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QUANTITATIVE METHODS IN
PROLACTIN-RECEPTOR RESEARCH

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Samuel Bacot Rhodes

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QUANTITATIVE METHODS IN PROLACTIN-RECEPTOR RESEARCH

By

Samuel Bacot Rhodes

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ABSTRACT

QUANTITATIVE METHODS IN
PROLACTIN-RECEPTOR RESEARCH

By

Samuel Bacot Rhodes

In this dissertation, the elements of the prolactin radioreceptor assay were critically examined. Emphasis was placed on the correlation between hormone iodination and receptor binding.

1. The lactoperoxidase (LPO) catalyzed iodination of prolactin (PRL) was studied using fluorescence spectroscopy and gel filtration. Experiments were designed to study the intrinsic tryptophanyl fluorescence of various mixtures of LPO, PRL, potassium iodide (KI) and H_2O_2. When the molar ratio of iodide to prolactin was less than or equal to 5, both the rate of decrease and the absolute decrease in fluorescence (maximally 40 $\pm$ 5%) varied directly in response to the initial concentration of iodide. At higher initial concentrations of iodide, only small additional fluorescence decreases occurred (maximally 54 $\pm$ 3% at a molar ratio of 50:1). Prolactin separated from the other products of iodination by gel filtration had a fluorescence 33 $\pm$ 3% less than native prolactin. This fluorescence decrease is the result of decreased energy transfer from tyrosine to tryptophan in response to the internal heavy atom effect and possible conformational changes in the prolactin molecule.

Using gel filtration, polyacrylamide gel electrophoresis and ^{125}I-PRL as a tracer, the stoichiometry of prolactin iodination was determined. Following a 25 minute incubation of PRL and KI (at a molar ratio of 5:1) with LPO and H_2O_2, iodide and monomeric prolactin coelute from a Sephadex G 100 gel in a molar ratio of 4.05:1. Sixteen percent (16 $\pm$ 4%) of the available iodide was incorporated into dimer and trimer prolactin, while only 7 $\pm$ 1% of the iodide was incorporated into lactoperoxidase. When the initial incubation molar ratio was increased to 14:1, iodide and monomeric prolactin coelute with a molar ratio of 4.35:1. We conclude that the maximum iodination stoichiometry of monomeric prolactin is about 4:1; the term 4-I-PRL is applied.

2. The relative binding affinity of 4-I-PRL and native prolactin (n-PRL) to rat liver membrane receptors was assessed using the radioreceptor assay. In two separate competitive displacement studies, 4-I-PRL was found to have a binding affinity significantly less than that of n-PRL. Using simultaneous equilibrium equations (derived in the text), n-PRL was found to have an apparent dissociation constant (K_d) of 3.51 $\pm$ 1.00 X 10^{-9}M where as 4-I-PRL had an apparent K_d of 10.9 $\pm$ 3.2 X 10^{-9} M (Scatchard analysis).

Despite the reduced binding affinity of 4-I-PRL, it retained significant biological activity. In a modified pigeon crop sac assay n-PRL and 4-I-PRL could not be distinguished by their ability to stimulate ^{14}C amino acid uptake into crop sac epithelium.

3. The dissociation of ^{125}I-PRL from liver membrane receptors was also studied. Using these and other published data, arguments were

developed for the rejection of the classical single step kinetic model previously used to describe prolactin binding. An alternative two-step model was developed which appears to be in excellent agreement with the present as well as previously published kinetic data. The two-step model was further corroborated by use of a specifically designed capacitative-resistance analog circuit.

Frances, we did it!

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TABLE OF CONTENTS

TABLE OF CONTENTS--continued

TABLE OF CONTENTS--continued

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In Germany in 1849, Arnold Berthold found that castration of roosters resulted in the loss of the comb and characteristic red pigmentation as well as a dramatic change in the animal's behavior. Transplanting testes into the abdomen of the castrated birds restored not only the appearance but also the vitality of the birds. This was the first demonstration that bloodborn "humors" (later identified as testosterone) could have such profound effects on animal physiology. In subsequent years, a large number of steroid, peptide and protein chemical messengers were isolated and characterized. Although these hormones were derived from such divergent organs as the gonads, the pancreas and the pituitary gland, they all shared one characteristic: they all facilitate integrative adjustments within the individual. That is, hormones help coordinate and synchronize the functions of the various organ and tissue systems.

It was soon found that in many cases, hormones occur throughout the general circulation, yet only specific target tissues seem to respond. This observation and a number of other studies of hormonal effects led to the concept that the binding of hormone to a specific recognition site initiates a sequence of steps culminating in the hormonal response (Hechter, 1955). The validity of this "receptor hypothesis" could only be demonstrated after methods were established

for producing highly purified, radiolabelled hormones. Radiolabelling techniques permit the researcher to detect hormones at picomolar concentrations which approach normal, physiological conditions. The second technological advance which helped establish the receptor hypothesis was the method of cellular fractionation pioneered by Palade and his co-workers (Meldolesi et al., 1971). Using differential centrifugation of tissue homogenates, endocrinologists could not only distinguish the tissue specificity but also the subcellular specificity of hormone binding.

Margaret Birkinshaw and I. R. Falconer (1972) were the first to demonstrate that ^{125}I-prolactin was concentrated from the circulation by the mammary gland of the rabbit. They further suggested that the plasma membrane was the initial site of hormone action. Presumably, this initial hormone binding gave rise to secondary intracellular responses as proposed by Sutherland (1970). In subsequent studies, the binding of ^{125}I-prolactin to membrane receptors was shown to be specific, saturable and could even be used as a sensitive and rapid assay for lactogenic hormones (Frantz et al., 1974; Shiu et al., 1974).

Quantitative analyses of receptor binding have evolved from the model that was developed for hormone-antibody interactions as conceived by Solomon Berson and Rosalyn Yalow (1959). The model assumes the simple interaction of a protein and ligand which are dissolved in a homogenous medium and free to associate and dissociate according to second order rate kinetics (Scatchard, 1949). Although this mathematical model has been applied with considerable success to the binding of ^{125}I-prolactin to suspensions of cell membranes, the model does not in

any way account for the coupling of hormone binding to a measurable biological response. In fact, this mathematical treatment assumes complete, unimpaired dissociation of hormone and receptor. This is not a very attractive model for a biological system which presumably involves a cascade of sequential biochemical events.

The analysis of prolactin binding to receptors is also limited by the assumption that native and iodinated hormone have the same biochemical properties. Radioiodination (Hunter and Greenwood, 1964; Thorell and Johansson, 1971) is an invaluable tool for monitoring the location and behavior of protein hormones but it is an invasive technique which has the potential of changing the physical-chemical properties of prolactin. Since iodide is not a native constituent of prolactin, the biochemical and biological consequences of its introduction into the hormone should be evaluated (Bolton, 1977).

A third difficulty associated with the quantitative analysis of prolactin-receptor interactions is the question of prolactin iodination stoichiometry. To the knowledge of the author, the stoichiometry of I-prolactin is unknown. Although relative changes in receptor populations can easily be detected, it is impossible to estimate with precision, the number of receptors which are bound to ^{125}I-prolactin at any point in time. On the other hand, if the molar ratio of iodide:prolactin was known, direct measurements of receptor density could be made from the specific radioactivity of the hormone.

In this manuscript, experiments are described which are intended to resolve three inter-related problems.

1) Fluorescence and chromatographic experiments are described which were designed to determine the stoichiometry of ovine prolactin iodination by the lactoperoxidase method.

2) Experiments were designed to evaluate relative biochemical differences between native and iodinated prolactin as detected in the radioreceptor assay and a modified pigeon crop sac assay.

3) ^{125}I-prolactin-receptor dissociation experiments and an analog computer circuit were used to derive a mathematical model of hormone-receptor interaction which includes a consideration of post-binding biochemical events.

Structurally, the manuscript is divided into five sections. The first section is a review of the pertinent literature of prolactin biology, receptor binding and iodination. The second section is a description of the materials, methods and commercial suppliers for the experiments in the remainder of the text. The third and fourth sections are the experimental sections which describe the results of iodination and binding studies respectively. Each chapter is introduced by a brief conceptual description of the rationale which underlies a given experiment. This format was chosen since later experiments were predicated on the results of earlier experiments. This presentation should therefore clarify the logical progression of the experiments. The final section presents summary discussions and concluding remarks.

LITERATURE REVIEW

I. Biochemical Properties of Prolactin

The first extensive preparation, identification and assay of prolactin was performed by Riddle, Bates and Dykshorn (1933) using extracts from sheep pituitary glands. In subsequent years, a great deal of research has been dedicated to unraveling the physical-chemical properties of ovine prolactin. Prolactin is a protein hormone secreted from the acidophil cells of the anterior pituitary gland. It is a single polypeptide chain consisting of 198 amino acids with no carbohydrate moieties (Li et al., 1969). There appears to be a random linear distribution of the twenty commonly occurring amino acids except that only two tryptophan residues are present. The hormone is isoelectric at pH 5.73 and has a molecular weight of about 22,550. Three disulfide bonds between cysteine residues 4 and 11, 173 and 58 and 190 and 198 produce a "triple horseshoe" secondary configuration (Li et al., 1970). It appears however, at least in the case of human chorionic lactogen, that reduction of disulfide bonds does not interfere with the hormonal biological activity (Bewley, 1977).

Although a series of lactogenic hormones have been isolated including bovine prolactin (Wallis, 1974), porcine prolactin (Bewley and Li, 1975) and human growth hormone (Li and Starman, 1964) all share more than 80% amino acid homology. The various lactogenic hormones also

share comparable biological activity as assayed in the pigeon crop sac (Niall, 1972) and the radioreceptor assay (Posner, 1974). That is, all lactogens are able to stimulate dose dependent growth of the pigeon crop sac epithelium and all appear to compete for the same class of membrane hormone receptors. One of the most interesting lactogens is human growth hormone. It appears to have two distinct biologically active sites: one possessing growth hormone activity and the other possessing lactogenic activity. These active sites were very eloquently demonstrated in a recent experiment using cultured rat liver cells (Ranke et al., 1976). It should be noted in contrast that lactogenic hormones from the lower vertebrates have little or no biological activity in the primates. The physical-chemical reasons for this species specificity are unknown (Niall et al., 1973).

It is now quite clear that ovine and probably other prolactins may occur in a variety of molecular forms. Multiple forms of ovine prolactin were first recognized by Emmant et al. (1963) who used electrophoresis to define three protein bands, all of which were immunoreactive with antibody formed against native prolactin. Subsequent studies identified the multiple electrophoretic forms as deamidation products (Lewis et al., 1970). Superimposed on this micro-molecular multiplicity, we also see macromolecular interactions of prolactin molecules. Squire et al. (1963) was the first to identify pH dependent, reversible aggregations of prolactin into dimers and trimers which could be separated using ultracentrifugation and exclusion chromatography. Follow-up investigations, however, showed that some aggregations were not reversible and not charge dependent. In other words, some covalent bonding

between prolactin monomers was suggested (Rogol et al., 1975). This hypothesis was supported by Dombroske who isolated "large forms" of prolactin from rat pituitaries and from in vitro pituitary culture medium (Dombroske et al., 1976). These researchers found that the relative abundance of monomeric, dimeric and trimeric prolactin in the culture medium was related to the rate of prolactin secretion from the pituitary. Furthermore, the large forms of the hormone could only be separated into monomers by using strong reducing and dissociating conditions (Dombroske, 1976). He concluded that aggregates may be bound by disulfide cross-links. The relative biological potency of each of the aggregate forms of prolactin remains unclear but even large aggregates (greater than 150,000 daltons) retain some immunochemical activity (Rogol and Rosen, 1974).

Extremely little is known about the three dimensional folding of monomeric prolactin. Attempts to perform X-ray difraction analysis on prolactin have been stifled by the difficulty of preparing pure crystals of the protein (personal communication from Alexander Tulinski). Li et al. (1972) used optical rotatory dispersion and circular dichroism to examine human chorionic somatomammotrophin, ovine prolactin and human prolactin. It is estimated that each lactogen consists of about 45-55% α-helix and less than 12-14% β-sheet configuration. Kawauchi (1973) used an interesting approach to localize the biologically active site of prolactin. He found that prolactin bioactivity, as assessed by the pigeon crop sac, was abolished by chemical modification of tryptophan 90 with o-nitrophenylsulfenyl (NPS). In contrast, substitution of NPS on tryptophan 149 caused only a 20% decrease in prolactin bioactivity.

These experiments suggest that tryptophan 90 is located in or near the biologically active site. The authors also argue that tryptophan 90 is probably buried inside of the tertiary folds of the protein since severe chemical methods were necessary (50% acetic acid) in order to accomplish substitution of NPS into the indole ring. Studies of the biological activity of human growth hormone have also been undertaken by Li and Bewley (1976). They found that cleavage of the carboxy-terminal fifty-one amino acids will destroy all biological activity as measured by the pigeon crop sac bioassay and in the tibia epiphyseal closure assay. Furthermore, the bioactivity is restored by non-covalent association of the cleavage fragment. They also report that reduction of the disulfide bonds does not reduce the biological activity.

II. Hypothalamic Control of Prolactin Release and Synthesis

The pituitary gland is located directly below the hypothalamus. The gland is surrounded by bone, the sella turica. Although the pituitary is completely composed of tissues of ectodermal origin, the gland can be divided embryologically and functionally into two distinct regions. Embryologically the anterior pituitary arises from Rathke's pouch (an outpouching from the pharynx), whereas the posterior pituitary is derived from the infundibulum (an extension of the basal diencephalon). In some species, the two pituitary lobes are separated by an intermediate lobe. The protein and glycoprotein hormones FSH, LH, ACTH, PRL, MSH, and GH are released from the anterior pituitary (in some species MSH is secreted by the intermediate lobe); the octapeptide

hormones oxytocin and ADH are secreted from the posterior pituitary (Turner and Bagnara, 1976).

Since the posterior pituitary is a direct outgrowth of the hypothalamus, its secretions are actually manufactured in hypothalamic nuclei. In fact, the hypothalamic-hypophysial nerve tract, from the supraoptic and paraventricular nuclei in the hypothalamus to the posterior pituitary, represents the actual transport route for oxytocin and ADH. The release of these octapeptides is in direct response to excitatory nerve impulses transmitted to the axon terminals in the posterior pituitary (Donovan, 1970).

The anterior lobe of the pituitary is in communication with the hypothalamus by a series of portal capillaries which arise in the median eminence of the basal hypothalamic area (Netter, 1968). An additional distinction of the adenohypophysis is that the hormones secreted from the gland are synthesized in the gland. The control of hormonal synthesis and secretion is therefore quite different from that which we find in the posterior lobe.

Historically, it was Geoffrey Harris who first proposed that "chemotransmitter" from the hypothalamus might actually be regulating adenohypophyseal function (Harris, 1955). Shortly thereafter Saffran and Schally (1955) demonstrated in vitro that a crude hypothalamic extract could cause the release of ACTH from cultured anterior pituitary glands. The general theory then developed that small peptides were released at the median eminence, carried via the portal capillaries to the anterior pituitary and in turn these "releasing factors" caused the secretion of the various pituitary hormones. Although this pathway

certainly is important, it has recently been demonstrated that pituitary hormones are also carried to the brain and the cerebrospinal fluid directly from the pituitary. The concept of a simple hypothalamic-hypophyseal axis is no longer an adequate model (Bergland and Page, 1979).

A. Prolactin Release Inhibiting Factors

The first demonstration of hypothalamic control of prolactin secretion was reported by Meites (1959). It appeared that the hypothalamus exerted a tonic inhibitory effect since pituitary glands surgically separated from the hypothalamus *in vitro* and *in vivo* would spontaneously secrete prolactin. Moreover, a hypothalamic extract was later shown to inhibit both synthesis and release of prolactin (Talwalker *et al.*, 1963). The extract was named prolactin inhibiting factor (PIF) and was assumed to be a small peptide. Although the mechanism of action of PIF at the pituitary is not clear, it has been suggested that PIF acts to prevent Ca^{++} entry into lactotrophic cells, thereby inhibiting the spontaneous release of prolactin secretory granules (Nicoll, 1971). To date, no peptide with PIF activity has been identified but Shaar and Clemens (1974) produced evidence that dopamine has an inhibitory effect on prolactin secretion. Schally's group in a thorough follow-up study demonstrated dopamine's transient effects were due to rapid degradation of the catecholamine in saline solutions. They also showed that PIF activity was insensitive to pepsin treatment; a strong argument against a peptidergic factor (Schally *et al.*, 1974). Recently dopaminergic receptors have been found on pituitary cells and

their function correlates well with dopaminergic control of prolactin release (Caron et al., 1977). In spite of this strong evidence for dopamine as PIF, there is some suggestion that prolactin secretion is inhibited even in the presence of the dopamine blocker haloperidol (Ojeda et al., 1974). The question of a PIF distinct from dopamine is still open to investigation.

B. Prolactin Releasing Factors

Prolactin, unlike the pituitary trophic hormones, is under the dual control of both inhibiting factors and releasing factors. The existence of a releasing factor for prolactin (PRF) became evident when a hypothalamic extract was able to stimulate lactation in estrogen primed rats (Meites et al., 1961). In avian species, PRF is more easily demonstrated. Kragt and Meites (1965) found that a hypothalamic extract was able to stimulate prolactin release in vitro from pigeon pituitary glands. It is suspected that PRF is the principle regulator of prolactin secretion in avian species.

During the late 1960's, the search for the chemical identity of the various hypophysiotrophic hormones (releasing factors) became very intense. By 1969, a group headed by Schally had isolated and identified the tripeptide (pyro-glu-his-pro-NH_2) as thyrotrophin releasing factor (Boler et al., 1969). The tripeptide (TRH) was a potent releasing factor for thyroid stimulating hormone (TSH) and, unexpectedly, TRH also had very strong prolactin releasing activity. The PRF activity of TRH had been demonstrated in the rat (Rivier and Vale, 1974), the cow (Convey et al., 1972) and in humans (Bowers et al., 1973). TRH is

extremely potent and picomolar concentrations are sufficient to cause a several fold increase in synthesis and release of prolactin from cultured pituitary cells (Hinkle and Tashjian, 1973; Tashjian et al., 1971). Since maximal prolactin secretion in vitro occurs 24 hrs after exposure to TRH, major changes in the cell protein synthetic machinery were suspected. The biochemical pathways associated with increased prolactin synthesis and release have been studied in depth by Tashjian whose group has identified membrane receptors for TRH on the surface of pituitary cells. The biological activity of TRH can be blocked by chemical analogs of the hormone (Hinkle et al., 1974). Furthermore, TRH stimulates the transcription of a specific messenger RNA for prolactin (Biswas and Tashjian, 1974; Dannies and Tashjian, 1976). However, the mechanism of TRH action is not dependent on cAMP as in other "second messenger" hormone systems (Hinkle and Tashjian, 1977).

The PRF activity of TRH caused some initial confusion because under normal physiological conditions, prolactin and thyroid stimulating hormone (TSH) are not always released at the same time. In fact, during various temperature stresses, serum levels of prolactin and TSH may fluctuate inversely (Mueller et al., 1974). It now appears that prolactin and TSH are subject to the combined influence of several hypophysiotrophic factors. For example, somatostatin (a tetradecapeptide produced in the hypothalamus) can selectively inhibit TRH induced TSH secretion, without altering prolactin secretion (Siler et al., 1974). In contrast, dopamine selectively inhibits prolactin release even in the presence of TRH without inhibiting the release of TSH (Chen and Meites, 1975). In summary, the release of TSH and prolactin is subject to the simultaneous

effects of TRH, dopamine and somatostatin. The quantitative aspects of this hormone interaction are still not clearly understood and the possibility of a PRF distinct from TRH cannot be completely ruled out.

C. Feedback Regulation

Just like the pituitary, the hypothalamus is also subject to regulation in accordance with the physiological state of the animal. That is, the release and synthesis of releasing factors will fluctuate according to the influence of various blood-born agents which exert their effect directly on the brain. For example, the steroid hormone testosterone (secreted by the testes) is known to inhibit the hypothalamic release of gonadotrophic releasing hormone (GnRH). Reduced portal capillary levels of GnRH cause a decreased release of luteinizing hormone from the pituitary which in turn causes reduced synthesis of testosterone at the testes (Turner and Bagnara, 1976). This closed loop control of hormone secretion is called long loop feedback regulation. The most extensively studied of the feedback systems involve the gonadotropins and sex steroids. For a review of the mechanisms of feedback regulation see McEwen (1976). The ultimate fate of feedback at the hypothalamus is a change in the levels or turnover rate of the various neutrotransmitters and the biogenic amines. These fluctuations in neurotransmitter release in the hypothalamus are directly responsible for the secretion of the hypophysiotrophic hormones at the median eminence. A detailed description of the mechanism is beyond the scope of this review but a good summary article has been written by Meites et al. (1977). It should be noted that some hormones may also have stimulating

or inhibitory effects which they exert directly at the pituitary gland. This phenomenon is referred to as short-short loop feedback.

Unlike the other adenohypophysial hormones, growth hormone and prolactin are not trophic hormones; that is they do not cause the secretion of another hormone from a distal gland. It was suspected that these protein hormones might be self regulating and exert inhibitory effects directly at the hypothalamus or possibly at the pituitary. Apropos to this line of reasoning, MacLeod and Abod (1968) implanted prolactin secreting tumors into intact rats. They found that when the rats' own pituitary glands were removed, they secreted less PRL in vitro and had a reduced pituitary content of prolactin. Similarly, Chen et al. (1970) found that rats with tumor implants exhibited increased PIF activity in the hypothalamus. An interesting study was also done with pseudopregnant rats. In this case, minute quantities of prolactin were implanted directly into the median eminence. The implant caused an increased secretion of LH and FSH but prolactin secretion was inhibited (Voogt and Meites, 1971). These authors however were not certain if the site of inhibition was the hypothalamus or the anterior pituitary. In order to clarify this question Voogt and Ganong (1974) incubated pituitary glands in the presence of excess prolactin in vitro. Under these conditions they were unable to cause a decreased secretion of prolactin. The conclusion was that prolactin autoregulates via the hypothalamus. Supportive evidence now exists for this feedback mechanism since prolactin binding sites have actually been identified in the brain (Walsh et al., 1978). Prolactin has also been detected in physiological levels in the cerebrospinal fluid of the rat and human (Jordan

and Kendall, 1978). In spite of the evidence against the pituitary as the site of negative feedback for prolactin, Frantz et al. (1975) found specific binding sites for prolactin on the membranes of cultured pituitary cells. Moreover, Payne (1975) found a positive correlation between the quantity of specific binding and the amount of prolactin secretion in primary cultures of pituitary cells. This membrane binding however could be related to the secretory function of the cells rather than as a regulatory process. The whole question of autoregulation of prolactin secretion directly at the pituitary has been reopened by Herbert et al. (1979). They found that a clonal strain of rat pituitary cells was very sensitive to the medium concentration of exogenous prolactin. Dose related inhibition of prolactin secretion resulted when 0 to 1000 ng of o-PRL was provided in the culture medium. We do not know what role the short-short loop feedback might play in vivo.

D. Effects of Other Hormones on Prolactin Secretion

As rats mature and reach puberty, their serum levels of prolactin also rise. Moreover, the serum levels for females is higher than in males (Yamamoto et al., 1970). In light of this observation, it was suspected that other hormones, especially the sex steroids, might influence the release of prolactin. This hypothesis was supported by the finding that prolactin serum levels and pituitary content was higher during estrus and proestrus as compared to diestrus in the rat (Ieiri et al., 1971). Furthermore, an injection of estrogen into ovariectomized rats caused increased secretion of prolactin (Chen and Meites, 1970).

It now appears that estorgen (and perhaps to a lesser extent progesterone) stimulates prolactin release by three separate mechanisms: 1) Fuxe and Hokfelt (1969) have produced good evidence showing that dopamine turnover rate varies inversely with the level of estrogen in the hypothalamus; 2) Estrogens also have a direct effect on cultured prolactin secreting pituitary cells (Haug and Gautvik, 1976); and 3) Labrie et al. (1976) found that estrogen modulates the number of TRH receptor sites on pituitary cells. In short, the interaction of estrogen and prolactin secretion is very complex and it has been suggested that puberty may be the biological signal for the onset of prolactin secretion (Meites et al., 1972).

The role of testosterone in prolactin secretion is not as clear. An intravenous injection of testosterone propionate in 0.5 to 2.0 mg doses causes increases in PRL secretion in castrate male rats and it was postulated that the site of action was the hypothalamus (Kalra et al., 1973). Nicoll and Meites (1964) were unable to see effects of either testosterone or glucocorticoids on prolactin secretion from cultured pituitaries in vitro, but more recent work shows that testosterone inhibits prolactin release in cell cultures (Haug and Gautvik, 1976). The response of the intact animal to testosterone may ultimately be related to the conversion of testosterone to estrogen in situ (McEwen, 1976).

Except for the older report by Nicoll and Meites (1964), we find that glucocorticoids have an inhibitory effect on prolactin secretion. Adrenalectomy causes increases in pituitary and serum prolactin levels and even potentiates the prolactin releasing effects of the drug

perphanazine (Ben-David et al., 1971). Dannies and Tashjian (1973) have shown direct inhibition of PRL secretion by 5 x 10^{6}M hydrocortisone in pituitary cell cultures but other reports suggest that the glucocorticoids may play a more complex role. For instance, Euker et al. (1975) showed that dexamethasone could block stress induced prolactin release in a dose dependent manner and Vanhaelst et al. (1974) reports that dexamethasone can suppress both basal and TRH stimulated prolactin release in humans. Although glucocorticoids are suspected to play a role in the hypothalamus, there are reports of specific glucocorticoid receptors in several regions of the brain including the cerebrum and medulla (McEwen, 1976).

E. Other Effectors of Prolactin Secretion

Some of the most interesting inhibitors of prolactin secretion are the natural and synthetic derivatives of the wheat ergot fungus. These drugs are closely related to the hallucinogen LSD but also seem to be specific inhibitors of prolactin secretion. The mechanism of action appears to be two-fold: 1) via increases in PIF activity in the hypothalamus (Wuttke et al., 1971), and 2) directly at the pituitary (Clemens et al., 1975). Due to the specificity of their effect, these ergot drugs have been widely used to inhibit prolactin secretion under experimental conditions (Brooks and Welsch, 1974).

Recently, the interest in "Brain opiates" has stimulated research concerning the role that they might play in hypothalamic control of the pituitary hormones. McCann et al. (1974) has found that morphine injected into the third ventricle will release prolactin. Whereas Blank

et al. (1979) has evidence that the endogenous opioid drugs may be essential for normal endocrinological maturation in the female rat. All effects seem to be mediated via the hypothalamus. An excellent review of the role of endogenous opioids has been written by Meites et al. (1979).

III. Physiological Activity of Prolactin

Prolactin occurs in practically all vertebrate species and is responsible for a staggering number of diverse physiological effects. The hormone is involved in osmoregulation in teleost fish and amphibians (Bern, 1975), nestbuilding and parenting behavior in several avian species (Nicoll et al., 1967) and milk production in mammals (Stricker and Gruter, 1928); just to name a few. To review all of the bioactivities of prolactin would be an unnecessary digression, but the author would like to consider the physiological roles of prolactin as they pertain to the rat and pigeon which are the animals used in the present research.

Stricker and Gruter (1928) published the first report to demonstrate the importance of the pituitary gland in milk production in mammals. It soon became evident however that milk production was not dependent on a single hormone but occurred in response to ovarian, adrenal and hypophyseal factors. In hypophysectomized, adrenalectomized and ovariectomized rats, Lyons et al. (1958) found that mammary duct development required GH, adrenal steroids and estrogen. But in order for lobuloalveolar growth to occur, progesterone and prolactin were

required. The temporal sequence of hormone action was better elucidated by Meites (1966). He found that prior to parturition, serum levels of estrogen rise followed by a rise in prolactin, ACTH and cortisone on the day of parturition. It is believed that these sequential events actually initiate lactation. The mechanism of prolactin action at the mammary gland is mediated by a membrane receptor (Shiu and Friesen, 1974; Frantz et al., 1972). The receptor binding appears to initiate phospholipase activity at the membrane (Rillema and Wild, 1977) and to activate urea cycle intermediates intracellularly (Rillema et al., 1977). The resulting interaction of prostaglandins, polyamines and possibly cyclic nucleotides enhance specific synthesis of casein messenger RNA (Turkington et al., 1973; Rosen et al., 1975). The end result is the elaboration of specific proteins into milk (Turkington, 1971). Under some conditions, prolactin is able to promote tumorigenesis in mammary tissue. The conditions which predispose the tissue to tumor growth are still poorly understood and this problem is an area of active research. For a current review see Welsch and Nagasawa (1977).

In rats, hamsters, ferrets and mice, prolactin is luteotrophic and has been shown to be one of the necessary hormones in the luteotrophic complex. For a review see Malbandov (1973). Everett (1954) reported that pituitary autographs beneath the renal capsule of rats would maintain corpora lutea for an extended period of time. Meites and Shelesnyak (1957) also found that prolactin was an essential component for the maintenance of pregnancy; presumably due to its ability to support progesterone secretion from the ovary. The supportive effect of prolactin however is selective such that corpora lutea which

no longer can secrete progesterone atrophy more rapidly in the presence of prolactin. That is, at least in the rat and mouse, prolactin is luteolytic to "older" corpora and luteotrophic to "younger" corpora (Malven, 1969).

In the male rat, prolactin plays a supportive role in spermatogenesis. Apparently prolactin synergizes with LH at the testes to cause an increase in testosterone production. Prolactin probably acts at the interstitial cells of Leydig and not directly at the Sertoli cells (Bartke, 1971). The precise interaction of LH and prolactin is not clear, especially since prolactin is able to inhibit the secretion of LH (Grandison et al., 1977).

The liver is effected in several diverse and interesting ways by prolactin. In many respects prolactin acts as a metabolic hormone at the liver since changes in carbohydrate, lipid and protein synthesis all occur in the presence of prolactin (Bern and Nicoll, 1968). Recently an in vitro chondrocyte stimulating assay was used to show that prolactin can stimulate the release of sommatomedin which is synthesized in the liver (Francis and Hill, 1975). Bala et al. (1978) also found that prolactin will regulate the release of sommatomedin from the liver. However, they find that adrenal factors may be involved. These studies would implicate prolactin as a trophic hormone. In addition, a maternal pheramone which is found in the feces of female rats is also "released" by prolactin. The maternal pheramone has its origin in the liver (Moltz and Leidahl, 1977). Because of these diverse physiological effects and the ease of liver tissue preparation, the liver has become a preferred organ for prolactin receptor studies. One of the most

interesting physiological findings of the receptor studies is that prolactin even regulates the number of its own receptors (Posner et al., 1974; Posner and Kelly, 1975; Costlow et al., 1975; Frantz et al., 1977)!

The role of prolactin in the mammalian kidney is really not understood but specific osmoregulatory functions have been reported for the hormone. For example, prolactin can increase the reabsorption of water in isolated renal tissue cultures from rats. This antidiuretic activity is overpowered by furosemide (Manku et al., 1973). In rats, the specific binding of prolactin at the kidney also is influenced by the hydration state and the circulating levels of prolactin (Marshall, 1975). These renal responses possibly could be coordinated by the osmosensitivity of the pituitary. In a study with cattle, Labella et al. (1975) demonstrated that prolactin release from the pituitary in vitro is inversely proportionate to the extracellular osmolarity of the medium. These data compliment and are consistent with the proposed osmoregulatory function of prolactin at the kidney. It should be noted though that the levels of other hormones are also effected but to a lesser extent by the osmolarity of the incubation medium.

While pigeons are nesting, the epithelial lining of the crop develops a secretory function. The milky fluid which is elaborated in the crop consists of desquamated cells and other materials which are fed to young squabs. The development of "crop milk" is in direct response to the circulating levels of prolactin and in fact this biological response is the basis of the classical bioassay for prolactin (Riddle et al., 1933). As expected, prolactin induced proliferation of pigeon crop sac epithelium is mediated by an increased RNA and protein

synthesis (Nicoll et al., 1967). Within physiological limits, the rate of amino acid uptake into epithelial cells is also proportional to the level of prolactin presented to the crop (Frantz and Rillema, 1968). The pigeon crop sac has also been shown to possess specific membrane receptors for prolactin (Kledzik et al., 1975).

IV. Lactogenic Membrane Receptors

A. General Properties of the Lactogenic Receptor

In recent years, the question of tissue specificity of hormones has been an area of active research. By definition, a target tissue for a hormone should be the only tissue which is able to respond to the hormone. This tissue specificity is believed to reside in a functional unit known as a receptor. The receptor, through its interaction with the hormone, provides the first step in the induction of a biological response. We require that the hormone-receptor interaction be an absolute prerequisite for all other cellular responses to the hormone (Cuatrecasas, 1974). Although the initial functional role of all receptors is the same, the physical and chemical properties of receptors for the various hormones, as well as their location in the cell may vary extensively. Indeed, it is these differences which will ultimately define the tissue specificity.

The first evidence of a prolactin receptor was derived from a study by Birkinshaw and Falconer (1972). They injected estrogen primed rabbits with ^{125}I labeled ovine prolactin and then performed autoradiography on tissue slices from the mammary gland. They found that the

radiograph silver grains were most abundant at the points corresponding to capillary-membrane surfaces. This was true regardless of the length of time allowed to pass before the radiographs were prepared. They concluded that the initial site of action for prolactin was at the plasma membrane. They also found that ^{125}I-prolactin disappeared from rabbit mammary with an average half-life of 52h. Since a membrane receptor for insulin had already been reported (Cuatrecasas, 1969), it seemed reasonable that membrane binding might be the initial step in prolactin action.

In subsequent studies, researchers relied heavily on preparative centrifugation methods described by Neville (1960) and Palade (Meldolesi et al., 1971) for the preparation of cell membrane fractions from tissue homogenates. These "membrane particles" were then used as substrate for prolactin binding studies. The prolactin binding activity in mouse mammary tissue was first analyzed by Frantz et al. (1972) and subsequently in the rabbit by Shiu and Friesen (1974a). Collectively, a great deal of physical-chemical information was obtained concerning the lactogenic receptor. The association of the hormone with receptor is both time and temperature dependent; optimal conditions being at 37°C. Furthermore, pH and ionic concentration also exert profound effects. The receptor activity is sensitive to both trypsin and phospholipase digestion; implicating a role for both protein and lipid. Presumably the receptor is fully operational only when embedded in the membrane. Binding was exclusive to hormones with known lactogenic activity (h-GH, o-PRL, sommatomammotropin) whereas other protein hormones (insulin, o-GH, LH, TSH) are not competitive for the receptor. Furthermore, the

binding is saturable and at least partially reversible. Estimates of equilibrium binding affinities range from 3.4 X 10^{-10}M to 9.0 X 10^{-9}M. The tissue distribution of receptors was unexpectedly broad and includes mammary, liver, adrenal, kidney and ovary. The most recently discovered binding site for prolactin is the ependyma of rat choroid plexus. This site of binding may be related to behavioral or regulatory actions of prolactin (Walsh et al., 1978).

Of particular interest to receptor studies is human growth hormone (h-GH). This protein has both lactogenic and growth stimulating activity. The question arises as to the specificity of the binding site. Posner (1976) used liver membrane particles to show that h-GH may have two distinct populations of receptors to which it can bind whereas ovine prolactin bound only to a single population of receptors. This experiment was confirmed by Ranke et al. (1976) using intact rat hepatocytes. In their experiment, preincubation of liver cells with prolactin could block lactogenic but not growth hormone sites whereas preincubation with bovine growth hormone had the converse effect. Since intact cells were used, effects of extraneous cytoplasmic enzymes were minimized. From these experiments we postulate that h-GH has two distinct biologically active sites. This is all the more remarkable since h-GH is the smallest of all the lactogens identified (Li and Bewley, 1976). Apparently, evolution has made the structural-functional activity of the hormone more efficient. The author would like to point out the good agreement between studies performed on intact cells and membrane preparations. It appears that cell fractionation does not introduce qualitative errors into receptor studies.

Although the qualities of binding have been described, the causal relationship between receptor binding and biological activity needed to be established. Appropriate to this task, Shiu and Friesen (1974b) solubilized and purified (approximately 1500 fold) the membrane receptor from rabbit and then used this protein as an antigen. The antibody formed against receptor was then injected into estrogen primed rabbits and was able to block the induction of amino acid incorporation into casein. In addition, the antiserum blocked prolactin binding to the mammary gland without effecting insulin binding (Shiu and Friesen, 1976). When this same antiserum is injected into postpartum female rats, the growth of their respective pups was significantly reduced as compared to control animals. Presumably, the antiserum injection resulted in reduced milk production (Bohnet et al., 1978). Clearly, receptor binding is a prerequisite of hormonal activity. Another, perhaps more interesting point is demonstrated by this experiment which apparently eluded the authors. Both antibody and hormone bind specifically with the lactogenic receptor yet only the hormone can induce biological activity. The "activating" step must therefore be more than a simple mechanical action and the initiation of a biological response must involve very subtle protein interactions. The experiment elucidates the subtlety of the hormone-receptor interaction.

Before leaving this topic, we should mention that specific receptor sites for prolactin and insulin have been identified in the Golgi apparatus of rat hepatocytes (Bergeron et al., 1978). The biological significance of this site of binding is by no means clear. However, it is currently believed that the Golgi is actually the source of plasma

membrane proteins and would quite logically have receptor sites for prolactin. That is, the receptor sites found in the Golgi apparatus may actually be "destined" for the plasma membrane (for a discussion of this concept see Lodish and Rothman, 1979 or Blobel, 1977). Very recently, intracellular binding has been demonstrated *in vivo* (Josefsberg *et al.*, 1979). These authors find that 40 to 50% of the binding of ^{125}I-PRL is to intracellular organelles. Furthermore, the uptake of prolactin into the cell is very rapid and occurs within 10 minutes of a single injection of labelled hormone. Since very thorough control experiments were performed, we must conclude that prolactin is actually carried within the cell following binding. This phenomenon may be peculiar to the liver.

B. Quantitative Analysis of Receptor Binding

The binding of hormone to receptor is generally measured using ^{125}I radiolabelled prolactin. The labelled hormone is incubated with membrane particles for a prescribed period of time and then the membrane component is separated from the medium by centrifugation or filtration (Frantz and Turkington, 1972; Shiu *et al.*, 1973). The quantity of hormone which adheres to the membrane is designated "total binding". Since non-specific interactions of the hormone with the glassware or by physical intrapment of the radiolabel in the membrane surely occurs, an effort to identify the "specific" binding fraction is made. Operationally, specific binding is defined as the fraction of the total binding which can be competitively displaced by a large excess of unlabelled hormone. This definition is a conventional adaptation of

the specific binding defined by Cuatrecasas for insulin (Lehmann, 1978). Although the exact nature of the "nonspecific binding" is unknown, in practice it usually represents a constant percentage of the initial radioactivity in the incubation medium and can therefore be uniformly subtracted from each datum. Specific binding is very sensitive to competitive displacement and provides the basis for a lactogenic assay with a resolving power of 5 ng/ml (Shiu et al., 1973).

Mathematical treatments of hormone binding have been based on the simple model of a reversible ligand binding such that:

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

where H = hormone, R = receptor and HR = bound complex. The overall forward rate expression is $(H)(R)k_1$ and the reverse rate expression is $(HR)k_{-1}$. At equilibrium, the forward and reverse rates are assumed to be equal and therefore,

(1b) $$\frac{k_{-1}}{k_1} = K_d = \frac{(H)(R)}{(HR)}$$

where K_d is called the dissociation constant.

Since the total number of receptors is equal to $(HR) + (R) = (R_T)$, we may substitute into equation (1b) to yield:

(2a) $$K_d = \frac{(H)\ (R_T) - (HR)}{(HR)}$$

This equation may be rearranged into two familiar forms:

(2b) $$\frac{(HR)}{(H)} = \frac{-(HR)}{K_d} + \frac{(R_T)}{K_d}$$ (Edsall and Wyman, 1958)

and

$$(2c) \qquad \frac{1}{(HR)} = \frac{1}{(H)} \quad \frac{K_d}{(R_T)} + \frac{1}{(R_T)} \qquad \text{(Rodbard, 1973a)}$$

Both equation (2b) and (2c) are in the form of straight lines and have the potential of generating values for (R_T) and K_d if (HR) and (H) can be measured accurately. The requirements are that (1) H and HR can be separated perfectly, (2) measurements must be made at equilibrium, (3) there is only one population of receptors with which the hormone may bind, (4) that no "product" is formed (HR dissociates only into H + R), and (5) that H and R interact in a single homogeneous phase. The consequences (mathematically) of violating these assumptions are discussed in detail by Rodbard (1973b). In practice, hormone binding cannot be detected by normal physical means due to the minute concentrations involved. Consequently, radiolabelled hormones are reacted with the receptor. This requires the additional assumption that labelled and unlabelled hormones behave identically (Kahn et al., 1974). Theoretical, mathematical treatments of the hormone-receptor system described above abound in the literature. Berson and Yalow (1959) describe in detail the quantitative methods and the data analysis of insulin and insulin antibody binding. They also present convincing kinetic data in support of a two-receptor-populations model. More recently, a number of graphical methods for binding analyses have been developed. The emphasis has been directed toward resolving heterogeneous binding sites and their respective binding affinities (Klotz and Hunston, 1971; Baulieu and Raynaud, 1970). Modifications of the simple model have also been suggested by several authors (Rodbard and Bertino, 1973;

Boeynaems and Dumont, 1975a), and the mathematical treatment of eight different models is reviewed by Boeynaems and Dumont (1975b). Their article is especially valuable because they include computer simulations for each of the model systems. With respect to hormone systems, they conclude that a straight linear Scatchard plot may lend support to a particular model but a curvilinear Scatchard plot is subject to several very distinct interpretations.

In order to establish the model in equations 2a-2c, kinetic studies should be undertaken to demonstrate that the "forward" reaction rate follows second order kinetics and that the "reverse" reaction rate follows first order kinetics (Maron and Lando, 1976). It is not sufficient that the equilibrium concentrations of bound and free hormone generate a linear Scatchard plot (Rodbard, 1973b). To date, only one detailed kinetic study of prolactin binding has been reported in the literature (Shiu and Friesen, 1974a). Their data are consistent with second order forward rate reactions but are not consistent with first order reverse rates. That is, the dissociation data do not conform to that which would be expected from a single population of dissociating receptors. All the more discouraging, this article set a precedent for subsequent researchers all of whom consider the binding to proceed as in equation (1) above. We should also point out that derived "equilibrium constants" vary vastly from laboratory to laboratory and even from experiment to experiment. For example, reported K_d values for prolactin binding in liver range from 10^{-8}M (Ranke et al., 1976) to 10^{-10}M (Costlow et al., 1975). In part, this range of values may represent the influence of preparative methods since "solubilized" receptor

is known to have an affinity five times that of receptor in membrane (Shiu and Friesen, 1974b). At best, we are forced to conclude that with present methods, "equilibrium constants" for prolactin are only valuable for simultaneous, relative comparisons.

C. Physiological Regulation of Receptors

Although the release of prolactin from the pituitary gland is under strict regulation, the responsiveness of specific tissues to prolactin is also variable. For example, prolactin does not always stimulate milk production in the mammary gland but only after pre-exposure to estrogen. It was suspected therefore that receptor activity might be regulated by humoral factors. The first insight as to the mechanism of the variable tissue response was supplied by Kelly et al. (1974). They found that the prolactin binding capacity of rabbit mammary and liver tissues increased as the animal matured. At puberty a nine-fold increase in prolactin binding capacity occurred and an additional increment in binding was observed during pregnancy. Throughout this maturation process the apparent binding affinity of the receptors remained the same. It was proposed that estrogen was responsible for the receptor "induction". This hypothesis was confirmed by Posner et al. (1974) who found that estrogen injections could induce lactogenic binding sites in the liver of rats. However, estrogen cannot induce receptors in hypophysectomized animals. In the intact animal, even subtle changes in the sex steroids could effect prolactin binding as evidenced by changes in receptor levels during the various stages of estrus. Furthermore, the receptor induction requires de novo protein synthesis

(Kelly et al., 1975). Although estrogen induces receptors in the liver, it reduces receptor levels in mammary tissue. That is, receptor activity decreases in mammary tissue in the presence of estrogen (Bohnet et al., 1977).

Since the pituitary gland played a role in the regulation of receptors, it was suspected that prolactin itself might induce receptors. Posner and Kelly (1975) found that pituitary implants under the renal capsule of hypophysectomized female rats caused a rapid restoration of receptor activity. This receptor restoration paralleled the increase in serum prolactin levels with a three day lag period. These findings were confirmed by Costlow et al. (1975). He also found that estrogen and prolactin given together have no greater effect than prolactin given alone. The conclusion was that the pituitary plays the principle regulatory role. Estrogen simply increases the release of prolactin.

The ability of prolactin to induce its own receptors has also been studied in dwarf mice. These animals are born with pituitary glands which secrete neither prolactin nor growth hormone. They also lack detectable levels of hepatic prolactin receptors. However, prolactin "therapy" results in the rapid production of prolactin binding sites (Frantz et al., 1977; Knazek et al., 1977). It has also been found that growth hormone can induce lactogenic receptors in dwarf mice (Knazek et al., 1978) as well as in hypophysectomized female rats (Bohnet et al., 1976).

As mentioned previously, estrogen's receptor inducing activity is dependent on an intact pituitary gland in the experimental animal. In contrast, testosterone is able to reduce the number of lactogenic

binding sites per mg of tissue and apparently has its effects without mediation of the pituitary. For example, testosterone injections into hypophysectomized male rats will reduce prolactin binding at the liver by 50% (Bohnet et al., 1976). Androgens can also block the estrogenic effect in spite of increased circulating levels of prolactin. That is, when estrogen and testosterone are given together, the net effect is a reduction in prolactin binding capacity at the liver. Comparable steroid effects have also been reported for renal and adrenal populations of prolactin receptors (Marshall et al., 1976).

At the molecular level, it is unknown how the number of receptors changes with respect to time but a number of interesting ideas for inactivation of receptors have been considered by Kolata (1977). These inactivating steps could involve conformation changes in the receptor or changes in the rate of receptor degradation. Alternatively, receptor inactivation could involve chemical modification of the binding site as is often the case with enzymes (Holzer and Duntze, 1971).

D. Membrane Receptors as Biological Transducers

Since the classic paper by Danielli and Davson (1935) the concept of the "bi-molecular leaflet membrane" has undergone considerable evolution. These authors visualized the plasma membrane as being a relatively static "sandwich" of lipid between protein. Today however, it is agreed that the surface membranes of animal cells undergo a number of structural and functional changes which are generally described in terms of degrees of fluidity. Furthermore, the membrane can alter its lipid-protein content under the influence of both external and internal

stimuli (Siekevitz, 1972).

In many respects a hormone receptor plays the role of a biological transducer. The receptor converts the chemical energy of the binding process into the activation of specific metabolic events within the cell. The extracellular events (binding) function as a stimulus and the hormone-receptor complex functions as a mediator of physical-chemical energy. Although we are limited by present day technology, as to the precise molecular events of this transduction, there is a growing body of pertinent literature which should be considered. I will briefly consider some of the important papers which are relevant to the hormone receptor concept.

The question of membrane fluidity was addressed by Devaux and McConnell (1972) by using spin labelled phosphatidylcholine. By considering changes in the electron spin resonance, it was estimated that lipids could diffuse through the plane of the membrane with a diffusion constant of about 2×10^{-8} cm^2/sec at room temperature. It was estimated that neighboring lipid molecules could therefore exchange at a rate of about 10^7 sec^{-1}. On the other hand, lipids are thought to "flip flop" from the outside to the inside of the membrane at a much slower rate. The average inside-outside transition in phospholipid vesicles is estimated to have a half life of 6.5 hours (Kornberg and McConnell, 1971). Embedded in this liquid crystal matrix we find the various functional proteins such as the prolactin receptor. It appears that these proteins have associated with them, predictable and characteristic quantities of membrane lipid. For example, Jost et al. (1973) has estimated that cytochrome oxidase has a boundary lipid complement

of about 0.2 mg phospholipid per mg of protein. They suggest that this bound lipid functions as an anchor. We should not, however, imply that the membrane proteins are immobilized since NMR, fluorescence studies and circular dichroism reports have all demonstrated that membrane proteins are capable of considerable conformational change in the plane of the membrane (Chapman, 1973; Bretscher, 1973). There is also more direct evidence that membrane proteins are capable of substantial movement in the plane of the membrane. Using lymphocyte cells, a number of investigators have found that membrane antibodies "diffuse" to one pole of the cell upon binding of antigen. For a review of this "capping" phenomenon see Raff (1976). Some membrane proteins are located only on the outside of the membrane, some are located only on the inside, and some appear to stretch through the lipid matrix. This membrane assymmetry can be determined by labeling the extracellular proteins with ^{125}I while the cell is still intact (Phillips and Morrison, 1971). The insertion of proteins, such as receptors, which extend through the membrane is believed to be controlled by the ribosomes at the time of receptor synthesis (Lodish and Rothman, 1979).

At the time of hormone binding to receptor, very specific conformational changes must occur. The binding forces probably include electrostatic attraction, hydrogen bonding, hydrophobic interactions and Van der Waals forces (Lehman, 1978). Here association of the hormone with membrane is not sufficient for initiation of a biological response. For example, Kohn (1978) has worked with purified membrane receptors for TSH. He found that although TSH will bind with a number of membrane proteins, only when TSH binds with its specific receptor

protein do conformational changes occur. Furthermore, these conformational changes (as measured by C D) are a requirement for changes in membrane permeability. Similarly, Sonenberg found that the binding of growth hormone to the membranes of erythrocytes was accompanied by a change in the intrinsic membrane fluorescence (Sonenberg, 1971). They went on to show that a greater negative ellipticity of the membrane fluorescence correlated with an increase in 5' nucleotidase activity (Postel-Vinay et al., 1o74; Rubin et al., 1973). Studies such as these have led several investigators to believe that hormone binding to receptors is directly responsible for activation of enzyme activity within the cell.

Another possible mechanism of receptor action involves receptor attachment to intracellular microfilaments. Van Obbergher et al. (1976) found that microfilament inhibitors also could inhibit the binding of human growth hormone and insulin to lymphocytes; whereas microtubule inhibitors had no effect on binding. They speculated that surface receptors were attached directly to microfilaments. However, Loor (1976) has shown that the relative sizes of receptors, microfilaments and microtubules are so drastically different that at best microfilaments could only play a peripheral role in hormone action. For the present, we are still uncertain of the precise mechanisms of energy transduction at the membrane but this remains a lively area of research.

V. Assays for Prolactin

Since the original report of Stricker and Gruter (1928) that pituitary extracts could stimulate milk production in the rabbit, a

number of investigators have searched for relatively simple and reproducible assays for prolactin. The most widely accepted assay methods have been based on the capacity of prolactin to stimulate "crop milk" production in the pigeon. The original procedure by Riddle and Bates (Riddle et al., 1933) was based on daily systemic injections of hormone preparations directly into the breast muscle. At the end of four days the animal was sacrificed and the entire crop was removed and weighed. Although this method was fairly quantitative, it lacked sensitivity and thus the "micro" technique was developed by Lyons (1937). This latter method involved intracutaneous injections of hormone preparations directly over the crop gland. In addition to its increased sensitivity, the "micro" method also provided a means for comparing two different hormone preparations in the same bird. In a comparison of three different pigeon crop assays and one rabbit mammary assay, Bergman et al. (1940) found that the "micro test" was the most sensitive and quite reliable. They also concluded that the same humoral factor was responsible for both crop development in pigeons and mammatrophic activity in the rabbit. For years, the crop sac assay remained essentially unchanged until Nicoll (1967) developed a more quantitative means of assessing crop sac epithelial growth. His technique was based on the localized "micro test" but relied on cutting standardized 4 cm discs of tissue from the crop, separating the epithelium and weighing the tissue. Nicoll could quantitate responses which were not visually detectable and therefore the sensitivity of the assay was improved to as little as 0.25 μg of prolactin. The method still required a total of four injections over a two day period and the increment in crop weight showed a

linear log-dose response. At the cellular level, the crop sac epithelial growth involves a rapid uptake of water and amino acids; the maximal rate of ^{14}C amino acid uptake occurring 19 h after a single intradermal injection of hormone (Frantz and Rillema, 1968). This amino acid uptake provides the basis for a semi-quantitative and very sensitive assay for prolactin.

In more recent years, tissue culture methods had improved such that prolactin assays based on biochemical and histological changes in the mammary gland were developed. Turkington (1971) for example developed an assay based on ^{32}P-casein synthesis in mouse mammary cultures in vitro. The assay was sensitive to the range of 2-1000 ng of prolactin and it was found that dilutions of human serum generated parallel dose-response curves to the standard. Similarly, Frantz et al. (1972b) described a bioassay for prolactin based on histological changes in cultured mouse mammary. The maximum sensitivity of the system was about 5 ng, but the coefficient of variability was 25-30%. Other tissue culture systems have been reported using various modifications of the techniques described above. Most are based on the uptake of a radioactive precursor of milk as a quantitative biological endpoint (Bullough and Wallis, 1974; Doneen, 1976). We should note that cross species (heterologous) assays for prolactin are sometimes variable or produce non-parallel dose-response curves, especially when human growth hormone is being tested. For this reason, completely homologous assays are recommended (Doneen, 1976; Leung et al., 1978).

The most commonly used assay method at present is the radioimmunoassay (RIA). This is certainly due to its relative ease and sensitivity.

Based on the pioneer work of Berson and Yalow (1959) with insulin Niswender et al. (1969) developed a RIA for rat prolactin. They found their assay to be highly specific and to correlate well with previously reported changes in serum levels of prolactin. Furthermore, the RIA was estimated to be 1000-2000 times more sensitive than the local pigeon crop sac method. Frantz et al. (1972b) also found good correlation between RIA and the mouse mammary bioassay, but at low doses (15-60 ng/ml) the correlation is poor. When a RIA for bovine prolactin was compared with the pigeon crop sac assay, some disappointing results were obtained (Raud and Odell, 1971). The pigeon crop was 2.5 times more responsive to crude pituitary extracts than to purified hormone. The RIA on the other hand generated parallel dose-response curves for both crude extracts and purified hormone. They attributed the discrepancy in the crop sac assay to the effect of ACTH on the crop gland. In contrast, Leung et al. (1978) found that the RIA for rat prolactin and a rat mammary bioassay had parallel slopes. However, the RIA detected only 25% of the bioassayable prolactin. Apparently when RIA and bioassays are compared, only completely homologous assay systems should be used.

Since the binding of hormone to receptor is the first requisite for a biological response, it would seem reasonable that receptor binding could be developed as an assay for prolactin. Shiu et al. (1973) have described such an assay (RRA) which has a sensitivity of 5 ng/ml for ovine production. They found that the assay is specific for lactogens and that the relative responsiveness of the receptors correlates well with the known relative potencies of h-GH, o-PRL and other

lactogens. Although others have reported good success with the receptor assay (Costlow, 1974; Posner, 1976), there are also reports of discrepancies of the RRA and bioassay (Nicoll, 1975). Ultimately the potential value of the RRA has been overshadowed by the widespread use of the RIA.

VI. Radioiodination of Prolactin

Steroids as well as the protein hormones circulate in the blood in minute quantities ranging from 10^{-15} to 10^{-9} moles per liter. For this reason, physiological quantities of hormones are very difficult to detect by the usual physical-chemical methods. During the past two decades, radiolabelling has been indispensable as a means of "tracing" and quantitating the various physiological roles of hormones. Although the steroid hormones are often labelled with ^{3}H or ^{14}C, the protein and glycoprotein hormones are most often labelled with radioiodide (^{125}I).

The first report of a lactogenic hormone being labelled was by Greenwood et al., (1963). They prepared ^{131}I-human growth hormone using the chloramine T method of iodination. The method involves a 60 sec incubation of hormone, chloramine T and radioiodide and is reported to yield protein products of high specific radioactivity. The method has been used with considerable success by a number of authors (for example, see Nicoll et al., 1973; Midgley, 1966) for the radioimmunoassay.

A second method of protein iodination is also in common use which involves the enzyme lactoperoxidase. The iodination reaction requires three substrates: peroxide, iodide, and the phenolic compound which is iodinated. Kinetic studies indicate that a quaternary complex is not

formed, but that a ping-pong-type mechanism is involved (Morrison and Bayse, 1970). The authors also found that the concentration of available iodide showed saturation kinetics but a large excess of iodide actually inhibited the rate of reaction. Thorell and Johansson (1971) were the first to use the lactoperoxidase method for the iodination of protein hormones. They found that insulin, glucagon, FSH, GH, LH, and TSH all retain their immunological reactivity and were used in radioimmunoassays with good results. They also found that lactoperoxidase was capable of self iodination. Frantz and Turkington (1972) also used ^{125}I and lactoperoxidase for the iodination of ovine prolactin to a specific activity of 130 μCi/μg. About 60% of the biological activity of the hormone was retained as determined by its ability to bind with mammary gland particles and to stimulate ^{32}P-casein synthesis. In contrast, they found that chloramine T iodination caused a greater than 90% loss of biological activity. This latter finding was corroborated by Rogol and Rosen (1974) who reported that the chloramine T method of iodination was associated with a profound loss of desirable immunochemical properties for radioiodination. They demonstrated that the change in bioactivity was also associated with a significantly altered sephadex chromatography elution pattern as compared to lactoperoxidase iodinated hormone. Similarly h-PRL and amniotic fluid prolactin were also damaged by chloramine T iodination. Several "new" species of the hormones were generated during the chemical treatment as observed by two dimensional polyacrylamide gel electrophoresis (Rogol and Chrambach, 1975).

Although the lactoperoxidase method of iodination of protein would appear to be the preferred method, it too has its associated

problems. Alexander found that "active iodide", which is generated by peroxide, can oxidatively cleave tryptophan peptide bonds and therefore damage the molecular structure of protein (Alexander, 1974). The damage is caused within one minute which would be a typical time frame for hormone iodination. Formation of dityrosine cross-links in protein during enzyme iodination also occurs. This could in part account for the formation of "large forms" of iodinated hormone which are observed in Sephadex elution chromatograms (Aeschbach et al., 1976). The authors also found that iodination of trypsin and chymotrypsin resulted in 85% loss of enzyme activity and this inactivation was the result of cross-link formation.

One should also be concerned about the site(s) of iodination with respect to the active site of the hormone. That is, it is quite possible that iodination at or near the active site could reduce, but not abolish, biological activity. Iodination has caused a 20-50% loss of binding activity in various antihapten antibodies (Grossberg et al., 1962) and substantially reduces the enzymic activity of arginine kinase (Fattoum et al., 1971). Koshland and his co-workers have even used iodination as a means of identifying the active site of anti-p-azobenzene-arsonic acid antibody (Koshland et al., 1963). Because of these potentially damaging effects of iodination, it should not be assumed that native and iodinated prolactins have the same biological activity nor that they will behave identically in assay systems. To my knowledge, no comprehensive study has been undertaken to evaluate the biological effects of prolactin iodination.

The methods of prolactin iodination by lactoperoxidase are also quite variable. For a comprehensive and detailed review see Bolton (1977). As a consequence, the stoichiometry of prolactin iodination is usually unknown. For example, reaction times for iodination vary from 2 sec (Thorell and Johansson, 1971) to 10-15 minutes (Frantz and Turkington, 1972; Rogol and Chrambach, 1975). Some authors use a single dose of peroxide and others use two to three (see Bolton, 1977). Furthermore, the stoichiometry of ^{125}I:prolactin:lactoperoxidase also varies from laboratory to laboratory. The only attempts to quantitate the incorporation of ^{125}I into prolactin are generally based on TCA precipitation (Posner et al., 1974) or planimetry of elution chromatograms (Frantz and Turkington, 1972; Rogol and Rosen, 1974; Bolton, 1977). Iodination of the lactoperoxidase, losses of iodide on glassware (which can be substantial) or iodination of albumen in the chromatography column are not accounted for. In short, the quantitative aspects of prolactin iodination are practically unknown.

VII. Fluorescence Analysis of Proteins

Following electronic excitation (photon absorption), molecules may undergo various relaxation processes that ultimately determine their luminescence properties. In many cases the sequel is not very interesting; the energy is transferred as heat to the surroundings. However, in some cases, reradiation occurs and this will usually be at lower frequency than that of the exciting light. This process is called fluorescence. Competitive with fluorescence we find vibrational

and geometrical relaxation as well as internal conversion and intersystem crossing. Furthermore, in aqueous solutions of complex macromolecules such as we find in biological systems, collisional quenching, non-collisional energy transfer and solvent effects may also be important.

A. Intramolecular Energy Transfer in Proteins

The tertiary structure of protein hormones is largely dependent on hydrophillic and hydrophobic interactions of the various amino acid residues with one another and with their environment. The indole and phenol groups are the side chains of the two most hydrophobic residues and contribute importantly to the stability of the native state. Within proteins, it is more often the rule than the exception to find three dimensional groupings of aromatic amino acids. It has been suggested that the delocalized electrons of aromatic side chains impart stability to the tertiary structure (Tulinski et al., 1973; Liljas, 1972). These aromatic residues are also the chromophores which are the most readily studied by fluorescence techniques and therefore are good structural probes (Edelhoch, 1976). Although each of these amino acids has its own absorption and fluorescence characteristics, they need not behave independently. Under favorable conditions, the excitation energy from one chromophore can be transferred to another which becomes the ultimate emitter. The requirements are (1) the possibility of a dipole-dipole interaction between chromophores and (2) an appreciable overlap of the donor fluorescence spectrum and the acceptor absorbance spectrum. Phenylalanine, tyrosine and tryptophan can satisfy these requirements

and consequently one often finds that protein solutions will only emit tryptophan fluorescence; tryptophan having the lowest energy first excited state (Van Holde, 1971). The efficiency of such energy transfer depends on the inverse sixth power of the distance between the two groups and in practice, energy transfer decreases rapidly as the separation between amino acids increases beyond 20 Å. Such sensitized fluorescence thus serves as a useful yardstock for the distances between groups in macromolecules (Chignell, 1970; Chen and Kenohan, 1967).

In a study of human serum albumin, bovine serum albumin and ovalbumin Weber (1961a) found that at neutral pH, only tryptophan fluorescence was observed. Following treatment with 8M urea, a second fluorescent component was distinguished which was identified as tyrosine. The denaturant had the affect of unraveling the protein tertiary structure and thereby separating tyrosine and tryptophan residues to a distance which vastly decreased the efficiency of energy transfer. In some cases, the tyrosine fluorescence in proteins can be "unmasked" without denaturing the protein by performing emission scans at two different excitation wavelengths (Weber, 1961b). This method takes advantage of the differential absorption manifolds of tyrosine and tryptophan. Excitation at 275 nm will produce greater fluorescence at 305 nm than excitation at 295 nm which excites only tryptophan.

Under alkaline conditions tyrosine ionizes. When this occurs, its absorption peak shifts from 274 nm to 295 nm. We now have an overlap of tryptophan fluorescence and tyrosine absorbance wavelengths. Energy transfer from tryptophan to tyrosine is now possible and tryptophan fluorescence quenching should therefore parallel tyrosine

ionization (Edelhoch, 1976). This phenomenon was clearly demonstrated by Steiner and Edelhoch (1963) using soybean trypsin inhibitor.

B. Iodide "Collisional Quenching"

Quenching of fluorescence occurs by any process that results in a decrease in the fluorescence efficiency of a molecule. "Collisional quenching" is a bimolecular process where the excitation energy of one species is dissipated by the close association of the second species (the quencher). In aqueous solution, a quencher will reduce the fluorescence efficiency of a protein by enhancing intersystem crossing of the excited aromatic residues. Since the triplet lifetime is much longer than the excited singlet lifetime solvent effects become much more significant and excitation energy is lost as heat (Wehry and Rogers, 1966). This quenching by a molecule in the environment of the fluorophore is also called the external heavy ion effect and is believed to have some charge transfer character (Berlman, 1973).

Iodide is known to be an efficient and specific quencher of tryptophanyl fluorescence and its effects have been studied in detail with model compounds as well as with lysozyme (Lehrer, 1971). It is interesting to note that "accessibility" of quencher to fluorophore in proteins is extremely sensitive to the three dimensional conformation of the protein. For this reason, active site determinations as well as tertiary structure may be studied by use of collisional quenchers (Lehrer, 1967).

C. Iodide Internal Heavy Ion Effect

Substitution of a heavy atom such as iodide or other halogen into the ring of an aromatic amino acid will cause a significant decrease in fluorescence. In order for the effects to be manifested, the electrons of the fluorophore must "see" the heavy atom. That is, the pi orbitals of the fluorophore must have considerable overlap with the orbitals on the heavy atom. As a result, spin-orbit coupling is attained (Berlman, 1973). Furthermore, many iodo-substituted aromatics exhibit "photodissociation" in liquid solution, reducing fluorescence efficiency even more (Wehry and Rogers, 1966). In a study using halogen substituted naphthalenes, McClure (1949) showed that fluorescence can be drastically reduced by a single insertion of iodide.

Iodination of tyrosyl residues to the 3-iodo or 3,5-diiododerivatives abolishes fluorescence of the iodinated residue and converts it to an energy sink (Cowgill, 1976). An energy sink is simply a non-fluorescent residue that is capable of accepting excitation energy from another tyrosyl (or tryptophanyl) residue and degrading that energy non-radiatively. The increased absorbance of iodotyrosine and the good overlap of tyrosine absorbance and emission manifolds accounts for the high sink efficiency in proteins. Using this principal, fluorescence quenching curves derived from iodination experiments on insulin and RNAase were used to determine the fluorescence efficiency of susceptible tyrosyl residues (Cowgill, 1965). Since iodination of tyrosine also causes a red shift in its absorbance manifold, the net flux of excitation energy in a protein may actually be reversed such that energy transfer from tryptophan to tyrosine occurs. This phenomenon was

eloquently demonstrated by Perlman et al. (1968) using thyroglobulin and human serum albumin. By quantitatively iodinating tyrosyl residues in these proteins, the critical transfer distances between tryptophan and "nearest neighbor" tyrosines were estimated. Rhodes (1976) has similarly shown that iodination of tyrosine residues in the protein hormone prolactin results in a 35% decrease in tryptophanyl fluorescence.

D. Environmental Influences on Protein Fluorescence

Although it is beyond the scope of this review to consider all effectors of protein fluorescence in detail, a brief mention of several environmental factors which are of particular interest will be presented.

1. Hydrogen bond formation in a non-polar environment causes tyrosyl fluorescence quenching. In fact, H-bonded tyrosyl residues have a fluorescence efficiency approaching 0. For example, Cowgill (1967a) found that two out of four tyrosines in pancreatic trypsin inhibitor were practically devoid of fluorescence. These tyrosyl residues also could not be iodinated and therefore are believed to be buried within the protein tertiary folds.

2. Sulfhydryl and cystinyl groups cause quenching of both tyrosine and tryptophan fluorescence in proteins. The mechanism is believed to involve extremely short range interactions between the aromatic ring and the sulfur atoms that facilitate vibrational dissipation of the excitation energy (Cowgill, 1967b).

3. Ionization of tyrosyl residues at low hydrogen ion concentrations ($pH > 8$) result in a loss of fluorescence and converts the amino acid into an energy sink (Cowgill, 1965). This radiationless loss of

energy also provides a pathway for energy transfer and a concommitant quenching of tryptophan fluorescence.

4. The tryptophanyl emission maximum is greatly effected by the polarizability of the fluorophore's environment. For example, proteins embedded in the lipid matrix of cell membrames tend to have emission spectra which are shifted to shorter wavelengths relative to aqueous solutions of the same proteins (Wallach and Zahler, 1966). This blue shift is the result of stabilization in the environment of the fluorophore.

E. Fluorescence Perturbations During Macromolecular Binding

Any macromolecular event which can change the charge distribution of the excited state orbitals will influence the fluorescence of the molecule. Since many biologically important reactions require the "binding" or intimate association of large molecules, changes in their intrinsic fluorescence might be anticipated. For example, the polyene antibiotic filipin, is known to fluoresce strongly in water solution. The addition of cholesterol to the medium however results in a 60% decrease in fluorescence efficiency (Schoreder et al., 1971). This finding was interpreted as evidence for pi*-pi interaction between the excited states of the fluorophore and the orbitals of cholesterol. In another system, it has been reported that colchicine becomes fluorescent only when bound to the protein tubulin. This phenomenon has been developed into a sensitive assay for colchicine binding (Bhattacharyya and Wolff, 1974). Similarly, the kinetics of the binding of various saccharides to lysozyme have been described based solely on changes in

the enzyme's tryptophan fluorescence. Using this method, specific tryptophan residues have been implicated at the enzymic active site (Halford, 1975). The activator of yeast pyruvate kinase, fructose 1-6 diphosphate causes dose-dependent decreases in kinase tryptophanyl fluorescence. This fluorescence interaction has been used to assess the relative binding affinity of activator and enzyme in the presence or absence of substrate (Kuczenski and Suelter, 1971). Sometimes the specific details of a binding process are not clearly understood yet fluorescence perturbations can provide insight as to possible mechanisms. For instance, the interaction of proteins and lipids in biological membranes may be studied by the application of fluorescent probes. For a good review see Wallach et al. (1970).

In summary, fluorescence studies of biological macromolecules and proteins in particular, may yield valuable information about structure and function of the compound. In general, fluorescence enhancement suggests energy transfer or stabilization in the environment of the fluorophore while fluorescence quenching may have several interpretations (Hercules, 1966).

MATERIALS AND METHODS

I. Iodination Methods

Ovine prolactin (n-PRL) for all phases of this study was obtained from the National Institute of Health (NIH-P-S-11 and NIH-P-S-12). Lactoperoxidase (LPO) lyophilized from milk was obtained from Calbiochem (San Diego, CA) and used as supplied without further purification. Potassium iodide (KI) and 30% H_2O_2 were obtained in analytical grade (Mallinckrodt Chemical Works, St. Louis, MO). Carrier free $Na^{125}I$ (14 mCi/μg) was purchased in 5 mCi samples dissolved in NaOH (Amersham-Searle). Samples of n-PRL, LPO and KI were weighed on a Cahn model 4100 electrobalance (Cahn Corp., Paramount, CA) and stored in siliconized Pyrex tubes at -20° C until the time of use. On the day of an iodination experiment, PRL samples were dissolved in minimal quantities of NH_4HCO_3 buffer (pH 8.3) and then diluted with 0.4 M sodium acetate (pH 5.3). Three microliters of 30% peroxide were dissolved in 50 mls of sodium acetate buffer; LOP and KI were also dissolved in appropriate quantities of sodium acetate buffer.

Radioiodination reactions for receptor studies were performed by a modification of the method of Frantz and Turkington (1972). Generally 5 μg n-PRL, 5 μgLPO, 5 nmoles KI, and 1 mCi ^{125}I were incubated in a siliconized 2 ml polyethylene tube. The reaction was initiated by the addition of 25 μl of the peroxide mixture. The reactants were agitated

continuously for either 15 or 25 minutes; the final reaction volume was 200 µl. At the end of this period, several crystals of sucrose were added to the mixture (to increase solution density) along with several crystals of dextran blue (Pharmacia Corp., Stockholm, Sweden). The iodination products were then drawn into polyethylene tubing (Clay Adams) using a 1 ml disposable syringe and subsequently applied to the top of a gel filtration column.

"Cold iodination" reactions were carried out by essentially the same procedures except the quantities and volumes of reactants were multiplied by 10, i.e., 50-100 µg of prolactin were iodinated in proportionate volumes of sodium acetate buffer. The fluorescence monitored iodination reactions were carried out directly in the cuvette of an Aminco-Bowman spectrofluorometer (American Instrument Co., Silver Spring, MD). In order to standardize the reaction conditions, a stock solution containing 900 µg LPO and 900 µg n-PRL was prepared as well as serial dilutions of KI ranging from 0 to 50 µg/ml. Before each experiment, 1.5 ml of the PRL-LPO solution was added to the cuvette followed by 0.25 mls of KI. The reaction was initiated by the addition of 0.25 mls of peroxide. The final concentration of PRL in each case was 25 µg/ml. Relative changes in fluorescence (excitation at 275 nm, emission wavelength at 348 nm) or emission scans (excitation at 275 nm) were recorded with an on-line X-Y recorder. In some cases, these reaction products were also subjected to gel filtration. In order to control the effects of gel filtration and to select prolactin species of known molecular weight, samples of n-PRL were also chromatographed under identical

conditions. Fluorescence spectra of n-PRL and iodinated prolactin (I-PRL) were then compared.

II. Gel Filtration

Gel filtration was performed using Sephadex G100 cellulose beads (Pharmacia) packed into a vertical 1.5 x 21 cm glass column. The gels were eluted with either 0.4 M sodium acetate (pH 5.6) or potassium phosphate (pH 7.4), at a constant flow of 9 mls/h. One (1) ml elution fractions were collected into siliconized 1 x 5 cm glass tubes using an automated fraction collector (ISCO Model 820, Lincoln, NE). Before hormone samples were applied to the column, the bed homogeneity was checked visually by watching the progress of a dextran blue dye marker pass through the gel. All hormone samples were preceded through the gel by 1 mg of bovine serum albumin (Sigma Chemical, St. Louis, MO) in order to minimize prolactin binding to glassware. Gel filtration was performed at 4° C. The K_{av} values were calculated by the method of Laurent and Killander (1964). By definition:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume, V_o = void volume and V_t = total gel volume.

In some cases it was desirable to know the protein content of I-PRL or n-PRL solutions eluting from gels. Protein determinations were performed by the method of Massey and Dean (1975). Tannic acid, HCl and gum arabic were obtained from Baker Chemical Co. (Philipsburg, NJ) and protein concentrations were based on standard solutions of

n-PRL. The turbidity of protein containing samples were read at 650 nm after zeroing against a reagent blank using a Beckman model DU spectrophotometer (Beckman Instrument Inc., Fullerton, CA).

III. Electrophoresis

Discontinuous polyacrylamide gel electrophoresis under dissociating conditions was carried out by the method of Weber et al. (1972). Twelve (12) cm gels were prepared in 14 cm glass tubes coated with dimethyldichlorosilane (Sigma) and soaked in 1% sodium dodecyl sulfate (SDS; Sigma) between uses. Acrylamide (Ames Co., Elkhart, IN), BIS (Sigma), ammonium persulfate (Canalco, Rockville, MD) and tetramethylethylenediamine (Canalco) were used to prepare the gels according to Shapiro et al. (1967). The electrophoretic apparatus was obtained from the Davis Company (Lincoln, NE) and an ISCO model 490 power supply was used.

Protein samples for electrophoresis were precipitated with two volumes of ice cold 10% trichloroacetic acid (Baker), incubated for one hour at 4° C, centrifuged at 750 x g for 10 minutes and then carefully aspirated. The pellets were resuspended in 100 µl of electrophoretic gel buffer containing 1% SDS and 1% mercaptoethanol (Sigma). The suspension was then heated for 3 minutes over a boiling water bath, the pH was adjusted to 7.5 and several crystals of sucrose were added to increase the sample density. Five (5) µl of 0.1% phenol red (Canalco) was added as a tracking dye. Forty (40) µl of prepared sample was then carefully applied directly to the top of the electrophoretic gels

(7.5% T) under 0.5 mls of buffer. The gels were electrophoresed at 2 mA/gel until the dye marker approached the bottom of the column (about 3.5 h; final potential = 370 volts). The gel buffer was 0.2 M sodium phosphate (pH 7.2) (see Weber et al., 1972) and the electrode buffer was a 1:10 dilution of gel buffer with 0.1% SDS added. Following electrophoresis, gels were stained with 0.01% Coomassie Brilliant Blue R 250 (Sigma) in methanol and acetic acid for 3 hours. Destaining was by diffusion in a mixture of 50 mls methanol:75 mls glacial acetic acid:1 liter water.

IV. Binding and Dissociation of ^{125}I-Prolactin and Receptors

A. Preparation of Binding Material

The methods described here were adapted from the methods of Shiu et al. (1973). Freshly excised livers from multiparous Sprague-Dawley rats (Spartan Research Animals, Okemos, MI) were hand homogenized at 4° C in heparinized 0.4% sodium citrate. The homogenate was filtered through cheesecloth and then centrifuged in five volumes of citrate at 1000 Xg at 4° C for 20 minutes to remove large fragments and nuclei. The supernatant was centrifuged at 100,000 Xg for 90 minutes to obtain the total microsomal pellet (Meldolesi et al., 1971). This cell fraction includes not only plasma membrane but also the Golgi apparatus and endoplasmic reticulum (Ehrenreich et al., 1973). The microsomal pellet was resuspended in 0.025 M tris-HCl buffer (Sigma), pH 7.4 containing 10 mM $CaCl_2$, and 0.1% BSA to a final concentration of 1500 µg/ml of protein. The resulting suspension was then thoroughly mixed and divided

into several 5 ml aliquots which were frozen and stored at -20° C. Binding by these fractions was shown to be stable for more than six months.

B. Binding and Dissociation Methods

All binding and dissociation experiments were carried out in 400 µl polyethylene tubes (Clay Adams) and all dilutions pertinent to binding experiments were done with the binding medium (tris-HCl-$CaCl_2$) described above. For each tube, a constant amount (either 60,000 or 45,000 CPM) of ^{125}I-PRL was provided plus 0 - 5000 ng n-PRL. Volumes were adjusted to 300 µl and the tubes agitated. To these tubes, 100 µl (150 µg protein) of membrane preparation was added. The tubes were thoroughly mixed by vortex and incubated at room temperature for three hours. After three hours, the tubes were centrifuged at 10,000 Xg for 5 minutes (Beckman Microfuge), the surface of the pellet washed once with binding medium, and the tips of the tubes cut off using a razor blade. The pellets were then dispersed in 1 ml of "Lowry A" solution (see Appendix C) and counted in a gamma spectrometer (Model 1085, Nuclear Chicago-Searle) for 20 minutes. Counting efficiency was estimated at 69% using an ^{129}I external standard. The total binding (B_t) in a tube is expressed as the total quantity of radioactive counts appearing in the pellet in the absence of any competitive hormone. Nonspecific binding (B_n) is the quantity of binding which occurs in the pellets of tubes incubated with an excess (5 µg) of n-PRL. Specific binding is operationally defined as: specific binding = $B_t - B_n$. Specific binding is adjusted to 150 µg of membrane protein using the

Lowry method (Lowry et al., 1951). The formulation of the "Lowry A" and "Lowry B" solutions are provided in Appendix C. Individual modifications of the binding procedures are described in later sections of this dissertation.

V. Pigeon Crop Sac Assay

The methods for this assay are essentially identical to those described by Frantz and Rillema (1968). Four to six week old White King squabs (Palmetto Pigeon Plant, Charleston SC) of either sex were maintained on a normal diet at room temperature and exposed to about 16 h of light per day. Two days before an experiment the feathers covering the crop area were removed. Into each bird 0.2 mls of chromatographed (K_{av} between 0.35 and 0.55) n-PRL or I-PRL was injected into opposite sides of the skin overlying the crop region. Similarly, a control injection of bird Ringer solution was made into each crop. The injections form a "bleb" which was marked with color coded pens. Thirty hours later, 5 μCi/Kg of an amino acid mixture (^{14}C-labelled protein hydrosylate amino acids, Schwartz/Mann, Orangeberg, NY) was injected into the wing vein of each bird. After an additional three hours, the birds were sacrificed by cervical dislocation and the crops were opened by a midline incision. Marked sections of the crop were then cut out using a 1.5 cm (diameter) cylindrical cork cutting tool. The epithelial tissue was separated from supporting tissue layers and sonified (Heat Systems - Sonifier, Plainville, NY) for one minute in 1 ml of "Lowry A" solution. One tenth (0.1) ml of the sonicate was

removed for protein determinations and the remainder was dispersed in 15 mls of a modified Bray's dioxane scintillation solution (see Appendix D). Scintillation counting was performed with a Nuclear Chicago model 6860 liquid scintillation counter. Quenching was determined using an external ^{133}Ba standard. ^{14}C counting efficiency was 42% and counts during each 20 minute period were adjusted to 1 mg protein content as determined by the Lowry procedure (Lowry et al., 1951).

VI. Electronic Circuitry

All component parts for the analog circuits were purchased from Radio Shack (Fort Worth, TX). Resistors and capacitors were rated at 50% tolerance; diodes were general purpose silicon type. Decade resistance boxes (Halicrafter Co.) and potentiometers (Radio Shack) were variable from 0 to 10 meg-Ohms. Operational amplifiers (type 741) and a regulated power supply ($\pm$ 12 V D.C.) were obtained from Poly Paks (S. Lynnfield MA). An on-line chart recorder (model SR, Sargent-Welch, Detroit, MI) was calibrated to 0-20 volts D.C. and used for analog circuit readout.

BIOCHEMICAL ANALYSIS OF PROLACTIN IODINATION

I. Fluorescence Studies of Prolactin Iodination

A. Objectives

In preliminary experiments (Rhodes, 1976), the author found that quantitative decreases in intrinsic tryptophanyl fluorescence from prolactin correlate with lactoperoxidase catalyzed iodination. These fluorescence decreases were shown to be distinct from collisional quenching and appear to parallel the ongoing iodination reaction. Ovine prolactin is a particularly interesting molecule to study by fluorescence since it possesses only two tryptophan residues and seven tyrosines. The sites of iodination are tyrosine and the concomitant decreases in tryptophanyl fluorescence presumably occur due to decreased energy transfer from tyrosine to tryptophan. It was proposed that the fluorescence quenching was the result of the following mechanisms: (1) an internal heavy ion effect, (2) a conformational change in the tertiary structure of prolactin, or (3) a combination of these effects. These mechanisms would result in diminished efficiency of energy transfer. A change in conformation could have serious effects on the biological activity of prolactin.

It was of interest to pursue a fluorometric study of prolactin iodination for three reasons. First, to investigate the fluorescence dose-response of prolactin with respect to iodide concentration.

This could yield information concerning the stoichiometry of iodoprolactin. Second, to compare fluorescence spectra of native and iodoprolactin to see if evidence exists for conformational changes in prolactin. Third, to establish methods of iodination which could yield a hormone preparation with predictable and reproducible biochemical properties. The methods could then be adapted to accommodate radioiodination procedures.

B. Experimental

Stock solutions containing equal quantities (w/w) of prolactin and lactoperoxidase were prepared to 0.4 M sodium acetate buffer (pH 5.6). A series of potassium iodide solutions (0-40 μg/ml) and a solution of 2.0 μ moles/ml H_2O_2 were also made in the same buffer. For each experiment, 1.5 mls of the prolactin-lactoperoxidase solution was added directly to the quartz cuvette of the spectrofluorometer followed by 0.25 mls of KI. An initial fluorescence reading was then taken with the excitation monochrometer set at either 275 nm or 295 nm and the emission monochrometer at 348 nm (the emission maximum for prolactin). At time t=0, 0.25 mls of the H_2O_2 solution was added and the time dependent fluorescence changes were recorded with an on-line X-Y recorder (sweep speed = 0.1 cm/sec). The final concentration of prolactin and lactoperoxidase was 25 μg/ml. The H_2O_2 was available at about a 300-fold excess (mole/mole). The volumes of buffer and quantities of reactant used in these experiments were selected to reproduce typical radioiodination conditions (Frantz and Turkington, 1972).

In some cases, the iodination products were separated using a 1.5 x 21 cm Sephadex G100 column eluted with sodium acetate buffer. The fractions were collected in 1 ml aliquots. The pooled products (K_{av} = 0.35 - 0.55) were scanned using the spectrofluorometer. Concentrations of chromatographed hormone were determined by the tannin reagent method (see Materials and Methods).

C. Results

Figure 1-A provides the emission spectra of equimolar solutions of native (n-PRL) and iodinated (I-PRL) prolactin. The iodination was carried out in the presence of a 20-fold excess (mole/mole) of iodide. These spectra were taken from chromatographed fractions of prolactin which eluted with a K_{av} between 0.35 and 0.55 (monomer). The excitation wavelength was 275 nm which should provide excitation energy directly to both tyrosine and tryptophan. The usual emission wavelength maximum of tyrosine in aqueous solution is about 305 nm; the spectra in Figure 1-A are both characteristic of tryptophan (emission maximum = 348 nm). Although there is a marked decrease in fluorescence intensity of iodinated prolactin as compared to native hormone, qualitatively the emission spectra are quite similar. Possibly there is a slight red shift in the iodinated form of the hormone (compare using vertical dotted line). This spectral shift could be the result of an exaggerated tyrosine quenching relative to tryptophan fluorescence. However, the effect is minor if real.

Figure 1-B presents the quantitative fluorescence analysis of three pairs of prolactin samples (K_{av} = 0.35-0.55). The bar graphs

Figure 1
Fluorescence Decreases Associated with Prolactin Iodination

A. Emission scans of iodinated and native prolactin: excitation=275 nm. Scans were performed on fractions eluting from a Sephadex G 100 column with K_{av} between 0.35 and 0.55. Dotted vertical line represents the emission maximum of n-PRL (348 nm).

B. Per cent decrease in fluorescence (275 nm ∫∫ 348 nm) of n-PRL and I-PRL in fractions eluting from a Sephadex G 100 column as compared to equimolar solutions of non-chromatographed n-PRL. * = significantly different from n-PRL fluorescence. n.s. = not significantly different from 0.

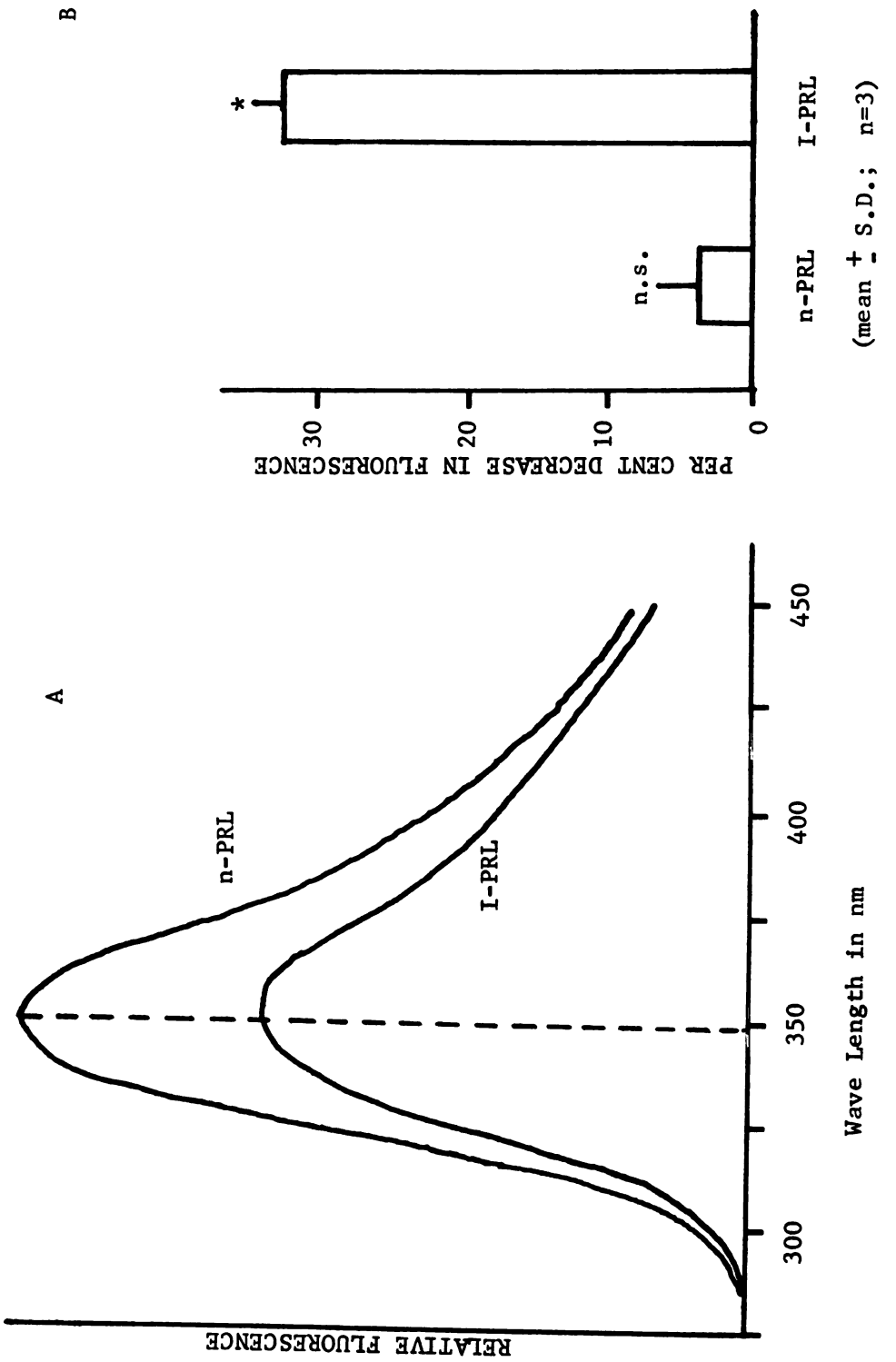


Figure 1
Fluorescence Decreases Associated With Prolactin Iodination

represent the percent decrease in fluorescence (± S.D.) of n-PRL and I-PRL relative to an equimolar solution of unchromatographed n-PRL. Excitation and emission were at 275 nm and 348 nm respectively. As above, iodination was carried out with a 20-fold excess of iodide (mole/mole). There was no significant effect of chromatography alone, but iodinated hormone had 33 percent less fluorescence than n-PRL.

Figure 2 illustrates the effect of iodide concentration on the total fluorescence decrease. In this graph "percent decrease in fluorescence" is defined as:

$$\frac{F_f}{F_H} \times 100$$

where F_f is the final fluorescence (275 // 348) following the addition of both iodide and H_2O_2 and F_H is the fluorescence of a prolactin lactoperoxidase solution following the addition of H_2O_2 alone. This method of data expression corrects for dilution effects as well as the oxidative effect that peroxide might have on tryptophan (Alexander, 1975). It should be noted that the results in Figure 2 refer to the combined fluorescence of prolactin and lactoperoxidase and not prolactin alone. With equal quantities of prolactin and lactoperoxidase, lactoperoxidase will contribute about 25% of the total fluorescence (Rhodes, 1976). We see in Figure 2 that the total fluorescence decreases are directly dependent on the initial concentration of iodide. That is, at low initial concentrations of iodide, the fluorescence decrease is linearly proportional to the initial concentration of iodide. In the range of 6 to 50 iodides per prolactin, the fluorescence decrease is nearly independent of the initial iodide concentration. The slow, gradual

Figure 2
Effect of Iodide Concentration on Fluorescence Decrease

Each point represents the per cent decrease in fluorescence (275 nm//348 nm) resulting from the addition of 300 nmoles of H_2O_2 to a mixture of n-PRL, lactoperoxidase and KI. The fluorescence decrease is corrected for dilution and non-specific fluorescence quenching by comparison to a control blank. In each case, the initial concentrations of n-PRL and lactoperoxidase are 25 μg/ml. The molar ratios of iodide to prolactin are given on the abscissa. In two cases, data were pooled (indicated by horizontal bars = ± S.D.).

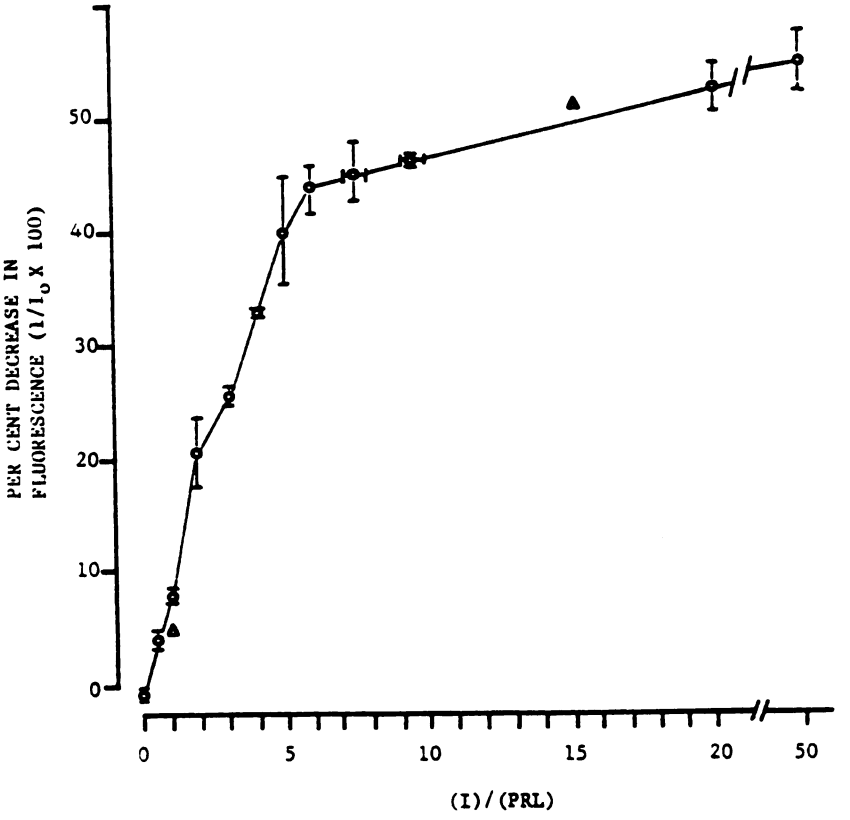


Figure 2
Effect of Iodide Concentration on Fluorescence Decrease

decrease (above I/P = 5) could be due to collisional quenching effects of iodide (Hercules, 1966). It should be pointed out that the fluorescent decrease at a molar ratio of 5:1 cannot be accounted for by fluorescence decreases associated with monomeric prolactin alone. If we assume that the prolactin species in Figure 1-B were fully iodinated and that they were the only species to suffer a loss in fluorescence, then the maximum expected fluorescence decrease would be: 33% x 0.75 = 24.7% (percent decrease in fluorescence x fractional contribution to initial fluorescence). There is a 10-20% fluorescence decrease which is unaccounted for. However, Thorell and Johansson (1971) have reported that lactoperoxidase is capable of significant amounts of self iodination which could contribute to the decrease in fluorescence. It is essential to recognize that lactoperoxidase will compete for available iodide. Also, prolactin aggregates and polymer formation (Aeschbach et al., 1976) as well as indole ring oxidation (Alexander, 1974) could quench fluorescence.

Figure 3 demonstrates the effect of the initial iodide concentration on the rate of decrease in fluorescence during iodination. The units are percent change in fluorescence per second and therefore are arbitrary. The actual ordinate axis corresponds to the reciprocal of the length of time required for one-half of the total fluorescence change to occur. The intention of the author was not to perform a rigorous kinetic study but only to establish the dependence of the rate of change in fluorescence on the initial concentration of iodide. The conditions of the iodination reaction (equal concentration of hormone and enzyme with limiting quantities of iodide) make accurate

Figure 3
Effect of Iodide Concentration on Rate of Decrease in Fluorescence

The rate of fluorescence decrease is defined as the reciprocal of the length of time (in seconds) required for one half (½) of the total fluorescence decrease to occur. Experimental conditions are described in legend of figure 2. The abscissa provides the initial iodide concentration in μmoles/liter and as the molar ratio of iodide to prolactin. * significant correlation coefficient ($p < 0.01$). ** slope ($\pm$ S.E. of the slope) significantly different from 0 ($p < 0.01$).

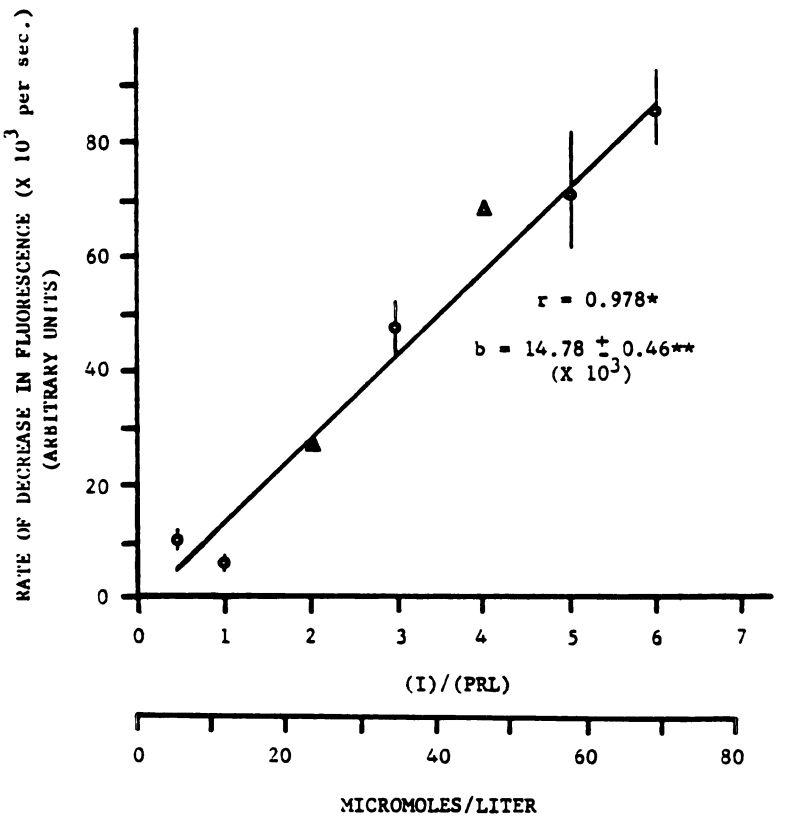


Figure 3
Effect of Iodide Concentration on Rate of Decrease in Fluorescence

kinetic measurements practically impossible. For example, with an initial iodide concentration of 68 μ moles (I/PRL = 6), the fluorescence change reaches 90% of its equilibrium value in 15 seconds. Calculations were therefore restricted to low concentrations of iodide and are approximate at best.

In spite of the approximate methods, we see in Figure 3 that the rate of change in fluorescence shows an excellent linear correlation with the initial concentration of iodide. Morrison and Bayse (1970), using other methods, report that iodide at low concentrations is rate-limiting for lactoperoxidase activity. It would be reasonable to conclude that the rate of decrease in fluorescence may reflect the rate of iodination.

D. Discussion

Iodination of ovine prolactin results in a 33% quenching of monomeric prolactin fluorescence. This fluorescence quenching is associated with only a small (if any) shift in the fluorescence maximum toward longer wavelengths. Although there is no direct evidence for tyrosine fluorescence in native prolactin, we must conclude that a significant portion of the n-PRL tryptophanyl fluorescence is obtained by energy transfer from tyrosine. Following iodination, tyrosyl excitation energy is dissipated due to increased intersystem crossing (Cowgill, 1965) and tryptophan fluorescence may even be quenched by energy transfer to tyrosine as suggested by Cowgill (1976). Since there are two tryptophanyl residues in ovine prolactin and seven tyrosines, the precise mechanism of quenching cannot be resolved using the results of these

experiments. More than one "excimer" complex may be responsible for the total fluorescence of the prolactin molecule. If this is the case, then a constant fraction of the initial n-PRL tryptophanyl fluorescence could be immune to the effects of tyrosine iodination. Since 67% of the initial fluorescence is not affected by iodination, this seems a probable explanation.

The total decrease in fluorescence associated with iodination varies as a linear function of available iodide (see Figure 2) up to a molar ratio of about 5:1. Additional increases in the available iodide cause only small decrements in fluorescence over the range of concentrations studied. If we make the assumption that all iodide is incorporated into prolactin, then we could speculate that the maximum level of iodination would be five iodides per prolactin. This assumes that the reaction is essentially irreversible. Our own fluorescence data as well as the data of Thorell and Johansson (1971) however indicate that some iodide is probably incorporated into lactoperoxidase and some may remain in solution. As a consequence, we may conclude that at least two tyrosine residues are not accessible for iodination. Since tyrosines can potentially contain up to four iodides per molecule (Morrison and Bayse, 1970), as many as six tyrosines may be inaccessible to the enzyme. If the sites of iodination could be identified precisely, we could obtain insight into the tertiary structure of prolactin. This task is left to future researchers.

We have also seen that both the rate of change in fluorescence and the total change in fluorescence depend on the initial concentration of iodide. This is strong evidence that fluorescence changes may

be used as a direct measure of lactoperoxidase catalytic activity. Unfortunately, iodide is incorporated into both prolactin and lactoperoxidase and both seem to respond fluorometrically. Consequently, fluorescence techniques do not offer a very promising means of determining prolactin iodination stoichiometry under the conditions of these experiments.

Figure 3 points out a fact which may be of interest to researchers who perform routine iodinations. Typically, hormone iodination mixtures consist of 5 μg PRL, 5 μg LPO and 1 mCi of ^{125}I. These conditions would correspond to an I:PRL ratio of 2.4:1. Referring to Figure 3, we would predict that one-half of the iodide would be incorporated into prolactin after 31 seconds of reaction. Rogol and Chrambach (1975) report that they stop the iodination reaction after 60 seconds. Others (Frantz and Turkington, 1972; Shiu and Friesen, 1973) either wait for up to 20 minutes or add additional peroxide. We would predict that various researchers are working with the hormone at various levels of iodination. Since the effects of iodination on binding or biological activity have not been rigorously assessed, various researchers may be working with hormones with different biochemical properties. If the intention of the investigator is to prepare a uniform and reproducible hormone preparation, it is recommended that the reaction be allowed to proceed for at least 15 minutes. Under the conditions described and with an initial dose of 3 iodides per prolactin, the reaction would be 95% complete in 11 minutes. In order to control the specific radioactivity of the iodinated prolactin, varying quantities of ^{127}I (stable isotope) could be added to the iodination mixture.

II. Chromatographic Analysis of Iodinated Prolactin

A. Objectives

In the preceding section we were able to use fluorescence spectroscopy to monitor the iodination reaction. We found that the initial iodide concentration could be used to regulate not only the rate of reaction but also the total change in fluorescence. The presumptive conclusion was that a maximum of five iodides could be incorporated into the protein fraction and that the initial iodide concentration and length of time allowed for reaction would determine the quantity of iodide incorporated into prolactin. However, it was also suggested that lactoperoxidase may be self-iodinated during the reaction. It therefore is important to be able to distinguish iodoprolactin from iodolactoperoxidase. If this differentiation of iodinated products can be made, then the real stoichiometry of iodinated prolactin could be calculated. This is a desirable result since it would greatly simplify the estimation of specific radioactivity of ^{125}I-PRL. If prolactin iodination stoichiometry were known, one could perform an iodination under controlled conditions, separate the products and determine the quantity of ^{125}I-PRL directly from the quantity of radioactivity present.

In the experiments described here, gel filtration and denaturing, discontinuous polyacrylamide gel electrophoresis are used to partition the products of iodination into the various components. The distribution of iodide into lactoperoxidase, prolactin and unbound components is quantitatively assessed using ^{125}I tracer. The conditions for iodination are five moles of iodide per mole of prolactin which is

believed to correspond to the maximal possible incorporation of iodide into prolactin.

B. Experimental

One hundred µg samples of n-PRL were incubated with 100 µg of lactoperoxidase, 2.9 µg Ki, 800 µCi ^{125}I (total iodide:PRL molar ration = 5:1) and 300 nmoles H_2O_2 in four mls of sodium acetate buffer (pH 5.6). The reaction was allowed to continue for 25 minutes at room temperature. According to calculations performed in the preceding section, this should be sufficient time for more than 95% iodide incorporation. In one experiment 9.4 µg of Ki + 800 µCi ^{125}I was used (total iodide:PRL ratio = 14:1). One-half (0.5) ml samples of the iodination products were applied to the top of a 1.5 x 21 cm Sephadex G100 column and eluted with potassium phosphate buffer (pH 7.5). The eluent was collected automatically in 1 ml aliquots. Selected, pooled gel filtration fractions (see Results) were then precipitated with two volumes of 10% trichloracetic acid, centrifuged, aspirated and resuspended in 100 µl of phosphate buffer containing 0.1% mercaptoethanol and 0.1% sodium dodecyl sulfate (SDS). The samples were then boiled, mixed with several sucrose crystals (to increase density) and bromophenol blue as a tracking dye; 40 µl of each sample was then applied to the top of 12 cm SDS gels (7.5% T). Gels were electrophoresed in phosphate buffer (pH 7.2) for about 3.5 h at 2 mA/gel; final voltage = 370. Gels were stained using Coomassie brilliant blue dye. Control gels containing either denatured lactoperoxidase or prolactin were also run simultaneously for comparison. Gels containing labelled hormone were cut by hand with a

razor blade into 5 mm slices, dispersed in H_2O_2 and counted in a gamma spectrometer.

C. Results

Figure 4-A provides the elution profile of two gel filtration experiments on the products of iodination reactions carried out at an initial I:PRL ratio of 5:1. The ordinate is the counts per minute of ^{125}I in each fraction, the abscissa is the partition coefficient (K_{av}). The four peaks labelled I, II, III, IV correspond to the elution peaks described by Rogol and Chrambach (1975) which had very similar K_{av} values. Rogol and Rosen (1974) found that 85% of the prolactin occurred in peak III and "larger forms" of prolactin (about 15%) were found in peak I. Iodolactoperoxidase elutes with a K_{av} of about 0.1. Peak IV is the salt peak (free iodide). The indistinct nature of peak II in the present study may reflect the specific methods of iodination. Since "peak II" is not clearly defined, we will simply take any fractions eluting with a K_{av} between 0.13 and 0.23. It should also be pointed out that these elution profiles are in good agreement with work done by other workers in our laboratory. Dombroske (1976) found that peak III corresponds to monomeric prolactin and peak I corresponds to heavier forms of prolactin (dimer, trimer, etc.). The author has found that lactoperoxidase (M.W. = 78,000) is confined to the peak I fractions (unpublished finding). Symmetrical curves were drawn to fit the peaks defined in Figure 4-A and the relative contribution of each peak to the total was determined by cutting out the curves and weighing them. These data are presented in Table 1.

Figure 4
Sephadex Chromatography of Iodination Products

Each graph represents the ^{125}I elution profile resulting from gel filtration of iodination products on a Sephadex G 100 column eluted with potassium phosphate buffer. In each case, 1 ml fractions were collected and counted in a gamma spectrometer for 0.1 minutes. The abscissa represents the partition coefficient (K_{av}) of each fraction. Peaks I, II, III and IV correspond to the fractions described by Rogol and Rosen (1974; see text).

A. Two separate elution profiles of iodination products which were iodinated under initial conditions of 5 iodides per prolactin.

B. Elution profile of a single iodination experiment; initial conditions were 14 iodides per prolactin.

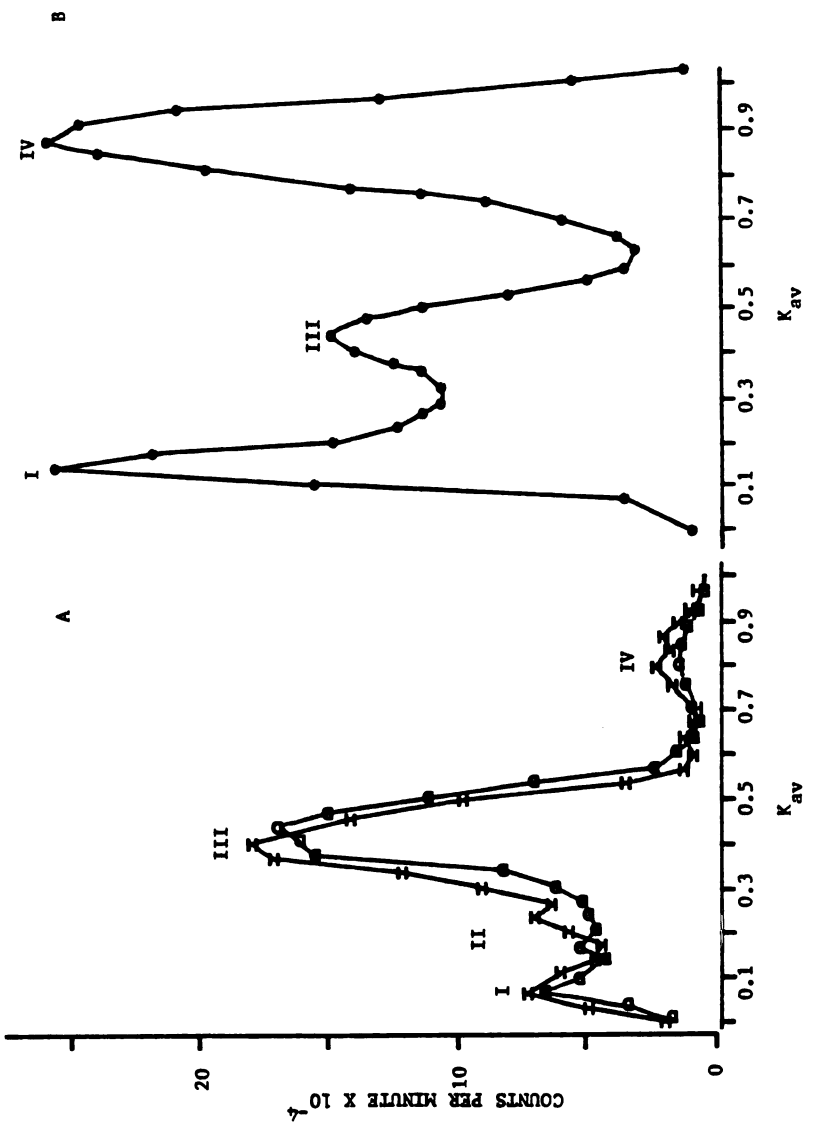


Figure 4
Sephadex Chromatography of Iodination Products

Figure 4-B is the elution profile of the iodination reaction carried out at an initial I:PRL ratio of 1:4. We are unable to detect a peak II under these conditions; the reason is unknown. As in Figure 4-A, the peaks were cut out, weighed, and the results compiled in Table 1. We see from Table 1-A that when iodide is supplied at a limiting level (5:1), all but 8% of the iodide is incorporated into protein. However, when iodide is present in excess (14:1), as much as 49% remains free in solution. Furthermore, it appears that when more iodide is available, more iodide is incorporated into lactoperoxidase (peak I).

Table 1. ^{125}I Content of Gel Filtration Fractions

	Chromatographic Peaks			
	I	II	III	IV
A.	13.4	8.2	70.0	8.5
	12.3	12.3	67.6	7.6
	12.85 ± .78	10.25 ± 2.9	68.8 ± 1.69	8.05 ± 0.64
B.	24.5	---	26.6	49.0

All values are percent of total eluted radioactivity (± S.D.).
A and B refer to graphs 4-A and 4-B, respectively.

In order to derive full benefit from these data, I would like to define a unit which I shall call a "molar unit". Since we start an iodination reaction with known ratios of iodide and prolactin, it is reasonable to assume that the total eluent of gel filtration

(or electrophoresis) will contain iodide and prolactin in the same ratio; even if absolute quantities are greatly diminished. We can say that the initial molar ratio multiplied by the relative iodide content of a gel filtration peak is equal to the "molar units" of iodide in that peak. For example, using the data in Table 1-A we see that peak III contains: 5 x 0.688 = 3.44 molar units of iodide. Similarly, in Table 1-B we see that peak III contains: 14 x 0.266 = 3.72 molar units of iodide. Notice that approximately the same quantity of iodide occurs in the prolactin monomer peak even though the quantity of available iodide was nearly tripled. This suggests that prolactin monomer is fully iodinated under conditions where five iodides per prolactin are provided. Rogol and Rosen (1974) have estimated that 85% of the prolactin which is iodinated emerges in the monomer fraction (peak III). This estimate is in good agreement with estimates in our laboratory (unpublished results). Accordingly, we estimate that the number of molar units of prolactin in peak III would be: 1 x 0.85 = 0.85 molar units. The ratio of iodide to prolactin which coelutes in peak III would be 3.44/0.85 = 4.05 for the experiments illustrated in Figure 4-A and 3.72/0.85 = 4.38 for the experiments illustrated in Figure 4-B. This is strong indirect evidence for a monomer iodination stoichiometry of 4 to 1.

In order to verify the identity of iodinated constituents of the gel filtration study, pooled samples (totalling 3 mls) corresponding to the maxima of peaks I, II, and III from the 5:1 iodination reactions were subjected to SDS disc polyacrylamide gel electrophoresis as described in Materials and Methods. Control samples of denatured n-PRL

and lactoperoxidase (LPO) were also electrophoresed. Staining bands of ^{125}I-labelled protein were cut out and counted with a gamma spectrometer. Only bands which correspond to LPO or n-PRL (control gels) were observed. Control slices (unstained regions) from each gel were also cut out and counted. The results of the electrophoresis are therefore classified by two criteria: (1) the relative mobility (R_f) of the protein band and (2) the percent of total radioactivity found in staining bands. Control (background) radioactivity was subtracted from each gel slice. The electrophoretic data are summarized in Table 2.

Table 2. SDS Electrophoresis of Selected Gel Filtration Fractions

R_f	I	II	III	PRL (control)	LPO (control)
0-0.1	triplet 55 ± 8	---	---	---	triplet
.3-.4	singlet 0 ± 0	singlet 3 ± 5	---	---	singlet
.75-.85	singlet 45 ± 8	singlet 97 ± 5	singlet 100 ± 0	triplet	---

All values are percent of total counts in protein bands (± S.D.)
n = 2 in each case

The control, n-PRL gels produced a single, dark staining band which had an R_f of about 0.8. This dark band was flanked on each side by single, very faint bands. The entire triplet was always within the range of R_f = 0.75-0.85. This is in good agreement with the findings of

other researchers (Lewis et al., 1963; Dombroske, 1976). The lactoperoxidase control gel produced a series of bands. The darkest staining and broadest component was largely excluded from the gel (R_f = 0-0.05). A second "doublet" at about R_f = 0.1 and a broad indistinct band at about 0.25-0.3 were also observed. Since the molecular weight of lactoperoxidase is 78,000 (Morrison and Bayse, 1970), it would be expected to be excluded from a 7.5% T gel (Weber et al., 1969; Shapiro et al., 1967). The author suspects that the 0.25 to 0.3 R_f band corresponds to contaminants. However, since they constitute a potential source of iodination, they should be accounted for. In Table 2 we see that peak III can be resolved into only a single component which coelutes with n=PRL. Peak II contains two bands but only the prolactin band possesses significant amounts of radioactivity. Peak I contained three visualized bands but only the LPO and prolactin bands contained radioactivity. In short, all radioactively labelled components in gels are associated with either prolactin or lactoperoxidase electrophoretic mobilities.

Notice that only 0.55 (from Table 2) x 12.85% (from Table 1) or 7% of the iodide is found in lactoperoxidase. Presumably a higher percentage would be found if the initial I:PRL ratio had been higher. If we assume a priori that "large form" prolactin is iodinated to the same extent as prolactin monomer then we can estimate the amount of prolactin found in fractions other than peak III.

Peak I, I-PRL = 0.45 x 12.85% = 5.8%

Peak II, I-PRL = 0.97 x 10.25% = 9.9%

Total percent of initial iodide = 15.7% in peaks I and II

All together there is 15.7% (from peaks I and II) and 68.8% (from peak III) = 84.5% iodide incorporation into prolactin species. Assuming equal iodination stoichiometry of large and monomeric prolactin then: 68.8/84.5 X 100 = 81% of iodoprolactin is monomer. This estimate is in reasonably good agreement with that of Rogol and Rosen (1974).

D. Discussion

The experiments presented here demonstrate that an average of four (4) moles of iodide are incorporated into each mole of prolactin when iodination is carried out at an initial I:PRL molar ratio of 5:1. About 7% of the available iodide is bound to lactoperoxidase and about 8% remains free in solution. Increasing the available iodide concentration by three-fold causes only a very small increase in iodide found in monomeric prolactin. These findings are entirely consistent with the fluorescence experiments described in the previous section. That is, the extent of prolactin iodination appears to correlate with relative decreases in fluorescence. However, fluorescence analysis alone is unable to account for the species of iodinated protein. Fluorescence decreases are the resultant of both prolactin and lactoperoxidase iodination and they cannot be distinguished photometrically. On the other hand, since increases in available iodide above a molar ratio of 5 iodides per prolactin do not increase the apparent level of monomer iodination, more iodide must be incorporated into lactoperoxidase. This shows up in gel filtration as an increase in the "peak I" fraction and may show up in fluorescent studies as a slower rate of fluorescence decrease (see Figure 2).

In summary, it should be reasonable to assert that monomeric prolactin attains an equilibrium level of iodination at four iodides per prolactin under the conditions of these experiments. Henceforth we shall use the designation 4-I-PRL to indicate the appropriate stoichiometry.

Once again I would like to point out that prolactin has seven tyrosines and therefore the potential for at least seven iodides per prolactin (maximally 28). That only four iodides are incorporated suggests that a minimum of three tyrosines are inaccessible to the lactoperoxidase enzyme. This fact could be used to help describe the three dimensional structure of prolactin and offers a potentially rewarding direction for future research.

ANALYSIS OF IODOPROLACTIN BINDING AND BIOLOGICAL ACTIVITY

I. Comparison of Native and Iodinated Prolactin Binding to Rat Liver Membrane Particles

A. Objectives

Prolactin is known to be physiologically effective in the range of 10^{-12} to 10^{-9} M and is therefore difficult to detect at these concentrations by classical chemical means. The study of binding of the hormone to membrane receptors has been aided by the use of ^{125}I labelled prolactin (^{125}I-PRL) as described in previous sections. We must consider iodination to be an invasive procedure which has the potential of damaging prolactin and not assume a priori that native (n-PRL) and iodinated hormone will behave identically (Bolton, 1977). Ultimately, descriptive parameters such as equilibrium constants (K_d, K_a, etc.) which are derived using iodinated hormone could differ significantly from the actual parameter of the native hormone.

Although it is well-documented that ^{125}I-PRL will bind with receptor, and can be competitively displaced by native hormone (Frantz and Turkington, 1972; Shiu and Friesen, 1974), there have been no attempts reported in the literature to compare the relative binding potentials of native and iodinated prolactin. The published equilibrium binding values for prolactin arise from studies using either ^{125}I-PRL or a mixture of native and iodinated hormone. In other words, one

one cannot know if these parameters are truly representative of the native hormone.

In this section, experiments were designed to assess the relative binding affinities of ^{125}I-PRL and n-PRL for membrane receptor in rat liver. The following logic was applied: since only ^{125}I-PRL can be detected (gamma radiation), we use this form of the hormone as a standard. We then perform competitive displacement experiments using native hormone or "cold iodinated" prolactin (^{127}I-PRL) as competitor. In all we have three species of prolactin: ^{125}I-PRL, ^{127}I-PRL (non-radioactive, stable isotope), and n-PRL. In one experiment we would compete ^{127}I-PRL with ^{125}I-PRL and in a second experiment we would compete n-PRL with ^{125}I-PRL. The results of such an experimental design should yield two pieces of information: (1) we should be able to estimate the relative binding affinities of native and iodinated prolactin and (2) we should be able to derive an estimate of the "true" binding affinity of native prolactin. The only underlying assumption throughout is that ^{125}I-PRL and ^{127}I=PRL have the same binding properties. That is, we assume no isotope effect and therefore measurements of their binding activities should be the same. In order to standardize the form of prolactin, iodination was performed under conditions to yield four iodides per molecule of prolactin as previously described. Henceforth, we shall refer to this as 4-I-PRL.

B. Experimental

Preparations of ^{125}I-PRL and ^{127}I-PRL were made as described in Materials and Methods. The ^{125}I-PRL was prepared from a reactant

mixture containing the following stoichiometric quantities: 3 moles ^{127}I-PRL:2 moles ^{125}I:1 mole PRL. The ^{125}I-PRL was made from a reaction mixture with a 5:1 iodide to prolactin ratio. As stated earlier, the term 4-I-PRL will be used henceforth. Native prolactin was exposed to lactoperoxidase and then chromatographed as if it were iodinated. As required by our protocol, only fractions eluting with a K_{av} between 0.35 and 0.55 were used. Concentrations of ^{127}I-PRL and n-PRL were determined by the tannin reagent method (see Materials and Methods). All hormone preparations were used within 48 h of their preparation. Receptor material for the evaluation of the specific binding was obtained by hand homogenization of minced liver from multiparous Sprague-Dawley rats. Receptor material for each experiment was pooled and mixed thoroughly by vortex in order to provide a uniform population of receptors for all binding tubes. It should be noted that this crude membrane fraction included not only plasma membrane but Golgi and other microsomal constituents. Two series of polyethylene tubes contained 0 to 2.5 μg of either n-PRL or 4-I-PRL mixed with ^{125}I-PRL such that each tube held 45,000 counts per minute (2.25×10^{-14} moles). To this mixture, receptor material was added. Following a 3 h incubation at room temperature, the particles were collected by centrifugation at 10,000 Xg for 5 minutes. The supernatant was carefully aspirated from the surface of the pellet which then was rinsed one time with binding medium (Materials and Methods), again aspirated and the tips of the tubes were cut just above the pellet and counted in a gamma spectrometer. In the second experiment, the entire procedure was repeated precisely as above

except that each incubation tube received 60,000 counts per minute (3.0×10^{-14} moles) of ^{125}I-PRL and a different liver membrane preparation was used.

C. Results

The specific binding, as defined in earlier sections, for the two competitive displacement experiments are given in Figure 5. Since different quantities of ^{125}I-PRL and different liver preparations were used in each experiment, the data for A and B are treated independently. Experiment A refers to the binding performed with 45,000 CPM's of ^{125}I-PRL and B refers to the second experiment. The total binding in A was 2550 $\pm$ 85 ($\bar{x} \pm$ S.D.) CPM and the maximum specific binding was 59% of the total (1526 $\pm$ 62 CPM). Total binding in Experiment B was 7630 $\pm$ 90 CPM and maximum specific binding was 63% of the total (2840 $\pm$ 108). The data in Figure 5 are normalized and expressed as specific binding/maximum specific binding (B/B_0). There was no significant difference in maximum specific binding for tubes incubated with 4-I-PRL as compared with n-PRL.

The results of these experiments were analyzed by two separate statistical methods. Initially the data in Figure 5 were analyzed by a factorial analysis of variance. The two factors were (1) dose and (2) form of competitor (n-PRL vs. 4-I-PRL). The ANOVA tables are presented on page 89. In all cases there is a very significant effect due to dose as well as a significant difference between native hormone and iodinated hormone as competitors. The factorial analysis of variance also provides information about the interaction of the two variables

Figure 5

Competitive Displacement of ^{125}I-PRL from Receptors

Each graph depicts the normalized specific binding (B/B_0) of ^{125}I-PRL to 150 µg of liver membrane protein in the presence of 0 to 2500 ng of competitor hormone. Competitor hormone was either n-PRL or 4-1-PRL (cold iodinated prolactin). Each datum represents the mean ± S.D. (n=3).
* = significant individual difference ($P < 0.05$) between n-PRL and 4-I-PRL tubes at the specified dose of competitor hormone. (A) = each tube incubated with 45,000 counts per minute (2.25×10^{-14} moles) ^{125}I-PRL.
(B) = each tube incubated with 60,000 counts per minute (3.0×10^{-14} moles) ^{125}I-PRL.

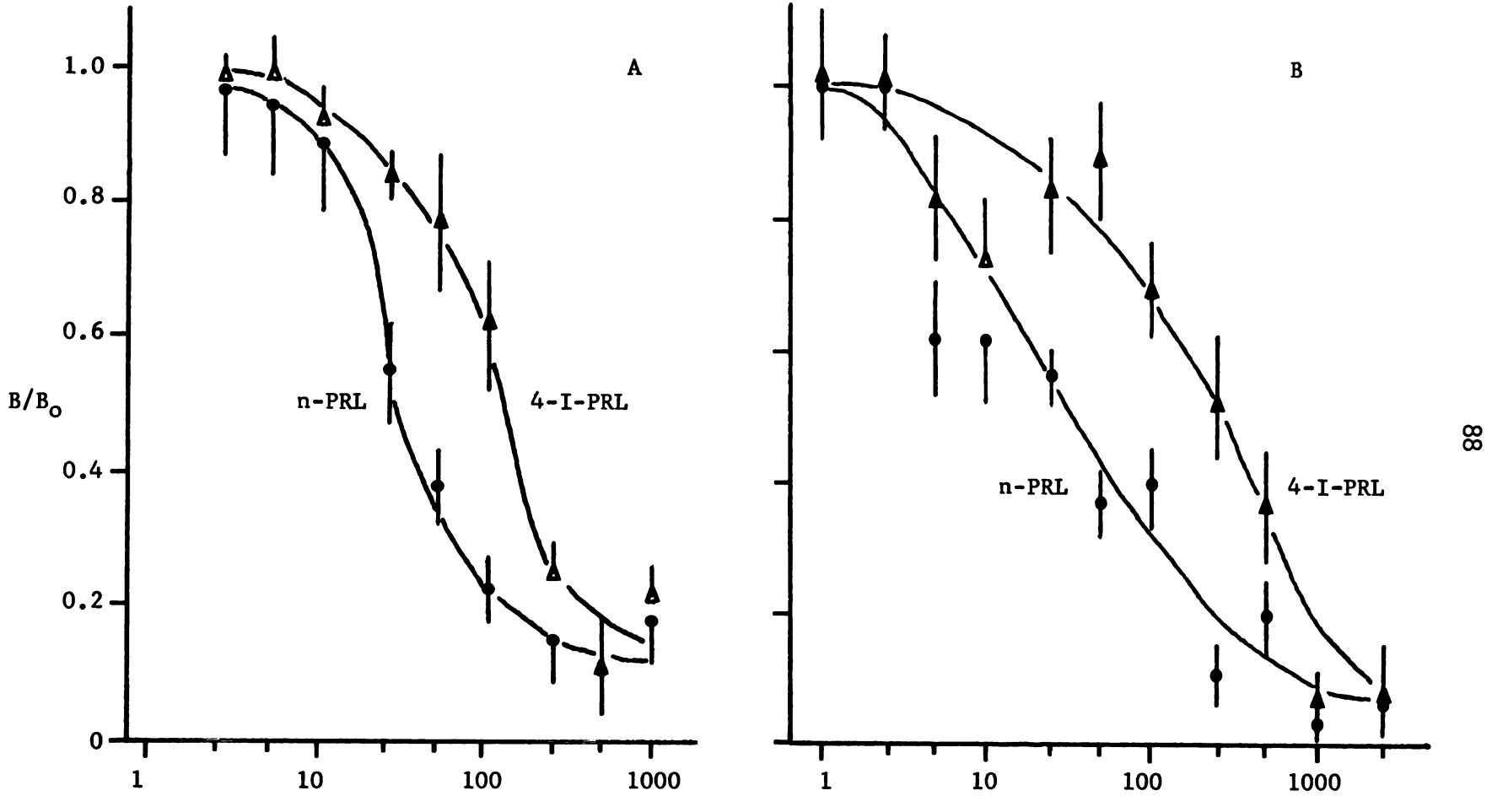


Figure 5
Competitive Displacement of ^{125}I-PRL From Receptors

Table 3. Analysis of Variance of Competitive Displacement Data

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
		(Experiment A)		
Form of PRL	1	308871	308871	21.69*
Dose	8	5897244	737155	51.79*
Interaction	8	324529	40566	2.85*
Error	36	512444	14234.6	
Total	53			
		(Experiment B)		
Form of PRL	1	21681408	21681408	291.9*
Dose	10	120645232	12064523	171.5*
Interaction	10	10373384	1037338	14.7*
Error	38	2673271	70349	
Total	59			

*$P < 0.05$

being tested. In this case we test for the independence of the two factors. We see in the table above that there is significant interaction of the two factors. In other words, the receptor response to dose depends on which form of hormone is used as competitor. This statistic tells us that curves fitted to the data will have different shapes. Therefore, there is evidence that both quantitative and qualitative differences exist between native and iodinated hormone. Since significant interaction exists between the factors, it is meaningful to test for the "simple" effects at each dose (Steel and Torrie, 1960). Significant independent differences are designated in Figure 1 by * over the relevant data ($P < 0.05$).

The Scatchard plot (Scatchard, 1949) is in such common use in the literature, it may be of value to examine the data by this method. The Scatchard plots of Experiments A and B are provided in Figure 6. The straight lines were fitted by linear regression to the mean values of bound hormone at each dose of competitor. Notice that in both experiments, the dissociation constants (K_d) as well as the predicted total number of receptors (R_T) is different for iodinated and native hormone (the differences are only significant in Experiment A). The statistical parameters for the linear regression are provided in the legend of Figure 6.

The mathematical method used to prepare the Scatchard plots (see legend of Figure 6) assumes identical behavior of tracer and competitor and therefore is extremely sensitive to hormones with different receptor binding affinities. If divergent slopes are generated, then competitor hormones have significantly different binding affinities. As a consequence, the apparent R_T values will also differ. This latter observation is entirely artifactual (Rodbard, 1973a). For example, if a competitor hormone has three times the binding affinity as another, then their respective slopes in the Scatchard plot will differ by a factor of three and the hormone with the greater affinity will appear to have only 1/3 the number of receptors.

D. Discussion

The experiments outlined in this section have provided evidence (both by analysis of variance and by linear regression analysis) that native prolactin and iodinated prolactin behave differently when allowed

Figure 6
Scatchard Analysis of Prolactin Binding

$B/F = 1\ K_d\ (B) + R_t/K_d$; when $B/F \longrightarrow 0$, $B \rightarrow R_t$

$$B/F = \frac{\text{Bound } ^{125}\text{I-PRL (moles/mg of liver protein)}}{\text{Free } ^{125}\text{I-PRL (moles/liter)}}$$

$$B = \left[\frac{\text{Bound } ^{125}\text{I-PRL (moles/mg)}}{\text{total } ^{125}\text{I-PRL (moles/l)}}\right] \text{ X (total } ^{125}\text{I-PRL + competitor)}$$

K_d = apparent dissociation constant

R_t = apparent total number of receptors (moles/mg)

A. Results of experiment A

(x) = 4-I-PRL as competitor, $K_{dI} = 10.9 \text{ X } 10^{-9}M^*$, $(R_t)_I = 11.3 \text{ X } 10^{-13}$ correlation coefficient (r) = -0.895*

(o) = n=PRL as competitor, $K_{dn} = 3.6 \text{ X } 10^{-9}M^*$, $(R_t)_n = 3.7 \text{ X } 10^{-13}$ correlation coefficient (r) = -0.946*

$(K_{dI} - K_{dn})^*$: difference in slopes is significant.

$(R_{tI} - R_{tn})^*$: difference in intercept is significant

B. Results of experiment B

(x) = 4-I-PRL as competitor, $K_{dI} = 38.0 \text{ X } 10^{-9}M^*$, $(R_t)_I = 9.2 \text{ X } 10^{-12}$ correlation coefficient (r) = -0.8596*

(o) = n-PRL as competitor, $K_{dn} = 6.7 \text{ X } 10^{-9}$ (n.s.), $(R_t)_n = 1.4 \text{ X } 10^{-12}$ correlation coefficient (r) = -0.7263 (n.s.)

* - values are significantly different from 0 ($p < 0.05$)

n.s. - values are not significantly different from 0

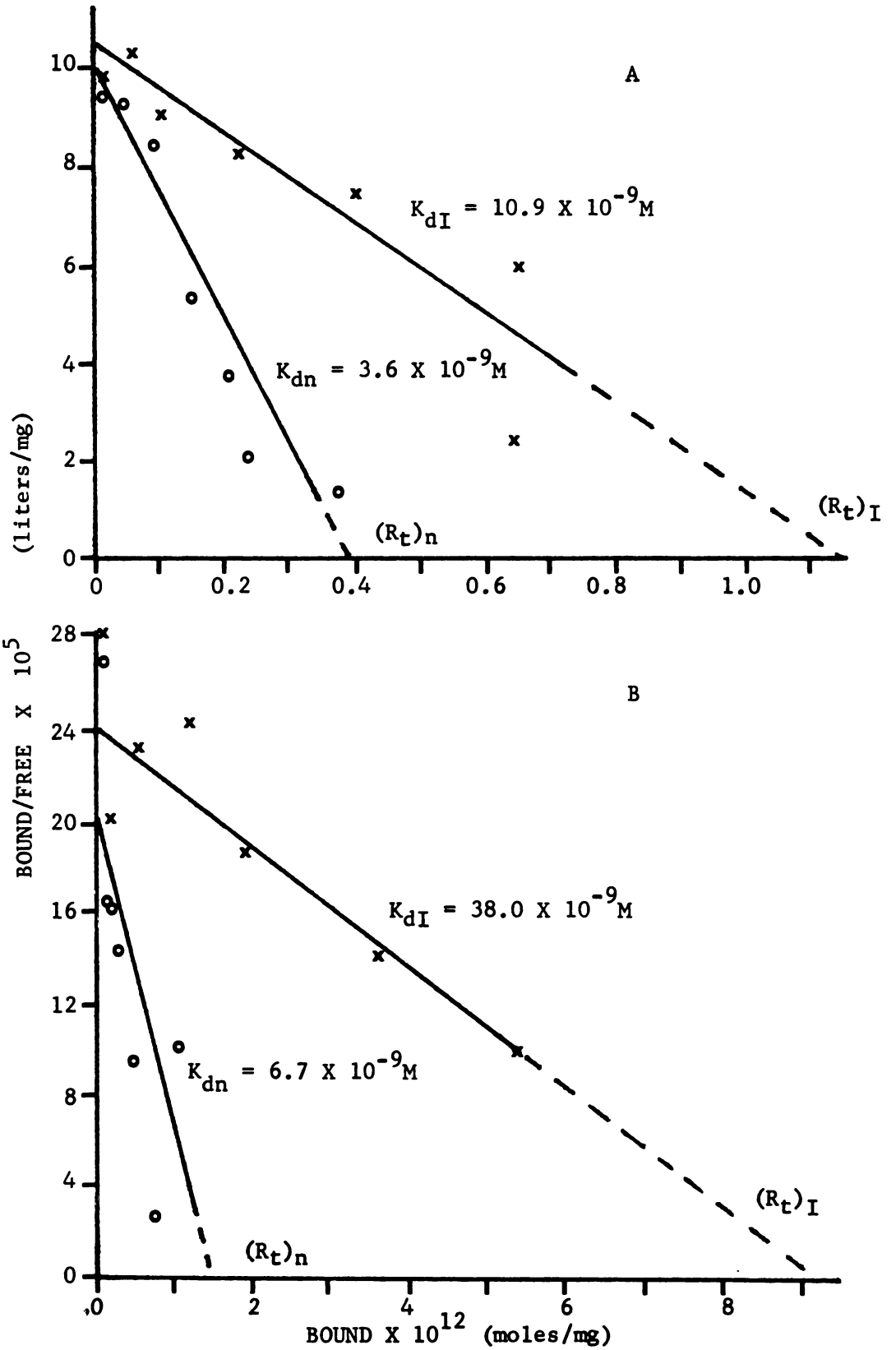


Figure 6
Scatchard Analysis of Prolactin Binding

to react competitively with ^{125}I-PRL and receptors. It appears that n-PRL has a binding affinity between 3 and 5 times greater than that of iodinated hormone when assessed by the Scatchard analysis (see Figure 2). We should point out that between-experiment precision is poor. For instance, compare the results of Experiment A and B. However, this appears to be the case throughout the literature as well (see Literature Review). The estimates of K_d and R_T are unique to the particular experimental conditions which prevail. Since Experiment A and B were performed on separate preparations of liver particles, the observed differences might be expected. On the other hand, consistent relative, "within experiment" effects are observed. That is, native prolactin is a more effective competitor than I-PRL for receptor binding. In short, in the hands of the author, the radioreceptor assay is only efficient at making relative quantitative measurements. The absolute, intrinsic binding parameters of the prolactin receptor remain a mystery.

Logistically, the experiments in this section are broken down into two cases. In one case, we compete ^{125}I-PRL and ^{127}I-PRL. That is, except for the isotopic difference, the system is homogenous. We could assume that the estimates of K_{dI} and $(R_T)_I$ for I-PRL are fairly accurate. In the second case, we compete n-PRL with ^{125}I-PRL and therefore have a mixed or heterogenous system. The resultant values of K_{dn} and $(R_T)_n$ are therefore not reliable.

An analytical solution for the relative binding affinity of n-PRL is possible if we make the simple assumption that iodinated and native hormone bind to the same population of receptors. This certainly must

be true since binding of ^{125}I-PRL is competitive with n-PRL. Thus, we can treat the second case as two simultaneous equilibria such that:

$$\text{n-PRL} + \text{R} \rightleftharpoons \text{n-PRL-R} \quad \text{and} \quad \text{I-PRL} + \text{R} \rightleftharpoons \text{I-PRL-R}$$

and at equilibrium:

$$(1)\ K_{dI} = \frac{(\text{I-PRL})(\text{R})}{(\text{I-PRL-R})} \quad \text{and} \quad K_{dn} = \frac{(\text{n-PRL})(\text{R})}{(\text{n-PRL-R})}$$

but both hormone species compete for the same population of receptors and if we substitute and rearrange we find that:

$$(2)\ K_{dn} = \frac{K_{dI}\ (\text{n-PRL})(\text{I-PRL-R})}{(\text{n-PRL-R})(\text{I-PRL})}$$

The iodinated species in Equation 2 can be measured directly since they are radioactive and K_{dI} can be solved precisely using the Scatchard analysis. Furthermore, we know that (n-PRL) + (n-PRL-R) must equal the total quantity of competitor prolactin provided in the binding medium. If we can produce an expression for either (n-PRL) or (n-PRL-R), then we can solve for both.

$$(3)\ (\text{n-PRL-R} = (R_T) - (\text{R}) - (\text{I-PRL-R})\ \text{(conservation of mass)}$$

and

$$(4)\ (\text{R}) = \frac{K_{dI}\ (\text{I-PRL-R})}{(\text{I-PRL})} \quad \text{(from Equation 1 above)}$$

and (R_T) can be solved precisely using the Scatchard analysis for case 1.

Based on these equations (1 through 4) and the derived constants in the legend of Figure 2, we can arrive at solutions for K_{dn} for each dose of n-PRL. Solutions are provided in Table 4, on the following page,

for doses of n-PRL which fall in the linear range of the dose-response curves.

Table 4. Simultaneous Equilibrium Solutions for K_{dn}

Dose of n-PRL	K_{dn}* Experiment A	K_{dn}* Experiment B
2.5 ng	2.77	----
5.0 ng	4.26	----
10.0 ng	5.21	0.118
25.0 ng	2.66	2.312
50.0 ng	2.88	2.30
100.0 ng	2.74	6.79
250.0 ng	4.05	3.00
500.0 ng	----	0.1439
	3.51 $\pm$ 1.00**	2.44 $\pm$ 2.45**

*All values x 10^{-9} M

**Mean $\pm$ S.D.

The average value of K_{dn} derived for Experiment A is identical to the K_{dn} value obtained by the Scatchard analysis in Figure 2. In fact they can be shown to be mathematically equivalent. The average value for Experiment B has such a large standard deviation associated with it that it does not differ significantly from zero. We note that the Scatchard plot for n-PRL in Experiment B also does not have a significant slope. Both the Scatchard plot and the simultaneous equilibrium model developed above assume a "linearized" dose-response. As a result,

both are sensitive to deviations from linearity; this shows up as an increase in variance in data which do not conform to a linear model. If the objective of the statistical analysis is to make a comparative statement about relative biochemical behavior, one's conclusions should not be encumbered by variance introduced by a mathematical data transformation. In the present case, a significant difference between n-PRL and I-PRL was detected in Experiment B using the factorial analysis of variance, whereas the difference was not significant using the least squares method of linear regression slope analysis. The analysis of variance is therefore the more efficient statistic.

In summary, we can state that the radioreceptor assay appears to be a reasonably sensitive means of assessing relative (but not absolute) binding affinities of various prolactin species. In particular, n-PRL has an apparent equilibrium binding constant (K_d) about 3 to 4 times smaller than that of I-PRL. Only 4-I-PRL was used in this experiment and it remains to be seen whether prolactin species at other levels of iodination have reduced receptor binding activity. Although the Scatchard plot (as used in these experiments) can provide reasonable estimates of equilibrium binding constants, based on a simple model of hormone binding, the Scatchard plot is not amenable to sensitive statistical analysis. The author has found that the factorial analysis of variance is a more valuable statistical tool for analyzing competitive displacement data since it is not effected by deviations from linearity and does not assume a linear dose-response.

II. Analysis of Prolactin Dissociation from Receptors

A. Objectives

If the model of a hormone binding with receptors as indicated below is to be accepted, then two kinetic criteria should be met. The forward rate of reaction

$$(1) \quad H + R \rightleftharpoons HR$$

(association) should follow second order kinetics overall and the reverse rate (dissociation) should follow first order kinetics. The forward reaction rate for prolactin binding was studied by Shiu and Friesen (1974) and found to be in good agreement with second order rate kinetics. That is, the rate of the reaction is a function of receptor concentration and hormone concentration. Furthermore they found that the rate constant increased as the temperature of the reactants increased; from an initial value of $2.6 \times 10^{10}\ M^{-1}\ min^{-1}$ at 23° C to $5.6 \times 10^{10}\ M^{-1}\ min^{-1}$ at 37° C. Dissociation data on the other hand have never been rigorously analyzed. In theory, the dissociation of receptor-hormone complex (HR) into free hormone and receptor in the presence of an infinite sink for H should follow a simple exponential decay such that:

$$(HR) = (HR)_0\ e^{-kt}$$

where $(HR)_0$ designates the concentration of complex at time = 0. The exponential "k" is the rate constant for the reaction. In practice, it is impossible to provide a true infinite dilution for H but this condition can be closely approximated by recording the dissociation of

radiolabelled hormone in the presence of a large excess of native hormone. The precision is improved in the following way: we define "zero percent" bound hormone as that quantity of ^{125}I-PRL which will bind to receptor in the presence of 5 μg of native prolactin. We define "100 percent" bound as the quantity of ^{125}I-PRL which binds in the absence of native hormone minus "zero percent" bound. That is, our base line is adjusted. At any point in time we therefore define "specific binding" as the total quantity of bound hormone minus "zero percent" bound hormone.

Once again we are confronted by the possibility of differential binding of iodinated and native prolactin. It is conceivable that iodinated and native prolactin bind to distinct populations of receptors. If this were true, then dissociation of ^{125}I-PRL in the presence of cold iodinated hormone would approach a different dissociation limit than dissociation in the presence of native hormone (n-PRL). In this section, experiments were designed to study the rate of dissociation of hormone from receptors and to help characterize the binding sites for iodinated and native prolactin. The protocol is to allow dissociation of ^{125}I-PRL from receptors either in the presence of n-PRL or in the presence of "cold iodinated" prolactin.

B. Experimental

Membrane particles (150 μg protein) were incubated in polyethylene tubes with 60,000 CPM's (3.0×10^{-14} moles) of ^{125}I-prolactin. Three tubes initially contained 5 μg of n-PRL. After 3 hrs. of pre-incubation, the tubes were centrifuged at 10,000 xg for 5 minutes. The difference

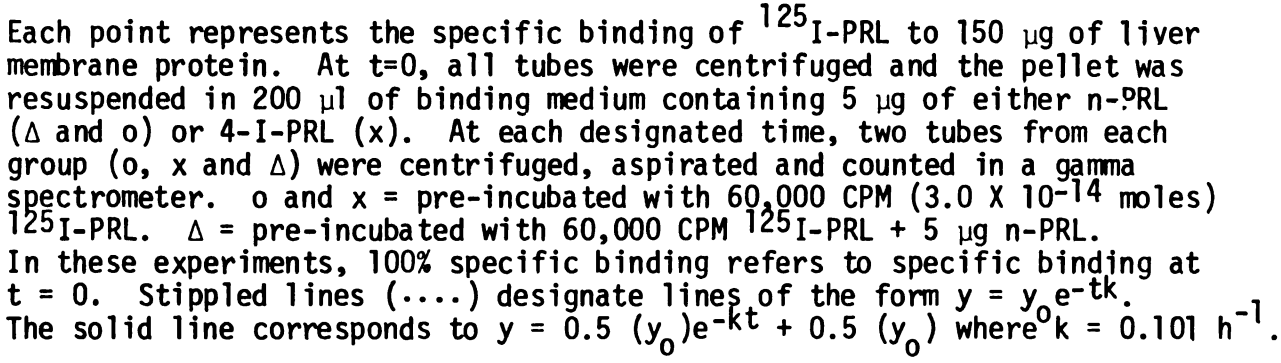
in bound ^{125}I between tubes incubated with and without n-PRL was defined as the "zero time" specific binding. Following a three hour pre-incubation with 60,000 CPM's of ^{125}I-PRL, three series of tubes were centrifuged and then subjected to one of the following treatments: (1) the pellet was resuspended in 5 μg n-PRL or (2) the pellet was resuspended in 5 μg "cold iodinated" prolactin (4-I-PRL) or (3) the pellet was resuspended in its original medium containing ^{125}I. One pair of tubes from each group was then recentrifuged at each of the following times: 15 min, 45 mins, 3.5 h, 8.5 h and 19.5 h. After the final centrifugation, the tips of the tubes were cut off, the pellets were dispersed in 1 ml of water and counted in a gamma spectrometer. The data were then expressed as percent of the "zero time" specific binding.

C. Results

Figure 7 is a graphical representation of the data from the experiment described above. The ordinate is expressed both as percent specific binding and as moles of bound ^{125}I-PRL. The vertical bars indicate $\pm$ S.D. The dashed line represents the control values; all of which did not differ significantly from zero. The significance of this finding is that "non-specific binding" did not increase over the zero time values during the 19.5 hours of the experiment. The (x) symbols refer to the tubes incubated in the presence of excess cold iodinated hormone and the (.) symbols designate the tubes incubated in the presence of n-PRL. The data are generally described by a rapid dissociation during the first 3.5 h and a much slower dissociation during the

Figure 7

Dissociation of ^{125}I-PRL from Receptors



Each point represents the specific binding of ^{125}I-PRL to 150 µg of liver membrane protein. At t=0, all tubes were centrifuged and the pellet was resuspended in 200 µl of binding medium containing 5 µg of either n-PRL (Δ and o) or 4-I-PRL (x). At each designated time, two tubes from each group (o, x and Δ) were centrifuged, aspirated and counted in a gamma spectrometer. o and x = pre-incubated with 60,000 CPM (3.0×10^{-14} moles) ^{125}I-PRL. Δ = pre-incubated with 60,000 CPM ^{125}I-PRL + 5 µg n-PRL. In these experiments, 100% specific binding refers to specific binding at t = 0. Stippled lines (....) designate lines of the form $y = y_o e^{-tk}$. The solid line corresponds to $y = 0.5\ (y_o)e^{-kt} + 0.5\ (y_o)$ where $k = 0.101\ h^{-1}$.

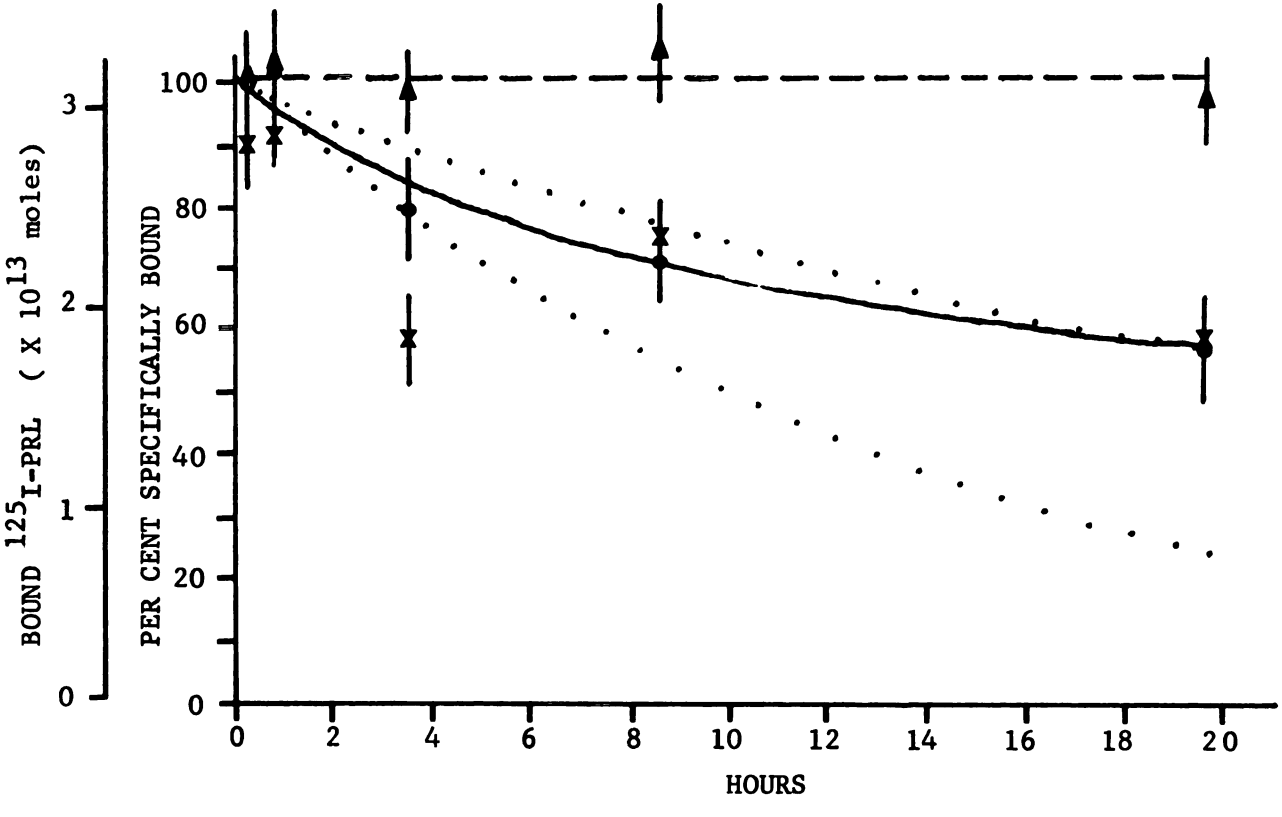


Figure 7
Dissociation of ^{125}I-PRL From Receptors

subsequent 16 h. It should be noted that after 19.5 h at 23° C, about 60% of the hormone remains bound to receptors and that both sets of data appear to be approaching the same limit. The two sets of data also overlap at all time periods except at 3.5 h; the significance of this is not understood.

Although the smooth line which passes through the data is a simple exponential, it appears that as much as 50% of the "bound" ^{125}I-PRL does not dissociate from receptors. In other words, the bound hormone seems to be differentiated into a dissociating and a non-dissociating component. If all hormone were dissociated according to the same kinetics, then we would expect the data to conform to curves such as the stippled lines in Figure 7. There is also evidence, at least in the case of the tubes incubated with cold iodinated prolactin, that the rapid, early phase of dissociation is somewhat more variable. Temperature or other experimental effectors could be responsible for these results or possibly subtle complexities of the dissociation may be operative. Any conclusions should await further experimentation.

D. Discussion

The dissociation of ^{125}I-PRL from liver membrane receptors was studied using competitive displacement of bound prolactin with n-PRL or cold iodinated prolactin. The results generated by dissociation in the presence of native prolactin can be fitted to a smooth continuous curve of the form $(PRL\text{-}R) = (0.5))PRL\text{-}R)_0 e^{-kt} + (0.5))PRL\text{-}R)_0$. In the presence of cold iodinated prolactin, the results are somewhat more erratic and difficult to interpret. Clearly the early stages of dissociation

are more variable than the later stages.

Both sets of data appear to converge on the same limit as time increases above eight hours. This suggests that iodinated and native prolactin compete for the same population of receptors because if iodinated hormone were bound to a different population of receptors, then incubation in the presence of cold iodinated hormone should produce more dissociation than incubation in the presence of n-PRL. There is no evidence for heterogeneity of receptors.

It is curious that the data are "fit" best to a curve other than a simple exponential decay. Since there is considerable variation and only five points on the graph, no conclusive statements can be made. However, there is a suggestion that as much as 50% of the specific binding is non-dissociable or at least dissociates very much more slowly than the first 50%. One cannot argue that rebinding of the ^{125}I-PRL in the incubation tube is significant because non-specific binding is subtracted from each tube at each time period. One would also find it difficult to argue in favor of two populations of binding sites for prolactin because the forward reaction rate studies performed by Shiu and Friesen (1974) gave no indication of receptor heterogeneity. Furthermore, equilibrium studies produce linear Scatchard plots for prolactin.

To investigate the nature of the differentially dissociating component (or non-dissociating component), I have used the data of Shiu and Friesen for prolactin dissociation from rabbit mammary membrane particles (Shiu and Friesen, 1974) and replotted them along with the data from Figure 7 on semi-log paper. In Shiu and Friesen's study,

^{125}I-PRL was allowed to bind to membrane particles for six hours at 23° C. The dissociation experiments were then performed at 4°, 23°, and 37° C essentially as described earlier in this section. The corresponding results are indicated in Figure 8. Note that practically no dissociation occurs during a 24 h period at 4° C. Furthermore, at 23° C the results for rat liver and rabbit mammary are practically identical. Both seem to cease dissociation beyond 50%. In contrast, at 37° C dissociation continues apparently toward "0" but at a slower rate. In no case (except at 4°) are linear plots obtained, suggesting that something other than a single step dissociation is occurring.

In order to describe the results in Figure 8 in a more meaningful, quantitative way, Q_{10} values were calculated using the rabbit mammary data. Q_{10} is the ratio of reaction rates per 10° C rise in temperature. This value is an indicator of the temperature sensitivity of a given reaction. Since the dissociation reaction seems to have two components, it is of interest to see if the two phases of dissociation respond equally to changes in temperature. The rates of reaction were measured during two intervals during the dissociation experiment at 23° and 37° by estimating the slopes of the lines in Figure 8. The first interval is between t=0 and t=5h. The second interval falls between t=10 and t=24h. In order to normalize the results to a common scale, the units of the estimated rate constants are expressed as percent^{-1} h^{-1}. In the first interval we calculate an average Q_{10} value of 2.5 and in the second interval a Q_{10} value of 5.6 (see Table 5, page 107). That is, the second component of dissociation is more than twice as sensitive to temperature change as the first component.

Figure 8

Graphical Anal-sis of ^{125}I-PRL Dissociation

The dissociation data of ^{125}I-PRL from rabbit mammary gland (Shiu and Friesen, 1974a) as well as the dissociation of ^{125}I-PRL from rat liver (replotted from figure 7) are plotted on a semi-log graph. Lines were visually fitted to the data at each temperature. Note that rabbit mammary (X) and rat liver (o) data are both used for the 23° determinations. Since the data are not well described by single straight lines, initial (0-5 hours) and secondary (10-24 hours) slopes were estimated. Based on the assumption that secondary slopes represent a distinct dissociating component, the initial concentration of the second component is estimated by extrapolation to t = 0 (dotted lines)

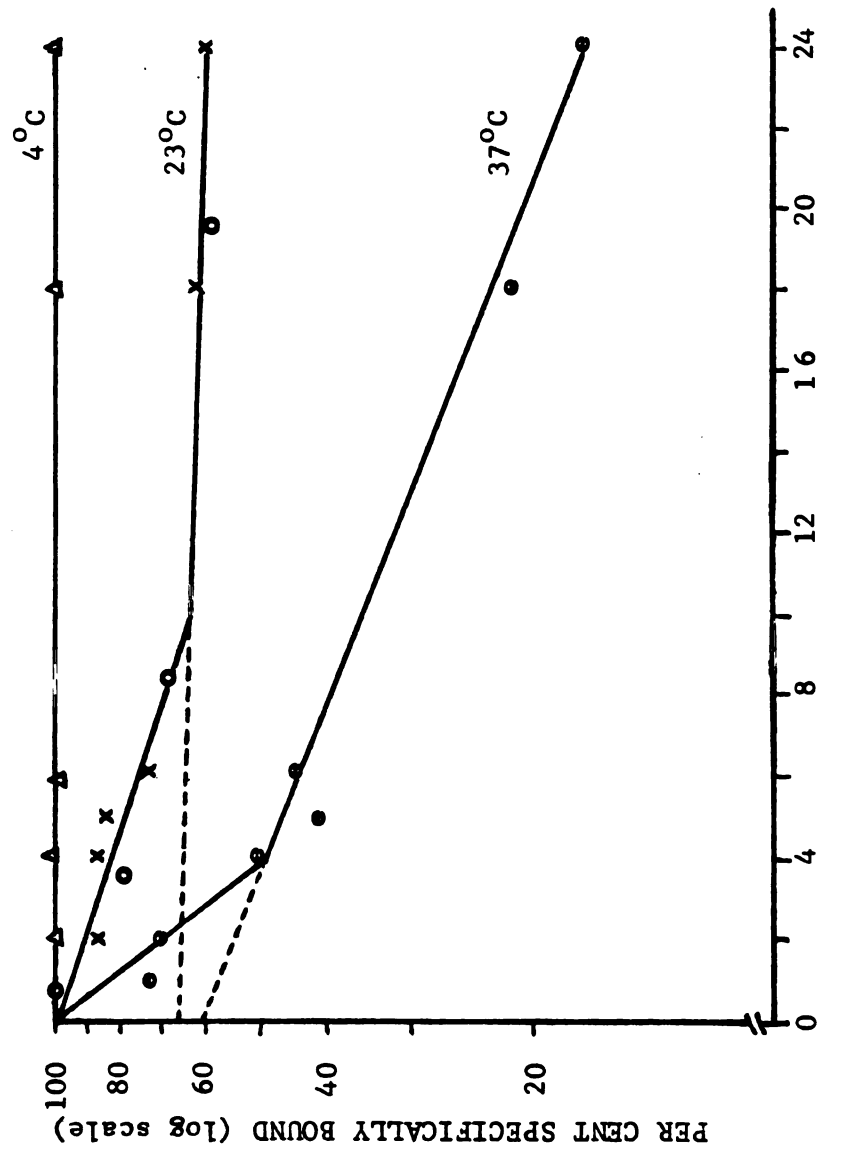


Figure 8
Graphical Analysis of ^{125}I-PRL Dissociation

Table 5. Estimates of Temperature Dependent Rate Constants

First Interval (0-5)		Second Interval (10-24)	
Temp.	Rate (k)	Temp.	Rate (k)
23°C	0.0446	23°C	0.0046
37°C	0.1597	37°C	0.0516
$Q_{10} = 2.5$		$Q_{10} = 5.6$	

where $Q_{10} = \frac{(k_1)}{(k_2)}^{10/t_1-t_2}$

From these calculations we see that the temperature sensitivity of the dissociation reaction can be divided into two time-dependent components. It seems extremely unlikely that such a phenomenon could be due to the action of a single step dissociation. The simplest model which could accommodate these data and still be consistent with second order forward reaction rates is illustrated below:

Step I Step II

$$(2)\ PRL + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PRL\text{-}R \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PRL\text{-}R'$$

where PRL = prolactin, R = receptor, PRL-R = primary complex and PRL-R' = secondary complex.

In this model, PRL-R' is simply the PRL-R complex following some energetic change such as a conformational change in the receptor protein or translocation of the receptor in the membrane. PRL-R' is separated

from PRL-R thermodynamically by the energy barrier $\Delta G = RT \ln(\frac{k_2}{k_{-2}})$, but may appear identical to PRL-R by current physical measurement techniques. It is proposed that the formation of PRL-R occurs on or in the membrane. In other words, the initial step of the hormone action is the binding to the membrane receptor. This is followed by an energetically favored conformational change which is a reversible reaction.

In essence, the binding process is represented by two sequential reversible processes both of which are temperature sensitive and each having characteristic rate constants. During dissociation, the first step (I) is a more rapid, less temperature dependent step and appears to predominate during the first 5 or 6 h of dissociation. After this initial stage, the second step (II) becomes rate limiting and the dissociation proceeds at a slower rate. The overall rates of dissociation will therefore depend on two variables: (1) the intrinsic rate constants of each step (k_{-1}, k_2, k_{-2}) and (2) the initial concentrations of PRL-R and PRL-R'. If we watch the dissociation of ^{125}I-PRL from receptors in the presence of excess native hormone, the only limiting factor for the first step of the reaction is k_{-1} (the intrinsic rate constant). That is, we assume that PRL-R $\longrightarrow$ PRL + R is essentially unidirectional due to the conditions of the experiment. As the concentration of PRL-R approaches zero, k_{-2} becomes the rate limiting step and the rate of dissociation slows. The model therefore requires that $k_{-2} < k_{-1}$ at all temperatures greater than 4° C; otherwise we could not detect two reaction rates. At 4° C, k_{-1} becomes essentially "0" and no dissociation occurs.

The differential expressions for the rates of the dissociation reaction can be written as:

$$(3)\ \frac{d(PRL\text{-}R')}{dt} = -k_{-2}\ (PRL\text{-}R') + k_2\ (PRL\text{-}R)$$

$$(4)\ \frac{d(PRL\text{-}R)}{dt} = -k_{-1}\ (PRL\text{-}R) = k_2\ (PRL\text{-}R) + k_{-2}\ (PRL\text{-}R')$$

It should be obvious that the two pools of receptors feed into each other and at any point in time the total quantity of bound receptor = (PRL-R) + (PRL-R') = ($PRL\text{-}R_T$). On theoretical grounds, the model is well-founded but in practice one cannot determine the relative concentrations of PRL-R and PRL-R' by normal physical measurements. However, if we return to the experimental data, we may be able to make approximate extrapolations to "0" time concentrations of PRL-R and PRL-R'. In Figure 8 the slopes of the "second interval" lines have been extrapolated back to t=0 and we find that the initial pool of PRL-R' appears to be about 60-65% of the total initial quantity. In each experiment depicted in Figure 8 the tubes of bound receptor were equilibrated at 23° C prior to initiation of dissociation. It is therefore reasonable to assume equivalent initial conditions for each experiment. If we take the equilibrium conditions to be 65% PRL-R' and 35% PRL-R then according to the model, $\frac{k_2}{k_{-2}}$ must equal 0.65/0.35 = 1.86. We now have an expression for k_2 in terms of k_{-2} such that $k_2 = (1.86)\ k_{-2}$. In principle we should be able to integrate equations (3) and (4), use the rate constants in Table 5 as first approximations of k_{-1} and k_{-2} and generate curves which are at least qualitatively like those in Figure 8. The author recognizes that the rates obtained from Figure 8 during the

two specified intervals are not strictly analagous to k_{-1} and k_{-2} but once again they provide a reasonable starting place for modelling.

In order to perform a simultaneous integration of the rate equation, LaPlace transforms and matrix algebra are employed (Simon, 1972). If we include the additional boundary conditions described above, the following equations result:

$$(5)\ \frac{(PRL\text{-}R) + (PRL\text{-}R')}{(PRL\text{-}R_T)} = \theta\, e^{-\lambda_1 t} + (1-\theta)\, e^{-\lambda_2 t}$$

$$\text{where } \theta = \frac{(PRL\text{-}R')_o\,(0.86\,k_{-2} + k_{-1} - \lambda_1) - (PRL\text{-}R)_o\,(0.86\,k_{-2} + \lambda_1)}{2\,\gamma\,(PRL\text{-}R_T)_o}$$

$$\gamma = \tfrac{1}{2}\,(2.86\,k_{-2} + k_{-1})^2 - 4\,k_{-2}k_{-1}$$

$$\lambda_1 = \frac{(2.86\,k_{-2} + k_{-1})}{2} - \gamma$$

$$\lambda_2 = \frac{(2.86\,k_{-2} + k_{-1})}{2} + \gamma$$

For a complete derivation as well as a generalized equation for any values of k_{-1}, k_{-2}, k_2, $(PRL\text{-}R)_o$ and $(PRL\text{-}R')_o$ see Appendix A. Although the exponents and coefficients are complicated functions of the two rate constants, the form of equation 5 is surprisingly simple.
We should note that the form of the equation is also the same as we would find with two distinct populations of receptors. We therefore cannot establish the model on the grounds of dissociation data alone. However, if two distinct populations of receptors were operative then both the forward rate reactions and the equilibrium reactions would provide some suggestion of their existence. This has not been the case in the literature for ovine prolactin. Although it is not obvious,

equation 5 will assume the form of $Y = (0.5)(PRL\text{-}R_T)e^{-kt} + (0.5)(PRL\text{-}R_T)$ if k_{-2} is taken as 0 and we start with equal quantities of PRL-R and PRL-R'. This would be in good agreement with the original curve fitting.

If we now use equation 5 and insert the values of k_{-1} and k_{-2} from Table 5, the graphs in Figure 9 are generated. The data from Figure 8 have also been replotted in Figure 9 for your reference (note that Figure 9 is a linear scale). In light of the approximate method used for obtaining the rate constants, a remarkably good fit results. It is a reasonable conclusion that the assumption of initial equilibrium conditions is valid. With computer modelling, an exact fit should be within easy reach.

If this model is accepted, some interesting consequences must also be considered. Since k_2 is taken as 1.86 x k_{-2}, then the forward rate of PRL-R $\longrightarrow$ PRL-R' must be very slow relative to k_1. This should be expected though because if PRL-R $\longrightarrow$ PRL-R' were rapid, then almost all bound hormone would be in the form of PRL-R'. This is clearly not the case at equilibrium. Furthermore, we would expect molecular transitions in a relatively viscous environment, such as the lipid membrane, to occur more slowly than in aqueous solution.

The model proposed above would fit well with other observations concerning membrane proteins. For example, the temperature sensitivity of the second component (PRL-R' $\longrightarrow$ PRL-R) suggests that the membrane fluidity may be the major controlling factor. Glasser et al. (1970) used proton magnetic resonance spectroscopy and circular dichroism (CD) to measure temperature dependent effects of membrane lipid-protein interactions in erythrocytes. At 18° C they found that methylene

Figure 9

Simulation of ^{125}I-PRL Dissociation

Using the two-step model and the rate constants from Table 5, the solid lines were generated by repetitive solution of equation 5 (see text and Appendix A). It was assumed that the receptors and hormone were initially (t = 0) at equilibrium, that the equilibrium concentrations of HR' and HR were 65% and 35% respectively and that the dissociation of hormone and receptor was irreversible. Experimental data for ^{125}I-PRL dissociation from rabbit mammary tissue (Shiu and Friesen, 1974a) is also provided.

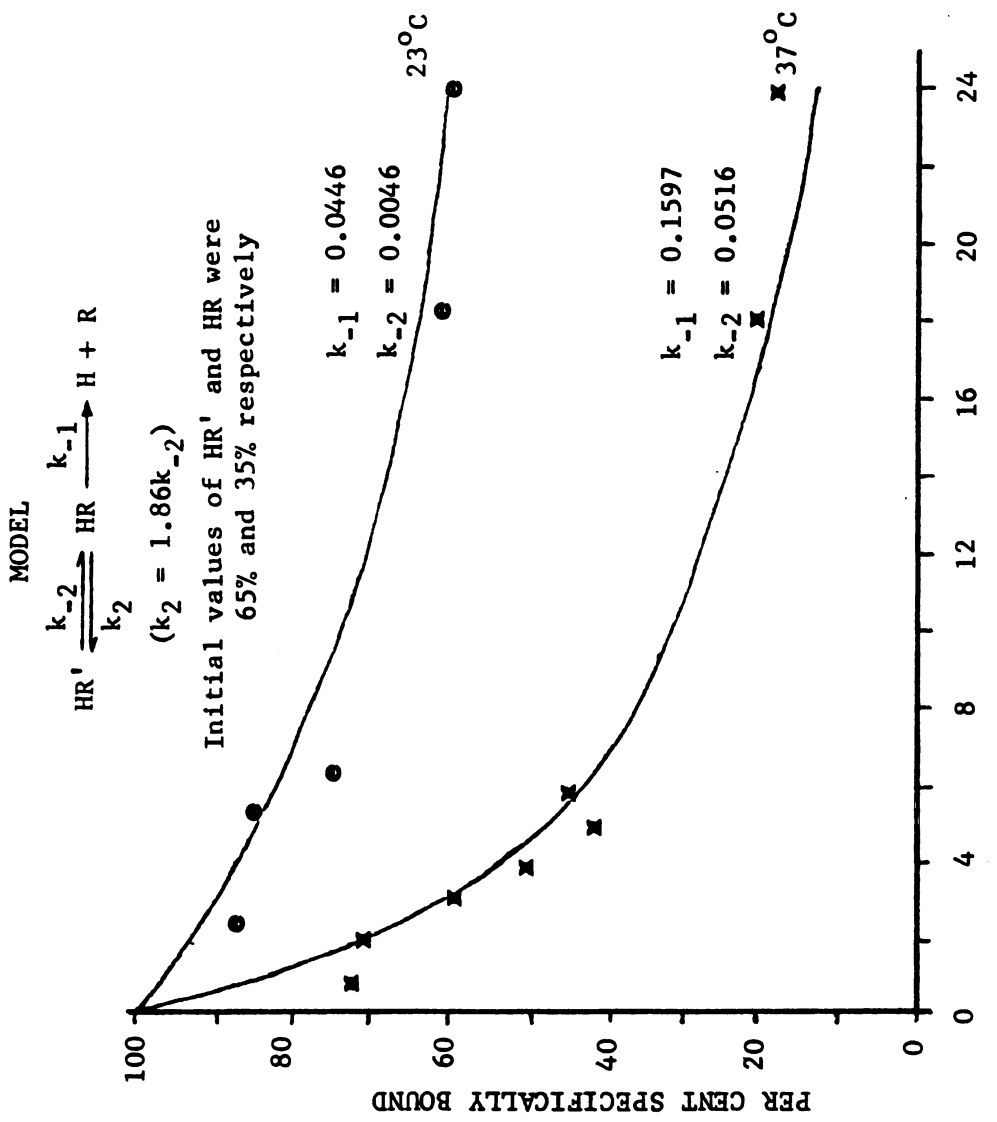


Figure 9
Simulation of ^{125}I-PRL Dissociation

resonance from membrane phospholipids was very slight, suggesting restricted lipid movement. At 40° C however, the lipid is much more fluid (increased methylene resonance) and a shift in CD is observed suggesting a change in the average α-helical content of the membrane proteins. Similarly, the well-documented "capping" phenomenon in lymphocytes is temperature dependent and can be stopped or severely retarded by cooling cells below 20° C (Raff, 1976). The relationship of the membrane proteins and lipids is certainly dynamic and involves biologically important changes. Dansyl galactoside, for instance, becomes more fluorescent as it is "carried" through the membrane of E. Coli cells. This fluorescence change is believed to be related to conformational changes in the β-galactoside carrier protein (Lee, 1978). It is also known that membrane phospholipids modulate the biological effects of many hormones including insulin (Cuatrecasas, 1971), TSH (Kohn, 1978) and prolactin (Rillema, 1977). Phospholipases can modify the membrane response to different hormonal effectors and the addition of specific phospholipids to solubilized adenylate cyclase preparations can restore their hormonal responsiveness. The two step model and the temperature sensitivity of the prolactin receptor response would therefore complement previously reported findings concerning the intimate relationship of the hormone receptor with the membrane. The PRL-R' complex also provides physical evidence for a role of the hormone after the initial binding condition. Supportive evidence is now appearing in the literature for a two compartmental model of prolactin binding. Posner's group has found that prolactin binding to liver results in the rapid uptake (endocytosis) of the prolactin-receptor complex

(Walsh et al., 1978; Josefsberg et al., 1979) into the cytoplasm of liver cells in vivo. If this process is in fact operative then we would essentially have two populations of receptors; those receptors on the cell surface and those associated with intracellular organelles. In a membrane preparation which includes both plasma membrane and microsomal components then we would anticipate two populations which are interconvertible. This concept fits our model very well. Whatever the functional significance of the two-step model, it seems to be a more precise quantitative treatment of prolactin binding studies.

III. An Electronic Analog Model of Hormone Dissociation from Receptors

A. Objectives and Theory

The study of prolactin binding and dissociation from receptors can involve difficult quantitative and analytical methods as suggested in the preceding section of this dissertation. If the two step model proposed here is accepted, then mathematical calculations become much more cumbersome. We even have reason to believe that a three or four-step model may be necessary in order to account for cytoplasmic phenomena as reported by Josefsberg et al. (1979). A mathematical treatment of multicompartmental models can become extremely complex and for many scientists prohibitive. Although in recent years digital computers are more and more accessible, they require knowledge of programming and often time-sharing.

An alternative to digital computations for data analysis is the use of analog devices. They often offer the advantage of lower expense and may provide a useful conceptual basis for understanding the dynamics and complexity of a model system. Electronic analog computers can be built in the laboratory and used "on line" for rapid data analyses without the inconvenience of "digitalization" of imperical results.

Analog computers also offer the potential for "hands on" simulation and hypothesis testing. Therefore they can be valuable teaching tools. In the case of hormone-receptor experiments, the technology and use of radioisotopes makes experimentation inaccessible to practically all undergraduate and many beginning graduate students. An analog computer which could simulate hormone-receptor interactions would afford many students the opportunity to investigate an actively growing field in physiology and biochemistry.

In this section of the dissertation, the theory and testing of an electronic analog circuit for the binding of prolactin is presented. The actual test circuit is designed to model the dissociation data presented in the preceding section of this manuscript. Variations and possible developments are also suggested. The principle component in the circuit is the capacitor which is a charge storage unit, the analog being that receptors are capable of momentarily "storing" hormone. The rate of discharge of a capacitor can be regulated by resistors and thus resistors are analogous to the kinetic rate constants of hormone binding. The theory is developed below.

Suppose that we have a parallel plate capacitor with one plate connected directly to ground and the other plate initially has q coulombs of charge. Now, we allow the capacitor to discharge through resistance R_1 to ground. At any point in time, the charge on the capacitor may be expressed as:

$$(1) \quad q = q_0 \, e^{-t/R_1C_1}$$

where R_1 is resistance in Ohms, C_1 is the capacitance of the capacitor (in farads), and t is the length of time in seconds during which the capacitor is allowed to discharge through R_1. In short, we have a simple exponential equation which is dependent on: (a) the initial charge, (b) the time constant of the circuit (R_1C_1), and (c) the length of time that the capacitor is allowed to discharge (t). If we make the substitution $\frac{1}{R_1C_1} = k$, then equation (1) becomes identical to the expression for the simple model of a hormone and receptor dissociation. The charge q is analogous to the instantaneous concentration of bound hormone (HR) and $k = \frac{1}{R_1C_1}$ is analogous to the intrinsic rate constant for dissociation of bound complex into free hormone and receptor. Note, that in order for the analogy to hold we must assume an infinite sink for hormone. That is, there is no possibility of the reverse reaction (no reverse potential for charging the capacitor). We have just described an analog circuit which could be used to model the dissociation of bound hormone into free hormone and receptor assuming the following:

$$(2) \quad HR \xrightarrow{k_{-1}} H + R \qquad \text{(infinite sink for H)}$$

The voltage difference across the two capacitor plates would be directly proportional to the charge on the capacitor since

$$(3)\quad V_C = \frac{q}{C_1}$$

where V_C = voltage drop across capacitor,

q = charge in coulombs, and

C = capacitance in farads.

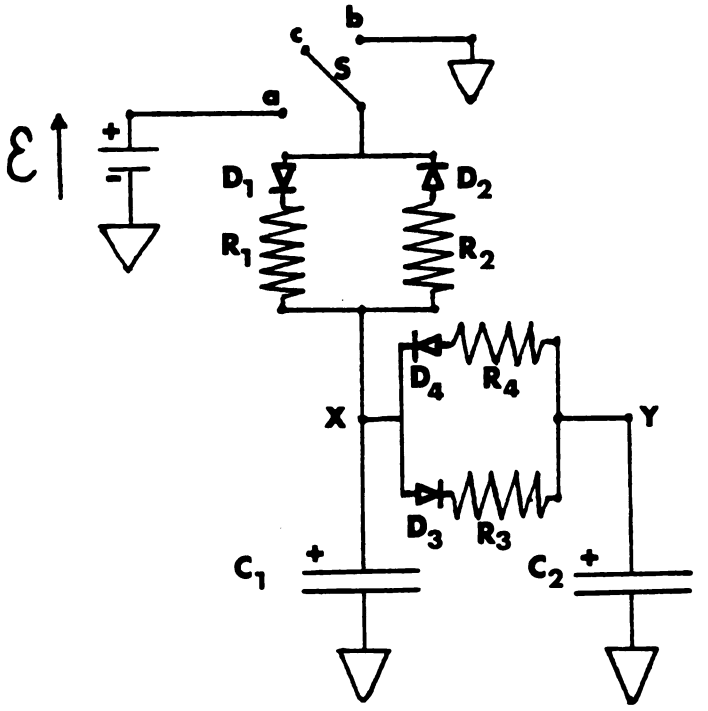
Thus, we need only measure the voltage at any point in time and multiply this value by the capacitance to obtain the charge remaining on the capacitor. If we select values of R, and C, such that $\frac{1}{R_1C_1} = k_{-1}$ (from equation 2 above) then both the capacitative discharge and the hormone dissociation would occur at the same rate.

If we differentiate equation (1) with respect to time, then we obtain the following rate expression

$$(4)\quad \frac{dq}{dt} = (q)\frac{1}{R_1C_1}$$

but $\frac{1}{R_1C_1}$ is equal to k and thus we obtain an expression analogous to the one step hormone-receptor dissociation expression, $\frac{d(HR)}{dt} = -k(HR)$. Furthermore, $\frac{dq}{dt}$ is simply equal to i, the current through the resistor R_1. Thus, the rate of hormone dissociation is directly analogous to the current through R_1.

Now consider the circuit in Figure 10. All symbols are standard electronic component symbols as found in Malmstadt et al. (1973). If the switch (S) is placed in position a, then capacitors C_1 and C_2 will charge through resistances R_1 and R_3 respectively until the



R_1 through R_4 = resistors

D_1 through D_4 = diodes

$\mathcal{E}$ an external electromotive force

S = three pole switch where a=charging, b=discharging, c=standby

C_1 and C_2 = electrolytic capacitors

Figure 10
Analog Circuit of Prolactin Dissociation From Receptors

charge on the capacitors are in equilibrium with the applied electromotive force (EMF). If the switch in Figure 10 is now moved to position b, then C_1 and C_2 may discharge through resistances R_2 and R_4. Note however, that the charge on C_2 continues to be augmented through the circuit D_3R_3 and the charge on C_1 continues to be augmented through D_4R_4 so long as there is charge on the capacitors. When the switch is in position b, the capacitors are connected to ground and there will be no current through R_1D_1. The instantaneous expressions for the rates of change in charge at C_1 and C_2 can be written as follows:

$$(5)\quad \frac{dq_1}{dt} = -(q_1)\frac{1}{C_1R_2} - (q_1)\frac{1}{C_1R_3} + (q_2)\frac{1}{C_2R_4}$$

and

$$(6)\quad \frac{dq_2}{dt} = -(q_2)\frac{1}{C_2R_4} + (q_1)\frac{1}{C_1R_3}$$

If we make the following substitutions: $\frac{1}{C_2R_3} = k_{-1}$, $\frac{1}{C_1R_3} = k_2$, $\frac{1}{C_2R_4} = k_{-2}$, $q_1 = (HR)$ and $q_2 = (HR')$ then equations (5) and (6) become:

$$(7)\quad \frac{d(HR)}{dt} = -k_{-1}(HR) - k_2(HR) + k_{-2}(HR)$$

and

$$(8)\quad \frac{d(HR')}{dt} = -k_{-2}(HR') + k_2(HR)$$

The latter form of the equations are identical to the expressions derived for the two-step dissociation model in the preceding section of this dissertation. The integrated forms of the equations have already been provided (see Appendix A).

It should be obvious that by choosing appropriate values of C_1, C_2, R_2, R_3 and R_4 that the behavior of hormone receptors can be modeled using electronic components as analogs. Referring to Figure 10, if we monitor the voltage at points x and y and multiply each voltage by C_1 and C_2 respectively, then we would obtain the respective values of q_1 and q_2. These, of course, are analogs to (HR) and (HR'). By summing q_1 and q_2 we obtain the analog of (HR_t), the total quantity of hormone bound to receptor at any point in time. Mathematically stated:

$$q_t = (V_1)(C_1) + (V_2)(C_2)$$
$$= q_1 + q_2$$

where V_1 and V_2 are the voltages measured at capacitors C_1 and C_2 at any point in time t and q_t is the total charge on the two capacitors. In practice, we do not need to know the actual charge in coulombs and can therefore normalize equation (9) by dividing through by the quantity $(C_1 + C_2)$ yielding:

$$(10) \quad V_t = \frac{q_t}{(C_1 + C_2)} = (V_1)\frac{(C_1)}{(C_1 + C_2)} + (V_2)\frac{(C_2)}{(C_1 + C_2)}$$

In an actual circuit, the values of C_1 and C_2 would be known and the coefficients in equation (10) would be constant fractions.

Before continuing to the experimental section, an important property of the circuit in Figure 10 should be pointed out. Imagine that capacitors C_1 and C_2 are allowed to charge for a period of time until:

$$(11) \quad \varepsilon = \frac{q_1}{C_1} = \frac{q_2}{C_2}$$

When the conditions in equation (11) occur, electrical equilibrium is obtained and there is no net current through the circuit. The electric potential (V) at any point in the circuit is therefore analogous to the Gibbs Free Energy (G) of the hormone system. That is, when $\Delta V = 0$, the circuit is at electrical equilibrium just as when $\Delta G = 0$, the hormone binding is at chemical equilibrium (the thermodynamics, though somewhat cumbersome, can be shown to be identical: see Daniels and Alberty, 1975). Now, if for example we wish to begin an experiment with 65% of the charge on C_2 and 35% on C_1, then we could select capacitance values of say 65 μf and 35 μf respectively. Assuming that we start our experiment at equilibrium ($V_1 = V_2$), then $k_2 = \frac{1}{(35\times10^{-6})(R_3)}$ and $k_{-2} = \frac{1}{(65\times10^{-6})(R_4)}$. However, in order for our analogy with the hormone system to hold, we also require that $K_d = \frac{q_1}{q_2} = \frac{k_{-1}}{k_2} = 1.86$. This will only be true if $R_3 = R_4$. By choosing equal values of R_3 and R_4, we do not limit our possibilities for experimentation though, since the capacitances of C_1 and C_2 may be varied at will.

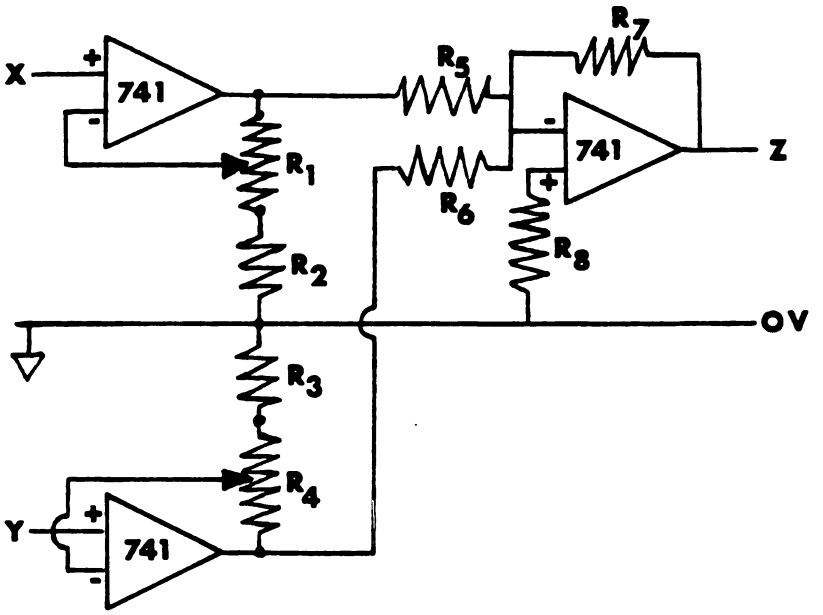
B. Experimental

In this section, introductory experiments were conducted using the circuit described in Figure 10. Only "discharge" experiments were undertaken in order to illustrate the capabilities of this analog device. All component values in these experiments were chosen in order to simulate prolactin dissociation data. Of particular interest is the choice of capacitance values. In these experiments the ratio of C_1:C_2 was chosen as 35:65 in order to model the initial concentrations of PRL-R and PRL-R' which we determined in the preceding section. In the

experiments which follow, a circuit identical to that in Figure 10 was constructed using commercially available resistors (5% tolerance), capacitors (5% tolerance) and diodes (general purpose silicon type). For the purpose of these experiments R_1 was eliminated ($R_1 = 0$) and R_2 was replaced by a 0-500 KΩ potentiometer. R_3 and R_4 were made variable by incorporating 0 to 10 MΩ decade resistance boxes into the circuit. Throughout the experiments reported here, C_1 was 70 μf and C_2 was 130 μf. The voltages at points X and Y were monitored using DC voltage amplifiers constructed from type 741 operational amplifiers (see Materials and Methods). The gain in the DC amp at X was set at unity (1) and the gain of the amp at Y was set at 1.86. The output of these amplifiers was summed at the input of a third (unity gain) amplifier whose output was fed to a chart recorder. The amplifier gains and summations were designed such that the readout on the chart recorder was directly proportional to the total charge on the two capacitors. The voltage monitoring circuit is schematically diagrammed in Figure 11. For each experiment, the capacitors were charged to 5.9 volts (total readout = 16.9 V). Resistance values were then imposed and the circuit allowed to equilibrate. The switch (S in Figure 10) was then moved to position C (standby) and the chart recorder was started (paper speed = 6 inches/min). At time t=0, the switch was moved to the b position and the changes in voltage (charge) were recorded.

C. Results

The circuit outlined in Figure 11 proved to effectively isolate the experimental circuit from the recording equipment since no change



R_1 - R_8 = Resistors

741 = type 741 operational amplifiers

X and Y refer to the respective points in figure

Z and OV are outputs to chart recorder

Figure 11
Voltage Following Circuit

in voltage could be detected during a 5 minute control experiment when the circuit was fully charged and the switch was in the standby position. This would be expected since the DC amplifiers as shown, should have input impediments of about 400 MΩ (manufacturer's specifications).

The results of six "discharge" experiments are presented in Figure 12, and the corresponding k values can be found in Table 6. The values of k correspond to those in equations (7) and (8) as derived above. In order to perform the experiments during a convenient time period, the abscissa of the graph was adjusted by an arbitrary factor of 0.7936 hours per second. Similarly, the k values ($\frac{1}{RC}$) were adjusted by a factor of $e^{1/0.7936}$ = 0.2347 seconds per hour. The actual length of time for each experiment was one minute. The Y axis in Figure 12 is intended to simulate percent specific binding and is mathematically equivalent to equation (10) above.

We see in Table 6 that graphs A-2 and C-2 have k values very similar to the 37° C and 23° C dissociation data presented in the previous section of this dissertation. These graphs also exhibit excellent agreement with the curves generated by digital computations in Figure 9. The graphs in part B of Figure 12 correspond to conditions (and k values) intermediate between A and C. Data such as these might be produced by a hormone dissociation experiment at temperatures intermediate between 23° and 37°.

D. Discussion

In this section, the theory and experimental performance of a simple electronic analog device are presented with respect to modelling

Figure 12
Analog Simulation of Prolactin Dissociation

Each graph is a simulation of data which might be produced in a prolactin-receptor dissociation experiment. The graphs were produced by a chart recorder coupled to a capacitor discharge analog device (see text). The analog computer design was based on a two-step dissociation model and the circuitry is presented in figure 10. The axes are labelled with the analog headings and therefore are inclosed in parentheses. The actual ordinate is 0 to 16.9 volts D.C. and the actual abscissa ranges from 0 to 60 seconds for each experiment. The rate of discharge of the capacitors was regulated by variable resistance components installed in the circuit. The capacitor-resistance time constants as well as the analogous kinetic rate constants for each experiment are given in Table 6.

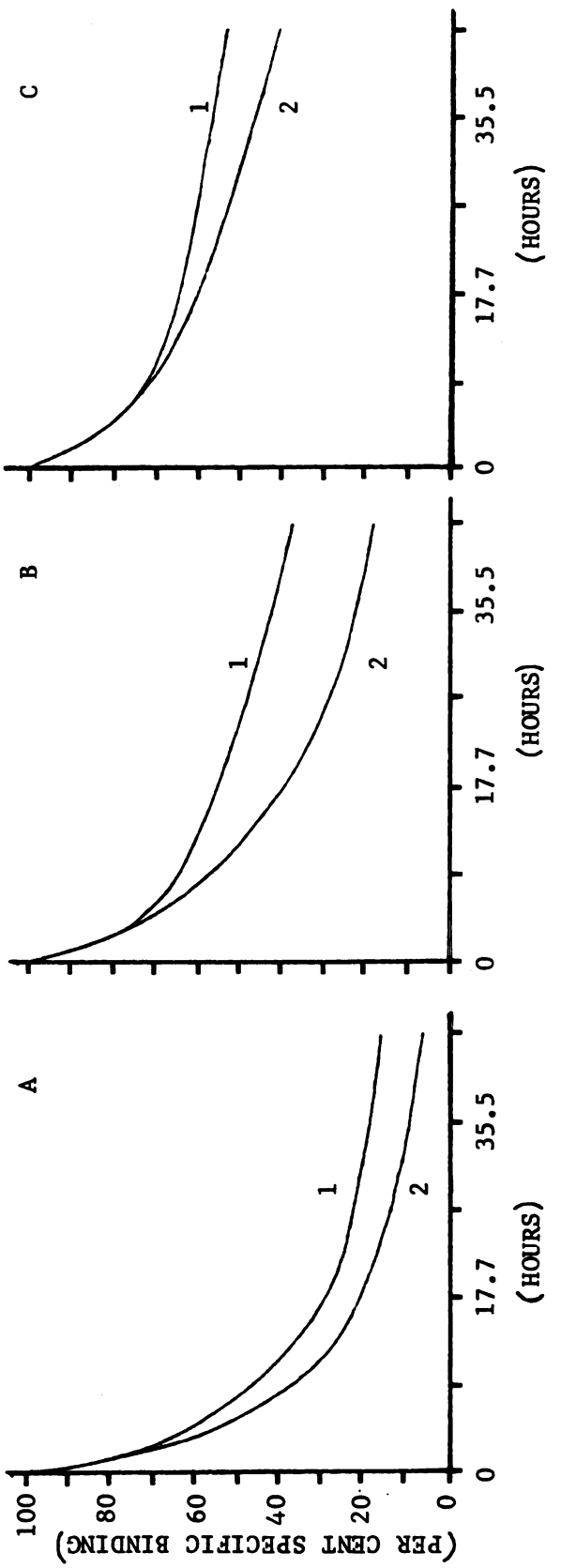


Figure 12

Analog Simulation of Prolactin Dissociation

Table 6. Analog of Hormone Dissociation Rate Constants

		Actual Resistance Values			Analog k Values		
		R_2	R_3 & R_4		k_{-1}	k_2	k_{-2}
A.	1.	21 K	80 K	1.	0.1597	0.0419	0.0225
	2.	21 K	40 K	2.	0.1597	0.0838	0.0451
B.	1.	43 K	400 K	1.	0.0780	0.0084	0.00451
	2.	43 K	100 K	2.	0.0780	0.0335	0.0180
C.	1.	77 K	1 M	1.	0.0435	0.00335	0.00180
	2.	77 K	400 K	2.	0.0435	0.00838	0.0045

All analog k values are based on the relationships $k_1 = \frac{1}{R_2C_1}$, $k_2 = \frac{1}{R_3C_1}$, and $k_{-2} = \frac{1}{R_4C_2}$ where C_1 = 70 μf and C_2 = 130 μf. The k values were then multiplied by 0.2347 s/hr in order to obtain a convenient time frame.

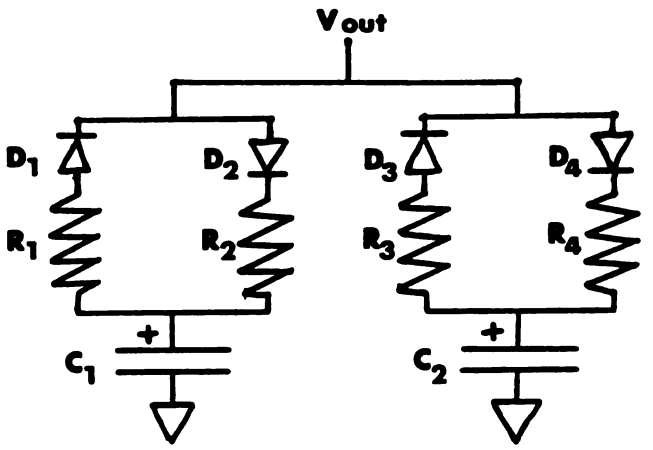
of hormone-receptor interactions. The analog circuit is centered around the charging and discharging of electrolytic capacitors in the presence or absence of an externally applied EMF. Since capacitative discharge through a resistance follows simple exponential decay, the mathematics of the electronic circuit can be shown to parallel the mathematics of hormone-receptor dissociation (see Rodbard, 1973a). The various rate constants of hormone binding are completely analogous to the time constants ($k = \frac{1}{RC}$) of capacitative discharge. The circuit constructed and tested in this section was designed to simulate the dissociation of a two-step hormone binding system and the curves generated using the analog device are in excellent agreement with curves constructed using digital computations.

The analog device described here has two significant potential applications: (1) provides analytical solutions for curves generated by experimental hormone data and (2) provides a valuable teaching tool for the concepts of chemical and/or hormone binding equilibria. In practice, it is a simple matter to plot the hormone dissociation data on a graph and simply vary the resistance values on the analog device until a curve is "fit" to the data. For example, the graphs in Figure 12 of this section were all produced in less than an hour. Once the capacitance and resistance values are known, the values for the analogous rate constants can be solved immediately by simple algebra. The analog circuit possibly could be improved by adding a logarithmic operational amplifier between the chart recorder and the presently described device. This would "linearize" the circuit output and make a two component system easier to identify. Even in its present form, the

analog device is an easier, and certainly less expensive tool to work with than a comparable digital computer. Furthermore, no programming experience or shared-time is required. The mathematics involved with analytical solutions for the two-step hormone dissociation model are prohibitive by hand and thus, the analog device provides a convenient and inexpensive alternative. If present theories are correct about protein hormones such as prolactin entering the cytoplasm (Posner et al., 1979), then two, three or even four compartmental models of hormone-cell interactions would be required for accurate analytical data evaluations. The mathematics of sequential compartmental analyses can be monstrous; the analog circuitry is quite simple.

The circuitry for the analog derive can also be adapted to accommodate other hormone-receptor models. For example, the two receptor population model proposed for insulin by Kahn et al. (1974) could be modeled as in Figure 13. In this case, C_1 and C_2 correspond to two separate populations of insulin receptors while R_1 through R_4 regulate the respective forward and reverse rate constants. The model could be taken one step further using four capacitors to simulate two populations of two-step receptors.

The analog device is especially attractive as a teaching tool. Because the technology of receptor binding studies is so sophisticated and involves the use of radioactive isotopes and chronically prepared animals, the methods are completely inaccessible to most undergraduate or even beginning graduate students. The analog device described here is capable of generating "hands on" data which illustrate not only the the basic principles but also the subtleties of hormone-receptor



R_1 through R_4 = Resistors

D_1 through D_4 = Silicon Diodes

C_1 and C_2 = electrolytic capacitors

Figure 13
Analog Circuit for Dissociation of Insulin from Receptors

interactions. The model therefore provides the opportunity for "actual" data collection and analysis without the expense and danger of using gamma spectrometers and radioisotopes in teaching laboratories.

IV. Biological Assay of Native and Iodinated Prolactin

A. Objectives

In previous sections of this manuscript, iodinated and native prolactin were clearly distinguished by use of the radioreceptor assay. Since iodinated hormone has reduced binding activity relative to native hormone, it is of interest to see if iodinated prolactin retains its biological activity. In this section, a modified pigeon crop sac assay (Frantz and Rillema, 1968) was used to compare the crop stimulating activity of iodinated and native prolactin. This assay method is based on the uptake of ^{14}C amino acids into hormone stimulated crop tissue. The method was selected because hormone quantities required are minimal (only a single injection is necessary) and the methods were readily adapted in our laboratory. Although the method is only semi-quantitative, it is specific to prolactin and gives negative results with non-specific stimuli such as serum albumin injections.

B. Experimental

"Cold iodination" techniques and gel filtration were used to prepare three distinct prolactin solutions: 4-I-PRL, 1-I-PRL and n-PRL as defined previously. Each of these solutions was chromatographed on Sephadex G 100 in potassium phosphate buffer (pH 7.4); 1 ml aliquots

were collected. Only fractions with a K_{av} between 0.35 and 0.55 were used for further experimentation. After chromatography, the concentrations of each hormone preparation were determined using the tannin reagent method (see Materials and Methods). Three dilutions of each stock solution were then made to yield 2.13, 4.25 or 8.5 µg/200 µl in bird Ringer solution.

Four to six-week-old White King squabs of either sex were maintained on a normal diet at 24° C and exposed to about 11 h of light daily. Forty-eight hours before experimentation, the feathers covering the crop area were removed. Into each animal 0.2 ml of n-PRL was injected intradermally over one lateral aspect of the crop sac (dosage was either 2.13, 4.25 or 8.50 µg). The other half of the crop sac received an equal volume of either 1-I-PRL or 4-I-PRL at the appropriate dose. Each bird also received a third intradermal injection of 0.2 ml of bird Ringer solution. The injections were randomly assigned to the left or right side of the crop. Each injection site was marked with a color coded pen. Four birds were used at each dose level. Four hours prior to sacrifice, each animal was injected in the wing vein with 5 µCi/kg of a ^{14}C-amino acid mixture as described previously. The birds were then killed, the crops were excised, sonicated and counted as in the Materials and Methods section. The counts per minute of ^{14}C in the crop samples were normalized to 1 mg protein.

C. Results

In the report by Frantz and Rillema (1968), the results were expressed as a ratio of experimental and control values. In the present

study, this method of data analysis yielded values with an unacceptable degree of heterogeneity of variance as detected by Bartlette's test (see Steel and Torrie, 1960). The raw data (expressed as corrected counts per minute) were also found to be heterogenous by the same test. However, when the logarithm of each datum is subjected to the Bartlette test, the variance is found to be not significantly different from a homogenous population. For this reason, further data analyses have been performed on the $\log_{10}$ of the corrected counts per minute of ^{14}C in crop sac samples. Parametric statistics are otherwise assumed to be appropriate. The log-transformation of data is a common statistical manipulation and in fact is recommended for "counting" data which are not normally distributed (Steel and Torrie, 1960). The raw (untransformed) data are provided in Appendix B for inspection by the reader.

Figure 14 is a histogram of the transformed data from the experiment described above. The data were analyzed using the completely randomized design analysis of variance and the Student-Neuman-Kuels (SNK) multiple range test. The ANOVA table is provided below.

Table 7. Analysis of Variance of Amino Acid Uptake

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Hormone Treatments	7	0.325721	0.046532	7.59*
Error	22	0.134893	0.006135	
Total	29	0.460614		

*P < 0.05

Figure 14
Uptake of Amino Acids Into Pigeon Crop Sac

Each bar denotes the quantity of ^{14}C-labelled amino acid incorporated into pigeon crop sac epithelium per mg of tissue protein, three hours following a single 5 μCi/kg systemic pulse injection. The crop sac tissue was pre-treated (*in vivo*) with either n-PRL (n-P), 4-I-PRL (4-I), 1-I-PRL (1-I) or bird Ringer solution (control) at the indicated doses. A log transformation of the counts per minute of ^{14}C radioactivity was used in order to obtain data with an homogenous variance. * indicates significantly different from control by the Student-Neuman-Kuels multiple range test ($p < 0.05$).

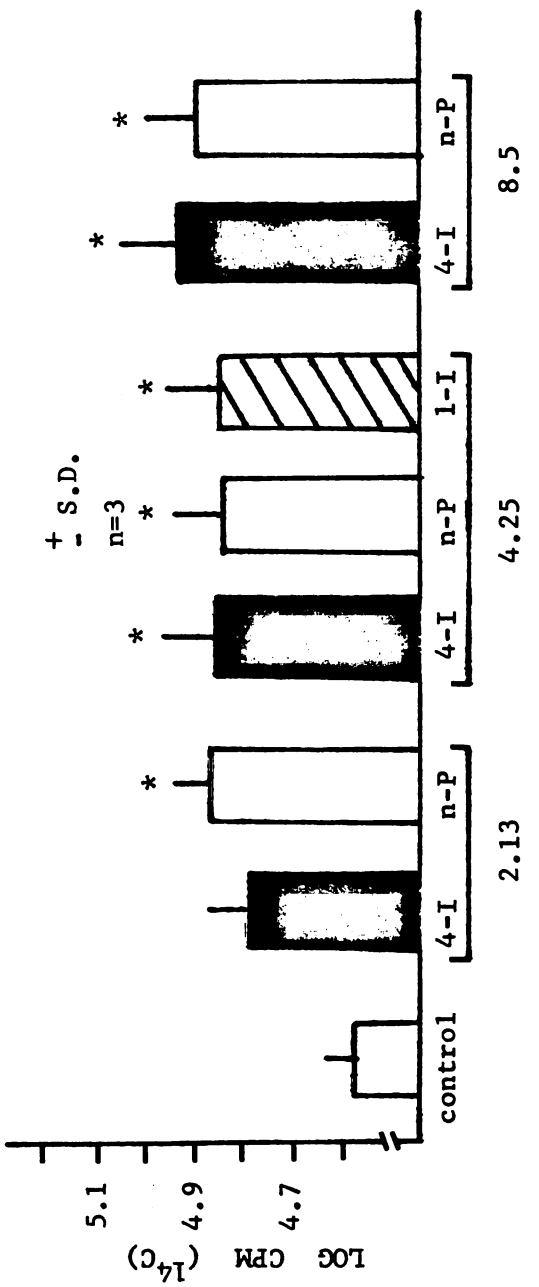


Figure 14
Uptake of Amino Acids Into Pigeon Crop Sac

We see from the ANOVA table that prolactin treatment produces a significant increase in amino acid uptake into the crop gland of the pigeon as compared to pigeons injected with vehicle alone. Furthermore, the SNK test shows us that each of the hormone treatments had a significant effect except for the lowest dose of 4-I-PRL (see Figure 14). Unfortunately, with this particular assay, there was not a significant dose-response. That is, we are unable to differentiate the response of the crop at the two microgram dose level from the response at the 8.5 dose level. The SNK test was also unable to detect any significant difference between iodinated and native hormone. The only comparative statement we can make is that at a dose of 2.13 μg, native prolactin will stimulate significant increases in amino acid uptake whereas 4-I-PRL will not.

D. Discussion

The uptake of ^{14}C-amino acids into the prolactin stimulated crop sac was used as a qualitative assay of lactogenic activity. In this experiment, we found that native prolactin, 4-I-PRL and 1-I-PR all were able to stimulate significant increases in the uptake of amino acids as compared to tissue injected with Ringer solution. These findings are of particular interest since similar hormone preparations could be distinguished by their differential ability to bind to rat liver receptors. Since the assay method used here could not make quantitative distinctions in the dose range used, our conclusion is limited. We can say with confidence that the 3-5 fold decrease in the apparent binding affinity of 4-I-PRL as compared to n-PRL does not result in the

complete loss of biological activity in the iodinated species. On the other hand, at doses less than 2.13 μg of 4-I-PRL, in the pigeon crop sac, there may be some loss of bioactivity. The quantitative aspects of this problem will need to be resolved by a more sensitive bioassay system.

Since the stoichiometry of iodination varies from laboratory to laboratory, two prolactin species at different levels of iodination were tested (I:P = 1:1 and 4:1). Both molecules produce comparable biological responses in this assay. These findings should be gratifying to researchers using the RIA and RRA since both assays assume identical behavior of native and iodinated prolactin. The author cautions that quantitative differences certainly exist for the RRA and appear to exist in the bioassay and RIA. For example, the relative sensitivity of the rat mammary bioassay and the RIA have been described by Leung et al. (1978). He found that the two systems produced parallel dose-response curves but that the bioassay was four times as sensitive as the RIA. This rat mammary assay could be a good system for assessing iodinated and native prolactin in future studies.

SUMMARY DISCUSSION AND CONCLUSIONS

The experiments in this dissertation have been directed toward the evaluation and quantification of the radioreceptor assay for prolactin. Special emphasis has been placed on the stoichiometry and biological effects of prolactin iodination by the lactoperoxidase method. We have also attempted to delineate the potentials and limitations of kinetic analyses of prolactin-receptor interactions.

From the results of the foregoing experiments, we have found that iodinated prolactin (^{125}I-PRL) may be successfully used for relative quantitative studies of receptor binding. However, the hormone has an apparent binding affinity (by Scatchard Analysis) 3-5 times less than that of n-PRL. The binding activity of n-PRL and I-PRL was assayed only in fractions eluting from Sephadex G 100 with a K_{av} of 0.33 to 0.55. Therefore, hormone which had been damaged by cross link formation (Aeschbach et al., 1976) or by aggregate formation (Squire et al., 1963) during iodination were excluded from the binding assay. The difference in binding is not simply a matter of relative potency of native and iodinated hormone but reflects a difference in the intrinsic "avidity" or kinetic rate constants of the two molecular species. The differences in the "specific" binding of native and iodinated hormone are therefore dose-dependent and non-uniform. In short, the two forms of the hormone produce non-parallel dose-response curves as evidenced by the factorial analysis of variance as well as linear regression analysis of Scatchard

plots. Under the conditions of the experiments described in this dissertation, the differential binding was greatest at doses of prolactin in the physiological range (10-250 ng/ml). In future investigations it is proposed that the relative binding affinities of n-PRL and I-PRL be accounted for when quantitative statements about receptors are made. Failure to account for these differences could lead to false conclusions if Scatchard analyses are employed (see section on binding experiments).

The comparative binding assay described above was repeated using different liver preparations and different quantities of radiolabelled hormone. In the second experiment, the same relative results were obtained. There were also significant quantitative differences between the two assays. That is between-assay variance was significantly greater than intra-assay variance. For example, in the first assay the apparent dissociation constants (K_d) of n-PRL and I-PRL were 3.6×10^{-9} M and 10.9×10^{-9} M respectively. In the second assay, the respective dissociation constants were 6.7×10^{-9} M and 38.0×10^{-9} M. In both experiments, relative affinities are consistent but absolute values are not reproducible. Although the receptor preparations in both experiments were derived from the livers of retired breeder female rats, it is possible that the animals were at different physiological states. It might therefore be argued that the quantitative differences in the two assays described above are the result of variant hormonal or other factors in the donor rats' physiology. Such an argument is not supported by the literature. Although prolactin receptor levels vary in response to hormones such as estrogen (Kelly et al., 1974), testosterone (Bohnet et al., 1978), growth hormone (Knazek et al., 1978) and prolactin

(Posner and Kelly, 1975), there are no reports known to the author which suggest that the affinities of the receptor change during various physiological states. That is, receptor numbers (per gram of tissue) may change but the binding properties of the receptors are not known to change. It seems very probable that the intra-assay differences in binding affinity described above are introduced by experimental procedures. This is true even though the experiments were carried out in the same laboratory and by the same investigators.

Due to the logic outlined above, the author is led to the conclusion that with the current methods, the radioreceptor assay cannot be used for reproducible, absolute evaluations of prolactin receptor properties. That is, comparisons of results from one laboratory to another are probably not justified. This conclusion has already been suggested by Brooks (1976). On the other hand, significant and meaningful results may be obtained within a given experiment as evidenced by the statistical analyses performed in this thesis. That is, statements about relative changes in receptor numbers as well as receptor affinities within a given experiment are justified.

The reduction in the apparent binding affinity of I-PRL as compared to n-PRL may be related to conformational changes in the hormone which result from the iodination process. The evidence for this statement is indirect and subject to several interpretations. We have found that iodination of ovine prolactin is accompanied by dose-dependent decreases in tryptophanyl fluorescence. This fluorescence quenching is probably not the result of direct chemical modification of tryptophan but instead occurs because of decreased energy transfer between tyrosine

and tryptophan (Rhodes, 1976). Tyrosine residues are the site of iodide fixation and therefore subject to the internal heavy ion effect, i.e., a decreased fluorescence due to spin-orbit coupling of the pi orbitals and the halide. However, the maximum number of iodides incorporated into a single prolactin molecule is four and the maximal fluorescence decrease due to iodination is 33%. The prolactin molecule has seven tyrosines and two tryptophans, and so the mechanism of fluorescence quenching is not easily discerned. Presumably iodotyrosines provide an energy sink for tryptophanyl fluorescence. Since 67% of the prolactin fluorescence is retained even in the presence of excess iodide suggests that more than one "excimer" center may exist in prolactin. One center would be sensitive to tyrosine iodination and the other independent of iodination caused quenching. Tyrosine residues which were not even iodinated might be separated from tryptophan and contribute to the fluorescence decrease. A possible means of testing for a conformational change in prolactin would be to perform tritium exchange experiments (Craig et al., 1975).

An alternative but purely speculative argument for the decreased binding affinity associated with I-PRL is that iodide attachment actually occurs at or near the active binding site of the prolactin molecule. In this way, iodination would result in a steric inhibition of hormone-receptor interaction. This is an equally plausible explanation but this argument tells us little about the active site and does not explain the dramatic decrease in tryptophanyl fluorescence which occurs during iodination.

In spite of the significant loss of receptor binding potential following prolactin iodination, the hormone retains its biological activity as detected by a modified pigeon crop sac assay. In fact, no significant differences were observed between n-PRL and I-PRL with the method employed in this study.

We are confronted with the issue of decreased binding activity and retained biological activity. Unfortunately, we have no real data upon which to build an explanation. It seems reasonable, nonetheless, to speculate that the in vivo biological response could be expressed as the integral of hormone dose, receptor activity, cytoplasmic and nuclear responses and time. Clearly there are maximal or minimal responses at the cellular level, but the biological response of the cell is not dependent on a single factor at any point in time. For example, significant differences in I-PRL and n-PRL might be discernible in the pigeon crop sac assay if the exposure of the crop to hormone was time or dose-limited. The only hint that this might be true is that the lowest dose of 4-I-PRL was unable to stimulate a significant increase in amino acid uptake. Unfortunately, the differences in native and iodinated prolactin cannot be resolved by the assay. Frantz and Turkington (1972) as well as Frantz et al. (1972a) have reported a 30-40% loss of biological activity due to iodination. These two groups of researchers however, used a more sensitive mouse mammary tissue culture assay for prolactin. They also used 1-I-PRL and not 4-I-PRL. If we accept this correlation between decreased binding affinity and decreased biological activity as cause and effect, then receptor binding is implicated as the biologically important rate limiting step.

However, such a conclusion is premature and should await further research.

We have also seen in this dissertation that quantitative analysis of prolactin binding is more effectively treated by use of a two-step model. The simple mathematics of a single ligand binding is not sufficient for the analysis of prolactin dissociation. This however presents a problem since the apparent forward reaction rates do agree with simple second order kinetics. This apparent paradox is actually resolved quite easily. By the methods used in receptor research, we are unable to distinguish between the first step of binding (HR) and the second step of binding (HR'). Once the hormone is attached to membrane, it is recorded as 'bound' whether it is in the HR state or the HR' state. Furthermore, a transition from HR to HR' does not free up a receptor. The receptor in essence is "carried" from the first step of binding to the second step of binding. The sum of HR and HR' is all that we can measure and the membrane particle functions as a "black box" which only tells us HR_t (the total quantity of bound hormone).

Although the receptor assay may generate data at equilibrium which is compatible with the simple model of hormone binding (Scatchard analysis, Lineweaver-Burk plot), the apparent equilibrium constants (K_d) will actually reflect the product of the first and second steps of binding. In terms of our model:

$$H + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HR'$$

and

$$K_d \text{ (apparent)} = \left[\frac{k_{-1}}{k_1}\right] \times \left[\frac{k_{-2}}{k_2}\right]$$

In principle, a third, fourth or fifth step could be added to the model, each exerting an effect under equilibrium conditions. This has the unfortunate consequence of adding another correction factor to our model of hormone-cell interaction. Notice that we also must make the assumption that I-PRL and n-PRL make the transition from HR to HR' with equal facility. Since dissociation of I-PRL proceeds identically in the presence of native hormone or in the presence of I-PRL, this assumption is probably reasonable.

I would also like to say a few words about possible biological interpretations of the two-step model. At present, there are two lines of argument which might be considered. The first model is simply the concept of the receptor undergoing a rotational or conformational change in the plane of the membrane. Possibly the hormone-receptor complex is actually pulled into the membrane or possibly the complex binds to an enzyme located within the cell. Supporting literature for such a theory has already been cited earlier in this manuscript.

The most current, and actively researched theory which should be considered, involves endocytosis of large segments of membrane following binding of prolactin to receptors. The theory as developed by Josefsberg et al. (1979) states that prolactin binding to liver membrane of female rats is followed by a rapid uptake of membrane into

the Golgi apparatus of the liver. They report that 40-50% of the specifically bound I-PRL is incorporated into Golgi fractions (Bergeron et al., 1978) within 5-20 minutes in vivo. The remainder of the bound ^{125}I-PRL in the microsomal fraction is found in "plasma membrane fractions" as defined by Ehrenreich et al. (1973). Furthermore, they report that ^{125}I-PRL which is dissociated from microsomal membranes retains its ability to bind to receptors (Josefsberg et al., 1979). In essence, their theory would constitute two populations of receptors (as a first approximation): one group of receptors would be found within Golgi vesicles, the second group would be found outside of the vesicles. Moreover, the two populations can be interconverted (i.e., the vesicles can turn inside out). One could go on to speculate that this vesicle opening and closing would be a temperature sensitive event. The membrane preparations which were used in the experiments reported in this thesis would include both Golgi and plasma membranes (Shiu et al., 1973; Meldolesi, 1971). Thus, the two-step dissociation which is reported here is entirely consistent with the evidence reported by Posner's group. Ultimately we are unable to discern whether the two-step model reflects rotational-conformational changes which occur in the plane of the membrane or more elaborate processes.

An electronic analog model for prolactin dissociation from receptors has also been described in this dissertation. We have demonstrated that the mathematics of hormone dissociation are identical to those of resistance-capacitance circuits. In addition to the practical aspects of the analog device (already described elsewhere in this manuscript), there is a philosophical appeal to this circuit. We can

now think of the hormone-receptor interaction as a "compliance" network. That is, receptors constitute an elastic element in the pituitary-tissue axis. This "elasticity" would have practical significance since it would tend to buffer the target cell against transients in circulating levels of hormone. A two-step model simply introduces another level of buffering.

It is now clear that prolactin receptor binding studies involve many complex and apparently interrelated factors. The quantitative analysis of this system is encumbered by a number of complicating factors which are both intrinsic to the biology of the animal and due to experimental methods such as hormone iodination. We are led to believe that the biology of prolactin is regulated at every level: the hypothalamus, possibly directly at the pituitary, at the receptor level and quite probably intracellularly. The road for future investigators is both challenging and awe inspiring.

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APPENDICES

APPENDIX A

DERIVATION OF AN INTEGRATED EQUATION FOR THE DISSOCIATION OF HORMONE FROM A TWO-STEP RECEPTOR SYSTEM

Suppose:

$$HR' \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} HR \xrightarrow{k_{-1}} H + R \text{ (an infinite sink for H is assumed)}$$

then:

$$\frac{d(HR')}{dt} = -k_{-2}(HR') + k_2(HR)$$

and

$$\frac{d(HR)}{dt} = k_{-2}(HR') - k_2(HR) - k_{-1}(HR)$$

$$= k_{-2}(HR') - (k_2 + k_{-1})(HR)$$

The following initial conditions are assumed:

$$\text{at } t=0 \quad (HR') = (HR')_o$$

$$(HR) = (HR)_o$$

$$\text{let} \quad (HR') = k_{1(t)}\,, \quad (HR')_o = x_o$$

$$(HR) = x_{2(t)}\,, \quad (HR)_o = \bar{x}_o$$

Thus:

$$\dot{x}_1 = -k_{-2}\, x_1 + k_2\, x_2$$

$$\dot{x}_2 = k_{-2}\, x_1 + (k_2 + k_{-1})\, x_2$$

$$x_{1(o)} = x_o \quad \text{and} \quad x_{2(o)} = \bar{x}_o$$

APPENDIX A--continued

Li

$$s\,x_1(s) - x_o = -k_{-2}\,x_1(s) + k_2\,x_2(s)$$

$$s\,x_2(s) - \bar{x}_o = k_{-2}\,x_1(s) + (k_2 + k_{-1})\,x_2(s)$$

or in terms of matrix algebra:

$$\begin{pmatrix} (s + k_{-2}) & k_2 \\ k_{-2} & s + k_2 + k_{-1}) \end{pmatrix} \begin{pmatrix} x_1(s) \\ x_2(s) \end{pmatrix} = \begin{pmatrix} x_o \\ \bar{x}_o \end{pmatrix}$$

which is equal to $A\,x(s) = x_o$

whose solution is $x(s) = A^{-1}\,x_o$

Now if we let $A = (s + k_{-2})(s + k_2 + k_{-1}0 - k_{-2}k_2$

$$= s^2 + s(k_{-2} + k_2 + k_{-1}) + k_{-2}\,k_{-1}$$

These equations will factor into:

$$s = \frac{-(k_{-2} + k_2 + k_{-1})}{2} \pm \tfrac{1}{2}\sqrt{(k_{-2} + k_2 + k_{-1})^2 - 4K_{-2}\,k_{-1}}$$

$$= -\alpha \pm \sqrt{\beta} \qquad \text{where } \alpha = \frac{(k_{-2} + k_2 + k_{-1})}{2}$$

and β = the quantity within the radical

Let $A = (s + \alpha - \sqrt{\beta}\,)(s + \alpha + \sqrt{\beta}\,)$

$$= (s + \lambda_1)(s + \lambda_2)$$

APPENDIX A--continued

therefore

$$A^{-1} = 1/(s + \lambda_1)(s + \lambda_2) \begin{pmatrix} (s+k_2+k_{-1}) & -k_2 \\ -k_{-2} & (s + k_{-2}) \end{pmatrix}$$

therefore

$$x_1(s) = \frac{x_0(s + k_2 + k_{-1})}{(s + \lambda_1)(s + \lambda_2)} - \bar{x}_0 \frac{k_2}{(s + \lambda_1)(s + \lambda_2)}$$

and

$$x_2(s) = - x_0 \frac{k_2}{(s + \lambda_1)(s + \lambda_2)} + \bar{x}_0 \frac{(s + k_{-2})}{(s + \lambda_1)(s + \lambda_2)}$$

and

$$x_1(s) = \left[\frac{sx_0}{(s + \lambda_1)(s + \lambda_2)} + \frac{(\bar{x}_0 - x_0) k_{-2}}{(s + \lambda_1)(s + \lambda_2)}\right]$$

$$x_2(s) = \left[\frac{s\bar{x}_0}{(s + \lambda_1)(s + \lambda_2)} + \frac{(\bar{x}_0 - x_0) k_{-2}}{(s + \lambda_1)(s + \lambda_2)}\right]$$

$\underline{L^{-1}}$:

$$x_1(t) = x_0 \left[\frac{\lambda_1 e^{-\lambda 1 t} - \lambda_2 e^{-\lambda 2 t}}{(\lambda_1 - \lambda_2)}\right] + \left[x_0 (k_2 + k_{-1}) - \bar{x}_0 k_2\right] \left[\frac{e^{-\lambda 1 t} - e^{-\lambda 2 t}}{\lambda_2 - \lambda_1}\right]$$

$$x_2(t) = \bar{x}_0 \left[\frac{1 e^{-\lambda 1 t} - 2 e^{-\lambda 2 t}}{(\lambda_1 - \lambda_2)}\right] + (\bar{x}_0 - x_0) k_{-2} \left[\frac{e^{-\lambda 1 t} - e^{-\lambda 2 t}}{(\lambda_2 - \lambda_1)}\right]$$

now:

$$(\lambda_1 - \lambda_2) = (\alpha - \sqrt{\beta}) - (\alpha + \sqrt{\beta}) = -2\sqrt{\beta}$$

$$(\lambda_2 - \lambda_1) = (\alpha + \sqrt{\beta}) - (\alpha - \sqrt{\beta}) = 2\sqrt{\beta}$$

APPENDIX A--continued

therefore:

$$x_1(t) = \frac{x_0}{2\sqrt{\beta}} \left(\lambda_2 e^{-\lambda_2 t} - \lambda_1 e^{-\lambda_1 t}\right) + \frac{(x_0(k_2 + k_{-1}) - \bar{x}_0 k_2)}{2\sqrt{\beta}} \left(e^{-\lambda_1 t} - e^{-\lambda_2 t}\right)$$

$$x_2(t) = \frac{\bar{x}_0}{2\sqrt{\beta}} \left(\lambda_2 e^{-\lambda_2 t} - \lambda_1 e^{-\lambda_1 t}\right) + \frac{(\bar{x}_0 - x_0)\, k_{-2}}{2\sqrt{\beta}} \left(e^{-\lambda_1 t} - e^{-\lambda_2 t}\right)$$

finally:

$$x_1 + x_2 = \frac{[x_0(k_2 + k_{-1}) - \bar{x}_0 k_2 - x_0\lambda_1 + (\bar{x}_0 - x_0) k_{-2} - \bar{x}_0\lambda_1]}{2\sqrt{\beta}} e^{-\lambda_2 t} - \frac{[x_0(k_2 + k_{-1}) - \bar{x}_0 k_2 - x_0\lambda_2 + (\bar{x}_0 - x_0) k_{-2} - \bar{x}_0\lambda_2]}{2\sqrt{\beta}} e^{-\lambda_2 t}$$

$$\lambda_1 = \frac{(k_{-2} + k_2 + k_{-1})}{2} - \tfrac{1}{2}\sqrt{(k_{-2} + k_2 + k_{-1})^2 - 4k_{-2}\, k_{-1}}$$

$$\lambda_2 = \frac{(k_{-2} + k_2 + k_{-1})}{2} + \tfrac{1}{2}\sqrt{(k_{-2} + k_2 + k_{-1})^2 - 4k_{-2}\, k_{-1}}$$

$$2\sqrt{\beta} = \sqrt{(k_{-2} + k_2 + k_{-1})^2 - 4k_{-2}\, k_{-1}}$$

APPENDIX B

Table 8. Non-transformed Data for Pigeon Crop Sac Assay

	Dose of Hormone		
	2. 3 µg	4.25 µg	8.5 µg
Control	51548	48928	41000
	39446	48058	53594
	38023	38149	51898
n-PRL	73682	70321	64741
	76344	58131	95651
	71582	75384	82614
4-I-PRL	74000	95780	62222
	57612	81415	97506
	54288	48471	103351
1-I-PRL		72443	
		74266	
		64117	

Each datum represents the number of counts per minute (scintillation counter) per mg tissue protein.

APPENDIX C

LOWRY PROTEIN DETERMINATION

Lowry A:

60 grams Na_2CO_3

12 gram NaOH

0.6 grams K,Na tartate

Water to make three liters

Lowry B:

5 grams $CuSO_4 \cdot 5H_2O$

Water to make one liter

Lowry C:

1 milliliter phenol reagent (Folin and Ciocalteu) plus water to make 20 milliliters

For Protein Determination:

1. Mix 1 part B with 49 parts A
2. Add 5 mls of A-B mixture to 1 ml of unknown
3. Allow to develop for 30 minutes
4. Add 1 ml C, allow to develop for 0.5-1.0 h
5. Read absorbance after zeroing against a blank at 660 nm.

APPENDIX D

THE SCINTILLATION COUNTING SOLUTION

Radioactive samples were counted in 15 ml of a solution made by the following recipe:

80 grams naphalene (Baker Chemical)

5 grams PPO (Sigma)

50 milligrams -NPO (Packard)

Add dioxane to make one liter

Counting solutions were used within three months following mixing.