to decrease hypothalamic PIF activity and hence raise serum prolactin levels in rats. Agents reported to increase hypothalamic PIF activity include prolactin itself (Chen et al., 1967; Clemens and Meites, 1968; Voogt and Meites, 1971), ergocornine (Wuttke et al., 1971), L-dopa and a variety of monoamine oxidase inhibitors (Lu and Meites, 1971). Furthermore L-dopa was reported to raise PIF activity in systemic blood of hypophysectomized and intact rats (Lu and Meites, 1972) and Kamberi et al. (1971b) reported that a single injection of dopamine into the third ventricle of rats increased PIF activity in hypophyseal portal blood.

B. Hypothalamic Stimulation

Unlike mammals, the predominant influence of the avian hypothalamus is stimulatory to prolactin release and apparently contains a prolactin-releasing factor (PRF). Whereas mammalian pituitaries autografted or cultured <u>in vitro</u> produced a marked secretion of prolactin, transplanted

chicken pituitaries (Ma and Nalbandov, 1963) or cultured pigeon pituitaries (Nicoll and Meites, 1962a) show no increase in prolactin release. Subsequently Kragt and Meites (1965) demonstrated that extracts of pigeon hypothalamic stimulated prolactin release by pigeon pituitaries in vitro. Likewise, crude hypothalamic extracts from chicken, quail (Meites, 1967), tricolored blackbird (Nicoll, 1965), duck (Gourdji and Tixter-Vidal, 1966), and turkey (Chen et al., 1968) induced in vitro prolactin release from their respective pituitaries.

Meites et al. (1960) first reported that crude rat hypothalamic extracts could induce lactation in estrogen-primed female rats. This was later confirmed by Mishkinsky et al. (1968). However, these studies could not definitely conclude that the extracts contained a PRF since many other agents, including cerebral cortical extracts, initiated lactation in these rats. The in vitro studies of Nicoll et al. (1970) provided evidence that rat hypothalamic extracts contained both PIF and PRF activities. Crude hypothalamic extracts added to rat pituitary incubations initially inhibited prolactin release and then later increased prolactin release. Using a somewhat different incubation system, Meites (1970) observed only inhibition of prolactin release over a similar time period. Krulich et al. (1971) sectioned rat hypothalamic and reported PRF activity predominantly in the median eminence and PIF

activity localized in the dorsolateral preoptic area. However, this report is suspected since numerous other studies indicate that median eminence lesions enhance prolactin release suggesting that this area contains PIF activity (Meites et al., 1972) and that lesions in the preoptic area have no significant effect on prolactin secretion (Everett and Quinn, 1966; Kordon, 1966).

Tashjian et al. (1971) first reported that synthetic pyro-glutamyl-histidyl-proline-amine (TRH) induced release of prolactin in vitro from clonal strains of rat pituitary tumor cells. Ensuing studies demonstrated that TRH markedly stimulates prolactin and thyrotropin secretion in humans (Hwang et al., 1971; Bowers et al., 1971; Jacobs et al., 1971), cows (Convey et al., 1972; Kelley et al., 1973), monkeys (Josimovich et al., 1974) and rats (Mueller et al., 1973; Dibbet et al., 1972). These results suggest that TRH might be responsible for hypothalamic PRF activity. However, under many physiological conditions, thyrotropin and prolactin are not released together (Meites, 1973) suggesting that prolactin releasing activity of the hypothalamus cannot be solely accounted for by the existence of TRH. Indeed, Valverde (1972) reported PRF activity in porcine hypothalamic distinct from TRH.

C. Role of Neurotransmitters

There is considerable evidence that synaptic mediators have an important role in control of anterior

pituitary function (Coppola, 1968; Wurtman, 1970). Substances generally thought to serve as neurotransmitters in the mammalian central nervous system include catecholamines (dopamine and norepinephrine), serotonin and acetylcholine (Cooper et al., 1974). Norepinephrine and serotonin are highly concentrated in the hypothalamus (Vogt, 1954; Brodie et al., 1959) and the median eminence is especially rich in dopaminergic nerve terminals (Anden et al., 1964; Fuxe and Hokfelt, 1969; Carlsson et al., 1962). Assay techniques for acetylcholine are much less sensitive and as a result, little is known about the central distribution of cholinergic neurons (Cooper et al., 1974). However, Shute (1969) demonstrated the presence of acetylcholine in the hypothalamus.

A role for catecholamines in regulating prolactin secretion was suggested by Kanematsu et al. (1963), who reported that reserpine, a recognized depleter of stored monoamines (Pletscher et al., 1955; Holzbauer and Vogt, 1956), induced lactation and depressed pituitary prolactin concentration in ovariectomized rabbits. Similarly, Mizuno et al. (1964) observed that iproniazid, an inhibitor of catecholamine metabolism (Jarvik, 1970) inhibited postpartum lactation in rats. Since lactation is not a specific response to prolactin alone, these early studies were of uncertain significance (Meites et al., 1963). However, subsequent work indicated that drugs that inhibit

catecholamine synthesis and release, including reserpine, chlorpromazine, alpha-methyl-para-tyrosine, alpha-methyl-metatyrosine, methyldopa and d-amphetamine, all significantly increased serum prolactin levels. Administration of catecholamine precursors and monoamine oxidase inhibitors resulted in a reduction of serum prolactin (Lu et al., 1970; Lu and Meites, 1971). These observations support the hypothesis that catecholamines act as neurotransmitters to increase release of hypothalamic PIF, which in turn depresses prolactin release (Meites et al., 1972).

An alternate hypothesis proposes that hypothalamic catecholamines may be released into hypophyseal portal blood vessels, transferred to the anterior pituitary and exert a direct effect on prolactin secretion (Fuxe and Nilsson, 1967; Anton-Tay et al., 1971; MacLeod, 1976). Since Fuxe (1964) and Hokfelt (1967) first identified dopaminergic nerve endings in the median eminence, there has been growing evidence that dopamine may be a physiological inhibitor of prolactin secretion. The work of MacLeod (1969), confirmed by Birge et al. (1970), Koch et al. (1970) and Shaar et al. (1974), demonstrated that dopamine directly inhibited in vitro secretion of prolactin. Although other catecholamines directly inhibited prolactin release in vitro, dopamine was most potent in this respect and was effective at concentrations known to exist in the hypothalamus (Shaar and Clemens, 1974b). Kamberi et al.

(1971) reported that catecholamines, principally dopamine, suppressed serum prolactin when injected into the third ventricle of rats but were ineffective when infused into hypophyseal portal blood. However, Takahara (1974) later suggested that the inability of infused catecholamines to alter prolactin release was related to their oxidative destruction. When dissolved in a glucose solution and infused into portal blood, dopamine markedly decreased serum prolactin. Systemically administered L-dopa, the immediate precursor of dopamine, reduced serum prolactin from pituitaries in situ (Lu and Meites, 1972) or when autografted to the kidney capsule (Lu and Meites, 1972; Donoso et al., 1974) and in rats with median eminence lesions (Donoso et al., 1973). Presumably, L-dopa is metabolically converted to dopamine which acts directly on the pituitary to inhibit prolactin release. Dopamine receptor blockers stimulate prolactin secretion in vivo (Meites and Clemens, 1972) and inhibit the action of dopamine in vitro (MacLeod, 1974), and dopamine agonists inhibit prolactin release in vivo and in vitro (Mueller et al., 1976; Smalstig et al., 1974). Recently Takalara et al. (1974) have reported that porcine hypothalamic extracts contain sufficient amounts of catecholamines to account for all the PIF activity. Similarly, Shaar and Clemens (1974a) showed that rat hypothalamic extracts subjected to monoamine oxidation or catecholamine adsorption

by aluminum oxide (alumina) had no PIF activity *in vitro*. However, these findings do not unequivocally mean that PIF is a catecholamine since other hypothalamic preparations showing PIF activity are devoid of catecholamines (Schally et al., 1973; Greibrokk et al., 1974). Also, there is some question as to whether treatment of hypothalamic extracts with alumina or monoamine oxidase may not have removed polypeptides (PIF, PRF, TRH, etc.) as well as catecholamines.

Serotonin and its metabolite melatonin act to increase prolactin release in contrast to catecholamines. Kamberi et al. (1971) observed a significant rise in serum prolactin after central administration of serotonin or melatonin, and Lu and Meites (1973) noted that systemic injections of serotonin precursors that pass the blood-brain barrier (tryptophan and 5-hydroxtryptophan) increase serum prolactin. Serotonin antagonists have been reported to depress the suckling-induced release of prolactin in rats (Kordon et al., 1973) and reduce serum prolactin in estrogen-primed female rats (Chen and Meites, 1974). These results suggest a role for serotonin in hypothalamic control of prolactin release.

Gala et al. (1970) implanted atropine, a cholinergic blocker, into the hypothalamus of rats and induced deciduomata formation suggesting that acetylcholine may be involved in control of prolactin secretion. Later Libertun and McCann (1973) and McLean and Nikitovitch-Winer (1975)

reported that atropine inhibited prolactin release. These results were challenged by Grandison et al. (1974) who showed that acetylcholine injected into cerebral ventricles or cholinergic drugs (pilocarpine and physostigmine) administered systemically, decreased serum prolactin. These authors dismiss the reports by the previous investigators as toxic and nonspecific excitatory effects of the large doses of atropine used. Earlier work has shown that acetylcholine has no direct effect on pituitary prolactin release (Talwalker et al., 1963). But recently, Grandison and Meites (1975) have suggested that the prolactin inhibiting action of the cholinergic system might be mediated by catecholamines. Thus prior administration of drugs that depress catecholamine activity prevented pilocarpine inhibition of prolactin release.

Several amino acids, including gamma-aminobutyric acid (GABA) and glycine have been proposed as possible central neurotransmitter agents (Krnjevic, 1974; Defeudis, 1975). Endogenous GABA is concentrated in the diencephalaic regions of the rat brain (Cooper et al., 1974) and when exogenously administered into lateral ventricles of rats increases serum prolactin levels (Mioduozewski, 1976). Ondo and Pass (1976) have reported similar effects of GABA and additionally noted that glycine also effectively elevated serum prolactin. The distribution of glycine in the central nervous tissue has not yet been established (Cooper

et al., 1974). It is noteworthy that a current abstract from Schally's laboratory (1976) has indicated that GABA may be responsible for hypothalamic PIF activity. Thus the reported effects of GABA on prolactin secretion are contradictory and the physiological significance of these observations are not yet established.

D. Prolactin Short Feedback Loop

Perhaps all pituitary hormones act to depress their own secretion by a "short feedback loop" (Motta et al., 1969). Sgouris and Meites (1953) first hypothesized that prolactin may act to inhibit its own secretion. Subsequent studies have supported this hypothesis albeit the mechanism of the feedback and its physiological significance remain to be determined. Systemically injected prolactin (Sinha and Tucker, 1968), transplanted pituitaries (Welsch et al., 1968) or prolactin secreting tumors (MacLeod, 1966, 1968; Chen et al., 1967), all significantly reduced the prolactin content of in situ rat pituitaries. Moreover, small implants of prolactin into the hypothalamus of rats decreased pituitary prolactin (Clemens and Meites, 1968) and reduced serum prolactin levels (Voogt and Meites, 1971). Similar implants inhibited lactation and caused mammary gland regression (Clemens et al., 1969a), prevented deciduomata formation and interrupted pseudopregnancy (Chen et al., 1968) and pregnancy (Clemens et al., 1969b). Rats bearing pituitary tumors or implants of prolactin in

the median eminence have been reported to increase hypothalamic PIF activity (Meites, 1970), and prolactin injections have been shown to activate tuberoinfundibular dopaminergic neurons in rats (Fuxe and Hokfelt, 1969). Nicoll (1971) has reported that prolactin added to pituitary incubates in vitro has no effect on prolactin release. These observations suggest that prolactin acts by way of the hypothalamus to inhibit its own secretion. However, a possible direct action of prolactin on the pituitary cannot be dismissed. Indeed Spies and Clegg (1971) have reported that pregnancy inhibited by pituitary or hypothalamic implants of prolactin in rats can be maintained by exogenous prolactin suggesting the pituitary as a possible site for prolactin feedback in autoregulation.

E. Exteroceptive Stimuli

Many exteroceptive stimuli can influence prolactin release. A variety of direct and indirect studies have suggested that the nursing stimulus produce a rapid release of pituitary prolactin. Early studies noted that suckling limited to a few nipples maintained milk secretion in all the mammary glands (Selye, 1934; Selye et al., 1934). A role for prolactin in the initiation and maintenance of lactation is now well established (Meites, 1961, 1966; Cowie, 1969). Within minutes after suckling, prolactin release increases and is maintained at high levels for 2-3 hours in rats (Amenomori et al., 1970). Frequent nursing

is necessary to sustain prolactin secretion during lactation (Folley, 1952; Turner, 1939). Although nursing serves as a major stimulus for prolactin release during lactation, other exteroceptive stimuli are also important (Neill, 1974). The mere presence of pups during the last half of lactation is sufficient stimuli to release prolactin (Grosvenor and Mena, 1971). Olfactory stimuli is the likely substitute for suckling since prolactin was not released when the mother could see and hear the pups but not smell them.

Physical stresses of many types have been reported to affect lactation and prolactin release (Nicoll et al., 1960). Restraint stress, ether anesthesia, continuous lighting or extreme heat are among those nonspecific stresses that elevate serum prolactin levels (Euker et al., 1975; Mueller et al., 1974; Wuttke et al., 1971; Kledzik et al., unpublished). Recently Mueller et al. (1976) observed that restraint stress raises hypothalamic serotonin and serum prolactin levels suggesting a causal relationship. Stresses such as cold (Mueller et al., 1974) and starvation (Campbell et al., 1975) lower serum prolactin. Riegle et al. (unpublished) has noted that restraint chronically applied, may also lower prolactin levels.

F. Effect of Estrogen, Testosterone and Ergot Derivatives on Prolactin Secretion

It has long been recognized that estrogens can markedly influence the secretion of prolactin. There is evidence that estrogens act directly upon the pituitary and/or indirectly through the hypothalamus to promote prolactin synthesis and release. Nicoll and Meites (1962b) first reported that estrogens directly stimulate prolactin release when added to rat pituitaries incubated in vitro. Other studies (Nicoll and Meites, 1964; Lu et al., 1971) subsequently confirmed these results. Estrogens stereotaxically implanted into the pituitary promote pseudopregnancy, mammary growth and lactation and mammary cancer growth, suggesting a direct stimulation of pituitary prolactin release (Ramirez and McCann, 1964; Kanematsu and Sawyer, 1963; Nagasawa et al., 1969). Moreover, estrogens have been reported to directly stimulate prolactin secretion from pituitaries autographed to the kidney capsule (Chen et al., 1970). The observation that estrogen injections reduce hypothalamic PIF activity in rats provides evidence for an indirect action of estrogen on pituitary prolactin release. Although estrogen implanted in the median eminence promote prolactin release (Ramirez and McCann, 1964; Nagasawa et al., 1969) the possibility that it is transported by portal blood to affect the pituitary directly cannot be excluded.

Ovariectomy decreases pituitary prolactin production albeit estrogen reverses this effect (Catt and Moffat, 1967; MacLeod et al., 1969). The proestrous surge of prolactin in rats seems to be dependent on estrogen since it is blocked by prior administration of antiestrogen compounds or estrogen antiserum (Neill et al., 1971). Other evidence suggests that estrogens sensitize the prolactin release mechanisms to exteroceptive stimuli. For example, the stress induced prolactin release (Neill, 1970) or the ease at which cervical stimulation induces pseudopregnancy (Everett, 1966) are greater during the estrogen-dominated phase of the estrus cycle.

Whereas estrogen can directly stimulate prolactin release from incubated pituitaries, Nicoll and Meites (1964) reported that testosterone had no effect on prolactin release in vitro. However, when injected into ovariectomized rats, testosterone reduced hypothalamic PIF content and increased pituitary prolactin concentration (Sar and Meites, 1968). Kalra et al. (1973) reported that testosterone proprionate significantly increased serum prolactin in castrated male and female rats.

Perhaps the most useful agents in suppressing prolactin release are the ergot alkaloids. A number of ergot derivatives (dopamine agonists) have been shown to inhibit prolactin secretion, as evidenced by inhibition of lactation and reduced serum prolactin (Shaar and

Clemens, 1972). Ergocornine increases hypothalamic PIF activity (Wuttke et al., 1971), but also can act directly on the pituitary to inhibit prolactin release (Lu et al., 1971). Rat pituitaries incubated in vitro with ergocornine show an increase in prolactin stores but a significant decrease in prolactin release (Lu et al., 1971). Additionally ergocornine has been reported to counteract estrogen stimulation of prolactin secretion in vitro and in vivo.

III. Functions of Prolactin

A. Mammary Gland

The role of prolactin in mammary gland development and secretory activities has been extensively studied. Many workers (Turner, 1939; Lyons et al., 1958; Meites and Hopkins, 1961) have noted that mammary glands of hypophysectomized animals show little or no growth response to ovarian hormones. The definitive studies of Lyons et al. (1958) and Nandi (1958, 1959, 1961) in rodents provided much of the information on the hormonal requirements of the mammary gland. Lyons and colleagues (1958) reported that in triply-operated rats (hypophysectomized, ovariectomized and adrenalectomized) normal mammary development could be induced by injections of growth hormone combined with adrenal steroids and estrogen, but progesterone and prolactin were necessary for lobulo-alveolar development.

The same hormones have been reported to produce mammary gland growth in hyposectomized mice (Nadi, 1959)--although in one strain of mice, growth hormone was found to be interchangeable with prolactin (Nadi, 1961). Subsequently Talwalker and Meites (1961) were able to induce moderate lubulo-alveolar growth in the absence of ovarian or adrenal steroids by multiple daily injections of growth hormone and prolactin. However, these observations do not negate a role for steroid hormones in mammogenesis but suggest that steroids may in some way sensitize the mammary tissue to the action of pituitary hormones. Nagasawa and Yanai (1971) have recently provided support for this idea. They reported that minute implants of estrogen over the mammary glands of ovariectomized rats resulted in localized lobulo-alveolar development when the levels of circulating prolactin were raised. However, if estrogen implants were too concentrated, mammary growth was, in many cases, retarded (Nagasawa and Yanai, 1972). Mammary gland tissue cultured in vitro containing hormone supplements have generally confirmed the in vivo studies on hormonal requirements for mammary gland growth (Rivera, 1964).

Stricker and Grueter (1928) demonstrated that anterior pituitary extracts injected into ovariectomized pseudopregnant rabbits initiated lactation. Ensuing studies with purified prolactin revealed some differences in lactogenic responses obtained with prolactin and with pituitary

extracts. Even though prolactin or adrenocorticoid hormoes alone can induce lactation in pregnant rabbits, a combination of these two hormones increases the intensity of lactation (Friesen, 1966; Meites et al., 1963). Prolactin and adrenal corticoids appear to be minimal requirements for lactation in guinea pigs and rats (Cowie and Lyons, 1959; Meites, 1966), while growth hormone and cortisol are as effective as prolactin and cortisol in C_3H mice (Nadi, 1958).

Hypophysectomy during lactation results in a complete cessation of milk production (Folley and Malpress, 1948). Hormonal replacement in hypophysectomized lactating rats has only been partially successful in restoration of milk secretion. Large doses of prolactin produce milk yields of 25% normal while prolactin administered with ACTH or corticoids results in 50% restoration (Cowie, 1957). Complete milk secretion was restored after hypophysectomy in rabbits injected with either prolactin or growth hormone (Cowie, 1969) and in hypophysectomized goats with prolactin, growth hormone, triiodothyrosmine, insulin and corticosteroid injections (Cowie, 1964).

Prolactin has little or no stimulatory effect on existing lactation in cows (Smith et al., 1974) but does increase milk production in goats (Meites, 1961) and rabbits (Cowie, 1969). Small weight gains have been

reported in rats injected with prolactin, suggestive of a galactopoietic effect (Meites, 1961).

B. Mammary Tumors

The two most important hormones in mammary tumorigenesis in mice and rats are believed to be prolactin and estrogen (Meites, 1972). Although carcinogen-induced rat mammary tumors can develop in the presence of normal serum prolactin levels (Meites, 1972), hypothalamic lesions (Clemens et al., 1968; Welsch et al., 1969), pituitary grafts (Welsch et al., 1968), or central acting drugs (Welsch and Meites, 1970; Quadri et al., 1973) that enhance prolactin secretion promote tumor growth. Drugs which inhibit prolactin release retard mammary tumor growth (Cassell et al., 1971; Quadri et al., 1973). However, if high levels of prolactin or ovarian hormones are present before the administration of a carcinogen the induction of the mammary tumor is inhibited in rats (Clemens et al., 1968; Welsch et al., 1969; Kledzik et al., 1974).

Growth of mammary tumors in rats can be maintained, at least temporarily, by prolactin alone even in the absence of the ovaries and adrenals (Pearson et al., 1969), but estrogen has no growth promoting action on mammary tumors in the absence of the pituitary (Sterental et al., 1963). Whereas low doses of estrogen are stimulatory to mammary tumor development and growth in the intact rat (Huggins et al., 1962), large doses of estrogen have an inhibitory

effect despite their ability to increase blood prolactin levels (Meites, 1972; Huggins et al., 1962). Recent studies have suggested that high doses of estrogen may interfere directly with the stimulatory action of prolactin on mammary tumor tissue (Meites et al., 1971). Welsch and Rivera (1972) reported that prolactin stimulated DNA synthesis in rat mammary tumor organ cultures but high doses of estrogen inhibited DNA synthesis and also suppressed -induced DNA synthesis. Moreover, a significant negative correlation has been reported between administered doses of estrogen and the subsequent binding of prolactin to mammary tumor cell membranes (Kledzik et al., 1976).

Frequently spontaneous mammary tumors appear in old female rats (Meites et al., 1972) and unlike carcinogen-induced mammary adenocarcinomas, they usually occur as single benign fibroadenomas. Apparently prolactin is also a major factor in growth and development of these mammary tumors. Thus bilateral median eminence lesions (Welsch et al., 1970) or multiple pituitary homografts (Welsch et al., 1970), significantly enhanced serum prolactin levels and spontaneous mammary tumor incidence in rats. Moreover, Quadri and Meites (1971) reported marked regression of spontaneous mammary tumors in old female rats treated with ergot drugs. Similar effects of hypothalamic lesions have been reported in mice (Bruni and Montemurro, 1971). In addition, Meites et al. (1972) have occasionally noted

spontaneous mammary tumors in rats bearing transplanted pituitary tumor that secrete large amounts of prolactin and growth hormone.

C. Ovaries

It is generally agreed that prolactin is necessary for progesterone secretion from the corpora lutea in the rat, mouse, hamster and ferret (Hilliard, 1973). In other species, a luteotropic role for prolactin has not been established. Recent studies have indicated that maintenance of progesterone secretion involves a luteotropic complex rather than a single hormone. In rats and ferrets, this complex apparently consists of prolactin and LH whereas in mice and hamster, prolactin and FSH are the minimally required luteotropins (Choudary and Greenwald, 1969; Greenwald, 1969).

In the absence of mating or surrogate cervical stimulation, the corpora lutea of the rat secrete only small amounts of progesterone during the estrous cycle and then regress. However, cervical stimulation prolongs the luteal phase and causes the corpora lutea to become fully functional. If the cervical stimulation does not lead to pregnancy, a "pseudopregnant" period of progesterone secretion lasts for 12-14 days (Hashimoto et al., 1968). Hypophysectomy during pseudopregnancy causes regression of functional corpora lutea but daily injections of prolactin will prevent this (Astwood, 1941). Moreover, during

pseudopregnancy, mammary gland growth and development is suggestive of sustained prolactin release (Freyer and Evans, 1923; Schutze and Turner, 1933). Confirming these earlier indirect studies, Freeman and Neill (1972) reported daily biphasic surges of prolactin release throughout pseudopregnancy. These surges of prolactin appear to maintain the morphological integrity of the corpora lutea and regulate the precursor pools of progesterone (Everett, 1954). Prolactin has been reported to increase the corpora luteal levels of sterol acyl-transferase and sterol esterase that function in the metabolism of cholesterol (Behrman et al., 1970). LH acts to convert the cholesterol to progesterone. Additionally prolactin may act to inhibit enzymes that catabolize progesterone (Weist and Kidwell, 1969; Zmigrod et al., 1972).

A similar pattern of pituitary prolactin release occurs during the first half of pregnancy in rats but after about day 12 prolactin secretion declines and is maintained at low levels until the end of gestation. Just prior to parturition there is a significant increase in prolactin secretion presumably as a result of increased estrogen secretion (Yoshinaga et al., 1969). This increase in prolactin release is believed to participate in the initiation of lactation occurring at parturition (Meites, 1961). During the second half of pregnancy in the rat the luteotropic function is assumed by a placental factor (Astwood

and Greep, 1938). This is evidenced by the failure of the corpora lutea to regress following hypophysectomy after day 12 of pregnancy (Pencharz and Long, 1933).

Paradoxically prolactin can also act to destroy the corpora lutea, at least in mice and rats. Malven (1969) reported that prolactin administered to hypophysectomized rats can have luteotropic or luteolytic actions, depending on the time of injection. When administered within 56 hours after hypophysectomy, prolactin had the luteotropic effect of progesterone stimulation. Yet when injections are delayed, prolactin had a luteolytic effect. Wuttke and Meites (1971) noted that ergot drugs that inhibit endogenous prolactin release do not interfere with normal estrous cycles or ovulation. However, the old corpora of the previous cycles do not degenerate. These observations suggested that the rise in prolactin seen during the estrous cycle serves to induce luteolysis of the older crop of corpora lutea. A similar role for prolactin has been reported in mice (Grandison and Meites, 1972).

D. Crop Sac

Members of the avian family Columbidae (doves and pigeons) have a large thoracic food storage organ, the crop sac (Nicoll, 1974). Near the end of the incubation period, the crop sac of the adult bird rapidly proliferates and thickens. The hypertropied mucosa cells gather fat

globules and are eventually sloughed into the crop sac lumen as "crop milk." Crop milk is used by the parent birds to feed their hatchlings.

Riddle and Braucher (1931) reported that crop sac proliferation is sensitive to anterior pituitary control. Subsequently, Riddle et al. (1932, 1933) identified and isolated prolactin as the pituitary hormone responsible for crop sac proliferation. Although the crop sac can respond to prolactin in the absence of gonadal, adrenal or thyroid hormones (Riddle and Dykshorn, 1932; Schooley et al., 1937) the crop response to prolactin in hypophysectomized pigeons is augmented by thyroxin, growth hormone or adrenocorticoid injections (Bates et al., 1962). Nicoll and Sherry (1967) reported that prolactin stimulates RNA and protein synthesis in pigeon crop sacs.

Over the past four decades the crop sac response to injected prolactin has become the classical bioassay for this hormone (Riddle et al., 1931, 1933; Lyons, 1937; Nicoll, 1967). In the methods most generally used, prolactin has been injected intradermally or systemically once daily and the crop sac removed on the 5th day and measured for growth response.

E. Male Accessory Sex Organs

Several reports have suggested a role for prolactin in controlling prostatic function. Prostatic atrophy was more marked after hypophysectomy than after castration

(Huggins and Russell, 1946; Lostroh and Li, 1957), and the prostatic growth response to exogenous androgen was smaller after hypophysectomy than after orchiodectomy (Grayhack et al., 1955; Vander Laan, 1953). Simultaneous treatment with prolactin enhanced the response of the prostate to testosterone in hypophysectomized rats (Grayhack et al., 1955; Grayhack, 1963) and prolactin and testosterone synergized in maintaining rat prostatic tissue in organ culture (Lasnitzki, 1972). Moger and Geschwind (1972) noted that prolactin alone was able to increase ^{65}Zn uptake by the prostates of castrated male rats. Asano (1965) reported that prostatectomy increased prolactin secretion and pituitary prolactin content in rats, and more recently (Asano et al., 1971) observed that injections of antiserum to prolactin decreased prostatic weights in rabbits. However, these interesting findings remain unconfirmed.

Similar activity of prolactin on the seminal vesicles has been described. Pasqualini (1953) reported a significant increase in seminal vesicle secretion of castrated rats after testosterone followed by prolactin, and Chase et al. (1957) observed growth of seminal vesicles of castrated rats with injections of prolactin alone or in combination with testosterone. Bengmark and Hesselsjo (1963, 1964) reported that prolactin stimulates the proliferation of rat seminal vesicle cells in tissue culture. However, Okamoto et al. (1960) reported that prolactin

increased prostatic weight but not seminal vesicle weights of hypophysectomized or hypophysectomized-castrated rats.

There is also ample evidence that prolactin influences testicular growth and function. In immature hypophysectomized rats, pituitary transplants increased testicular growth (Negro-Vilar and Saad, 1972). Bartke (1965, 1966a, 1966b) studied strains of infertile mice genetically deficient in growth hormone and prolactin. Injections of prolactin into these mice increased their spermatoza yield and rendered them fertile (Bartke and Lloyd, 1970). Similar effects on spermatogenesis were noted in hypophysectomized genetically normal mice (Bartke, 1971) and a synergism between LH and prolactin was observed. Hafez et al. (1971, 1972a, 1972b) reported that prolactin raised enzymatic activity in the testes of dwarf mice and that prolactin injections combined with LH into hypophysectomized rats raised testosterone secretion in vivo and testosterone synthesis in vitro. It is also noteworthy that Boynes et al. (1972) injected an ergot drug that lowered endogenous prolactin while increasing LH release and observed a lowered plasma testosterone level in male rats.

Recently McCann et al. (1974) noted that an initial elevation in serum prolactin at day 25 in male rats is associated with the beginning growth of accessory sex organs and that a second rise in prolactin after day 50 was associated with further accessory organ growth.

F. Osmoregulation

Many studies have indicated that prolactin has important osmoregulatory functions. In seagulls and ducks, prolactin stimulates secretion by nasal or orbital glands concerned with elimination of excess salt (Nicoll, 1974). In certain fish and amphibians, prolactin influences the permeability of gills and the functional activities of kidney and bladder to promote sodium retention (Nicoll and Bern, 1972; Nicoll, 1974).

Recent studies have suggested that prolactin may also facilitate sodium reabsorption by the mammalian kidney. Thus Lockett et al. (1965) reported that prolactin injections reduced urinary water and sodium excretion in rats and cats. Relkin and Adachi (1973) reported increased plasma prolactin levels in rats maintained on a low sodium diet. Marshall et al. (1975) reported that whereas unilateral nephrectomy and water deprivation elevated circulating prolactin levels significantly, salt loading had no effect. Ensor et al. (1972) noted that prolactin injections stimulated drinking and furthered water retention in dehydrated rats. Moreover, Ensor et al. (1972) observed that lactating rats were more resistant to dehydration than nonlactating rats. Humans have also been shown to decrease water and sodium loss and to increase plasma sodium levels in response to prolactin injections (Horrobin et al., 1971). However, hypertonic solutions have been reported

to increase blood prolactin levels and hypotonic solutions were reported to have the opposite effect in rats (Relkin, 1974) and man (Buckman and Peake, 1973a, b). These foregoing observations suggested that prolactin may have a more complex osmoregulatory role in mammals than just promotion of sodium retention by the kidney.

Burstyn et al. (1972) reported prolactin and aldosterone synergism in sheep. Very high salt intake abolished the sodium retaining effect of aldosterone but prolactin injections restored aldosterone activity. Similarly, Horrobin et al. (1973) reported that prolactin injections could restore the water retaining ability of ADH previously blocked by cortisol treatment.

G. Adrenals and Liver

Although prolactin is not generally regarded as a regulator of adrenal and liver function, some evidence has accumulated suggesting a trophic influence of prolactin on these organs. The reported presence of high affinity prolactin binding sites in liver (Posner et al., 1974) and adrenals (Marshall et al., 1975) supports this view.

Many hormones have been reported to inhibit adrenal 5α-reductase activity (Witorsch and Kitay, 1972). Adrenal 5α-reductase converts corticosterone to reduced metabolites. Accordingly, hormones which inhibit 5α-reductase activity promote an increase in corticosterone output. Witorsch et al. (1972) reported that estrogen lowers adrenal

5α-reductase activity in ovariectomized rats only in the presence of the pituitary. Of several pituitary hormones observed to lower reductase activity in hypophysectomized rats, only prolactin was inhibitory in castrated females (Witorsch and Kitay, 1972). This suggests that prolactin is involved in ovarian control of adrenal reductase activity. More recently, Lis et al. (1973) reported that prolactin or ACTH injected into hypophysectomized rats partially restored the capacity of adrenal cells to synthesize corticosterone in vitro and combinations of prolactin and ACTH were more effective than either hormone alone. Furthermore, Piva et al. (1973) demonstrated that prolactin enhances adrenal progesterone secretion in dexamethasone treated castrated female rats and Relkin et al. (1973a, b) reported that prolactin enhances aldosterone secretion in response to sodium deprivation.

Many hepatic functions have been attributed to prolactin in a variety of species. These include effects on carbohydrate, lipid and protein metabolism (Riddle, 1963; Bern and Nicoll, 1968). In general, prolactin has been reported to stimulate protein synthesis in mice (Chen et al., 1972) and rats livers (Turkington, 1972); to reduce hepatic lipid content in lizards (Licht and Hoyer, 1968), pigeons (Goodridge and Ball, 1967) and dogs (Winkler et al., 1971); to promote hepatic glycogen

synthesis in lizards (Callard and Chan, 1972) and mice (Elghamry et al., 1966).

IV. Peptide Hormone Binding to Receptors

A. Hormone Labeling

In order to detect a small number of receptor sites in a cell preparation, a hormone must be labeled to high specific activity without destroying its biological activity. Most receptor studies involving protein hormones have used radioiodine labeling. ^{125}I has been the isotope of choice for a number of practical reasons (Yalow et al., 1968). It is available virtually in a carrier-free state, has a long useful half-life, and is efficiently detected. ^{3}H-labeled peptide hormones have also been used successfully in receptor studies. However, the theoretical advantage of a substituted atom rather than an added iodine is outweighed by a much lower specific activity. Similarly, ^{14}C and ^{35}S do not provide labeled hormones of high specific activity even where it is possible to substitute many atoms (Roth, 1973).

Until recently, radioiodination of hormones were carried out using high concentrations of strong oxidizing agents such as chloramine-T (Hunter et al., 1962) or iodine monochloride (Kahn, 1965). Although these methods can produce radioiodinated hormones of high specific activity, the strong oxidizing agents may drastically alter

protein structure and biological activity. Nevertheless, these methods have been used by many investigators for studies of receptor-hormone interactions. More recently, gentler techniques of radioiodination, employing enzymes such as lactoperoxidase have been developed (Marchalonis, 1969; Thorell et al., 1971). These enzymatic methods can yield labeled hormones with high specific activity while preserving the biological activities.

B. Receptor Preparations

A variety of cell preparations have been the source of "receptors" for protein hormone binding studies. Whole tissue homogenates (Danzo et al., 1972; Leidenbergee et al., 1972) as well as intact cells isolated from the circulation (Lin et al., 1970; Galvin et al., 1972) grown in culture (Lesniak et al., 1973; Krug et al., 1972; Roth et al., 1972) or dispersed by mechanical (Dufau et al., 1971; Catt et al., 1971) or enzymatic treatment (Cuatrecasas, 1971; Kahn et al., 1973) have been used successfully. Cell preparations previously exposed to enzymes such as trypsin or phospholipase show a significant loss in receptor activity suggesting that receptors are proteins associated with membrane lipids (Posner, 1975). Receptors obtained from subcellular sources have been especially concentrated in membrane fractions (Freychet et al., 1971; Cuatrecasas, 1972). For some studies, receptors have been solubilized and freed from the membranes by detergents. However, many

detergents are difficult to remove from proteins and occasionally cause irreversible denaturation or micelle artifacts capable of entrapping labeled hormone (Blecher et al., 1974; Galvin et al., 1972). Solubilized receptors have been partially purified by column chromatography and estimates of their molecular weights range from 40,000 for the epinephrine receptor to several million for the ACTH receptor (Lefkowitz et al., 1969, 1972).

It has been shown that several hormones bind to saturable sites in their target tissues. However, virtually all radio-labeled hormones can bind nonspecifically to many biological and inert materials as well as to specific receptor sites (Cuatrecasas et al., 1975). Specificity is demonstrated when the binding of a labeled hormone is competitively inhibited only by the native hormone or closely related analogs. The degree of inhibition is predictable by the relative biological potencies of the unlabeled competitor. This approach is similar to hapten inhibition used to show the specificity of antigen-antibody reactions.

C. Incubation of Hormone and Receptor

The binding of a labeled hormone to its specific receptor site is dependent on concentration, temperature and time (Cautrecasas et al., 1975; Posner, 1975; Roth, 1973). Increasing the concentration of either labeled hormone or receptors increases the rate and extent of association. Maximal binding occurs sooner at higher

temperatures, but can be achieved at lower temperatures provided the incubation period is prolonged. A narrow pH range of 7.0-7.4 is generally optimal for binding. Dissociation of labeled hormone from receptor sites can be demonstrated by dilution, addition of excess unlabeled hormone or a change in pH and accelerated at increased temperature. Although hormone-receptor interactions should ideally be studied in physiologic conditions (37°C, pH 7.4, etc.), practical considerations often dictate otherwise. Incubation temperatures below 37° reduce hormone and/or receptor degradation and slow dissociation of hormone from receptor. Thus at lower temperatures the separation of bound and free radioactivity can be accomplished with little dissociation.

D. Affinity Constants and Binding Capacity

The affinity of a hormone for its receptor as well as the number of receptor sites in a membrane preparation have been estimated from competitive dose-response curves by a variety of mathematical methods (Feldman, 1972; Weber, 1965; Weder et al., 1974). These methods are based on the following assumptions: (1) labeled and unlabeled hormone react identically; (2) all binding sites are equivalent; (3) there is no cooperativity effect between binding sites; (4) the binding follows the law of mass action. Of the various available methods, one of the most commonly used

is that described by Scatchard (1949). At equilibrium, a Scatchard analysis of hormone-receptor interaction can be described as: $\frac{HR}{H_o-HR} = K_a\ (R_o-HR)$ where H_o is the initial concentration of hormone; R_o, the initial concentration of receptor; HR is the concentration of hormone-receptor complexes and K_a is the affinity constant. A graphical representation plots the ratio of bound/free hormone as a function of that bound. For a single class of binding sites this yields a straight line. The binding parameters can be easily determined from the Scatchard plot. The affinity constant, K_a, equals the negative value of the slope and the number of binding sites corresponds to the intercept on the abscissa. When there is a second class of binding sites, a curved relationship is seen. This is usually the case in hormone binding experiments since radio-labeled hormones bind to specific receptor sites and also to nonspecific sites having lower affinities and higher capacities. However, when the nonspecific binding is subtracted at each point, a linear plot of the specific binding can be resolved (Chamness et al., 1975). Nonspecific binding can be determined as the binding of labeled hormone in the presence of excess unlabeled hormone used to saturate high affinity receptor sites.

E. Prolactin Binding

The tissue distribution of radiolabeled prolactin was first reported by Cox (1951). Twenty minutes after an

intravenous injection into C_3H mice, the highest concentrations of radioactivity were found in the liver and kidney and appreciable amounts in mammary glands, adrenals and ovaries. A later autoradiographic study (Birkinshaw et al., 1972) noted similar results in rabbits with the highest uptake ratios of tissue plasma iodoprolactin in kidneys and lactating mammary glands. In the lactating mammary tissue the radioactivity was found to be localized on or near the alveolar secretory cell membrane proximal to the vascular supply. At about the same time, Mishkinsky et al. (1972) observed high concentrations of radioactivity in mammary glands of lactating rats after the injection of ^{125}I-labeled prolactin or $Na^{125}I$. In both cases, the uptake was suppressed by $KI\text{-}I_2$ indicating that the radioactivity did not necessarily represent labeled-prolactin. However, injections of either ^{125}I-labeled prolactin or ^{125}I-labeled HCG but neither ^{125}I-labeled albumin nor $Na^{125}I$ into pigeon crop sacs resulted in a significant retention of radioactivity. Although both iodoprolactin and iodo-HCG were bound to the crop sac, mucosa proliferation was elicited by prolactin and inhibited by HCG. This was interpreted as evidence for the binding of prolactin and HCG to the same receptor sites.

Rajaniemi et al. (1974) reported the localization of injected iodoprolactin in various tissues of mice and rats. In the kidney, radioactivity was confined to the

proximal tubular cells whereas in the liver the labeling was diffuse over parenchymal and Kupffer cells. In the testis, labeling was localized around Leydig cells with no indication of tubular distribution. The ventral prostate epithelium was strongly labeled but the labeling was weak in epithelium of preputial gland and seminal vesicles. The radioactivity concentrated in the ovary was localized in the corpora lutea and the mammary epithelium showed some labeling during pregnancy and lactation.

Midgley (1973) applied radioiodinated prolactin topically to rat ovarian sections and noted that the localization of radioactivity was affected by the functional state of the corpora lutea, that is, newly formed corpora bound more radioactivity than old corpora.

In this section, each of the foregoing studies have been limited to the identification of prolactin receptor activity by its ability to bind radioactivity presumably representing iodoprolactin. Recently, other investigators have focused attention on the following properties of prolactin receptors predicted from theoretical considerations: (1) they have strict structural specificity for prolactin; (2) they exhibit high affinity binding in accordance with the physiological concentrations of prolactin; (3) they exist in limited numbers in target cells and are easily saturated. Turkington et al. (1972, 1973) and Frantz (1974) identified specific prolactin binding

of high affinity and low capacity in plasma membranes of mouse or rat mammary gland, liver, kidney, midbrain, ovary, adrenal and seminal vesicle cells. The prolactin binding to mammary cell membranes was further characterized and found to be sensitive to trypsin or heating at 70°C indicating a receptor of protein nature. Furthermore, the prolactin concentration at which specific binding was observed was similar to that which induces casein synthesis. This provides supportive evidence for a physiological role of prolactin binding. The fact that the greatest specific binding was detectable in plasma membranes is consistent with an earlier study showing that sepharose-coupled prolactin retains its biological activity, indicating a membrane site of action (Turkington, 1970). Shiu and Friesen (1974a, 1976) have successfully blocked the biological action of prolactin with an antiserum to purified prolactin receptors from rabbit mammary glands. Apparently, the antibodies block the receptor sites and render them inaccessible to prolactin. Other studies have identified and characterized the prolactin binding in various tissues and species (Posner et al., 1974; Shiu et al., 1974b).

Several studies have hinted that prolactin receptor levels may be regulated by endocrine factors. This regulation may serve to determine the degree of tissue sensitivity to prolactin and provide a partial explanation for hormonal synergism and antagonism. Whether a hormone

induces or represses prolactin binding sites seems to depend upon the tissue involved and the existing hormonal milieu. Kelly et al. (1974) observed significant developmental changes in prolactin binding sites in rabbit and rat liver membranes. Prolactin binding activity was found to be similar in male and female rabbit livers and developed gradually before the onset of puberty. In contrast, liver membranes obtained from female rats showed a marked increase in specific prolactin binding during a time associated with the start of puberty and further increased during pregnancy. Liver membranes from male rats at all ages studied showed much lower binding activity than corresponding female livers. This suggests a role for ovarian hormones in prolactin receptor development. Indeed, Gelato and colleagues (1975) subsequently reported that ovariectomy reduces and estrogen increases prolactin binding to liver membranes of female rats. Other workers (Posner et al., 1974b) induced prolactin binding sites in male livers by daily injections of estrogens or prevented this induction by prior administration of anti-estrogen compounds (Kelly et al., 1975). A noteworthy correlation has been reported between the plasma estrogen levels and the number of ovarian prolactin binding sites during the rat estrous cycle (Saito et al., 1975). Binding was lowest during metestrus, increased during diestrus and reached a maximum at proestrus.

Unlike the ontogeny of prolactin receptors in rat livers, binding sites in the kidney and adrenals of rats decreased as puberty approached (Gelato, 1975). Supportive data showing that estrogen has an inhibitory effect on prolactin binding in kidneys and adrenals was provided by Marshall et al. (1976). Similarly, estrogen tended to depress prolactin binding in ventral prostates (Kledzik et al., 1976; Aragona et al., 1975) and mammary tissue (Gelato, 1975) of rats. Differential effects of testosterone on the binding of prolactin in male rats have also been observed. Testosterone reduces receptor activity in kidney and adrenals (Marshall et al., 1976), but increases it in ventral prostates (Kledzik et al., 1976; Aragona et al., 1975). Evidence suggesting a role for prolactin receptors in salt and water regulation has been reported (Marshall et al., 1975).

A concept that multiple hormone interactions are involved in control of prolactin receptors is supported by a number of recent experiments. Ovariectomy (OVX) and/or thyroidectomy (THX) significantly reduced hepatic prolactin binding activity. The combined surgery decreased binding more than either alone. Injections of thyroxin restored binding in THX rats to intact control levels and in OVX-THX rats to OVX values (Gelato et al., 1975). Hypophysectomy results in a loss of prolactin receptor activity in female livers and prevented an estrogen-induced increase in male

livers (Posner et al., 1974). A kidney pituitary implant, capable of maintaining circulating prolactin levels in hypophysectomized rats, partially prevented the decrease in prolactin receptor levels in female rats and induced the receptors in males consequent to hypophysectomy (Posner et al., 1975). Multiple injections of estrogen, progesterone, hydrocortisone, triiodotyronine (T_3) or estrogen plus T_3 had no effect on hepatic prolactin binding in the absence of the pituitary, but a single injection of prolactin to hypophysectomized rat produced a marked increase within 18 hours. Repeated injections of prolactin alone or combined with estrogen and T_3 had no greater effect than a single prolactin injection (Costlow et al., 1975). Administration of prolactin to intact rats did not further increase the normal prolactin binding activity measured in livers (Costlow et al., 1975). These results suggest that prolactin regulates its own receptor activity in rat livers and that part of the inductive effect of estrogen is through an ability to stimulate prolactin.

The binding of prolactin to membrane fractions of pigeon crop sacs has been shown to correlate well with the growth response of the crop sac to prolactin (Kledzik et al., 1975b). A similar relationship between the growth of carcinogen-induced rat mammary cancers to prolactin and subsequent prolactin binding has been reported (Kelly et al., 1974). Other studies (Turkington, 1974; Costlow et

al., 1974, 1975) have noted the existence of high affinity prolactin receptor sites in mammary tumors responsive to prolactin and little, if any, receptor activity in prolactin-independent tumors. Growth of rat mammary tumors in response to endocrine ablation has been better correlated to a combination of estrogen and prolactin receptor levels than to either receptor concentration alone (DeSombre et al., 1976). This supports earlier studies (Meites, 1972; Bradley et al., 1976) suggesting that rat mammary tumors may be dependent upon one or both of these hormones for growth.

The high affinity binding sites in mammary cell membranes have been utilized to develop a specific radioligand-receptor assay for prolactin and other lactogenic hormones (Turkington, 1971; Shiu et al., 1973). This assay offers important advantages that complement the conventional prolactin bioassays. Technically, radioligand assays are more sensitive and precise than bioassays and more conveniently applied to the measurement of large numbers of samples. Moreover, receptor assays allow the biological activity at target cells to be evaluated without the effect of metabolism in vivo (Catt et al., 1972). Shiu et al. (1973) used such a prolactin receptor assay to determine the serum concentrations of prolactin in many species and the potency of various prolactin preparations.

Additionally he was able to identify and measure a placental lactogen secreted during pregnancy in rats.

F. Receptors for Other Polypeptide Hormones

In the past few years many reports have appeared describing membrane receptors for other polypeptide hormones in various tissues. Like those involving prolactin receptors, most of these studies have determined whether the specificity, affinity and number of binding sites are compatible with the known biological activities of the hormones. This section will not attempt a comprehensive review of all such work but rather illustrate a general survey of hormone-receptor interactions studied to date.

Lefkowitz and colleagues (1969) first described the specific binding of radiolabeled ACTH to adrenal tissue extracts. Especially important was the demonstration that ACTH binding was quantitatively related to biological potency. At about the same time, Goodfriend and Lin (1969) identified angiotensin receptors in adrenal, aorta and uterine tissue. Subsequently, these authors (Goodfriend and Lin, 1970) noted that analogs inhibited the binding of labeled angiotensin proportional to their ability to stimulate smooth muscle contraction. Ensuing studies demonstrated the specific binding of labeled calcitonin (Marx et al., 1972), ADH (Campbell et al., 1972) and parathyroid hormone (Malbon and Zull, 1974) to renal

plasma membranes, glucagon to liver and fat cells (Rodbell et al., 1971; Robinson et al., 1971), and oxytocin to uterine fractions (Soloff and Swartz, 1974). Hintz et al. (1974) reported the binding of somatomedin to skeletal, liver and placenta membranes while others noted TRH (Bardon and Labrie, 1973) and LHRH receptors (Spona, 1972) in pituitary cells. Often the hormone-receptor interactions were correlated with adenyl cyclase activity suggestive of cellular stimulation.

Insulin receptors have been well characterized in hepatic (Freychet et al., 1972; Roth et al., 1975), placental (Posner, 1974) and adipose tissue (Cuatrecasas, 1971, 1972). Kahn et al. (1973) noted that livers from hyperglycemic obese mice bound less insulin than livers from thin litter mates. Although the cause of this reduced insulin binding is not yet clear, it does provide some physiological support for these studies. More recently Posner et al. (1974) demonstrated insulin binding activity in normal mammary tissue and Kelly et al. (1974) demonstrate insulin binding in carcinogen induced mammary tumors. Tissues reported to contain growth hormone receptors include liver (Posner et al., 1974), lymphocytes (Lesniak et al., 1973) and mammary tumors (Kelly et al., 1974). A growth hormone radio-receptor assay using liver membranes has revealed the existence of so-called "big" and "little" growth hormones. Apparently they are indistinguishable in

a radioimmunoassay system but the "big" growth hormone has much less binding activity to a receptor preparation. The thyroid has been reported to have binding sites for thyrotropin (Winand and Kohn, 1972) and the gonads contain receptors for follicle stimulating hormone (Means and Vaitukaitus, 1972), luteinizing hormone (Catt et al., 1971) and chorionic gonadotropin (Dufau and Catt, 1973).

Many of the studies cited above undoubtedly reflect true hormone-receptor interactions; there is good correlation between biological and physiochemical data. However, unexpected hormone binding activity was found in tissues not generally regarded as targets. These studies have created interesting possibilities for unknown endocrine functions likely to be elucidated by further work.

MATERIALS AND METHODS

I. Animals

Rats used for all studies, except Experiment V (Serum Prolactin, Testosterone and Prolactin Receptors in Ventral Prostates in Aging Male Rats) were of the Sprague-Dawley strain purchased from Spartan Research Animals Inc. (Haslett, Michigan). Male rats of the Long-Evans strain obtained from the Blue Spruce Farms (Altamont, New York) were used in Experiment V. All rats were housed in a light (14 hours/day) and temperature (25C ± 1C) controlled environment, and fed a diet of Purina Rat Chow (Ralston Purina Co., St. Louis, Missouri) and tap water ad libitum. All castrations, intravenous injections and mammary tumor measurements were performed under ether anesthesia.

Pigeons used in Experiment VI (Prolactin Binding Activity in Crop Sacs of Juvenile, Mature, Parent and Prolactin Injected Pigeons) were of the White Carneau strain obtained from Meadowbrook Farms (Fenton, Michigan). Pigeons that received daily injections were housed in a temperature (25C ± 1C) and light (14 hours/day) controlled

room and maintained on Rydes Pigeon Feed (Rydes Inc., Mason, Michigan) and tap water ad libitum.

II. Prolactin Radioreceptor Assay

A. Preparation of Prolactin-Binding Subcellular Fractions

All tissues were excised from decapitated animals, weighed and immediately frozen on dry ice. Subsequently the tissues were thawed and homogenized at 4°C in .3 M sucrose using a Brinkman Polytron-PT10 or ground glass homogenizer. The homogenates were centrifuged at 14,500 x g for 20 minutes in a Sorvall RC-2B centrifuge and the resultant supernatant centrifuged at 105,000 x g for 90 minutes in a Sorvall OTD-2 ultracentrifuge to obtain a membrane rich pellet (Gray and Whittaker, 1960; Shiu et al., 1973). Each pellet was resuspended in tris buffer (25 mM tris, 10 mM $CaCl_2$, pH 7.6) and its protein content colorimetrically determined by the method of Lowry et al. (1951). A detailed description of this protein assay is presented in Appendix A.

B. Preparation of ^{125}I-labeled Prolactin

Ovine prolactin (NIH S-10, 25.6 IU/ug) was radioiodinated at room temperature by a lactoperoxidase-H_2O_2 method similar to that reported by Thorell and Johansson (1971). A description of the reagents used and the iodination procedures is presented in Appendix B. The

reaction mixture was fractionated on a column of Sephadex G 50 (0.9 cm x 20 cm) eluted with .025 M tris-HCl containing 10 mM $CaCl_2$ at pH 7.8. Fractions of approximately 0.5 ml were diluted with 1% bovine serum albumin (BSA)-tris buffer to give approximately 70,000 cpm per 100 ul in a Nuclear Chicago automatic gamma counter with a 3 inch scintillation detector. Each fraction was tested for binding activity to stock membrane preparations of rat liver and kidney. For the purpose of choosing the best radioactive fraction for binding studies, it was previously determined that liver and kidney membranes would yield essentially the same results as any tissue showing specific binding. The amount of radioactivity that could be displaced by excess unlabeled prolactin (1 ug/tube) was considered to represent specific binding. The fractions demonstrating the highest specific binding were repurified on a Sephadex G-100 column (0.9 cm x 50 cm) and again tested for binding activity. Only the repurified fractions with the highest specific binding were used in the subsequent experiments.

C. Assay Procedure

Subcellular fractions were assayed in quadruplicate in 12 x 75 mm disposable culture tubes. Each tube contained 0.1 ml (^{125}I) iodoprolactin diluted in 1% BSA-tris buffer and a subcellular preparation (usually the 105,000 x g particulate membrane fraction) containing a

predetermined protein concentration in 0.4 ml of tris buffer. Parallel incubations were performed containing the same reactants together with excess unlabeled ovine prolactin (1 ug/tube). Again the final incubation volume was 0.5 ml. The incubations were terminated by the addition of 3 ml tris buffer and the bound and free (^{125}I) iodoprolactin was separated by centrifugation at 800 x g for 30 minutes. The resultant pellets were counted in an automatic gamma counter for 60 seconds each. Specific prolactin binding was the difference between cpm bound in the absence of excess unlabeled prolactin and that bound in its presence.

III. Hormones and Drugs

The following hormones and drugs were used: ovine prolactin (NIH-S-10, 25.6 IU/mg); ovine GH (NIH-S-11, 0.56 IU/mg); ovine LH (NIH-S-15, 0.99 NIH-LH-S-1 units/mg); ovine FSH (NIH-S-7, 1.15 NIH-FSH-S-1 units/mg); ovine TSH (NIH-S-6, 2.47 USP units/mg); estradiol benzonate (EB) and testosterone propionate (TP) (Nutritional Biochemicals Corporation, Cleveland, Ohio) and ergocornine methanesulfonate (ERG) (Sandoz Pharmaceuticals, Hanover, New Jersey). The 7,12-dimethylbenz (a)-anthracene (DMBA) was kindly provided by Dr. Paul Schurr, The Upjohn Co., Kalamazoo, Michigan.

Purified rat prolactin (H-10-10-B) obtained from Dr. S. Ellis (NASA, Ames Research Center, Moffett Field,

California) was iodinated and used for the determination of serum prolactin by the radioimmunoassay method of Niswender et al. (1969). Serum prolactin values are expressed in terms of NIAMDD-rat-prolactin-RP-1. ^{3}H-1,2 testosterone (New England Nuclear, Cambridge, Mass.) was the labeled ligand used for the testosterone immunoassay established by Smith and Hafs (1973) with values expressed in terms of Sigma Chemical Company (St. Louis, Missouri) testosterone.

IV. Statistical Analysis

Unless otherwise stated, all data were statistically evaluated by analysis of variance and individual means compared by the Student-Newman-Keuls test at a 1% level of significance.

EXPERIMENTAL

I. Prolactin Binding Activity in Ventral Prostates, Seminal Vesicles and Testes of 40 and 70 Day Old Male Rats: Effects of Testosterone

A. Objectives

Many reports have suggested a role for prolactin alone or as a synergist with testosterone in maintaining the weight and functional integrity of male accessory sex organs (see Review of Literature, III. Functions of Prolactin, E. Male Accessory Sex Organs). Since the binding of prolactin to high affinity membrane sites is believed to initiate prolactin dependent cellular events, it was of interest to determine whether specific prolactin binding sites existed in ventral prostates, seminal vesicles and testes of immature and mature male rats and, if so, to investigate the effects of testosterone on prolactin binding activity.

B. Materials and Methods

Intact male rats 30 or 60 days of age were injected SC with either 1 mg testosterone propionate (TP) in 0.1 ml corn oil or with vehicle alone (controls) for 10 days.

On the 11th day all rats were killed and their testes, seminal vesicles and ventral prostates removed, dissected free of fat and other adherent tissue and frozen on dry ice. Just prior to homogenization, the capsule surrounding each testis was removed and discarded. Subcellular fractions of decapsulated testes, seminal vesicles and ventral prostates were prepared and the specific binding of labeled prolactin was determined as previously described (see Materials and Methods, I. Prolactin Binding Assay). Using a pooled source of prostatic membranes the time course of binding at 4C, the effect of membrane protein concentration and the binding specificity for prolactin were determined.

C. Results

Figure 1 illustrates the time course of specific binding of labeled prolactin to prostatic membranes. Since a high level of specific binding at 4C was observed at 48 hours, this time was selected for subsequent incubations. Figure 2 shows that the specific binding of (^{125}I) iodo-prolactin increased linearly with the amount of membrane protein added up to 400 ug protein per reaction tube. In order to insure an adequate number of assay replications for each tissue sample, 200 ug of membrane protein was chosen for the routine incubations. Figure 3 shows that levels of TSH, LH or FSH as high as 1000 ng each were unable to displace ^{125}I labeled prolactin from the prostatic

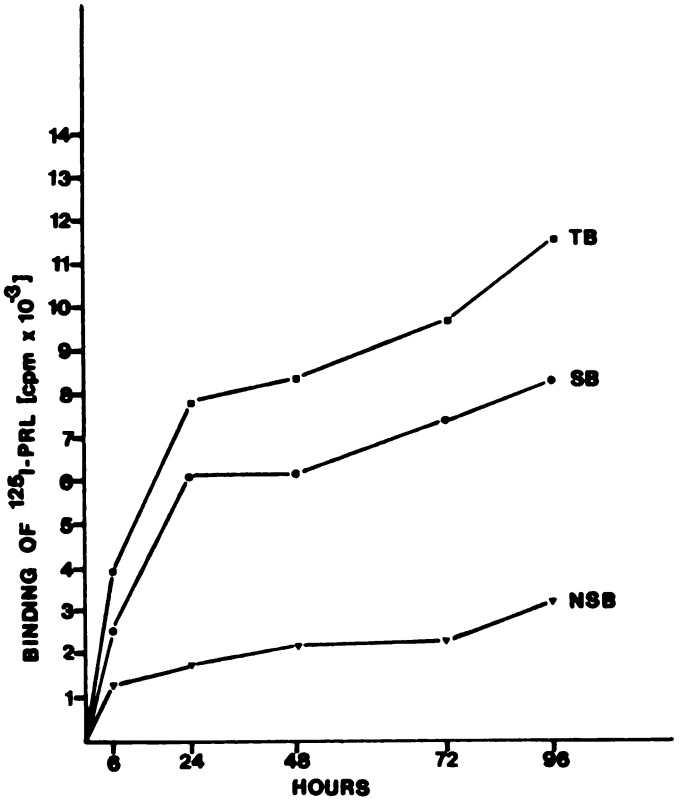

Fig. 1.--Time course of the binding of (^{125}I) iodoprolactin to prostatic membrane preparations. Approximately 72,000 cpm of (^{125}I) iodoprolactin were incubated with 200 ug membrane protein at 4C. Specific binding (SB) was the difference between cpm bound in the absence of excess unlabeled prolactin (TB) and that bound in its presence (NSB).

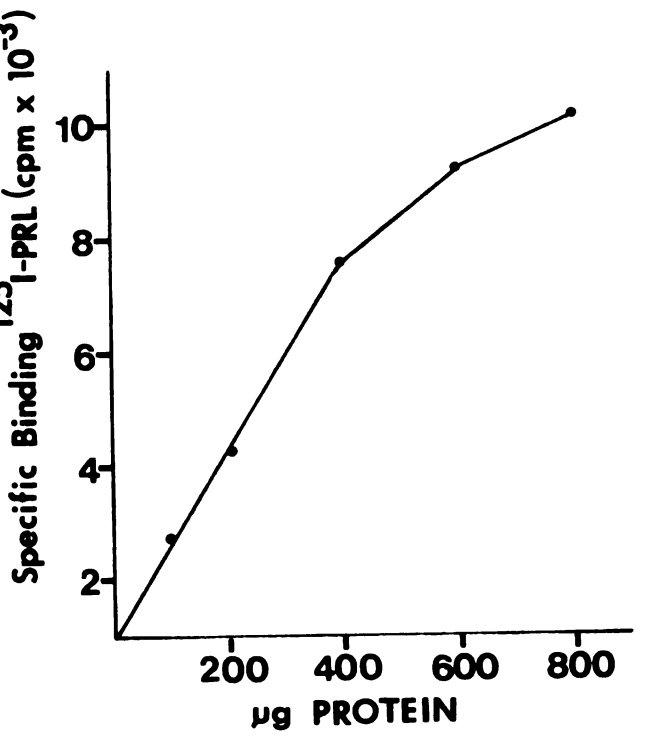

Fig. 2.--Effect of membrane protein concentration on the specific binding of ^{125}I-labeled prolactin. Approximately 62,000 cpm of (^{125}I) iodoprolactin were incubated with prostatic membranes at 4C for 48 hours. Determination of specific binding was as described in Figure 1.

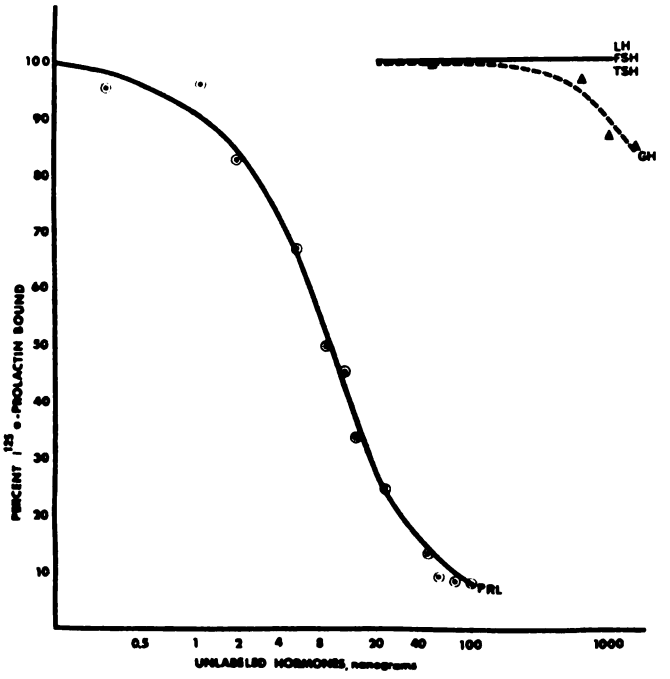

Fig. 3.--Competitive displacement of (^{125}I) iodoprolactin binding to prostatic membranes by various concentrations of unlabeled hormones. The ordinate reflects the amount of labeled-prolactin specifically bound expressed as the percent of that bound in the absence of competing hormone. The abscissa represents the log of the amount of unlabeled hormone present in each reaction tube. All membranes were incubated at 4C for 48 hours with approximately 72,000 cpm (^{125}I) iodoprolactin.

membrane preparation. By contrast, unlabeled prolactin at concentrations greater than 0.4 ng per reaction tube readily displaced the labeled prolactin. The competition seen with levels of GH greater than 500 ng is believed to be due to slight prolactin contamination in this NIH preparation.

The effects of TP injections on prolactin binding activity in testes, seminal vesicles and ventral prostates is shown in Figure 4. In each tissue the highest binding per 200 ug of protein was observed in the 105,000 x g membrane fraction. Injection of 1 mg TP for 10 days into intact 30 and 60 day old rats had little effect on prolactin binding activity in testes or seminal vesicles but significantly increased prolactin binding per 200 ug of protein derived from ventral prostates. There was no significant difference between the binding values of the two age groups. It is noteworthy that relative binding differences between control and TP treated groups were seen in equal amounts of protein from the 14,500 x g pellets although fewer counts were bound.

D. Conclusions

This study shows that particulate membrane fractions of rat ventral prostates have a greater prolactin binding ability than rat testes or seminal vesicles. No significant binding difference was noted in preparations obtained from 40 or 70 day old rats. Testosterone

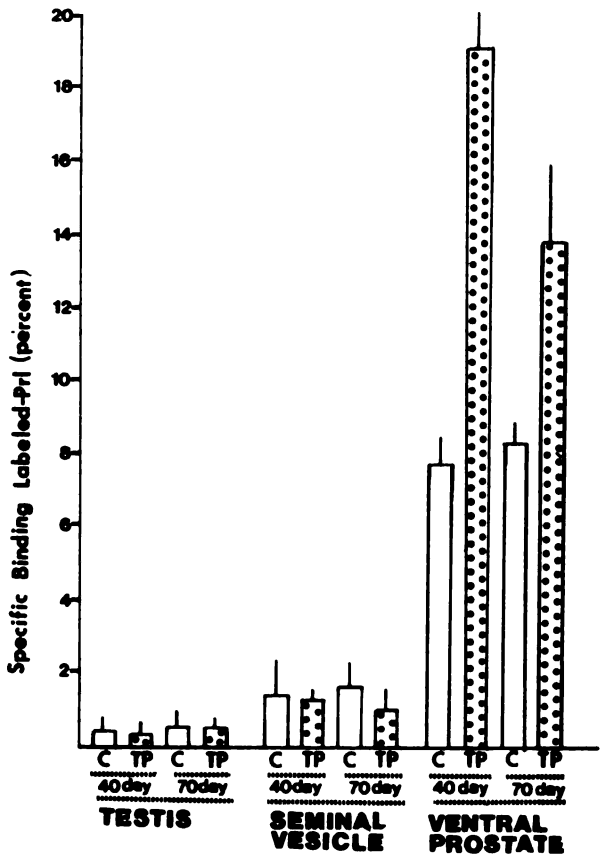

Fig. 4.--The effects of testosterone propionate (TP) injections on specific prolactin binding activity in testes, seminal vesicles and ventral prostates of 40 and 70 day old rats. Each bar represents the mean binding activity in tissues obtained from 10 animals. Two ventral prostates from rats within the same treatment group were pooled before homogenization and subsequently treated as a single sample. Testes and seminal vesicles were assayed individually. Each sample was assayed in quadruplicate. The standard error of the mean is indicated by the ventricle line in the middle of the bar. Membranes were incubated with approximately 62,000 cpm of (^{125}I) iodoprolactin for 48 hours at 4C.

increased specific prolactin binding in ventral prostates but had no significant effect on binding in testes or seminal vesicles. The specific binding of prolactin at 4C was shown to be time dependent and to increase linearly with the amount of membrane protein. The binding specificity for prolactin was demonstrated by displacement with unlabeled prolactin and inability of TSH, LH, FSH or GH to readily compete with labeled prolactin.

II. Effects of Castration, Testosterone, Estradiol and Prolactin on Specific Prolactin Binding Activity in Ventral Prostates of Male Rats

A. Objectives

The previous study indicated that testosterone increases specific prolactin binding to ventral prostates of rats. In order to further verify any testosterone influence on prolactin binding activity in ventral prostates it was necessary to demonstrate an effect of testosterone deficiency (castration). Since it was proposed that prolactin synergizes with testosterone in controlling prostatic function, the effect of prolactin alone or combined with testosterone on prolactin binding activity was ascertained. Other workers (Posner et al., 1974; Gelato et al., 1975) have reported that estrogen increases specific prolactin binding in male and female rat livers, therefore it was also of interest to determine the effects of

estrogen on prolactin binding activity in ventral prostates.

B. Materials and Methods

In the first experiment, intact male rats 30 days of age were injected SC with either 1 mg unlabeled prolactin dissolved in 0.85% NaCl (saline) adjusted to pH 8.2 with 0.1 N NaOH, or with saline at pH 8.2 (controls) for 5 days. The control rats were killed 2 hours after the last injection. The rats injected with prolactin were killed 2, 26 or 74 hours after their last injection. All ventral prostates were excised and frozen.

In the second experiment mature male rats, 200-225 g were castrated and treatment was begun on the following day. The castrated rats were injected SC for 10 days as follows: (a) castrate controls, 0.1 ml corn oil; (b) 1 mg prolactin/0.1 ml saline at pH 8.2; (c) 0.5 mg testosterone propionate (TP)/0.1 ml corn oil; (d) 1 mg TP/0.1 ml corn oil; (e) 1 mg TP/0.1 ml corn oil and 1 mg prolactin/0.1 ml. An intact control group received injections of 0.1 ml corn oil. All rats were killed 26 hours after the last injection.

In the third experiment, 5 groups of rats were treated as in experiment 2, except that 1 mg prolactin was given together with 0.5 mg TP, and treatment with 1 mg TP alone was deleted. Also, the rats were injected for 5 instead of 10 days. These rats were killed 26 hours after

the last injection and their ventral prostates were removed and frozen. Four additional groups in this experiment were treated as follows: (a) intact controls, 0.1 ml corn oil; (b) castrated controls, 0.1 ml corn oil; (c) castrated and 2 ug estradiol benzoate (EB)/0.1 ml corn oil; (d) castrated and 25 ug EB/0.1 ml corn oil. Each of these 4 groups also received 3 injections of 1 mg ergocornine/kg BW over a 26 hour period following their treatments in order to reduce endogenous blood levels of prolactin. Ergocornine (a dopamine agonist) has previously been shown to counteract an estrogen stimulation of prolactin release (Lu et al., 1971). These rats were killed 4 hours after the last ergocornine injection, and blood was collected from the decapitated trunk. The ventral prostates were excised and frozen. The blood was allowed to clot at 4C and the serum separated and stored at -20C until radioimmunoassayed for prolactin by the method of Niswender et al. (1969).

Specific prolactin binding activity was determined in each of the foregoing experiments before the beginning of succeeding experiment. Data was evaluated by analysis of variance and individual means compared by the Student-Newmans-Keuls test.

Scatchard functions were derived from competitive displacement of specifically bound (^{125}I) iodoprolactin by graded doses of unlabeled prolactin. The slopes (generated by linear regression) of the bound/free prolactin ratio as

a function of free prolactin yielded the negative reciprocal of the apparent dissociation constant (-1/Kd). The x-intercept produced the total labeled prolactin binding capacity.

C. Results

Table 1 shows that injection of 1 mg unlabeled prolactin for 5 days into intact 30 day old rats resulted in a significant decrease in ^{125}I labeled prolactin bound when the rats were killed 2 hours after the last injection. Prolactin binding in prostates from intact rats killed 26 or 74 hours after the last prolactin injection did not differ significantly from the controls. There were no differences in the weight of the ventral prostates among the 4 groups of experiment 2.

Castration for 10 days (Table 2) resulted in a significant decrease in prolactin binding per 200 ug particulate membrane protein. Injections of 1 mg prolactin for 10 days into castrates had no significant effect on prostatic binding of labeled prolactin. However, daily injections of 0.5 or 1.0 mg TP into orchidectomized rats significantly raised prolactin binding. The prostatic prolactin binding activity in castrated rats injected daily with the combination of 1 mg TP and 1 mg prolactin was not significantly different from that seen with 1 mg TP alone. Scatchard analysis of competitive displacement of labeled prolactin by graded doses of unlabeled prolactin

Table 1.--Effects of prolactin injections on prolactin specific binding activity in ventral prostates of intact rats.

Treatment (Hours after last injection)	No. of Rats	Ventral Prostate Weight mg/100 g BW	cpm bound[b] / 200 ug protein
Controls (2)	10 (5)[a]	46.0 ± 2.3	4726 ± 840
Prolactin, 1 mg (2)	10 (5)	49.0 ± 2.2	1890 ± 946[c]
Prolactin, 1 mg (26)	10 (5)	47.3 ± 1.6	3570 ± 316
Prolactin, 1 mg (74)	10 (5)	50.5 ± 2.7	3360 ± 526

All values expressed as mean ± SEM

[a]Number of pooled samples; each assayed in quadruplicate.

[b]Membranes were incubated with approximatelt 52,500 cpm of (^{125}I) iodoprolactin for 48 hours at 4C.

[c]Significantly different from other means at $P < 0.01$.

Table 2.--Effects of testosterone propionate (TP) and/or prolactin (PRL) or estradiol benzoate (EB) on specific[a] PRL binding activity in ventral prostates of castrated (Cast) rats.

Treatment for 10 days	No. of Rats	Ventral Prostate Weight[c] mg/100 g BW	cpm bound[c] / 200 ug protein
Intact, controls	10 (5)[b]	74.7 ± 3.6	3793 ± 553[d,e]
Cast, controls	10 (3)	9.6 ± 0.5[d]	632 ± 79[f]
Cast, 1 mg PRL	10 (3)	10.9 ± 0.7[d]	1039 ± 316[f]
Cast, 0.5 mg TP	10	165.4 ± 8.7[e]	3556 ± 474[d]
Cast, 1 mg TP	10	160.2 ± 10.6[e]	4821 ± 316[e]
Cast, 1 mg TP + 1 mg PRL	10	153.7 ± 4.1[e]	5058 ± 237[e]

[a]Membranes were incubated with approximately 49,000 cpm of (^{125}I) iodoPRL for 48 hours at 4C.

[b]Number of pooled samples; each assayed in quadruplicate.

[c]Mean ± SEM.

[d,e,f]Means with different superscripts are significantly different from each other at $P < 0.01$.

revealed the presence of a low concentration (8.8×10^{-15} moles/200 ug protein) of binding sites with high apparent affinity (Kd = 7.1×10^{-11} M) in the intact control membrane preparations. The concentration of high affinity binding sites was greatly reduced by castration (1.2×10^{-15} moles/200 ug protein), and treatment for 5 days with 0.5 mg testosterone propionate returned the concentration of binding sites towards normal values (5.7×10^{-15} moles/200 ug protein). The dissociation constants were not significantly altered in the castrated groups.

Table 3 again shows that castration reduced prolactin binding, and that injections of 0.5 mg TP for 5 days into castrates returned prolactin binding to intact values. Prolactin injections alone clearly had no significant effect on the binding of labeled prolactin and again failed to augment the action of TP. Administration of ergocornine to intact or castrate controls did not influence the binding of labeled prolactin, and injections of 2.0 ug EB for 5 days into castrate rats followed by 3 injections of ergocornine in 26 hours also did not significantly influence prolactin binding in the ventral prostate. However, injections of 25 ug EB into castrates followed by ergocornine decreased prostatic prolactin binding at a 5% level of significance. Neither dose of EB followed by ergocornine had a significant effect on the ventral prostate weight of castrated rats. The circulating levels of

Table 3.--Effects of prolactin (PRL), testosterone propionate (TP), ergocornine (ERG) and estradiol benzoate (EB) on specific prolactin binding activity in ventral prostates of castrated (Cast) rats.

Treatment for 5 days	No. of rats	Prostate weight, mg/100 BW	Serum PRL, ng/ml	cpm bound[b] / 200 ug protein
Intact controls	8	75.5 ± 4.0[c]	17.8 ± 2.6	5476 ± 463[c,d]
(Cast) controls	6 (3)[a]	12.3 ± 0.7[d]	24.8 ± 5.6	848 ± 540[e]
(Cast) 0.5 mg TP	6	112.8 ± 10.2[e]		7481 ± 540[f]
(Cast) 1 mg PRL	6 (3)	13.0 ± 0.9[d]		463 ± 154[e]
(Cast) 0.5 mg TP + 1 mg PRL	6	108.6 ± 7.4[e]		6324 ± 540[c,f]
Intact controls + ERG[h]	6	66.2 ± 2.1[c]	11.3 ± 1.2	4550 ± 386[d]
(Cast) controls + ERG	6 (3)	13.6 ± 0.8[d]	11.7 ± 0.6	540 ± 154[e]
(Cast) 2 ug EB + ERG	6 (3)	18.7 ± 2.0[d]	18.1 ± 2.3	462 ± 231[e]

Table 3.--Continued.

Treatment for 5 days	No. of rats	Prostate weight, mg/100 BW	Serum PRL, ng/ml	cpm bound[b] / 200 ug protein
(Cast) 25 ug EB + ERG	6 (3)	19.4 ± 3.1[d]	23.6 ± 1.3	154 ± 77[e,g]

All values expressed as Mean ± SEM.

[a]Number of pooled samples; each assayed in quadruplicate.

[b]Membranes were incubated with approximately 77,100 cpm of (^{125}I)-iodoprolactin for 48 hours at 4C.

[c,d,e,f]Means with different superscripts are significantly different from each other at $P < 0.01$.

[g]The mean is significantly different from controls at $P < 0.05$.

[h]One mg/kg BW ergocornine was injected 3 times over a 26 hour period following the 5 day treatment period.

prolactin were consistently low (< 25 ng/ml) in the groups given ergocornine as well as in the intact and castrate controls not given ergocornine.

D. Conclusions

This study demonstrates that castration decreases and testosterone increases prolactin binding to a particulate membrane fraction of rat ventral prostate. Scatchard analysis revealed that the concentration of high affinity prolactin binding sites was decreased by castration and returned toward normal by testosterone treatment.

The first experiment clearly demonstrates that injections of unlabeled prolactin can inhibit in vitro binding of labeled prolactin. However, since the binding of labeled prolactin was at the control level 26 and 74 hours after the last prolactin injection, it appears that prolactin does not markedly alter the detection of prostatic prolactin binding activity after 26 hours. This does not preclude the possibility that prolactin can influence prolactin binding in other tissues or in prolactin deficient rats. Indeed recent studies (Posner et al., 1975; Costlow et al., 1975) have suggested that prolactin can increase prolactin binding to hepatic membranes of hypophysectomized rats.

Experiments 2 and 3 show that prolactin in combination with TP did not increase prolactin binding or prostatic weight over that produced by TP alone. It is

possible that the doses of TP used were too large and masked any stimulatory action by prolactin. However, it seems unlikely that 0.5 mg alone (experiment 3) stimulated maximal prolactin binding since 1.0 mg TP produced even greater prolactin binding activity (experiment 2).

The results of experiment 3 suggest that large doses of estrogen injected into castrates can decrease prostatic prolactin binding. Since other studies (Posner et al., 1974; Gelato et al., 1975) have suggested that estrogen increases prolactin binding in rat livers, it appears that the mechanisms regulating prolactin binding sites can be different in different tissues. It subsequently has been shown that estrogen in female rats and testosterone in male rats decrease specific prolactin binding activity in kidneys and adrenal glands while castration increased binding in kidney of both sexes and in adrenals of female rats (Marshall et al., 1976).

III. Effects of Ergocornine Injections on Specific Prolactin Binding Activity in Ventral Prostates of Male Rats

A. Objectives

The preceding study suggested that high levels of circulating prolactin can conceal the detection of prolactin binding sites. Presumably the binding of endogenous prolactin renders the receptor sites inaccessible for *in vitro* binding of labeled prolactin. The previous work also

suggested that 26 hours after the last injection of prolactin, the available prolactin binding sites have returned to control values. However, unlike injected prolactin that wanes with a half-life of approximately 5 minutes (Koch et al., 1971), other agents, notably estrogen, will stimulate the release of endogenous prolactin many hours after its last injection. Ergocornine has been reported to inhibit prolactin release and to counteract estrogen stimulation of prolactin secretion (Wuttke et al., 1971; Lu and Meites, 1971). Although it was shown in the previous study that injections of ergocornine given over 26 hours did not significantly alter prolactin binding activity, it was not known whether these injections altered the binding capacity or affinity for prolactin. An increase in one of these parameters might compensate for a reduction in the other. As a result a change in prolactin binding activity might not be observed. The present study was undertaken to see the effects of once daily injections of ergocornine on prolactin binding activity and to determine whether multiple injections of ergocornine given over 26 hours influenced the binding capacity or affinity for prolactin in ventral prostates.

B. Materials and Methods

Intact male rats, 200-225 g, were injected once daily with either 1 mg or 2 mg ergocornine/kg BW or with the vehicle (0.87% NaCl containing 3% ethanol) alone

(controls) for 2 or 20 days. All rats were killed approximately 2 hours after the last injection and blood collected from the decapitated trunks. The ventral prostates were removed and frozen on dry ice. Serum was separated from the blood and later radioimmunoassayed for circulating prolactin (Niswender et al., 1969). Particulate membrane fractions of the ventral prostates were assayed for specific prolactin binding activity.

In a second experiment, 3 injections of 1 mg ergocornine/kg BW or vehicle alone (controls) were administered to intact male rats, 200-225 g, at 8 hour intervals. All rats were killed 4 hours after the last injection. Blood was collected and later assayed for prolactin. Ventral prostates were removed and subsequently assayed for prolactin binding activity. Membranes from ergocornine treated or control rats were separately pooled and used for Scatchard analysis. Binding capacity and affinity for prolactin were derived from the competitive displacement of (^{125}I) iodoprolactin binding by unlabeled prolactin.

C. Results

Table 4, experiment 1 shows that once daily injections of 1 mg or 2 mg ergocornine/kg BW for 2 or 20 days had no significant effect on the specific binding of labeled prolactin to prostatic membranes. Moreover, 3 injections of 1 mg ergocornine/kg BW given within 24 hours had no significant effect on specific prolactin binding

Table 4.--Effects of ergocornine injections on serum prolactin and specific prolactin binding activity in ventral prostates.

	No. of Rats	Serum Prolactin (ng/ml)	cpm bound[a] / 200 ug Protein
Experiment 1: Injected Once Daily for 20 Days (dose/kg BW)			
Controls	10	15.7 ± 0.4	4679 ± 213
Ergocornine (1 mg)	10	9.5 ± 0.8[b]	5220 ± 736
Ergocornine (2 mg)	10	8.7 ± 0.5[b]	4814 ± 209
Injected Once Daily for 2 Days (dose/kg BW)			
Ergocornine (1 mg)	10	9.8 ± 0.5	4571 ± 530
Ergocornine (2 mg)	10	10.1 ± 0.8	4496 ± 22
Experiment 2: Injected Every 8 Hours for 24 Hours (dose/kg BW)			
Controls	10	16.2 ± 0.9	4665 ± 153
Ergocornine (1 mg)	10	8.1 ± 1.2[b]	4846 ± 484

All values expressed as Mean ± SEM.

[a]Membranes were incubated with approximately 60,000 cpm (^{125}I) iodoprolactin for 48 hours at 4C.

[b]Means are significantly different from controls at $P < 0.01$.

(experiment 2) and no apparent effect on the binding capacity or affinity for prolactin. The dissociation constants of the (^{125}I) iodoprolactin binding to membrane preparations of control and ergocornine treated rats were 2.0×10^{-11} M and 2.6×10^{-11} M and the prolactin binding capacities per 200 ug protein were 3.1 and 3.9 fmoles respectively. In all cases serum prolactin levels were significantly lower in ergocornine treated rats than in control animals at the time of killing.

D. Conclusions

This study demonstrates that a daily reduction in circulating prolactin induced by single injection of ergocornine does not significantly influence the detection of prolactin binding sites. However, this does not preclude the possibility that a more extreme or prolonged reduction in circulating prolactin has an effect on prolactin binding activity. Deprivation of prolactin may contribute to the marked reduction in prolactin binding sites observed in hypophysectomized rats (Posner et al., 1974).

Most importantly this study shows that injections of ergocornine decrease serum prolactin levels for 24 hours but do not alter the membrane affinity for prolactin or the number of prolactin binding sites. This supports the use of ergocornine in those studies where high levels of circulating prolactin could mask a treatment effect. For example, it was previously shown that large doses of

estrogen reduced prolactin binding activity in ventral prostates of castrated rats. If ergocornine had not been used to offset estrogen stimulation of prolactin release, the reduced binding of labeled prolactin may have been interpreted as representing competition by endogenous prolactin and not the effect of estrogen.

IV. Time Course Effects of Single Injections of Testosterone or Prolactin on Prolactin Binding Activity in Ventral Prostates of Intact Rats

A. Objectives

The foregoing studies indicated that testosterone stimulated and high levels of circulating prolactin apparently masked prolactin binding sites in ventral prostates. The present study was undertaken to investigate the time course of these effects.

B. Materials and Methods

Intact male rats, 225-250 grams, were given a single tail vein injection of 1 mg ovine-prolactin dissolved in 0.85% NaCl at pH 8.2 (saline) or with saline alone (controls). Control rats were killed by decapitation either 5 minutes or 8 days later. Rats injected with prolactin were killed at various time intervals from 5 minutes to 8 days. All ventral prostates were removed,

frozen and subsequently assayed for binding of ^{125}I-labeled prolactin.

In a second study, intact male rats were injected SQ with 1 mg testosterone propionate (TP) dissolved in corn oil or corn oil alone (controls). Control rats were killed either 5 minutes or 20 days later. Rats given TP were killed at various times from 12.5 hours to 20 days after injection. Ventral prostates were excized, frozen and later assayed for prolactin binding activity.

C. Results

Figure 5 shows that the in vitro binding of (^{125}I) iodoprolactin was significantly reduced in membrane preparations obtained 5, 15 or 30 minutes after an intravenous injection of 1 mg unlabeled prolactin. The binding of labeled prolactin to ventral prostates removed 1 hour to 8 days after an injection of unlabeled prolactin was not significantly different from control values. Figure 6 shows that a single, subcutaneous injection of 1 mg TP significantly increased the binding of labeled prolactin to ventral prostates removed 3, 4 or 5 days later. The binding of labeled prolactin to ventral prostates removed from 12.5 hours to 2 days and from 6 days to 20 days after TP injection did not differ significantly from control values.

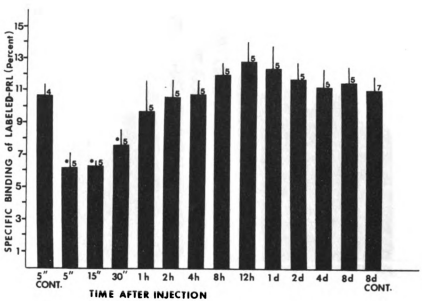

Fig. 5.--Time course effects of a single intravenous injection of unlabeled prolactin on in vitro binding of labeled prolactin. Specific binding is expressed as a percentage of the approximately 68,000 total cpm (^{125}I) iodoprolactin used in each incubation. A * indicates a significant difference as compared to controls ($p < 0.01$), while the vertical line at each dose represents the SEM. The numbers atop each bar indicate the number of individual prostates, each assayed in quadruplicate.

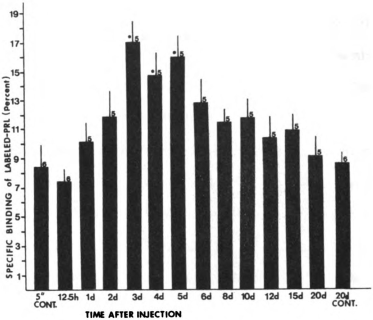

Fig. 6.--Time course effects of a single subcutaneous injection of testosterone propionate on _in vitro_ binding of labeled prolactin. Specific binding is expressed as a percentage of the approximately 68,000 total cpm (^{125}I) iodoprolactin used in each incubation. A * indicates a significant difference as compared to controls ($p < 0.01$) while the vertical line at each dose represents the SEM. The numbers atop each bar indicate the number of individual prostates, each assayed in quadruplicate.

D. Conclusions

These results demonstrate the time course effects of single injections of prolactin on testosterone on prolactin binding activity in ventral prostates. In agreement with an earlier study (Kledzik et al., 1976), high levels of circulating prolactin reduced the subsequent binding of labeled prolactin. The present study suggests that unlabeled prolactin is bound to prostatic membranes *in vivo* within minutes and that the available prolactin binding sites are at control values within an hour. An explanation for time discrepancies between this and an earlier study (Kledzik et al., 1976) in which injected prolactin decreased the binding of labeled prolactin to prostates removed 2 hours after injection, is the way in which unlabeled prolactin was administered. In the current study, unlabeled prolactin was injected intravenously whereas earlier prolactin was administered by a slower subcutaneous route. Shiu and Friesen (1974) have reported that 50% of labeled prolactin is dissociated from mammary gland binding sites *in vitro* at 37C within 5 hours. If injected prolactin had increased its own binding sites and then masked any immediate detection of them, it seems likely that an increase in the binding of labeled prolactin would have been observed at one of the many intervals tested. In general, I believe these results lend further support to the view that prolactin injections do not

markedly influence prolactin binding activity in ventral prostates beyond that of masking the detection of the binding sites.

The time course of testosterone stimulation of prolactin binding is consistent with reported effects of testosterone in ventral prostates. Fujii and Viller (1967, 1968) have noted that a single SC injection of TP produces maximal increases in protein synthesis and prostatic weight 3 to 4 days after injection. The present results show maximal stimulation of prolactin binding activity in prostates 3 to 5 days after TP injection. Since prolactin binding sites have been reported to be largely protein in nature (Posner, 1975), it is encouraging to find that testosterone stimulation of prolactin binding activity coincides temporally with stimulation of protein synthesis.

V. Serum Prolactin, Testosterone and Prolactin Receptors in Ventral Prostates of Aging Male Rats

A. Objectives

Senescence has been regarded by many investigators (Roth and Adelman, 1975) as altered cellular responsiveness to biochemical stimuli. The binding of a hormone to specific receptor sites is thought to initiate the cellular response to that hormone. Recent studies (Kelly et al., 1974; Gelato, 1975) demonstrated early developmental changes in prolactin binding sites, however, prolactin

binding activity in old aged rats has not yet been determined. The purpose of the present study was to investigate the effects of aging upon the prolactin binding activity in ventral prostates and to correlate the binding with serum prolactin and testosterone levels.

B. Materials and Methods

Long-Evans male rats, 2½, 10 or 20 month old were killed by decapitation. Blood was collected from the trunk and the ventral prostates excised and frozen. The blood was allowed to clot at 4C and the serum separated and stored frozen until assayed (in collaboration with H. H. Huang and S. Marshall) for prolactin by the method of Niswender et al. (1969). Serum testosterone was determined (in collaboration with J. F. Bruni) by the method of Smith and Hafs (1973). Specific prolactin binding activity was determined in membrane fractions of each ventral prostate.

In a later experiment, 3 injections of 1 mg ergocornine/kg BW were given to 2½ and 20 month old rats over a 24 hour period before killing. A 2½ month old control group received vehicle (0.87% saline-3% ethanol) injections only. All rats were killed 4 hours after the last injection. Blood collected from the decapitated trunks was later assayed for serum prolactin levels. Ventral prostates were removed, and subsequently assayed for prolactin binding activity in membrane fractions.

C. Results

Table 5 (experiment 1) shows that a specific prolactin binding in ventral prostates of 10 and 20 month old rats was reduced to approximately 50% and 3% of that seen in 2½ month old rats. There was no difference in serum prolactin between 2½ and 10 month old groups but by 20 months, the circulating prolactin had risen 3-fold. Serum testosterone was significantly less in 10 month old rats as compared to 2½ month old animals, and an even greater reduction of serum testosterone was found in 20 month old rats.

Table 5 (experiment 2) shows again that prolactin binding activity was markedly reduced in ventral prostates of 20 month old rats as compared to 2½ month old rats. Serum prolactin levels were similar in these groups that received ergocornine injections. As can be seen in the 2½ month old groups, ergocornine alone had no significant effect on prolactin binding activity.

D. Conclusions

These results demonstrate that aging is associated with significant decreases in binding of labeled prolactin to ventral prostates, increases in serum prolactin and reductions in serum testosterone. It seems unlikely that the decrease in specific prolactin binding was a result of increased endogenous prolactin occupying the binding sites. A significant reduction in binding of labeled

Table 5. Serum prolactin, testosterone and specific prolactin binding activity in ventral prostates of aging male rats.

Age	No. of Rats	Serum Prolactin (ng/ml)	Serum Testosterone (ng/ml)	cpm bound[a] / 200 ug Protein
Experiment 1				
2.5 month	8	41.2 ± 11.7	9.4 ± .53	3295 ± 234
10 month	6	38.6 ± 15.9	3.75 ± .52[b]	1416 ± 392[b]
20 month	5	133.9 ± 48.0[b]	2.24 ± .8[b]	130 ± 64[c]
Experiment 2				
2.5 month, controls	7	34.3 ± 5.6	9.76 ± 2.43	5812 ± 383
2.5 month, ERG injected[d]	7	29.5 ± 8.2	14.72 ± 4.52	6215 ± 437
20 month, ERG injected	5	36.0 ± 6.4	0.86 ± 0.21[b]	901 ± 141[b]

All values expressed as Mean ± SEM.

[a]Membranes were incubated in quadruplicate with approximately 50,000 cpm (experiment 1) and 85,000 cpm (experiment 2) of (^{125}I)-iodoprolactin for 48 hours at 4C.

[b,c]Means with different superscripts are significantly different from each other at $P < 0.01$.

[d]Three injections of 1 mg ergocornine/kg BW were administered over a 24 hour period before killing.

prolactin was noted in ventral prostates from 10 month old rats as compared to prostates from 2½ month old animals although no difference in serum prolactin was found. Moreover, an age related decrease in prolactin binding was seen in rats injected with ergocornine used to minimize endogenous prolactin competition. Since previous experiments established that castration decreases and testosterone increases prolactin binding in ventral prostates, the reduction in serum testosterone observed in 10 and 20 month old rats probably was a major factor in the reduction of prolactin binding activity observed in these old age groups.

VI. Prolactin Binding Activity in the Crop Sacs of Juvenile, Mature, Parent and Prolactin Injected Pigeons

A. Objectives

Prolactin is the endogenous stimulus for the formation of "crop milk" near the end of the pigeon's incubation cycle, and prolactin injections have been shown to cause rapid proliferation of the epithelial lining of the crop sac (Beans and Meyer, 1931; Riddle and Braucher, 1931). The crop sac response to systemic or locally injected prolactin has become the traditional bioassay for this hormone (Riddle et al., 1933; Lyons, 1937; Nicoll, 1967). Since specific prolactin binding sites have been reported to be present in a variety of prolactin responsive tissues (Turkington and Frantz, 1972; Posner et al., 1974), it was

of interest to determine the prolactin binding activity of pigeon crop sacs. In this study crop sacs from juvenile, mature, parent pigeons with crop milk, and pigeons injected with prolactin, were removed and assayed for specific binding of radioiodinated prolactin.

B. Materials and Methods

All birds were of the White Carneau Strain. No sex determination was made, as it has been shown that sex does not influence the crop sac response to prolactin (Riddle and Braucher, 1931). The juvenile pigeons used in this investigation were 4 weeks old, the mature pigeons were 6 months old and 8-12 month old parent pigeons were used 7-10 days after hatching of their young.

Six juvenile, 5 parent and 6 mature pigeons were killed on the day of arrival by cervical dislocation. The crops were excised, washed in cold tap water, frozen on dry ice and stored at -20C for less than 7 days. The remaining 8 mature birds were injected subcutaneously in the loose skin of the lower abdomen once daily for 4 days with 250 mg ovine prolactin dissolved in 0.85% NaCl adjusted to pH 8.3 with 0.1 N NaOH. On the fifth day, the 8 pigeons given prolactin injections were killed, and their crop sacs removed and frozen. All frozen crop sacs were later thawed, minced in .3 M sucrose with a Waring minicup blender and particulate membrane preparations prepared as

previously described (see Materials and Methods, I. Prolactin Binding Assay).

The time course of binding at 4C, the effect of membrane protein concentration and the binding specificity for prolactin were determined in pooled membrane fractions obtained from proliferated crop sacs.

C. Results

Figure 7 shows that the highest level of specific prolactin binding at 4C was at 48 hours and Figure 8 shows that the specific binding of labeled prolactin increased linearly with the amount of membrane protein. Figure 9 shows the results of competitive displacement of labeled prolactin by various concentrations of unlabeled hormones. Only unlabeled prolactin readily displaced the binding of labeled prolactin to 600 ug of membrane protein. Similar effects of incubation time, membrane protein concentration and binding specificity were previously described for prolactin binding activity in ventral prostates.

Table 6 shows the specific cpm bound per 600 ug membrane protein in crop sacs of juvenile, mature, parent and mature pigeons injected with prolactin. Crop sacs from juvenile pigeons contained approximately twice as much binding activity as an equivalent amount of protein from mature pigeons. Neither group showed visible proliferation of the crop sac epithelium. Injections of prolactin for 4 days into mature pigeons produced a 5-6 fold increase in

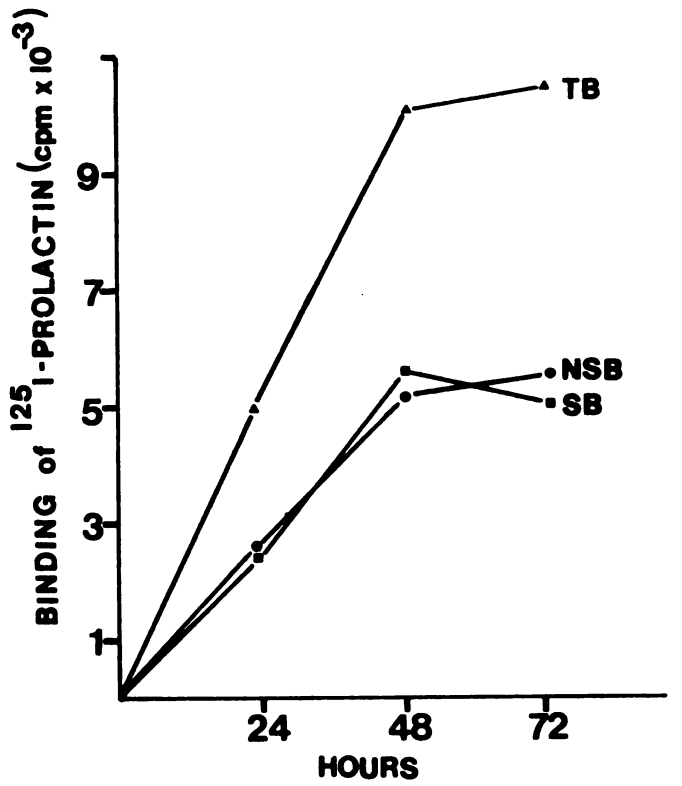

Fig. 7.--Time course of the binding of (^{125}I) iodoprolactin to pigeon crop sac membrane preparations. Approximately 79,000 cpm of (^{125}I) iodoprolactin were incubated with 600 ug membrane protein at 4C. Specific binding (SB) is the difference between cpm bound in the absence of excess unlabeled prolactin (TB) and that bound in its presence (NSB).

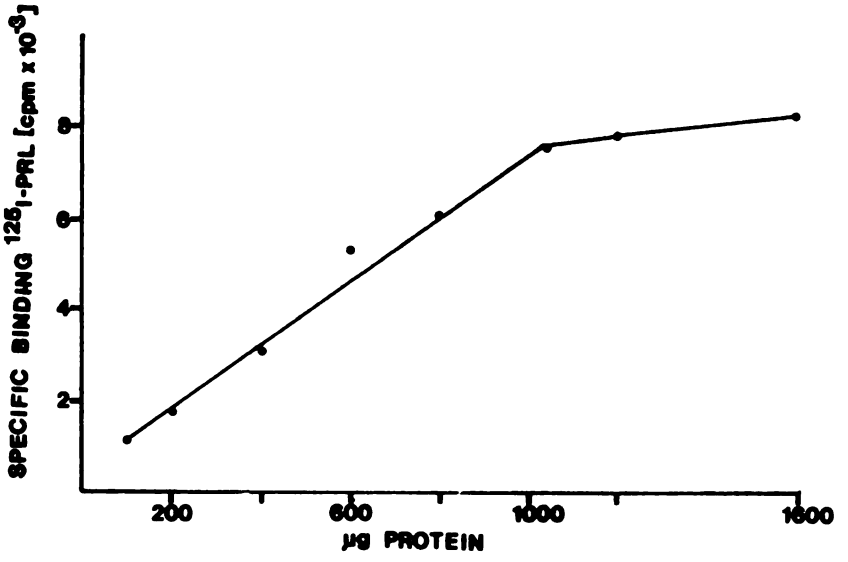

Fig. 8.--Effect of membrane protein concentration on the specific binding of (^{125}I) iodoprolactin. Approximately 79,000 cpm of (^{125}I) iodoprolactin were incubated with crop sac membranes at 4C for 48 hours. Specific binding was determined as described in Figure 7.

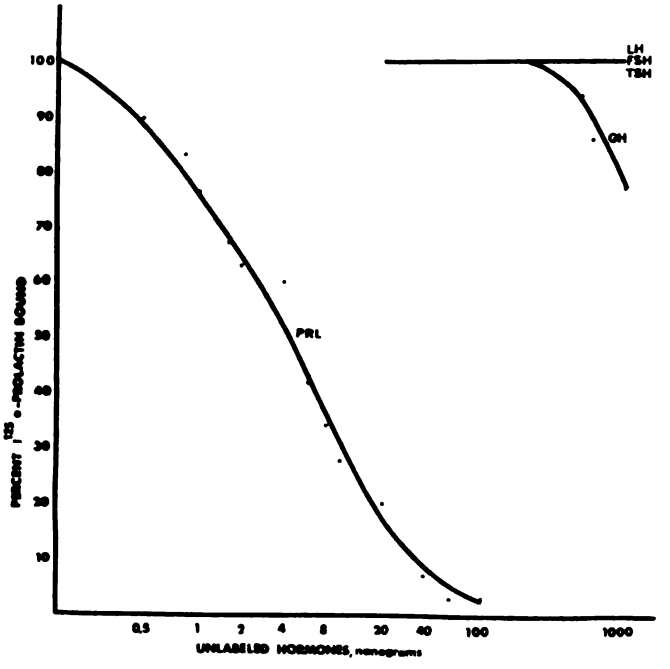

Fig. 9.--Competitive displacement of specific (^{125}I) iodoprolactin to 600 ug of particulate membrane protein from proliferated crop sacs by various concentrations of unlabeled hormones. The ordinate represents the amount of radioactivity specifically bound to the membranes as a % of control in which no unlabeled hormone was present. The abscissa represents the log of the amount of unlabeled hormone present in each reaction tube. All membranes were incubated at 4C for 48 hours with approximately 79,000 cpm of (^{125}I) iodoprolactin.

Table 6.--Specific prolactin binding activity in 600 ug of crop sac microsomal protein.

Type of Pigeon	No. of Pigeons	cpm bound[a,b] / 600 ug protein
Juvenile	6	2204 ± 466*
Mature	6	1153 ± 316
Parent	5	5072 ± 529**
Prolactin-Injected	8	6739 ± 467**

[a]Mean ± SEM

[b]Membranes were incubated at 4C for 48 hours with approximately 79,000 cpm ^{125}I-o-prolactin.

*P < 0.05 as compared to mature pigeons.

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

specific cpm bound and the crop sacs appeared to be as well proliferated as in the parent pigeons. Proliferated crops from parent pigeons had prolactin binding activity considerably higher than in crops from unstimulated juvenile or mature birds, although less than in crop sacs of the prolactin injected pigeons.

D. Conclusions

These results show that the pigeon crop sac contains specific binding sites for prolactin, and that proliferation of the crop sac in response to prolactin is associated with an increase in prolactin binding activity.

Crop sacs from mature pigeons have been reported to be more responsive to prolactin than crop sacs of young birds (Riddle et al., 1933). However, the present study demonstrated that unstimulated crop sacs from adult pigeons had less binding activity than in the crops of juvenile birds. Difference in crop response between adult and juvenile birds to prolactin may have little to do with changes in membrane binding, but may reflect an immaturity of the intracellular mechanisms governing crop epithelial proliferation in the juvenile pigeon.

VII. Effects of High Doses of Estrogen on Prolactin Binding Activity and Growth of Carcinogen Induced Mammary Cancers in Rats

A. Objectives

Prolactin and/or low doses of estrogen are stimulatory to mammary tumor growth in intact rats (Huggins et al., 1962; Meites, 1972). Large doses of estrogen increase serum prolactin but have an inhibitory effect on mammary tumor growth (Meites, 1972). Recent studies have suggested that large doses of estrogen may directly interfere with the stimulatory action of prolactin on mammary tumor tissue (Meites et al., 1971; Welsch and Rivera, 1972). Prolactin binding sites have been shown to be present in carcinogen-induced rat mammary tumors and a direct relationship has been reported between the growth response of these tumors to prolactin and their prolactin binding activity (Kelly et al., 1974). Since the action of prolactin on target tissues is thought to begin with the specific binding to membrane receptor sites, it was of interest to determine the effects of large doses of estrogen on prolactin binding activity in mammary tumor tissue.

B. Materials and Methods

Mammary tumors were induced in 55-60 day old virgin female rats by a single intravenous injection of a lipid emulsion containing 5 mg of 7,12-dimethylhenz(a)anthracene (DMBA). Approximately 2½ months later, when each rat had

developed at least one mammary tumor 1 cm in diameter or larger, the rats were randomly divided into groups and given daily SC injections for 10 days as follows: 1, 0.1 ml corn oil (controls); 2, 2.0 ug estradiol benzoate (EB) in 0.1 ml corn oil; 3, 25.0 ug EB in 0.1 ml corn oil. Immediately prior to, and at 5 day intervals during the treatment period, each mammary tumor was measured with calipers to the nearest mm for length, width, and depth. The sum of these measurements was determined for each tumor. Differences between diameter sums before and after the treatment period were recorded as the growth index. Each rat also received 3 injections of 100 ug ergocornine/100 g BW during a 24 hour period after the 10 days of treatment in order to reduce circulating levels of prolactin and thereby minimize competition for prolactin binding sites by the endogenous prolactin. All rats were killed approximately 4 hours after the last ergocornine injection and blood was collected from the decapitated trunk. Each palpable mammary tumor was excised, frozen on dry ice and stored at -20C until assayed 2 weeks later for prolactin binding activity. The blood was allowed to clot at 4C and the serum was separated and stored at -20C until radioimmunoassayed for circulating prolactin. The entire experiment was subsequently repeated with the following treatments: Group 1, 0.1 ml corn oil (controls); Group 2, 0.2 ug EB in 0.1 ml corn oil; Group 3,

10 ug EB in 0.1 ml corn oil; Group 4, 25 ug EB in 0.1 ml corn oil.

C. Results

Analysis of covariance disclosed that the data obtained from the two experiments could be combined for further statistical treatment, and hence the results are presented as from a single experiment. Figure 10 shows that mammary tumors in control rats increased .46 ± .09 cm in their growth index during the 10 day treatment period. Daily injections of 0.2 ug EB resulted in a gain in tumor growth index of .63 ± .70 cm, and rats given 2.0 ug EB daily showed an increase of .39 ± .10 cm. By contrast, rats injected with 10.0 ug EB or 25.0 ug EB daily showed a significant decrease in tumor growth index of .31 ± .13 cm and .39 ± .11 cm, respectively, during the treatment period.

The time course of specific binding of (^{125}I) iodoprolactin to tumor membranes at 37C, 24C and 4C is shown in Figure 11. Because the highest level of specific binding observed was between 45 and 69 hours at 4C as well as at 14 hours at 24C, a convenient time of 48 hours at 4C was selected for subsequent incubations. Figure 12 shows that of the various polypeptide hormones tested, only unlabeled prolactin readily displaced the binding of (^{125}I) iodoprolactin to tumor membranes. A Scatchard plot of a competitive displacement response curve revealed the

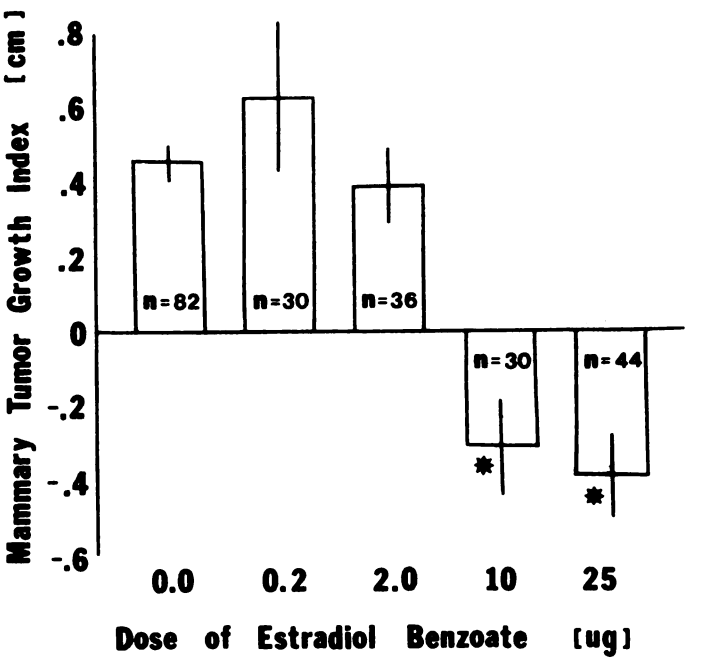

Fig. 10.--Effects of estrogen treatment on tumor growth. The growth index is the difference between the sums of tumor length, width and depth before and after treatment. Analysis of variance was used to determine variations among groups, and the least-significant-difference test was used for all comparisons between treated and control groups; a * indicates a significant difference vs. controls at $p < 0.01$. The vertical lines at each dose represent the SEM.

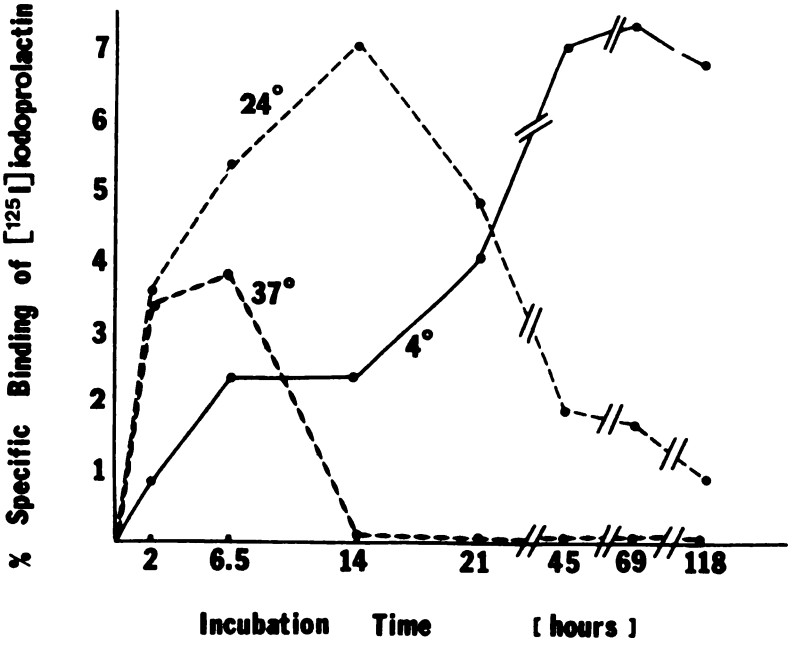

Fig. 11.--Time course of specific binding of (^{125}I) iodoprolactin to tumor membranes at 37C, 24C and 4C. The membrane preparations used for incubations were derived from a pooled source of 38 untreated DMBA-induced mammary tumors. Each reaction tube, incubated in quadruplicate, contained 300 ug membrane protein and approximately 100,000 cpm (^{125}I) iodoprolactin. Parallel incubations were performed in the presence of excess (1 ug/tube) unlabeled oPRL. Specific binding, expressed as a % of total radioactivity used in each incubation, is the difference between cpm bound in the absence of excess unlabeled PRL and that bound in its presence. Since all values were replicates of a common membrane source the SEM at each point was negligible (< 0.1%).

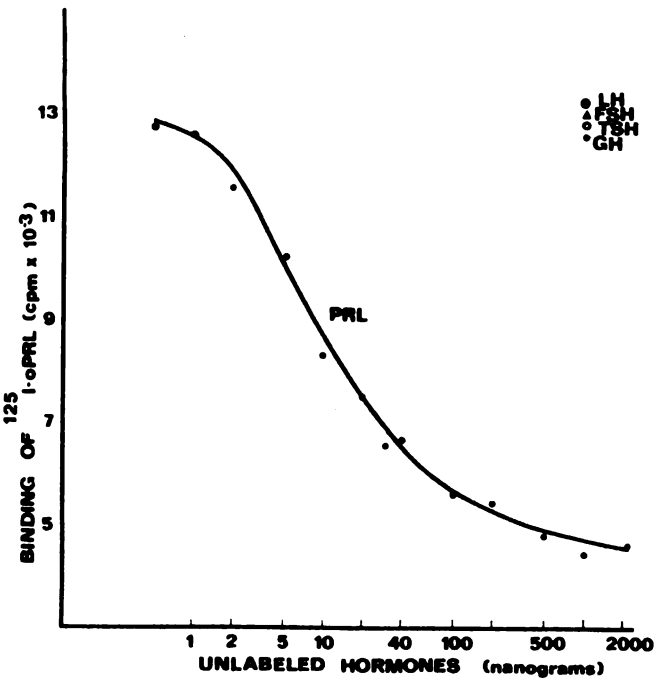

Fig. 12.--Competition of (^{125}I) iodoprolactin and unlabeled hormones for binding to tumor membranes obtained from a pooled source. LH, FSH, TSH and GH were tested only at 1,000 ng per reaction tube. Incubations were carried out in quadruplicate at 4C for 48 hours.

presence of prolactin binding sites in tumor membranes with a dissociation constant (Kd) of 4.0×10^{-9} moles and a binding capacity of 130 femtomols per 300 ug protein (Figure 13).

The effects of the administered doses of EB on specific prolactin binding activity in the DMBA-induced mammary tumors is shown in Figure 14. A linear regression analysis indicated a significant negative correlation ($P < 0.01$) between estrogen dose and the specific binding of (^{125}I) iodoprolactin to tumor membranes. However, further statistical analysis showed that only the two largest doses of EB significantly reduced specific prolactin binding activity as compared to controls ($P < 0.01$). The circulating levels of prolactin in the ergocornine treated rats at the time of killing were determined to be consistently low (< 20 ng/ml) in all groups.

D. Conclusions

Specific binding sites for prolactin were detected in membrane preparations obtained from DMBA-induced mammary tumors in this study, in agreement with a previous report (Kelly et al., 1974). The binding of (^{125}I) iodoprolactin was time and temperature dependent and sensitive to competitive displacement by as little as 0.5 ng unlabeled prolactin. A significant negative correlation was noted between administered doses of estrogen and the subsequent binding of prolactin to tumor cell membranes.

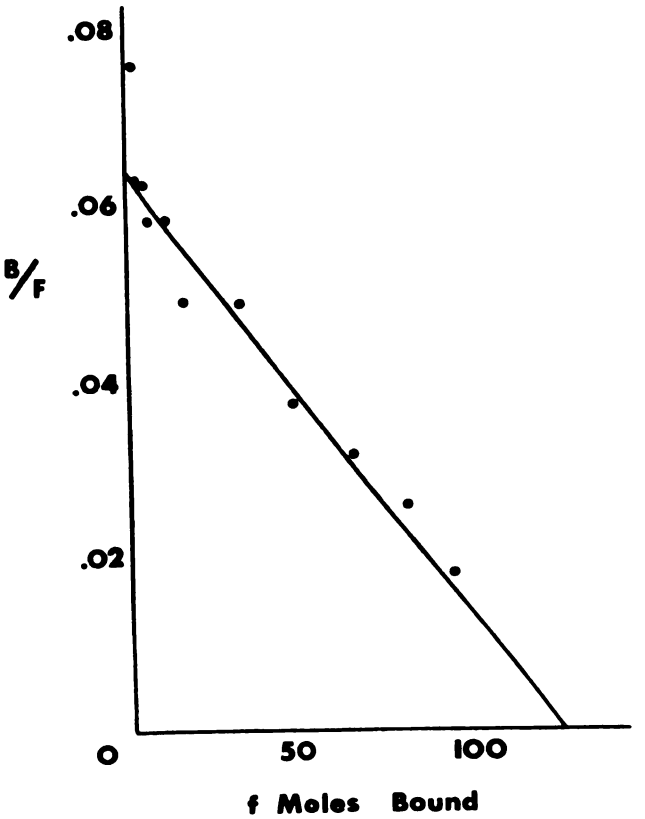

Fig. 13.--Scatchard analysis derived from a competitive inhibition curve. The ordinate represents the ratio of bound/free oPRL and the abscissa the number of femtomoles oPRL bound to tumor membranes. The abscissa intercept represents the total oPRL binding capacity for 300 ug of membrane protein.

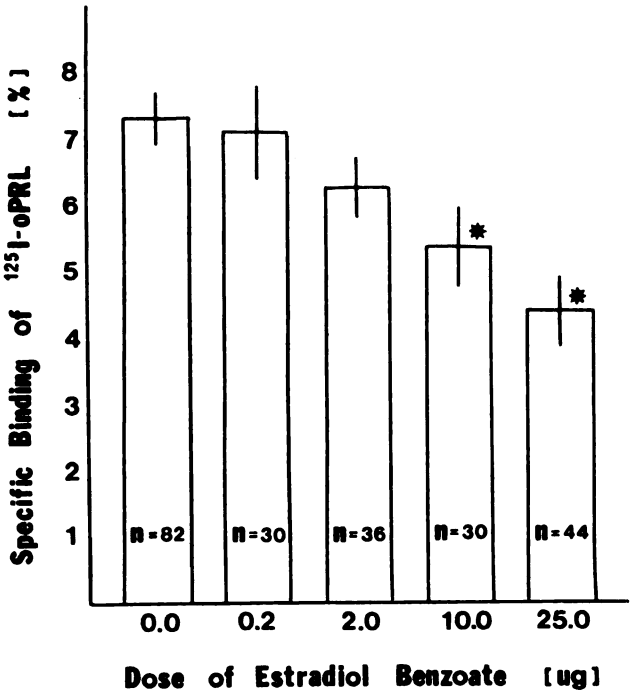

Fig. 14.--Effects of graded doses of EB on the specific binding of (^{125}I) iodoprolactin to mammary tumor membranes. Binding ranged from low (< 2%) to high (> 10%) in each group with no single tumor showing more than 18% specific binding. Variations among groups and mean comparisons were statistically analyzed as described in Figure 10. A * indicates a significant difference vs. controls at $p < 0.01$ while the vertical lines at each dose represent the SEM.

Injections of 10 or 25 ug estradiol benzoate daily for 10 days effectively inhibited mammary tumor growth and significantly reduced specific prolactin binding to mammary tumor membranes. Thus, despite elevated circulating prolactin levels which normally result from high estrogen administration, the growth promoting action of prolactin on mammary tumors appears to be diminished.

GENERAL DISCUSSION

These data indicate that particulate membrane fractions of rat ventral prostates, pigeon crop sacs and DMBA-induced mammary tumors in rats contain specific binding sites for (^{125}I) iodoprolactin. In contrast, membranes obtained from rat testis or seminal vesicles did not appreciably bind labeled prolactin.

Several reports have suggested that prolactin accentuates the effects of androgens in stimulating growth and function of prostates (Grayhack et al., 1955; Grayhack, 1963; Chase et al., 1957) and there is evidence that prolactin increases prostatic binding of testosterone. Thus, Lawrence and Landau (1965) observed that hypophysectomy decreased *in vivo* uptake of radioactive testosterone by rat prostates, and Farnsworth (1972) reported that prolactin increased binding of testosterone by prostatic slices *in vitro*. The data presented in this thesis demonstrates that castration decreases and testosterone increases the concentration of high affinity prolactin binding sites in ventral prostates. The influence of testosterone on the binding activity of prolactin, and of prolactin on

that of testosterone lends support to the view that they exert synergist actions on prostates.

Although other studies have indicated that prolactin increases prolactin binding activity in livers of hypophysectomized rats (Posner et al., 1975; Costlow et al., 1975), the present data suggests no such effect on ventral prostates of intact or castrated rats. Moreover, prolactin in combination with testosterone did not increase prolactin binding over that produced by testosterone alone. However, injections of unlabeled prolactin were able to significantly decrease the detectable prolactin binding sites within minutes following an intravenous injection. Presumably as a result of the higher levels of circulating prolactin, more prolactin binding sites were occupied and thus unavailable for the binding of labeled prolactin. Ergocornine was used to counteract any stimulation of prolactin release that would conceal an effect of treatment on prolactin binding activity.

The pioneering studies of Huggins et al. (1940, 1941) established the efficacy of estrogen therapy in androgen-dependent prostatic cancer. Although it has been shown that estrogen depresses blood testosterone levels (Alder et al., 1968), estrogen also is a potent stimulator of pituitary prolactin release (Chen and Meites, 1970; Nagasawa et al., 1969). Since prolactin has been reported to increase the affinity of the prostate for testosterone

(Farnsworth, 1972), it seems possible that prostatic tissue exposed to high circulating prolactin can more effectively concentrate the lower levels of androgen. The present data demonstrates that prolactin binding in the ventral prostate is reduced after a decrease in testosterone, i.e., after castration. Hence, if prolactin binding reflects biological activity, the ability of the prostate to concentrate testosterone may decrease with estrogen therapy even in the presence of high circulating prolactin. In addition, large doses of estrogen injected into castrated rats tend to further decrease prolactin binding. A role for prolactin in prostatic cancer has not been clearly established, but it is possible that the effectiveness of estrogen therapy on prostatic cancer is mediated in part by influencing prostatic prolactin binding.

Unlike ventral prostates, prolactin significantly stimulates prolactin binding activity in membrane fractions of pigeon crop sacs. The increase in prolactin binding activity in response to prolactin is associated with proliferation of the crop mucosa normally occurs during the second half of the incubation period. Near the end of incubation, and continuing approximately 2 weeks after hatching, secretion of "crop milk" is produced by desquamation of fat-laden epithelial cells (Beams and Meyer, 1931; Riddle and Braucher, 1931). Proliferation of the crop sac

mucosa and formation of "crop milk" in parent pigeons occurs in response to release of endogenous prolactin (Beams and Meyer, 1931; Riddle and Braucher, 1931). Using a cholchicine method, Lahr and Riddle (1938) reported increased mitotic rates in the pigeon crop sac throughout the 18 days of incubation. Injections of prolactin to virgin pigeons produced a crop response not unlike that in parent pigeons, and resulted in an increased mitotic rate. Whether the increase in mitosis is a direct consequence of prolactin binding has not yet been established. However, it appears to be significant that the increased prolactin binding activity is associated with proliferation of crop mucosa.

In recent years, the existence of high affinity estrogen receptor proteins in mammary cancers has been demonstrated (Jensen et al., 1972) and the presence of such receptors appears to be of value in determining estrogen dependency of such cancers. Although Braunsberg et al. (1973) has indicated that tumor regression induced by high estrogen therapy cannot be correlated with estradiol uptake and retention in human mammary tumor tissue, other workers (McGuire et al., 1975) have suggested a good correlation with tumor estrogen receptor values. Because the other important hormone in rat mammary tumor development and growth is prolactin, it was assumed for many years that the effectiveness of high estrogen treatment was

mediated by an inhibition of pituitary prolactin release. However, such an assumption was shown to be incorrect when Chen et al. (1970) demonstrated that large as well as small doses of estrogen elevated serum prolactin levels in rats. Subsequently, Meites (1972) suggested that high estrogen doses may inhibit mammary tumor growth by interfering with the peripheral action of prolactin on the tumor tissue. The present results clearly indicate that large doses of estrogen significantly reduce prolactin binding to tumor membranes. Previous work in our laboratory also suggested that high doses of estrogen in vitro can decrease prolactin binding to slices of DMBA-induced mammary tumor (Bradley et al., 1975). Thus, despite elevated circulating prolactin levels which normally result from high estrogen administration, the growth-promoting action of prolactin on mammary tumors appears to be diminished.

Prolactin binding activity in DMBA-induced mammary tumors previously has been shown to be correlated with the growth response to administered prolactin (Kelly et al., 1974). In general, the tumors with the greatest amount of prolactin receptor activity exhibited the greatest growth response to administered prolactin, and vice versa. If prolactin receptors are reduced in the mammary tumors by administration of high doses of estrogen, then the circulating prolactin would be expected to be less effective in promoting growth of the tumors, as actually observed in the

present work. This could then result in less tumor DNA synthesis from labeled thymidine, as reported by Welsch et al. (1972). However, we also have observed that administration of relatively large doses of prolactin can overcome the inhibition by high doses of estrogen on DMBA-induced mammary tumor growth (Meites et al., 1971). The mechanism(s) involved is not readily apparent at present. It is possible that large doses of administered prolactin increase prolactin receptors or favorably alter estrogen receptor activity in the mammary tumor tissue. Prolactin has been reported to increase estrogen receptors in DMBA-induced mammary tumors in rats (Vignon and Rochefort, 1974).

Throughout these studies I was careful to describe the specific binding of prolactin by membrane fractions as receptor or binding activity rather than receptors per se. This was to stress the uncertainty of any cellular events subsequent to binding and to emphasize that tissue binding of prolactin does not necessarily mean that prolactin receptors are present in every cell. Although it is generally assumed that there is a direct relationship between the amount of receptor binding activity and the magnitude of a target tissue response to a hormone, it is possible that target cells have spare or redundant receptors. Moreover binding activity may not reflect absolute biological activity, especially in wasting tissue (e.g., regressing mammary tumors), since the relation of membrane fractions to viable cells is known. It also is possible that prolactin

responsive tissues contain a mixed population of cells with respect to prolactin receptors and that the effects of testosterone, estrogen and prolactin on prolactin binding activity were to alter the relative number or composition of receptor containing cells. For example, a change in intracellular membranes relative to plasma membranes can alter the receptor content expressed on the basis of membrane protein. Thus binding differences in response to hormones may reflect changes in cells without prolactin receptors and/or cells with prolactin receptors. Likewise, binding comparisons of different tissues may reflect the tissue heterogeneity of cell type or composition and not the cellular sensitivities to prolactin. It seems likely that unequivocal results could be obtained by utilizing pure cell preparations and comparing the number of sites per specific binding cell. These possibilities need to be explored.

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APPENDICES

APPENDIX A

COLORIMETRIC PROTEIN ASSAY

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COLORIMETRIC PROTEIN ASSAY

This is the colorimetric assay of Lowry et al. (1951) in which a phosphomolybdute-phosphotungstate complex is reduced by a copper-protein complex producing a blue color. All reagents are prepared from stock solutions immediately prior to use.

Reagents:

1. Alkaline copper tartarate; mix 2% $CuSO_4$ 5 H_2O (2 ml), 4% tartarate (2 ml) and 3% Na_2CO_3 in 0.1 NaOH (96 ml).
2. Phenol reagent (Folin and Ciocalteu) diluted with equal volume of distilled H_2O.
3. Stock protein standard (bovine serum albumin) 2 mg/ml tris buffer (25 mM tris, 10 mM $CaCl_2$, pH 7.6).

Assay:

1. Each protein standard or unknown sample was assayed in triplicate. 20-300 ug of protein standard or the unknown solution is diluted in tris buffer so as to have a total volume of 0.5 ml. Blank tubes contain tris buffer alone.

2. Five ml of alkaline copper tartarate was added, mixed and allowed to stand at room temperature for 10 minutes.
3. 0.5 ml of diluted phenol is added to each tube, immediately mixed and allowed to react for 20 minutes at room temperature.
4. The optical density of each sample was measured at 750 mu in a Beckman DB-G spectrophotometer initially zeroed with the blank solution.

APPENDIX B

RADIOIODINATION OF PROLACTIN

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RADIOIODINATION OF PROLACTIN

Reagents:

1. Ovine-prolactin (NIH-S-10; 25.6 IU/mg)
2. Carrier free $Na^{125}I$ (Amersham/Searle, Chicago, IL).
3. Lactoperoxidase (grade A, Calbiochem, La Jolla, CA).
4. 30% hydrogen peroxide (Mallinckrodt Chemicals, St. Louis, MO).
5. Sephadex G-50, G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) expanded in tris-HCl buffer containing 10 mm $CaCl_2$ at pH 7.8.

Procedure:

1. Add 1.0 mCi $Na^{125}I$ to serum vial containing 5 µg o-prolactin in 20 µl distilled water.
2. Add 30 ng lactoperoxidase in 10 µl of distilled water and 10 µl of 30% hydrogen peroxide diluted 1:30,000. Shake gently for 50 seconds.
3. Add 200 µl of 16% sucrose solution. Withdraw entire reaction mixture and carefully layer on a sephadex G-50 column (.9 x 20 cm) coated with 1% egg albumin in tris buffer.

4. Collect .5 ml aliquots in disposable culture tubes (12 x 75 mm) containing .5 ml 1% bovine serum albumin (BSA)-tris buffer.
5. Count each tube in a Nuclear Chicago well scintillation counter (DS 303V) for 15 seconds to obtain iodination profile.
6. Dilute the peak fractions in 1% BSA-tris buffer so that 100 µl gives approximately 60-70,000 cpm in a Nuclear Chicago automatic gamma counter (Model 1085, with 3 inch crystal).
7. Test each fraction for its ability to bind specifically to stock membranes of rat kidney and liver.
8. Repurify the best binding fraction on a Sephadex G-100 column (.9 x 50 cm). Again collect .5 ml aliquots, dilute to 60-70,000 cpm and test each peak fraction for specific binding. Use only the fractions with the highest specific binding to assay receptor activity.

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58th Annual FASEB Meeting	1974	Reinitiation of Estrous Cycles in Light-Induced Constant Estrous Rats by Drugs
57th Annual Meeting of The Endocrine Society	1975	Effects of Castration, Testosterone, Estradiol and Prolactin on Specific Binding Activity in Ventral Prostates of Male Rats
1976 Meeting of The American Association of Cancer Research	1976	Effects of High Doses of Estrogen on Prolactin Binding Activity and Growth of Carcinogen-Induced Mammary Cancers in Rats

PUBLICATIONS

PAPERS:

Quadri, S. K.; G. S. Kledzik; J. Meites. 1973. Effect of L-Copa and Methyldopa on Growth of Mammary Cancers in Rats. Proc. Soc. Exp. Biol. Med. 142:759-761.

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ABSTRACTS:

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