QUANTIFICATION OF PROLACTIN RECEPTORS IN VARIOUS MURINE MAMMARY TUMORS AND THE EFFECT OF IN VIVO BOUND PROLACTIN ON THE ^{125}I-PROLACTIN RADIORECEPTOR ASSAY

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
CHARLES L. BROOKS
1976

This is to certify that the

thesis entitled

Quantification of Prolactin Receptors in Various Murine Mammary Tumors and the Effect of In Vivo Bound Prolactin on the ^{125}I-Prolactin Radioreceptor Assay

presented by

Charles L. Brooks

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Anatomy (Oncology)

Clifford W. Welsch
Major professor

Date 8/13/76

O-7639

ABSTRACT

QUANTIFICATION OF PROLACTIN RECEPTORS IN VARIOUS MURINE MAMMARY TUMORS AND THE EFFECT OF IN VIVO BOUND PROLACTIN ON THE ^{125}I-PROLACTIN RADIORECEPTOR ASSAY

By

Charles L. Brooks

The number of prolactin receptors in murine species was assessed through the use of an ^{125}I-prolactin radioreceptor assay. This assay was constructed in a similar manner to that first used by Shiu [Science 180: 986 (1973)].

The relative number of prolactin receptors in 7,12-dimethylbenzanthracene (DMBA)-induced rat mammary tumors, DMBA-induced rat mammary tumors from hypophysectomized-ovariectomized rats and spontaneous mammary tumors from C3H/HeJ mice correspond to the known in vivo and in vitro growth response to prolactin. Prolactin dependent DMBA-induced rat mammary tumors bind 2.94 ± 0.46% of the total ^{125}I-prolactin in the assay: while prolactin independent C3H/HeJ spontaneous mouse mammary tumors bind 0.71 ± 0.21%, DMBA-induced mammary tumors from hypophysectomized-ovariectomized rats bind 2.75 ± 1.17%. These tumors although prolactin independent are still prolactin sensitive.

The association constants for rat tissues are approximately $10^{9}M^{-1}$ while for mouse tissues approximately $10^{10}M^{-1}$. The binding is shown to be time and temperature dependent and specific for prolactin.

Charles L. Brooks

The assay of prolactin receptors in rat tissues is effected by endogenously bound prolactin. Increases in serum prolactin block the assay of prolactin receptors by an ^{125}I-prolactin radioreceptor assay. This problem can be partially overcome by the use of long incubation times, but more effectively dealt with by preassay removal of the endogenous prolactin. Finally, a two-step time-dependent model for prolactin-receptor interaction is suggested.

QUANTIFICATION OF PROLACTIN RECEPTORS IN VARIOUS MURINE MAMMARY TUMORS AND THE EFFECT OF IN VIVO BOUND PROLACTIN ON THE ^{125}I-PROLACTIN RADIORECEPTOR ASSAY

By

Charles L. Brooks

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Anatomy

1976

ACKNOWLEDGMENTS

Thanks to my committee, to the graduate students and fellows of the Departments of Physiology, Biochemistry, Dairy Science and Anatomy and faculty in many departments. With their help I have been able to produce this thesis and the maturation which it symbolizes. Special thanks to Dr. Clifford W. Welsch who allowed me to begin this process and has supported me throughout it.

Thank you Sue Willson Brooks.

TABLE OF CONTENTS

TABLE OF CONTENTS--continued Page

TABLE OF CONTENTS--continued

TABLE OF CONTENTS--continued

LIST OF TABLES

LIST OF TABLES--continued

LIST OF FIGURES

LIST OF FIGURES--continued Page

INTRODUCTION

The mechanism of action of hormones has been the subject of intensive study for decades (Hector and Halkerson, 1964). In the last ten years receptors for polypeptide hormones have been detected, quantified and characterized (Cuatrecasas, 1974; Posner, 1975; Lefkowitz, 1973). These receptors are located in the plasma membrane (Turkington, 1970; Schwartz et al., 1973; Shiu and Friesen, 1976). The reaction of polypeptide hormones with plasma membrane receptors is the initial interaction in a series of events involved in the control of a specific set of cellular reactions which are induced by a specific hormone. The hormone-receptor interaction is the subject of this thesis. The work presented here is a prerequisite to determining the normal and pathological function of the molecular biology of endocrine target cells.

The subject was limited to the interaction of prolactin and its receptor in various target tissues and mammary tumors. A number of experiments were performed to validate the radioreceptor assay for plasma membrane receptors which bind prolactin. Several experiments involving murine mammary tumors were next completed. These tests discerned if the presence of plasma membrane receptors for prolactin correlate to the known growth response of these mammary tumors to the hormone. Finally, a series of experiments were undertaken to consider the specific interaction of prolactin and its receptors. These experiments tested whether prolactin which bound to the receptor in vivo

effected the subsequent assay of prolactin receptors. In addition, data which myself and others have produced are examined to perceive which type of molecular models of hormone and receptor interaction are most likely.

The format of the Experimental Section combines the objectives, procedures and the results sections for each experiment. Experiments are discussed in groups or individually.

LITERATURE REVIEW

The mode of action of hormones upon their target cells begins by a specific interaction of the hormone and receptor. In general, each hormone has a unique receptor. The hormones acting by this mechanism can be classified into several groups depending upon where the initial interaction with the receptor occurs.

The first type of interaction occurs in the cytoplasm and involves steroid hormones and soluble protein receptors. The second type of interaction occurs at the plasma membrane. It is believed that the polypeptide hormones combine with protein receptors that laterally migrate within the cell's exterior membrane. Finally, thyroid hormone, a modified dipeptide, combines with receptors located within the target cell nucleus.

Steroid hormones diffuse into the cell because they are somewhat hydrophobic in nature and have a low molecular weight. These properties allow these molecules to diffuse into and through membranes (Jackson and Chalkley, 1974). The cytoplasmic binding of estradiol was the first and most complete mechanism to have been demonstrated. It is used as a model for steroid hormone-receptor interaction in this review. Estradiol combines with a protein which specifically binds the C-17 dihydroxy-steroid hormone. This binding accounts for the concentration and retention of ^{3}H-estradiol, first detected by Jensen and Jacobsen (Jensen and Jacobsen, 1960). Toft and Gorski (Toft and Gorski, 1966)

demonstrated two types of estrogen receptors by sucrose density centrifugation. These two types of estrogen receptors are called cytoplasmic and nuclear due to their intracellular location. Jensen (Jensen et al., 1968) demonstrated that the cytoplasmic receptor is transformed in a temperature-dependent reaction. The transformed receptor is only then allowed entry to the nucleus. Once inside the nucleus the transformed hormone-receptor complex can combine with specific RNA polymerases (Mohla et al., 1972), chromatin (Yamamoto, 1974; King et al., 1966), or chromatin associated acidic proteins (O'Malley et al., 1973; O'Malley and Schroder, 1976). Due to these interactions ribosomal RNAs (O'Malley and Means, 1974) and specific messenger RNAs (Tsai et al., 1975; Schwartz, 1975; O'Malley et al., 1975; McKnight et al., 1975) are induced. Several labs have pure preparations of specific mRNA induced by estradiol in the chick oviduct (Woo et al., 1975; Rhoads, 1975) and have translated this mRNA into the physiological end-product of estradiol stimulation, ovalbumen (O'Malley et al., 1975). This subject has recently been reviewed by Jensen (Jensen and DeSombre, 1973) and O'Malley (O'Malley and Means, 1974; O'Malley, 1976).

The same general interaction of steroids binding cytoplasmic receptors and their transformation to nuclear receptors have been demonstrated for corticosteroids (Tucker et al., 1971; Funder et al., 1973a; Funder et al., 1973b; Baxter et al., 1971; Baxter and Tomkins, 1971), androgens (Mainwaring, 1969; Unhiem et al., 1969; Fang et al., 1969), and progesterone (Milgrom and Baulieu, 1970; Feil et al., 1972; Sherman et al., 1970). The mode of action of these latter steroid hormones is thought to be similar to the mechanism of estradiol (O'Malley, 1976) but the set of end-results for each hormone is unique.

An example of the experimental problems is the difficulty encountered in understanding the complete mechanism for the induction of ovidin by progesterone. Problems arose because of progesterone's cross-reactivity to corticosteroid receptors and a serum binding globulin for progesterone (Goral and Wittliff, 1975; Milgrom and Baulieu, 1970). The discovery of synthetic progestins which demonstrate no cross-reactivity has solved such problems (Smith et al., 1974).

Another case of difficulty involved in investigating steroid hormone-receptor interactions is that testosterone is not the active male sex hormone. This steroid needs to be converted to 5α-dihydroxy-testosterone before binding to target organs (Fang et al., 1969; Gordon et al., 1974). Recently, in studies using synthetic androgens, the compounds which have a Δ^4-3-ketosteroid configuration and do not contain angular C-10 methyl groups are the best binders to target tissues (Laio et al., 1973).

A second mechanism of hormone action is that of thyroxine (T_3) and tetraiodothyronine (T_4). These hormones act directly upon nuclear binding sites in target cells (Samuels and Tsai, 1973). The receptors bind T_3 and T_4 in nuclear isolates (Samuels et al., 1974). The hormone-receptor complex binds to DNA (MacLeod and Baxter, 1975; Charles et al., 1975) and nucleolar chromatin (Gardner, 1975).

The polypeptide hormones exert their action upon the target cell by a different mechanism. The polypeptide hormones act by binding to a proteinacious receptor associated with the plasma membrane. The hormones do not enter the cell (Turkington, 1970; Schwartz et al., 1973) to exert their action. A large number of polypeptide hormones and

other substances are known to act via this mechanism. It is interesting to note that such diverse substances with relatively large and small molecular weights, such as gonadotropins and catacholamines, respectively, act by this mechanism. They are listed along with typical target tissues below.

Plasma Membrane Binding Hormones

Hormone	Target Tissue
ACTH	adrenal cortex
angiotensin II	adrenal cortex
vasopressin	kidney
oxytocin	fat cells
bungarotoxin(cholinergic receptor)	electric eel, torpedo, rat skeletal muscle
insulin	liver, fibroblasts, adipose tissue, lymphocytes
glucagon	liver
LH	ovary
HCG	testis, ovary
FSH	testis, ovary
prostaglandins	adipose tissue
TRH	anterior pituitary
norepinephrine	heart, liver
epinephrine	erythrocyte, spleen capsule
growth hormone	lymphocytes, liver
prolactin	mammary gland, prostate, liver
parathyroid hormone	kidney

One of the problems encountered in conducting radioreceptor assays for polypeptide hormones is the radiolabeling of hormones. Presently there are four methods (Hunter and Greenwood, 1962; Vaitukaitis et al., 1971; Thorell and Johansson, 1971; Bolton and Hunter, 1973). Each method has characteristics that leave the hormone in an altered state This produces a complex situation when using such preparations to interpret kinetic and affinity data. Most polypeptide radioreceptor systems make the assumption that labeled and nonlabeled hormones have identical binding properties (Rodbard, 1973). Reviews describing receptors for

polypeptide hormones have recently been published by Lefkowitz (Lefkowitz, 1973), Cuatrecasas (Cuatrecasas, 1974) and Posner (Posner, 1975).

Stricker and Grueter were the first to demonstrate the existence of a lactogenic hormone (Stricker and Grueter, 1928). Since then lactogenic hormones have been found in many species (Nicoll and Bryant, 1972) and have come to be called prolactin. Li and his colleagues were the first group to purify (Li et al., 1942) and eventually sequence (Li et al., 1970) a mammalian prolactin. Prolactins of lower species have been reviewed recently by Nicoll (Nicoll, 1974).

Until recently there have been doubts as to the existence of a human prolactin. Now it has been shown that human prolactin can be extracted (Apostolakis, 1965), purified (Hwang et al., 1973), and sequenced (Lewis et al., 1972). This molecule possesses many homologies with human growth hormone and human placental lactogen (Niall, 1972). Thus, it is not surprising that the latter two hormones show lactogenic actions. These actions are mediated through the binding of human growth hormone and human placental lactogen to receptor sites which bind prolactin (Posner et al., 1974; Robertson and Friesen, 1975).

There are three methods to label prolactin. Prolactin labeled by the strong oxidant Chloramine-T was found not to bind to prolactin receptor sites (Frantz and Turkington, 1972). Prolactin labeled by the lactoperoxidase method (Frantz and Turkington, 1972) was, in comparison, capable of binding to prolactin receptors (Rogel and Rosen, 1974). This latter method of iodination subjected the polypeptide to less harsh oxidants. In this laboratory and others, 25 to 60% of the prolactin labeled by such methods is bindable. A third method to radiolabel

prolactin eliminates the direct exposure of oxidants to the hormone and was shown to be superior to chloramine-T iodination when compared by immunoprecipitation (Bolton and Hunter, 1973). In this procedure the iodine is attached to a small acylating agent by chloramine-T. This ^{125}I-acylating agent is separated by solvent extraction and subsequently allowed to react with the free amino groups of the protein to form amides. Although not done with prolactin, this method may provide the least damaged ^{125}I-prolactin.

As with estradiol, the first attempts to localize prolactin receptors have been accomplished by injecting the labeled hormone into an animal and monitoring which organs concentrate and retain the radioactivity (Falconer, 1972). Subsequently these apparent target tissues have been fractionated and it has been determined that a microsomal fraction contains proteins capable of specifically binding prolactin (Shiu and Friesen, 1974). Additional evidence has been produced by autoradiographic methods (Rajaniemi et al., 1974), in vitro tissue slice incubations (Birkinshaw and Falconer, 1972), and sepherose-bound prolactin studies (Turkington, 1970) that indicate the cell surface is the location of the prolactin receptor. Finally, Shiu (Shiu and Friesen, 1976) has blocked the action of prolactin by an antibody to the purified (Shiu and Friesen, 1974) plasma membrane receptor. Shiu concludes that his results are direct evidence for an obligatory role for cell surface receptors. It must be pointed out that none of these methods provide unquestionable proof for the plasma membrane location of the prolactin receptor. In contrast, a nuclear prolactin receptor has recently been postulated (Chomcznski and Topper, 1974) in the rat and mouse mammary gland.

The earliest report of plasma membrane receptors for prolactin was by Turkington and Frantz (Turkington and Frantz, 1972). The binding activity of the mouse mammary gland was further characterized by Frantz and co-workers (Frantz et al., 1974). This work has recently been confirmed (Sakai et al., 1975).

The original purpose of the prolactin radioreceptor assay was to quantify serum and pituitary prolactin levels (Guyda, 1975). However, the common use of radioreceptor assays to access plasma (Kelly et al., 1974a) prolactin is complicated by serum factors that interfere with binding.

Friesen's laboratory had done extensive work with rabbit mammary gland plasma membrane (Posner, 1975; Friesen et al., 1973). The mammary gland prolactin receptor of the rabbit was initially used as an assay for lactogenic hormones (Shiu et al., 1973). With this tissue Shiu demonstrated hormone specificity, studied the kinetics, affinities, time course, enzyme sensitivities and optimal incubation conditions (Shiu and Friesen, 1974a). He also determined the tissue distribution and the efficiency of plasma membrane separation for prolactin plasma membrane receptors in the pregnant rabbit (Shiu and Friesen, 1974a). Finally, he solubilized, partially purified, and characterized the prolactin receptors of the pregnant rabbit mammary gland (Shiu and Friesen, 1974b). More recently, Shiu induced a specific antibody to the prolactin receptor (Shiu and Friesen, 1976).

Work has been done at a rapid pace on the species and organ distribution of prolactin receptors. Prolactin receptors have been demonstrated in the rabbit mammary gland (Shiu et al., 1973), mouse mammary gland (Frantz et al., 1974; Sakai et al., 1975; Sheth et al., 1974), and

in various tissues of the monkey, rat, mouse, guinea pig, rabbit, sheep, pigeon and frog (Posner et al., 1974; Kledzik et al., 1975). In the rat, prolactin receptors have been demonstrated in the mammary gland (Holcomb et al., 1976), liver (Kelly et al., 1974b), prostate (Aragona and Friesen, 1975; Kledzik et al., 1976), ovary (Saito and Saxena, 1975; Rolland and Hammond, 1975), adrenals, kidney (Marshall et al., 1975), pituitary (Frantz et al., 1975) and neoplastic tissue from both the mammary gland (Turkington, 1974; Costlow et al., 1974; Kelly et al., 1974c; Costlow et al., 1975b; DeSombre et al., 1976) and pituitary (Frantz et al., 1975). Receptors for prolactin have been demonstrated in the liver, kidney, adrenal, ovary, midbrain and mammary gland in the mouse (Frantz et al., 1973; Posner, 1976).

The greatest amount of physiological work has been done on prolactin receptors in the rat liver. The prolactin receptor in the rat liver was low in male and immature female rats. In female rats at puberty, prolactin receptors increased to a high level (Kelly et al., 1974b). The rat's hormonal milieu was involved in regulating liver receptor levels for prolactin. The injection of estradiol increased receptors in the liver of male (Posner et al., 1975) or female rats (Gelato et al., 1975). The presence of an intact pituitary was necessary for estrogen to be stimulatory. Hypophysectomy lowered prolactin receptors in the liver to barely detectable levels in the female rat (Costlow et al., 1975a). Ovariectomy also decreased the prolactin receptors in the liver, but not to the low levels of hypophysectomy (Kelly et al., 1975). Prolactin injections induced prolactin receptors in livers of hypophysectomized rats (Costlow et al., 1975a). It has been suggested that prolactin regulates its own receptor in the liver (Posner et al.,

1975) and estrogen increases prolactin receptors by acting through the pituitary and increasing prolactin secretion (Meites et al., 1972). Thyroid hormone has also been shown to be involved in regulating prolactin receptor levels in the female rat liver (Gelato et al., 1975). Estriol, progesterone, human placental lactogen and testosterone have not induced prolactin receptors in the liver of the female rat (Posner et al., 1974b). Castration of male rats resulted in a rise in prolactin receptors in the liver (Aragona and Friesen, 1975). Finally, the control of prolactin receptors in the rat liver was controlled at the level of production of the receptor protein and messenger RNA (Kelly et al., 1975).

A specific prolactin receptor has been demonstrated in the rat testis and prostate. In the testis binding was low at 20 days of age but increased until 70 days of age (Aragona and Friesen, 1975). On the other hand, the concentration per milligram plasma membrane protein of prostate prolactin receptors dropped from 20 days to 270 days of age and the castration of male rats lowered the prostatic binding of prolactin (Aragona and Friesen, 1975). Injection of testosterone proprionate increased the prostatic prolactin binding back to normal levels in castrated rats (Kledzik et al., 1975). Injections of prolactin did not effect prostate binding of prolactin (Kledzik et al., 1975), but there were conflicting reports as to the effect of estrogen on prolactin binding by the prostate. Kledzik injected estradiol benzoate for 5 days and reported no effect (Kledzik et al., 1975), while Aragona administered estradiol for 11 days and saw a 50% reduction of prolactin receptors in the prostate (Aragona and Friesen, 1975). In the male rat

reproductive tract the seminal vesicle and epididymis also exhibited low concentrations of binding.

The ovary also has been shown to specifically bind prolactin. Rat, cow, mouse, pig and human ovaries are the species in which this binding has been demonstrated (Frantz et al., 1973; Saito and Saxena, 1975; Rolland and Hammond, 1975). The rat ovary bound the most prolactin during proestrus, and the least during metestrus (Saito and Saxena, 1975). Radioautographic methods have demonstrated that prolactin binds specifically to corpora lutea and interstitial tissue (Rajaniemi and Vanha-Perttula, 1973; Midgley, 1973). It is believed that prolactin receptors mediate the increase in LH receptors (Holt and Richards, 1975). Subsequently, the LH receptors are believed to mediate the corpora's response to LH, which is an increased progesterone production (Lee and Ryan, 1973).

Prolactin is involved in body salt and water regulation (Nicoll, 1974). The kidney and adrenal are the two main organs involved in this process, and both specifically bound prolactin in male rats. The prolactin receptors in the adrenal increased after unilateral nephrectomy and salt loading (Marshall et al., 1975).

In an article by Frantz and colleagues, cultured normal and neoplastic anterior pituitary cells specifically bound prolactin (Frantz et al., 1975). In addition to the implications involving feedback systems to the adenohypophysis, the authors suggested these cell lines to be a stable source of prolactin receptors useful in the establishment of a prolactin radioreceptor assay.

Sheth showed that human placental lactogen binds to the lactogenic receptors in the mouse mammary gland and that prolactin binding in mice of high mammary tumor incidence (C3H/Jax) was significantly greater than that in mice of a low mammary tumor incidence (C57/BL) (Sheth et al., 1974).

Perry and Jacobs suggested the affinity for human growth hormone and ovine prolactin increased during late lactation in the rabbit mammary gland (Perry and Jacobs, 1975). They believe that the affinity change is due to an alteration in receptor protein.

Prolactin is involved in mammary cancer of murine species (Pearson et al., 1972) and perhaps human breast cancer (Pearson, 1967; Hobbs et al., 1974; Salih et al., 1972; Holdaway and Worsley, 1975; Welsch et al., 1976b). Due to prolactin's role in these tumors, the levels of prolactin receptors have been determined in several tumor types. Working with tissue slices, Costlow et al. has shown that the R3230AC rat mammary carcinoma (Costlow et al., 1974) contains slightly fewer prolactin receptors than lactating rat mammary gland. The prolactin receptors in both tissues have similar association constants. Turkington (Turkington, 1974), using a membrane particle assay, reported a similar association constant to Costlow's. His data do not agree with Costlow's in regard to the relative number of receptors in the R3230AC mammary tumor and rat mammary gland. In addition, Turkington demonstrated prolactin receptors in 7,12-dimethylbenzanthracene (DMBA)-induced rat mammary tumors and the lactating mouse mammary gland. He failed to demonstrate binding in spontaneous mammary tumors of C3H mice. This data is consistent with the concept that prolactin sensitive murine

mammary tumors contain measurable prolactin receptors while prolactin insensitive murine mammary tumors do not.

The number of prolactin receptors in DMBA-induced rat mammary tumors has been correlated with in vivo tumor growth (Kelly et al., 1974c). The number of prolactin receptors in 34 tumors correlated well with both growth response (r=0.69) and final tumor size in rats which have been injected for a period with prolactin. This relation was prolactin specific as there was no correlation of tumor growth and insulin receptor concentration. Finally, the prolactin binding in the livers of these animals was correlated inversely with the growth response of the tumors (r=-0.69).

Costlow and colleagues developed two sublines of the MTW9 transplantable rat mammary tumor (Costlow et al., 1975b). One line grew well in both ovariectomized or hypophysectomized rats. This line was considered hormone independent. A second cell line grew in intact rats, but regressed after ovariectomy. The first line contained measurable estrogen and prolactin receptors, while the second had lost more than 75% of the receptors for both hormones. Thus, the number of receptor sites in these cell lines appears to be related to the cell's hormonal requirements for growth.

Prolactin receptors in DMBA-induced rat mammary tumors have been significantly reduced by ovariectomy (Delgado et al., 1975). Those data implicate estrogen and/or prolactin as possible controlling factors in regulating the tumor's sensitivity to prolactin by changing the number of prolactin receptors. These findings are strikingly similar to the data for the induction of prolactin receptors in rat liver.

Human breast tumors have been tested for prolactin, insulin and growth hormone receptors (Holdaway and Worsley, 1975). Twenty of 28 breast cancers bound insulin significantly while no tumors bound human growth hormone. Human prolactin bound to 7 of 34 tumors as determined by a membrane particle assay. This study was repeated by a tissue slice method and similar results were obtained. The major difficulty of this work is that the binding was low and thus, difficult to distinguish from nonspecific background. In future ^{125}I-prolactin binding tests to receptors in human tumors it will be necessary to more clearly differentiate the specific and nonspecific binding of prolactin. Additionally, this binding needs to be correlated with the in vivo response of the patient to endocrine treatment or to in vitro tests which detect prolactin responsive tumors (Welsch et al., 1976b). Although a lot of progress has been made in identifying tissues which specifically bind prolactin, the physiological processes influencing and influenced by the prolactin-receptor interaction require a greater understanding.

MATERIALS AND METHODS

I. Animal Resources

Three murine species were used in these studies: female Sprague-Dawley rats and female Swiss-Webster mice were obtained from Spartan Research Animals (Haslett, MI), and female C3H/HeJ mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The C3H/HeJ mice are a high mammary tumor incidence strain, originally purchased for another experiment (Welsch, et al., 1976a), and used only to provide the mouse mammary tumors of Experimental Section Part II, Experiment I.

The Swiss-Webster mice and Sprague-Dawley rats were used in the remainder of the experiments. In experiments where pregnant or lactating rats or mice were used the animals were purchased from the breeder during gestation or lactation.

All animals were housed in plastic cages. The lighting and temperature conditions vary from a light (14 hours light/day) and temperature (23 ± 1°C) controlled environment at the Center for Laboratory Animal Resources (Michigan State University) to the conditions in the laboratory. Water and Lab Blox (Allied Mills, Chicago, IL) were available ad libitum.

II. Murine Mammary Tumor Sources

C3H/HeJ mouse mammary tumors were provided as a portion of another study being conducted in the laboratory (Welsch et al., 1976a). These tumors were randomly selected from animals of control and

2-bromo-α-ergocryptine (CB-154) treated groups. The control group tumors (7 tumors) were from animals treated from 1 month of age with bi-weekly injections of 0.9% NaCl with 1% ethanol. The mouse mammary tumors from the CB-154 treated group were from animals treated from 1 month of age with daily injections of CB-154. CB-154 is suspended in ethanol and diluted to 1.0 mg/ml with 0.9% NaCl. Both groups received 0.1 ml doses (0.1 mg/mouse CB-154). All tumors were removed after decapitation. CB-154 is an effective inhibitor of prolactin secretion (Brooks and Welsch, 1974).

DMBA-induced rat mammary tumors were induced by gastric intubation of 20 mg of DMBA into fifty 60-day-old female Sprague-Dawley rats (Huggins et al., 1961). After 10 weeks, 15 rats, each containing one mammary tumor, were randomly selected. The animals were decapitated and the tumor removed. The remaining 35 rats, each with one or more DMBA-induced mammary tumors, were hypophysectomized at the Hormone Research Laboratories (Chicago, IL) and returned to the laboratory. The following day all the rats were ovariectomized. The tumor's regression was followed by periodic observation. Several months later tumors began to redevelop and, after 4 months, four tumors were rapidly growing. These tumors were removed from the animals after decapitation.

III. ^{125}I-prolactin Radioreceptor Assay

A. Principles of the ^{125}I-prolactin Radioreceptor Assay

The ^{125}I-prolactin radioreceptor assay is a method to compare the capacity of tissues to specifically bind radiolabeled prolactin. ^{125}I-prolactin is believed to be specifically bound to prolactin receptors in various target tissues. A specific prolactin receptor is a protein molecule that is present in limited numbers on plasma membranes of

target tissues. These molecules have a high affinity for prolactin and other lactogenic hormones. It is believed that the interaction of prolactin with these receptors initiates the response which is specific for a particular target tissue.

A second group of molecules capable of binding prolactin are present in all tissues. These sites are called nonspecific sites and are believed to have a low affinity for prolactin. In addition, they are almost unlimited in number. The technical problem in determining the specific binding in tissues is to accurately assess both the specific- and nonspecific-bound ^{125}I-prolactin and to quantitatively express the specific-bound prolactin in a standard form.

This problem is approached by taking advantage of the fact that the specific prolactin receptors are limited in number. Two series of assay tubes are used in the ^{125}I-prolactin radioreceptor assay. The first series of tubes contains buffered receptor preparation and ^{125}I-prolactin. Both specific and nonspecific receptors are bound by ^{125}I-prolactin. The second series of tubes have buffered receptor preparation, ^{125}I-prolactin and an excess of unlabeled prolactin. In this second series only nonspecific binding sites are bound by ^{125}I-prolactin. Due to the limited number of specific prolactin receptors and essentially an unlimited number of nonspecific receptors the excess unlabeled prolactin can only effectively compete with ^{125}I-prolactin for the specific sites. Thus, the radioactivity emitted from the membrane preparations of the first series of tubes minus the radioactivity emitted from the membrane preparations of the second series of tubes is an estimate of the amount of prolactin bound to specific prolactin membrane receptors.

B. Tissue Processing

Tissue samples to be examined for ^{125}I-prolactin binding were removed from the animal and quickly frozen in 0.3M sucrose. The samples were stored at -20°C for up to 6 months before they were processed.

Tissue samples were thawed in warm water and immediately cooled to 4°C, the temperature for the remaining tissue processing steps. The tissue sample was minced and placed in a 50 ml polyethylene tube with between 3 and 5 volumes of 0.3M sucrose. This mixture was ground 30 seconds with a Polytron Model PT-10 (Brinkman Instruments, Westbury, NY). The tube with homogenate was placed in a SS-34 head of a refrigerated Sorvall Centrifuge Model RC2-B (Ivan Sorvall Inc., Norwalk, CT) and spun at 1,500g for 20 minutes. The supernatant was decanted into a glass grinder (Corning Glass Works, Corning, NY) and worked with four strokes. This supernatant was placed in a second 50 ml polyethylene tube and spun at 15,000g for 20 minutes. The supernatant was decanted and placed into one or two 13 ml tubes. They were capped, placed in a ti50 rotor, and spun at 100,000g for 90 minutes in a L5-50 Ultracentrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was discarded and the pellet resuspended in Tris-HCl buffer (25mM Tris-HCl, 10mM $MgCl_2$, pH 7.6). The resuspended pellet was an enriched plasma membrane fraction and contained up to 76% of the prolactin receptors when tested with lactating rabbit mammary gland (Shiu et al., 1973). This suspension was tested for protein content by the Lowry method (Lowry et al., 1951) and diluted to a concentration of 300 µg protein per 100 µl. The standardized preparation of plasma membranes was put in 3 ml aliquots into 12x75 mm culture tubes, capped and frozen at -20°C until assayed.

C. ^{125}I-iodination of Ovine Prolactin by the Lactoperoxidase Method

Ovine prolactin was iodinated by the method of Thorell and Johansson (Thorell and Johansson, 1971). The following ingredients were placed in order into a 1 ml polyethylene tube.

1. 20 µl Tris-HCl buffer
2. 5 µg ovine prolactin (NIH-P-5-9) in 20 µl of Tris-HCl buffer
3. 10 µg of lactoperoxidase (E.C. No. 1.11.1.7) (Sigma Chemical Company, St. Louis, MO, approximately 30 purpurogallin Units/mg)
4. 1.0 mCi of carrier free ^{125}I (Searle Analytic Inc., Des Plaines, IL)
5. 20 µl of 1:30,000 dilution of hydrogen peroxide (Mallinckrodt Chemical Works, St. Louis, MO) (3 µl of 30% H_2O_2 into 90 ml water)

Timing the reaction began when the H_2O_2 was added. It was allowed to continue for 2½ minutes at room temperature. At the end of this period 100 µl of 16% sucrose with bromthymol blue (Sigma Chemical Company, St. Louis, MO) was added. The contents of the tube were aspirated and placed on a G-50 (Course) Sephadex column (0.8x23 cm) (Pharmica Fine Chemicals Inc., Piscataway, NJ) and eluted by Tris-HCl buffer in 1 ml fractions into tubes each containing 1 ml Tris-HCl-BSA buffer (25mM Tris-HCl, 10mM $MgCl_2$, 0.1% by weight bovine serum albumen fraction V, Grand Island Biological Company, Grand Island, NY). A 10 µl sample was removed from all fractions and counted in an 1185 Series Automatic Gamma Counter (Searle Analytic Inc., Des Plaines, IL) (65% efficiency) to identify the radioactive prolactin. The ^{125}I-prolactin was normally detected in fraction 3, 4 or 5. This fraction was then repurified on a

second G-100 Sephadex column (1.3x45 cm) (Pharmica Fine Chemicals, Inc., Piscataway, NJ). The second column was eluted by Tris-HCl buffer and 2 ml fractions were collected into tubes each containing 1 ml of Tris-HCl-BSA buffer. Ten µl samples were removed from each fraction and counted. Three peaks were identified and tested for binding. The middle peak (usually fractions 18-26) was the only bindable material. These fractions were pooled and diluted in Tris-HCl-BSA buffer to a concentration of 50,000-100,000 cpm per 100 µl. The ^{125}I-prolactin was stored at 4°C in a BSA coated plastic beaker.

D. ^{125}I-prolactin Radioreceptor Assay of Prolactin Receptors in Enriched Plasma Membrane Preparations

This procedure was the standard assay procedure used in all assay experiments except when noted. It is similar to the assay developed by the group in Friesen's laboratory (Shiu et al., 1973).

For each enriched plasma membrane sample to be assayed a series of six 12x75 mm disposable culture tubes were used. Into three of these tubes (total binding tubes) 300 µl of Tris-HCl-BSA buffer were placed. Second, 100 µl of ^{125}I-prolactin (approximately 50,000-100,000 cpm) were added. Finally, 100 µl (300 µg protein) of enriched plasma membrane preparation were added.

In the second set of three tubes (nonspecific binding tubes) only 200 µl of Tris-HCl-BSA buffer were used. One µg of competing hormone contained in 100 µl of Tris-HCl-BSA buffer was added. The competing hormone normally was ovine prolactin (NIH-P-S-9) but in tests for specificity either ovine luteinizing hormone (NIH-LH-S-18) or ovine thyrotrophin (NIH-TSH-S-6) was used to compete with ^{125}I-prolactin.

Next, 100 µl of ^{125}I-prolactin (approximately 50,000-100,000 cpm) were added to these three tubes, Finally, 100 µl (300 µg protein) of enriched plasma membrane preparation were added. The incubation time began with the addition of this last component.

After 8 hours of incubation at room temperature 3 ml of Tris-HCl-BSA buffer at 4°C were added to each tube. The tubes were centrifuged in an International Model K centrifuge (International Equipment Company, Needham, MA) at 2,400 rpm (1,200g) for 30 minutes, decanted and blotted on absorbent paper. These tubes, along with a set of 6 total count tubes, were each counted for 60 seconds. The total count tubes contained 100 µl of the ^{125}I-prolactin used in the assay.

The percent specific binding was calculated from the counting data by the following equation.

$$\text{Percent Specific Binding} = \frac{\text{Average Counts of 3 Total Binding Tubes} - \text{Average Counts of 3 Nonspecific Binding Tubes} \times (100\%)}{(\text{Average Counts of 6 Total Count Tubes})}$$

IV. ^{125}I-prolactin Radioimmunoassay Procedure

The rat prolactin radioimmunoassay kit used in this lab was kindly provided by N.I.A.M.D.D. The assay procedure was that outlined in the kit's instructions. The standard curve of the assay used between 0.5 and 75 ng of standard prolactin (NIH-Rat Prolactin-RP-1, approximately 11 I.U. per mg). The assay used 20, 40 and 80 µl of serum for each blood sample tested.

The antibody dilutions for the rat prolactin radioimmunoassay were determined by double antibody titration. The total binding was always between 30 and 45%. The second antibody (sheep antirabbit gamma globulin) was provided by the laboratory of Dr. Joseph Meites, Department of Physiology.

EXPERIMENTAL PROCEDURES

This section is composed of two parts each containing multiple experiments. The first part contains experiments conducted to determine the validity and characteristics of the prolactin radioreceptor assay. Many of the procedural details involved in conducting this assay have been developed in other laboratories, thus, these procedures are found in the materials and methods section and only referred to in this section. These experiments are important because they show the validity of the assay when conducted by myself. The second part of this section is a series of confirmatory and original experiments both formulated and conducted by myself.

Part I of Experimental Section

I. The Effect of Increased Concentrations of Plasma Membrane Preparations on the Specific Binding of ^{125}I-prolactin

A. Objectives

The specific binding of ^{125}I-prolactin follows a Michaelis-Menten curve with a horizontal asymptote when the amount of receptors is varied over a wide range and the ^{125}I-prolactin is held constant. At high levels of receptor the curve is asymptotic and the percent specific binding serves as an estimate of the percent of radioactive hormone that is able to specifically bind. At lower receptor levels the percent specific binding varies in a nonlinear manner. It is in this lower range of receptor concentration that a radioreceptor assay gives percent specific binding results most closely proportional to the actual receptor number. The objectives of the experiments in this section were to:

1. determine the amount of enriched plasma membrane preparation most effective in ^{125}I-prolactin radioreceptor assays, and
2. determine the percent of ^{125}I-prolactin that is bindable after the first (G-50 sephadex column) and second purification (G-100 sephadex column) of the radiolabeled hormone.

B. Procedures

Several experiments were conducted using rat liver plasma membranes and the standard prolactin radioreceptor assay except as modified below. The membrane preparation used was an extremely high prolactin binder. The first experiment involved increasing amounts of enriched

plasma membranes varying from 0 μg to 1350 μg membrane protein (300 μg protein/100 μl) incubated with 10 μl ^{125}I-prolactin which had been purified twice. The total volume of each tube was 0.5 ml. The second experiment used values of 0 to 750 μg plasma membrane protein (300 μg/100 μl) of enriched liver plasma membranes with 100 μl of ^{125}I-prolactin which was purified twice and was conducted in a total volume of 0.5 ml. The last experiment used 100 μl of ^{125}I-prolactin that was purified once, and 0 to 8,000 μg plasma membrane protein (1168 μg/100 μl). These tubes contained a total volume of one milliliter.

An experiment was also conducted using lactating mouse mammary gland, a low prolactin binder. Three hundred to 900 μg of plasma membrane protein were incubated with 100 μl ^{125}I-prolactin that had been purified twice. The total volume of these assay tubes was 0.5 ml.

C. Results

^{125}I-prolactin binding followed a Michaelis-Menten curve in all studies (Figures 1-4). The specific binding reached asymptote at levels of receptor which were much higher than those used in the standard prolactin radioreceptor assay. The amount of plasma membrane protein used in the standard radioreceptor assay produced binding in a portion of the curve where differing binding capacities produced a nearly proportional change in percent specific binding (Figures 2 and 3).

The maximal percent specific binding was obtained from ^{125}I-prolactin that was purified twice (Figure 1). Approximately 28% of such preparation bound specifically to prolactin receptors. The maximal percent specific binding obtained from ^{125}I-prolactin that had been purified

once was 25%. Although the second purification of ^{125}I-prolactin did not greatly increase the percent specific binding, it did remove remaining lactoperoxidase and ^{125}I.

Figure 1. Maximal Percent Specific Binding of ^{125}I-prolactin to Varying Concentrations of Rat Liver Membrane Preparations (0-1350 μg membrane protein).

Approximately 0.14 ng of ^{125}I-prolactin and varying amounts of an enriched plasma membrane preparation were incubated for 8 hours at 23°C. The membrane preparation was obtained from a liver of a female rat. The ^{125}I-prolactin was twice purified.

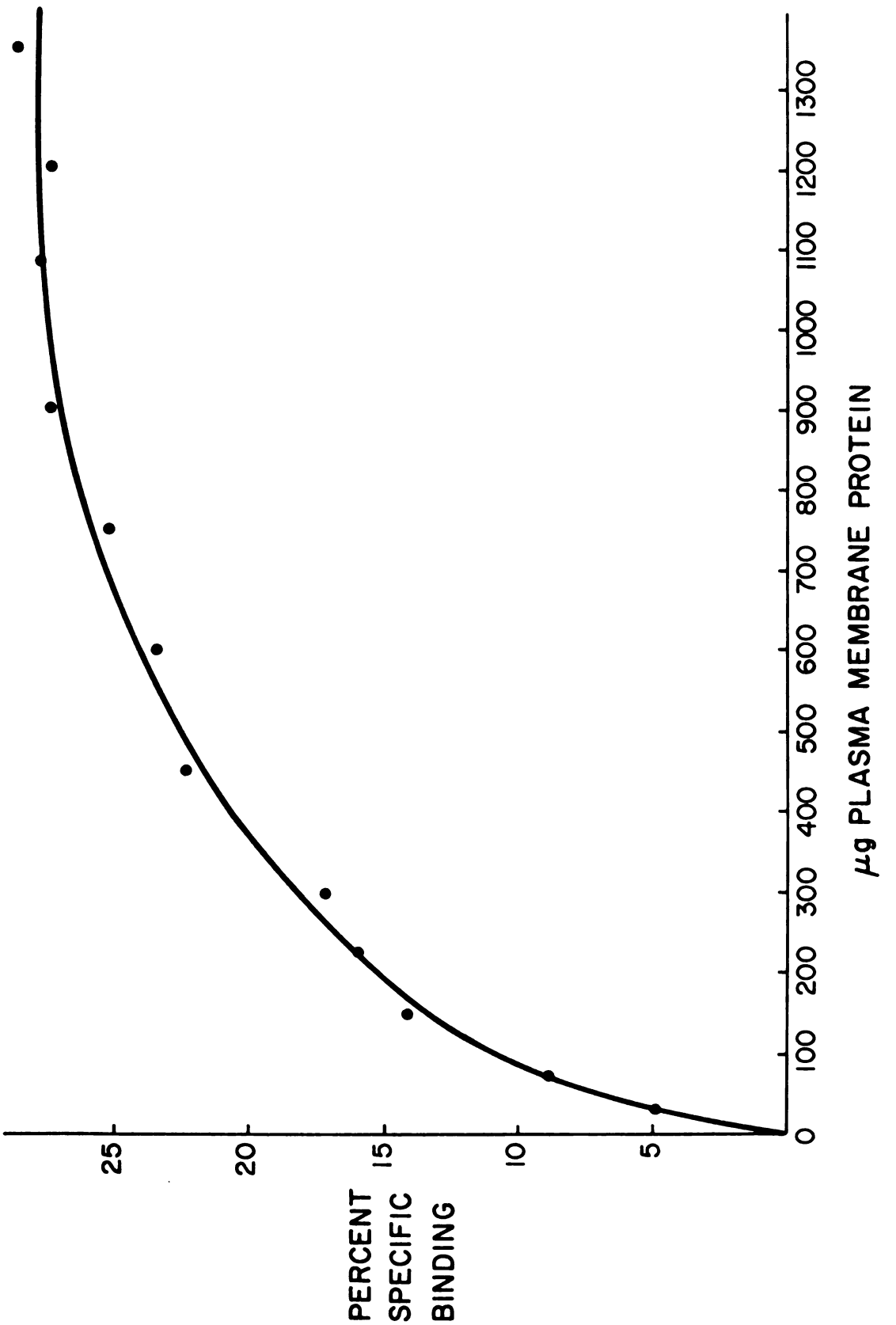

PERCENT SPECIFIC BINDING
25
20
15
10
5
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
μg PLASMA MEMBRANE PROTEIN

Figure 2. Maximal Percent Specific Binding of ^{125}I-prolactin to Varying Concentrations of Rat Liver Membrane Preparations (0-750 μg membrane protein).

Approximately 0.14 ng of ^{125}I-prolactin and varying amounts of an enriched plasma membrane preparation were incubated for 8 hours at 23°C. The membrane preparation was obtained from a liver of a female rat. The ^{125}I-prolactin was twice purified.

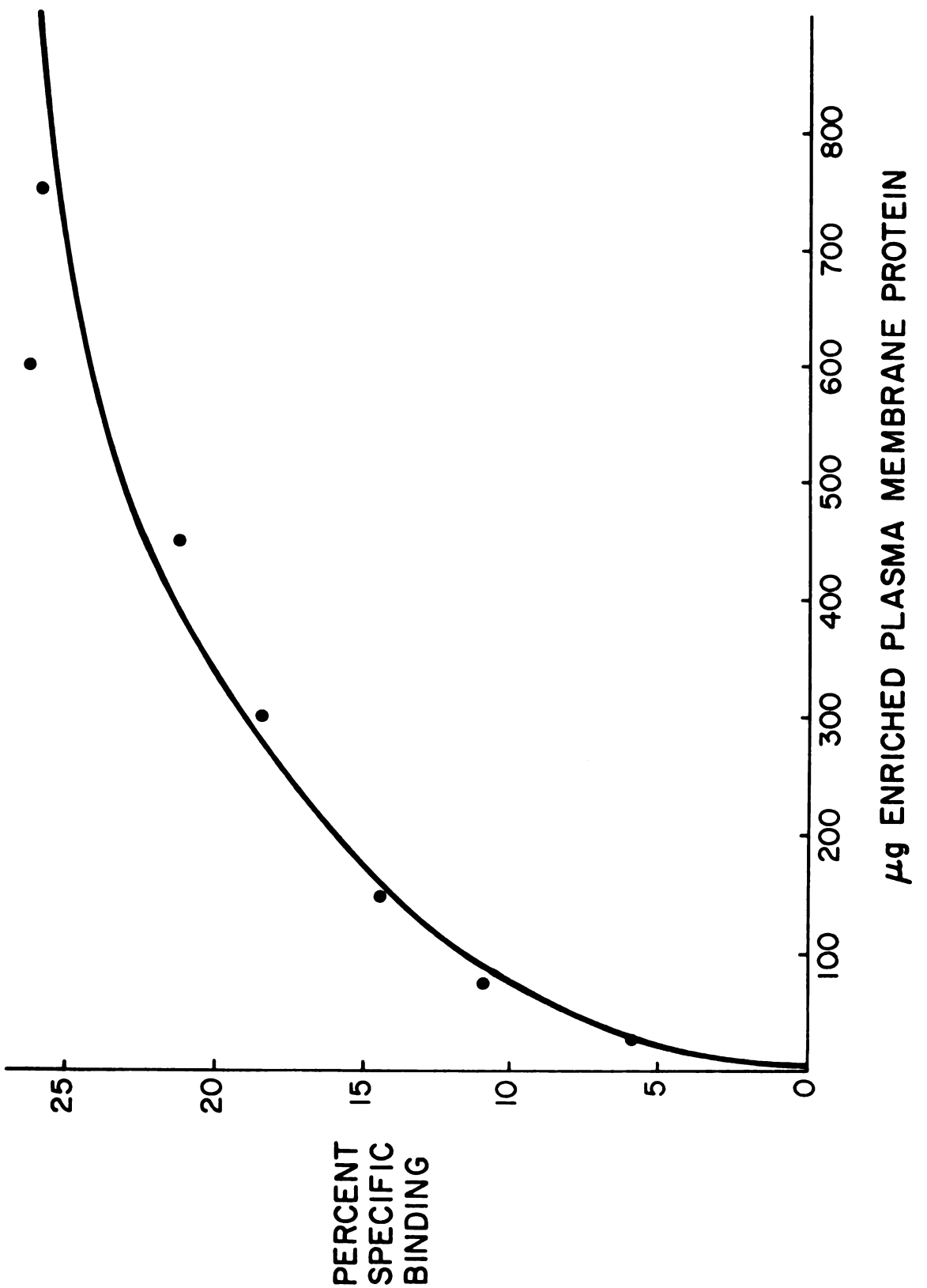

PERCENT SPECIFIC BINDING
25
20
15
10
5
0
100
200
300
400
500
600
700
800
μg ENRICHED PLASMA MEMBRANE PROTEIN

Figure 3. Maximal Percent Specific Binding of ^{125}I-prolactin to Varying Concentrations of Rat Liver Membrane Preparations (0-800 μg membrane protein).

Approximately 0.14 ng of ^{125}I-prolactin and varying amounts of an enriched plasma membrane preparation were incubated for 8 hours at 23°C. The membrane preparation was obtained from a liver of a female rat. The ^{125}I-prolactin was once purified.

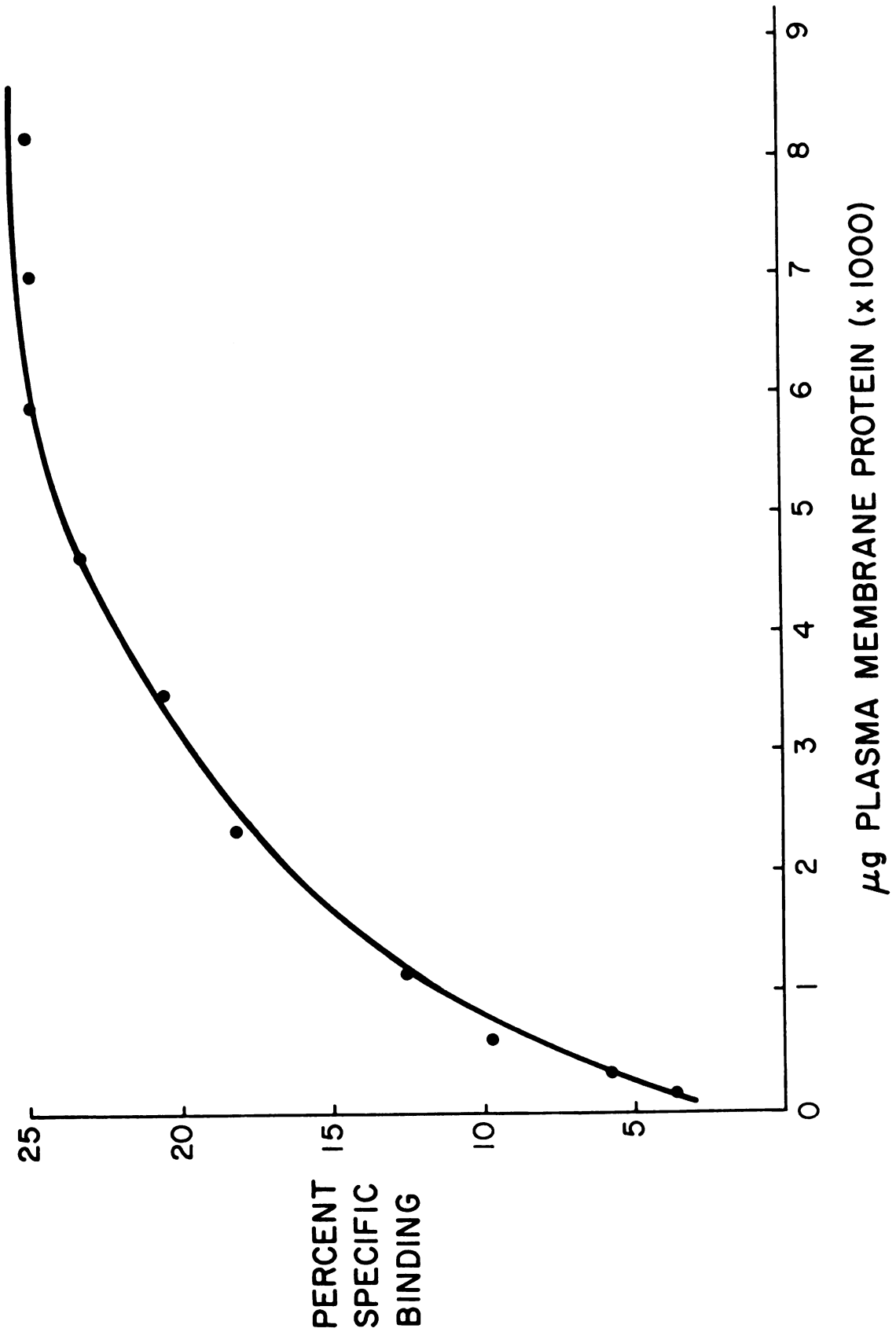
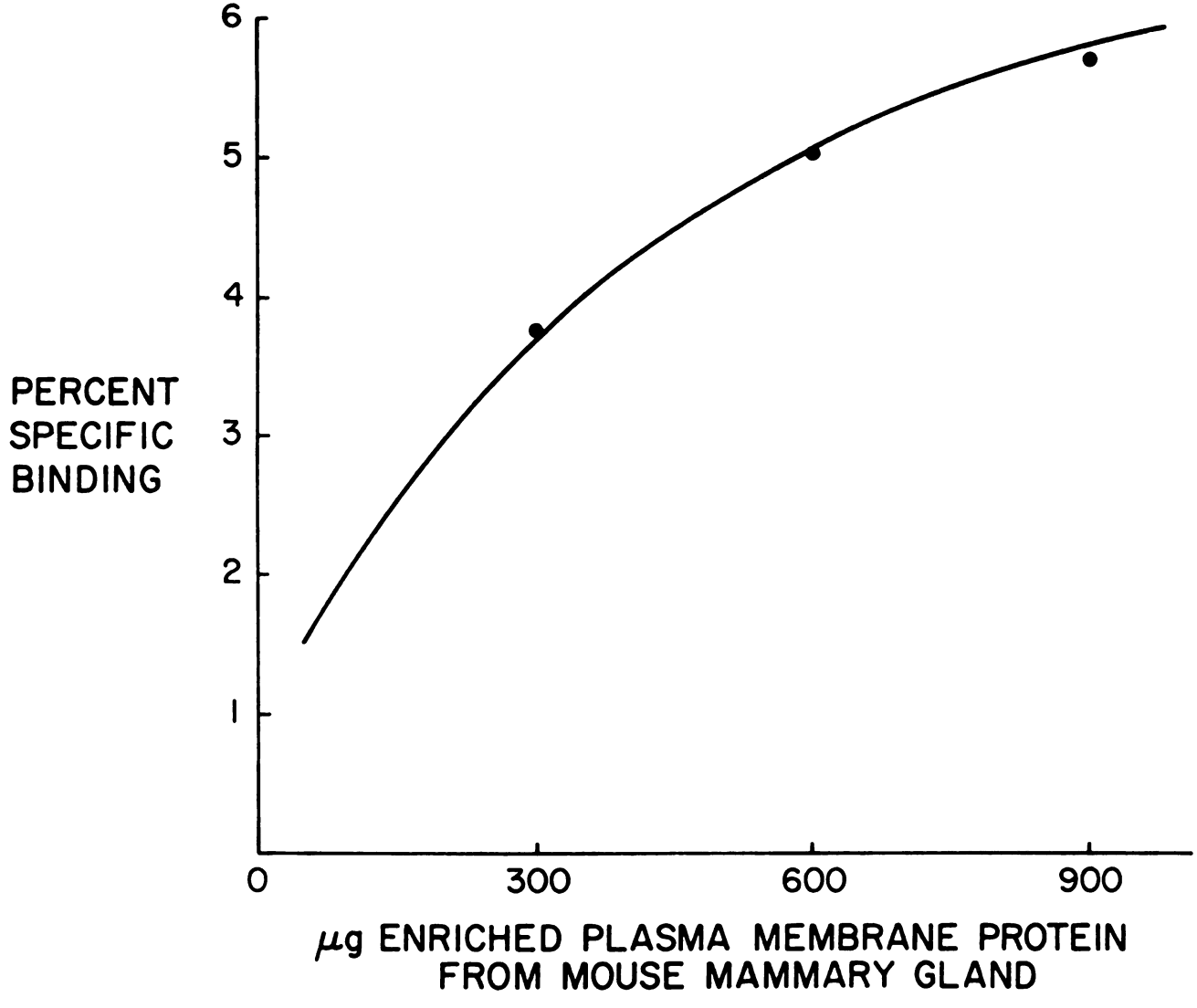

PERCENT SPECIFIC BINDING
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
μg PLASMA MEMBRANE PROTEIN (x 1000)

Figure 4. Percent Specific Binding of ^{125}I-prolactin to Varying Concentrations of Mouse Mammary Gland Membrane Preparations.

Approximately 0.14 ng of ^{125}I-prolactin and varying amounts of an enriched plasma membrane preparation were incubated for 8 hours at 23°C. The membrane preparation was obtained from a mammary gland of a lactating mouse. The ^{125}I-prolactin was twice purified.


6
5
4
3
2
1
PERCENT
SPECIFIC
BINDING
0
300
600
900
μg ENRICHED PLASMA MEMBRANE PROTEIN
FROM MOUSE MAMMARY GLAND

II. The Effect of Increased Concentrations of ^{125}I-prolactin on the Specific Binding of ^{125}I-prolactin

A. Objectives

Increasing ^{125}I-prolactin to very high levels can demonstrate whether or not the binding of ^{125}I-prolactin to receptor sites is a saturable process. The saturability of a specific set of prolactin receptors is a manifestation of the properties of the prolactin receptors. Certain properties of these receptors are that they appear to be unique types of protein and that the binding sites of these proteins all have identical affinity for the hormone. Thus the hormone receptor should follow a Michaelis-Menten curve when combined with its specific hormone. It is this Michaelis-Menten curve that one should observe when one studies increases of ^{125}I-prolactin with a constant amount of receptor protein.

Nonspecific binding is random binding to multiple sets of sites with varied low affinities. It is assumed that the number of such nonspecific sites is so large that as a matter of practicality they are unsaturable. Thus, it should be demonstrable that nonspecific binding should increase in direct proportion to increases in ^{125}I-prolactin.

B. Procedures

A series of standard prolactin radioreceptor assays were conducted in the normal manner but with the following procedural exceptions. First, the total volume of all tubes was 900 µl. The large volume was required to contain the increasing doses of ^{125}I-prolactin. Second, each assay set was one point of the ^{125}I-prolactin range, and has its

own total count tubes. Third, with each assay set an increased dose of ^{125}I-prolactin was given. In this experiment doses of 0 through 700 µl ^{125}I-prolactin were used. A relatively undiluted preparation was used in this experiment (313,512 cpm/100 µl). 300 µg of rat liver plasma membrane protein was the source of the prolactin receptors.

The ^{125}I-prolactin counts specifically bound verses the total counts of each assay set are plotted in Figure 5. The nonspecific ^{125}I-prolactin counts of each assay set are plotted against the total counts (^{125}I-prolactin) of the sets in Figure 6.

C. Results

The specific binding of ^{125}I-prolactin to rat liver membranes followed a Michaelis-Menten curve (Figure 5). This indicates that the prolactin receptors are a saturable set of protein binders. Figure 5 also demonstrated that in the normal prolactin radioreceptor assay system the receptors were not saturated, but only a portion were bound to ^{125}I-prolactin. Finally, with a very high number of counts in the incubation tube, the specific binding became variable. Rodbard predicts this type of effect when near saturating levels of ^{125}I-prolactin are used (Rodbard, 1973).

The nonspecific binding (Figure 6) was linear throughout the entire range of total counts. This indicates that the nonspecific sites were not saturable at these levels. The nonspecific binding became so large at high total counts that the variability of such numbers may mask moderate specific binding. Thus, using a moderate level of total counts is the best to discern specific ^{125}I-prolactin binding accurately. It appears that 50,000 cpm to 100,000 cpm per 100 µl in the standard assay

sets is optimal. It is high enough to be convenient and provide efficient counting, but small enough to keep the amount and the variability of the nonspecific binding at a fairly low level.

Figure 5. Percent Specific Binding of ^{125}I-prolactin to Rat Liver Membrane Preparations. Influence of Varying Concentrations of ^{125}I-prolactin.

300 μg of enriched plasma membrane protein were incubated for 8 hours at 23°C with varying doses of ^{125}I-prolactin. The membrane preparation was obtained from a liver of a female rat.

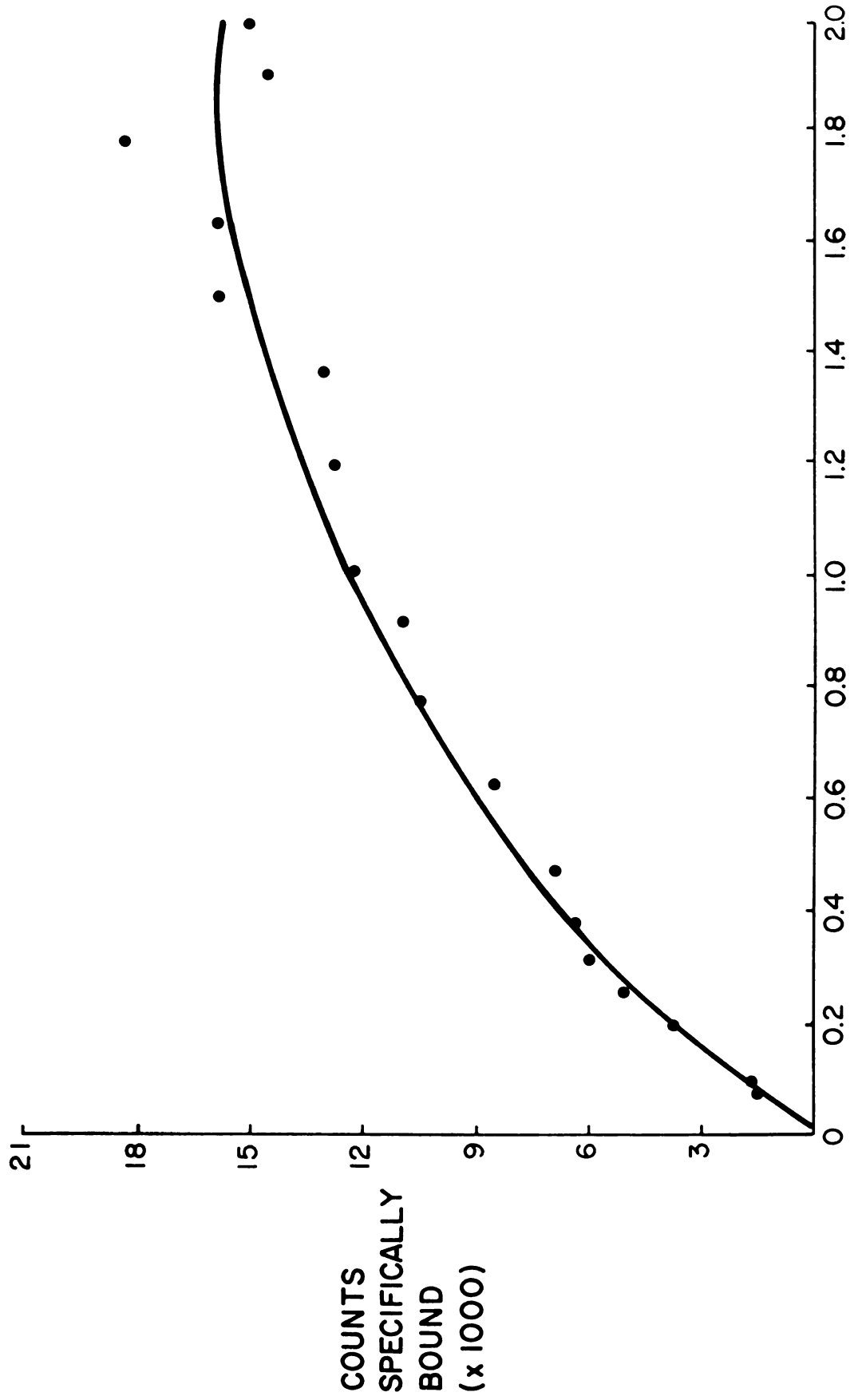
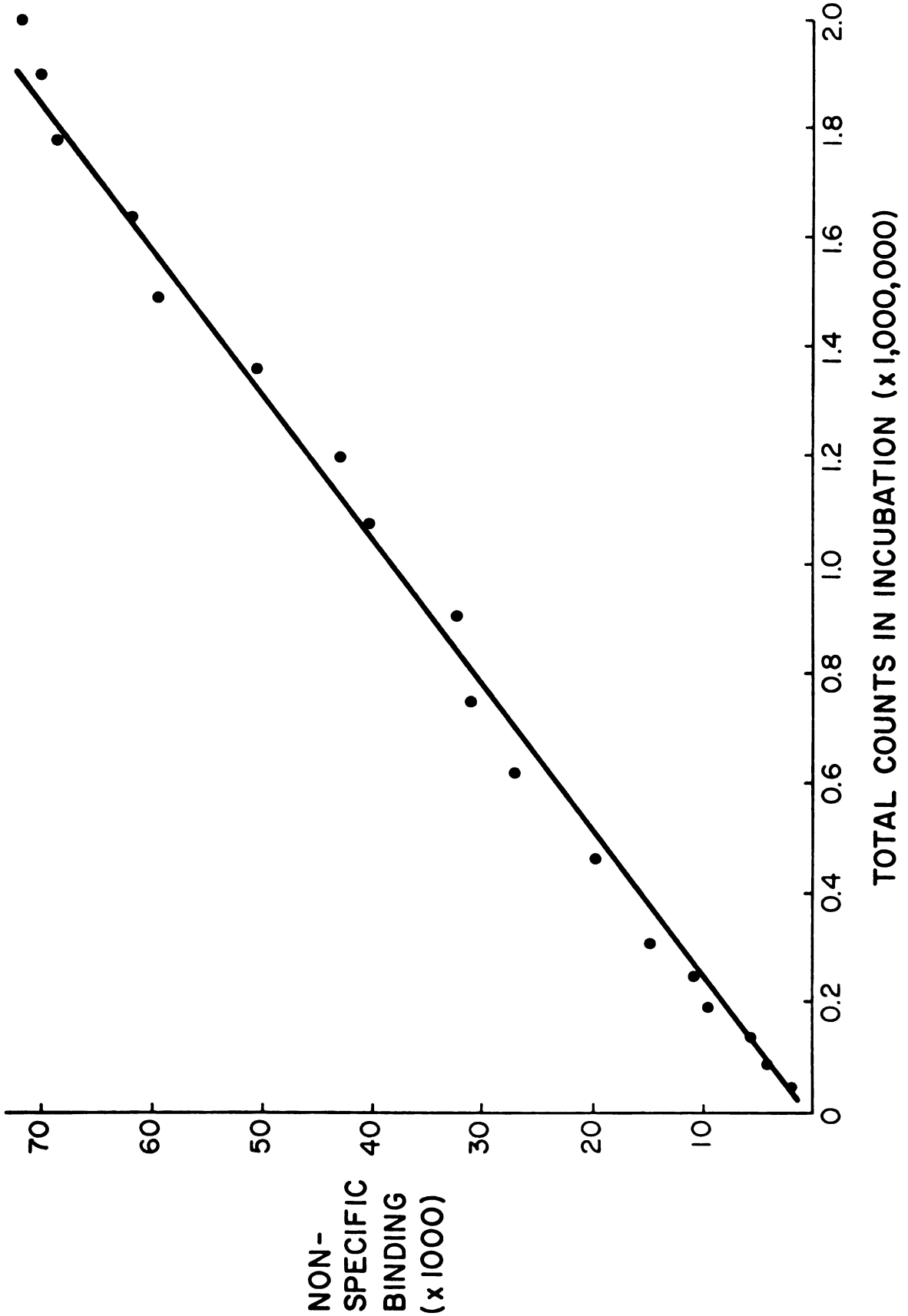

COUNTS SPECIFICALLY BOUND (x 1000)
21
18
15
12
9
6
3
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
TOTAL COUNTS IN INCUBATION (x 1,000,000)

Figure 6. Nonspecific Binding of ^{125}I-prolactin to Rat Liver Membrane Preparations. Influence of Varying Concentrations of ^{125}I-prolactin.

300 μg of enriched plasma membrane protein were incubated for 8 hours at 23°C with varying doses of ^{125}I-prolactin. The membrane preparation was obtained from a liver of a female rat.


NON-
SPECIFIC
BINDING
(x 1000)
70
60
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
TOTAL COUNTS IN INCUBATION (x 1,000,000)

III. Estimate of the Amount of Bindable ^{125}I-prolactin Used in the Prolactin Radioreceptor Assay

A. Objectives

A knowledge of the amount of bindable prolactin used in the radioreceptor assay is an important piece of data. This amount must be known to conduct kinetic studies and Scatchard analysis. The accuracy of such studies depends on an exact estimate of ^{125}I-prolactin. One method that quantifies prolactin is the radioimmunoassay. Radioimmunoassays bind on the basis of antigenic site, which is a sequence of several amino acid residues. Thus, the radioimmunoassay cannot give an accurate value of biologically bindable prolactin but only gives an accurate estimate of the total immuno-reactive prolactin. The amount of prolactin with a specific three dimensional structure needs to be known to estimate bindable prolactin. But radioimmunoassay data on total prolactin can be used in conjunction with the maximal percent binding of ^{125}I-prolactin (Figure 4) to estimate the amount of bindable hormone. In this experiment this type of procedure is done to estimate the nanograms of bindable prolactin of a sample identical in size (100 µl) to that normally used in radioreceptor assays and Scatchard analysis.

This study is limited because of the use of rat prolactin rather than ovine prolactin. This is due to the lack of an ovine prolactin radioimmunoassay in this lab. This may be overcome if the behavior of rat prolactin can be shown to be similar to the ovine prolactin. The similarity of behavior of the rat and ovine prolactins may be compared by using the ^{125}I-rat prolactin in a Scatchard analysis and by comparing the K_a and total receptors to those from Scatchard analysis conducted with ^{125}I-ovine prolactin.

B. Procedures

Five micrograms of rat prolactin (N.I.A.M.D.D.-rat prolactin-I-1, 30 IU/mg) was iodinated with ^{125}I in an identical manner to the ovine prolactin normally used in radioreceptor assays (Materials and Methods). The ^{125}I-prolactin was not diluted to the normal 50,000-100,000 cpm/100 µl. Thus, an amount of ^{125}I-prolactin of an assayable size and in a reasonable volume could be used in an ^{131}I-rat prolactin radioimmunoassay. The ^{125}I-rat prolactin had 256,057 cpm/100 µl. Aliquots of 20 µl (53,975 cpm), 40 µl (100,925 cpm), 80 µl (200,141 cpm) and 160 µl (402,981 cpm) of the ^{125}I-rat prolactin were tested six times each in a radioimmunoassay where the competing prolactin was labeled with ^{131}I. The assay was conducted as outlined in the Materials and Methods section of the thesis. The cross-over of ^{125}I counts to the ^{131}I settings was 0.0% when using the proper windows on a Nuclear Chicago 1185 Series Gamma Counter. The fraction of bindable ^{125}I-prolactin was discerned in a previous experiment as 0.28, and this value was used to determine the ng/100 µl of bindable ^{125}I-prolactin.

To demonstrate the similarity of ^{125}I-rat prolactin to the ^{125}I-ovine prolactin a Scatchard plot was also developed using the ^{125}I-rat prolactin diluted to 99,101 cpm/100 µl and unlabeled ovine prolactin.

C. Results

The ^{131}I-rat prolactin radioimmunoassay produced a value of 1.45 ng prolactin per 100 µl of the undiluted (256,057 cpm/100 µl) ^{125}I-rat prolactin. In a normal prolactin radioreceptor assay where approximately 90,000 cpm/100 µl are used the prolactin levels would have been approximately 0.51 ng/100 µl. 28% of this prolactin (Figure 1) was bindable.

Therefore, 0.14 ng/100 µl of ^{125}I-prolactin was calculated to be the amount involved in the binding of prolactin receptors (Table 1). This was the value that was used in calculating the Scatchard plots in this thesis. The radioimmunoassay competitive binding curve is illustrated in Figure 7.

The Scatchard plot (Figure 8) provided an association constant similar to those produced by ovine prolactin and a total prolactin binding in the normal range for 300 µg of rat liver plasma membrane protein. These values were a good indication that rat prolactin behaved similarly to ovine prolactin in this assay system and that 0.14 ng/100 µl was a good estimate of the bindable prolactin used in the radioreceptor assay.

Table 1. Calculation of the Bindable ^{125}I-prolactin in a Typical 90,000 cpm Sample

1. ^{131}I-radioimmunoassay value for a 256,057 cpm Sample of ^{125}I-rat Prolactin.

   1.45 ng

2. Adjusted ^{131}I-radioimmunoassay Value for a 90,000 cpm Sample of ^{125}I-rat Prolactin.

   0.51 ng

3. Adjustment of Amount of Bindable ^{125}I-rat Prolactin by the Assumption of 28% Specifically Bindable Prolactin.

   0.14 ng

4. It is reasonable to conclude that approximately 0.14 ng of prolactin in a typical 100 µl (90,000 cpm) volume of ^{125}I-prolactin is capable of specific binding to prolactin receptors. This is the value which will be assumed to be contained in each assay tube in subsequent studies reported in this thesis.

Figure 7. Competitive Binding Curve for ^{131}I-rat Prolactin.

This curve was used in the radioimmunoassay of ^{125}I-rat prolactin.

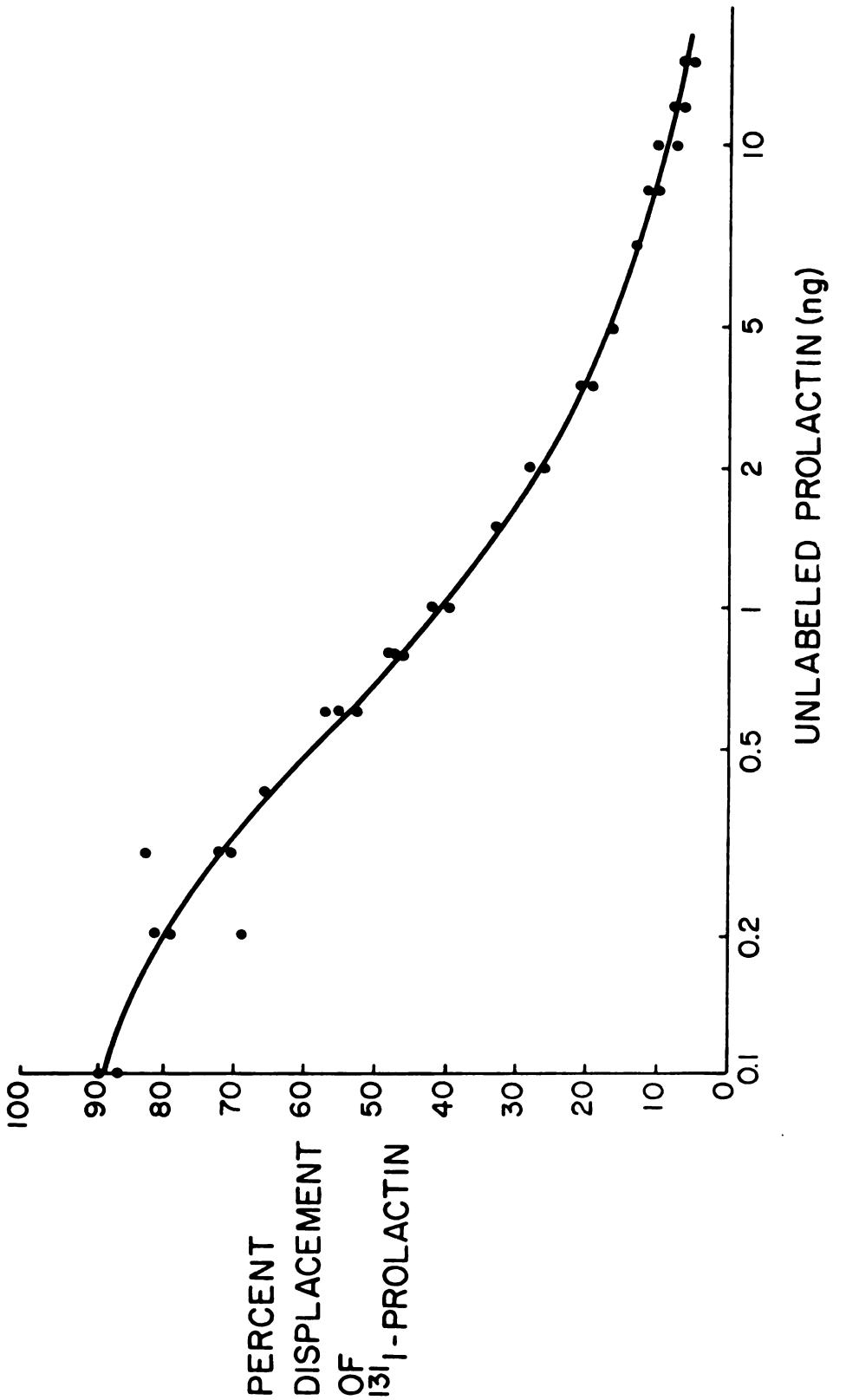
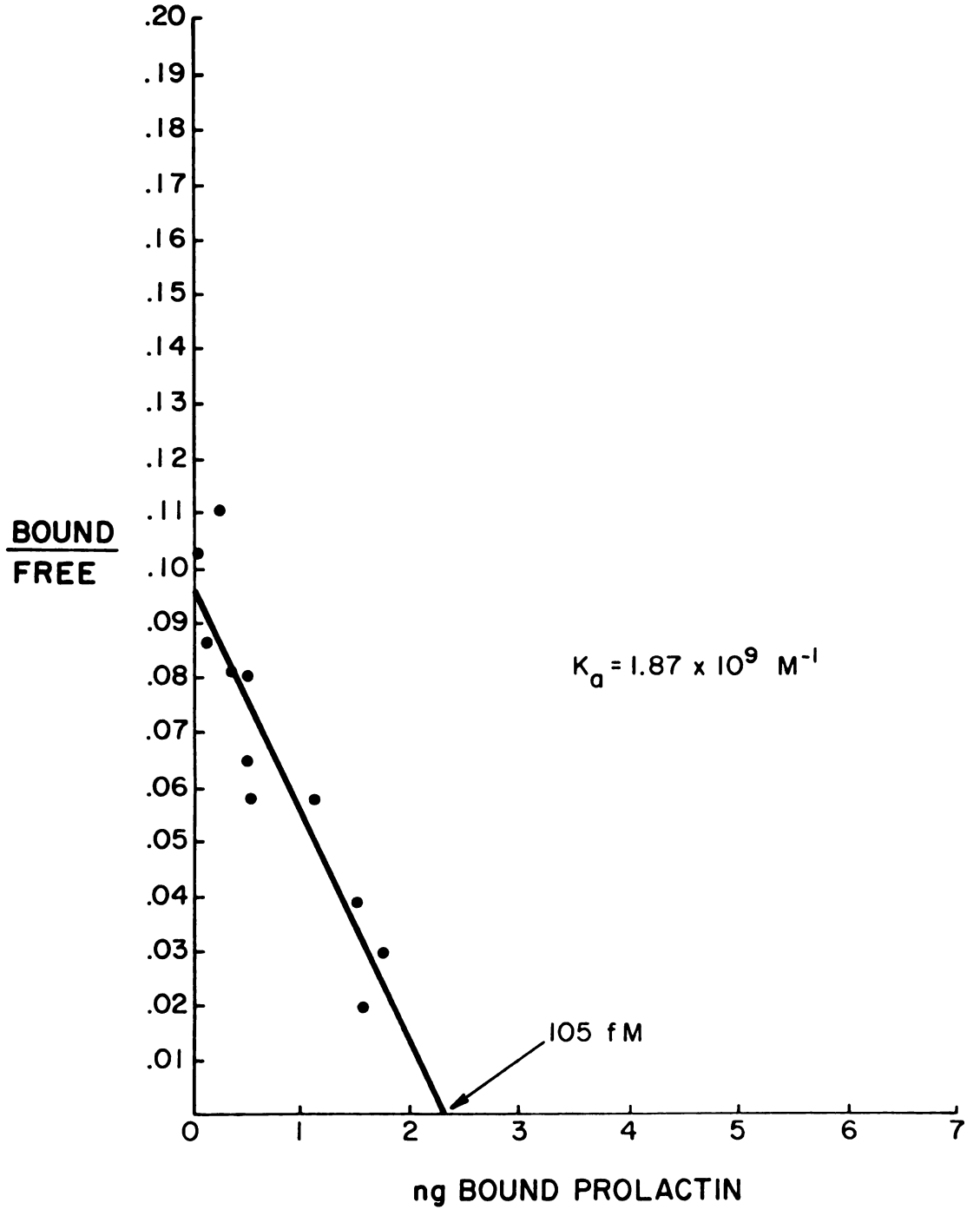

PERCENT
DISPLACEMENT
OF
^{131}I-PROLACTIN
100
90
80
70
60
50
40
30
20
10
0
0.1
0.2
0.5
1
2
5
10
UNLABELED PROLACTIN (ng)

Figure 8. Scatchard Plot for Prolactin Receptors of an Enriched Plasma Membrane Preparation From a Female Rat Using ^{125}I-rat Prolactin and Ovine Prolactin.

^{125}I-rat prolactin and ovine prolactin compete for prolactin receptors to generate the competitive binding curve from which this Scatchard plot is produced. Note the similar K_a of this and the Scatchard plots produced in the next experiment. The similar K_a's suggests equivalent affinities for the rat liver prolactin receptor and a similar three-dimensional structure of rat and ovine prolactin.


.20
.19
.18
.17
.16
.15
.14
.13
.12
.11
.10
.09
.08
.07
.06
.05
.04
.03
.02
.01
BOUND
FREE
$K_a = 1.87 \times 10^9\ M^{-1}$
105 f M
0
1
2
3
4
5
6
7
ng BOUND PROLACTIN

IV. Scatchard Plots and Hormone Binding Specificity

A. Objectives

The Scatchard plots in this thesis were generated from competitive binding curve data. The competitive binding curve is a generally accepted method to show specificity of the receptor population. Specificity is shown by the progressive diminution of ^{125}I-prolactin binding with increasing amounts of nonlabeled prolactin while other hormones fail to promote this process.

Scatchard plots produced from the competitive binding data provide three pieces of information. First, the negative slope of the plot is an estimate of the association constant of the hormone and receptor. Second, the intercept of the plot on the abscissa is an estimate of the total moles of receptor. Third, the shape of the plot can give an indication as to whether the receptor is one set of homogenous binding sites (straight line) or whether more complex hormone receptor interactions are occurring. Several complex reactions are: multiple classes of receptors, negative cooperativity among receptors, an exchange or two-step binding reaction (all concave upward plots) (Boeynaems et al., 1975; Boeynaems and Dumont, 1975).

In this experiment competitive binding curves and Scatchard plots are constructed for all tissues in this research which exhibit significant ^{125}I-prolactin binding. These tissues are livers (from mice and rats), mammary glands (from pregnant rats and lactating mice) and mammary tumors (from carcinogen treated female rats).

B. Procedures

Competitive binding curves and Scatchard plots were produced for every tissue by using 0.1 to 1000 ng of competing hormone. In addition a series of Scatchard plots were produced using a single rat liver membrane preparation with the same ^{125}I-prolactin preparation 1,3,7 and 9 days after iodination. This effort provided an estimate of the usefulness of ^{125}I-prolactin preparations over several days and estimates of the variability of association constants and total receptors. All Scatchard plots conducted during the course of this research were consolidated and are listed in tabular form on Table 3.

C. Results

The competitive binding curves and Scatchard plots for the various tissues are presented in Figures 9-18. The ^{125}I-prolactin association constants were consistent in all rat tissues and in all mouse tissues, but the mouse tissues ($10^{10}M^{-1}$) bound the hormone with an order of magnitude greater than that of the rat tissues ($10^{9}M^{-1}$). The results of the Scatchard plots of an ^{125}I-prolactin preparation tested on various days after iodination are displayed in Table 2. Use of ^{125}I-prolactin from 1-9 days after iodination did not appear to markedly influence the binding capacity of the iodinated hormone.

The competitive binding curves all showed specificity. Only prolactin effectively reduced the binding of ^{125}I-prolactin. Luteinizing hormone, thyroid stimulating hormone, and growth hormone did not reduce ^{125}I-prolactin binding.

The lactating mouse mammary gland was the most difficult tissue to demonstrate specific prolactin binding. This tissue possessed approximately 10 times less prolactin receptors than the other tissues tested. Table 3 is a complete list of prolactin association constants and fM of prolactin receptors derived from Scatchard plot analyses.

Table 2. Scatchard Plot Data for Prolactin Membrane Receptors of a Female Rat Liver Using an ^{125}I-prolactin Preparation on Various Days After Iodination

Day	fM per 300 µg protein	K_a ($\times 10^9 M^{-1}$)
1	221	3.73
3	172	2.04
7	235	4.22
9	156	2.90

Mean ± S.E.M. for fM receptor: 196 ± 19 fM

Mean ± S.E.M. for K_a: $3.22 \pm 0.48 \times 10^{9} M^{-1}$

Table 3. Cumulative Data for All ^{125}I-prolactin Scatchard Plots

Rat Tissues	fM Receptors per 300 µg Protein	K_a (x 10^9 M^{-1})
Livers (9) (obtained from female rats)	105	1.87
	241	4.69
	221	3.73
	172	2.04
	235	4.22
	156	2.90
	159	5.78
	195	2.60
	173	3.90
Mammary Gland (1) (obtained from a pregnant rat)	223	1.06
Mammary Tumors (4) (obtained from DMBA-treated female rats)	97	4.44
	51	3.77
	55	2.01
	110	1.21

Mouse Tissues	fM Receptors per 300 µg Protein	K_a (x 10^{10} M^{-1})
Livers (2) (obtained from female mice)	168	3.92
	239	3.51
Mammary Glands (4) (obtained from lactating mice)	13	1.12
	14	6.01
	11	2.50
	18	2.55

Figure 9. ^{125}I-prolactin Competitive Binding Curve for Prolactin Membrane Receptors From a Liver Obtained From a Female Rat

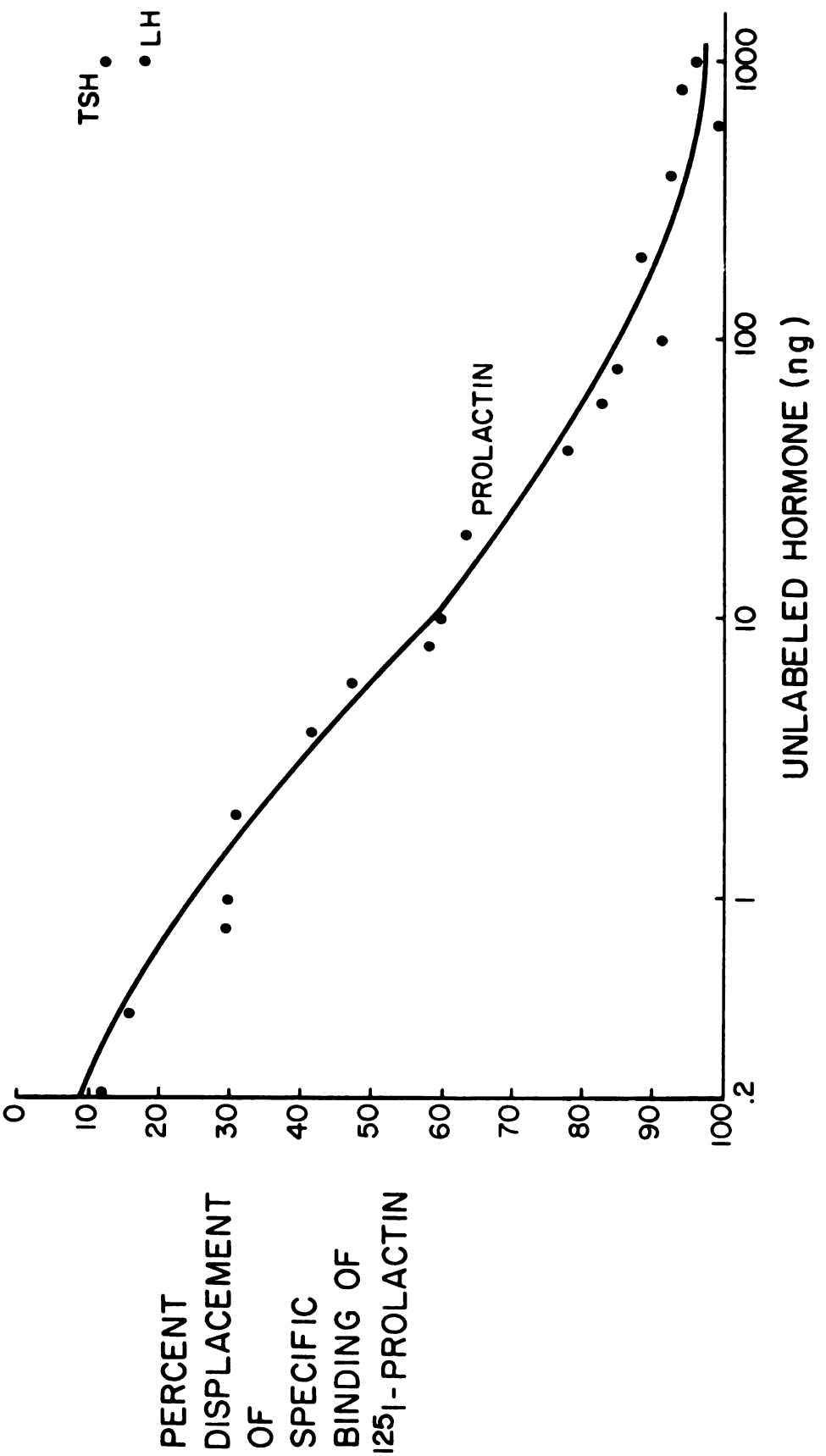

TSH
LH
PROLACTIN
PERCENT DISPLACEMENT OF SPECIFIC BINDING OF 125I-PROLACTIN
0
10
20
30
40
50
60
70
80
90
100
.2
1
10
100
1000
UNLABELED HORMONE (ng)

Figure 10. ^{125}I-prolactin Scatchard Plot for Prolactin Membrane Receptors From a Liver Obtained From a Female Rat

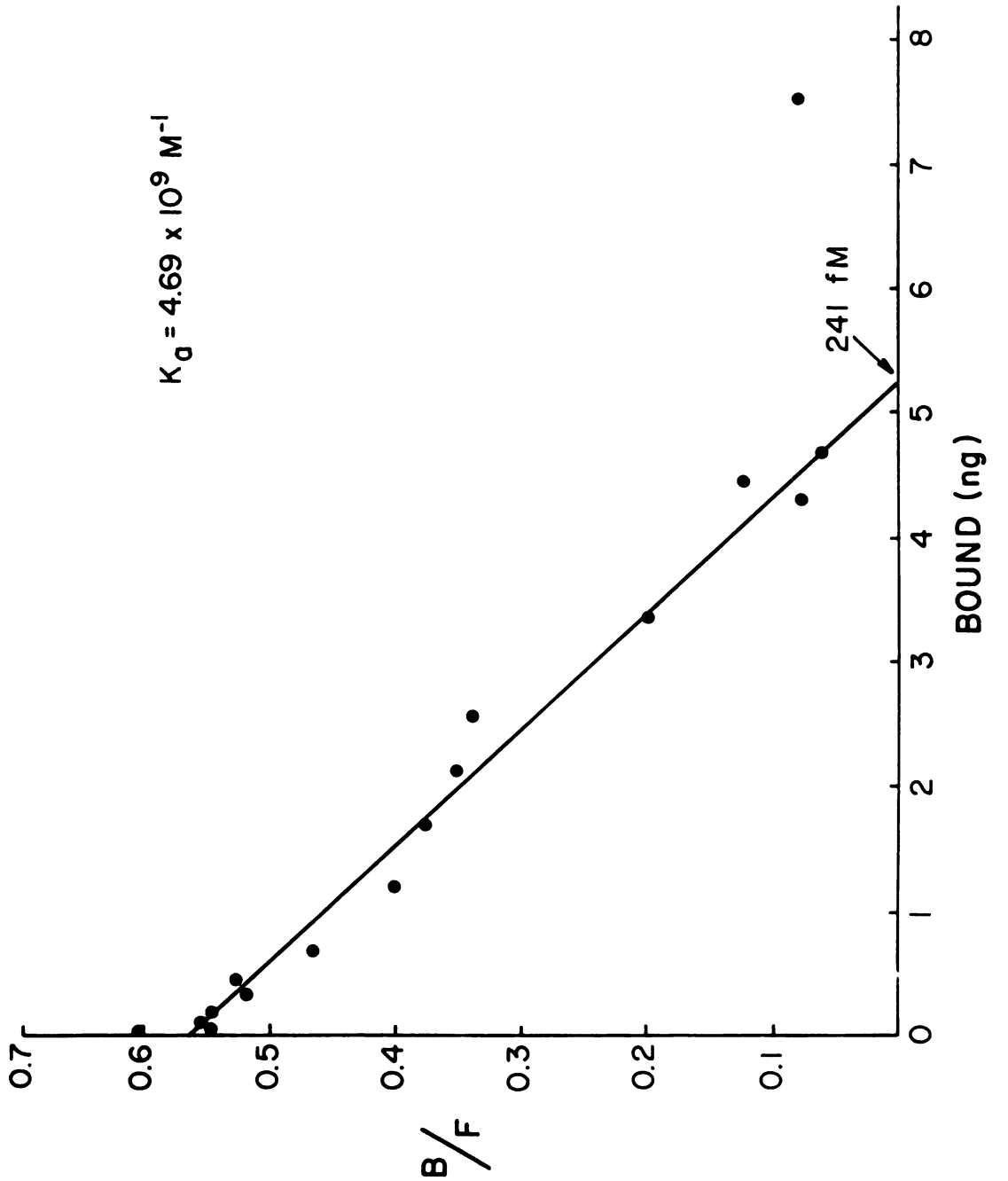

$K_a = 4.69 \times 10^9\ M^{-1}$
241 fM
B/F
BOUND (ng)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8

Figure 11. ^{125}I-prolactin Competitive Binding Curve for Prolactin Membrane Receptors From a Mammary Gland Obtained From a Pregnant Rat

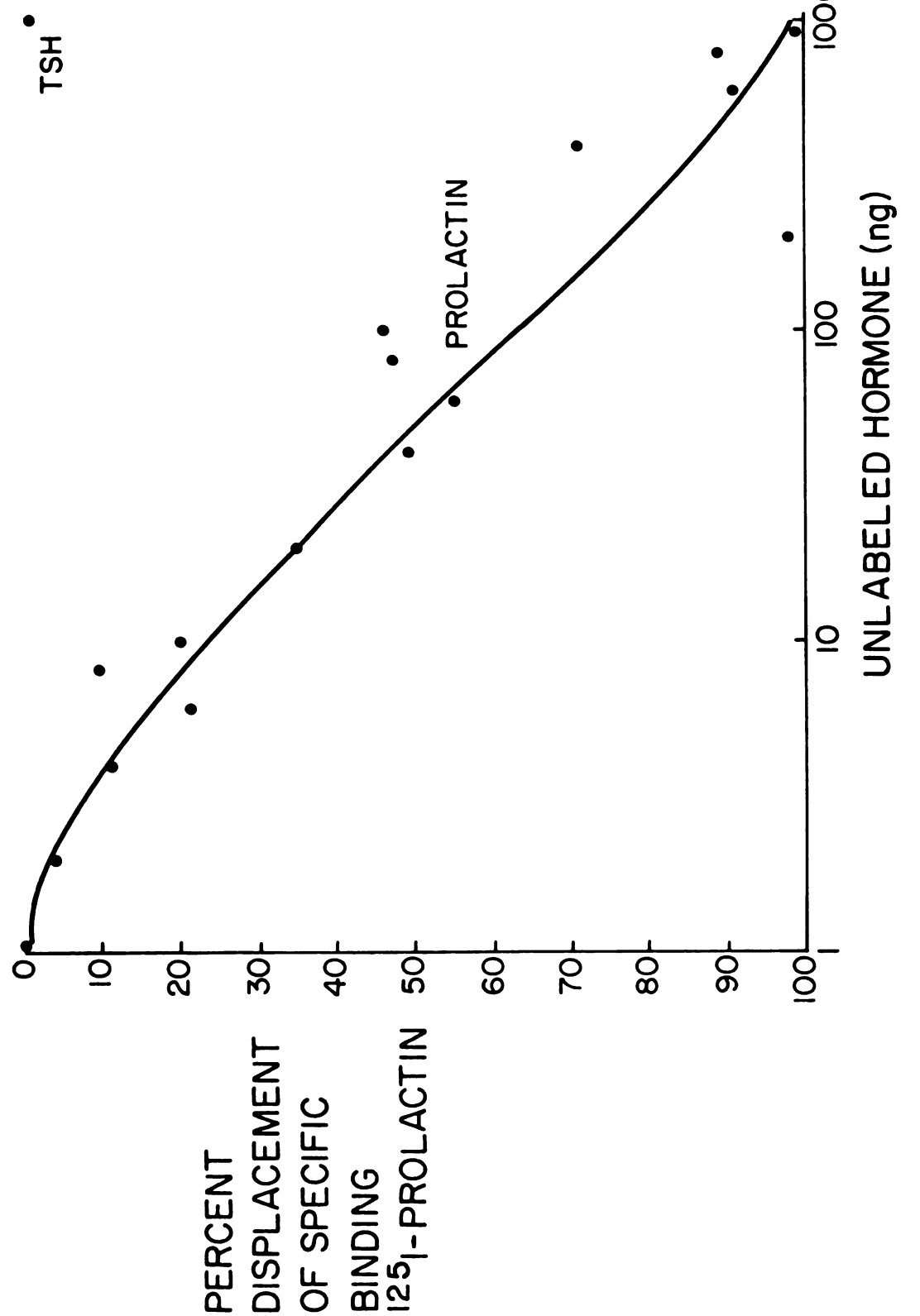

TSH
PROLACTIN
PERCENT DISPLACEMENT OF SPECIFIC BINDING ^{125}I-PROLACTIN
0
10
20
30
40
50
60
70
80
90
100
1
10
100
1000
UNLABELED HORMONE (ng)

Figure 12. ^{125}I-prolactin Scatchard Plot for Prolactin Membrane Receptors From a Mammary Gland Obtained From a Pregnant Rat

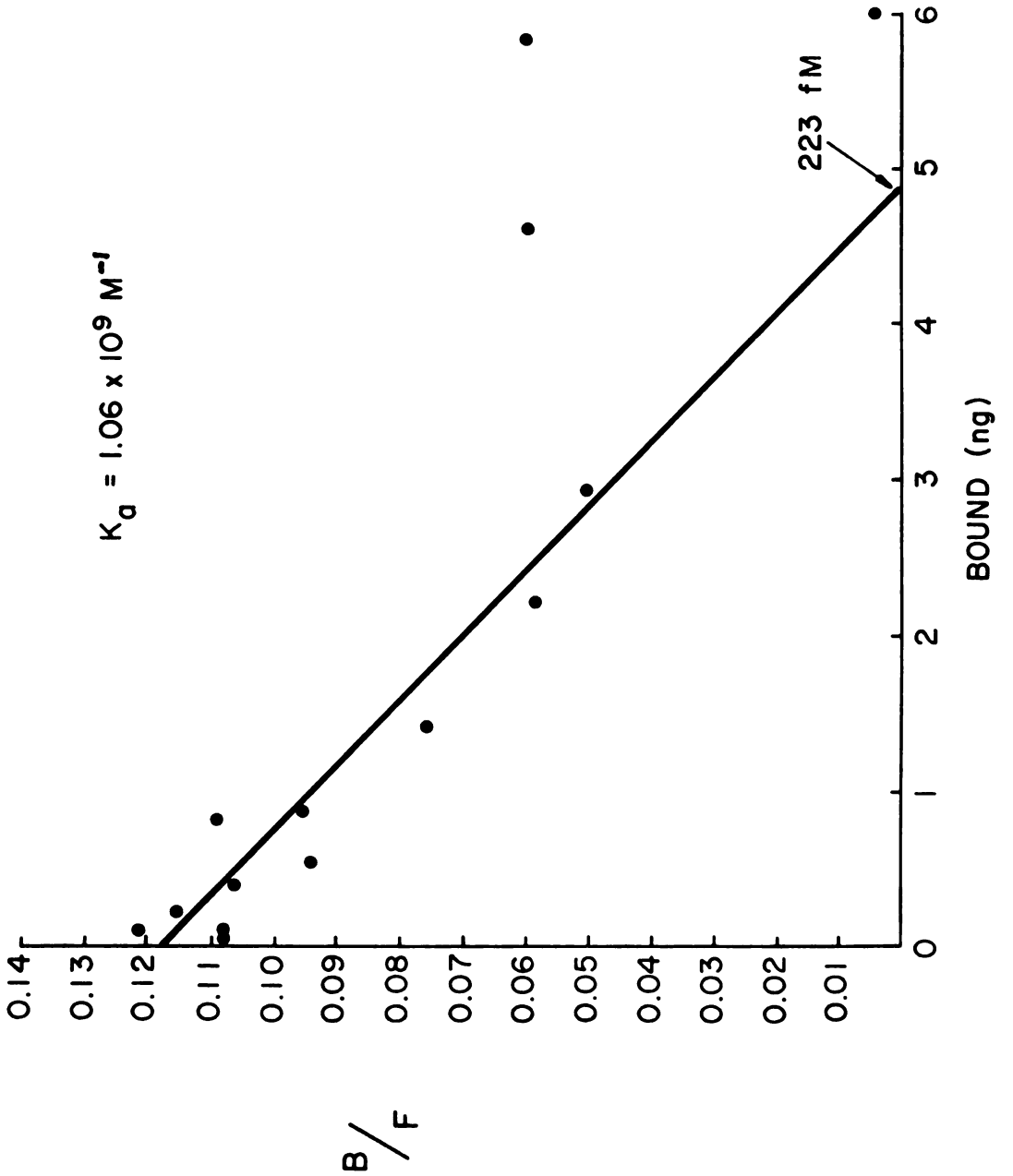

K_a = 1.06 x 10^9 M^{-1}
223 fM
B/F
BOUND (ng)
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1
2
3
4
5
6

Figure 13. ^{125}I-prolactin Competitive Binding Curve for Prolactin Membrane Receptors From a DMBA-induced Mammary Tumor Obtained From a Female Rat

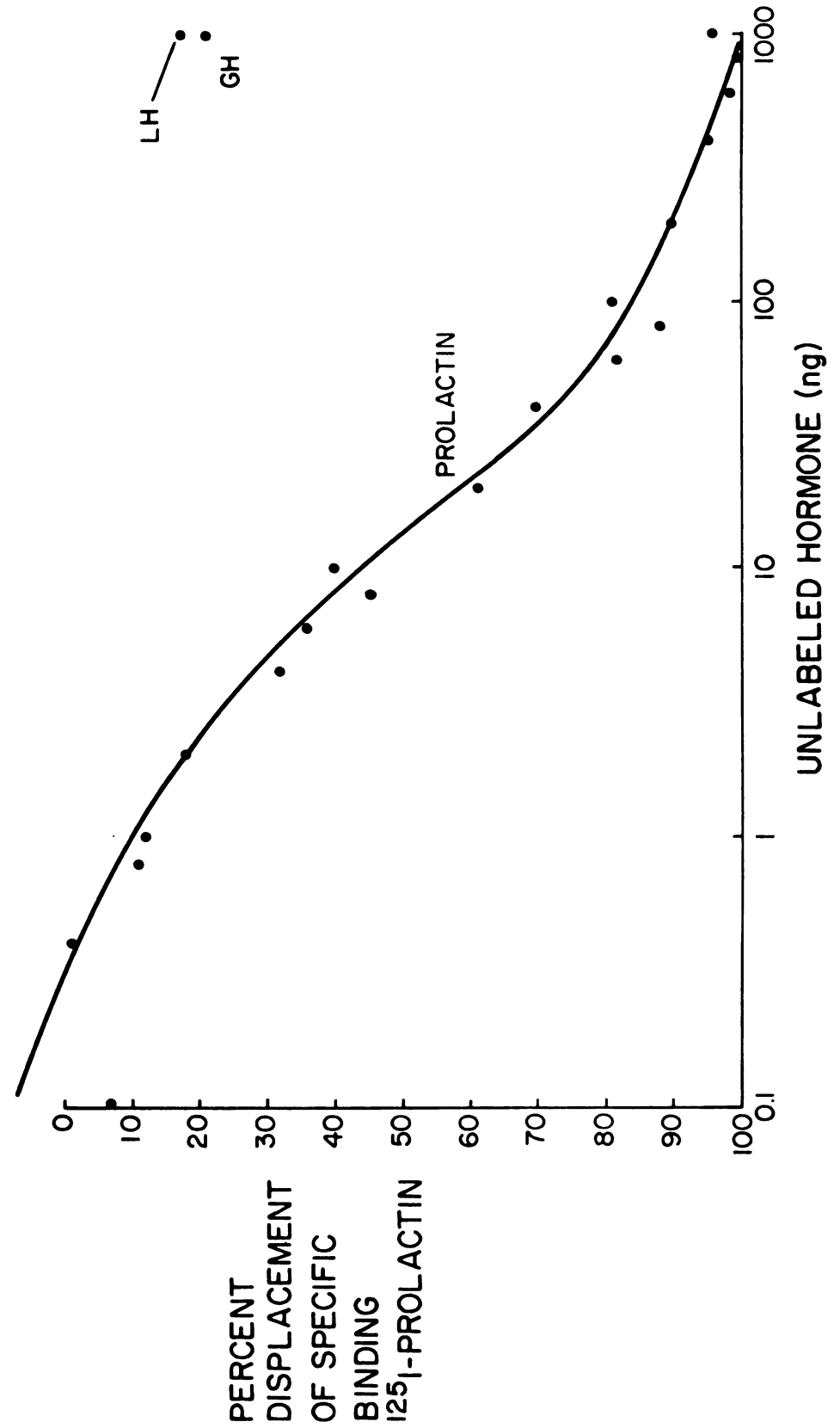

LH
GH
PROLACTIN
PERCENT DISPLACEMENT OF SPECIFIC BINDING ^{125}I-PROLACTIN
0
10
20
30
40
50
60
70
80
90
100
0.1
1
10
100
1000
UNLABELED HORMONE (ng)

Figure 14. ^{125}I-prolactin Scatchard Plot for Prolactin Membrane Receptors From a DMBA-induced Mammary Tumor Obtained From a Female Rat

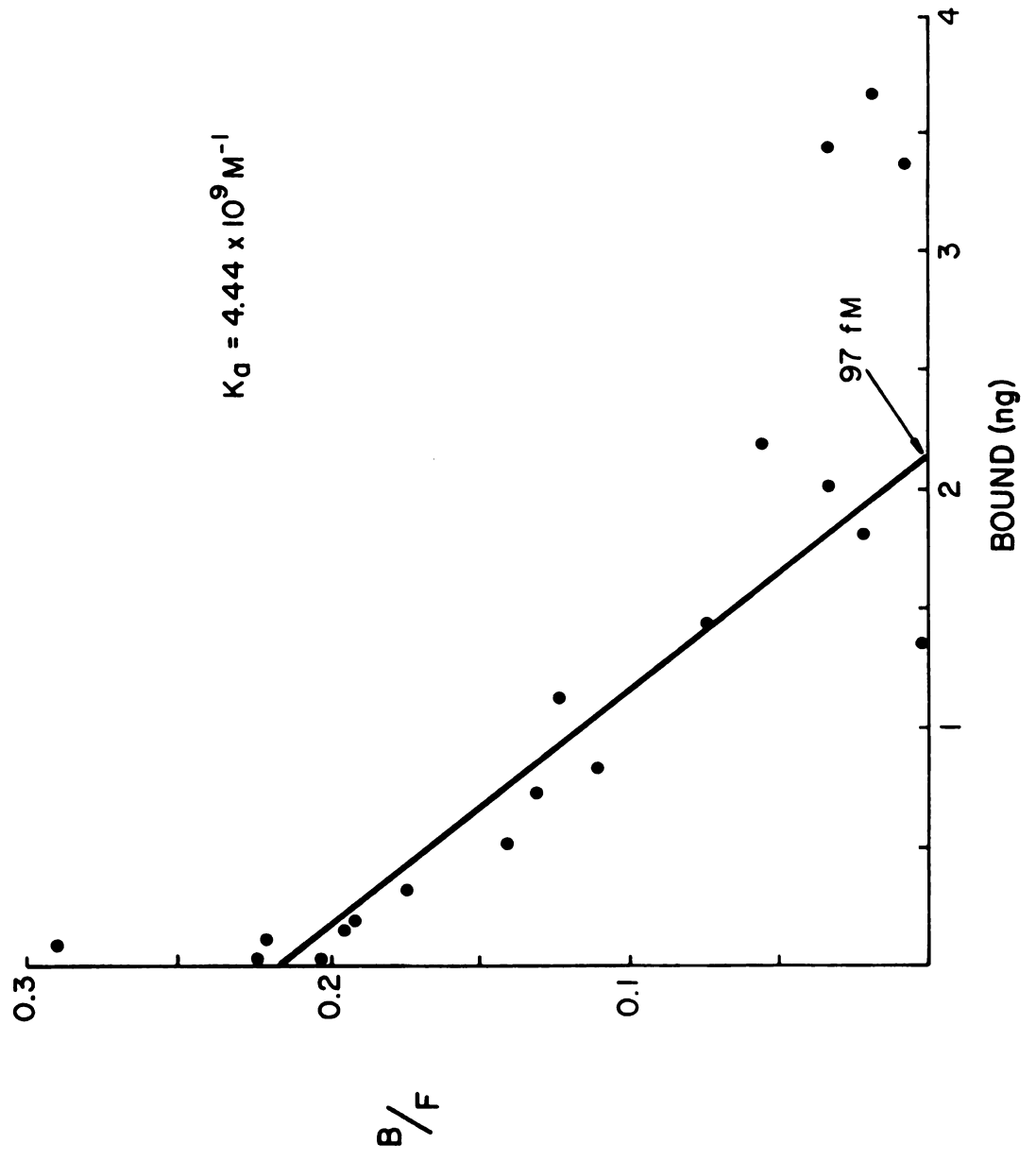

$K_a = 4.44 \times 10^9 M^{-1}$
97 fM
B/F
0.3
0.2
0.1
1
2
3
4
BOUND (ng)

Figure 15. ^{125}I-prolactin Competitive Binding Curve for Prolactin Membrane Receptors From a Liver Obtained From a Female Mouse

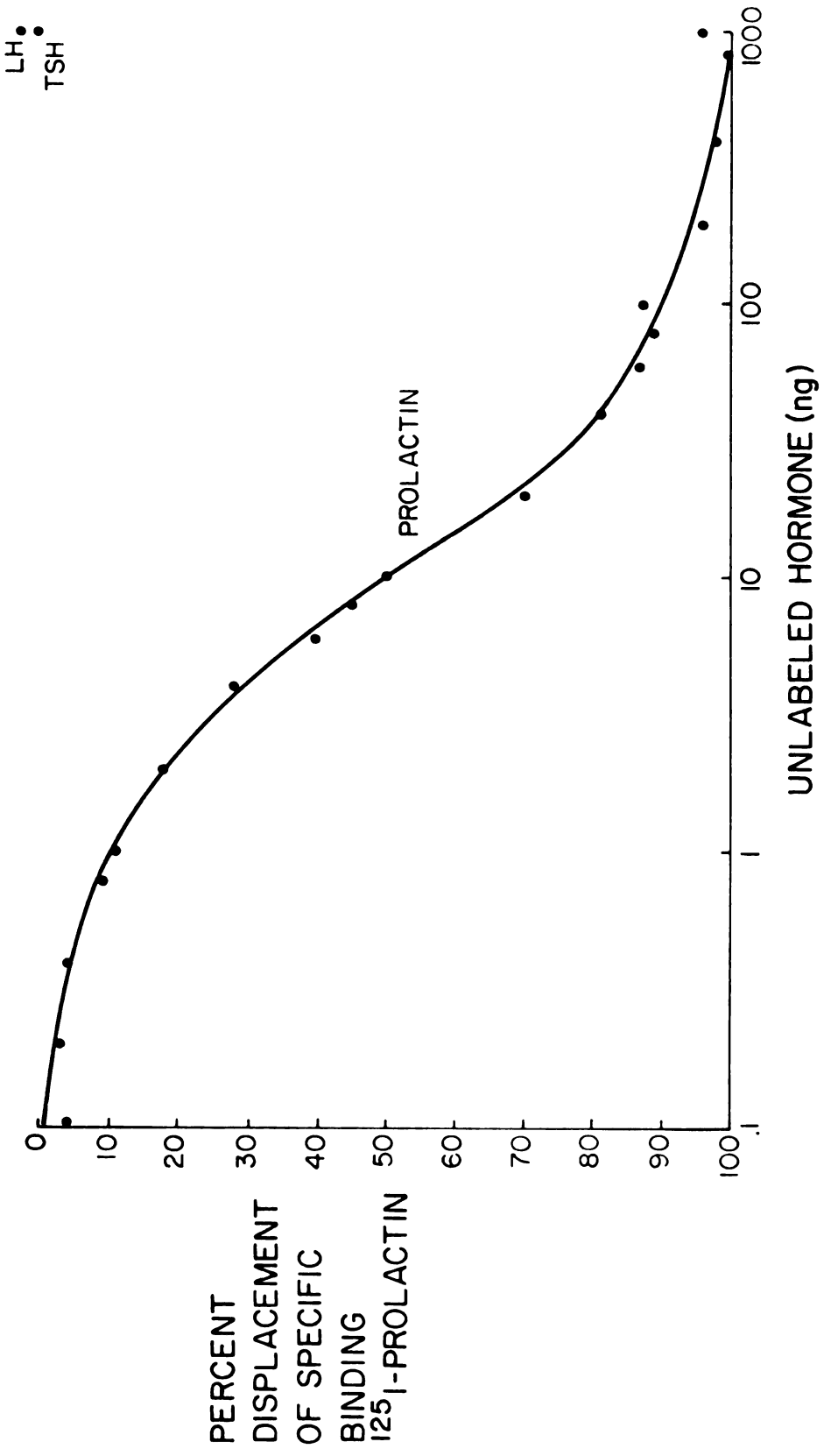

LH
TSH
PROLACTIN
PERCENT DISPLACEMENT OF SPECIFIC BINDING ^{125}I-PROLACTIN
0
10
20
30
40
50
60
70
80
90
100
.1
1
10
100
1000
UNLABELED HORMONE (ng)

Figure 16. ^{125}I-prolactin Scatchard Plot for Prolactin Membrane Receptors From a Liver Obtained From a Female Mouse

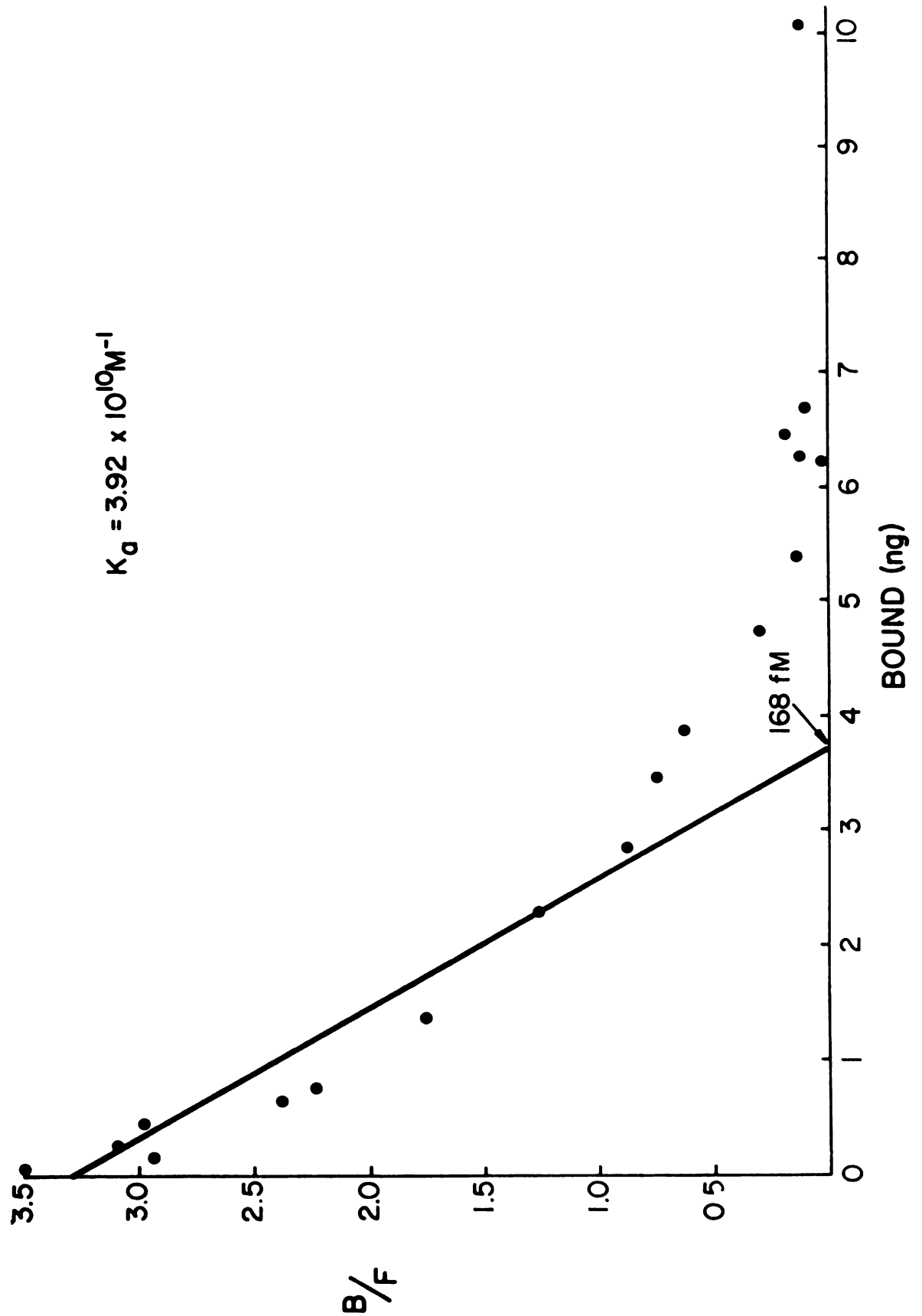

$K_a = 3.92 \times 10^{10} M^{-1}$
B/F
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
7
8
9
10
BOUND (ng)
168 fM

Figure 17. ^{125}I-prolactin Competitive Binding Curve for Prolactin Membrane Receptors From a Mammary Gland Obtained From a Lactating Mouse

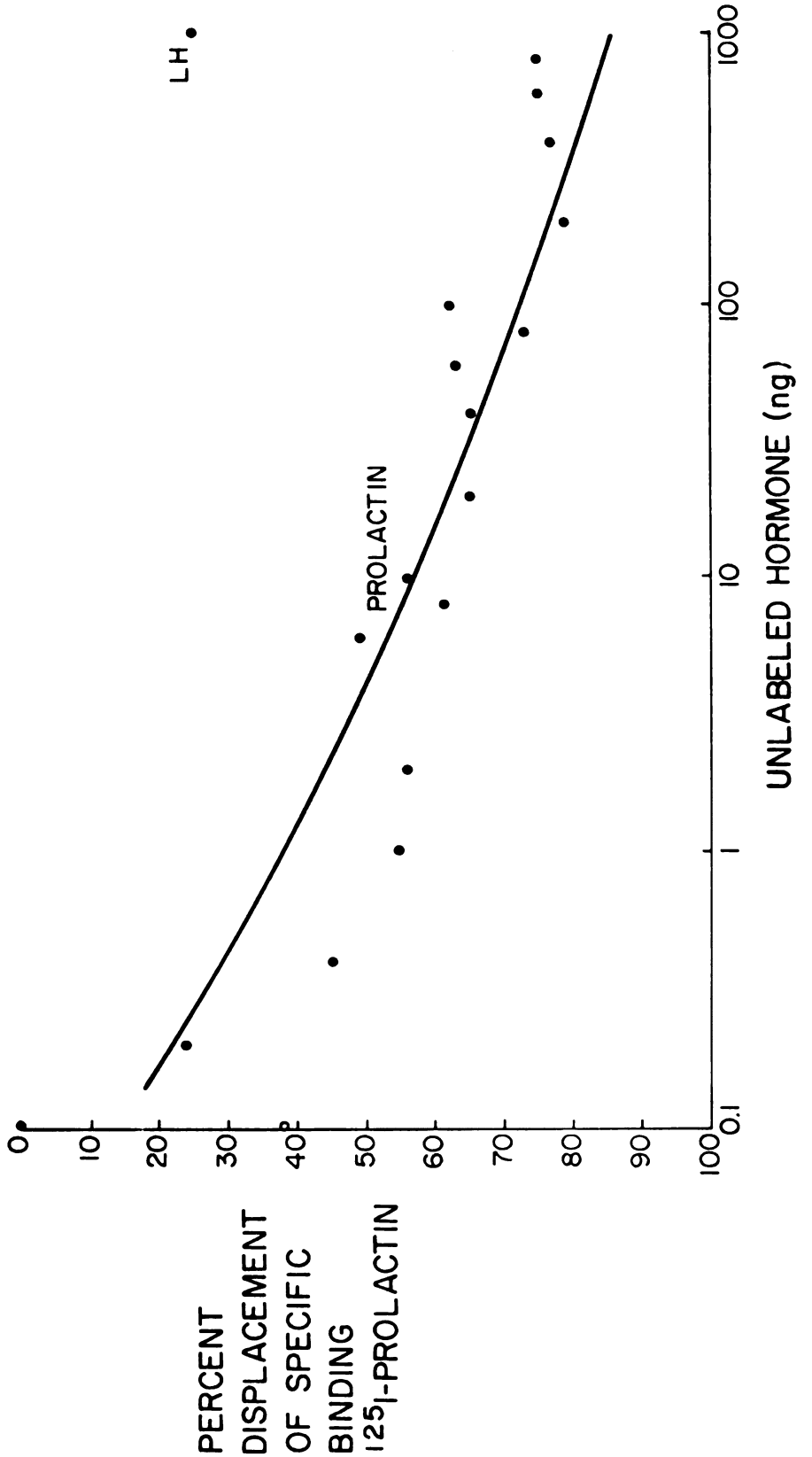
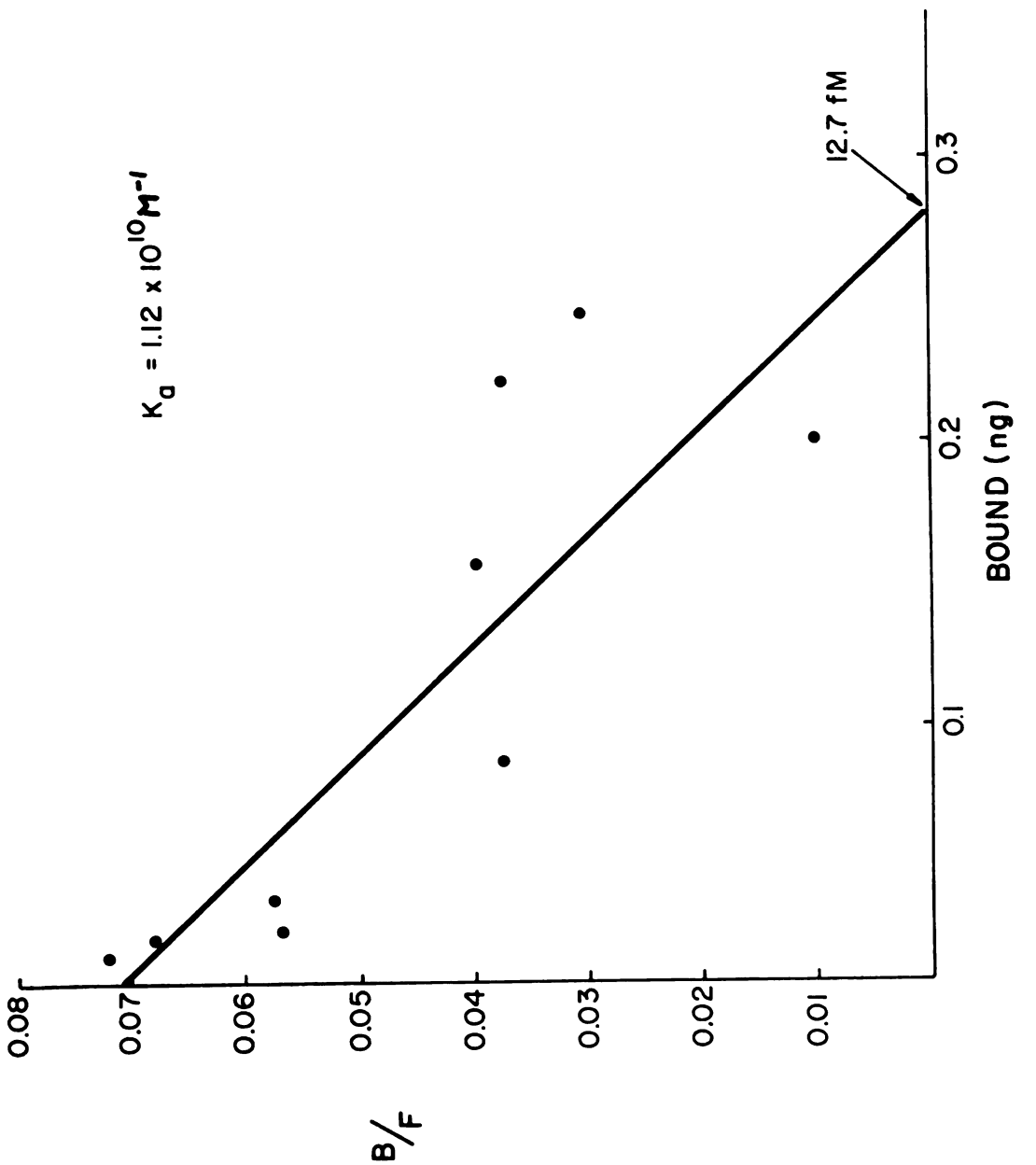

LH
PROLACTIN
0
10
20
30
40
50
60
70
80
90
100
0.1
1
10
100
1000
UNLABELED HORMONE (ng)
PERCENT DISPLACEMENT OF SPECIFIC BINDING ^{125}I-PROLACTIN

Figure 18. ^{125}I-prolactin Scatchard Plot for Prolactin Membrane Receptors From a Mammary Gland Obtained From a Lactating Mouse


$K_a = 1.12 \times 10^{10} M^{-1}$
12.7 fM
B/F
BOUND (ng)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.1
0.2
0.3

V. Effects of Time and Temperature on Specific Binding of ^{125}I-prolactin to Prolactin Membrane Receptors

A. Objectives

The binding of prolactin to its receptors is a time dependent process (Shiu and Friesen, 1974a). By varying the length of incubation time, one can determine the time required to reach a maximal percent specific binding. The maximal percent specific binding time is not the time required to reach equilibrium because there are two processes occurring during the incubation: both the binding reaction and receptor degradation occur simultaneously.

The binding process is also temperature dependent (Shiu and Friesen, 1974a). The rate of binding varies in a direct relation with the temperature.

The purpose of these experiments was to plot the time and temperature variables of the prolactin-receptor interaction, and from these data discern an optimal time and temperature to incubate the standard assay.

B. Procedures

These studies utilized a single liver membrane preparation obtained from a female rat. Standard assay sets were incubated at either 4°C, 23°C or 37°C. At specific times assay sets were spun-down and counted. The experiments were carried out for up to 48 hours. Some of these experiments were conducted by Jane Aldrich, an undergraduate student who worked under my direction.

C. Results

The time-temperature binding data are displayed in Figures 19-21. The specific binding of ^{125}I-prolactin to its receptor was a time and temperature dependent process. These data confirmed similar studies done by others (Shiu and Friesen, 1974a). The maximal binding was achieved at 23°C and not 37°C due to what appears to be a rapid degeneration of the receptors at 37°C. The greatest binding occurred at about 24 hours in the 23°C and 37°C incubation, while the 4°C incubation did not reach a maximum even at 48 hours. Only at 37°C was a receptor degradation reaction discernable.

A variability in specific ^{125}I-prolactin binding was perceptible. At all three temperatures the points in the first few hours of binding formed a smooth curve and at later times showed increasing amounts of variation about the hand-drawn curve. This effect was more pronounced at 37°C.

Figure 19. Time-temperature Binding Study of ^{125}I-prolactin to Prolactin Membrane Receptors of a Liver Obtained From a Female Rat. Incubated at 4°C for 8 hours.

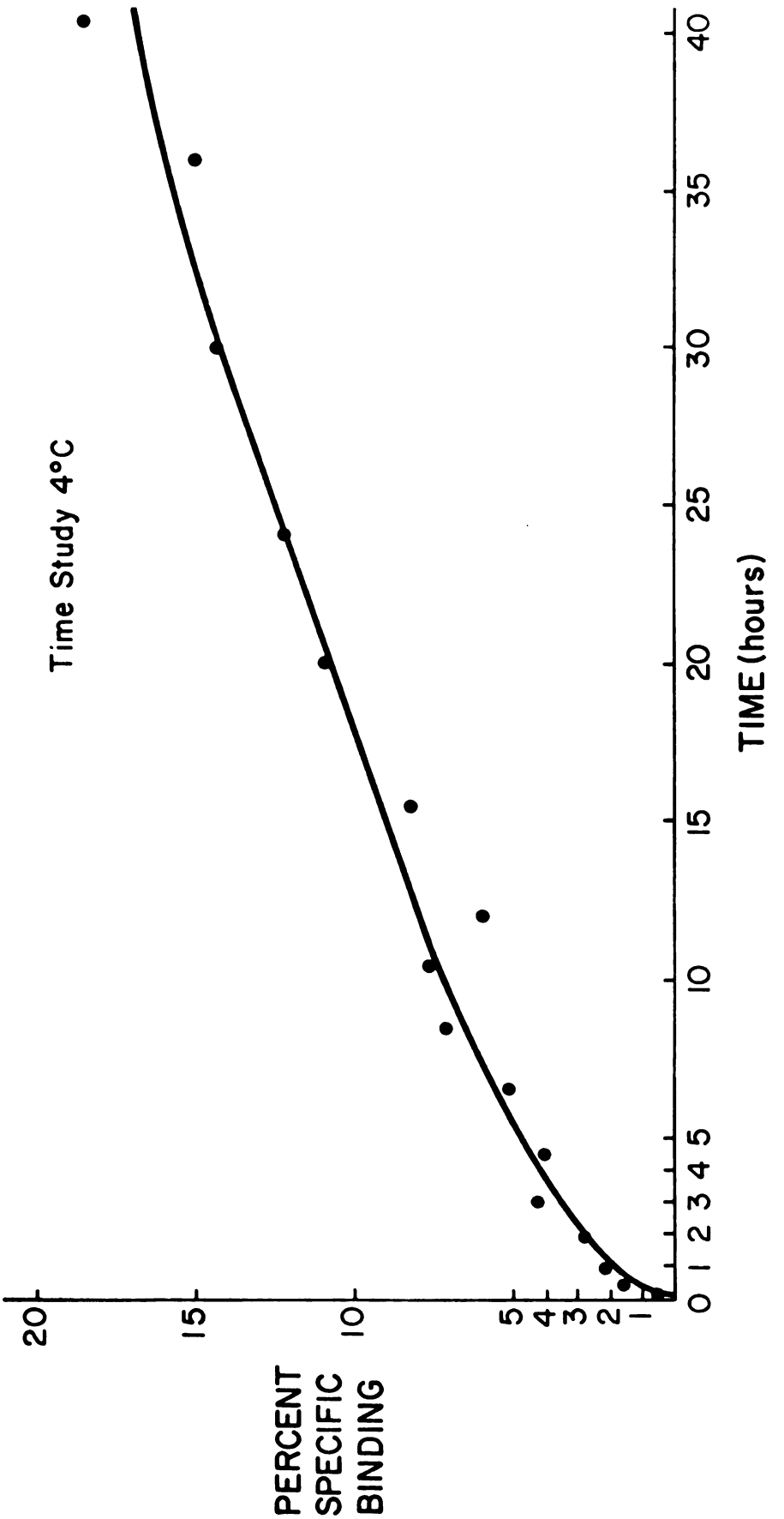

Time Study 4°C
PERCENT SPECIFIC BINDING
20
15
10
5
4
3
2
1
0 1 2 3 4 5 10 15 20 25 30 35 40
TIME (hours)

Figure 20. Time-temperature Binding Study of ^{125}I-prolactin to Prolactin Membrane Receptors of a Liver Obtained From a Female Rat. Incubated at 23°C for 8 hours.

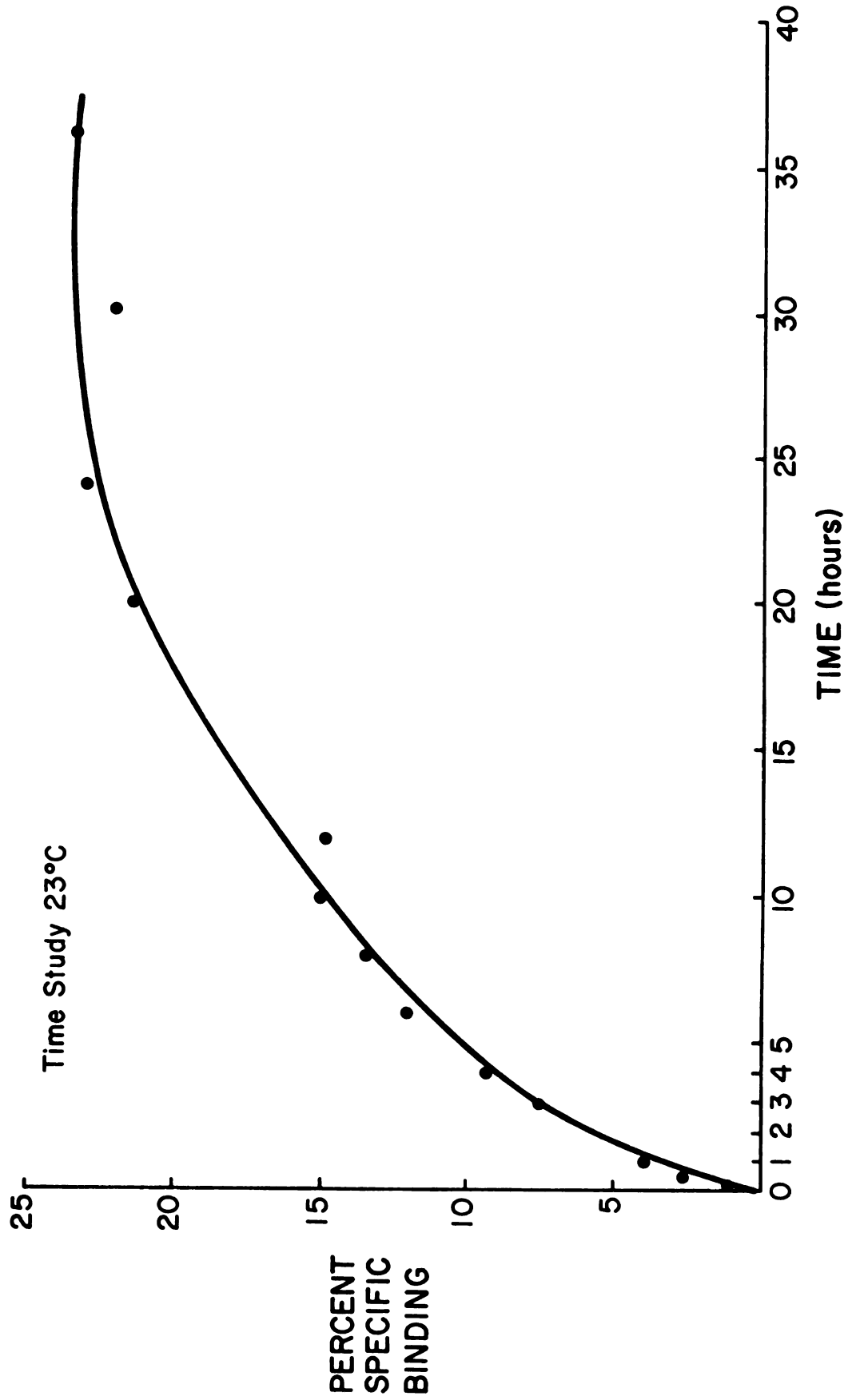
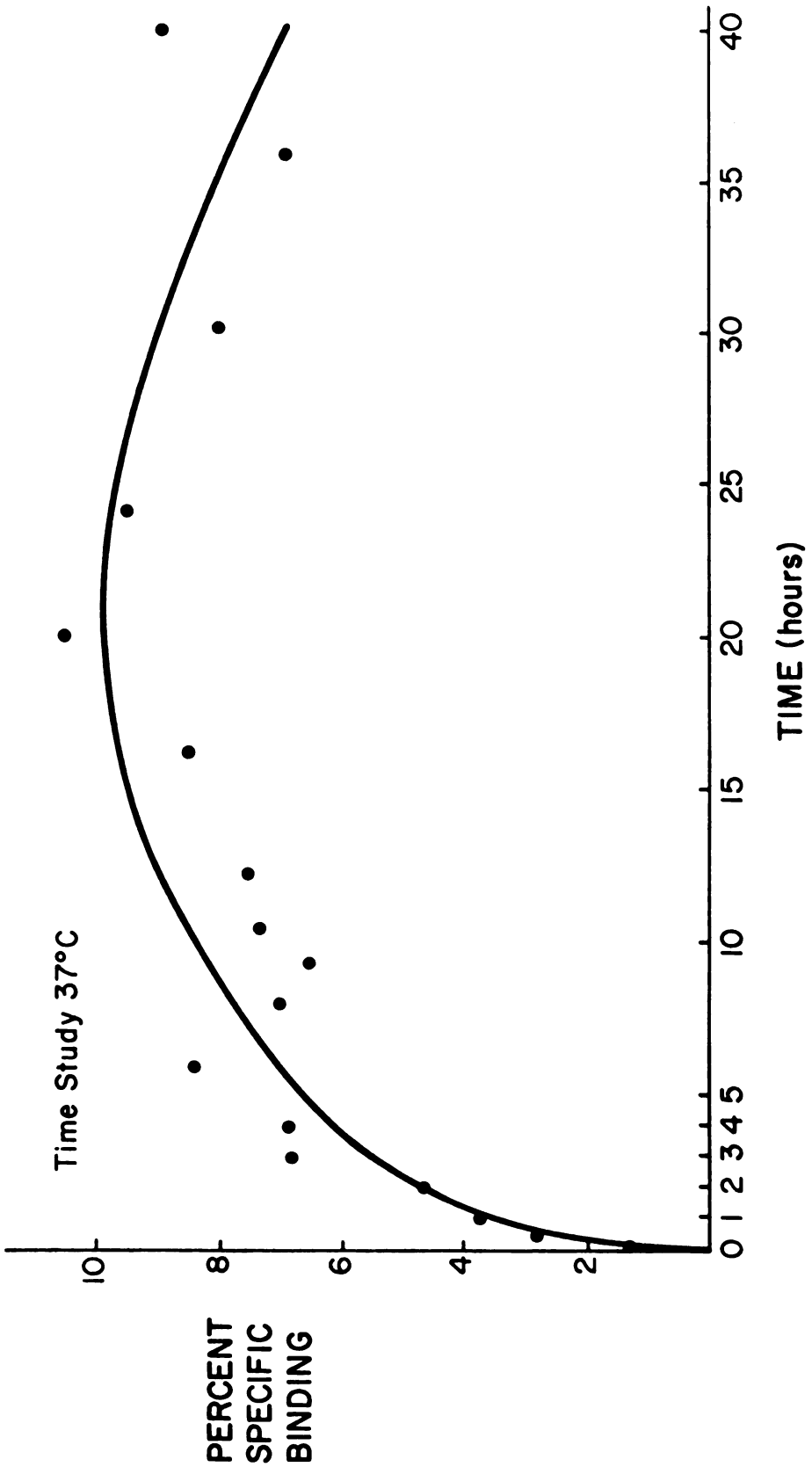

Time Study 23°C
PERCENT SPECIFIC BINDING
25
20
15
10
5
0 1 2 3 4 5
10
15
20
25
30
35
40
TIME (hours)

Figure 21. Time-temperature Binding Study of ^{125}I-prolactin to Prolactin Membrane Receptors of a Liver Obtained From a Female Rat. Incubated at 37°C for 8 hours.


Time Study 37°C
PERCENT SPECIFIC BINDING
2
4
6
8
10
0 1 2 3 4 5
10
15
20
25
30
35
40
TIME (hours)

D. Discussion

The results of these studies are in general agreement with the parameters of similar assay systems (Shiu and Friesen, 1974a) except for the time required to reach binding equilibrium. The rat and mouse prolactin-receptor association constants were within a broad experimental error of the data of others ($K_a = 10^8$-$10^{10} M^{-1}$).

One reason for the variance in K_a's could have been the difference in the time of assay incubation used in various labs. Friesen's group originally used assay incubation times of 1.5 hours (Shiu et al., 1973), but their latest publication used a 12 hour incubation time at 24°C (Aragona and Friesen, 1975). Using a tissue slice method (Costlow et al., 1975a) or a plasma membrane particle method (Costlow et al., 1975b), Costlow and co-workers only incubated an assay for 3 hours at 23°C. Frantz and Turkington incubated at 4°C for only 30 minutes (Turkington, 1974; Frantz et al., 1973). Other investigators have used incubation times for 2 hours at 37°C (Saito and Saxena, 1975), 5 hours at 24°C (Rolland and Hammond, 1975) and 24 hours at 4°C (Gelato et al., 1975). All of the investigators stated they reached equilibrium under their incubation conditions. They believe equilibrium was reached because the time-binding course leveled off at that time.

The criticism of these works is threefold. First, once equilibrium was said to be reached, the assay should have been carried for a considerably longer time, for only long incubation times could provide evidence which shows that slower binding rates do not continue. Second, two or three closely spaced time points whose binding were not statistically different were used to determine that binding curves become

asymptotic (Shiu and Friesen, 1975). This reasoning is questionable for the more variance one has in assay points the sooner one can see a line with zero slope. Finally, one is not dealing with solely a binding system; receptor and hormone degradation also occur. Thus, by the methods used no true time value for reaching equilibrium could be obtained.

The apparent faulty belief in equilibrium causes error in the estimation of association constants, but that all the K_a's were similar even with the differing incubation times suggests that the effect of non-equilibrium on the K_a is small. On the other hand, not having achieved equilibrium should introduce error to the estimate of total number of prolactin receptors. This error should be in direct proportion to the disequilibrium of the system.

The fact that so many investigators found a change in the rate of binding at various times prior to what I showed as equilibrium may mean that a different binding phenomenon occurred after an initial period of rapid binding. The first burst of binding may be the combination of ^{125}I-prolactin with receptors that are free of endogenous prolactin. The various times the leveling effect occurred may be proportional to the number of prolactin receptors that in vivo were not bound. The varying times noted to reach "equilibrium" were only relative measures of the prolactin free receptor population in the particular tissue sample. The slow later portion of binding (beyond the other investigator's equilibrium times) may be the combination of the ^{125}I-prolactin with the receptors that are currently becoming available due to the dissociation of endogenous nonlabeled prolactin. Thus, the later hours of my time-temperature study may have been an estimate of the dissociation

of the endogenous prolactin-receptor complexes. The best published dissociation rates (Shiu and Friesen, 1974a) are similar to the later rates of binding in my work. This picture is complicated by the amount of endogenous prolactin released in these studies. If 100 f moles of endogenous prolactin were released from binding sites into the reaction mixture, 2.2 ng of competing hormone would be released into a pool of less than 0.14 ng of ^{125}I-prolactin. Thus, an unquantified addition of unlabeled hormone could cause a changed equilibrium to that expected in the assumed simple assay system.

VI. Nonspecific Glass Binding of ^{125}I-prolactin in the Assay System

A. Objectives

Nonspecific ^{125}I-prolactin binding has been examined in the whole binding mixture and was found to be a total count dependent linear process (Figure 6). In this experiment the effects of time and the presence or absence of excess unlabeled prolactin on ^{125}I-prolactin binding to glass were examined.

B. Procedures

Two series of tubes were filled to a total volume of 0.5 ml. Both series contained 100 µl ^{125}I-prolactin, while one series contained in addition 1 µg of ovine prolactin in 100 µl of 0.1% BSA-Tris-HCl buffer. At various times, sets of 6 tubes were removed from each series and counted. The times of incubation varied from 1 to 24 hours. The two sets of tubes at each time were compared by the student's "t" test (Goldstein, 1964).

C. Results

The glass binding of ^{125}I-prolactin (Table 4) was shown to be a time-dependent process. It extended to 6.6 percent of the total counts within 24 hours at room temperature. The addition of 1 µg of ovine prolactin to the incubation mixture failed to reduce the ^{125}I-prolactin binding. It was concluded that there were a very high but finite number of very low affinity ^{125}I-prolactin acceptor sites. These sites could have involved the glass silica matrix or the bovine serum albumen that coats the glass.

Table 4. Nonspecific Glass Binding of ^{125}I-prolactin With and Without the Presence of Unlabeled Prolactin

	Avg. Percent Binding		
Time (hr.)	Without 1.0 µg Prolactin	With 1.0 µg Prolactin	Difference
1	3.17	3.10	0.07
2	3.49	3.39	0.10
4	3.52	3.45	0.07
6	4.05	3.91	0.14
8	4.12	4.05	0.07
12	4.49	4.46	0.03
24	6.60	6.03	0.57

VII. Reproducibility of the ^{125}I-prolactin Radioreceptor Assay for Prolactin Membrane Receptors

A. Objectives

The purpose of this experiment was to estimate the standard error of the mean of ^{125}I-prolactin binding to a single rat liver plasma membrane preparation conducted under standard assay conditions. An estimate of this sort was necessary to gain a realistic insight into the reproducability of the assay.

B. Procedures

Five separate standard assay sets were conducted for 8 hours at 23°C. The mean, variance, standard deviation, and standard error of the mean were calculated (Sokal and Rohlf, 1969).

C. Results

The mean, variance, standard deviation and standard error of the mean are listed in Table 5. Two standard deviations equal 0.70%. Due to the size of this assay variability I consider binding of 1.0% or more as areas of significant binding and binding of less than 1.0% as not significant. Other laboratories use this value for their criterion (Posner et al., 1974a).

Table 5. Statistics of Variance of Percent Specific Binding of a Standard ^{125}I-prolactin Radioreceptor Assay for a Rat Liver Membrane Preparation

Statistic	Value
Number Trials	5
Mean	6.81%
Variance	0.12%
Standard Deviation	0.35%
Standard Error of the Mean	0.16%

Part II of Experimental Section

I. Comparison of the Relative Number of Prolactin Receptors in Various Murine Mammary Tumors

A. Objectives

In recent years the effect of prolactin in promoting the growth of murine mammary tumors has been defined in terms of the tumor's in vivo or in vitro response to this hormone (Meites et al., 1972; Welsch et al., 1976b). The levels of prolactin in vivo have been controlled by methods such as administration of drugs (Flückiger, 1972) and hormones, pituitary transplants and removal of the pituitary or removal of other endocrine glands (Clemens and Meites, 1974). Cells and explants of mammary tissue have been cultured in vitro with and without prolactin (Dilley and Kister, 1975; Welsch et al., 1976b). In the last few years the effect of prolactin on growth of DMBA-induced mammary tumors and spontaneous mammary tumors from C3H mice have been determined (Welsch and Meites, 1974; Welsch and Gribler, 1973; Elias and Rivera, 1959; Nagasawa et al., 1966; Nagasawa et al., 1967).

With the advent of prolactin radioreceptor assays, intermediate steps in the action of the prolactin on murine mammary tumors can be evaluated. The purpose in quantifying prolactin receptors in various murine mammary tumors was to relate the relative number of prolactin receptors to the known in vitro and in vivo growth responses of these tumors to this hormone. These data may indicate whether the production of prolactin receptors by these tissues is an effective control mechanism for subsequent responsiveness to prolactin. The purpose of these experiments was to compare relative amounts of prolactin receptors in various murine mammary tumors and to relate the prolactin receptor number to prolactin induced growth responses of these tumors in vitro.

B. Procedures

Standard prolactin radioreceptor assays were conducted for the following murine mammary tumors:

1) DMBA-induced Rat Mammary Tumors. Generally, 90% of these tumors are dependent on prolactin for their growth. As the age of the tumors increases, fewer tumors display this dependency (Bradley et al., 1976).

2) DMBA-induced Mammary Tumors From Hypophysectomized-Ovariectomized Rats. These tumors have been shown not to require prolactin for growth, however the possibility remains that some of these hormone-independent tumors may still be prolactin sensitive.

3) Spontaneous C3H/HeJ Mouse Mammary Tumors. Most of these tumors did not grow when exposed to increased prolactin by injection (Nagasawa, 1966) or pituitary transplants (Nagasawa, 1967) in vivo or to high prolactin levels in vitro (Elias and Rivera, 1969). Neither did they respond to a drug-induced decrease in serum prolactin (Welsch and Gribler, 1973). Only rarely would a tumor of this sort respond to the growth promoting effects of prolactin (Welsch et al., 1976b).

All animals with tumors were killed by decapitation. The tumors were processed and assayed by the standard assay system.

C. Results

The binding data for all groups of tumors are displayed in Figure 22. Prolactin sensitive DMBA-induced rat mammary tumors showed the

largest range in percent specific binding of ^{125}I-prolactin (0.0-7.1, av. 2.94±0.46%). Since 1.0% specific binding was defined as significant, then 13 of 15 tumors tested contained appreciable prolactin binding. This is in accord with the data on growth response to prolactin derived from *in vivo* and *in vitro* studies (Welsch and Meites, 1974; Welsch et al., 1976b).

The ^{125}I-prolactin binding of the prolactin-independent spontaneous C3H/HeJ mouse mammary tumor was low (av. 0.71±0.21%). Only 1 of 9 tumors bound ^{125}I-prolactin greater than 1.0%. Six of these tumors were cultured by the method of Welsch and Rivera to determine their *in vitro* growth response to prolactin (Welsch and Rivera, 1972). There was no discernable correlation between prolactin binding and *in vitro* response to prolactin.

The four prolactin-independent DMBA-induced rat mammary tumors which grew in hypophysectomized-ovariectomized rats showed a widely scattered range of prolactin binding (0.0-5.6, av. 2.75±1.17%). These tumors were also cultured by the method of Welsch and Rivera (Welsch and Rivera, 1972). The ^{125}I-prolactin binding, tumor histology, and percent incorporation of ^{3}H-thymidine compared to controls are listed in Table 6. The test cultures contained 5.0 µg/ml insulin and 10 µg/ml ovine prolactin while the control cultures contained only 5.0 µg/ml insulin. These results indicate that prolactin-independent DMBA-induced rat mammary tumors can still be prolactin responsive even after a long period in hypophysectomized-ovariectomized rats. Furthermore, there appears to be a positive correlation between prolactin binding and prolactin-induced DNA synthesis *in vitro* of the fibrous mammary tumors.

Figure 22. Percent Specific Binding of ^{125}I-prolactin to Various Murine Mammary Tumors

Each block represents the assay of one tumor by the standard assay procedure.

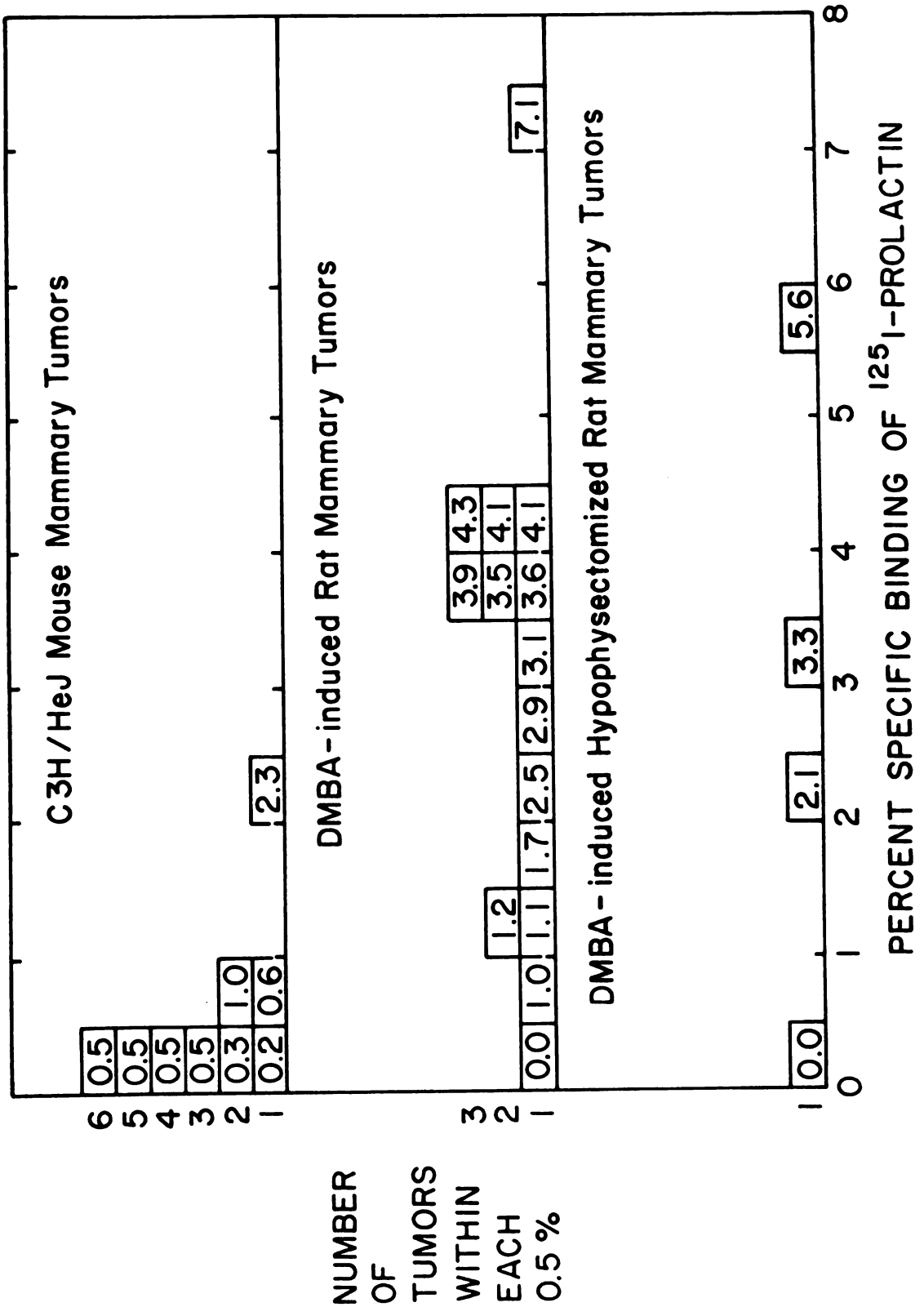

C3H/HeJ Mouse Mammary Tumors
DMBA-induced Rat Mammary Tumors
DMBA-induced Hypophysectomized Rat Mammary Tumors
NUMBER OF TUMORS WITHIN EACH 0.5 %
PERCENT SPECIFIC BINDING OF 125I-PROLACTIN
0.5
0.5
0.5
0.5
0.3
1.0
0.2
0.6
2.3
0.0
1.0
1.1
1.2
1.7
2.5
2.9
3.1
3.9
4.3
3.5
4.1
3.6
4.1
7.1
0.0
2.1
3.3
5.6

Table 6. Prolactin Binding, Histological Evaluation and Prolactin-induced Incorporation of ^{3}H-thymidine into DNA of DMBA-induced Mammary Tumors from Hypophysectomized-Ovariectomized Rats

Tumor Number	Percent Specific ^{125}I-prolactin Binding	Histology	Percent of Controls of H^{3}-thymidine Incorporation into DNA
1	5.51	fibrous	505
2	2.08	fibrous	214
3	0.00	fibrous	33
4	3.28	epithelial	97

[a]Control Culture: Insulin.

Test Culture: Insulin and Prolactin.

Culture results provided by Dr. C.W. Welsch.

D. Discussion

^{125}I-prolactin binding to prolactin receptors was studied in three types of murine mammary tumors. The DMBA-induced rat mammary tumor was shown to specifically bind ^{125}I-prolactin over a range of 0.0 to 7.1%. Only two tumors of 15 did not bind greater than 1%. If one selects to define prolactin dependence as specific binding greater than 1.0%, then this proportion of prolactin dependent tumors (13/15) compares favorably with *in vitro* (Welsch et al., 1976b) and *in vivo* (Bradley et al., 1976) data on the growth response of DMBA-induced rat mammary tumors to prolactin.

Of nine C3H/HeJ mouse mammary tumors only one tumor bound prolactin greater than 1.0% (2.3%). These results also compare favorably with *in vitro* (Welsch et al., 1976b; Elias and Rivera, 1959) and *in vivo* (Nagasawa et al., 1966) studies. The prolactin binding results in DMBA-induced rat and spontaneous mouse mammary tumors confirm the data of Turkington (Turkington, 1974), where prolactin receptors could be shown in DMBA tumors but not in C3H mouse mammary tumors.

^{125}I-prolactin binding to DMBA-induced rat mammary tumors which grew in hypophysectomized-ovariectomized female rats was demonstrated for the first time in this thesis. These tumors bound ^{125}I-prolactin quantitatively similar to the DMBA-induced rat mammary tumors from intact rats (0.0-5.6% versus 0.0-7.1%). Although only four tumors from these rats were analyzed, 3 of these showed significant prolactin binding. Hypophysectomy of rats causes a reduction in prolactin membrane receptors of liver (Posner et al., 1974b; Costlow et al., 1975a). This suggests that at least in this selected group of DMBA-induced rat mammary tumors the mechanism for control of prolactin receptors is different than that in the rat liver.

The results of these studies indicate that the concentration of prolactin membrane receptors may be predictive for the tumors' ability to respond to prolactin. This concept is in accord with that of Kelly et al. who have positively correlated prolactin receptor concentration and *in vivo* growth response of DMBA-induced mammary tumors to prolactin treatment (Kelly et al., 1974c). Recently, the number of both prolactin and estrogen receptors was found to be a better predictor of a DMBA-induced tumor response to hormone ablation (DeSombre et al., 1976).

II. Induction of Prolactin Membrane Receptors in the Livers of Hypophysectomized-Ovariectomized Female Rats

A. Objectives

Several laboratories have reported that the number of prolactin receptors in female rat livers is controlled by the level of serum prolactin (Posner et al., 1975). A significant reduction in prolactin binding in female rat liver within 24 hours after hypophysectomy, and an almost complete disappearance within 48 hours was recently reported (Costlow et al., 1975a). Additionally, ovariectomy also reduced the prolactin binding in female rat livers (Kelly et al., 1975), but was dependent upon the presence of the pituitary.

In this experiment liver prolactin receptors of one group of female rats were assayed 8 months after hypophysectomy-ovariectomy. A second group of rats, hypophysectomized-ovariectomized 8 months earlier, were given injections of prolactin to determine whether or not it is possible to induce liver prolactin receptors after 8-month hormone deprivation. These groups were compared to the liver ^{125}I-prolactin binding of a group of normal age-matched female rats.

B. Procedures

Eleven hypophysectomized-ovariectomized rats were divided into two groups. Group I (6 rats) was injected s.c. with 1.0 mg of ovine prolactin (NIH-P-S-9) twice daily for 5 days. The prolactin was suspended in 0.9% saline at a concentration of 1.0 mg/ml. Group II (5 rats) was given the saline vehicle at the same interval. Group III (6 rats) was composed of normal intact age-matched female rats and was also given the saline vehicle. Twenty-four hours after the last injection

all animals were sacrificed by decapitation. All hypophysectomized rats were checked for a complete pituitary removal. The livers were removed, processed and assayed by standard procedure. Standard errors were calculated for the specific ^{125}I-prolactin binding of each group. The three groups were compared by an Analysis of Variance and a Least Significant Range Test (Sokal and Rohlf, 1969).

C. Results

Eight months after hypophysectomy-ovariectomy the untreated rat livers specifically bound only 0.5% of the ^{125}I-prolactin (Table 7). After injection of prolactin for 5 days the livers of hypophysectomized-ovariectomized rats bound 7.1% of the ^{125}I-prolactin. This latter value (7.1%) was not significantly different than that obtained from the normal control rats (11.0%). This is the first evidence to my knowledge that prolactin can induce its own hepatic receptor in hypophysectomized-ovariectomized female rats.

Table 7. Induction of Prolactin Membrane Receptors in the Liver of 8-month Hypophysectomized-Ovariectomized Female Rats by Injection of Ovine Prolactin

Group	Number of Animals	Treatment	^{125}I-prolactin Percent Specific Binding ± SEM.
I	6	Hypox.-Ovx. + Prol.	7.1 ± 1.2%[a]
II	5	Hypox.-Ovx. + Saline	0.5 ± 0.1%[b]
III	6	Age-matched Controls	11.0 ± 2.2%[a]

[a/b]Statistically significant at $P<0.05$ by Analysis of Variance and Least Significant Range tests.

SEM = Standard error of the mean.

III. Effect of Lowered Serum Prolactin Levels (by CB-154) on the Quantity of Prolactin Membrane Receptors in Liver and Mammary Gland of Normal and Lactating Rats

A. Objectives

Prolactin is involved in the induction of its own receptor in the rat liver (Posner et al., 1975). The data which support this concept are derived from the use of hypophysectomy which suppresses ^{125}I-prolactin binding and injection of hyperphysiological doses of prolactin or transplantation of a pituitary to the kidney capsule, both of which can return ^{125}I-prolactin binding to normal levels (Posner et al., 1975).

In this experiment I determined whether a drug-induced reduction of serum prolactin to near hypophysectomized levels would cause a parallel reduction of prolactin receptors in the liver. CB-154 has been shown to effectively lower serum prolactin to near hypophysectomized values in rats (Brooks and Welsch, 1974).

B. Procedures

Two experiments were performed: one on mature female rats and a second on 10-day-lactating rats.

1) Eleven female Sprague-Dawley rats were divided into groups of 4 and 7 rats. The group of 4 rats received 0.5 ml of 0.9% saline-1% ethanol 50, 26 and 2 hours before decapitation. The group of 7 rats received 0.5 mg of CB-154 in 0.5 ml of 0.9% saline-1% ethanol 50, 26 and 2 hours before decapitation. At decapitation all livers were removed and blood collected from the trunk. Livers were processed and assayed for prolactin receptors by the

standard assay method. All blood samples were tested for serum prolactin by the NIH-rat-prolactin radioimmunoassay procedure.

2) Eleven 10-day-lactating female Sprague-Dawley rats were divided into two groups of 5 and 6 rats each. The first group of 5 rats was given injections of 0.9% saline-1% ethanol 24 and 12 hours before decapitation at a dose of 2 ml/kg body weight. The second group of lactating rats was given injections of CB-154 at a dose of 4 mg/kg body weight in 0.9% saline-1% ethanol at a concentration of 2 mg/ml. The drug was given 24 and 12 hours before decapitation. The livers, mammary glands, and blood were taken at the time of decapitation. The blood and organs were processed as in the first experiment.

The CB-154 was prepared by suspending a weighed amount of the drug in ethanol and bringing it to volume with 0.9% saline. The ^{125}I-prolactin binding was statistically analyzed by the student's "t" test (Goldstein, 1964).

C. Results

Administration of CB-154 to either group of animals did not significantly change the quantity of prolactin receptors in the livers and mammary glands. CB-154 effectively lowered serum prolactin levels in both treated groups (Table 8).

Table 8. Effect of CB-154 on Prolactin Membrane Receptors in Liver and Mammary Glands of Normal and Lactating Female Rats

Experiment I, Normal Female Rats

Treatment (# animals)	Serum Prolactin (ng/ml ± SEM)	Percent Specific Binding of ^{125}I-prolactin to: Liver (% ± SEM)
Saline (4)	28.5 ± 9.6[a]	2.95 ± 0.65[c]
CB-154 (7)	4.5 ± 0.7[b]	4.21 ± 0.39[d]

Experiment II, 10-day-lactating Female Rats

Treatment (# animals)	Serum Prolactin (ng/ml ± SEM)	Percent Specific Binding of ^{125}I-prolactin to: Liver (% ± SEM)	Mammary Gland (% ± SEM)
Saline (5)	101.0 ± 45.0[a]	6.68 ± 0.67[c]	2.48 ± 0.16[c]
CB-154 (6)	10.1 ± 0.8[b]	8.23 ± 1.44[d]	3.52 ± 0.39[d]

a/b, $P<0.01$

c/d, $P>0.05$ (not statistically significant)

SEM = Standard error of the mean

D. Discussion

The control of the number of prolactin membrane receptors in the rat liver has been shown to involve the concentration of serum prolactin (Posner et al., 1975). The work reported in the study has shown that hypophysectomy-ovariectomy reduced liver prolactin receptors and that prolactin receptors were induced in hypophysectomized-ovariectomized rats by large doses of ovine prolactin even after long periods of prolactin deprivation. This confirmed the work of others (Posner et al., 1975; Costlow et al., 1975) but also showed that there was no permanent change in the liver's ability to generate prolactin receptors 8 months after hypophysectomy-ovariectomy. It also demonstrated that prolactin did not need the presence of the ovary or ovarian steroids to retain the ability to induce receptors.

Kelly (Kelly et al., 1975) demonstrated that ovariectomy reduced by 41% the relative number of prolactin receptors in the female rat liver. The effect of the reduction of steroids by ovariectomy may have been to reduce the estrogen-induced release of prolactin (Clemens and Meites, 1974) which resulted in a reduced prolactin stimulus to induce prolactin receptors in this tissue (Posner et al., 1975). An experiment to discern if this relationship is correct was conducted by lowering serum prolactin levels in normal and lactating female rats by the use of CB-154, a drug which effectively suppresses serum prolactin in rats (Brooks and Welsch, 1974).

As shown (Table 8), CB-154 did not significantly reduce the number of prolactin membrane receptors. These data do not support the previously published conclusion (Kelly et al., 1975; Posner et al., 1975) that prolactin directly determines the relative number of prolactin

receptors in the liver and mammary gland. It is possible that changes in quantity of prolactin receptors may be due to lowered serum prolactin which would allow the equilibrium of hormone and receptor to shift to the left and thus free receptors from endogenous prolactin leaving them open for assay by ^{125}I-prolactin.

IV. Removal of *In Vivo* Bound Prolactin from the Prolactin Membrane Receptors of Mouse Mammary Gland by Treatment with Low pH or High $MgCl_2$ Concentration

A. Objectives

If *in vivo* bound prolactin could mask the prolactin receptor from subsequent assay then it may be possible to dislodge the bound hormone by partial denaturation of the receptor protein. Following removal of such hormone the receptor protein may be renatured and if undamaged, assayed.

The lactating mouse mammary gland consistently bound very low levels of prolactin. The association constant was $10^{10}\ M^{-1}$, indicating a powerful binding between hormone and receptor. This high affinity may have promoted the binding of a large proportion of the prolactin receptors in this tissue by the normal serum prolactin concentrations and thus blocked their assay. Because of these characteristics this tissue was used in an attempt to remove endogenously bound prolactin.

Previous work had suggested two potentially successful methods to remove prolactin from its receptor. The first method was to use a short term pH 2.0 wash. This was suggested by Saxena (Saxena, personal communication) who used this procedure to routinely remove bound ^{125}I-HCG from receptors on bovine corpora luteal plasma membranes (Haour and Saxena, 1974; Landsman and Saxena, 1974; Saxena et al., 1974). The second method was to use 5.0 M $MgCl_2$ to remove the endogenous prolactin. This method was used successfully by Shiu (Shiu and Friesen, 1974b) to remove solubilized prolactin receptors of rabbit mammary gland from an

affinity column prepared with ovine prolactin ligands. In this experiment, both methods were tested for ability to remove endogenously bound prolactin from mouse mammary gland.

B. Procedures

Four Swiss-Webster mice on day 10 of lactation were used in this experiment. All mice were removed from their litters 24 hours before decapitation. The mammary glands and livers were removed and processed by the standard assay procedure. The enriched plasma membrane of the mammary glands from the mice were pooled for each experiment. The livers were also pooled and used as a control in each assay.

The membrane enriched fractions were spun down at 100,000g for 90 min. and resuspended vigorously in either 5.0 M $MgCl_2$ (pH 7.6) or 25mM Tris Buffer with 10mM $MgCl_2$ adjusted to pH 2.0. The solution was immediately centrifuged at 100,000g for 120 min. and resuspended in the normal Tris-HCl buffer at pH 7.6 and a concentration of 300 μg/100 μl of plasma membrane protein. The treated mammary gland preparation, a portion of the untreated preparation and the mouse liver preparation were each assayed for 6 hours at 23°C.

C. Results

The results of the binding experiments are shown in Table 9. The 5.0 M $MgCl_2$ wash failed to increase the binding from the control levels. This may have been due either to the salt's failure to remove the endogenous hormone or to its permanent denaturation of the receptor. One major difference between this work and that of Shiu and Friesen's was that the mouse mammary gland receptors in this study were membrane-bound while in the study of Shiu they were solubilized by triton X-100.

It is known that divalent cations have profound effects on various physical properties of membranes, and thus it may have been the effect of the Mg^{++} on the membrane that prevented positive results.

The pH 2.0 wash, on the other hand, was successful in that binding was increased from 0.94% to 5.38% by the acid wash. These data support the concept that a large portion of mouse mammary gland prolactin receptors are indeed occupied by endogenous prolactin.

Table 9. Removal of *In Vivo* Bound Prolactin from the Prolactin Membrane Receptors of Lactating Mouse Mammary Gland by Treatment with Low pH or High $MgCl_2$ Concentration[a]

I. Test of 5.0 M $MgCl_2$ Wash[b]

Treatment	^{125}I-prolactin Percent Specific Binding
5.0 M $MgCl_2$	1.09
Control	0.93

[b]percent specific ^{125}I-prolactin binding of control mouse liver: 13.2.

II. Test of pH 2.0 Wash[c]

Treatment	^{125}I-prolactin Percent Specific Binding
Ph 2.0	5.38
Control	0.94

[c]percent specific ^{125}I-prolactin binding of control mouse liver: 20.9.

[a]assay conditions: 6 hours at 23°C

D. Discussion

The results of exposing mouse mammary gland prolactin receptors to low pH indicated that the standard assay procedure may not have been capable of detecting total prolactin receptors. The brief exposure of mouse mammary gland to a pH 2.0 bath increased the assayable prolactin receptors by 572%. The most straightforward interpretation of these data is that the low pH treatment partially denatured the receptor protein and/or prolactin, thus allowing any endogenously bound prolactin to dissociate.

A second interpretation is that the low pH caused the exposure of prolactin receptors that had been buried in the plasma membrane. This idea of hidden receptors within the membrane was suggested by the work of Cuatrecasas on the insulin receptor (Cuatrecasas, 1971). He showed that a 3- to 6-fold increase in the assayable insulin receptors in fat and liver cell membranes was induced by treatment with phospholipase.

Previous work presented in this thesis suggests the interference by endogenously bound prolactin on the standard prolactin radioreceptor assay. In addition, the work of Saxena supports this concept. Saxena routinely used low pH to remove close to 100% of ^{125}I-HCG from bovine corpora lutea plasma membranes (Saxena, personal communication). This procedure of removing hormone from the receptors allowed him to reuse the gonadotropin receptors in an HCG assay (Saxena et al., 1974) to determine early pregnancy (Landesman and Saxena, 1974).

This complex situation perhaps can be clarified by conducting a further experiment. A pH 2.0 bath should be used to dissociate the endogenous prolactin from mouse mammary gland prolactin receptors. The

freed prolactin could be concentrated by dialysis and quantified by ^{125}I-prolactin radioimmunoassay. The moles of assayed prolactin should be equivalent to the moles of additional prolactin receptor assayable after the treatment. This protocol assumes the low pH does not destroy the immunoreactivity of the free prolactin or the receptor activity of the free prolactin receptors.

V. Effect of an Injection of a Large Dose of Bovine Prolactin on the Quantity of Assayable Prolactin Membrane Receptors in Livers and Mammary Glands of Female Rats

A. Objectives

The results of the last few experiments raised the question as to whether or not a significant portion of prolactin membrane receptors in various tissues were bound *in vivo* by the animal's endogenous prolactin thus masking the radioreceptor assay. If one assumes the binding mechanism to be

$$\text{Prolactin} + \text{Receptor} \rightleftharpoons \text{Prolactin-Receptor}$$

and also assumes the association binding constant (K_a) to be those obtained by the previously produced Scatchard plots (10^9 to $10^{10} M^{-1}$), then a significant portion of receptors should be occupied by prolactin from the animal's blood. The lack of kinetic data prevents resolution of this problem. The best dissociation data for prolactin and its receptor was the work published by Shiu (Shiu and Friesen, 1974a, 1974b) involving the dissociation of ovine-prolactin from rabbit mammary glands. At 4°C only 10% was lost after 45 hours of incubation. At 23°C, 50% was lost by 45 hours. At 37°C, 50% was lost by 5 hours of incubation. This slow dissociation indicated that binding of prolactin to tissue receptors was a relatively long lasting process and, thus, not easily lost during long incubations.

The experiment in this section was designed to determine if an unusually large rise in serum prolactin can block a portion of the prolactin receptors in various tissues to subsequent assay. In addition, an 8 hour incubation period was used to test the *in vivo* bound hormone's ability to dissociate during the normal assay incubation times.

B. Procedures

The experiment was performed on midpregnant and 10-day-lactating rats. All animals received subcutaneous injections of 4.0 mg/kg body weight of CB-154. The drug was at a concentration of 2.0 mg/ml and was suspended in 0.9% saline with 1% ethanol. Injections of CB-154 were given 12 and 24 hours prior to decapitation. The purpose of the CB-154 injections was to lower the rat's blood prolactin concentrations to similar low values, thus eliminating blood prolactin as a variable in these experiments. The weights of the midpregnant rats varied between 310 and 360 grams, while the lactating rats weighed between 300 and 450 grams.

One hour prior to decapitation the rats were randomly divided into two groups. Group I received 1.0 ml of 0.9% saline by an intraperitoneal route. Group II received 1.0 ml of 0.9% saline containing 2.0 mg of bovine-prolactin (NIH-B-3) also by intraperitoneal route. One hour later all rats were killed by decapitation. Blood was collected from the trunk, processed and stored at -20°C until assayed for both rat prolactin by the NIH rat prolactin radioimmunoassay kit and bovine prolactin (RIA) through the courtesy of Dr. H.A. Tucker of the Departments of Dairy Science and Physiology. The liver and mammary glands were also excised from all rats, processed and assayed by the standard assay procedure. These data were analyzed by Analysis of Variance and Least Significant Range test (Sokal and Rohlf, 1969).

C. Results

The prolactin binding data from both the midpregnant rat and 10-day-lactating rat experiments are presented in Tables 10 and 11. A substantial rise in bovine serum prolactin for one hour reduced the

assayable prolactin receptors in both liver and mammary gland by 42 to 62%. All prolactin binding was significantly reduced at a $P<0.05$ level of significance except the mammary glands of the midpregnant rats where only a 25% reduction in prolactin binding was noted. This difference was small and statistically insignificant due to the very low levels of binding in the control mammary glands.

The radioimmunoassay data for both rat and bovine prolactin are presented in Table 12. In both midpregnant and 10-day-lactating rats the rat serum prolactin levels were reduced and showed a low variance between rats. The serum prolactin levels (rat prolactin) in both groups which received 2.0 mg bovine prolactin were slightly higher than the controls. This may have been due to a slight cross reactivity of bovine prolactin to the rabbit anti-rat prolactin antibody. The bovine prolactin radioimmunoassay demonstrated that the injection of 2.0 mg of bovine prolactin one hour prior to blood sampling raised the average bovine serum prolactin concentration to above 4000 ng/ml.

The results of this study provide evidence that an extremely large rise in serum prolactin reduces the relative amounts of assayable prolactin membrane receptors. The target tissue prolactin receptors were bathed in high levels of bovine prolactin for approximately one hour and during this period a substantial portion of receptors were bound by this hormone. The dissociation of bovine prolactin from these *in vivo* bound receptors did not negate this masking effect even after several hours of incubation at 4°C during tissue processing and 8 hours of incubation at 23°C. Thus, the dissociation of the bovine prolactin from the receptor was slow, and the assay blocking effect of this hormone was not lost within 8 hours of assay incubation.

Table 10. ^{125}I-prolactin Binding to Liver and Mammary Gland of Midpregnant Rats: Effect of 2.0 mg Bovine Prolactin Injected One Hour Prior to Decapitation

I. Liver (midpregnant rats)

Treatment (# animals)	^{125}I-prolactin Percent Specific Binding*	P-Value
CB-154 + Saline (6)	12.8 ± 1.4	P<0.05
CB-154 + Prolactin (6)	7.5 ± 0.6	

II. Mammary Gland (midpregnant rats)

Treatment (# animals)	^{125}I-prolactin Percent Specific Binding*	P-Value
CB-154 + Saline (6)	1.2 ± 0.1	not sig.
CB-154 + Prolactin (6)	0.9 ± 0.2	

*Mean ± standard error

assay conditions: 8 hour incubation at 23°C

Table 11. ^{125}I-prolactin Binding to Liver and Mammary Gland of 10-day-lactating Rats: Effect of 2.0 mg Bovine Prolactin Injected One Hour Prior to Decapitation

I. Liver (lactating rats)

Treatment (# animals)	^{125}I-prolactin Percent Specific Binding*	P-Value
CB-154 + Saline (6)	8.2 ± 1.4	P<0.05
CB-154 + Prolactin (6)	3.2 ± 0.3	

II. Mammary Gland (lactating rats)

Treatment (# animals)	^{125}I-prolactin Percent Specific Binding*	P-Value
CB-154 + Saline (6)	3.5 ± 0.4	P<0.05
CB-154 + Prolactin (6)	1.6 ± 0.2	

Table 12. Rat and Bovine Serum Prolactin Levels in Midpregnant and Lactating Rats Treated With CB-154 or CB-154 and Bovine Prolactin

I. Midpregnant Rats

Treatment (# animals)	Rat Prolactin (ng/ml)*	Bovine Prolactin (ng/ml)*
CB-154 + Saline (6)	19.2 ± 1.3	nondetectable
CB-154 + Prolactin (6)	23.2 ± 2.0	>4000

II. Lactating Rats

Treatment (# animals)	Rat Prolactin (ng/ml)*	Bovine Prolactin (ng/ml)*
CB-154 + Saline (6)	11.5 ± 2.4	nondetectable
CB-154 + Prolactin (6)	22.5 ± 2.4	>4000

*Mean ± standard error

D. Discussion

The results of this experiment demonstrated that a large rise in serum prolactin can block the assay of a substantial portion of prolactin receptors in rat liver and mammary gland. It was probable that prolactin receptors became saturated by bovine prolactin after a 2.0 mg injection of the hormone. Although this experiment was not designed to assess the kinetic parameters of prolactin binding, the demonstration of approximately half of the receptors being blocked from assay indicated that the dissociation of prolactin from receptors was slow. This blocking was evident even after an 8 hour incubation at 23°C.

The percent of the total receptors which should be bound by the levels of serum prolactin can be predicted. If one assumes that the binding of cellular membrane receptors and prolactin reaches equilibrium, and if one assumes the reaction to be:

$$\text{Prolactin} + \text{Receptor} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Prolactin-Receptor},$$

then one can calculate a binding curve. The association constant is equal to:

$$K_a = \frac{(\text{Bound Receptor})}{(\text{Free Prolactin})(\text{Free Receptor})}.$$

Rearrangement of this statement produced:

$$(K_a)(\text{Free Prolactin}) = \frac{(\text{Bound Receptor})}{(\text{Free Receptor})}.$$

Thus, by knowing the prolactin concentration (by RIA) and the association constant (approximately $10^9 M^{-1}$ in the rat), one can calculate the ratio of bound to free receptors. The data shown in Table 13 were developed by assuming a $10^9 M^{-1}$ association constant and varying free

prolactin concentrations. One can see that, if at equilibrium, the levels of bovine prolactin seen in this experiment (>4000 ng/ml) should essentially bind all prolactin receptors contained on the target cells.

If this relationship is correct the receptors were all bound at the beginning of the assay incubation. After 8 hours at 23°C approximately ½ of the total receptors were still being bound by endogenous prolactin. Using these data, 8 hours would be a crude estimate of the half-time of dissociation at 23°C.

The relationship of rate constants (or reaction half-times) and the association constant can be used to derive a theoretical half-time of the association rate. The change to half times from rates introduces an element of error into our mathematics. The binding curve follows a Michaelis-Menten curve, while our mathematical model follows a hyperbolic curve. After several half-times there is a difference in the two curves as an asymptote is approached. I feel justified in using this formulation involving half-time because the area of interest is the first few half-times, and because the purpose of this analysis is to follow an intuitive argument, not to construct a mathematical proof. A more rigorous argument can be constructed by the use of the Langmuir equation (Bull, 1971) but the more intuitive argument is presented here. The statement of association:

$$K_a = \frac{k_1}{k_2}$$

where k_1 and k_2 are the association and dissociation rates, can be transformed to a relation of half-times ($t_{½}$).

Table 13. Binding Isotherm of Prolactin and Receptors Which Have a $10^9 M^{-1}$ Association Constant

Prolactin Concentration*		Bound Receptor / Free Receptor	% of Receptors Occupied
0.22 ng/ml	10^{-11}M	0.01	1
2.2 ng/ml	10^{-10}M	0.1	9
22 ng/ml	10^{-9}M	1	50
220 ng/ml	10^{-8}M	10	91
2200 ng/ml	10^{-7}M	100	99

*prolactin = 22,000 M.W.

Since, $$k = \frac{0.693}{t_{\frac{1}{2}}}$$

then, $$K_a = \frac{\frac{0.693}{t_{\frac{1}{2}}\text{ (forward)}}}{\frac{0.693}{t_{\frac{1}{2}}\text{ (reverse)}}}$$

and $$K_a = \frac{t_{\frac{1}{2}}\text{ (reverse)}}{t_{\frac{1}{2}}\text{ (forward)}}.$$

A theoretical half-time of the forward reaction can be calculated with $K_a = 10^9 M^{-1}$, and a $t_{\frac{1}{2}}$ (reverse) = 8 hours.

$$t_{\frac{1}{2}}\text{ (forward)} = \frac{t_{\frac{1}{2}}\text{ (reverse)}}{k_a} = \frac{8 \text{ hours}}{10^9} = 28.8 \text{ μsec.}$$

A half-time of a forward reaction rate of this size would indicate that the prolactin binding to its receptor is complete in less than a millisecond. The binding of prolactin is not believed to be completed this quickly. The fastest binding to be shown for prolactin and its receptor is in the mouse mammary gland where several minutes are required to achieve equilibrium (Frantz et al., 1974).

If one uses another $t_{\frac{1}{2}}$ (reverse) of 45 hours (Shiu and Friesen, 1974) the half-time of association is still 162 μsec. This is a value which is many orders of magnitude faster than the observed binding reaction.

Another way to look at this relationship is to assign a forward reaction half-time, derive a reverse half-time and compare it to values found in the literature. If a half-time of association of 1.0 min is chosen, the dissociation half-time is 166,666,667 hours or about 19,000 years. Clearly these values do not correspond to the results presented in this thesis or by other investigators (Shiu and Friesen, 1974; Frantz et al., 1974; Sakai et al., 1975).

The kinetic analysis presented above suggests that the simple model of:

$$\text{Prolactin} + \text{Receptor} \rightleftharpoons \text{Prolactin-Receptor}$$

is not the proper model. The range of the estimates of k cannot account for a K_a of $10^9 M^{-1}$. An additional k or k's which would be incorporated into the statement for K_a may be able to account for the discrepancy, but the correct binding reaction will have to be more complex than that presently postulated.

VI. Effects of an Ether Stress Induced Rise in Serum Prolactin on the Quantity of Assayable Prolactin Membrane Receptors in Livers of Female Rats

A. Objectives

The purpose of these experiments was to elucidate whether or not a moderate rise in serum prolactin induced by ether stress, would shift the equilibrium of prolactin-receptor binding so that a portion of these receptors would be blocked from assay. If the action of prolactin is transmitted via the binding event, then an increase in binding should be observed when a physiological increase in serum prolactin occurs. If this does not happen then it may be questioned as to how a physiological rise of prolactin could induce such processes as lactation by the plasma membrane mechanism. Further, these experiments crudely examined the time required for these freshly bound receptors to dissociate from prolactin. Finally, the time period that increased serum prolactin concentrations were allowed to bathe the target tissue was examined in relation to the time which the freshly bound prolactin and receptors required to dissociate. This last test may indicate if the prolactin-receptor binding is the simple one-step process it has been assumed to be, i.e.,

$$\text{Prolactin} + \text{Receptor} \rightleftharpoons \text{Prolactin-Receptor Complex}$$

or if a more complicated mechanism is required to explain prolactin binding. If the simple process is correct, then the half-time of dissociation of the prolactin-receptor complexes should require the same amount of time regardless of the period of time allowed for prolactin and receptor to form complexes.

To conduct this type of experiment the binding variation between animals needed to be minimized. This was accomplished in these experiments by using a paired "t" statistical experimental design. The data were gathered by comparing the binding of ^{125}I-prolactin to two pieces of liver from the same rat. One piece was exposed and a second piece was not exposed to an ether stress induced increase in serum prolactin. Ether anesthesia and surgical stress sharply increases serum prolactin (Simonel et al., 1975).

B. Procedures

Groups of 75-day-old female Sprague-Dawley rats were examined daily to determine their estrous cycles. Animals in proestrous were not used in this study. A rat was placed in a saturated ether jar. As soon as the animal was immobile it was removed and placed in an etherized nose cone. As quickly as possible the rat was cut open by a ventral midline incision. A lobe of the animal's liver was removed. Next a 0.5 to 1.0 ml blood sample was taken by cardiac stab. The time was noted. The animal was then allowed to lie on the table under ether anesthesia for a total ether exposure of either 5 (Study I) or 30 minutes (Study II). At the end of this period a second blood sample was removed by cardiac stab, and a second lobe of the liver was removed. A vaginal washing was taken to confirm the stage of the estrous cycle. The blood was processed and assayed for prolactin by the NIH rat prolactin radioimmunoassay procedure. The liver samples were processed and assayed for prolactin binding by the standard assay procedure, except that each pair of liver samples was incubated in the assay for varying lengths of time. The assay results of early and late samples were compared statistically

by a paired "t" analysis (Goldstein, 1964). Study I, where the increased prolactin levels were allowed to bathe the liver for 5 minutes, was done twice with a total of 20 rats. These results were pooled. Study II, where the increased prolactin levels were allowed to bathe the liver for 30 minutes, was done once with 10 rats. The protocol for Studies I and II are shown in Table 14.

C. Results

Study I. (5 minute etherization)

The prolactin radioreceptor assay results of the 1 minute and 5 minute liver samples are shown in Table 15. The liver samples were assayed for incubation times of 1 or 2 hours. At one hour the 5 minute liver samples bound 22% less ^{125}I-prolactin than the 1 minute liver samples. This reduction of prolactin receptors available for assay in the 5 minute liver samples was assumed to be due to the statistically significant increase in serum prolactin induced by the ether anesthesia and surgical stress. After 2 hours of assay incubation the difference in ^{125}I-prolactin binding was lost. This may have been due to the rather rapid dissociation of the prolactin receptors in the 5 minute liver samples which had become occupied during their 5 minute bathing in high serum prolactin concentrations.

The results of the prolactin radioimmunoassay are presented in Table 16. There was a statistically significant rise ($P<0.01$) in serum prolactin when comparing the 1 minute and 5 minute blood samples. Although the average time that the early sample collection was completed was 76 seconds, it was a good estimate of the prolactin level prior to the ether stimulus. Simonel (Simonel et al., 1975) had shown that

the appearance of an ether-stress-induced rise in serum prolactin has between a one and two minute lag from the commencement of etherization. Between the early and late blood samples serum prolactin rose 610%.

Study II. (30 minute etherization)

The prolactin radioreceptor assay results of the 1 minute and 30 minute liver samples are shown in Table 15. The liver samples in this study were incubated for assay incubation times of 1, 2, 4 and 10 hours. A significant reduction of assayable prolactin receptors in the late liver samples was noted at 1, 2 and 4 hours of incubation. The reduction was approximately 17% at these three time periods. It was not until 10 hours of assay incubation that the prolactin binding of early and late liver samples was equivalent. The eventual loss of the difference in binding of 1 minute and 30 minute liver samples was due to the dissociation of the prolactin receptors that had become occupied as a result of the 30 minutes of increased serum prolactin levels.

The rat prolactin radioimmunoassay results are presented in Table 16. The rise of serum prolactin between the 1 minute and 30 minute blood samples was significant at $P<0.05$ and was a 254% increase. The early blood sample collection had been completed by an average of 50 seconds.

The results of both Study I and Study II show that a substantial endogenous rise in serum prolactin can cause a portion of the target tissue receptors to be bound within a short time and thus block receptors from the ^{125}I-prolactin of a prolactin radioreceptor assay. The blocking of the prolactin radioreceptor assay by this endogenously bound prolactin lessens with time due to the dissociation of the endogenous prolactin

from the receptor. Thus, it may be possible to overcome this assay problem by using long incubation times in prolactin radioreceptor assays. Finally, the time required for the endogenously bound prolactin to dissociate from the prolactin receptor is different in Study I and Study II. After the liver prolactin receptors are bathed in increased serum prolactin levels for 5 minutes the endogenous prolactin can dissociate within one to two hours of assay incubation. This is in contrast to Study II where the high prolactin levels are exposed to the liver prolactin receptors for 30 minutes. Once exposed for 30 minutes the dissociation requires between 4 and 10 hours to complete.

Table 14. Protocol for Experiment VII

Study I

Begin Etherization	Remove Blood and Liver Samples	Remove Blood and Liver Samples	Vaginal Epithelial Smear
0	1	5	

Time (minutes)

Study II

Begin Etherization	Remove Blood and Liver Samples	Remove Blood and Liver Samples	Vaginal Epithelial Smear
0	1	30	

Time (minutes)

Table 15. ^{125}I-prolactin Binding to Liver of Rats: Effect of an Ether Stress Induced Rise in Serum Prolactin

Study I (5 minute etherization)*[a]

Assay Incubation time (hours)	^{125}I-prolactin Percent Specific Binding liver (1 min)	liver (5 min)	% Reduction of liver binding	Statistical difference@
1	0.90	0.71	-22	P<0.01
2	2.11	2.01	- 5	none

Study II (30 minute etherization)*[b]

Assay Incubation time (hours)	^{125}I-prolactin Percent Specific Binding liver (1 min)	liver (30 min)	% Reduction of liver binding	Statistical difference@
1	1.40	1.18	-16	P<0.05
2	1.79	1.48	-17	P<0.05
4	2.75	2.21	-16	P<0.05
10	6.23	6.45	+ 4	none

*Assay conditions: variable time, 23°C

[a]20 75-day-old female rats

[b]10 75-day-old female rats

@Paired "t" test

Table 16. Effect of Ether Stress on Serum Prolactin Levels of Female Rats

Study I (5 minute etherization)[a]

Sample time (min)	Prolactin (ng/ml)
1	80[c]
5	488[d]

Study II (30 minute etherization)[b]

Sample time (min)	Prolactin (ng/ml)
1	59[e]
30	150[f]

[a]average time early blood drawing completed, 76 sec.

[b]average time early blood drawing completed, 50 sec.

[c/d]$P<0.01$ by paired "t"

[e/f]$P<0.05$ by paired "t"

D. Discussion

The results of these experiments demonstrate that a moderate rise in serum prolactin can result in the blocking of approximately 15-20% of the total assayable prolactin membrane receptors. This phenomena adds credence to the concept that the action of prolactin on target cells is initiated by the binding of prolactin to plasma membrane receptors. Although these data support this concept, further work needs to be conducted to demonstrate a direct relationship between the level of binding and the physiological response to the hormone.

The persistence of the binding of endogenous prolactin causes specific problems in the standard radioreceptor assay. The fact that unquantified amounts of endogenous prolactin are carried into the assay incubation on in vivo bound receptors can introduce error in two ways. First, if the reaction is not run until all endogenously bound receptors are allowed to liberate their prolactin the total amount of prolactin receptors cannot be accurately determined. Second, if equilibrium is attained, an unknown quantity of prolactin is liberated into the assay. This causes a situation identical to the first few points of a competitive binding curve, where doses of less than one nanogram can effectively compete for a discernable number of prolactin receptors.

An illustrative example is a plasma membrane preparation that contains 200 fM of prolactin receptor ($K_a = 10^9 M^{-1}$) per 300 µg protein and is removed from a rat with a serum prolactin level of 22 ng/ml. An estimated (Table 13) 50% of the prolactin receptors are bound at equilibrium by the 22 ng/ml of rat prolactin suggesting that 100 fM of endogenous prolactin can be released into the incubation. This is equivalent to 2.2 ng of prolactin capable of being released into the

assay, an amount that is in excess of the estimated amount of ^{125}I-prolactin (0.14 ng) in each assay tube. Thus, in the standard assay procedure, underestimates of the relative numbers of prolactin receptors can be substantial in animals which have differing prolactin levels either immediately before or at the time of tissue sampling.

The K_a obtained from Scatchard plots is effected by incorrect estimates of total hormone in the incubation. Additionally, failure of the reaction to reach equilibrium with the endogenous prolactin pool and the added ovine prolactin pool in Scatchard analysis produces error in the estimate of the total number of receptors. Because of these two factors, estimates of K_a and total prolactin receptor population cannot be obtained from the same Scatchard plot. The best method to obtain valid binding data may be to treat the plasma membrane preparations prior to assay with low pH to remove this troublesome endogenous prolactin.

Although these experiments were not designed to produce values for prolactin receptor dissociation rates, the results indicate that after a five minute exposure of rat liver prolactin receptors to a moderate increase in serum prolactin the dissociation of prolactin from the freshly bound receptors is more rapid than if the exposure is for 30 minutes. It is possible that the initially bound prolactin-receptor complex has a different affinity than the prolactin-receptor complex after 30 minutes, and that the mechanism for the cellular interaction of prolactin is at least a two-step process. The concept of prolactin binding in a two-step process is more compatible with the results of the kinetic analysis of binding presented in the preceding experiment. This

type of binding once properly analyzed, could yield the rate constants presumed to be missing in the statement of the association constant (K_a).

There are presently at least five explanations for nonlinear Scatchard plots. These are: receptor heterogeneity, negative cooperativity, dissociation of a receptor subunit during binding, and a two-step mechanism (Boeynaems and Dumont, 1975; Boeynaems et al., 1975) or altered affinity of labeled ligand (Taylor, 1975).

Receptor heterogeneity, $K_1 \neq K_2$

$$R_1 + H \overset{K_1}{\rightleftharpoons} HR_1$$

$$R_2 + H \overset{K_2}{\rightleftharpoons} HR_2$$

Negative Cooperativity, $K_1 > K_2$

$$R_2 + H \overset{K_1}{\rightleftharpoons} R_2H$$

$$R_2H + H \overset{K_2}{\rightleftharpoons} R_2H_2$$

Dissociation Mechanism

$$RE + H \overset{K}{\rightleftharpoons} RH + E$$

Two-Step Mechanism*

$$R + H \overset{K_1}{\rightleftharpoons} RH$$

$$RH + E \overset{K_2}{\rightleftharpoons} RHE$$

*Other two-step mechanisms can also be invoked.

Altered affinity of labeled ligand $K_1 \neq K_2$

$$H^* + R \underset{}{\overset{K_1}{\rightleftharpoons}} H^*R$$

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

If these various mechanisms are applied to the data which suggest two different association constants being seen in sequential order the most plausible explanation is the two-step mechanism. There are several reasons for this selection.

First, there are two differing dissociation rates of prolactin from receptor, and so two distinct species of hormone receptor complex should exist. This eliminates the dissociation mechanism for it only proposes one species of hormone-receptor complex. The altered affinity of labeled ligand mechanism must also be dismissed because the in vivo binding and the dissociation only involves unlabeled ligand.

Second, the rapid dissociation of prolactin from its receptor occurs only after the five minute exposure. After a 30 minute exposure there must be both a fast and slow dissociation if the receptor heterogeneity mechanism is a reality. Since this did not occur, receptor heterogeneity probably does not exist. Another piece of data that disqualified the receptor heterogeneity mechanism is the shape of the competitive binding curves. All competitive binding curves of prolactin receptors are not biphasic as a large heterogeneity of receptors demands. Instead, they are single phase smooth curves which imply homogeneity of binding sites and hormone.

* Other two-step mechanisms can also be invoked.

Third, if negative cooperativity is involved in the prolactin-receptor interaction the amount of unlabeled prolactin required to competitively displace ^{125}I-prolactin should be greater than the amount required in a noncooperative system. In a noncooperative binding system the proper range of unlabeled hormone effective in displacing ^{125}I-prolactin is 4 orders of magnitude as seen in Table 18. This range should reduce the bound ^{125}I-prolactin from 99% of the total receptors bound by ^{125}I-prolactin to 1% bound. The range of unlabeled hormone for an equivalent competition in a negatively cooperative system would have to be greater than 4 orders of magnitude. This phenomena is due to the negative cooperative effects on binding, i.e., it takes a greater range of hormone to saturate a negative cooperative system when compared to a noncooperative system. Thus, when applied to a competitive binding experiment a greater range of unlabeled prolactin is required to saturate all receptors and effectively prevent all ^{125}I-prolactin from binding. Examination of the competitive binding curves in this thesis reveals that displacement of essentially all ^{125}I-prolactin occurs in four or slightly less than four orders of magnitude of unlabeled prolactin concentration. The competitive binding curve of mouse mammary gland is an exception. I believe that the difference is due to error in working with such a low binding tissue. Despite the mouse mammary gland, data from rat and mouse liver, rat mammary gland and DMBA-induced rat mammary tumors do not support the idea of a negatively cooperative binding mechanism. These data have not eliminated the possibility of negative cooperativity where the association constants are similar, but are an indication that negative cooperativity where the association constants are very dissimilar is not a possible mechanism for prolactin binding.

A two-step mechanism fits the data in many regards. First, it does provide two distinct species of hormone receptor complexes. This is compatible with the data indicating two dissociation rates which appear in a time sequence. Second, no cooperative effects are needed to explain this mechanism. So if allowed to achieve equilibrium, receptors that use a two-step mechanism are compatible with the majority of competitive binding curve data. Third, the two-step mechanism provides at least 4 rate constants that can be involved in the K_a measured from Scatchard analysis.

A two-step mechanism requires the presence of a second intramembrane or intracellular moiety to be involved in the second-step reaction. Although no direct evidence of the existence of such a species has been produced in the prolactin receptor system, the idea has been recently presented to explain the coupling of adenylate cyclase with epinephrine, glucagon and ACTH (Helmreich, 1976; Cuatrecasas, 1974).

A two-step mechanism adds a second control point to the responses elicited by prolactin. The level of prolactin receptor protein is one level of the cellular modulation of sensitivity to prolactin. The modulation at this level is shown to be related to estrogen (Posner et al., 1974b), prolactin (Costlow et al., 1975a; Posner et al., 1975) and thyroid hormone (Gelato et al., 1975) in the rat liver; by dehydration in the kidney; by dehydration or salt loading in the adrenal (Marshall et al., 1975); or by testosterone, prolactin or castration in the ventral prostate of the rat (Kledzik et al., 1976).

The second level of control may be the production of the molecular species involved in a second-step of binding. The production of this second molecular moiety may be linked to hormonal changes at parturition in the mammary gland. Prolactin receptors are present in the rat mammary gland throughout pregnancy and lactation. Yet despite the presence of lactogenic hormones during both these phases the mammary epithelial responds by lactation only in the later period (Holcomb et al., 1976). This suggests that a second control mechanism involving the transferal of prolactin's action to the cell may be present.

These data are compatible with a two-step model of prolactin binding in target cells. The seeming plausability of such a mechanism to explain experimental observations makes this model an ideal candidate for further research. More complicated steps in this mechanism may exist, but will remain unknown until more work is completed.

CONCLUSION

1. Certain variables of the ^{125}I-prolactin radioreceptor assay, as conducted in this thesis, are consistent with those reported in other laboratories. For example, association constants, receptor concentrations, time and temperature binding dependence, tissue specificity and assay reproducibility are all comparable to previous published values.
2. ^{125}I-prolactin bound specifically to plasma membranes of prolactin dependent and independent DMBA-induced rat mammary tumors whereas the hormone generally failed to bind to plasma membranes of spontaneous C3H/HeJ mouse mammary tumors. It is well known that growth of the DMBA-induced rat mammary tumor is stimulated by prolactin whereas growth of the C3H/HeJ mouse mammary tumor is not affected by this hormone. Thus, an excellent correlation appears to exist between the capacity of the tumor to bind prolactin and its growth responsiveness to this hormone. This suggests that the concentration of prolactin membrane receptor might be predictive for the growth sensitivity of a tumor to this hormone.
3. The administration of prolactin to hypophysectomized-ovariectomized rats resulted in an increase in the specific binding of ^{125}I-prolactin to liver plasma membranes. This suggests that prolactin can regulate its own receptor concentration.
4. Marked increases in blood prolactin in rats by administration of the hormone or by ether stress sharply reduced the specific binding of

^{125}I-prolactin to plasma membranes of liver and mammary glands. Increased blood prolactin levels apparently cause an increase in the quantity of endogenous prolactin that binds to available membrane receptor sites, reducing assayable ^{125}I-prolactin binding sites. Thus, the level of prolactin in the blood appears to be a critical factor in the quantification of prolactin membrane receptors by the ^{125}I-prolactin radioreceptor assay.

5. The specific binding of ^{125}I-prolactin to plasma membrane receptors of the mouse mammary gland can be sharply increased by prior washing of the membrane preparation with a pH 2 bath at 4°C. This procedure appears to release endogenously-bound prolactin from its receptor thus increasing assayable binding sites. Such a procedure should increase the accuracy and sensitivity of the ^{125}I-prolactin radioreceptor assay.
6. Evidence is presented in this thesis which indicates that the binding of prolactin to its plasma membrane receptor is not a simple single step phenomenon, but a complex multiple-step, time-dependent process.

BIBLIOGRAPHY

BIBLIOGRAPHY

Aragona, C. and H. G. Friesen. Specific prolactin binding sites in the prostate and testis of rats. Endocrinology, 97: 677 (1975).

Apostolakis, M. The extraction of prolactin from human pituitary glands. Acta Endocrinologica, 49: 1 (1965).

Baxter, J. D. and G. M. Tomkins. Specific cytoplasmic glucocorticoid hormone receptors in hepatoma tissue culture cells. Proc. Nat. Acad. Sciences, 68: 932 (1971).

Baxter, J. D., A. W. Harris, G. M. Tomkins and M. Cohn. Glucocorticoid receptors in lymphoma cells in culture: Relationship to glucocorticoid killing activity. Science, 171: 189 (1971).

Birkinshaw, M. and I. R. Falconer. The localization of prolactin labelled with radioactive iodine in rabbit mammary tissue. J. Endocrinology, 55: 323 (1972).

Boeynaems, J. M., S. Swillens and J. E. Dumont. A negative control model of hormone receptor interaction. Biosystems, 7: 206 (1975).

Boeynaems, J. M. and J. E. Dumont. Quantitative analysis of the binding of ligands to their receptors. J. Cyclic Nucleotide Res., 1: 123 (1975).

Bolton, A. E. and W. M. Hunter. Labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J., 133: 529 (1973).

Bradley, C. J., G. S. Kledzik and J. Meites. Prolactin and estrogen dependency of rat mammary cancers at early and late stages of development. Cancer Res., 36: 319 (1976).

Brooks, C. L. and C. W. Welsch. Reduction of serum prolactin in rats by 2 ergot alkaloids and 2 ergoline derivatives: A comparison. Proc. Soc. Exptl. Biol. and Med., 146: 863 (1974).

Bull, H. B. An introduction to physical biochemistry. F. A. Davis Co., Philadelphia (1971), p. 242.

Charles, M. A., G. U. Ryffel, M. Obinata, B. J. McCarthy and J. D. Baxter. Nuclear receptors for thyroid hormone: Evidence for nonrandom distribution within chromatin. Proc. Nat. Acad. Sci., 72: 1787 (1975).

Chomcznski, P. and Y. J. Topper. A direct effect of prolactin and HPL on mammary epithelial nuclei. Biochem. Biophys. Res. Comm., 60: 56 (1974).

Clemens, J. A. and J. Meites. Hypothalamic control of prolactin secretion. In Mammary Cancer and Neuroendocrine Therapy. (B.A. Stoll, ed.). Butterworth and Co., London (1974) p. 160.

Costlow, M. E., R. A. Buschow and W. L. McGuire. Prolactin receptors in estrogen receptor deficient mammary carcinoma. Science, 184: 85 (1974).

Costlow, M. E., R. A. Buschow and W. L. McGuire. Prolactin stimulation of prolactin receptors in rat liver. Life Sciences, 17: 1457 (1975a).

Costlow, M. E., R. A. Buschow, N. J. Richert and W. L. McGuire. Prolactin and estrogen binding in transplantable hormone-dependent and autonomous rat mammary carcinomas. Cancer Res., 35: 970 (1975b).

Cuatrecasas, P. Unmasking of insulin receptors in fat cells and fat cell membranes. J. Biol. Chem., 246: 6532 (1971).

Cuatrecasas, P. Membrane receptors. Ann. Rev. Biochem., 43: 169 (1974).

Delgado, C., J. Pensky and O. H. Pearson. Prolactin receptors in rat mammary tumors and liver. 57th Annual Meeting of the Endocrine Society, p. 83, (1975) (Abstract).

DeSombre, E., G. Kledzik, S. Marshall and J. Meites. Estrogen and prolactin receptor concentration in rat mammary tumors and response to endocrine ablation. Cancer Res., 36: 354 (1976).

Dilley, W. G. and S. J. Kister. In vitro stimulation of human breast tissue by human prolactin. J. Natl. Cancer Inst., 55: 35 (1975).

Elias, J. J. and E. Rivera. Comparison of the response of normal, precancerous and neoplastic mouse mammary tissues to hormones in vitro. Cancer Res., 19: 505 (1959).

Falconer, I. R. Uptake and binding of ^{125}I-labelled prolactin by mammary tissue. Biochem. J., 126: 8 pp. (1972) Abstract.

Fang, S., K. M. Anderson and S. Liao. Receptor proteins for androgens. J. Biol. Chem., 244: 6584 (1969).

Feil, P. D., S. R. Glasser, D. O. Toft and B. W. O'Malley. Progesterone binding in the mouse and rat uterus. Endocrinology, 91: 738 (1972).

Flückiger, E. Drugs and the control of prolactin secretion. In Prolactin and Carcinogenesis. (A. R. Boyns and K. Griffiths, eds.). Alpha Omega Alpha Publishing, Cardiff (1972) p. 162.

Frantz, W. L. and R. W. Turkington. Formation of biologically active ^{125}I-prolactin by enzymatic radioiodination. Endocrinology, 91: 1545 (1972).

Frantz, W. L., J. H. MacIndoe and R. W. Turkington. Prolactin receptors: Characteristics of the particulate fraction binding activity J. Endocrinology, 60: 485 (1974).

Frantz, W. L., P. Payne, O. Dombroski and Carlos Sonnenschine. Binding of ovine ^{125}I-prolactin to cultured anterior pituitary tumor cells and normal cells. Nature, 225: 636 (1975).

Friesen, H. G., G. Tolis, R. Shiu and P. Hwang. Studies on human prolactin: Chemistry, radioreceptor assay and clinical significance. In Human Prolactin. (J. L. Pasteels and C. Robyn, eds.). American Elsevier Publishing, N.Y. (1973) p. 11.

Funder, J. W., D. Feldman and I. S. Edelman. Glucocorticoid receptors in the rat kidney: The binding of tritiated-dexamethasome. Endocrinology, 92: 1005 (1973b).

Funder, J. W., D. Fieldman and I. S. Edelman. The roles of plasma binding and receptor specificity in the mineral corticoid action of aldosterone. Endocrinology, 92: 994 (1973a).

Gardner, R. S. Nuclear thyroid hormone receptors: Evidence for association with nucleolar chromatin. Biochem. Biophys. Res. Comm., 67: 625 (1975).

Gelato, M., S. Marshall, M. Boudreau, J. Bruni, G. a. Campbell and J. Meites. Effects of thyroid and ovaries on prolactin binding activity in rat liver. Endocrinology, 96: 1292 (1975).

Goldstein, A. Biostatistics. The MacMillan Company, N.Y. (1964) p. 51.

Goral, J. E. and J. L. Whittliff. Comparison of glucocorticoid binding proteins in normal and neoplastic mammary tissue in the rat. Biochemistry, 14: 2944 (1975).

Gordon, J., J. A. Smith and R. J. B. King. Metabolism and binding of androgen by mouse mammary tumor cells in culture. Mol. and Cellular Endo., 1: 259 (1974).

Guyda, H. J. Heterogeneity of human growth hormone and prolactin secreted in vitro: Immunoassay and radioreceptor assay correlation. J. Clin. Endo. Metab., 41: 953 (1975).

Haour, F. and B. B. Saxena. Characterization and solubilization of gonadotropin receptor of bovine corpus luteum. J. Biol. Chem., 249: 2195 (1974).

Hector, O. and I. D. K. Halkerston. On the action of mammalian hormones. In The Hormones. (G. Pincus, K. V. Thimann and E. B. Astwood, eds.). Vol. V, Academic Press, N.Y. (1964) p. 697.

Hobbs, J. R., I. DeSouza, H. Salih and P. Raggatt. Selection of hormone-dependent breast cancer. British J. Surgery, 61: 785 (1974).

Holcomb, H. H., M. E. Costlow, R. A. Buschow, and W. L. McGuire. Prolactin binding in rat mammary gland during pregnancy and lactation. Biochem. Biophys. Acta, 428: 104 (1976).

Holdaway, I. M. and I. Worsley. Specific binding of human prolactin and insulin to human mammary carcinomas. 57th Annual Meeting of the Endocrine Society (1975) p. 160 (Abstract).

Holt, J. A. and J. S. Richards. Effects of ergocryptine and prolactin on ovarian binding of ^{125}I-HCG and ^{125}I-prolactin in FSH-primed LH-treated immature rats. 57th Annual Meeting of the Endocrine Society (1975) p. 82 (Abstract).

Huggins, C., L. C. Grand and F. P. Brillantes. Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. Nature, 189: 204 (1961).

Hunter, W. M. and F. C. Greenwood. Preparation of iodine-131 labelled human growth hormone of high specificity activity. Nature, 194: 495 (1962).

Hwang, P., M. Robertson, H. Guyda and H. G. Friesen. The purification of human prolactin from frozen pituitary glands. J. Clin. Endo. Metab., 36: 1110 (1973).

Jackson, V. and R. Chalkley. The binding of estradiol-17β to the bovine endometrial nuclear membrane. J. Biol. Chem., 249: 1615 (1974).

Jensen, E. V. and H. I. Jacobsen. Fate of steroid estrogens in their target tissues. In Biological Activities of Steroids in Relation to Cancer. (G. Pincus and G. P. Vollmer, eds.). Academic Press, N.Y. (1960) p. 161.

Jensen, E. V., T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jurgblunt and E. R. DeSombre. A two-step mechanism for the interaction of estradiol with rat uterus. Proc. Nat. Acad. Sci., 59: 632 (1968).

Jensen, E. V. and E. R. DeSombre. Estrogen receptor interaction. Science, 182: 126 (1973).

Kelly, P. A., H. A. Robertson and H. G. Friesen. Temporal pattern of placental lactogen and progesterone secretion in sheep. Nature, 248: 435 (1974a).

Kelly, P. A., B. I. Posner, T. Tsushima and H. G. Friesen. Studies of insulin, growth hormone and prolactin binding: Ontogenesis, effects of sex and pregnancy. Endocrinology, 95: 532 (1974b).

Kelly, P. A., C. Bradley, R. P. C. Shiu, J. Meites and H. G. Friesen. Prolactin binding to rat mammary tumor tissue. Proc. Soc. Exptl. Biol. Med., 146: 816 (1974c).

Kelly, P. A., B. I. Posner and H. G. Friesen. Effects of hypophysectomy, ovariectomy and cycloheximide on specific binding sites for lactogenic hormones in rat liver. Endocrinology, 97: 1408 (1975).

King, R. J. B., J. Gordon, D. M. Cowan and R. D. Inman. The intranuclear localization of [6,7-^{3}H]-Oestradiol-17β in Dimethylbenzanthracene-induced rat mammary adenocarcinoma and other tissues. J. Endocrinology, 36: 139 (1966).

Kledzik, G., S. Marshall, M. Gelato, G. Campbell and J. Meites. Prolactin binding activity in the crop sacs of juvenile, mature, parent and prolactin-injected pigeons. Endocrine Res. Comm., 2: 345 (1975).

Kledzik, G. S., S. Marshall, G. A. Campbell, M. Gelato and J. Meites. Effects of castration, testosterone, estradiol, and prolactin on specific prolactin-binding activity in ventral prostate of male rats. Endocrinology, 98: 373 (1976).

Landsman, R. and B. B. Saxena. Radioreceptor assay of HCG as an aid in miniabortion. Fertility and Sterility, 25: 1022 (1974).

Lee, C. Y. and R. J. Ryan. Luteinizing hormone receptors in luteinized rat ovaries. In Receptors for Reproductive Hormones. (B.W. O'Malley and A. R. Means, eds.). Plenum Press, N.Y. (1973) p. 419.

Lefkowitz, R. J. Isolated hormone receptors. New England Journal of Medicine, 288: 1061 (1973).

Lewis, U. J., R. N. P. Singh and B. K. Seavey. Problems in the purification of human prolactin. In Prolactin and Carcinogenesis. (A. R. Boyns and K. Griffiths, eds.). Alpha Omega Publishing, Cardiff (1972) p. 4.

Li, C. H., H. M. Evans and M. E. Simpson. Studies on pituitary lactogenic hormone. VII. A method of isolation. J. Biol. Chem., 146: 627 (1942).

Li, C. H., J. S. Dixon, R.-B. Lo, K. D. Schmidt and Y. A. Pankov. Studies on pituitary lactogenic hormone XXX. The primary structure of sheep hormone. Arch. Biochem. Biophys., 141: 705 (1970).

Liao, S., T. Liang, S. Fang, E. Castaneda and T.-C. Shao. Steroid structure and androgenic activity. J. Biol. Chem., 248: 6154 (1973).

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin Phenol reagent. J. Biol. Chem., 193: 265 (1951).

MacLeod, K. M. and J. D. Baxter. DNA binding of thyroid hormone receptors. Biochem. Biophys. Res. Comm., 62: 577 (1975).

Mainwaring, W. I. P. A soluable androgen receptor in the cytoplasm of the rat prostate. J. Endocrinology, 45: 531 (1969).

Marshall, S., M. Gelato and J. Meites. Serum prolactin levels and prolactin binding activity in adrenals and kidneys of male rats after dehydration, salt loading and unilateral nephrectomy. Proc. Soc. Exptl. Biol. Med., 149: 185 (1975).

McKnight, G. S., P. Pennequin and R. T. Schimke. Induction of ovalbumen mRNA in chick oviduct as measured by hybridization to complementary DNA. J. Biol. Chem., 250: 8105 (1975).

Meites, J. K., H. Lu, W. Wuttke, C. W. Welsch, H. Nagasawa and S. K. Quadri. Recent studies on functions and control of prolactin secretion in rats. Recent Progress in Hormone Res., 28: 471 (1972).

Midgley, A. R., Jr. Autoradiographic analysis of gonadotropin binding to rat ovarian tissue sections. In Receptors for Reproductive Hormones. (B. W. O'Malley and A. R. Means, eds.). Plenum Press, N.Y. (1973) p. 365.

Milgrom, E. and E.-E. Baulieu. Progesterone in uterus and plasma I. Binding in rat uterus 105,000g supernate. Endocrinology, 87: 276 (1970).

Mohla, S., E. R. DeSombre and E. V. Jensen. Tissue specific stimulation of RNA synthesis by transformed estradiol receptor complexes. Biochem. Biophys. Res. Comm., 46: 661 (1972).

Nagasawa, H., K. Kuretani and F. Kanzawa. Effect of Prolactin on the growth of spontaneous mammary tumor in mice. Gann, 51: 637 (1966).

Nagasawa, H., R. Yanai, H. Iwahashi and M. Fujimoto. Effect of pituitary isographs on the growth of spontaneous mammary tumors in mice. Gann, 58: 337 (1967).

Niall, H. D. The chemistry of human lactogenic hormones. In Prolactin and Carcinogenesis. (A. R. Boyns and K. Griffiths, eds.). Alpha Omega Alpha Publishing, Cardiff (1972) p. 13.

Nicoll, C. S. and G. D. Bryant. Physiological and immunological properties of prolactin. In Prolactin and Carcinogenesis. (A. R. Boyns and K. Griffiths, eds.). Alpha Omega Alpha Publishing, Cardiff (1972) p. 28.

Nicoll, C. S. Physiological actions of prolactin. In Handbook of Physiology. (R. O. Greep and E. B. Astword, eds.). American Physiological Society, Washington, D.C. (1974), Vol. IV, Section 7, part II, p. 253.

O'Malley, B. W., W. T. Schrader and T. C. Spelsberg. Hormone-receptor interactions with the genome of eukaryotic target cells. In Receptors for Reproductive Hormones. (B. W. O'Malley and A. R. Means, eds.). Plenum Press, New York (1973) p. 174.

O'Malley, B. W. and A. R. Means. Female steroid hormones and target cell nuclei. Science, 183: 610 (1974).

O'Malley, B. W., S. L. C. Woo, S. E. Harris, J. M. Rosen and A. R. Means. Steroid hormone regulation of specific messenger RNA and protein synthesis in eukaryotic cells. J. Cell Physiology, 85: 343 (1975).

O'Malley, B. W. and W. T. Schrader. The receptors of steroid hormones. Scientific American (February 1976) p. 32.

Pearson, O. H. Prolactin-dependent rat mammary cancer: A model for man? Transactions of the Association of American Physicians, 82: 225 (1969).

Pearson, O. H., R. Murry, G. Mozaffarian and J. Pensky. Prolactin and experimental breast cancer. In Prolactin and Carcinogenesis. (A. R. Boyns and K. Griffiths, eds.). Alpha Omega Alpha Publishing, Cardiff (1972) p. 154.

Perry, H. M. and L. S. Jacobs. Mammary prolactin receptors: Heightened affinity in the late puerpuerium. 57th Annual Meeting of the Endocrine Society, p. 84 (1975) (Abstract).

Posner, B. I., P. A. Kelly, R. P. C. Shiu and H. G. Friesen. Studies of insulin, growth hormone and prolactin binding: Tissue distribution, species variation and characterization. Endocrinology, 95: 521 (1974a).

Posner, B. I., P. A. Kelly and H. G. Friesen. Induction of a lactogenic receptor in rat liver: Influence of estrogen and the pituitary. Proc. Nat. Acad. Sci., 71: 2407 (1974b).

Posner, Barry I. Polypeptide hormone receptors: Characteristics and applications. Canadian Journal of Physiol. and Pharmacol., 53: 689 (1975).

Posner, B. I., P. A. Kelly and H. G. Friesen. Prolactin receptors in rat liver: Possible induction by prolactin. Science, 188: 57 (1975).

Posner, B. I. Characterization and modulation of growth hormone and prolactin binding in mouse liver. Endocrinology, 98: 645 (1976).

Rajaniemi, H., and T. Vanha-Perttula. Attachment to the luteal plasma membranes: An early event in the action of luteininzing hormone. J. Endocrinology, 57: 199 (1973).

Rajaniemi, J., A. Oksanen and T. Vanha-Perttula. Distribution of ^{125}I-prolactin in mice and rats studies with whole-body and micro-autoradiography. Hormone Res., 5: 6 (1974).

Rhodes, R. G. Ovalbumen messenger ribonucleic acid. J. Biol. Chem., 250: 8088 (1975).

Robertson, M. C. and H. G. Friesen. The purification and characterization of rat placental lactogen. Endocrinology, 97: 621 (1975).

Rodbard, D. Mathematics of hormone receptor interaction. In Receptors for Reproductive Hormones. (B. W. O'Malley and A. R. Means, eds.). Plenum Press, N.Y. (1973) p. 289.

Rogol, A. D. and S. W. Rosen. Alteration of human and bovine prolactins by a chloroamine-T radioiodination: Comparison with lactoperoxidase-iodinated prolactins. J. Clin. Endo. Metab., 39: 379 (1974).

Rolland, R. and J. M. Hammond. Demonstration of a specific receptor for prolactin in procine granulosa cells. Endocrine Res. Comm., 2: 281 (1975).

Saito, T. and B. B. Saxena. Specific receptors for prolactin in the ovary. Acta Endocrin., 80: 126 (1975).

Sakai, S., K. Kohmoto and T. Johke. A receptor site for prolactin in lactating mouse mammary tissues. Endocrin. Japon, 22: 379 (1975).

Salih, H., H. Flax, W. Brander and J. R. Hobbs. Prolactin dependence in human breast cancers. The Lancet II: 1103 (1972).

Samuels, H. H. and T. S. Tsai. Thyroid hormone action in cell culture: Demonstration of nuclear receptors in intact cells and isolated nuclei. Proc. Nat. Acad. Sci., 70: 3488 (1973).

Samuels, H. H., J. S. Tsai and J. Casanova. Thyroid hormone action: In vitro demonstration of putative receptors in isolated nuclei and soluable nuclear extracts. Science, 184: 1188 (1974).

Saxena, B. B., S. H. Hasan, F. Haour and M. Schmidt-Gallwitzer. Radioreceptor assay of HCG: Detection of early pregnancy. Science, 184: 739 (1974).

Schwartz, J., D. F. Nutting, H. M. Goodman, J. L. Kostyo and R. E. Fellows. Growth hormone covalently bound to sepharose. II. Study of the biological activity of a growth hormone-sepharose complex in adipose tissue and diaphragm muscle. Endocrinology, 92: 439 (1973).

Schwartz, R. J., M.-J. Tsai, S. Y. Tsai and B. W. O'Malley. Effect of estradiol on gene expression in the chick oviduct. J. Biol. Chem., 250: 5175 (1975).

Sherman, M. R., P. I. Corvol and B. W. O'Malley. Progesterone-binding component of chick oviduct. J. Biol. Chem., 245: 6085 (1970).

Sheth, N. A., K. J. Ranadive and A. R. Sheth. In vitro binding of radioiodinated HPL to murine mammary gland. Europ. J. Cancer, 10: 653 (1974).

Shiu, R. P. C., P. A. Kelly and H. G. Friesen. Radioreceptor assay for prolactin and other lactogenic hormones. Science, 180: 968 (1973).

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

Shiu, R. P. C. and H. G. Friesen. Solubilization and purification of a prolactin receptor from the rabbit mammary gland. J. Biol. Chem., 249: 7902 (1974b).

Shiu, R. P. C. and H. G. Friesen. Blockade or prolactin action by an antiserum to its receptors. Science, 192: 259 (1976).

Simonel, C. E., C. L. Brooks and C. W. Welsch. Effects of ether anesthesia on plasma prolactin sampling. Experientia, 31: 688 (1975).

Smith, H. G., R. Smith, D. O. Toft, J. R. Neergaard, G. P. Burrows and B. W. O'Malley. Binding of steroids to progesterone receptor proteins in the chick oviduct and human uterus. J. Biol. Chem., 249: 5924 (1974).

Sokal, R. R. and F. J. Rohlf. Biometry. W. H. Freeman and Co., San Francisco (1969) p. 40.

Stricker, P. and G. Grueter. Action de lobe anterieur de l'hypophyse sur la montee laiteuse. Compt. Rend. Soc. Biol., 99: 1978 (1928).

Taylor, S. I. Bindings of hormones to receptors. An alternative explanation of nonlinear Scatchard plots. Biochemistry, 14: 2357 (1975).

Thorell, J. I. and B. G. Johansson. Enzymatic iodination of polypeptides with ^{125}I to high specific activity. Biochem. Biophys, Acta, 251: 363 (1971).

Toft, D. and J. Gorski. A receptor molecule for estrogens: Isolation from the rat uterus and preliminary characterization. Proc. Nat. Acad. Sci., 55: 1574 (1965).

Tsai, M.-J., R. J. Schwartz, S. Y. Tsai and B. W. O'Malley. Effect of estrogen on gene expression in the chick oviduct. J. Biol. Chem., 250: 5165 (1975).

Tucker, H. A., B. L. Larson and J. Gorski. Cortisol binding in cultured bovine mammary cells. Endocrinology, 89: 152 (1971).

Turkington, R. W. Stimulation of RNA synthesis in isolated mammary cells by insulin and prolactin bound to sepharose. Biochem. Biophys. Res. Comm., 41: 1362 (1970).

Turkington, R. W. and W. L. Frantz. The biochemical action of prolactin. In Prolactin and Carcinogenesis. (A. R. Boyns and K. Griffiths, eds.). Alpha Omega Alpha Publishing, Cardiff (1972) p. 39.

Turkington, R. W. Prolactin receptors in mammary cancer cells. Cancer Res., 34: 758 (1974).

Unhjem, O., K. J. Tveter and A. Aakvaag. Preliminary characterization of an androgen-macromolecular complex from the rat ventral prostate. Acta Endocrinologica, 62: 153 (1969).

Vaitukaitis, J. L., R. Sherins, G. T. Ross, J. Hickman and G. Ashwell. A method for the preparation of radioactive FSH w/ preservation of biological activity. Endocrinology, 89: 1356 (1971).

Welsch, C. W. and G. M. Rivera. Differential effects of estrogen and prolactin on DNA synthesis in organ cultures of DMBA-induced rat mammary carcinoma. Proc. Soc. Exptl. Biol. Med., 139: 623 (1972).

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

Welsch, C. W. and J. Meites. Neuroendocrine control of murine mammary carcinoma. In Mammary Cancer and Neuroendocrine Therapy. (B. A. Stoll, ed.). Butterworth and Co. (1974), p. 25.

Welsch, C. W., C. C. Hassett, C. Adams, L. K. Lambrecht and C. L. Brooks. 17β-estradiol and enovid-induced mammary tumorigenesis in C3H/HeJ female mice: Counteraction by concurrent treatment with 2-bromo-α-ergocryptine. International J. Cancer, submitted (1976a).

Welsch, C. W., G. C. Iturri and M. J. Brennan. DNA synthesis of human, mouse and rat mammary carcinomas in vitro: Influence of insulin and prolactin. Cancer, submitted (1976b).

Woo, S. L. C., J. M. Rosen, C. D. Liarakos, Y. C. Choi, H. Busch, A. R. Means, B. W. O'Malley and D. L. Robberson. Physical and chemical characterization of purified ovalbumen messenger RNA. J. Biol. Chem., 250: 7027 (1975).

Yamamoto, K. Characterization of the 4s and 5s forms of the estradiol receptor protein and their interaction with DNA. J. Biol. Chem., 249: 7068 (1974).