STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF THE RABBIT MAMMARY GLAND PROLACTIN RECEPTOR

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ABBREVIATIONS

BSA - bovine serum albumin

All other abbreviations are according to the Journal of Biological Chemistry.

CHAPTER 1

INTRODUCTION

Over the past ten years, considerable progress has been made in the study, identification, and purification of a variety of membrane-hormone receptors (review 1 - 7). A hormone receptor may be defined as that membrane-associated structure which performs the function of hormone recognition and binding, so as to initiate the chain of events leading to a hormonal response (4).

In general, the approach has been to study the interaction of a radioactively labeled ligand and the plasma membrane, either in the intact cells or with isolated membrane preparations. In a few instances, the hormone receptor has been solubilized from the plasma membrane by nonionic detergents and partially purified. In these studies, it is essential that a number of criteria be satisfied to establish that the binding measurements truly reflect a hormone-receptor interaction. These are:

(1) The labeled ligand used must be fully biologically active.

(2) Bound labeled ligand must be displacable by unlabeled ligand.

(3) Binding must be saturable within a physiological concentration range.

(4) Binding must be of high affinity.

(5) Binding should be restricted to tissues known to be physiologically sensitive to the ligand.

(6) Binding must be reversible.

The labeled hormone must have a high specific activity (2 μC/μmole) and this is usually obtained by iodination with carrier-free 125-Iodine. Highly labeled ligands are necessary in order to assay extremely minute amounts of receptor. For example, only 0.0004% of liver membrane is the insulin receptor (3).

To date, most receptors for polypeptide hormones appear to be proteins or glycoproteins since they are susceptible to proteolytic enzymes; and at least one, the insulin receptor, is a glycoprotein since it binds to the lectins Concanavalin-A and wheat germ agglutinin (3). Recent work by Kohn (9,10,11) has shown that the receptor for thyrotropin may be a ganglioside.

Most of the polypeptide hormones function by binding to a specific protein receptor on the plasma membrane surface; this complex then activates or inhibits adenyl cyclicase. Cyclic-AMP activates protein kinases to phosphorylate cytoplasmic or nuclear proteins which in turn leads to a physiological response (3).

At present, the mode of action of prolactin is not well understood. There is no evidence that adenyl cyclase is activated or inhibited when prolactin binds to its plasma membrane receptor (12,13). In mammals, the biological actions of prolactin are mammotropic (growth and different-

iation of breast tissue), lactogenic, and luteotrophic. Ovine prolactin was shown to have 82 separate actions in different species (14). These include all of the major metabolic actions identified for growth hormone, though in most cases the potency of prolactin was so weak relative to growth hormone that it is questionable whether prolactin contributes significantly to any general growth promoting process in mammals (14).

Nicoll, in 1972 and 1974 (15,16), speculated that most of the actions of prolactin seem to be modifications of other hormones' actions. Horobin (12) has suggested that prolactin acts by regulating the synthesis of prostaglandin in all its target tissues. Deis, in 1971 (17) showed that prostaglandin F_2 alpha given to pregnant rats could cause parturition and initiate lactation. Rellema, in 1975 (18), using explanted mouse mammary glands and RNA synthesis as a measure of prolactin action, showed that cyclic-GMP initiated the early action of prolactin on RNA synthesis while cyclic-AMP or phosphodiesterase inhibitors abolished the cyclic-GMP effect.

In mouse mammary gland, the effect of prolatin can be blocked by indomethacin (which inhibits prostaglandin synthesis activity) and imitated by PGF_2 (12,18). In rat blood vessels, the effect of prolactin can be blocked by indomethacin and imitated by PGS (12,13,19). Further, prolactin stimulates PG synthesis by over 100-fold in this tissue. In lithium-treated rats, the effect of prolactin in restoring responsiveness to vasopressin can be imitated

by PGA_2 (12,20,21).

In each of these cases, the prostaglandins were required for the action of the second messenger, calcium in the case of the blood vessels, and cyclic AMP in the other two. The prostaglandins neither stimulated the production of the second messenger nor was stimulated by it. Calcium ions which were made available by vasopressin seemed to be unable to activate the contractile mechanism in the absence of prostaglandin E_2. Prolactin regulated the amount of prostaglandin and thus modulated the response to calcium ion. Thus, in this example, prolactin did not modify the supply of second messenger but modified the responsiveness to second messenger made available by other stimuli by regulating the prostaglandin level in the cell.

In the mammary gland, there is evidence that prolactin acts by stimulation of prostaglandin synthesis. Prolactin also acts to increase the amount of protein kinase available for activation by cyclic-AMP. Prostaglandin could be the intermediate which is responsible for this result. Prolactin and, hence, prostaglandin may act to induce new protein kinase synthesis or modify existing protein kinase so that it is capable of being activated by cyclic-AMP.

The first committed step in the mode of action of prolactin is binding to its protein receptor in the plasma membrane. The purpose of this investigation was to purify and to characterize the prolactin receptor from rabbit mammary tissue in order to permit future investigation regarding the details of the interaction between prolactin and its receptor.

CHAPTER II

LITERATURE REVIEW

Iodination and Assay of Protein Hormones

Detection of protein hormone receptors is difficult due to the extremely small number of receptors present on the cell surface. For example, Cuatrecasas (22) found 10^4 insulin receptors per fat cell, 8×10^4 epidedimal growth factor receptors per rat mammary gland cell.

Because of these extremely low levels of receptors, it is necessary to use radioactively labeled compounds of very high specific activity (1000-3000 Ci/mmole). The simplest method of achieving this high specific activity is to label the peptide or protein with ^{125}I or ^{131}I. There are four procedures commonly used to covalently attach a ^{125}I or ^{131}I to a protein.

Hunter and Greenwood (25) developed the chloramine-T sodium metabisulfite technique protein labeling. Briefly, a protein, sodium 125-Iodide, and chloramine-T are mixed. The chloramine-T reacts with ^{125}I to form $^{125}I_3^-$ which will iodinate a tyrosyl residue meta to the hydroxyl group. Since monoiodotyrosine is more reactive than tyrosine, often the final product will be diiodotyrosine. The disadvantage of this technique and other chemical iodination techniques

is destruction of histidine or tryptophan residues by the strong oxidants necessary to generate the reactive iodine species (26).

Iodination using iodinemonochloride (26) as the iodinating species gives results similar to chloramine-T iodination and like chloramine-T can lead to protein cleavage.

A chemical labeling technique which does not use a strong oxidant was recently developed by Hunter and Bolton (27). An inherently reactive molecule, the N-hydroxysuccinimide ester of p-hydroxylphenylpropionic acid, is iodinated using ^{125}I and chloramine-T and purified. This iodinated compound can acylate the ϵ-NH_2 groups of lysine. The advantages of this technique are the avoidance of strong oxidants, a high specificity for only free amino groups, and the labeling of a normally noncritical residue. The disadvantage is preparation and purification of the Hunter-Bolton reagent which is difficult and time consuming.

An enzymatic iodination technique is also available (28). Lactoperoxidase will iodinate tyrosyl residues in the presence of sodium iodide and hydrogen peroxide over a pH range of 4 to 8. Since monoiodotyrosine reacts only at one third the rate of tyrosine, reaction conditions can be adjusted so that monoiodotyrosine derivatives are principally formed (28). Normally, small concentrations of hydrogen peroxide are used which minimize the destructive effects on histidine or tryptophan residues.

Other methods of preparing radioactively labeled

proteins exist, although these do not achieve the very high specific activities obtained with 125-Iodine or 131-Iodine. Among the more useful are tritium exchange using microwave discharge activation of tritium gas (28); chemical modification of sulfhydryl groups with radioactive organomercurials (29); substitution of free amino groups with (^{3}H)-acetic anhydride (30); oxidation of terminal galactose residues followed by reduction with sodium borotritide (31); and incorporation of [35-S]-sulfate (32).

Once labeled, it is necessary to purify the labeled hormone to remove degradation products, hormone aggregates, denatured hormone, or unreacted label. Numerous methods exist to separate labeled hormone from unreacted label. For example, small columns of cellulose that do not adsorb protein but do remove iodide (4), gel filtration chromatography (4), small columns of ion-exchange resins to which proteins adsorb (4), microsilica (4), and talc adsorbtion have all been used to separate free from hormone-bound label. ^{125}I-labeled insulin and glucagon were purified by adsorbtion to talc which not only removed unreacted iodide but also damage hormone since undamaged hormone was adsorbed much more tightly to the talc (4). Other hormones such as ^{125}I-labeled prolactin and ^{125}I-labeled growth hormone, both of which tend to form aggregates, were purified on small DEAE-cellulose columns or by gel filtration chromatography on G-100 (24,33). Damaged and undamaged luteinizing hormone were separated by affinity chromatography on antiluteinizing

hormone antiserum-agarose (4).

Normally, the procedure used to assess the effect of labeling is to remove and assay samples from a single labeling mixture as progressively greater substitution is obtained (4). The assay may be for biological activity or binding activity. If no progressive fall in activity is observed, it may be concluded that labeling does not modify activity (4).

For insulin, Cuatrecasas (24) has established criteria for undamaged ^{125}I-labeled insulin which may be useful for other proteins (24). Precipitation of radioactivity by 8% trichloroacetic acid, performed in 0.1 M sodium phosphate buffer, pH 7.4, containing 1% (w/v) albumin, must be at least 97% (25). Adsorption of radioactivity to talc (25 mg/ml), performed in the same buffer must be greater than 97% (26). Adsorption of radioactivity to microfine silica (QUSO 5 mg/ml), performed in 0.1 M sodium acetate buffer, pH 4.5, and containing 0.1% (w/v) albumin, must be greater than 97% (27). The nonspecific binding of ^{125}I-labeled insulin to rat fat cells or membranes and to the Millipore filters must together be less than 0.4% of the total ^{125}I-labeled insulin present in the incubation mixture, and less than 20% of the total uptake of radioactivity of the sample (4).

Generally, receptors are assayed by incubation of a membrane preparation containing the receptor or a soluble fraction containing the receptor with the labeled hormone until a steady-state is reached. The bound hormone

is separated from the free hormone and counted to determine the degree of binding. Normally, a rapid separation technique such as centrifugation (34) or filtration (22) is used to avoid dissociation of the complex. Since the rate of dissociation is lowered by decreased temperature, separations are normally performed at 4° (22).

Ideally, when assaying receptors, only the hormone which binds to the highly specific receptor would be detected. However, it is frequently difficult to determine the quantity of the total binding that relates specifically to the hormone's interaction with its receptor. This difficulty may be overcome by incubation in the presence and absence of displacing concentration of unlabeled hormone (22). It is assumed that the labeled hormone displaced by unlabeled hormones is specifically bound and that not displaced is bound in a nonspecific manner (22). This nonspecifically bound labeled hormone may be due to binding to glass, filters, connective tissue, or other non-receptor material (35). This nonspecific binding may in some cases (for example, nonspecific binding of insulin) exhibit very high affinity. Nonspecific binding because of binding mass will always have a high capacity such that saturability of binding cannot be demonstrated (35). Nonspecific binding in most cases is rapid and does not exhibit the longer time course observed for receptor binding. (35).

Insulin Receptors

The binding of insulin to a variety of cells and

membranes has been studied in detail in various laboratories (5,9,36,37). Considerable evidence that insulin receptors are on the surface of the cell has accumulated over the past few years. Trypsin (30,38) and insoluble trypsin and chymotrypsin derivatives (30) destroy the insulin response in fat cells. Also, insulin attached covalently to agarose can exert insulin effects on fat cells (39).

The insulin receptor is assayed by measuring the binding of ^{125}I-labeled insulin to isolated membranes, whole cells or soluble membrane protein (22,40). The ^{125}I-labeled insulin used was labeled with chloramine-T (44) and a specific activity of 500-2000 Ci per mmole was obtained. ^{125}I-labeled insulin was shown to be biologically active and to have less than 1 mole of iodine per mole of insulin.

Particulate, membrane-associated insulin binding activity was isolated from fat cell (40) and rat liver (42). The binding of ^{125}I-labeled insulin to fat cell membranes and liver membranes was shown to be saturable and time-and-temperature-dependent (40,42). The ^{125}I-labeled insulin membrane complex dissociates spontaneously and the rate of dissociation increases with increasing temperature (40). At 25°, the rate of association is 8.5×10^{6} mole^{-1} sec^{-1} and the rate of dissociation is 4.2×10^{-4} sec^{-1} (46). At 24°, the dissociation constant is 7.5×10^{-11} M .

Maximum binding occurs at pH 7.5 (40) and in the presence of 2 M sodium chloride (40). High concentration

of salts gave similar increase in binding.

A variety of protein modification reagents have been reacted with insulin receptors (40). N-Ethylmaleimide and iodoacetamide have no effect on binding activity, indicating a free sulfhydryl is not involved in binding. The tryptophan modifying reagents, 2-hydroxy-5-nitro-benzylbromide and 2-methoxy-5-nitrobenzylbromide do not effect binding activity. Tetranitromethane, which in the absence of sulfhydryl groups is relatively specific for tyrosyl residues (43), caused a drastic reduction in insulin binding activity. Also, diazonium-1-H teriazole, which reacts primarily with histidyl and tyrosyl groups, totally inactivated the insulin receptor. Thus, it appears that tyrosyl and possibly histidyl groups are involved in binding.

Digestion of fat cell membrane and liver cell membrane with phospholipase A or phospholipase C led to a 3-6 fold increase in binding of ^{125}I-labeled insulin (44). Phospholipase D had no effect. This increase could not be reversed by adding a variety of exogenous phospholipids to membrane previously digested with phospholipases C and A (44).

Some lipid-soluble compounds can also cause this increased insulin binding (44). Digitonin, vitamin K, filipin (a polyene antibiotic), and millitin (a protein found in bee venom with amphipathic properties) all increase insulin binding, presumably due to perturbation of membrane lipids (44). Simple extraction of fat cell

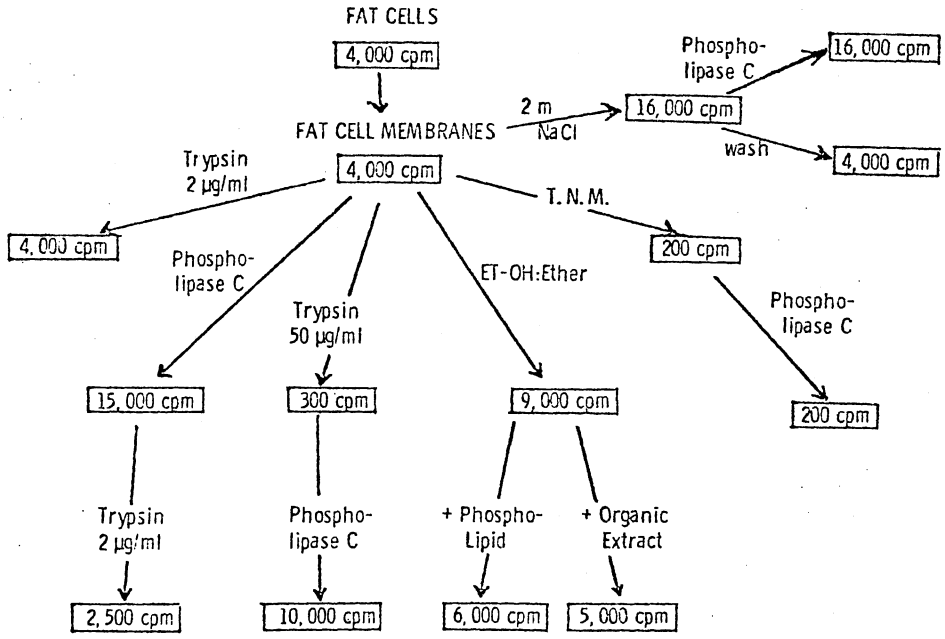

Figure 1. Summary of the Nature of "Buried" Insulin Receptor (4).

membrane with ethanol-ether (3:1 v/v) also causes an increase in insulin binding. If fat cell membranes are digested with trypsin (which destroys all insulin binding activity), then the trypsin is inactivated with soybean trypsin inhibitor, and these trypsin-digested membranes are incubated with phospholipase C, insulin binding reappears at a level equal to approximately the increase found upon phospholipase C digestion of intact (non-trypsin treated) membranes (44). These results, along with the increase in insulin binding activity of fat cells upon treatment with phospholipases A and C and lipid soluble compounds, suggests that the increase in insulin binding represents exposure or unmasking of receptors which are normally inaccessible to insulin (44). Since trypsin digestion does not modify those receptors which can be uncovered by phospholipase digestion, these normally buried receptors are not only inaccessible to insulin but also to other large molecules (44). Identity between these new receptors and receptors on the surface is postulated since the kinetics of complex formation of both is the same (44). A summary of these effects is shown in Figure 1.

Cuatrecasas (22) has solubilized the insulin receptor from fat cell membranes and liver cell membranes by incubation with the nonionic detergent Trition X-100. The loss of binding of insulin to membrane upon detergent extraction with Trition X-100 was accompanied by the appearance of insulin binding activity in the high speed supernatant of the extract (42). Maximum solubilization occurs at

0.5% v/v Triton X-100 (42). This soluble receptor was detected by precipitation of the insulin-receptor complex with polyethylene glycol 6000 in the presence of gammaglobulin (42). The precipitated complex was then separated from free ^{125}I-labeled insulin by collecting the precipate on Millipore EH filters (42). Ten percent polyethylene glycol was found to precipitate the complex and leave free ^{125}I-labeled insulin in solution (42). Less than 0.5% of the total free insulin was precipitated at 10% polyethelene glycol. At 8% polyethylene glycol, the complex did not precipitate; and at]2% polyethylene glycol, free insulin began to precipitate (42). Triton X-100 above 0.2% in the assay mixture and pH above 8 or below 7 interfered with precipitation (42).

There was a loss of binding activity to solubilized insulin receptor upon incubation with increasing concentrations of sodium dodecyl sulfate, urea, guanidine-HCl, and glycerol (45). At 0.2% sodium dodecyl sulfate, 5 M urea, and 4.0 M guanidine-HCl, essentially no binding activity remianed (45). Treatment with 20% glycerol resulted in a loss of 50% of the insulin binding activity (45). Treatment with 0.16% sodium dodecyl sulfate, 3 M urea, and 20% glycerol were reversible (45). At higher concentrations of sodium dodecyl sulfate and urea, there was a permanent loss of activity (45). Treatment with urea from 2 M to 4 M was only partially reversible (45).

Soluble insulin receptors from fat cells and liver were found to retain 80% of their activity when stored at 4^{o} in 50 mM Tris, 1% Trition X-100 after 30 days and

and indefinitely at -20°C (45).

Sodium chloride concentrations up to 3.0 M, incubation with neuraminidase, phospholipase C and phospholipase A had no effect on binding activity of the soluble receptor (45). Trypsin, however, destroyed all binding activity (45).

The sedimentation coefficient ($S^{o}_{20,w}$) for the soluble liver insulin receptor in the absence of detergent was 15.8 S and for the soluble fat insulin receptor the $S^{o}_{20,w}$ was 17.4 S (45). In the presence of 0.5% Triton X-100 the sedimentation coefficients were 11.2 S for the soluble liver receptor and 10.8 S for the soluble fat cell receptor (45).

The association rate constant for the soluble liver receptor was 2.3×10^{6} $mole^{-1}$ sec^{-1} and for the soluble fat cell receptor was 2.9×10^{6} $mole^{-1}$ sec^{-1} (45). The dissociation rate constant for liver receptor was 3.8×10^{-4} sec^{-1} and 4.4×10^{-4} sec^{-1} for fat cell receptor (45). The dissociation constant, determined by Scatchard analysis for the liver receptor was 1.3×10^{-10} M and 1.8×10^{-10} M for fat cell receptor (45).

The soluble insulin receptor does not appear to be a lipoprotein. The molecular properties of the soluble receptor, especially the sedimentation coefficient in cesium chloride of density 1.298 g ml^{-1} suggested a low lipid content (45). The soluble insulin binding activity appears to be independent of membrane lipids since it's binding properties are similar to those of membrane-

associated insulin receptor (45).

In 1973, Cuatrecasas (46) observed that wheat germ agglutinin enhanced the binding of insulin to fat cells and liver cell membrane at a concentration of 1 μg/ml. The lectin increased insulin binding by increasing the rate of complex formation without altering the rate of dissociation of the insulin receptor complex or altering the total number of binding sites for insulin (46).

Wheat germ agglutinin or Concanvalin-A concentrations from 1 μg/ml to 100 μg/ml blocked the binding of insulin to fat cells (46). The enhancement of insulin binding caused by low concentrations of wheat germ agglutinin was probably due to the binding of the lectin at a site distinct from the receptor; while at higher concentrations, the lectin bound to the receptor (46).

Both wheat germ agglutinin and Concanavalin-A mimic the effect of insulin on conversion of glucose to carbon dioxide (48). Maximal effects occurs at 1 to 2 μg/ml (48).

Cuatrecasas (47,48) has attempted to purify the liver insulin receptor using a variety of conventional and affinity chromatographic techniques. Rat livers were homogenizied with a polytron PT35St for three minutes at a setting of 3.5 in 0.25 M sucrose and centrifuged at 600 x g, 12,000 x g, and 40,000 x g (47). The insulin receptor activity was found in the 40,000 x g pellet (47). This material was then solubilized with 1% Triton X-100 (47).

Ammonium sulfate fractionation of the Triton X-100 extract resulted in insulin binding activity in the 0-20% fraction and the 20-40% fraction with approximately a 3-fold purification (47). The insulin binding protein could be chromatographed on DEAE cellulose with buffers that contained Triton X-100 (47). A linear gradient of 0.1 M ammonium acetate, pH 6.3 to 1 M ammonium acetate, pH 6.3, eluted the receptor with a 20-fold purification (47).

A variety of insulin-agarose derivatives were synthesized (Figure 2) and tested for their ability to bind the insulin receptor (47). The adsorbants containing a spacer arm were the most effective in binding of the insulin receptor (47). The only exception was derivative F, which was ineffective in either the predominantly azohistidyl form or in the form containing a mixture of azohistidyl and azotyrosyl bonds (47). Using derivative C, 20 to 90% of the binding activity could be retained with such columns. Elution was with 50 mM sodium acetate, 4.5 M urea, pH 6.0, which dissociated the insulin-receptor complex. Urea concentrations greater than 4.5 M resulted in lower recoveries. Elution could not be achieved by simply low pH. Urea was removed by dialysis and the receptor was active (47). Between 50 to 80% of the adsorbed receptor was recovered by the urea elution (47). Approximately an 8000-fold purification was achieved over insulin receptor solubilized with Trition X-100 or a 250,000-fold purification from the crude liver homogenate (47). Cuatrecasas

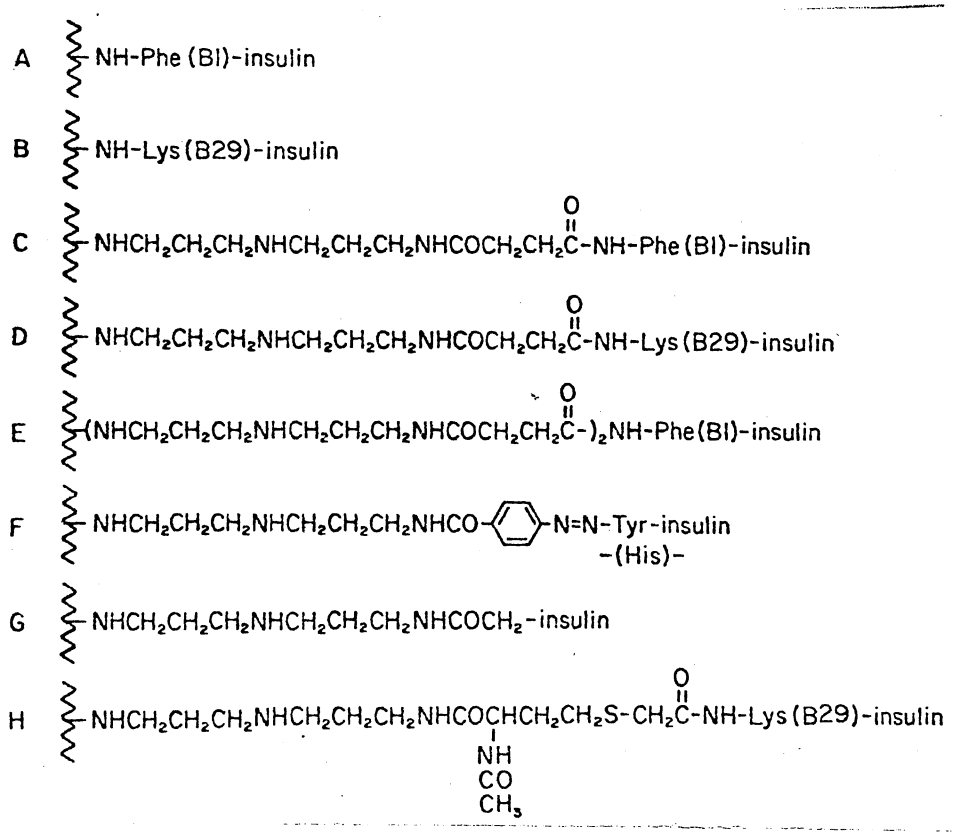

Figure 2. Agarose-Insulin Derivatives Used to Purify the Insulin Receptor (4).

(47) has calculated that a purification of 400,000-fold is necessary, assuming a molecular weight of 300,000 for the receptor and 6,000 for insulin. There has been no large scale purification of insulin receptor using this procedure for two reasons. The concentration of insulin receptor present in the crude liver homogenate is extremely low, 2×10^{-4}%, and the capacities of insulin-agarose columns are low (47).

Cuatrecasas and Tell (48) have also used immobilized wheat germ agglutinin and immobilized Concanavalin-A to partially purify insulin receptor (48). Wheat germ agglutinin and Concanavalin-A were coupled directly to cyanogen bromide activated Sepharose or to Sepharose with an extention arm of diaminodipropylaminosuccinylate (48). The insulin receptor solubilized from liver membranes would bind to either type of lectin column and could be eluted with 0.3 M α-methylmannoside if bound to Concanavalin-A-Sepharose or with 0.3 M N-acetylglucosamine if bound to wheat germ agglutinin-Sepharose (48). Both types of lectin columns gave a 3,000-fold purification of the receptor when compared to the Triton X-100 extract of liver membranes and the recovery was over 90% (48). The use of lectin-agarose columns has three advantages over insulin-agarose columns. These are 1) ease of elution, 2) high capacity for the binding proteins, and 3) the avoidance of possible contamination by insulin (48)

There has been considerable controversy over whether the binding of insulin to its receptor exhibits negative

cooperativity (35,51,52). In 1973, DeMeyts, et al. (49) studied the dissociation rate of ^{125}I-labeled insulin from its receptor under two conditions in order to detect cooperative interactions: first, the rate of dissociation of the hormone-receptor complex was measured by diluting the complex sufficiently to prevent rebinding of the dissociated ^{125}I-labeled insulin and second, the rate of dissociation of the complex was measured by dilution to the same extent in a buffer containing an excess of unlabeled hormone.

Cultured human lymphocytes were reacted with ^{125}I-labeled insulin under conditions such that at equilibrium only a minority of the receptor sites were occupied. The cells were washed to remove free ^{125}I-labeled insulin, resuspended in an identical volume, divided into aliquots and diluted 100-fold into fresh medium. Half of the cells were in hormone free buffer (dilution only) and the other half were in buffer containing an excess of unlabeled insulin. The rate of dissociation of insulin was then determined. The results showed that filling the empty site by cold insulin increased the dissociation of labeled insulin from the other sites. These results implied that filling the sites produced site to site interactions which increased the dissociation rate constant of other sites, which is consistent with the existence of negative cooperativity (49).

Cuatrecasas and Hollenberg (35) disagreed with these results. They showed that insulin will bind to non-receptor materials such as talc, silica, protein-agarose

derivative, and glass test tubes and that these interactions, at least superficially, show saturability, specificity, high affinity, and reversibility. Artifacts due to the binding of 125-I-labeled insulin to non-receptor materials could be interpreted as negative cooperativity. Most of the observed effects were attributed to dimerization or aggregation of 125-I-labeled-insulin.

DeMeyts, Bianco, and Roth (50) in 1976 presented further evidence that the insulin receptor of human cultured lymphocytes did indeed display negative cooperativity. Scatchard plots were curvilinear, concave upwards, which indicates the presence of either site-site interaction of the type defined as negative cooperativity or binding sites of different affinities (21). Kahn, et al. (51,52) have critically reviewed the possibility of multiple classes of insulin binding sites of differing affinities and found discrepancies between the results obtained from steady-state data and the results obtained from kinetic analysis. However, a cooperative model had not been explored. Roth, et al. (50) looked for negative cooperativity in the binding of insulin to human cultured lymphocytes by the dilution technique. ^{125}I-labeled porcine insulin of specific activity 180 to 250 μCi/μg was incubated with human cultured lymphocytes to 5% saturation of the total insulin receptors under steady-state conditions. The lymphocytes were concentrated by centrifugation and diluted 100-fold the original assay volume. The dilution buffer in half of the tubes contained insulin from 10^{-10} M to 10^{-7} M. At fixed

times, the tubes were filtered on Millipore filters and counted. Thus, only ^{125}I-labeled insulin remianing bound to the lymphocytes was detected. In all cases, the lymphocyte-^{125}I-labeled insulin complex exposed to cold insulin dissociated at a faster rate than the complex not exposed to cold insulin. The rate of dissociation was markedly affected by temperature. The dissociation rate at 4^{o} was first order and slow (20% dissociated in 3 hours). At 37^{o}, 95% had dissociated in 3 hours and the dissociation curve became multiexponential. As temperatures were increased from 4^{o} to 37^{o}, the dissociation curves of dilution alone and dilution with cold insulin more closely resembled one another. Concentration of urea as little as 1 M resulted in a 4-fold decrease in the half-life of the insulin-receptor complex. Divalent cation, Ca^{++} or Mg^{++}, caused a decrease in dissociation by dilution only (no cold insulin). The effect of divalent cations on dissociation by dilution with cold insulin was to shift the enhanced dissociation caused by cold insulin to higher concentration of cold insulin.

Dimerization, as the cause of negatively cooperative effects, was dismissed for four reasons. (1) Insulin, at concentrations where dimerization was essentially nonexistant still accelerated dissociation. (2) The cooperative effect actually decreased at insulin concentrations above 10^{-7} M where it is known that dimers become a significant proportion of the molecular species. (3) Nondimerizing forms of insulin, tetranitrotyrosine-

insulin (51) and guinea pig insulin (52) induced accelerated dissociation. (4) Desalanine-desasparagine insulin, which dimerizes with an association constant 100 times lower than insulin, at concentrations 10,000 times higher than insulin, does not accelerate dissociation of ^{125}I-labeled insulin.

Human Chorionic Gonadotopic and Luteinizing Hormone

The gonadal receptor for luteinizing hormone and chorionic gonadotropin (hCG) has been solubilized and partially characterized (53,57).

The receptor was extracted in soluble form by treatment of a particulate binding fraction of the interstitial cells of rat testes with 1% Triton X-100 (1). The soluble receptors were assayed using ^{125}I-labeled human chorionic gonadotropin, labeled using lactoperoxidase. Free and bound forms of the hormone were separated with polyethylene glycol precipation (53).

The initial rate of binding of hCG by soluble receptors was higher at 34^{o} than at 24^{o} or 40^{o}, but degradation of receptors occurred more rapidly at the higher temperature with a corresponding loss of binding activity. The equilibrium association constant of the soluble hormone-receptor complex at 24^{o} was 0.5 -1 x 10^{10} M^{-1} which was lower than that of the particulate receptor complex (2.4 x 10^{10} M^{-1}) (53). The optimim pH for binding was 7.4 and no effects of buffer composition, ionic strength

or calcium concentration upon binding was demonstrable (53). Scatchard analysis gave straight lines, indicating that all sites were of the same affinity and there were no cooperative effects (54).

Trypsin caused loss of gonadotropin binding activity in both particulate and soluble receptors, indicating that the receptors are proteins. More interesting, phospholipase A caused a 6-fold decrease in binding to particulate receptor and a 20% decrease in binding to soluble receptor. Phospholipase C treatment of soluble receptors caused aggregation. These two observations indicated a significant role of phospholipid in the structural and functional properties of the receptor (53).

Treatment of the soluble receptor with neuraminidase caused a 2 to 3-fold increase in binding, but this was thought to be due to a desialation of gonadotropin during subsequent incubation since asialo-hCG has been shown to have a higher affinity for gonadal binding sites than the mature molecule (53,57,60).

The presence of multiple forms of the receptor, solubilized from rat testis, was demonstrated by gel-filtration chromatography and sucrose density gradient centrifugation (55). Unchanged receptors and the receptor-hCG complex formed after extraction with Triton X-100 had sedimentation coefficients of 6.5 S and 7.5 S, respectively (55). Additional forms of the hormone-receptor complex, with sedimentation coefficients of 7.0 S and 8.8 S, were identified in extracts of prelabeled interstitial cell

fragments treated with detergents such as Lubrol PX, Lubrol WX, and sodium deoxychlolate (55). The 8.8 S forms of the receptor-hormone complex could also be obtained by dialysis of the 7.5 S complex. This increase in sedimentation coefficient could be due to removal of significant proportion of the bound detergent during dialysis and the maintenance of the soluble complex by the highly hydrophilic sialated glycoprotein hormone (55). The increased sedimentation coefficient of the complex extracted by Triton X-100 from previously labeled particles (8.8 S) compared to the complex formed by solubilizing the receptor and then reacting with ^{125}I-hCG (7.5 S) could be caused by extraction of a larger or less asymmetric species or one containing less phospholipid than that extracted from unlabeled particulate receptors (55).

Exposure of the 7.5 S receptor and its dialyzed 8.8 S form to phospholipase A or C had little effect. In contrast, phospholipase A decreased binding to the 6.5 S unoccupied soluble receptor and phopholipase C caused aggregation (55). It was concluded that phospholipids form an essential component of the receptors and that binding activity was strongly influenced by a phospholipid moiety which was susceptible to hydrolysis by phospholipase A (55)

Glycoprotein Hormones, Toxins, and Ganglioside Receptors

Recently, gangliosides have been shown to be the

cell surface receptors or a part of the receptor for a number of protein ligands (9,13,59-62). Cuatracasas (23,63-67) has shown that the cholera toxin receptor on rat fat cell or liver cell membranes is the monosialoganglioside, GM_1. Kohn and co-workers (9-11,59-62) have demonstrated that thyrotropin, human chorionic gonadotropin, and perhaps luteinizing hormone and follicle stimulation hormone all may have membrane receptors that are gangliosides or oligosaccharides similar in structure to gangliosides.

Kohn (60) has reported that there is an amino acid sequence similarity between the B subunit of cholera toxin and the B subunit of glycoprotein hormones. At present, it is believed that the B chain binds specifically to a membrane ganglioside which serves as its receptor and that the binding of the B chain causes a conformation change of the intact toxin molecule with the resultant formation of an active A subunit. The active A subunit may then translocate within the cell membrane and activate adenylate cyclase by direct interactions (59).

In 1973, Cuatrecasas (63-66) reported on the interaction of Vibrio chloerae enterotoxin with fat cell membranes and liver cell membranes. Binding to membranes occured very rapidly, being complete in 5 minutes at 24^{o}. A single fat cell could bind 2×10^{4} molecules of cholera toxin, and liver membranes bound a maximum of 0.8 mg of toxin per mg of protein. The dissociation

constants for formation of the toxin-fat cell and toxin-liver cell complexes were 4.6×10^{-10} M and 1.1×10^{-9} M, respectively. Certain glycoproteins, fetunin and thyroglobulin were found to bind the toxin and inhibit toxin binding to membranes. A variety of glycosphingolipids were tested as to their ability to inhibit toxin binding to membranes. The ganglioside, GM_1, was the most potent inhibitor, inhibiting at concentrations as low as 1 ng/ml. Toxin binding was lost when membranes were extracted to remove glycolipids, but could be recovered in the ganglioside fraction of the extracts. More definite proof that gangliosides were the receptors for cholera toxin was the observation that when exogenous gangliosides were incorporated into whole cells membranes, both binding of cholera toxin and the biological response of cholera toxin were greatly enhanced.

Recent evidence has demonstrated that the thyrotropin receptor, like the cholera toxin receptor, is a ganglioside. Tate, et al. (68) found that tryptic digestion of bovine thyroid plasma membranes yielded a soluble thyrotropin receptor that exhibited specific thyrotropin binding and had properties similar to membrane associated thyrotropin receptor. The soluble receptor had a molecular weight of 25,000-30,000 and contained 30% carbohydrate and 10% sialic acid (68). The sialic acid was vital to receptor function since neuraminidase treatment destroyed the ability of the receptor to bind thyrotropin.

In 1976, Mullin, et al. (9,10) demonstrated that

certain gangliosides inhibited ^{125}I-labeled thyrotropin binding to the thyrotropin receptors on bovine thyroid membranes. It was found that the ability of the ganglioside to inhibit was greatly affected by the number and location of the sialic acid residues within the ganglioside structure. The order of efficacy of inhibition was $G_{D1B} > G_{T1} > G_{M1} > G_{M3} > G_{D1a}$. Fluorescense studies indicated that inhibition was associated with a conformational change of the thyrotropin molecule. The ganglioside inhibition appeared to be hormone specific since it was not affected by albumin, glucagon, insulin, prolactin, follicle stimulating hormone, growth hormone, or corticotropin. However, cholera toxin inhibited ^{125}I-labeled thyrotropin binding about 40% at 1nM but enhanced thyrotropin binding at lower concentrations. This observation was explained by the following scheme: Thyrotropin binds preferentally to receptor sites composed of G_{D1B} or G_{T2} sites more accessible to thyrotropin. The toxin thus behaved as a positive cooperative ligand. At higher toxin concentration, toxin binding was to other gangliosides (G_{D1a} or G_{T1}) resulting in inhibition of thyrotropin binding.

Mullin, et al. (9) and Tate, et al. (68) hypothesised that TSH and cholera toxin may be analogous in their mode of interaction with the plasma membrane. Support for this argument were the observations that both the B component of cholera toxin and B subunit of thyrotropin have sequence homologies and determinants which dominate the binding of their respective proteins. These determinants

interact with a receptor that is a ganglioside or whose structure is similiar to a ganglioside with a unique number and location of sialic acid residues. A specific conformational shift is induced in thyrotropin and cholera toxin upon binding (10,11) and a second subunit (the subunit or A protein) translocates within the membrane to interact with adenyl cyclase.

Since sequence homologies exist between luteinizing hormone, human chorionic gonadotropin, thyrotropin, and cholera toxin (9,60) it seems likely that luteinizing hormone and human chorionic gonadotropin also have a similar mechanism of receptor interaction; but that each of these hormones recognizes carbohydrate sequences distinct from those recognized by thyrotropin and cholera toxin. Each target organ must, therefore, have a receptor with a specific carbohydrate sequence on a ganglioside-like structure. The interaction of the appropriate hormone with its specific oligosaccharide would result in a unique conformational change such that the subunit would be placed in a favored position for adenyl cyclase activation in that particular cell. Interaction with the wrong hormone would result in a different conformation, an unfavorable position, and no interaction with adenyl cyclase (9,10).

Recently, evidence has accumulated that suggests the binding of interferon to specific cell surface receptors is necessary for the development of its antiviral activity and that these receptors are gangliosides or ganglioside-like structures (11,62). The evidence for this

concept is that Phaseoulus vulgaris lectin (specific for N-acetyl-D-galactosamine) blocks interferon action (69). Sepharose bound interferon loses its antiviral activity after preincubation with gangliosides (70), interferon binds to ganglioside-Sepharose (70), interferon binding to ganliosides is inhibited by the lectin (70), neuraminidase destroys the ability of gangliosides to inhibit the action of interferon (71), and thyrotropin and cholera toxin inhibit interferon action (11,62).

Both cholera toxin and thyrotropin, when added together with interferon, inhibited approximately 2-fold the development of antiviral activity in mouse L-cells (11). However, inhibition did not occur when either cholera toxin or thyrotropin were added after interferon (11). Inhibition by the two agents differed in two ways: Maximal effects were obtained at 1 nM cholera toxin and 10 nM thyrotropin, and the thyrotropin effects were reversible, whereas cholera toxin effects were not (11). These findings, along with the other data, suggest that interferon has a ganglioside or ganglioside-like surface receptor whose structure might be similar to that of the cholera toxin receptor (11,62).

Prolactin

To date, numerous mammalian prolactins have been purified and structurally characterized. Porcine (72), ovine (73), human (74) and monkey (74) prolactin all are monomers with a molecular weight of approximately 23,000. Ovine and porcine prolactin have been sequenced and of the

198 residues, 162 are identical, as shown in Figure 3 (72-74).

Prolactin from all sources shows a high degree of homology when compared to growth hormone (Figures 3 and 4) (74). There is approximately 50% homology in primary structure, especially near the COOH-terminal half. Both have monomeric molecular weights of near 22,000. Both have a high α-helix content (45%-55%) which is unusually stable at extreme pH. Both have a tryptophan residue occurring slightly NH_2-terminal to the center of the primary structure which is not exposed to the external solvent in the native conformation. Both compete for each others receptor binding site in receptor binding assays (75).

There are, however, critical structural differences between prolactin and growth hormone. Two of the three disulfide bonds in prolactin are homologous with similar disulfide bonds in growth hormone. The third disulfide in prolactin forms a small loop near the amino-terminus and has no counterpart in growth hormone. Also, prolactin contains two tryptophan residues rather than the single tryptophan found in growth hormone. The far ultraviolet circular dichroism profiles differ for prolactin and growth hormone.

The question of how much of the amino acid sequences of these hormones are necessary for biological action has received much study. There have been no reports, to date,

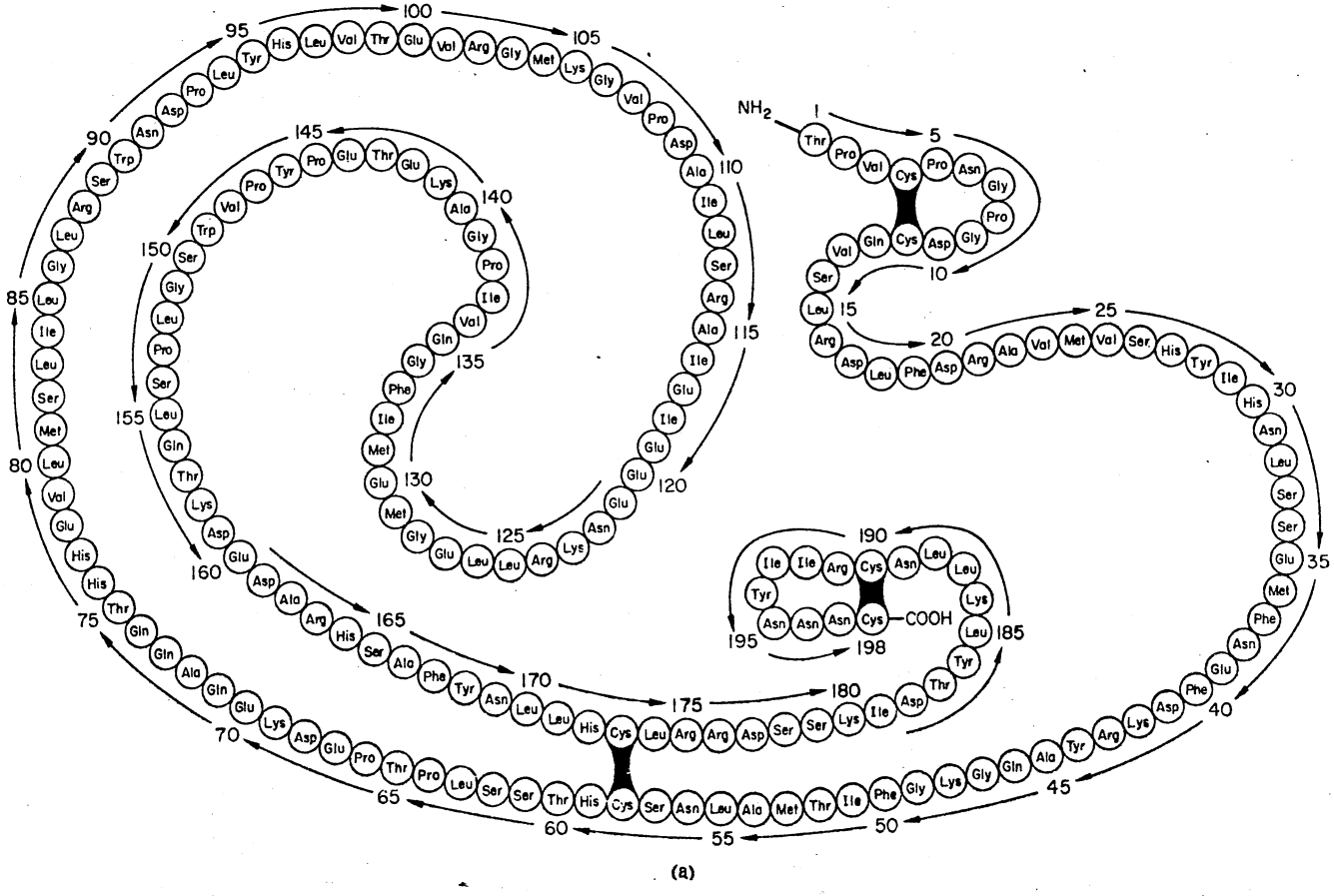

Figure 3. The Complete Amino Acid Sequence of Ovine Prolactin (73)

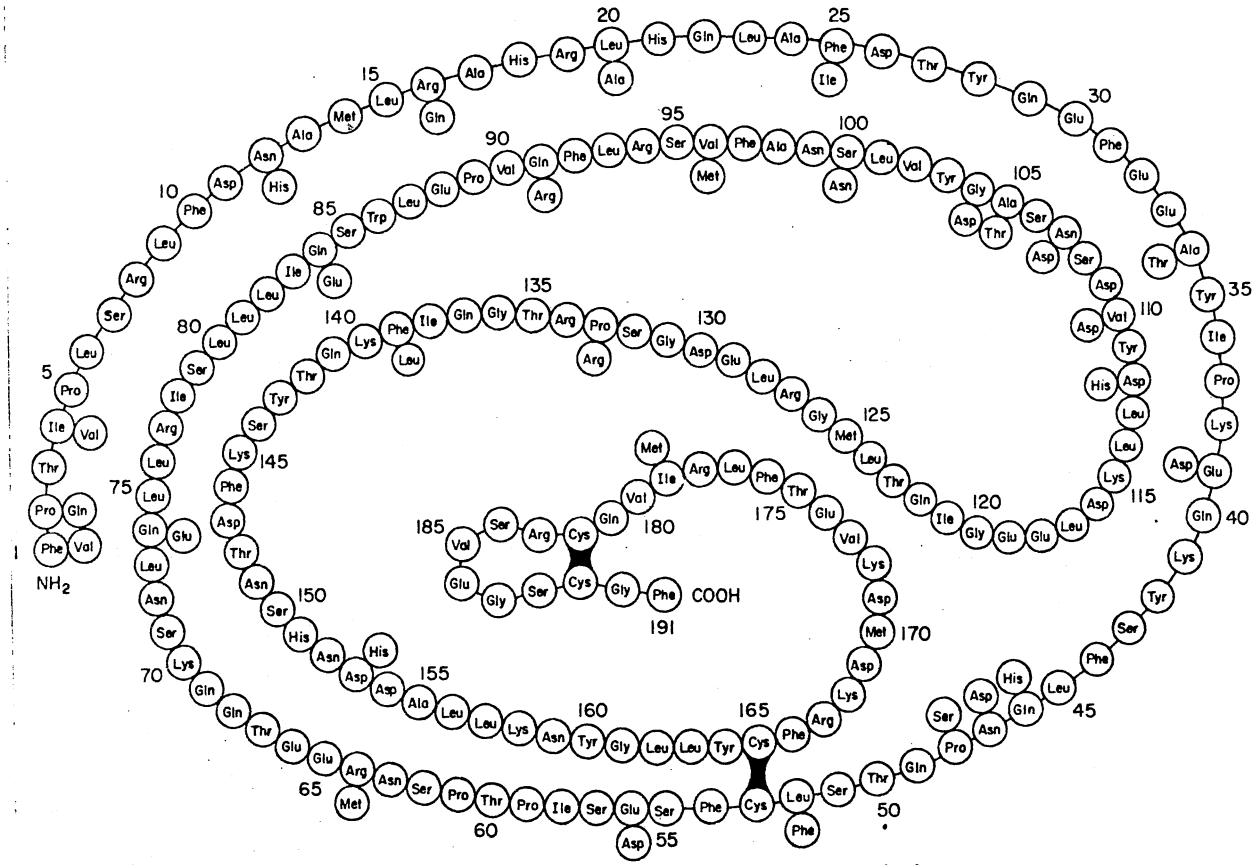

Figure 4. The Complete Amino Acid Sequence of Human Growth Hormone (73)

TABLE I

SOME BIOLOGICAL ACTIONS OF HUMAN GROWTH HORMONE, HUMAN PLACENTAL LACTOGEN, AND HUMAN PROLACTIN (14)

I. General metabolic actions—associated chiefly with hGH
   (1) Increased statural growth in children, through stimulation of epiphysial cartilage growth
   (2) Increased growth of the following tissues: connective tissue, including bone (due to periosteal growth); muscle; skin and accessory skin structures; viscera, including heart, lungs, liver, kidneys, adrenals, intestines, pancreas
   (3) Nitrogen
      (a) Increased uptake of amino acids by muscle
      (b) Increased protein synthesis
      (c) Increased RNA synthesis
      (d) Increased DNA synthesis
      (e) Decreased protein catabolism; positive nitrogen balance
   (4) Fat
      (a) Increased lipolysis; increased concentration of free fatty acids in plasma; increased fatty acid oxidation (chronic effect)
      (b) Increased lipogenesis (acute 'insulin-like' action, seen only transiently after hGH administration)
   (5) Carbohydrate
      (a) Decreased glucose utilisation
      (b) Increased gluconeogenesis
      (c) Antagonism of insulin action (plasma glucose may remain normal if pancreas normal because of increased insulin secretion; in diabetics, hGH causes increased plasma glucose and increased ketogenesis)
      (d) Transient decrease in plasma glucose after intravenous hGH (acute 'insulin-like' action)
   (6) Calcium and phosphorus
      (a) Increased absorption of calcium by intestine
      (b) Increased urinary excretion of calcium
      (c) Positive calcium balance (usually)
      (d) Increased renal tubular reabsorption of phosphate
      (e) Positive phosphorus balance
      (f) Increased serum phosphorus
   (7) Sodium and potassium
      (a) Decreased sodium excretion; positive balance
      (b) Decreased potassium excretion; positive balance
   (8) Connective tissue
      (a) Increased chondroitin sulphate synthesis
      (b) Increased collagen synthesis and degradation
      (c) Increased urinary hydroxyproline
   (9) Renal function
      Increased glomerular filtration rate; decreased blood urea concentration
   (10) Hepatic function
      Increased conjugating capacity for many substances

II. Mammotropic and other effects—associated chiefly with prolactin, but seen to a considerable extent with hPL and hGH (non-primate growth hormones exhibit little or no prolactin-like activity)
   (1) Mammotropic—growth and differentiation of breast tissue
   (2) Lactogenic—during later pregnancy and puerperium
   (3) Luteotrophic (in rodents)
   (4) Crop sac-stimulating (in doves)
   (5) Parental behaviour-stimulating (birds, some mammals, some fishes; hGH little investigated in this respect)

of an active fragment of prolactin being produced after proteolysis. Sengh, et al. (76) have digested human growth hormone with either plasmin or naturally occuring proteases retained after isolation. Digestion resulted in the loss of a 6-12 amino acid fragment around residue 140 to yield a molecule with some increase in growth-promoting activity and a marked increase in prolactin activity. The modified molecule did not cross react immunologically with human prolactin.

Both hormones are produced in the pituitary gland, by eosinophils, with growth hormone (in the human gland) in far greater abundance. Human growth hormone is present at approximately 13 mg/gland while human prolactin is present at only 135 μg/gland (77). In the human, prolactin is more actively synthesized and released than is growth hormone, the gland content being turned over several times a day for human prolactin compared to a fraction of the gland content per day for human growth hormone (77).

The biological actions of prolactin are extremely diverse (Table I) (14). To some extent, human prolactin possesses all of the biological activities associated with human growth hormone. In most cases, the potency of prolactin is so low compared to growth hormone; it is of little consequence (14). The most characteristic actions of prolactin, however, are those listed in Part II of Figure 3. The lactogenic activity of prolactin is perhaps the best studied characteristic. Mouse mammary tissue from mid-pregnant animals will respond in organ culture

with secretory changes and the onset of milk production when exposed to as little as 10^{-10} M ovine prolactin (78,79). These effects have allowed the development of a bioassay using mouse organ culture and monitoring production sensitive enough to detect prolactin in unextracted human serum (81,82).

Secretion of prolactin is regulated by both physiological and pharmacological stimuli. Normal prolactin levels are about 5 mg/ml for males and 8-10 mg/ml for nonpregnant females (8). Prolactin levels in humans are elevated 2-4 fold by sleep and stress and 10-fold by nursing or pregnancy (14). Unlike the other anterior pituitary hormones, the dominant hypothalmic regulation of prolactin inhibitory factor (PIF) is dopamine (12). Perhaps the best proof was the observation that rat hypothalamic extracts lost the PIF activity after incubation with monomine oxidase (83) with eliminated catecholamines. Evidence that a peptide PIF does not exist was the finding that pepsin failed to destroy PIF activity in hypothalamic extracts (80). Thus, it would seem inhibition of prolactin release is controlled primarily by catecholamines.

There are, however, protein or peptide factors which control the release of prolactin. Both thyrotropin-releasing hormone and prolactin releasing hormone stimulate prolactin secretion. These factors have been isolated from hypothalami and are distinct from one another (81). Thyrotropin-releasing hormone was found to stimulate prolactin secretion when added to cloned pituitary cells

in vitro (82) and also caused release in humans (83,84). Prolactin releasing factor was found in acetic acid extracts of bovine pituitary stalk-median eminence and the activity was distinct from and more potent than thyrotropin releasing factor (81). The chemical nature of prolactin releasing factor has not been determined.

Prolactin Receptor

In 1973 Shiu et al. (85) described the preparation and assay for membrane associated prolactin receptor for midpregnant rabbit mammary gland following injections of human placental lactogen and hydrocortisone. Their procedure involved homogenizing the tissue with a Virtis homogenizer and centrifugation at 1500 x g, 15,000 x g and 100,000 x g. Receptor activity was found in the 100,000 x g pellet. Ovine prolactin iodinated by lactoperoxidase was used in their assay. A radioreceptor assay for prolactin was developed with a sensitivity of approximately 10 μg/ml. Prolactin from various sources (rat, monkey, ovine, human) would displace bound ^{125}I-labeled prolactin. Human growth hormone and human placental lactogen would also effectively compete. However, rat, ovine, and bovine growth hormone, insulin, lactoperoxidase, human follicle-stimulating hormone, luteininzing hormone, and glucagon did not compete with ^{125}I-labeled prolactin even at concentration above 1 mg/ml.

Costlow, et al. in 1974 (24) detected prolactin receptors in tissue slices of rat mammary gland and

R-3230AC rat mammary tumors using ^{125}I-labeled ovine prolactin prepared by the lactoperoxidase method. The mammary gland receptor had a K_d of 1.0×10^{-9} M. These were 3800 and 2600 sites per cell for normal tissue and tumor tissue, respectively.

Shiu and Friesen (86) have reported some of the properties of prolactin receptor from the rabbit mammary gland. The association and dissociation of ^{125}I-labeled prolactin were time and temperature-dependent processes, both being maximal at 37^{o}. Iodination using lactoperoxidase produced ^{125}I-labeled prolactin which had more specific binding than prolactin labeled using chloramine-T. Assaying with ^{125}I-labeled human prolactin iodinated by lactoperoxidase, out of 100,000 cpm used, 5000 counts were specifically bound and 1,500 cpm were nonspecifically bound. Assaying with ^{125}I-labeled human prolactin iodinated by chloramine-T, out of 100,000 cpm used, 2500 cpm were specifically bound and 2000 were nonspecifically bound.

The dissociation constant 3.4×10^{-10} M. The association constant was 2.9×10^{9} M^{-1}. They found that the specific binding of ^{125}I-labeled human prolactin to receptors occured over a narrow pH range with maximal binding at pH 7.3. Divalent cations doubled the binding. Treatment of the particulate receptor with 5.0 mg/ml trypsin reduced specific binding by one-half. Treatment with 5.0 mg/ml phospholipase C reduced specific binding by one-third and indicated the possibility of a lipid moiety involved in binding or membrane stability. Treat-

ment with neuraminidase, deoxyribonuclease or ribonuclease had no effect. A distribution study of prolactin binding activity in narrow organs showed adrenal membranes highest followed by mammary gland, ovary, liver, and kidney.

Shiu and Friesen (87), in 1974, successfully solubilized and partially purified rabbit mammary gland prolactin receptor. The receptor was solubilized with 1% Triton X-100 from a crude plasma membrane fraction.

Triton X-100 at concentrations higher than 0.01% affected the physical properties of ^{125}I-labeled prolactin but did not affect ^{125}I-labeled human growth hormone. Therefore, ^{125}I-labeled human growth hormone was used in the binding studies and assays for prolactin receptor. The altered prolactin molecule had a molecular weight of 80,000 determined by gel filtration. This large prolactin was precipitated by polyethylene glycol. As a result, the precipitation of large amounts of free ^{125}I-labeled ovine prolactin mimicked the formation of prolactin and hence, the detection of the ^{12} I-labeled ovine prolactin-receptor complex. Triton X-100 did not affect the ability of native prolactin to displace labeled hormone from the receptor, which suggested that this large prolactin can still bind to the receptor. Since detergents are known to bind to proteins with the formation of micelles, it is possible that more detergent was bound to the prolactin molecule such that a bigger micelle was formed. Shiu and Friesen (87) found that human growth hormone was not affected by the

detergent which suggested that less detergent binds to this hormone resulting in the formation of a smaller micelle.

Receptor ^{125}I-labeled human growth hormone complex could be detected in the void volume of a Sephadex G-100 column or by precipitation with 12.5% polyethylene glycol. Scatchard analysis demonstrated that the affinity of the soluble receptor (Ka = 16 x 10^9 M^{-1}) for human growth hormone was 5-fold greater than that of the particulate receptor (Ka = 3 x 10^9 M^{-1}). The soluble receptor was purified approximately 1500-fold by affinity chromatography on human growth hormone-Affigel-10 (Bio-Rad). Recovery of activity was 8%.

Discontinuous polyacrylamide gel electrophoresis of purified receptor revealed at least seven distinct protein bands. The receptor activity coincided with one or two of the major protein bands of RF 0.12. By gel filtration chromatography, the receptor had a molecular weight of 220,000.

Recently, a prolactin receptor in liver has been detected (88-92). The receptor was absent in prepuberal female rats and increased towards adult levels at the time of puberty (88,89). Pregnancy was found to substantially increase these receptors (88,89). This raised the possibility that sex steroids might play a role in determining the presence of the lactogenic receptors. This was indeed the case since treatment of male rats with estrogen induced the appearance of the receptor to approximately the same concentration as that present in pregnant rats (90).

There is some evidence that prolactin may induce its own receptor in the liver (93). Hypophysectomy diminished receptor levels in female rats, and males became unresponsive to estrogen. A renal pituitary implant halted the decrease in hypophysectomized females and induced the receptor in hypophysectomized males. The increased receptor level in hypophysectomized males with a renal pituitary implant was preceded by an elevated level of circulating prolactin. Thus, it seems possible that prolactin induces its own receptor.

Hypophysectomy of female rats resulted in an extremely rapid loss of liver prolactin receptor, 70% within 24 hours and 95% within 48 hours (91). Further evidence that prolactin modulates the level of its own receptor in rat liver was the observation of prolactin receptors between 12 and 18 hours after injection (91).

Antibodies have been prepared to prolactin receptor partially purified by affinity chromatography (94). A 1/100 dilution of guinea pig antiserum in the prolactin binding of ^{125}I-labeled ovine prolactin to membrane particles. The antiserum effectively blocked the biological effect of prolactin upon casein synthesis. These results support the hypothesis that the membrane structures which bind prolactin are essential for mediating action of prolactin.

CHAPTER III

PREPARATION OF PARTICULATE AND SOLUBLE RABBIT MAMMARY GLAND PROLACTIN RECEPTOR AND ASSAY FOR PARTICULATE AND SOLUBLE RECEPTOR

Materials

Sucrose, Triton X-100, Tergitol NPX, bovine serum albumin, Trizma, and acetobromoglucose were from Sigma. Octanol was from Aldrich. Petroleum ether (BP 35-60^0), benzene, and methanol were from Mallinckrodt. Silver nitrate was from Fisher. Purex bleach was used as a source of sodium hypochloride and was purchased at a local market. Lactating rabbits (16 days) were obtained from a local supplier.

Synthesis of Octyl-Glucoside

The nonionic detergent octyl-glucoside was synthesized by a modification of the procedure of Barton and Thompson (95) and Noller (96). Silver oxide was prepared by reacting silver nitrate in 80° water with a slight molar excess of sodium hydroxide. The precipitated silver oxide was collected by filtration and washed with 80°

water (2 liters), hot methanol (1 liter), and 80° water (2 liters). The moist silver oxide cake was then dried in a vacuum oven at 45°. Normally, about 0.03 moles were prepared for each synthesis. To prepare tetraacetyl-alkylglucoside, a mixture of 0.15 mole of octanol, 0.02 mole of acetobromoglucose, 0.25 mole of freshly prepared silver oxide, 0.1 mole of Dririte, and 200 ml of benzene in a tightly stoppered flask were shaken mechanically overnight at room temperature. The solution was then filtered through 2 layers of Whatman 51 filter paper to remove most of the silver oxide and Dririte. Benzene was removed from the filtrate by rotary evaporation at 50°. The liquid remaining was centrifuged at 15,000 x g to remove any remaining silver oxide. Excess octanol was removed by steam distillation. The crude tetraacetyloctylglucoside congealed upon cooling and was collected by filtration. It was recrystallized twice by dissolving in methanol, cooling, and adding water until crystals formed. The purified tetraacetyloctylglucoside was dried overnight in a vacuum oven at room temperature. Deacetylation was performed by reaction with sodium methylate. The tetraacetylglucose was dissolved in 100 ml of a 0.1 N solution of sodium methoxide in absolute methanol, tightly stoppered, and allowed to react for 24 hours. The sodium methoxide was destroyed by addition of a large amount of dry ice. After the solution had warmed to room temperature, it was applied to a Dowex 50 (1.5 cm x 20 cm) column and equilibrated with methanol to remove the

sodium carbonate. Bubbles formed in the column because of carbon dioxide release but this did not interfere with chromatography. The methanol was removed by rotary evaporation. Crystallization of the octyl-glucoside was difficult since there was a tendency to form a gel. Supposedly, octyl-glucoside can be recrystallized from acetate or acetone by the addition of petroleum ether. Acetone seemed to give the best crystals but often they were amorphous. If this were the case, they were dissolved in water and lypholized. The final product was greater than 98% octyl-glucose as shown by GLC analysis on a 180 cm x 0.25 mm SP 2340 coulmn.

Preparation of Particulate Rabbit Mammary Gland Prolactin Receptor

Membrane fragments which specifically bind prolactin were prepared from the mammary gland of lactating rabbits (85). Lactating (16-25 days) rabbit mammary glands were excised, frozen in liquid nitrogen, and stored at -20^0 until used. To prepare membranes, 100 gm pieces were thawed, cut into small pieces, and washed with 0.3 M sucrose to remove most of the milk. The glands were then homogenized in 0.3 M sucrose (200 ml/100 gm of tissue) at a setting of 6 using a 50 ml chamber in a Sorvall omnimixer for 5 minutes at 0^oC or a setting or 8 using a 200 ml chamber. At the end of each minute the omnimixer was stopped and the

tissue adhering to the blades was added back to the homogenizing misture. After a total homogenizing time of 5 minutes, the homogenate was passed through 4 layers of cheese cloth to remove whole pieces of tissue. The filtrate was centrifuged at 15,000 x g for 20 minutes in a SS 34 Rotor in a Sorvall RC2B centrifuge. The pellet was discarded and the supernatant solution was centrifuged at 100,000 x g for 90 minutes in a 30 or 42.1 rotor in a Beckman L5-65 centrifuge. This 100,000 x g pellet was resuspended in 0.3 M sucrose and stored at -20°. A summary of the preparation precedure is presented in Table 2.

The receptor could then be solubilized from the crude membrane preparation by nonionic detergents (1% Triton X-100, 1% Tergitol NPX, or 50 mM octyl-glucoside) or 30 mM cholic acid. The effects of these detergents are shown in Figures 5, 6, and 7. A stock solution of 10% Triton X-100 in 10 mM Tris pH 7.6 or 10% Tergitol NPX in 10 mM Tris pH 7.6 was added to a 20-40 mg/ml suspension of rabbit particulate receptor in 0.3 M sucrose to a final detergent concentration of 1%. The suspension was stirred for one hour at 4° and then centrifuged at 100,000 x g for 2 hours. Activity was found predominantly in the supernatant solution using either detergent, indicating that the prolactin receptor had been solubilized from the plasma membrane. The detergent, octyl-glucoside, was added in solid form to the particulate receptor in 0.3 M sucrose and

TABLE II

PREPARATION OF PARTICULATE RABBIT MAMMARY GLAND PROLACTIN RECEPTOR

Sample	Vol. (ml)	Protein (mg)	S. A. ($\frac{\text{cpm bound}}{\text{mg protein}}$)	Total Activity ($\frac{\text{cpm bound}}{\text{sample}}$)
Homogenate	48	1838.0	1,902	3.49×10^6
15,000 x g pellet	11	424.6	4,210	1.78×10^6
100,000 x g pellet	9	163.8	28,010	4.59×10^6

11.2 gm of rabbit mammary gland were prepared and assayed as described in the text.

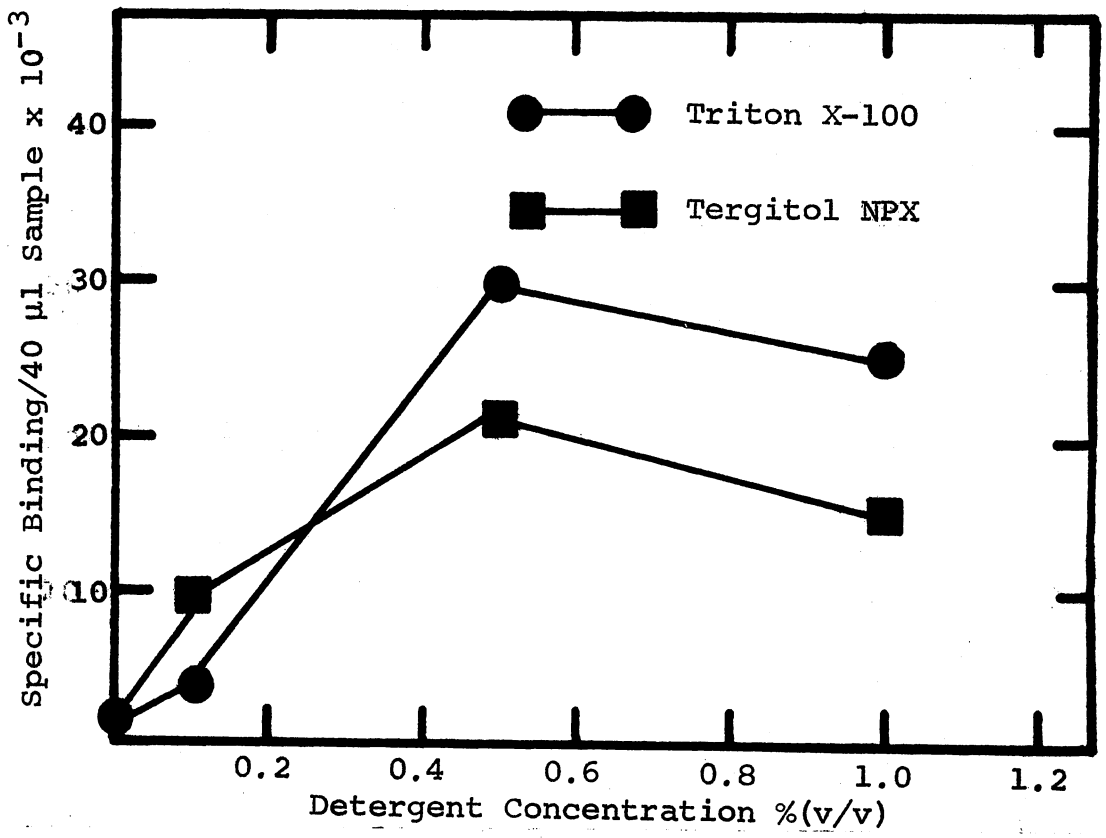

Figure 5. Solubilization of Prolactin Receptor with Triton X-100 and Tergitol NPX

Triton X-100 or Tergitol NPX were incubated with rabbit mammary gland particulate prolactin receptor at various detergent concentrations for 30 minutes at 4° in a total volume of two ml. The samples were centrifuged at 100,000 x g for 90 minutes and the supernatant solutions were assayed. 40 µl of nonsolubilized receptor bound 12,500 dpm.

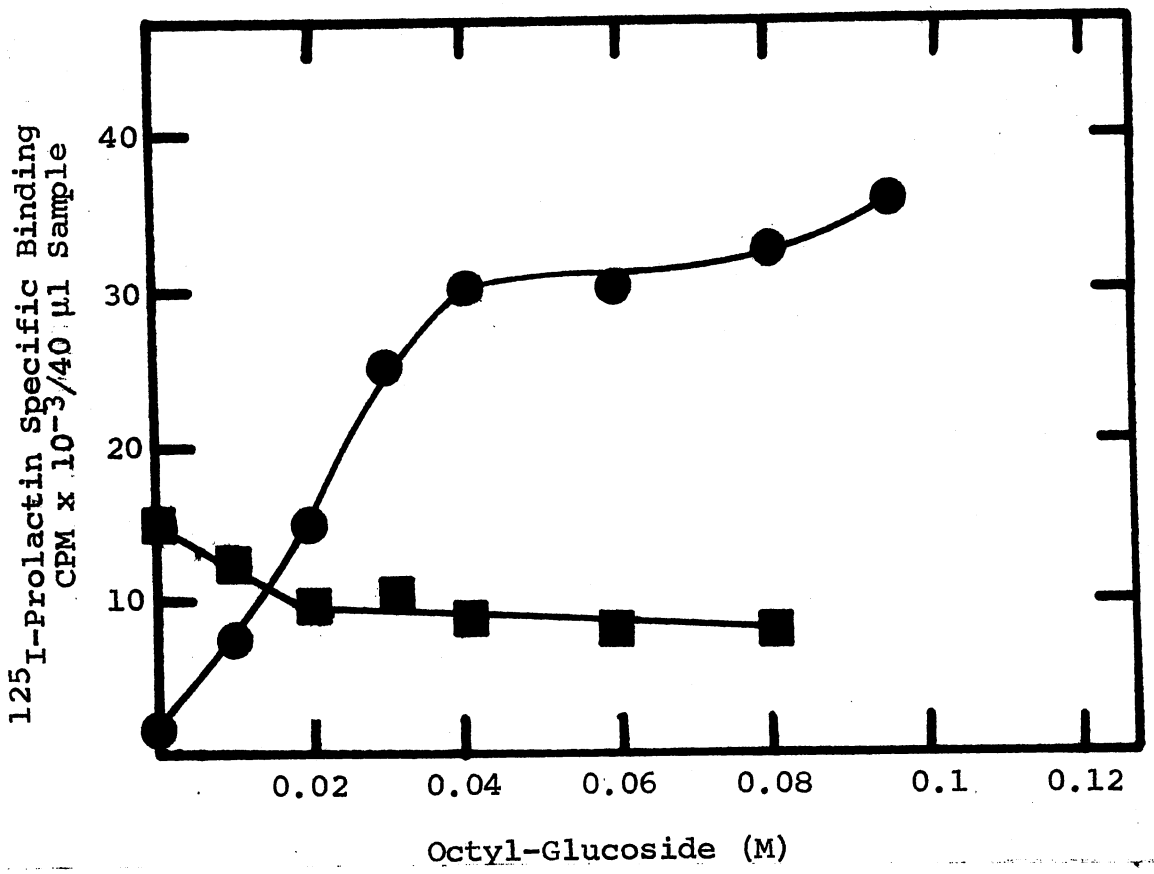

Figure 6. Solubilization of Prolactin Receptor by Octyl-glucoside

To particulate prolactin receptor was added various concentrations of octyl-glucoside. After mixing for four hours at 4°, the samples were centrifuged at 100,000 x g for 90 minutes and the supernatant solutions and pellets assayed. The pellets were resuspended in the original sample volume of 0.3 M sucrose.

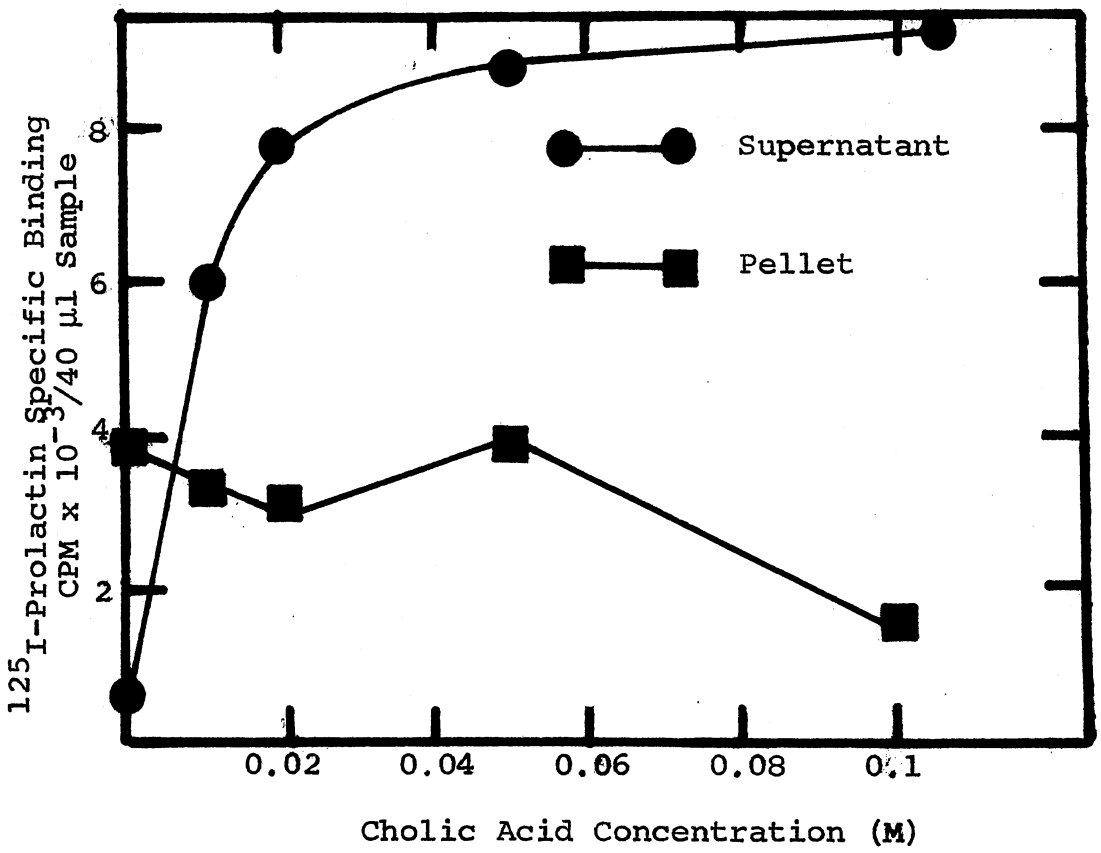

Figure 7. Solubilization of Prolactin Receptor by Cholic Acid

Particulate rabbit mammary gland prolactin receptor was incubated with increasing concentrations of cholic acid by addition of a 0.2 M stock solution of cholic acid in 20 mM Tris, pH 8.0. The total volume of the incubation was two ml. Incubations were for 30 minutes at 25°. Samples were centrifuged at 100,000 x g for 90 minutes. The pellets were redissolved in two ml of 0.3 M sucrose. Both the supernatant solutions and the resuspended pellets were assayed.

stirred for at least 4 hours at 4°. At the end of this time, the suspension was centrifuged at 100,000 x g for 2 hours. Most of the activity was found in the supernatant solution. The receptor was solubilized with cholic acid by the addition of a 0.1 M cholic acid, 50 mM Tris, pH 8.0 solution to a solution of the particulate receptor in 0.3 M sucrose to a final detergent concentration of 30 mM. The suspension was stirred at 4° for 4 hours and then centrifuged at 100,000 x g for 2 hours. Most of the activity was found in the supernatant solution. A summary of the preparation and solubilization of rabbit prolactin receptor is shown in Fiqure 8. No activity was found in lactating guinea pig mammary gland when prepared as above. Also, no activity was found in rat or rabbit milk or cream.

Assay for Particulate Prolactin Receptor

The assay for the membrane-associated prolactin receptor from rabbit mammary gland was similar to that used by Friesin et al. (85). The buffer was 40 mM Tris, 20 mM magnesium chloride, 2% BSA, pH 7.6. The total volume of the assay was 0.5 ml. 0.25 ml or buffer, 0.01 ml ^{125}I-labeled prolactin (100,000 cpm), 10-100 µl of receptor, and deionized water to 0.5ml were mixed and incubated to equilibrium (4 hours at 25°). Specific binding was determined by including a duplicate set of assays containing a displacing amount of unlabeled prolactin (1-10 µg/assay).

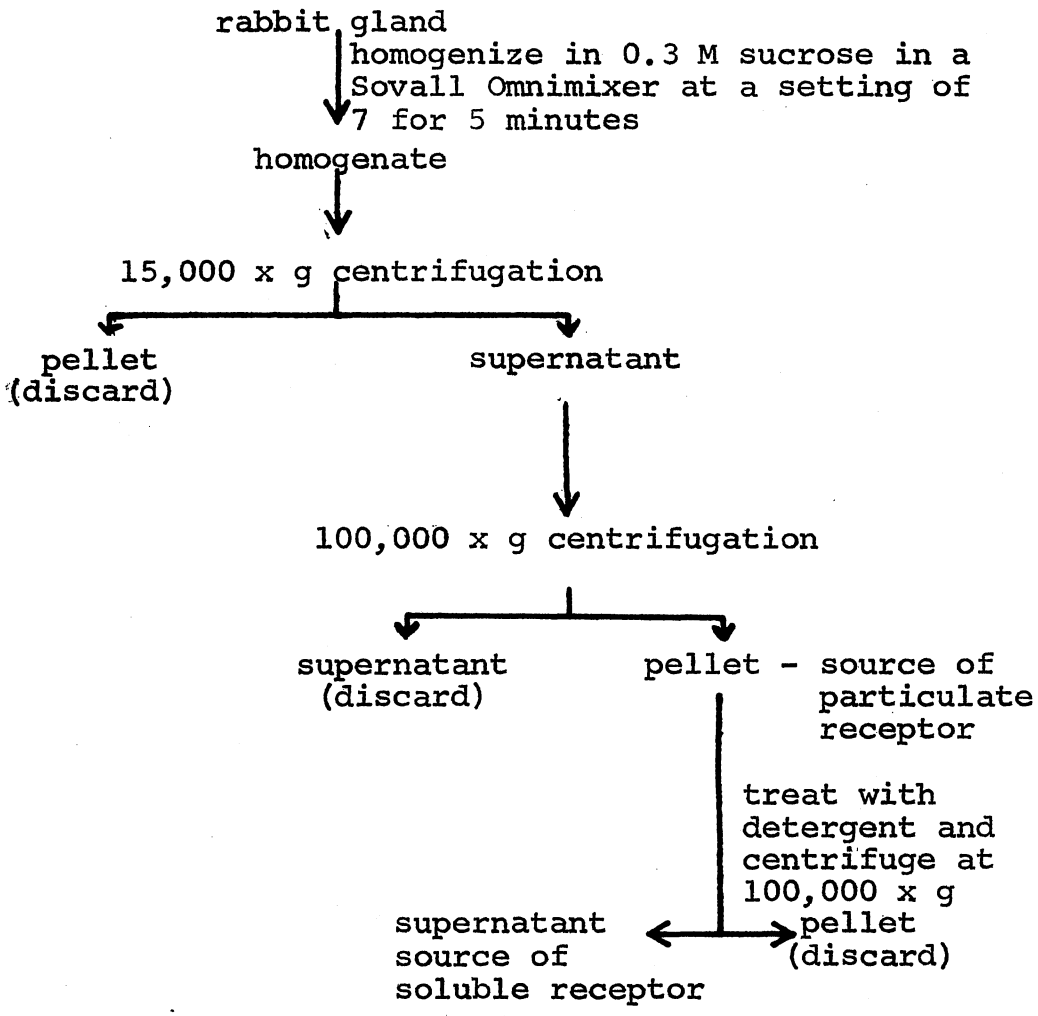

Figure 8. Preparation of Rabbit Mammary Gland Prolactin Receptor

At the end of the incubation period, bound and free labeled hormone were separated by filtration on Millipore EHWP02500 filters. The filters were first soaked for 1 hour in assay buffer to mask nonspecific absorption sets. The assays were then filtered through the treated filters, washed with 5 ml of cold 20 mM Tris, 10 mM magnesium chloride, 0.1% BSA, pH 7.6, and counted in a Beckman Biogamma counter. The filters were counted by first wrapping in a small (4 x 4 cm) piece of aluminum foil to avoid contamination of the counting tubes. Specific binding was defined as the difference in counts between the counts bound in the presence and absence of unlabeled hormone. Bound and free hormone may also be separated by centrifugation in a Beckman Microfuge B for 2.5 minutes. After pelleting, the membrane particles were washed with 1 ml of a 1:1 dilution of assay buffer and centrifuged for an additional 2.5 minutes. The entire centrifuge tube was counted after removal of the cap.

Assay for Soubilized Prolactin Receptor

Detergent solubilized prolactin receptor was assayed by a polyethylene glycol precipitation procedure similar to that used Cuatrecasas to assay solubilized insulin receptor (22). The assay conditions were the same as for the particulate receptor. To separate bound and free prolactin, the assay tubes were first cooled in an ice-bath, then to each was added 0.5 ml of 0.1% bovine gammaglobulin, in 0.1 M sodium phosphate, pH 7.0, and 0.7 ml of 25% polyethylene

glycol (MW 6000-7500). The tubes were vortexed until the contents were thoroughly mixed and allowed to precipitate for 10 minutes in an ice-bath. The receptor-hormone complex co-precipitates with gammaglobulin while the free ^{125}I-labeled prolactin which remains soluble. The tube contents were filtered through Millipore EHWP02500 filters which had been soaked for 1 hour in the assay buffer. The assay tube was washed with 2 ml of 10% polyethylene glycol (MW 6000-7500) in 0.1 M Tris, pH 7.6 which was added to the filter. Finally, the filter was washed with 2 ml of 10% polyethylene glycol (MW 6000-7500) in 0.1 M Tris, pH 7.6. The filter was wrapped in aluminum foil and counted. Specific binding was determined as before.

Friesen (75) reported that Triton X-100 at concentrations as low as 0.01% interfered with the detection of prolactin receptor when ^{125}I-labeled prolactin was used as the ligand. It appeared ^{125}I-labeled prolactin aggregated and greatly increased backgrounds. To over-come this problem, Friesen assayed with ^{125}I-labeled human growth hormone instead of ^{125}I-labeled prolactin. It was found, in this study, that Triton X-100 under 0.1% had little or no effect upon ^{125}I-labeled prolactin or backgrounds of the the assay.

CHAPTER IV

IODINATION OF PROLACTIN

Materials

Tris(hydroxylmethyl)aminomethane and bovine serum albumin were from Sigma. Carrier-free sodium 125-Iodide was from New England Nuclear, Schwarz/Mann and Amersham/Searle. Chromatography gels were from Bio-Rad and Pharmacia Fine Chemicals. Ion exchange resins were from Pharmacia Fine Chemicals and Whatman. Bovine lactoperoxidase was purified from skim milk by the procedure of Rombauts, et al. (97) up to the reverse ammonium sulfate gradient. The A_{450}/A_{280} ratio was greater than 0.9. Prolactin (ovine - NIH-P-S-10, HIH-P-S11) was provided by the Pituitary Hormone Distribution Program of the National Institute of Arthritis and Metabolic Diseases. Choramine-T was synthesized from p-toluenesufonamide and sodium hypochloride (98).

Methods and Results

A variety of iodination methods were used to label prolactin with 125-Iodine. Methods using the oxidant chloramine-T, such as that used by Cuatrecasas (22) to label insulin, resulted in near total inactivation of ovine prolactin. Briefly, prolactin was iodinated at room temper-

ature by addition of 5 μg of ovine prolactin in 10 μl of 0.1 M sodium phosphate buffer, pH 7.4, to 100 μl of 0.25 M sodium phosphate buffer containing 1.0 mCi Sodium 125-iodide. 20 μl of chloramine-T (5 mg/ml in water) were added and, after 40 seconds, 20 μl of sodium metabisulfite (10 mg/μl in water) were added. ^{125}I-labeled prolactin and free sodium 125-iodide were separated by chromatography on a 15 cm x 40 cm Bio-Gel P-6 column which had been swollen in column buffer which had been made 1% in bovine serum albumin in order to mask nonspecific adsorption sites. The column buffer was 20 mM Tris-(aminoethyl)aminomethane (Tris) and 150 mM NaCL, pH 7.6. Specific activities of the iodinated prolactin were 30 μCi/μg to 70 μCi/μg. When used in the binding assay, this material showed little or no specific binding and extremely low nonspecific binding, indicating that large aggregates had not formed and were not the reason for the inactivation. Prolactin may have a histidyl residue essential for activity, and inactivation could possibly be due to oxidation of this residue by chloramine-T.

Lactoperoxidase was used by Frantz and Turkington (99) to label ovine prolactin. Lactoperoxidase from 0.1 to 10 μg, ovine prolactin 1 to 10 μg, sodium 125-iodide 1 to 5 mCi and hydrogen peroxide, 100 to 1250 ng, were mixed in a total volume of 0.220 to 0.75 ml for 10 minutes at room temperature. This mixture was then applied to a DE32 column to separate labeled from unlabeled hormone and

unreacted 125-iodide. Using this procedure under the conditions given above, it was observed that prolactin aggregated to the extent that it chromatographed in the void volume of a 1 cm x 100 cm Bio-Gel P-100 column. The aggregated prolactin, when used in the assay for rabbit mammary gland prolactin receptor, collected on the Millipore filters giving backgrounds too high to determine if any of the aggregated ^{125}I-labeled prolactin bound to prolactin receptor. Various attempts to dissociate the aggregates with pH 10, 50 mM Tris; pH 3, 50 mM sodium acetate; 30% ethanol, 6 M urea, 1% sodium dodecyl sulfate, and 2 M NaCL failed to dissociate more than 10-20% of the aggregates. The best results were obtained with 6 M urea (Figure 9). Free and bound sodium 125-iodide were separated on a 0.5 cm x 40 cm Bio-Gel P-6 coulmn. When DE32 that had been pretreated with BSA (24) was used to separate bound and free 125-iodide, the bound peaks were extremely broad and recoveries were low, possible due to hydrophobic or nonspecific adsorption centers on the cellulose-diethylaminoethyl gel. Specific activities ranged from 10 μCi/μg to 30 μCi/μg with essentially no nonspecific binding.

A modification of the lactoperoxidase methods of Thorell and Johansson (100) and Costlow, **et al.** (91) was used to successfully iodinate ovine prolactin to a high specific activity. The iodination reaction mixture contained: 5 μg ovine prolactin NIH P-S-10 or 11 (5 μl), 20 μl 200 mM sodium phosphate pH 7.0, 1 mCi sodium 125-iodide

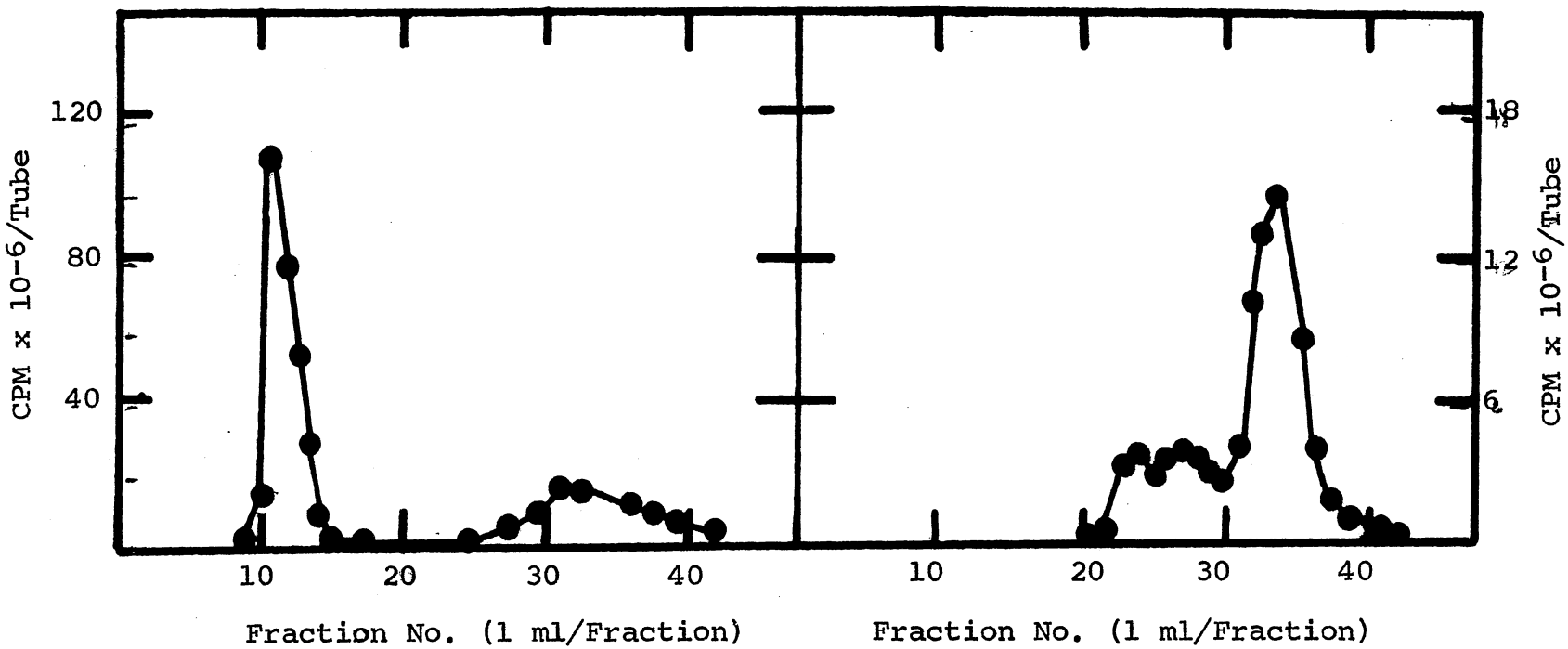

Figure 9. Gel Filtration of Prolactin Iodination Mixture on Bio-Gel P-6 and Purification of the 125-I-Labeled-Prolactin on Bio-Gel P-100

The iodination reaction mixture (0.5 ml) plus rinse (0.6 ml) was applied to a Bio-Gel P-6 column (0.5 cm x 40 cm) and eluted with 20 mM Tris, 150 mM sodium chloride, pH 7.6. Fractions 10-17 were pooled, precipitated with ammonium sulphate, and applied to a Bio-Gel P-100 column (1 cm x 120 cm) and eluted.

(10 µl), 1 µl lactoperoxidase (7.7 mg/ml), 3 µl of H_2O, and 1 µl of 0.88 mM hydrogen peroxide. The reaction was carried out in a 1.5 ml Beckman Microfuge tube with magnetic stirring using a 2.4 mm piece of acid-washed paper clip as a stirring bar. The reaction time was 90 seconds at room temperature. The reaction was stopped by diluting the mixture with 0.5 ml of cold column buffer (20 mM Tris, 150 mM magnesium chloride, pH 7.6). The column used to separate bound and free 125-iodide was Bio-Gel P-6, 0.5 cm x 40 cm column previously treated with bovine serum albumin to mask nonspecific adsorption sites. Bio-Gel P-6 (5 gm) was allowed to swell overnight at 4° in 50 ml of water containing 1 gm of bovine serum albumin. The column was then poured and allowed to equilibrate for 2 hours or longer with 20 mM Tris, 150 mM sodium chloride, pH 7.6 before use. A new column was prepared for each iodination. Fractions, 0.5 ml to 0.75 ml, were collected into 0.2 ml of 1% BSA-column buffer using Beckman polyethylene gamma counting vials as collection tubes (Figure 10). The tubes were counted in a Beckman Biogamma counter. Disintegrations per minute were determined by comparing the 27.5 Kev and 55 Kev peaks (104). Specific activity was determined by counting 5 µl or 10 µl aliquot of the ^{125}I-labeled prolactin peak and assuming a 100% protein recovery from the column. Normally, this material was used for assay purposes without further purification. If a high amount of aggregate were present as indicated by high backgrounds in the assay, the ^{125}I-labeled

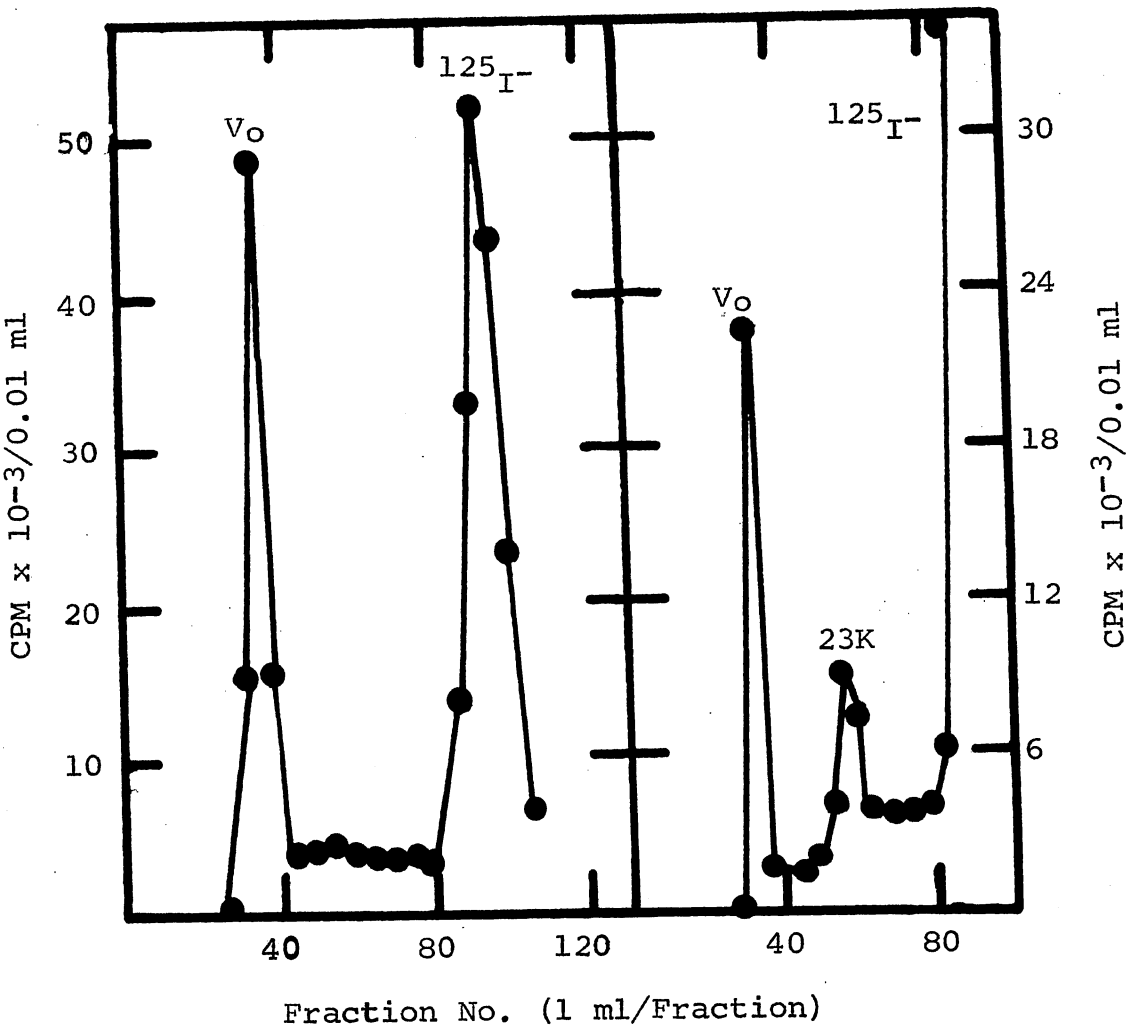

Figure 10. Iodination of Ovine Prolactin with Lactoperoxidase and the Effect of 6 M Urea upon the Dissociation of the Aggregated Product

5 µg of ovine prolactin were iodinated as by Frantz and Turkington (103) and applied to a 1 cm x 100 cm Sephadex G-100 column equilibrated in a 50 mM Tris, pH 7.6 buffer. A 0.5 ml aliquot of the peak tube was made 6 M in urea, incubated for 30 minutes and re-applied to the Sephadex G-100 column.

could be further purified by chromatography on a 1 cm x 120 cm Bio-Gel P-100 column.

The ^{125}I-labeled prolactin peak was pooled and made 80% in ammonium sulfate by addition of saturated ammonium sulfate, pH 7.6 and allowed to precipitate overnight at 4°. The precipate was collected by centrifugation at 100,000 x g for 1 hour using a 30 rotor in a Beckman L5-65 centrifuge. The precipitate contained approximately 90-100% of the original counts. The precipate was redissolved in a minimum volume of 20 mM Tris, 150 mM sodium chloride, pH 7.6 buffer and applied to a Bio-Gel P-100 column which had been saturated with BSA to mask nonspecific adsorption centers. One to two ml samples were collected into 0.2 ml of 1% bovine serum albumin and counted. Normally, three peaks were present: one near the void volume which was possible ^{125}I-labeled lactoperoxidase or highly aggregated ^{125}I-labeled prolactin, one of molecular weight 50,000 which could be a prolactin dimer, and one large peak at 23,000 which was the prolactin monomer (Figure 10). ^{125}I-labeled prolactin off the P-6 column was approximately 60-70% bindable, ^{125}I-labeled prolactin off the P-100 was approximately 80-90% bindable. Specific activities varied from 45 μCi/μg to 100 μCi/μg. The labeled prolactin was stored at 4° in the same tubes used for collection off the column and then diluted to the desired concentration when needed. The labeled prolactin was stable for approximately 1 to 2 months. Table III is a summary of the lactoperoxidase iodination

TABLE III

IODINATION OF OVINE PROLACTIN TO A HIGH SPECIFIC ACTIVITY

Stock Solution	Vol. (μl)	Final Concentration
20 mM PO_4 pH 7.0	20	100 mM
100 mC/ml $Na^{125}I$	10	1 mC
1 mg/ml prolactin	5	5 μg
7.5 mg/ml lactoperoxidase	1	7.5 μg
H_2O	3	
0.88 mM H_2O_2	1	0.22 mM

React for 90 seconds

Desalt over P-6 column

Purify on P-150 column if necessary

S. A. = 60 μC/μg

procedure.

Another procedure which yielded active labeled prolactin was the Bolton-Hunter reagent used to modify the free amino groups in prolactin (29). Briefly, the iodinated hydroxyphenylpropionic acid ester was made by reacting N-succinimidyl-3(4-hydroxyphenyl)propionate (0.2-9.25 μg in 1 ml of pH 4.5 50 mM sodium acetate buffer), 2 mCi sodium 125-iodide in 20 ml water, and 50 μg of chloramine-T in 10 l of 0.25 M phosphate buffer pH 7.5. The reaction was immediately terminated by the addition of 120 μg of sodium metabisulphite in 10 μl of 0.05 M phosphate, pH 7.5, after which 200 μg of carrier potassium iodide in 10 μl of this buffer was added. The iodinated product was extracted twice with 0.25 ml of benzene and recovered by evaporation of the solvent under vacuum. Five μl dimethylforamamide was added before the benzene extraction to insure quantitative recovery. This procedure must be completed in as short a time as possible (20-30 seconds). The specific activity of the iodinated ester, assuming 100% recovery in the benzene extraction, was 200 Ci/mmole. The iodinated ester was used without further purification.

To label prolactin, 5 μg ovine prolactin in 50 μl of 0.1 M sodium borate buffer, pH 8.5, was added to the dried ester in a conical centrifuge tube and agitated for 15 minutes at 0°. Conjugated and nonconjugated ester were separated by gel chromatography on a Bio-Gel P-6 column. Incorporation into prolactin was low (specific activity =

10 μCi/μg of prolactin. This was possibly due to hydrolysis of the ester during the iodination process. The labeled prolactin was, however, active in the receptor assay with 50% of the label specifically binding to prolactin receptor. Bolton and Hunter (27) reported specific activities ranging from 50 μCi/μg to 200 μCi/μg for a variety of protein.

CHAPTER V

PARTIAL CHARACTERIZATION OF RABBIT MAMMARY GLAND PROLACTIN RECEPTOR

Effect of Magnesium Chloride on Prolactin Binding

Materials

Tris(hydroxymethyl)aminomethane, dithiazone, and bovine serum albumin were purchased from Sigma Chemical Company. Magnesium chloride was purchased from Mallinckrodt. Carbon tetrachloride was purchased from Fisher.

Methods

Magnesium chloride was purified as described by Morrison and Uhr (103). An aqueous solution of 50% (w/v) of magnesium chloride was extracted five times with an equal volume of 0.001% (w/v) solution of dithiazone in carbon tetrachloride. The aqueous layer was then extracted four times with an equal volume of carbon tetrachloride to remove any dithiazone, after which the carbon tetrachloride was removed from the aqueous layer by bubbling nitrogen through

the solution. Trans-1,2-diaminocycohexane-N,N,N',N'-tetraacetic acid was added to a final concentration of 0.005 M. The salt solution was crystalized by evaporation in a vacuum oven at 40° and used without further recrystallization.

Prolactin receptor was assayed under the standard assay conditions except that the magnesium chloride concentration of the assay buffer was varied.

Results

Prolactin binding, as determined under assay conditions, increased linearly and was maximum at 0.01 M magnesium chloride. Formation of the complex then decreased with increasing magnesium chloride concentrations and at 0.5 M little or no binding could be detected. Similar results were obtained with both the soluble and particulate receptor (Figure 11). These results are in agreement with those reported by Friesen (86).

The decrease in prolactin binding can at least partially be explained as an ionic strength effect and not specific to magnesium chloride since high concentrations of sodium chloride also cause a decrease in prolactin binding (Figure 12).

Effect of Digestion by Proteolytic Enzymes on the Activity of Prolactin Receptor

Materials

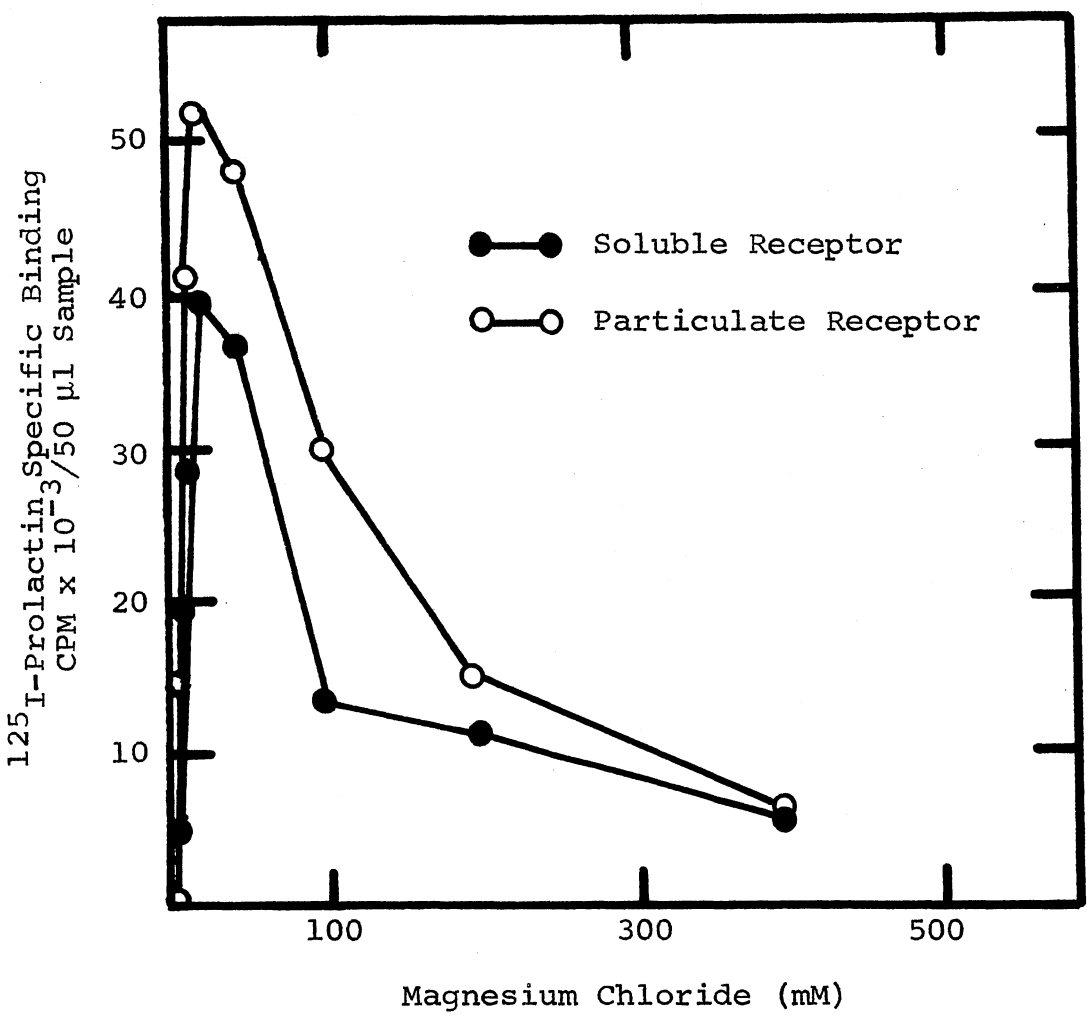

Figure 11. Effect of Magnesium Chloride on Receptor Activity

Particulate and soluble prolactin receptor were assayed in the presence of increasing concentrations of purified magnesium chloride. 300 µg of particulate or 200 µg of soluble receptor, 200,000 dpm of 125-I-labeled prolactin, and varying concentrations of magnesium chloride in the presence and absence of unlabeled prolactin were incubated together for four hours at 25°. Bound and free 125-I-labeled prolactin were separated as previously described.

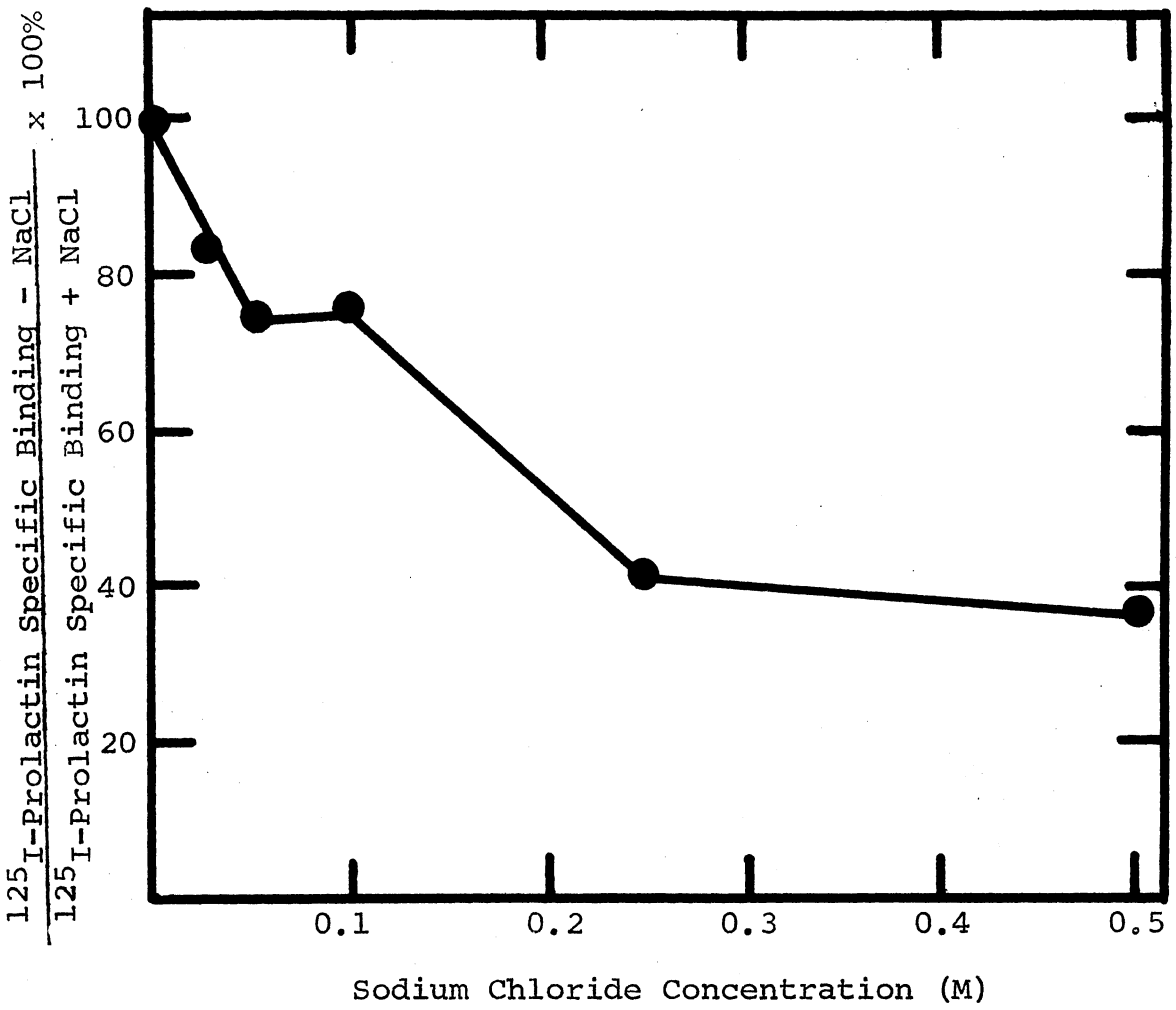

Figure 12. Effect of Sodium Chloride on the Formation of the Prolactin-Receptor Complex

Particulate prolactin receptor was assayed in the presence of increasing concentrations of sodium chloride. 400 µg of particulate receptor, 200,000 dpm of 125-I-labeled prolactin, and varying concentrations of sodium chloride were incubated together in the presence and absence of displacing amounts of unlabeled prolactin for four hours at 25°. Bound and free 125-I-labeled prolactin were separated as described in the text.

Trypsin, chymotrypsin, soybean trypsin inhibitor, and phenylmethylsulfonylfloride were purchased from Sigma Chemical Company.

Methods

The effects of trypsin and chymotrypsin on the binding activity of soluble prolactin receptor were determined by incubating 0.5 ml of soluble receptor (10 mg protein) with varying concentration (0.1 μg/ml to 50 μg/ml) of each proteolytic enzyme in 50 mM Tris, 10 mM $CaCl_2$ buffer, pH 7.6, for 30 minutes at 25°C. The reactions were stopped by addition of a minimum of the two molar excess of either soybean trypsin inhibitor or phenylmethylsulfonylfluoride to the trypsin or chymotrypsin incubations, respectively. Aliquots of each incubation were then assayed and compared to a control which had been incubated in the absence of proteolytic enzymes.

Results

Both proteolytic enzymes abolished binding activity (Figure 13) with detectable effects observed at 0.5 μg/ml and complete loss of binding activity at 20 μg/ml. It appears that the binding activity is susceptible to trypsin and chymotrypsin.

Inactivation by both trypsin and chymotrypsin appeared to be first order (Figure 14). A correlation coefficient was calculated for each first order plot. The correlation

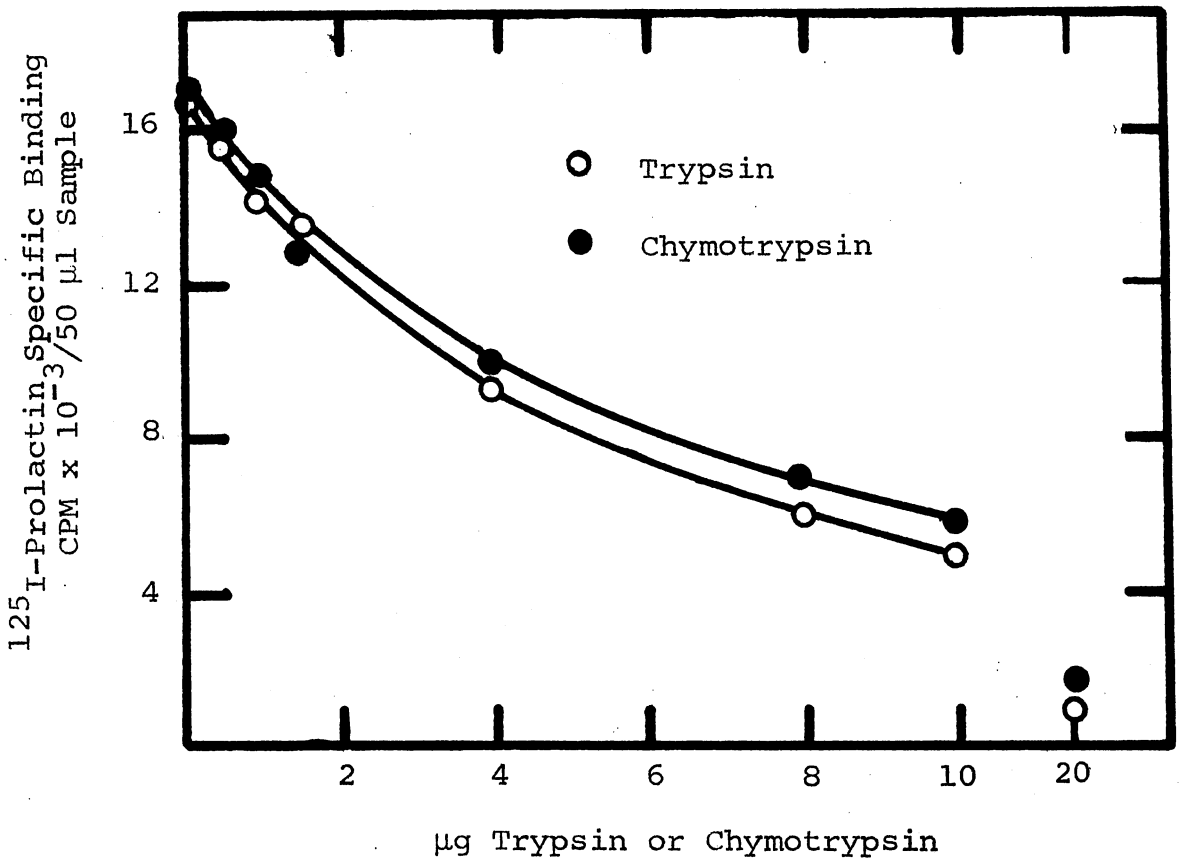

Figure 13. Effect of Trypsin or Chymotrypsin Digestion on Prolactin Receptor Binding Activity

Soluble prolactin receptor (0.5 ml) and 0.5 ml of 0.1 M Tris, 20 mM calcium chloride, pH 7.4, containing varying amounts of trypsin or chymotrypsin were incubated together for 30 minutes at 37°. The reactions were stopped by the addition of 1 mg of soybean trypsin inhibitor or 10 µg of p-methylsulfonylfluoride. 50 µl aliquots were assayed as described in the text.

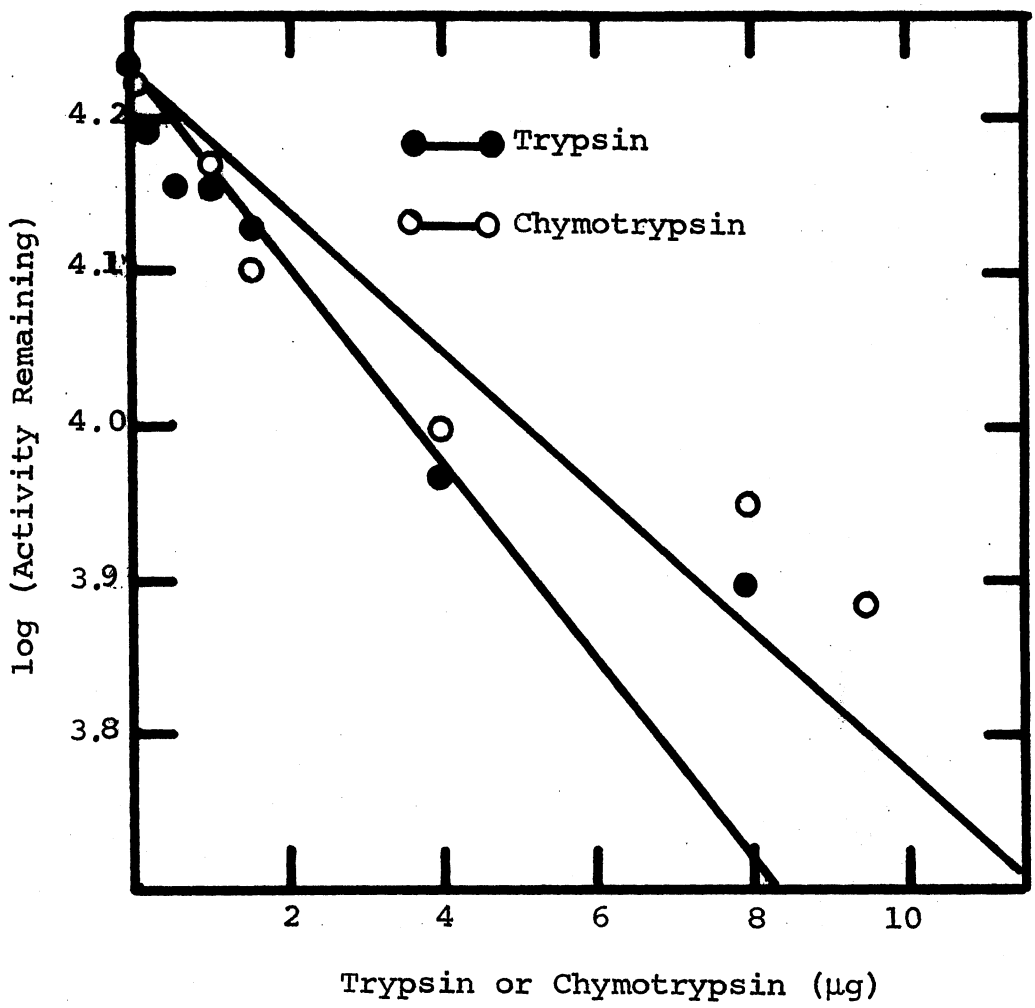

Figure 14. First Order Inactivation Plot of Soluble Receptor by Trypsin or Chymotrypsin

The data from Figure 13 was replotted in semilog form. The correlation coefficient of the trypsin inactivation curve was 0.98. The correlation coefficient of the chymotrypsin inactivation curve was 0.97.

coefficient was 0.97 for chymotrypsin inactivation and 0.98 for trypsin inactivation where 0 is no fit and 1 is a perfect fit of the data plots on a straight line.

Effect of Temperature on the Kinetics of Prolactin Binding

Materials and Methods

The binding assay was done as described previously.

Results

Each assay contained 400 g of particulate receptor or 200 g of soluble receptor and 2×10^5 dpm of ^{125}I-labeled prolactin. Assays were removed from the incubation at various times and bound and free ^{125}I-labeled prolactin were separated. Equilbrium was reached after 5.5 hours at 25°, 2-3 hours at 37°, and not reached after 8 hours at 4° (Figure 15). Nonspecific binding was maximal after 1 hour and increased from 2×10^3 cpm at 4° to 4.5×10^3 cpm at 37^{0}.

Scatchard Analysis of Prolactin Receptor Binding

Materials and Methods

Binding assays were done as previously described with the following exceptions. A fixed concentration of ^{125}I-labeled prolactin was added to each assay and unlabeled

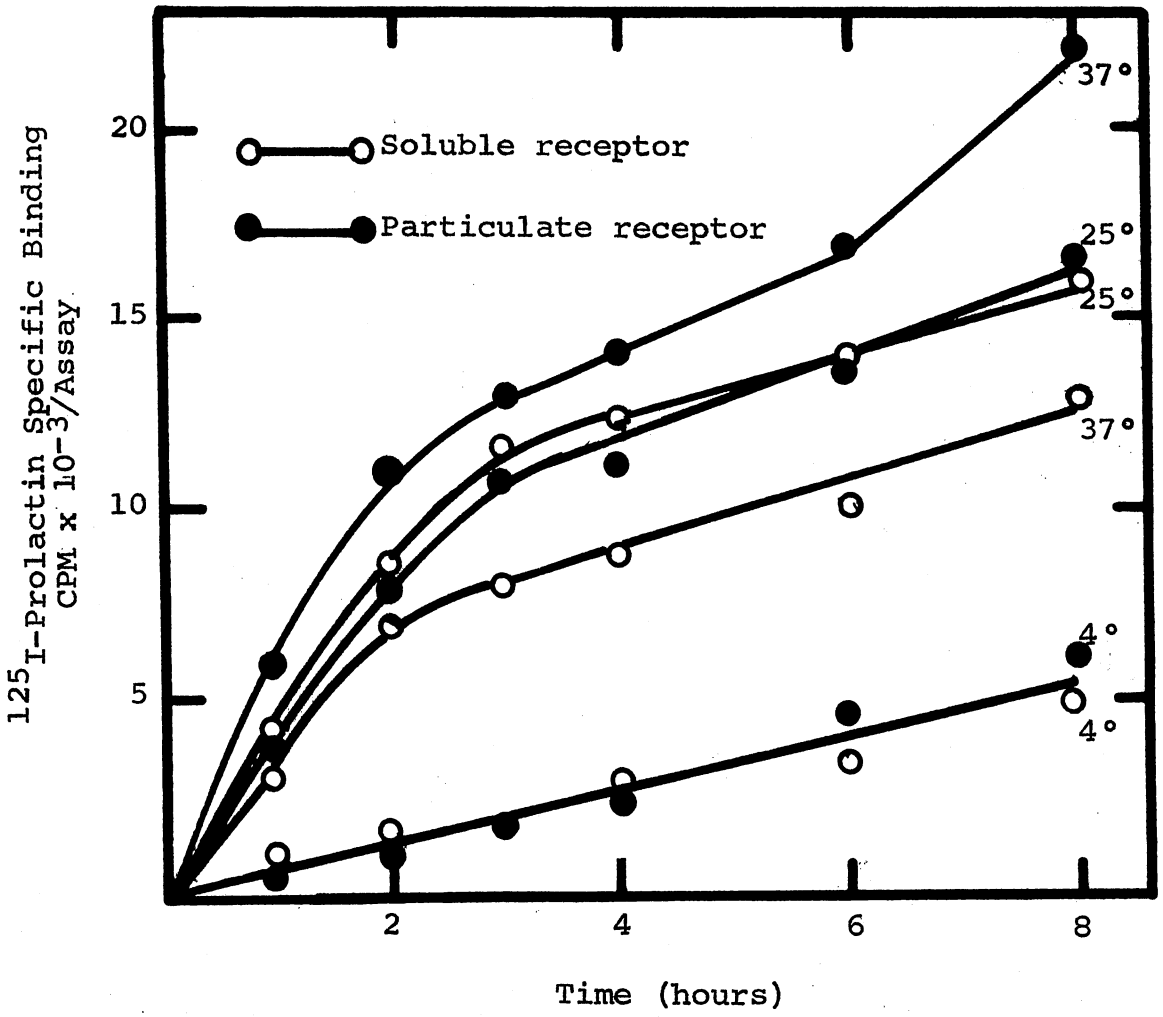

Figure 15. Kinetics of Prolactin Binding as a Function of Temperature

Assays were run as described in Methods for the particulate receptor and soluble receptor, except the length of the incubation and the temperature of the incubation were varied. Data was taken from more than one experiment using different prolactin receptor preparations.

prolactin was added to vary the final prolactin concentrations from 10^{-11} M to 10^{-9} M. Incubations were six hours at 25°. The bound and free ^{125}I-labeled prolactin were separated, and the amount of bound prolactin was directly determined by counting on a Beckman Biogamma counter. The concentration of free prolactin was determined indirectly by subtraction of the bound from the total.

Results

A graph of bound prolactin versus the ratio of bound/free prolactin gave linear plots for both soluble and particulate receptor-prolactin binding, which indicates one class or one affinity of binding sites (Figures 16 and 17). The dissociation constant determined for the binding of prolactin to soluble receptor was 1×10^{-10} M.

Effect of Phospholipases on Prolactin Receptor Binding Activity

Materials

Bee venom phospholipase A, Clostridium welchii and Bacillus corus phospholipase C, and cabbage phospholipase D were purchased from Sigma Chemical Company.

Methods

To determine if phospholipids were required for binding activity, the prolactin receptor was incubated with bee

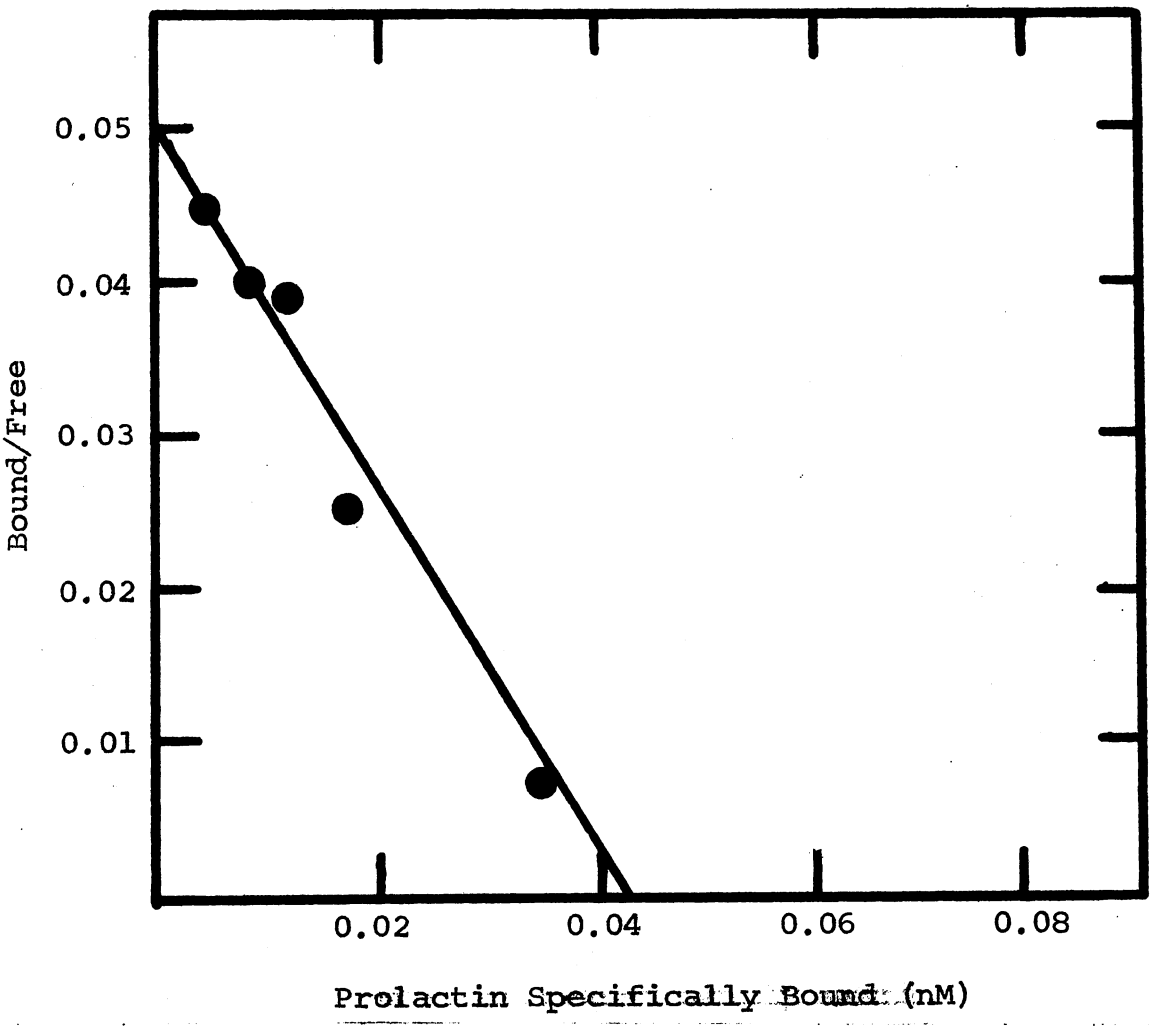

Figure 16. Scatchard Analysis of the Binding of Prolactin to Soluble Prolactin Receptor

300 µg of soluble receptor, a fixed concentration of 125-I-labeled prolactin (400,000 dpm of 33 µC/µg) and unlabeled prolactin to increase the total prolactin to the desired concentration were mixed together and incubated for 6 hours at 25°. Bound and free 125-I-labeled prolactin were separted by precipitation and filtration. Bound prolactin was determined directly by counting and free prolactin was determined by subtracting the bound from the total. The Kd determined from this plot was $1X10^{-9}$ M . The Kd from 2 plots was $9.3X10^{-10}$ M . A correlation coefficient of 0.87 was calculated.

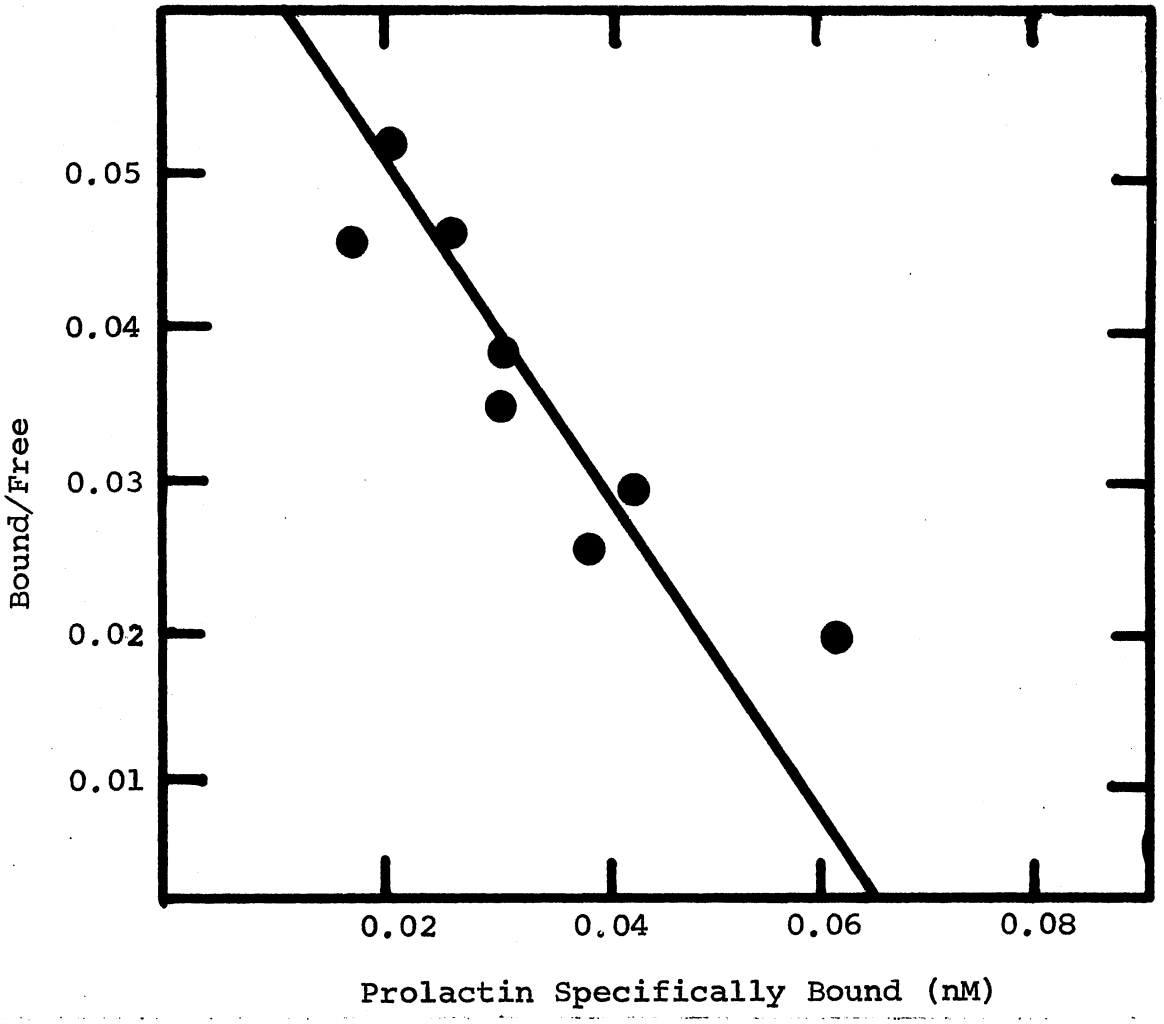

Figure 17. Scatchard Analysis of Binding of Prolactin to Particulate Prolactin Receptor

200 μg of particulate receptor, a fixed concentration of 125-I-labeled prolactin (400,000 dpm of 36 μC/μg), and unlabeled prolactin to increase the total prolactin concentration to the desired concentration were mixed together and incubated for six hours at 25°. Bound and free prolactin were separated by centrifugation and bound determined deirectly by counting. Free prolactin was determined indirectly by subtracting the bound prolactin from the total. The Kd determined from this plot was $9.3X10^{-10}$ M . The Kd calculated from two other plots and this one was $9.6X10^{-10}$ M . A correlation coefficient of 0.87 was calculated.

venom phospholipase A, Clostridium welchii phospholipase C, and cabbage phospholipase D. The soluble receptor was incubated with each enzyme separately and with a combination of the three enzymes. Phospholipase A was dissolved in 100 mM Tris, 10 mM calcium chloride pH 7.4 buffer (1 mg/ml, 1500 units/ml). Phospholipase D was dissolved in 100 mM sodium acetate, pH 5.6 buffer (2 mg/ml, 50 units/ml). Phospholipase C was dissolved in 100 mM Tris 10 mM calcium chloride, pH 7.4 buffer (2 mg/ml 10 units/ml). Each incubation contained 0.5 ml of soluble receptor (20 mg/ml in 0.3 M sucrose, 0.1% Triton X-100), 0.1 ml of buffer and 0.1 ml of the enzymatic solution. Incubation was at 37°. Activity remaining was assayed with time and compared to a control receptor solution that was incubated in the appropriate buffer without the enzyme.

Results

Figure 18 shows the time course of activity remaining. After three hours phospholipase A digestion resulted in a 5% loss of activity, phospholipase C an 8% loss of activity, phospholipase D a 20% loss of activity, and all three a 15% loss of activity. Since the error in the assay for soluble receptor is 10-20% and no further loss of activity was observed after 30 minutes, the slight decrease in activity may not be considered significant. It should be noted that enzymatic activity was not assayed. Other investigators have used phospholipases in the presence of Triton X-100 (45)

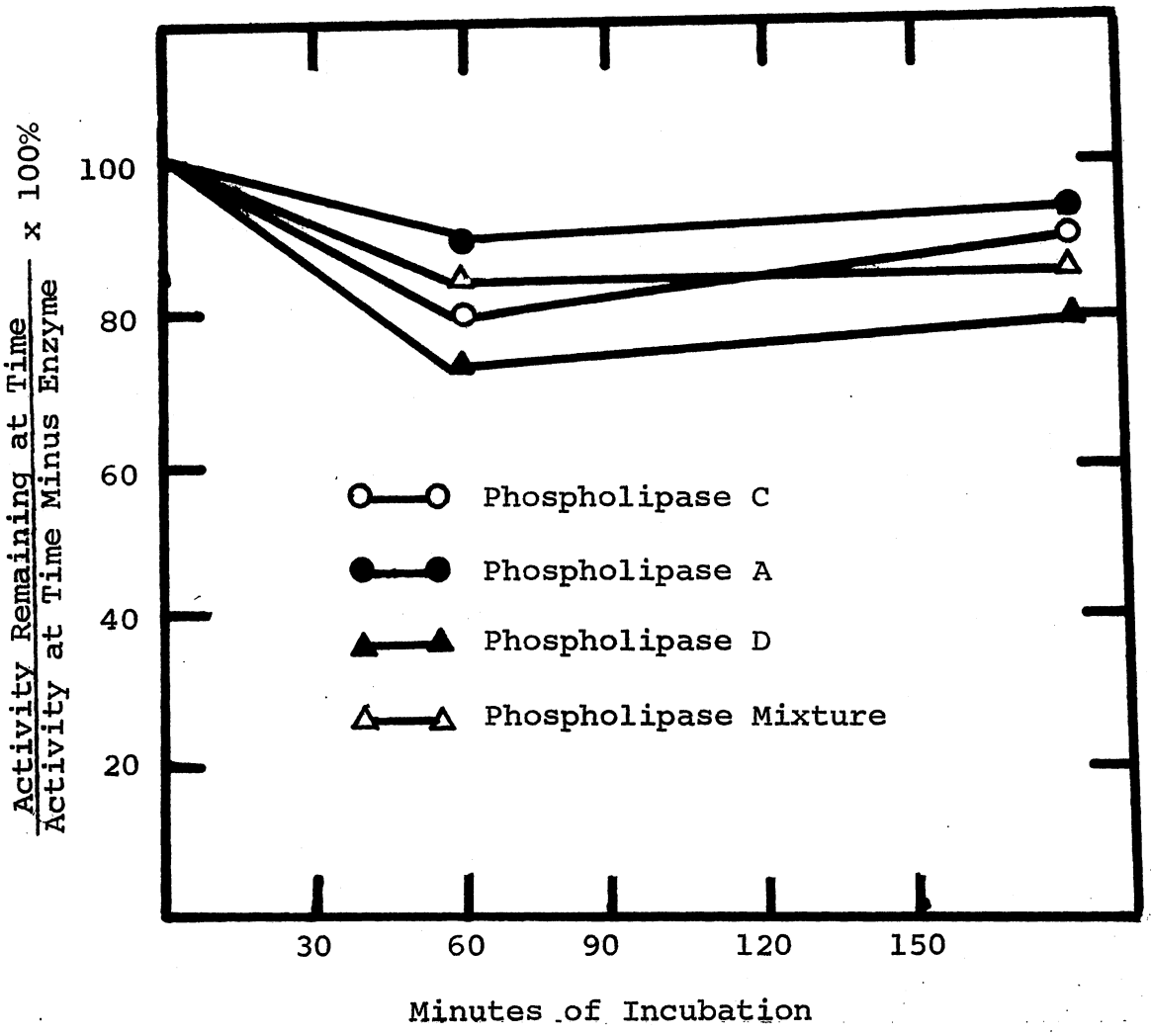

Figure 18. Effect of Phospholipase Digestion on Prolactin Receptor Binding Activity

0.5 ml of soluble receptor (20 mg/ml) in 0.3 M sucrose, 0.1% Triton X-100 and 0.5 ml of phospholipase solution (phospholipase A, 75 units; phospholipase C, 5 units; phospholipase D 15 units; or all three enzymes) were mixed together and incubated at 37°. At the indicated times, activity remaining was assayed (50 µl sample per assay in duplicates) and compared to receptor co-incubated in the appropriate buffer minus the phospholipase.

and the effect of the detergents on enzymatic activity was not reported.

Effect of Glycosidases on Prolactin Receptor Activity

Materials

Type IX neuraminidase from Clostridium perfingens, type III α-mannosidase, and type β-galactosidase were purchased from the Sigma Chemical Company.

Methods

To determine if carbohydrates were critical for prolactin binding activity, the soluble receptor was digested with α-mannosidase, β-galactosidase, and neuraminidase, and a combination of all three enzymes. α-Mannosidase was dissolved in 0.1 M sodium actate pH 6.0 buffer (2 mg/ml, 40 units/ml). β-Galactosidase and neuraminidase were dissolved in 0.1 M Tris pH 7.4 buffer (2 mg/ml, 1700 units/ml 10 units/ml, respectively). Incubation mixtures consisted of 0.5 ml of soluble receptor in 0.3 M sucrose, 0.5% Triton X-100, 0.4 ml of the enzyme buffer, and 0.1 ml of the enzymatic solution. When all three enzymes were added, the buffer was 0.1 M tris pH 7.4. Incubations were at 37°. At various times, samples from the incubation mixtures were removed and assayed for binding activity. Activity was compared to a sample that was co-incubated in the absence of enzyme.

Results

Figure 19 shows the time course of inactivation. After three hours of incubation, the neuraminidase digestion resulted in a 2% loss of activity, α-mannosidase digestion resulted in a 19% loss of activity, β-galactosidase digestion resulted in a 10% loss of activity, and digestion with all three resulted in a 15% loss of activity. Again, it was difficult to ascertain if these enzymes caused a significant loss of activity.

Effect of Modification of Sulfhydryl Residues on the Activity of Prolactin Receptor

Materials

N-Ethylmaleimide, p-chloromercuribenzoate, and 2-mercaptoethanol were purchased from Sigma Chemical Company.

Methods

To determine if a sulfhydryl residue(s) was necessary for activity of the prolactin receptor, the soluble receptor was incubated with various concentrations of two sulfhydryl modifying reagents. Conditions for modification with N-ethylmaleimide were similar to those described by Means and Feeney (103). Soluble receptor, 0.5 ml, in 0.3 M sucrose 0.5% Triton X-100 was mixed with 0.5 ml of 0.1 M

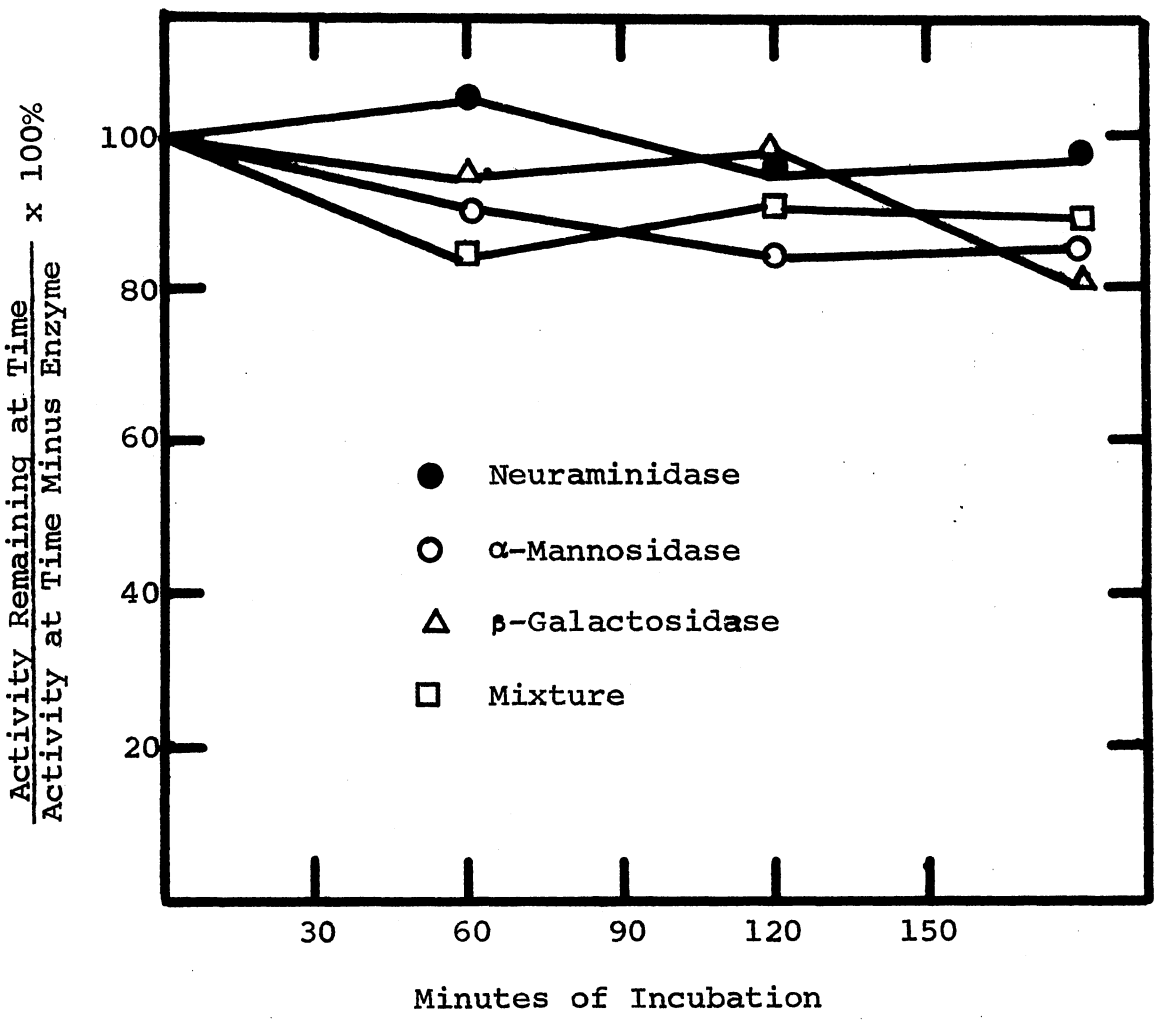

Figure 19. Effect of Digestion with Glycosylases on Prolactin Binding Activity

0.5 ml of soluble receptor in 0.3 M sucrose, 0.1% Triton X-100 and 0.5 ml of enzyme solution containing 4 units of α-mannosidase, 170 units of β-galactosidase, 1 unit of neuraminidase, or a combination of all three were mixed together and incubated at 37°. At the indicated times, duplicate 50 µl aliquots were removed and assayed.

sodium phosphate pH 7.0 buffer containing varying amounts of N-ethylmaleimide. Incubation was for 30 minutes at 25° in a temperature controlled water bath. The reaction was stopped by addition of a two molar excess of 2-mercaptoethanol, 50 µl of the reaction mixture was assayed for prolactin binding activity. Modification with p-chloromercuribenzoate was performed as described by Means and Feeney (103). p-chloromercuribenzoate was dissolved by adding sodium hydroxide to a suspension of p-chloromercuribenzoate and water until all of the reagent was dissolved (approximately pH 12-13). Solid glycylglycine was added to lower the pH to 7.8-8.0. In this manner, a stock solution of 0.1 to 0.2 M p-chloromercuribenzoate was prepared. Soluble receptor, 0.5 ml, (15 mg/ml in 0.3 M sucrose 0.5% Triton X-100) and 0.5 ml of 0.2 M Tris containing various concentrations of p-chloromercuribenzoate were mixed and incubated for 30 minutes at 25° in a temperature controlled water bath. The reaction was not terminated by addition of 2-mercaptoethanol since the p-chloromercuribenzoate-sulfhydryl reaction is reversible. Instead, the receptor was precipitated by the addition of 0.7 ml of 25% polyethylene glycol 6000 and 0.5 ml of 0.1% bovine gammaglobulin. The precipitate was collected by centrifugation at 100,000 x g for 30 minutes. The pellet was redissolved in one ml of 20 mM Tris, 0.1% Triton X-100, pH 7.6 buffer. Duplicate 50 µl samples were assayed for prolactin binding activity and compared to a control that was co-incubated without the modifying reagent.

Results

Figure 20 shows the inactiviation plots for both N-ethymaleimide and p-chloromercuribenzoate treated receptor. In both cases, the receptor was essentially totally inactivated. Maximal inactivation under the above conditions was at 10 mM p-chloromercuribenzoate and 20 mM N-ethylmaleimide.

The reactions were first order (Figure 21). A correlation coefficient was calculated for each first order plot. The first order plot of p-chloromercuribenzoate inactivation had a correlation coefficient of 0.98 and for N-ethylmaleimide the correlation coefficient was 0.99.

Effect of a Variety of Dissociation Agents on the Receptor-Prolactin Complex

Materials

2-Mercaptoethanol, p-chloromercuribenzoate, sodium chloride, magnesium chloride, and dimethylformamide were purchased from Schwarz/Mann. Dioxane, methanol, and glycerol were purchased from Curtin-Matheson Chemical Company. Absolute ethanol was supplied by the University of Kansas Medical Center Pharmacy. Ovine prolactin (NIHPS 12) was supplied by the National Institute of Arthritic and Metabolic Diseases Pituitary Hormone Distri-

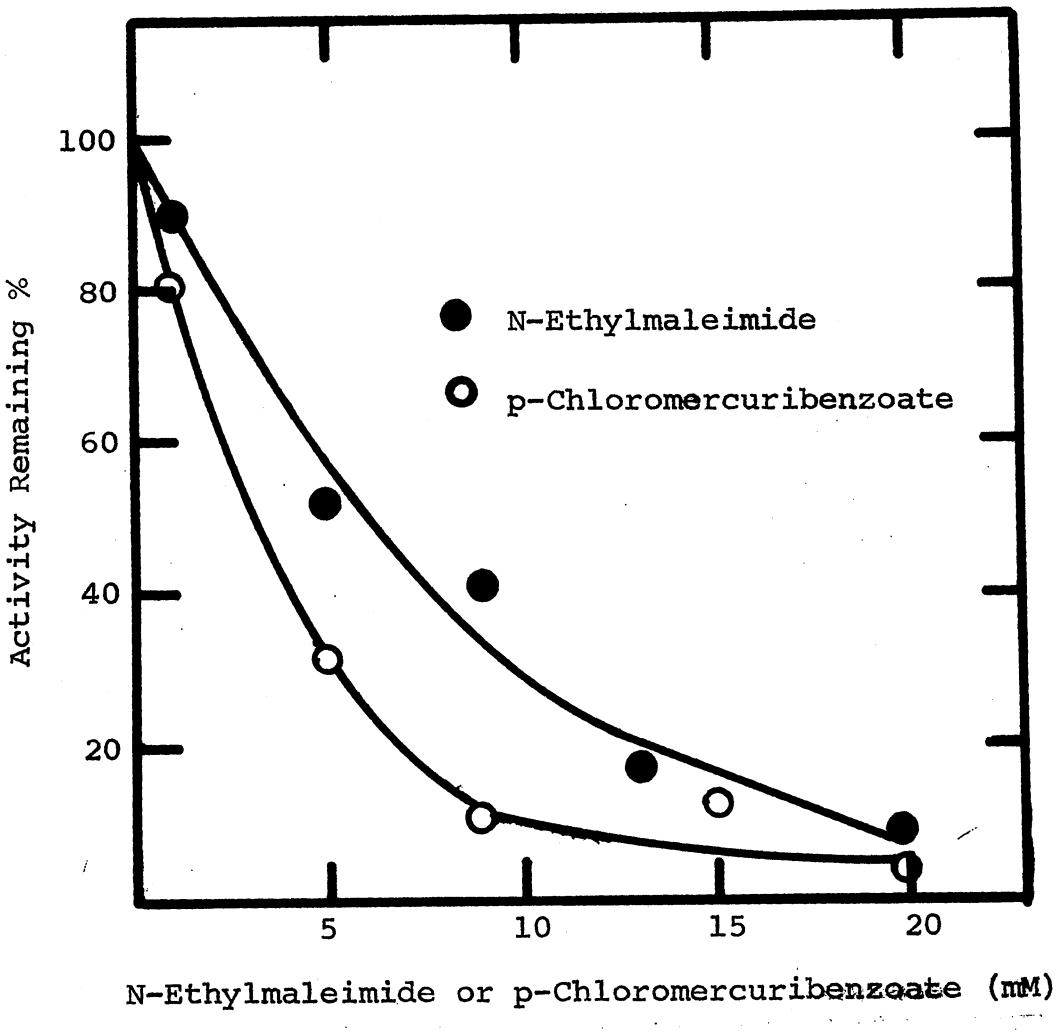

Figure 20. Effect of Sulfhydryl Reagents on Prolactin Binding Activity

0.5 ml of soluble receptor in 0.3 M sucrose, 0.5% Triton X-100 and 0.5 ml of buffer containing either N-ethylmaleimide or p-chloromercuribenzoate were incubated together for 30 minutes at 25°. The N-ethylmaleimide reaction was stopped by the addition of a 2 molar excess of 2-mercaptoethanol. The PCMB reaction was terminated by precipitation of the protein with polyethylene glycol 6000. The precipitate was resuspended in 1 ml of buffer and assayed.

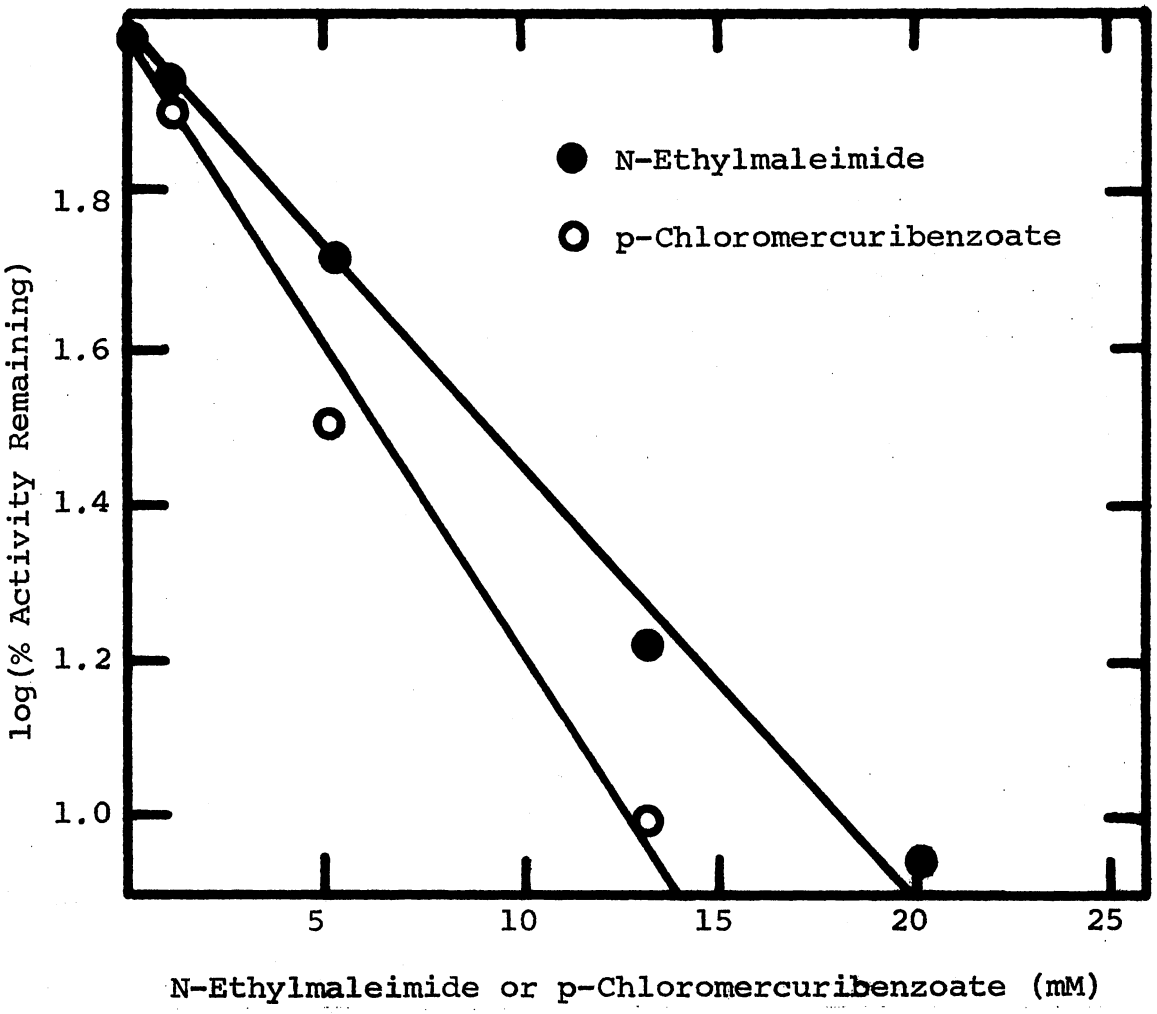

Figure 21. First Order Inactivation Plot of Soluble Receptor by p-Chloromercuribenzoate and N-Ethylmaleimide

The data from Figure 20 was replotted to show first order inactivation. The correlation coefficient of the N-ethylmaleimide plot was 0.99. The correlation coefficient of the p-chloromercuribenzoate plot was 0.98.

bution Program.

Methods

To determine if the prolactin receptor exhibited either positive or negative cooperativity upon the binding of prolactin, the experimental approach developed by Roth, et al. (50) was used. Particulate receptor and ^{125}I-labeled prolactin were incubated together at concentrations such that only 10% to 20% of the available binding sites were filled. Normally, 1-5 mg of receptor were incubated with 4×10^5 dpm of ^{125}I-labeled prolactin for 4 hours at 25^{o} in the presence and absence of unlabeled prolactin in a total volume of 1 ml. The buffer was 20 mM Tris, 10 mM magnesium chloride, 0.5% bovine serum albumin pH 7.6.

Depending upon the number in the dissociation experiments, multiples of the binding mixture were incubated in Beckman Microfuge tubes. The pellets, which contained both free receptor and receptor-prolactin complex, were resuspended in 20 mM Tris, 10 mM magnesium chloride, 0.1% bovine serum albumin, pH 7.6 buffer. The resuspended pellets were pooled into two tubes, one containing those incubated in the absence of unlabeled prolactin and one containing those incubated in the presence of unlabeled prolactin.

Aliquots of the resuspended pellets were then diluted 100-fold into buffer (20 mM Tris, 10 mM magnesium chloride, and 0.1% bovine serum albumin pH 7.6) containing 10^{-6} M

prolactin. 0.1 ml of the resuspended pellets were diluted into 10 ml of buffer. Part of the resuspended pellet was diluted into prolactin-free buffer. The dilution tubes were then incubated at 25° or 37°. At various times, the tubes were removed and filtered on a Millipore filter. The filter was then counted in a Beckman Biogamma counter. The amount of specifically bound complex remaining was determined by subtracting the counts remaining in the complex in the dilution tubes that had previously been incubated with receptor, ^{125}I-labeled prolactin, and unlabeled prolactin from those incubated with only receptor and ^{125}I-labeled prolactin. The time courses of dissociation of the complex in the presence and absence of prolactin were then compared. If the rate of dissociation was faster in the presence of prolactin, the explanation would be negative cooperativity. If the rate of dissociation was slower in the presence of saturating amounts of prolactin positive cooperativity could exist. If the rates were the same in the presence or absence of saturating amounts of prolactin, prolactin binding could be considered to be a non-cooperative process.

A variety of organic and inorganic compounds were tested on the dissociation of the prolactin-receptor complex in a similar manner with the following exceptions. Incubation after dilution was for 30 minutes at 37° and often more than 10-20% of the available sites were filled. Results are expressed as prolactin bound after 30 minutes/ prolactin bound at zero time x 100% versus concentration of

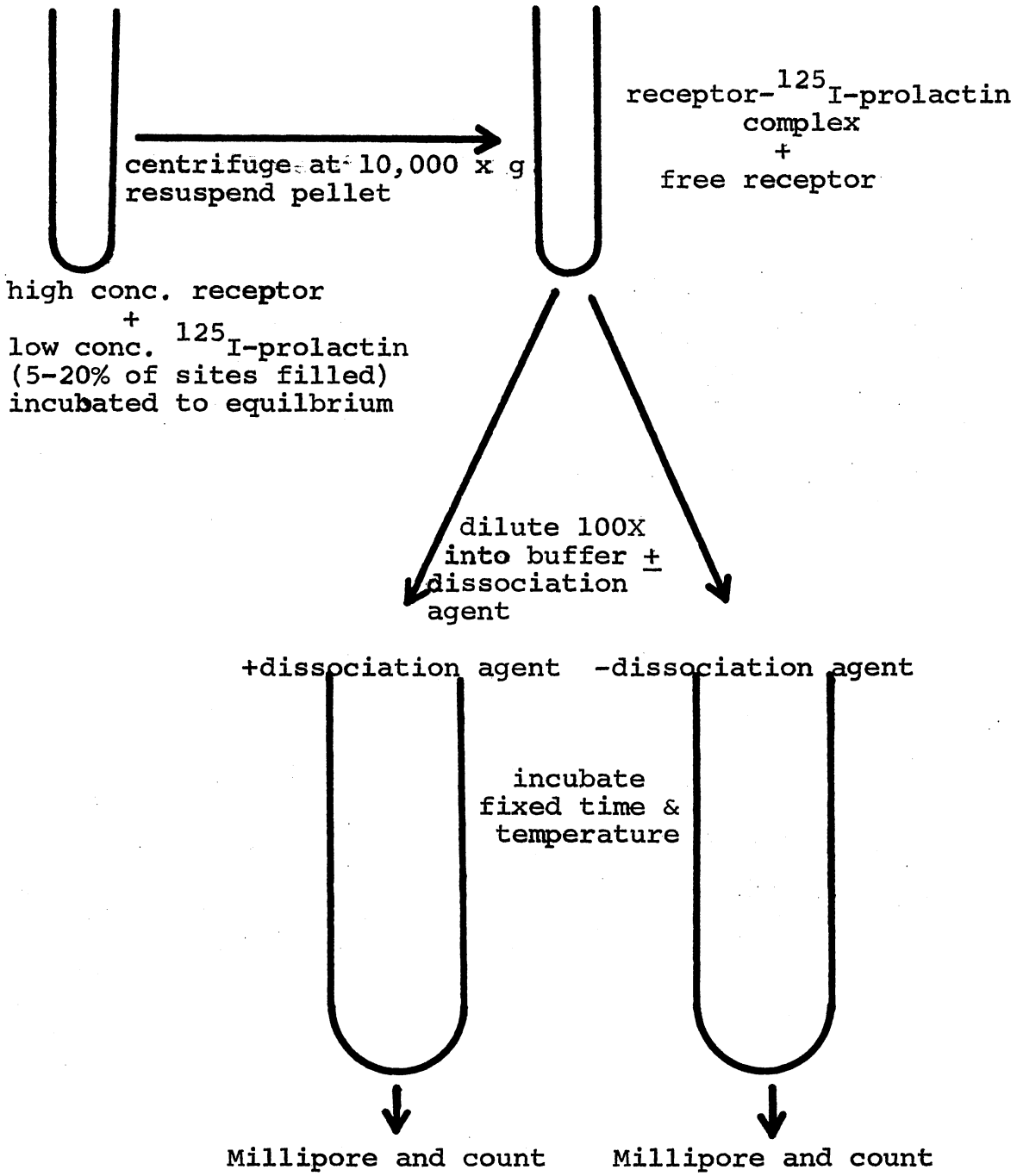

Figure 22. The Experimental Procedure for the Dissociation Experiments

dissociation agent. Specific activities were determined as previously described. Figure 22 gives a graphic summary of the experimental procedure used in the dissociation experiments.

Results

There was no difference in the rates of dissociation of the prolactin-receptor complex in the presence or absence of saturating amounts of prolactin (Figure 23). When the remaining sites were allowed to fill in the presence of 10^{-6} M prolactin or when the sites were left unfilled, 13% of the complex had dissociated after 2 hours at 25^{o}. The membranes used in this study had been frozen at -20^{o}. When fresh membranes were used, there was no detectable cooperativity effect (Figure 24).

Figures 25 and 26 show the effect increasing temperatures on the rate of dissociation. Dissociation was extremely slow at 4^{o} and increased with temperature.

Since the receptor has a sulfhydryl group necessary for activity, the effect of a sulfhydryl modifying reagent on the rate of dissociation was determined. When one set of dilutions was made 10 mM in p-chloromercuribenzoate, no increase in the rate of dissociation could be detected (Figure 27). This result indicates that the sulfhydryl group necessary for activity is protected by bound prolactin either by prolactin directly masking it at the binding site or through conformational changes which occur upon

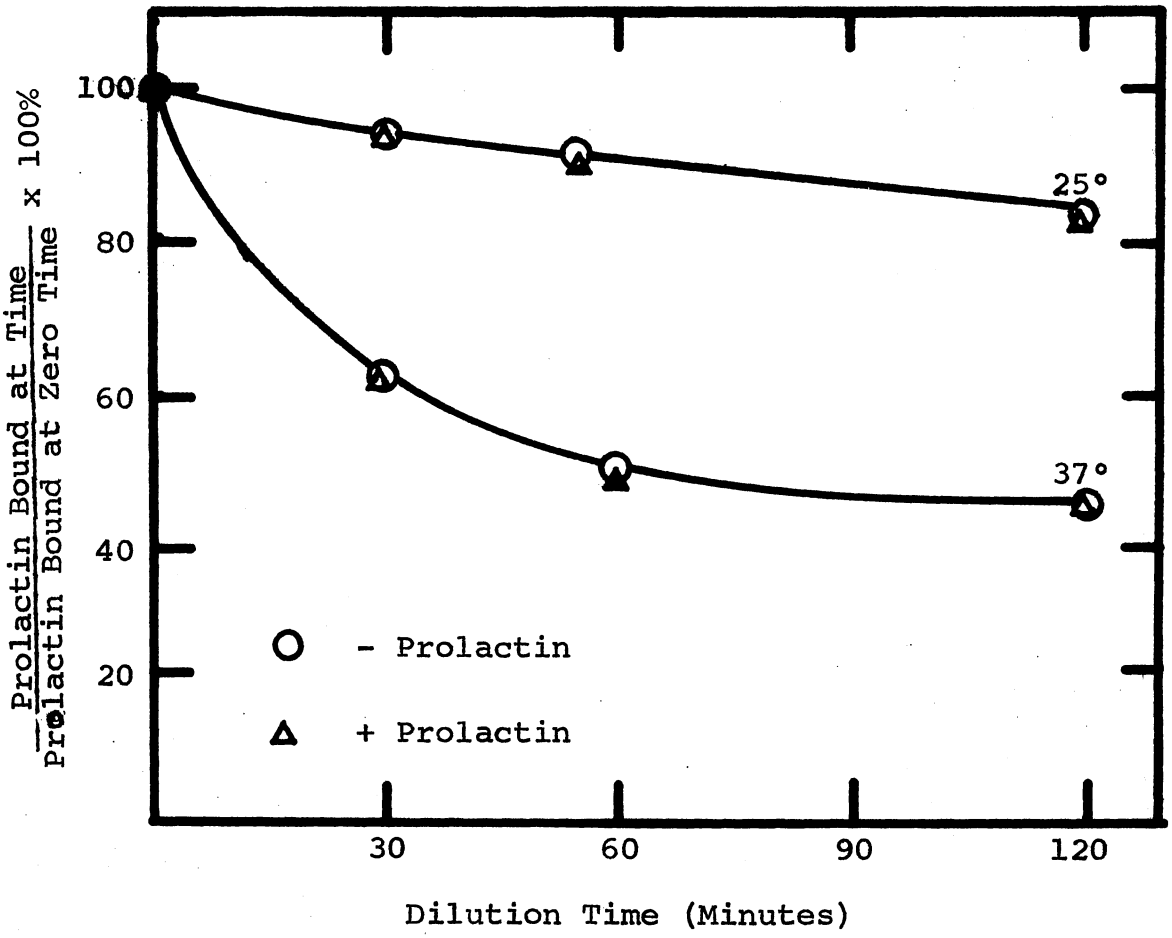

Figure 23. The Effect of 10^{-6} M Prolactin on the Rate of Dissociation of the Receptor-Prolactin Complex

Particulate receptor and 125-I-labeled prolactin were incubated together for four hours at 25° in the presence and absence of unlabeled prolactin as described in the text. Bound and free 125-I-labeled prolactin were separated by centrifugation and the pellets redissolved in 20 mM Tris, 10 mM magnesium chloride, 0.1% BSA, pH 7.4. At zero time, duplicate 0.1 ml aliquots were diluted into ten ml of buffer ± 10^{-6} M prolactin and incubated at 25° or 37°. At various times, tubes were removed from incubation and filtered on EHWP Millipore filters. Specifically bound prolactin remaining was determined by counting the Millipore filter.

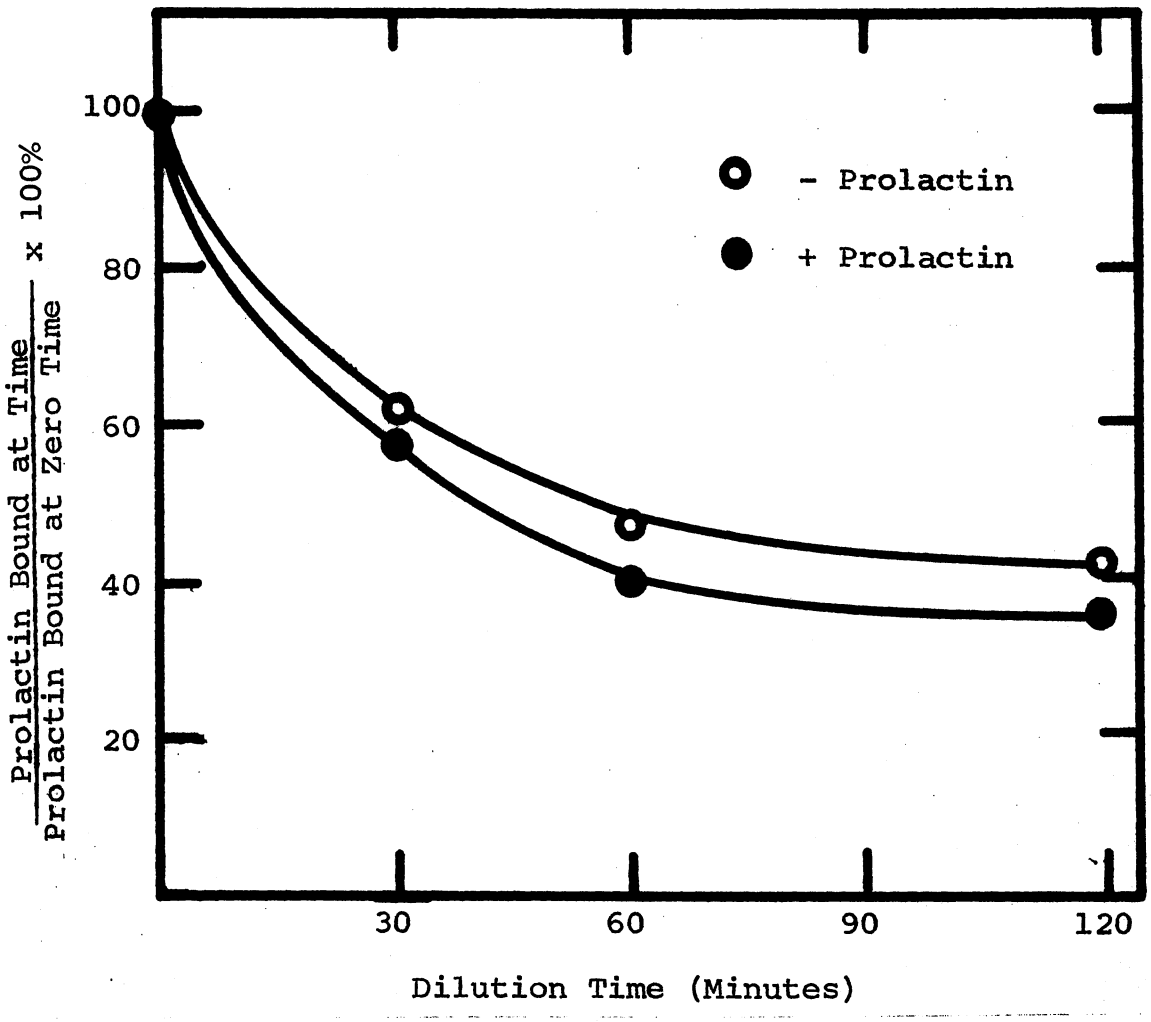

Figure 24. Effect of 10^{-6} M Prolactin on the Rate of Dissociation of the Prolactin-Prolactin Receptor Complex Using Freshly Prepared (Nonfrozen) Receptor

Freshly prepared particulate prolactin receptor and 125-I-labeled prolactin were incubated together for four hours at 25° in the presence and absence of unlabeled 125-I-labeled prolactin as described in the text. Bound and free 125-I-labeled prolactin were separated by centrifugation and the pellets redissolved in 20 mM Tris, 10 mM magnesium chloride, 0.1% BSA, pH 7.6. At zero time duplicate 0.1 ml aliquots were diluted into ten ml of buffer $\pm$ 10^{-6} M prolactin and incubated at 37°. At various times, tubes were removed from incubation and filtered on EHWP Millipore filters. The amount of complex remaining was determined by counting and specific binding determined.

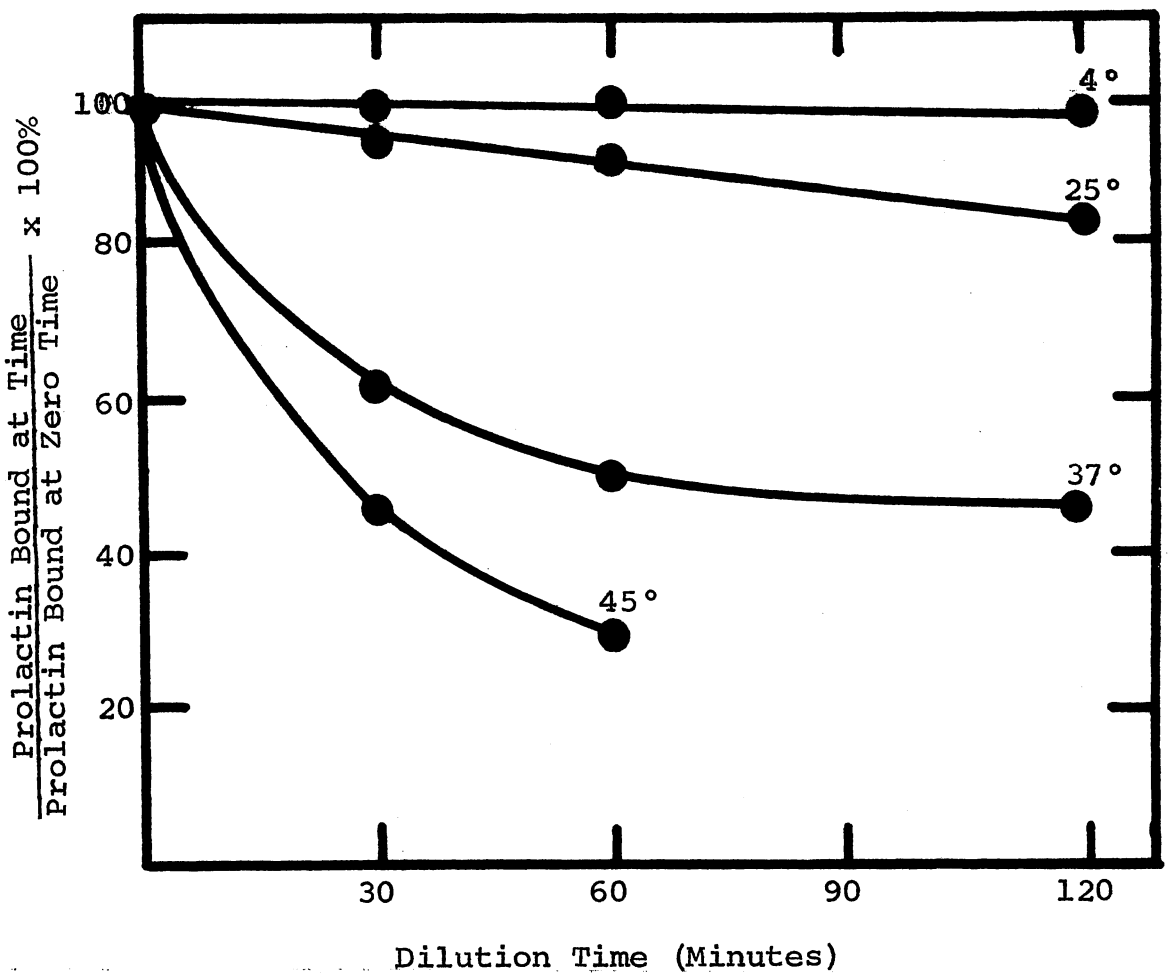

Figure 25. Effect of Varying the Temperature on the Rate of Dissociation of the Prolactin-Receptor Complex

Particulate receptor and 125-I-labeled prolactin were incubated together for four hours at 25° in the presence and absence of unlabeled prolactin as described in the text. Bound and free 125-I-labeled prolactin were separated by centrifugation and the pellets resuspended into 20 mM Tris, 10 mM magnesium chloride, 0.1% BSA, pH 7.6. At zero time, 0.1 ml aliquots were diluted into ten ml of buffer and incubated at 4°, 25°, 37°, or 45°. At various times, tubes were removed from incubation and filtered EHWP Millipore filters. Specifically bound 125-I-labeled prolactin remaining was determined by counting.

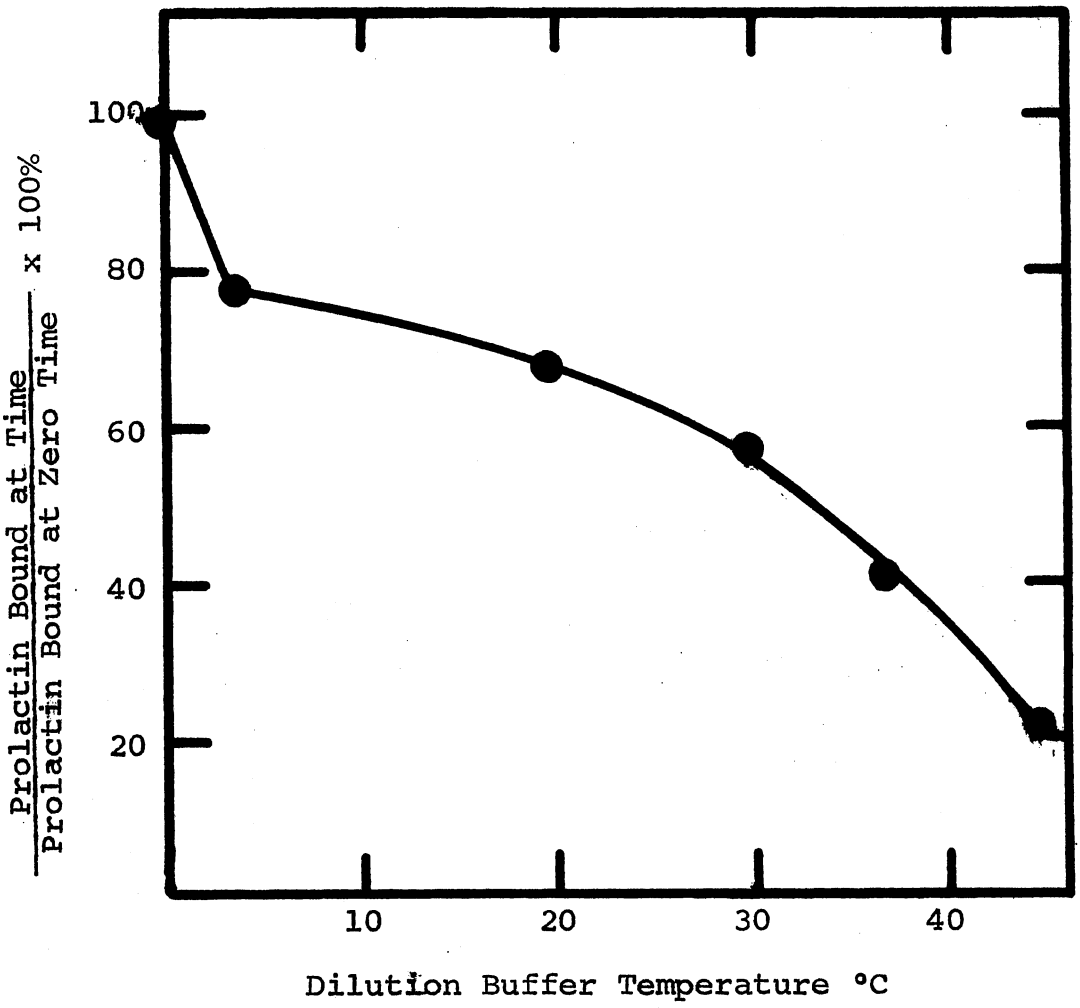

Figure 26. Effect of Temperature on the Dissociation of the Prolactin-Prolactin Receptor Complex

Particulate receptor and 125-I-labeled prolactin were incubated together for four hours at 25° in the presence and absence of unlabeled prolactin as described in the text. Bound and free 125-I-labeled prolactin were separated by centrifugation and the pellets resuspened in 20 mM Tris, 10 mM magnesium chloride, 0.1% BSA, pH 7.6. At zero time, duplicate 0.1 ml aliquots were diluted into 10 ml of buffer and incubated at 0°, 4°, 20°, 30°, 37°, and 45° for 30 minutes. At the end of the incubation time, the tubes were removed and filtered on EHWP Millipore filters. The amount of complex remaining was determined by counting and specific binding was determined.

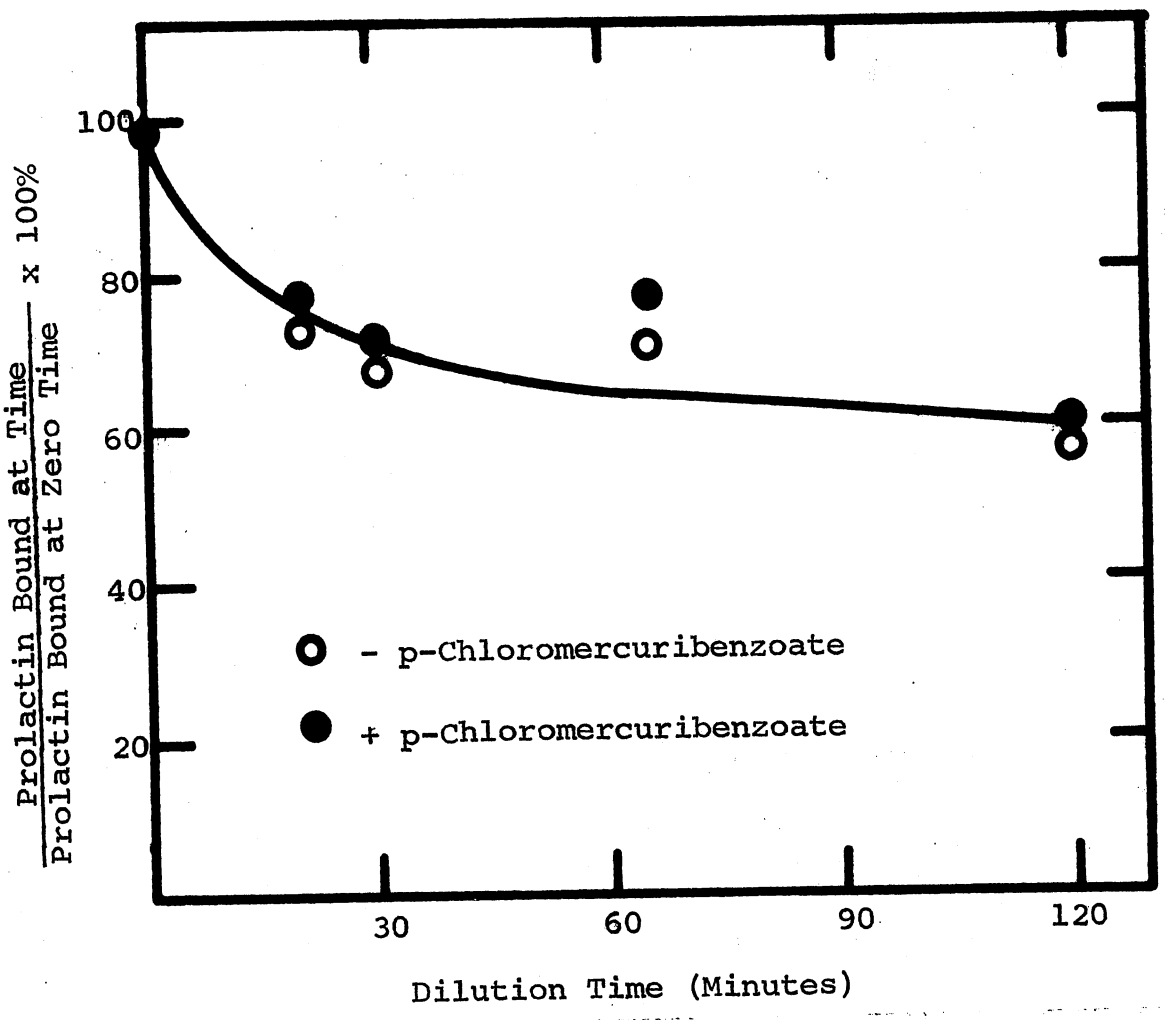

Figure 27. Effect of p-Chloromercuribenzoate on the Rate of Dissociation of the Prolactin-Prolactin Receptor Complex

Particulate receptor and 125-I-labeled prolactin were incubated together for four hours at 25° in the presence and absence of unlabeled prolactin as described in the text. Bound and free 125-I-labeled prolactin were separated by centrifugation and the pellet was resuspended in 20 mM Tris, 10 mM magnesium chloride, 0.1% BSA, pH 7.6. At zero time duplicate 0.1 ml aliquots were diluted into ten ml of buffer ± 10 mM p-chloromercuribenzoate and incubated at 37°. At various times, tubes were removed from incubation and filtered on EHWP Millipore filters. The filters were counted and specifically bound 125-I-labeled prolactin determined.

binding and thus, masking it at another site.

A variety of other agents were tested for their effect upon dissociation of the complex. Figure 28 shows the effect of these agents on dissociation. High concentrations of sodium chloride and magnesium chloride increase the rate of dissociation. At 2.0 M sodium chloride, there was a 30% decrease in the amount of hormone bound after a 30 minute incubation as compared to that bound at zero time. However, at 0.5 M sodium chloride, the complex was slightly more stable with 15% more complex remaining after 30 minutes. Treatment with magnesium chloride caused an increase in dissociation at all concentrations. AT 2.0 M magnesium chloride there was a 50% decrease in the amount of complex after a 30 minute incubation as compared to that bound at zero time.

The organic solvents dioxane, ethanol and acetone all had little effect on dissociation. At 30% (w/v) concentration in the dilution buffer, dioxane and ethanol caused only a slight increase in dissociation while treatment with acetone at the same concentration resulted in a slightly greater increase in the rate of dissociation (Figures 29 and 30).

When urea was added to the dilution buffer, the rate of dissociation increased linearly with urea concentration (Figure 30).

Glycerol stablized the complex (Figure 31). At 50% (v/v) glycerol in the dilution buffer, 35% more of the complex was remaining after the 30 minute incubation time.

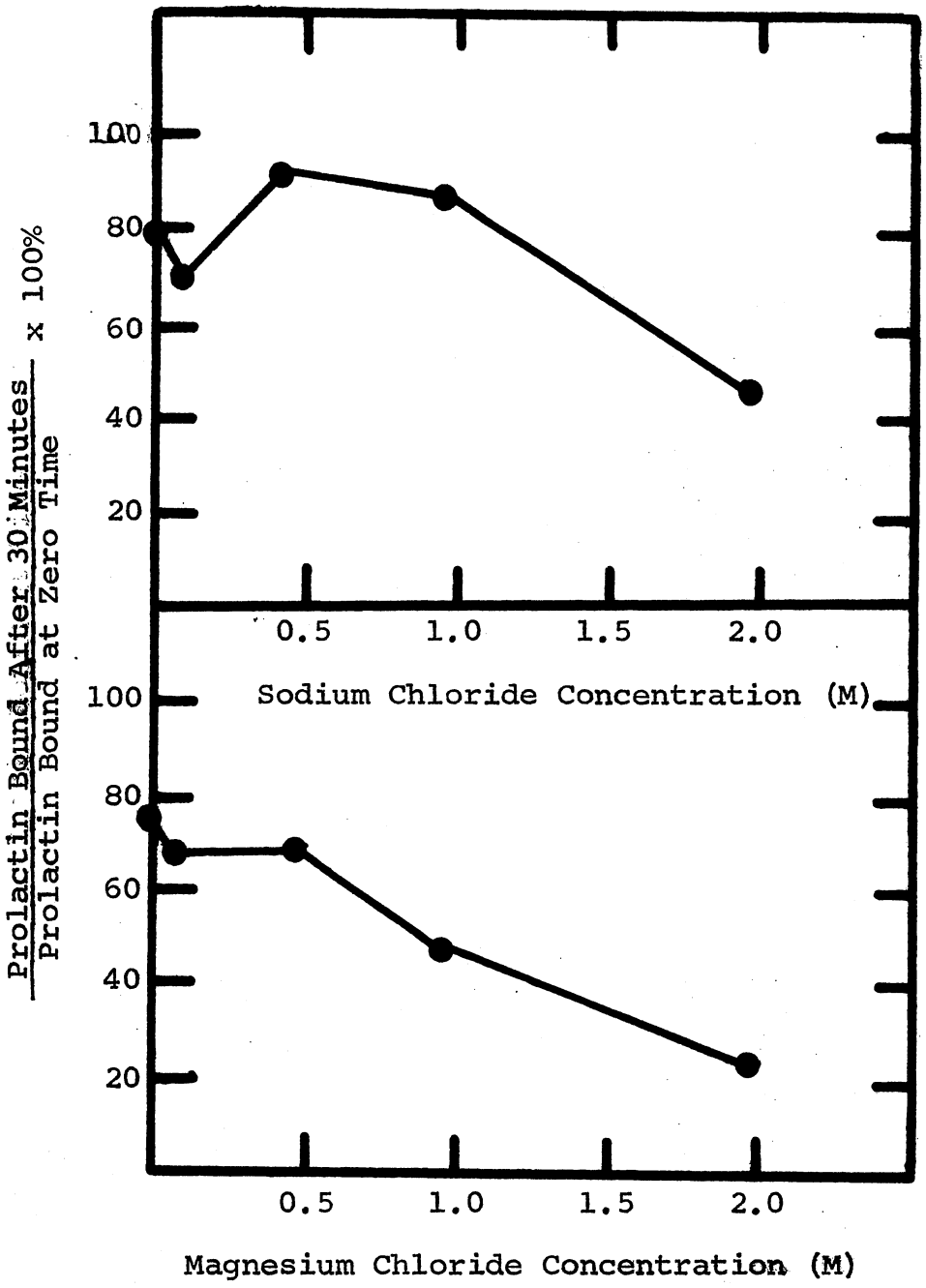

Figure 28. Effect of Magnesium Chloride and Sodium Chloride on the Rate of Dissociation of the Prolactin-Prolactin Receptor Complex

Experimental conditions were as described in the text. At zero time, duplicate 0.1 ml aliquiots were diluted into ten ml of buffer containing increasing concentrations of sodium chloride or magnesium chloride and incubated for 30 minutes at 37°. The tubes were then removed and filtered on Millipore EHWP filters. The filters were counted and specifically bound 125-I-labeled prolactin determined.

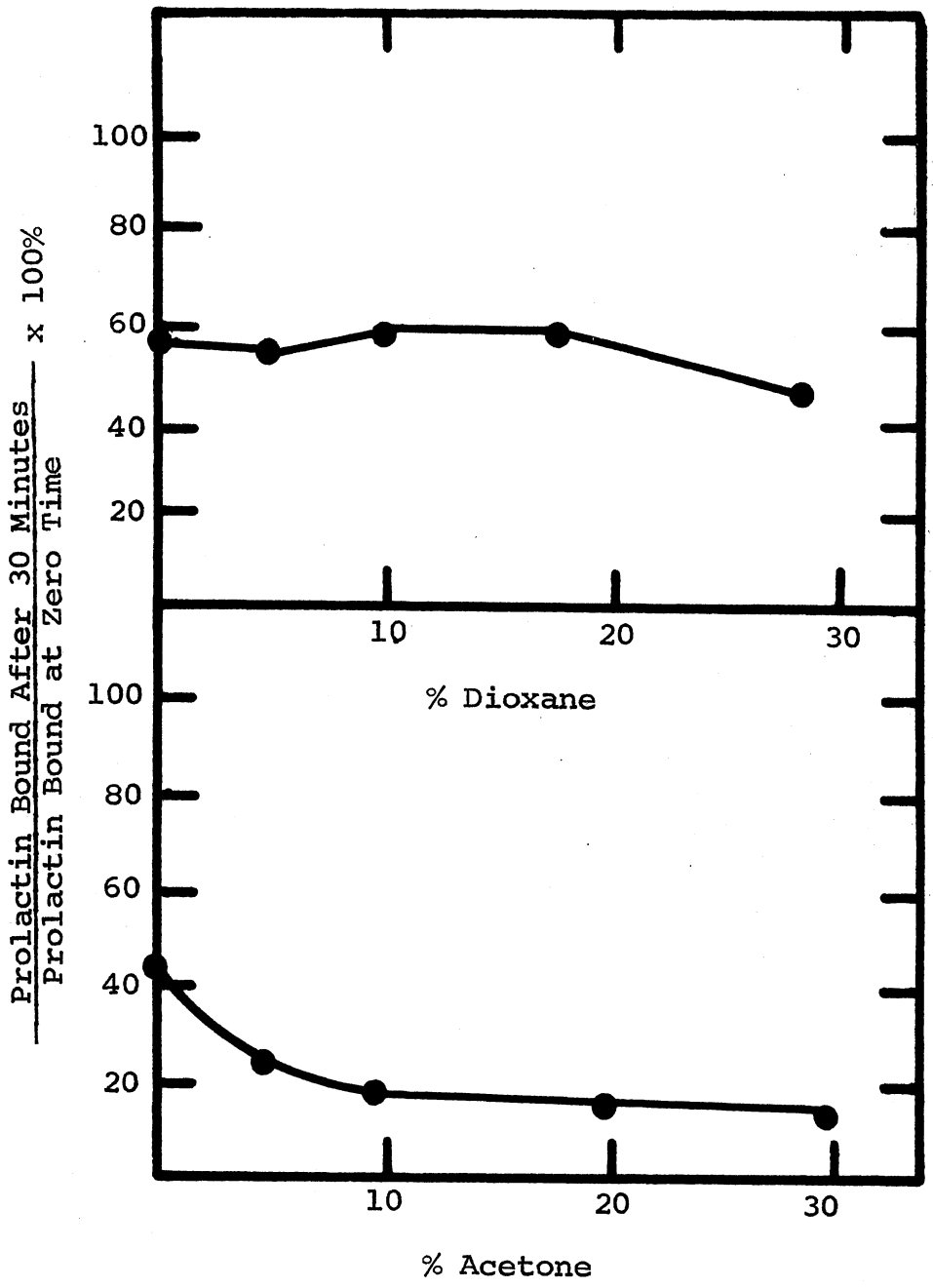

Figure 29. Effect of Dioxane and Acetone on the Rate of Dissociation of the Prolactin-Prolactin Receptor Complex

Experimental conditions were as described in the text. At zero time duplicate 0.1 ml aliquiots were diluted into ten ml of buffer containing increasing concentrations of dioxane or acetone and incubated at 37° for 30 minutes. At the end of the incubation, the tubes were removed and filtered on EHWP Millipore filters. The filters were counted and specifically bound 125-I-labeled prolactin determined

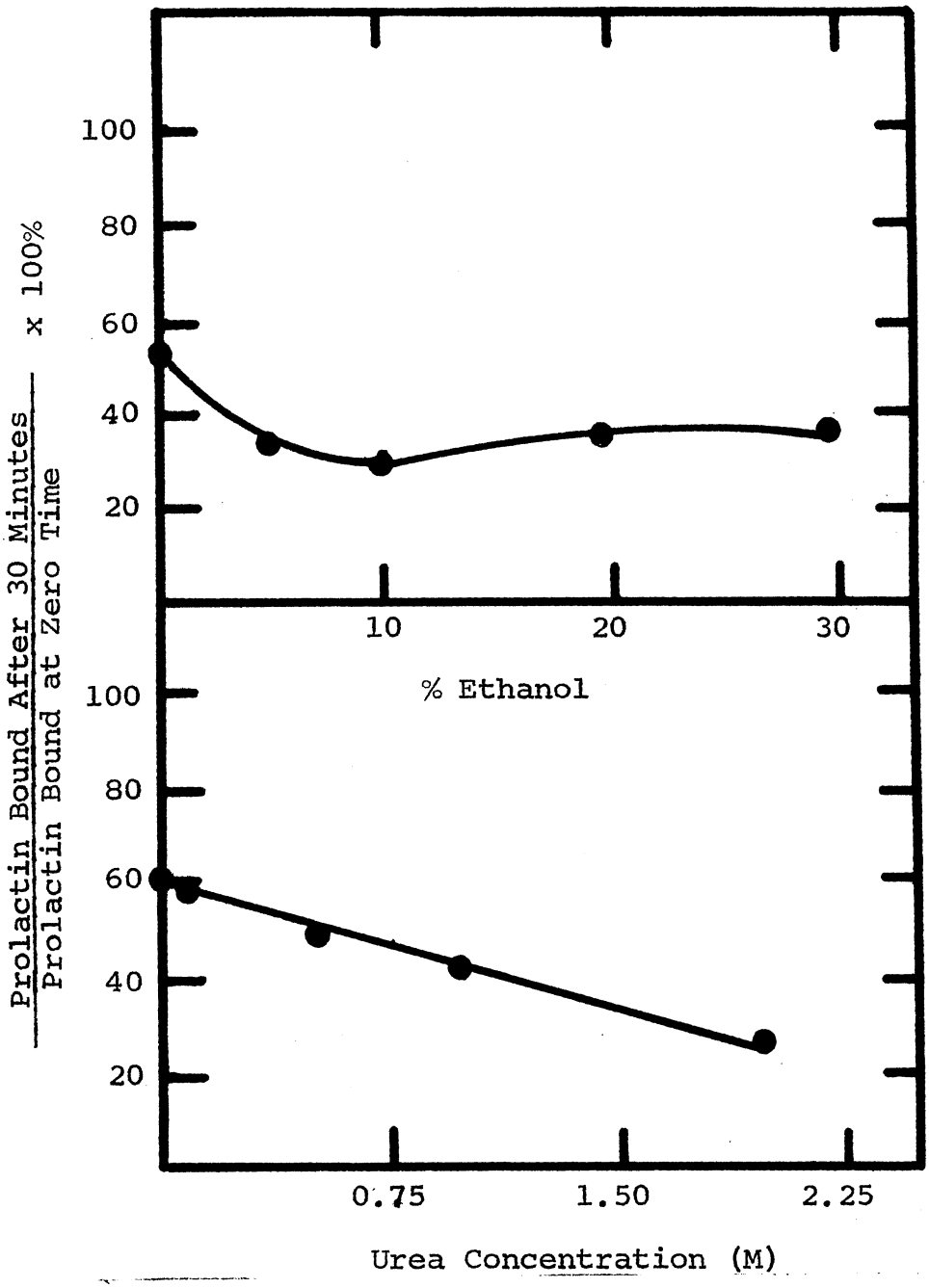

Figure 30. Effect of Ethanol and Urea on the Rate of Dissociation of the Prolactin-Prolactin Receptor Complex

Experimental conditions were as described in the text. At zero time duplicate 0.1 ml aliquiots were diluted into ten ml of buffer containing increasing concentrations of ethanol or urea and incubated for 30 minutes at 37°. The tubes were then removed and filtered on EHWP Millipore filters. The filters were counted and specifically bound 125-I-labeled prolactin determined.

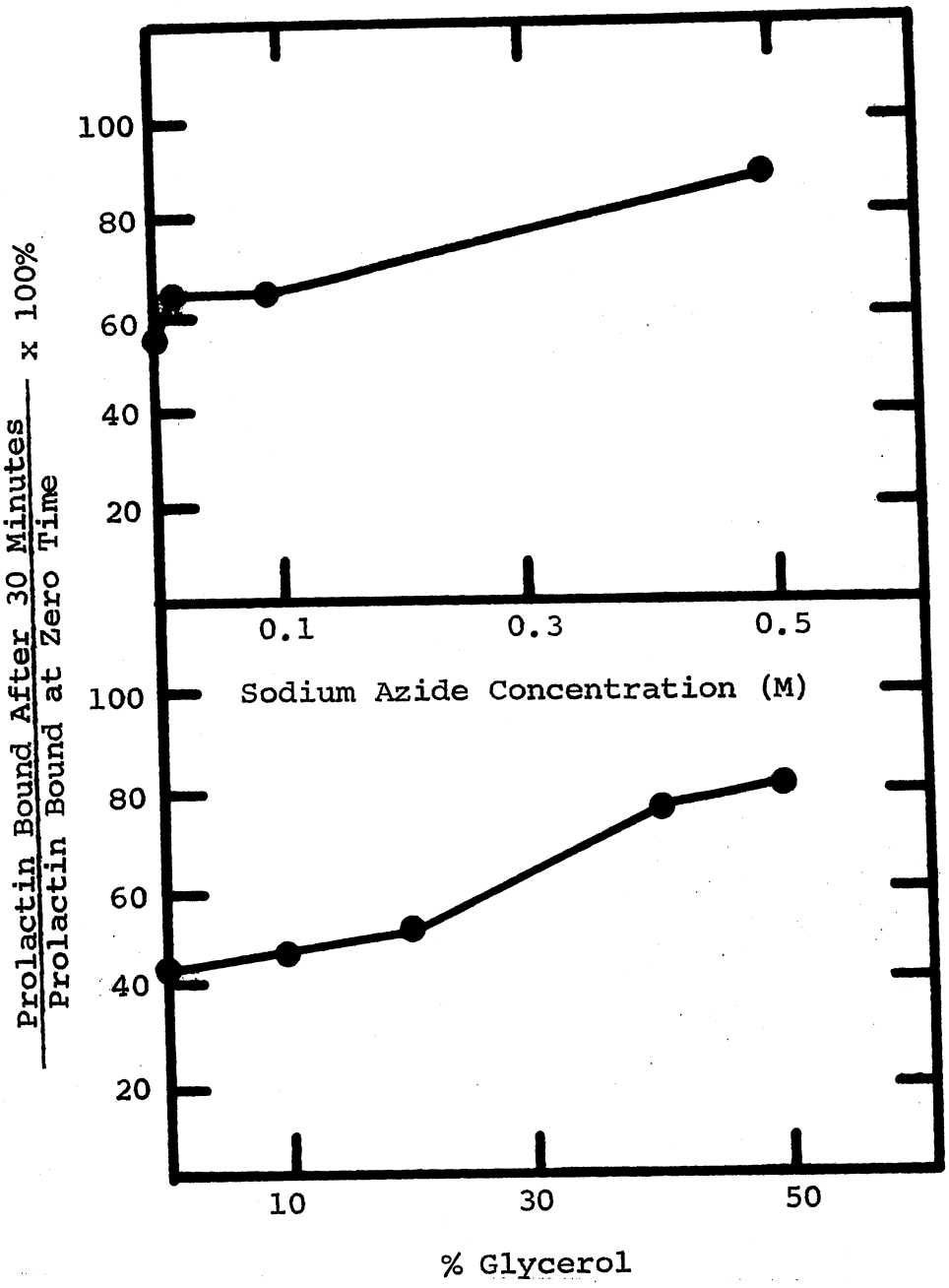

Figure 31. Effect of Sodium Azide and Glycerol on the Rate of Dissociation of the Prolactin-Prolactin Receptor Complex

Experimental conditions were as described in the text. At zero time duplicate 0.1 ml aliquiots were diluted into ten ml of buffer containing increasing concentrations of sodium azide or glycerol and incubated at 37° for 30 minutes. The tubes were then removed and filtered on EHWP Millipore filters. The filters were counted and specifically bound 125-I-labeled prolactin determined.

The effects of the various agents on the dissociation of the prolactin-receptor complex are summarized in Table IV. Also, included in Table IV are effects of 0.5 M mercaptoethanol and 0.5 M lithium chloride both of which increased the rate of dissociation.

From these results, it appears that the forces stablizing the complex are ionic in nature and not to any large degree hydrophobic, since the complex can be dissociated by salts and not by less polar organic solvents.

Radioreceptor Assay for Prolactin

Materials

Ovine prolactin (NIH-P-S11) was obtained from the National Institute of Arthritis and Metabolic Diseases Pituitary Hormone Distribution Program. Particulate rabbit prolactin receptor was prepared as described previously.

Methods

Ovine prolactin was dissolved in 50 mM sodium phosphate buffer, pH 8.0, and diluted to 100 µg/ml, 10 µg/ml, 1 µg/ml and 0.1 µg/ml by serial diltuion into 20 mM Tris, 0.1% bovine serum albumin, pH 7.6 buffer.

Particulate rabbit prolactin receptor (20 µl, 400 µg), 250 µl of the standard binding buffer, ovine prolactin (1 µl-100 µl, 1 ng-100 µg), and water to a total volume of 0.5 ml were mixed together and incubated for 30 minutes at 25° with shaking. After this initial incubation, 10^6 dpm of

TABLE IV

SUMMARY OF THE EFFECTS OF A VARIETY OF AGENTS ON THE DISSOCIATION OF THE PROLACTIN-PROLACTIN RECEPTOR COMPLEX

Treatment	Effect on Dissociation
2.0 M Sodium Chloride	increases
2.0 M Magnesium Chloride	increases
0.5 M Lithium Chloride	increases
0.5 M Mercaptoethanol	increases
2.0 M Urea	increases
pH 10	increases
37°	increases
4°	decreases
20% Dioxane	no effect
30% Ethanol	no effect
20% Acetone	no effect
50% Glycerol	decreases
0.02 M p-Chloromercuribenzoate	no effect
0.5 M Sodium Azide	decreases
10^{-6} M Prolactin	no effect

^{125}I-labeled prolactin were added to the assay tubes and incubation was allowed to continue for 4 hours. Bound and free ^{125}I-labeled prolactin were separated by centrifugation in a Beckman Microfuge B. The pellet was washed with 1 ml of a 1:1 solution of binding buffer and counted.

Results

A graph of percent ^{125}I-labeled prolactin bound versus nanograms of ovine prolactin in the assay is shown in Figure 32. Competition of unlabeled prolactin and ^{125}I-labeled prolactin for receptor sites was linear from 0 to 9 ng ovine prolactin. The minimum detectable level of ovine prolactin was 0.5 ng.

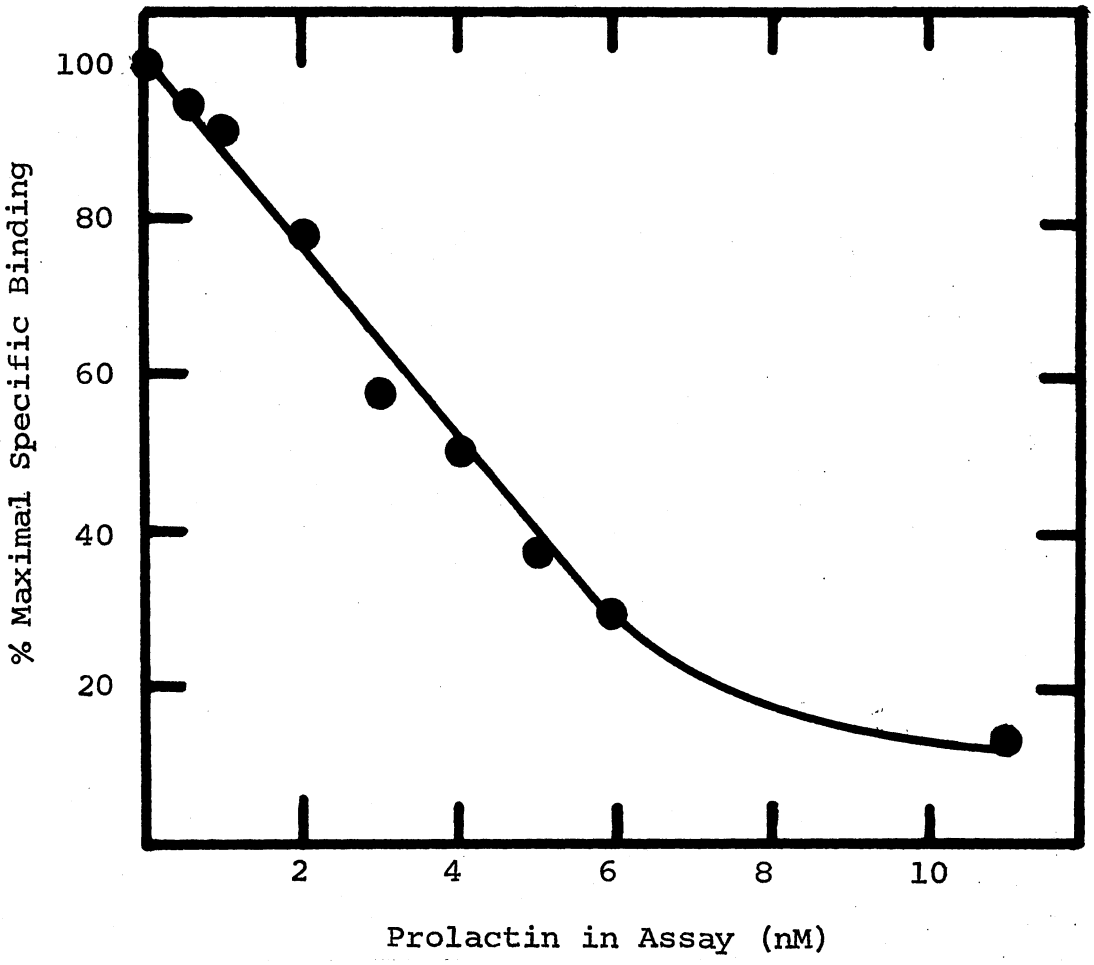

Figure 32. Radioreceptor Standard Curve for Ovine Prolactin

Particulate receptor (20 µl, 450 µg), 250 µl of the standard binding buffer, ovine prolactin (1-100 µl, 0.1 ng-100 µg), and water to a total volume of 0.5 ml were mixed together and incubated for 30 minutes at 25°. 100,000 dpm of 125-I-labeled prolactin were added to the assay tubes and incubation continued for four hours. Bound and free prolactin were separated by centrifugation. Specific binding of 125-I-labeled prolactin was assigned 100% and the competition of unlabeled prolactin was expressed as a percentage of this.

CHAPTER VI

PURIFICATION OF RABBIT MAMMARY GLAND PROLACTIN RECEPTOR

Gel Filtration

Materials

Sepharose 4B was purchased from Pharmacia. Agarose 0.5 m, Agarose 1.5 m, Bio-Gel P-100 and P-150 were purchased from Bio-Rad.

Methods

Gel filtration columns were poured and handled as described in technical manuals supplied by the producers (104,105). Prolactin receptor was solubilized using Triton X-100 as described previously.

Results

Soluble prolactin receptor was chromatographed on Agarose 1.5 m, Bio-Gel P-100, and Bio-Gel P-150. The column buffer was 20 mM Tris, 10 mM $MgCl_2$, 0.1% BSA, 0.1% Triton X-100 pH 7.6. The column material had previously been treated with 1% bovine serum albumin to mask non-specific protein absorption sites. Figure 33 shows the elution

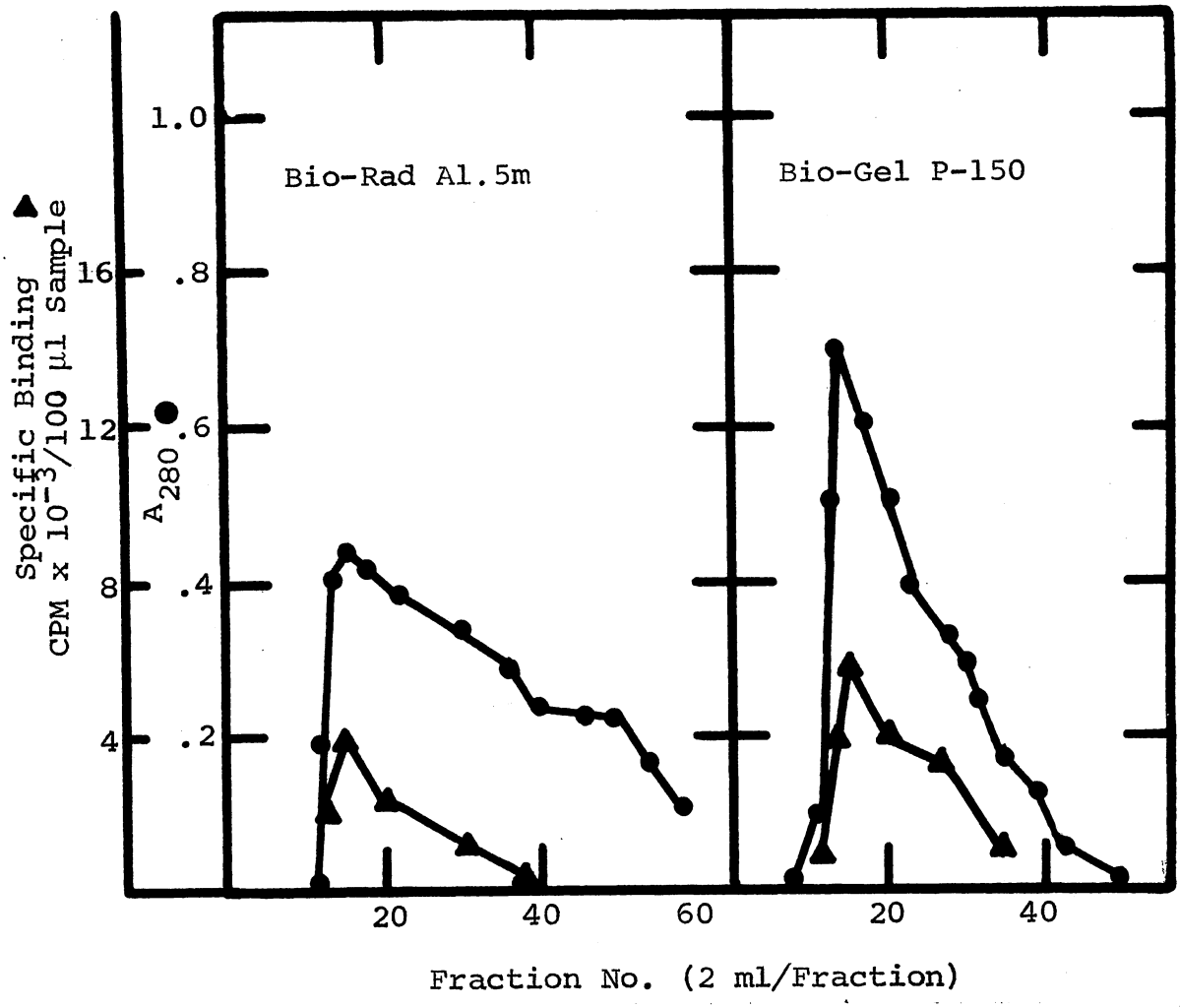

Figure 33. Elution Profile of Triton X-100 Solubilized Receptor on Bio-Rad A1.5m and Bio-Gel P-150

Two ml of Triton X-100 solubilized receptor was applied to a 0.7 cm x 120 cm Bio-Rad A1.5m chromatography column and a 0.7 cm x 110 cm Bio-Gel P-150 chromatography column, both equilibrated in 20 mM Tris, 0.1% Triton X-100, pH 7.6 buffer. Two ml fractions were collected and the protein profiles determined by monitoring at 280 nm. Fractions were assayed for prolactin receptor activity by the soluble binding assay (methods). Recovery of starting activity was approximately 33% upon chromatography on either column.

profile of the solubilized receptor on an Agarose 1.5 m column and a Bio-Gel P-150 column. Protein was determined by the method of Lowry, et al. (106). One severe handicap in attempting gel filtration in the presence of Triton X-100 is, due to the high molecular weight of the Triton X-100 micelle, approximately 100,000 (107), the pore size of the gel is effectively reduced. However, the soluble prolactin receptor did migrate ahead of bovine gammaglobulin, which has a molecular weight of 160,000, on an Agarose 1.5 m column. Recovery of activity was low (33%) perhaps due to the time required to elute the receptor (1-2 days).

Ion Exchange Chromatography

Materials

Carboxylmethylcellulose-32 and diethylaminoethyl-cellulose-32 were purchased from Whatman. Sephadex A-50 and C-50 were purchased from Pharmacia Fine Chemicals. Cellex-P and Dowex-50 were purchased from Bio-Rad.

Methods and Results

Procedures and methods recommended by the manufacturer of each resin were used (108,109). Before use, each column was saturated with bovine serum albumin in buffer of low ionic strength. Albumin bound to exchange sites was then removed by equilibration with the highest ionic strength buffer in the experiments.

The cation-exchange resin columns, 1.5 cm x 5 cm,

were equilibrated overnight with 20 mM Tris, 0.1% Triton X-100, pH 7.6. The columns were then equilibrated with 10 ml of a 1:1 dilution of Triton X-100 solubilized receptor and 40 mM Tris pH 7.6, resulting in a sample buffer of 20 mM Tris, 0.5% Triton X-100, 0.15 M sucrose pH 7.6. Normally, only a small amount of activity would elute with the wash (5-10%). Elution with buffer plus 50 mM sodium chloride, followed by buffer plus 0.2 M sodium chloride, then by buffer plus 0.5 M sodium chloride, and finally by buffer plus 1 M sodium chloride, resulted in only a fraction of the total activity being recovered (under 10%). All chromatographic procedures were at 4^{o}.

The anion exchange resins were in similar columns of 1.5 cm x 5 cm and equilibrated overnight with 20 mM sodium acetate, 0.1% Triton X-100, pH 6.0. The columns, both the Sephadex C-50 and Whatman CM-32, were loaded with a 1:1 dilution of Triton X-100 solubilized receptor and 40 mM sodium actate, pH 6.0, resulting in a sample buffer of 20 mM sodium acetate, 0.5% Triton X-100, 0.15 M sucrose, pH 6.0. Between 40-60% of the initial activity and 30-70% of the total protein was eluted in the wash. The remaining activity could not be eluted with salt concentrations up to 1 M sodium chloride.

Two other exchange resins were tried, the strong anion exchange, Cellex-P, and the strong cation exchanger, Dowex 50. Both resins were used under conditions described for the weak ion exchanger. The results were similar to those described for the weak anion and cation exchanger.

It should be noted that all the ion-exchange resins gave highly variable results. Results varied from essentially all the activity eluting with the wash to no activity being recovered in any of the wash or eluted fractions. One possible explanation was the variability in the receptor preparation since receptor was prepared from tissue that was fresh or had been frozen up to 2 months. Another possible explanation was that the presence of Triton X-100 interfered with the ability of the exchange resin to bind protein. Cuatrecasas (76) used a DEAE resin to partially purify insulin receptor and he stated that it was fortuitous that insulin would bind under the conditions described.

Purification by Affinity Chromatography on Wheat Germ Agglutinin-Sepharose and Concanavalin-A-Sepharose

Materials

Raw wheat germ was purchased from Sigma Chemical Company. Concanavalin-A was purchased from Sigma Chemical Company and Miles Laboratories, Inc.

Methods

Wheat germ agglutinin was purified from raw wheat germ by the method of DeVine, et al. (110).

Briefly, wheat germ was defatted by repeated extraction with petroleum ether (BP 40-60^{o}) in a soxhlet.

500 gm of defatted wheat germ were then extracted into 2 liters of 0.1 M sodium chloride with stirring, overnight, at 4°. The suspension was filtered through four layers of cheese cloth and then centrifuged at 10,000 x g for 30 minutes. The supernatant solution was made 70% in ammonium sulfate and allowed to precipitate overnight at 4°. The pellet was collected by centrifugation at 10,000 x g for one hour and redissolved in deionized water. The resuspended pellet was centrifuged at 20,000 x g for 30 minutes to remove any insoluble material. The pH of the supernatant solution was adjusted to 7.0 by addition of solid Tris. This solution (200 ml) was then applied to a 2 cm x 30 cm ovomucoid-Sepharose affinity column that was equilibrated with 0.1 M sodium chloride. After washing with 0.1 M sodium chloride, the bound wheat germ agglutinin was eluted by 0.1 M acetic acid. Yields or purity were not determined; but from 500 gm of defatted wheat germ, 30-60 mg of protein bound to and could be eluted from an ovomucoid-Sepharose column. Concanavalin-A and wheat germ agglutinin affinity gels were synthesized by modification of the method of Cuatrecasas (50). The ligand support was either Affi-Gel-10 or Sepharose with a diaminodipropylaminosuccinyl extention arm (synthesis described later). Water-soluble Concanavalin-A was dissolved in 0.1 M sodium bicarbonate, 0.1 M α-methyl-mannoside, 0.1 M magnesium chloride, pH 8.0 to a final concentration of 5 mg/ml. Wheat germ agglutinin was dissolved in 0.1 M sodium phosphate, pH 7.2, 0.1 M N-aceytl-glucosamine.

Wheat germ agglutinin is relatively insoluble at neutral or basic pH and not all of the lectin was in solution. Ten ml of the Concanavalin-A or 10 ml of the wheat germ agglutinin solution were reacted with 0.5 gm of Affi-Gel 10 or 0.5 gm of the N-succinimide ester of Sepharose-diaminodipropylaminosuccinate at 4° with gently shaking for 24 hours. Glycine (0.5 gm) was added and the mixture was held at room temperature for 4 hours. The gels (7-8 ml) were then poured into chromatography columns and washed at room temperature for 24 hours. The Concanavalin-A columns were washed with 0.1 M Tris, 10 mM calcium chloride, 10 mM magnesium chloride, 0.1% Triton X-100, ph 7.8. A samll amount of insoluble calcium carbonate formed which was removed from the top of the column. The wheat germ agglutinin columns were washed with 0.1 M Tris, pH 7.4, 0.1% Triton X-100. Ligand concentrations on the gels were estimated by hydrolyzing aliquots of the gels in 7 N sodium hydroxide at 80° for 4 hours and then determining Lowry protein. The Concanavalin-A columns contained between 2-4 mg Concanavalin-A per ml of Sepharose. The wheat germ agglutinin columns contained between 0.3-0.5 mg wheat germ agglutinin per ml of Sepharose.

Results

Small affinity columns (1-2 ml) were poured containing either Concanavalin-A-Sepharose or wheat germ agglutinin-Sepharose and equilibrated with either 0.1 M Tris, 10 mM

calcium chloride, 10 mM magnesium chloride, 0.1% Triton X-100, pH 7.8 (Concanavalin-A) or 0.1 M Tris, 0.1% Triton X-100, pH 7.4, (wheat germ agglutinin). Triton X-100 solubilized receptor (2 ml) was diluted with 2 ml of the corresponding equilibration buffers and applied to the two affinity columns at room temperature. The flow rate was drastically reduced. Elution was by addition of either 0.3 M α-methylmannoside or 0.3 M N-acetyglucosamine to the column buffers of the Concanavalin-A or wheat germ agglutinin columns, respectively. Once equilibrated with the elution buffer, flow was stopped for 30 minutes after which fraction collection was resumed.

The wheat germ agglutinin-Sepharose column did not bind the receptor. Results from chromatography on Concanavalin-A-Sepharose were highly variable. In a few instances, receptor appeared to bind to the resin and could be eluted with 0.3 M α-methylmannoside. The fold purification in these instances was approximately 60 with an 80% recovery of activity. However, in most attempts, Concanavalin-A-Sepharose failed to bind receptor. New Concanavalin-A columns were synthesized containing 0.1 mg and 10 mg Concanavalin-A per ml of Sepharose. Varying ligand concentration did not improve the reproducibility of the results. Twice, Concanavalin-A-Sepharose Chromatography was attempted, substituting 10 mM octyl-glucoside for 0.1% Triton X-100; but no receptor activity bound. The reasons for the irreproducibility of these results was not determined.

Hydrophobic Chromatography

Materials

Tyrosine, norleucine, leucine, alanine, tryptophan were purchased from the Sigma Chemical Company (1,4). Diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, cyanogen bromide, butylamine, hexylamine, and octylamine were purchased from the Aldrich Chemical Company. Sepharose 4B was purchased from Pharmacia Fine Chemicals.

Methods

Sepharose was activated with cyanogen bromide by the buffer method of March, et al. (111). Sepharose 4B was washed with ten volumes of deionized water and dryed by filtration on a vacuum funnel to a moist cake. The Sepharose 4B was then diluted with an equal volume of deionized water. Two volumes of 2 M sodium carbonate were added to the Sepharose 4B slurry. The mixture was stirred rapidly on a magnetic stirrer and 0.1 volume of cyanogen bromide in acetonitrile (2 gm cyanogen bromide per ml of acetonitrile) was added. After reacting for two minutes, the mixture was filtered quickly by suction through Whatman No. 54 filter paper, washed with ten volumes of 0.1 M sodium bicarbonate, pH 9.5, ten volumes of deionized water, and ten volumes of the buffer in which the ligand was dissolved. The moist Sepharose cake was then transfered to the solution containing the ligand (which was cooled to 4^{o}) and allowed

to react for 24 hours at 4° with minimal magnetic stirring. The buffer used to couple the diamino compounds and the monoamine compounds was 0.1 M sodium bicarbonate, pH 9.5. The concentration of the ligands was 2 M in the coupling reaction and the volume of the coupling solution was twice that of the Sepharose 4B. Since the free base form of the amino compounds was used, the simplest method to adjust the solution to pH 9.5 was to first adjust the undiluted diamine to pH 9.5 with concentrated hydrochloric acid, add sodium bicarbonate to 0.1 M, readjust the pH, and bring the solution to volume with deionized water or 0.1 M sodium bicarbonate.

The buffer used to couple tyrosine and tryptophan to Sepharose 4B was also 0.1 M sodium bicarbonate, pH 9.5. Two volumes of either 0.2 M tryosine or 0.2 M tryptophan were used in the coupling reaction.

The buffer used to couple leucine or norleucine to Sepharose 4B was a 1:1 dilution of 0.2 M sodium carbonate, pH 9.5, and dioxane. Two volumes of 0.2 M leucine or 0.2 M norleucine were used in the coupling reaction. Neither leucine nor norleucine were completely soluble in the coupling buffer.

Sepharose-diaminoalkyl and Sepharose-aminoalkyl chromatography were performed as described by Shartiel, et al. (112,113). Hydrophobic chromatography on Sepharose-amino acid gel was performed as described previously (114).

Results

The order of increasing hydrophobicity of amino acid-Sepharose gels is leucine, norleucine, tyrosine, and tryptophan. This type of hydrophobic chromatography has also been called phosphate-induced hydrophobic chromatography (114) since the sample to be chromatographed is made 1-2 M in phosphate or sulfate.

When 5 ml of soluble receptor (approximately 100 mg of Lowry protein) in 1% Triton X-100, 0.3 M sucrose, were made 1.5 M in sodium phosphate pH 7.4 by a 1:1 dilute with 3 M sodium phosphate, pH 7.4, or 1.5 ammonium sulfate, pH 7.4, small droplets formed, which were probably Triton X-100. This resulted in the solution becoming turbid. When this solution was applied directly to any of the amino acid-Sepharose columns (2 cm x 15 cm column equilbrated with either 1.5 M sodium phosphate, pH 7.4, or 20 mM Tris, 1.5 M ammonium sulfate, pH 7.4), there was no distinct peak of material in the wash but rather there was a long trail of turbid material. The material in the eluted fractions was very dilute, but activity (10-40%) could be detected. The tops of the columns had a layer of precipatated material. Upon elution with 20 mM Tris, 0.1% Triton X-100, more activity could be detected in the eluted fractions (10-20% but with no purification. If the sample were centrifuged at 100,000 x g for 1 hour before coupling to the columns, a small pellet at the bottom of the centrifuge tube and a thin low density layer floating on the surface were formed. Approximately 20% of the original activity was found in the

supernatant solution and another 40% of the original activity was found in the combined pellet and lipid layer (redissolved in 20 mM Tris, 0.1% Triton X-100, pH 7.6). It appeared, therefore, that the prolactin receptor, when in Triton X-100, was not soluble in high enough concentrations of phosphate or sulfate ions to allow chromatography on amino acid-Sepharose columns. The lowest concentration of phosphate or sulfate in which the receptor remained soluble was 0.75 M. At this concentration, the receptor and other proteins would not bind to the columns. Other salts which proved ineffective were 0.1 M sodium pyrophosphate and 2 M sodium chloride.

Hydrophobic diaminoalkyl-Sepharose resins proved more effective. Initially, chromatography on these columns was performed by placing a 1:1 dilution of the sample in 0.3 M sucrose, 1% Triton X-100 with 40 mM Tris, pH 7.6. The columns were then washed with 20 mM Tris, 0.1% Triton X-100 until the A_{280} material was zero. Elution was by an increasing gradient of 20 mM Tris 0.1% Triton X-100, pH 7.6 to 20 mM Tris, 1.0 M NaCl, 0.1% Triton X-100, pH 7.6. Subsequently, it was found that prolonged exposure to Triton X-100 masked the hydrophobic sites on these columns, resulting in a drastic loss of their ability to bind protein. When Triton X-100 was present in the chromatography buffers or the sample, the diaminoalkyl columns could be used only once or twice. Washing the resin with 0.1 M sodium bicarbonate, 2 M sodium chloride, pH 9.5, deionized water, or 0.1 M

sodium bicarbonate, 2 M sodium chloride, pH 9.5, buffer saturated with chloroform would not restore capacity to the gel.

This problem was eliminated by solubilizing the receptor with the nonionic detergent, octyl-glucoside (synthesis of octyl-glucoside and solubilization of prolactin receptor is described in Chapter III). Using receptor solubilized with Triton X-100 and this detergent, three diaminoalkyl columns, C_4-diamino-Sepharose, were tested for their ability to bind prolactin receptor. A 1.5 cm x 15 cm column of each of the diamino-alkyl gels was prepared and equilbrated with 20 mM Tris, pH 7.4. Five ml of the octyl-glucoside solubilized receptor or five ml of Triton X-100 solubilized receptor were diluted 1:1 with 40 mM Tris, pH 7.4, and applied to each column. Elution was by 20 mM Tris, 1 M sodium chloride, pH 7.4.

The C_4-diamine-Sepharose (Figure 34) failed to bind a significant percentage of the prolactin receptor (less than 10%). The C_6-diamine-Sepharose column (Figure 34) bound 80-90% of the total receptor activity but activity could not be eluted with 1 M sodium chloride. The C_5-diamine-Sepharose columns bound 70% of the total activity. This activity could be eluted with 1 M sodium chloride. The failure of activity to elute from the C_6-diamine-Sepharose could be for at least two reasons: 1) The interaction of receptor with C_6-diamine may be, for all practical purposes, irreversible, or 2) the lack of the detergent in the elution

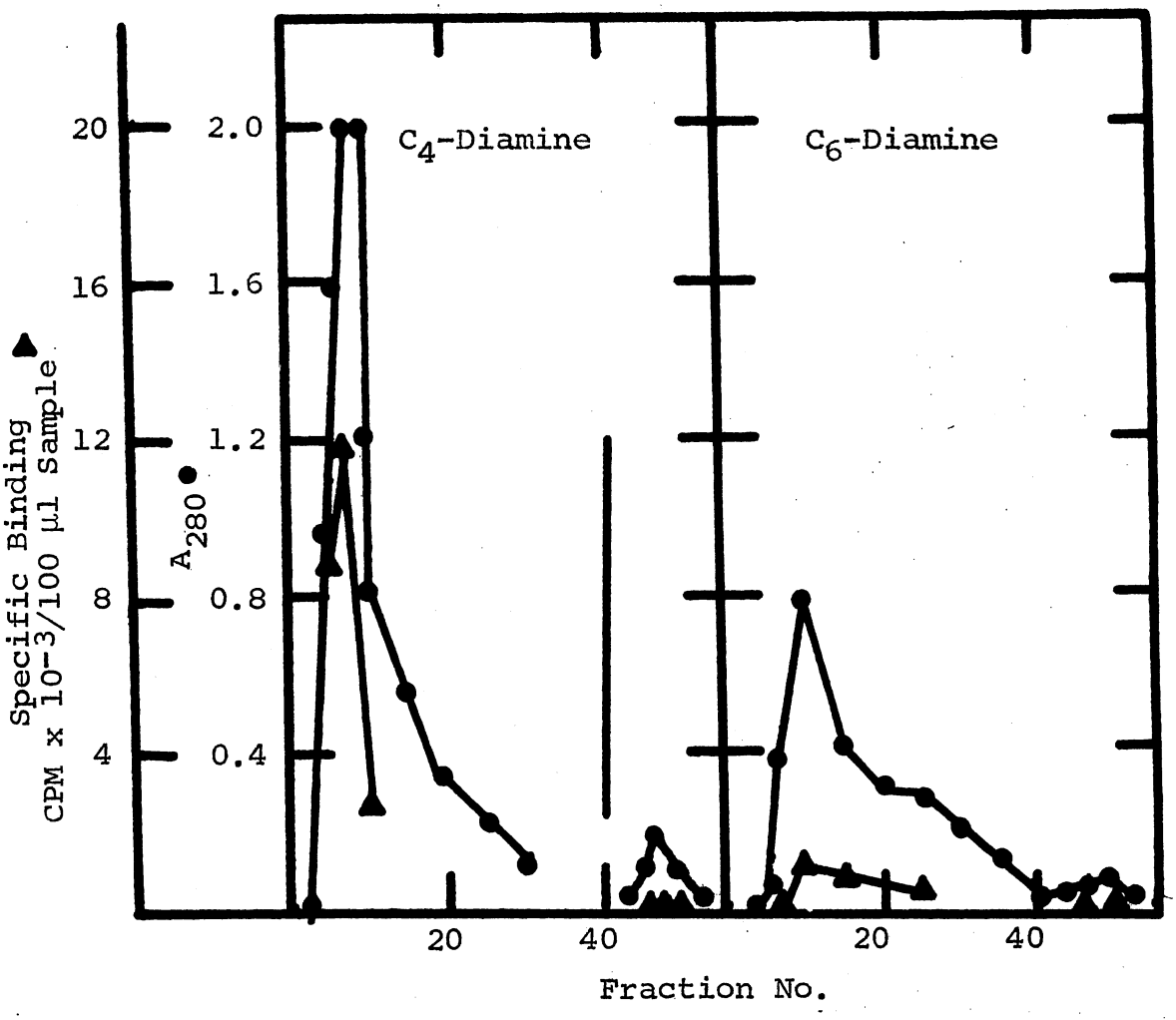

Figure 34. Chromatography of Triton X-100 Solubilized Receptor on C_4-diamine-Sepharose and C_6-diamine-Sepharose

Five ml of soluble receptor (diluted 1:1 with 40 mM Tris, pH 7.4) were applied to a 1.5 cm x 15 cm C_4-diamine-Sepharose column and to a 1.5 cm x 15 cm C_6-diamine-Sepharose column. The columns were washed with 20 mM Tris, pH 7.4. Five ml fractions were collected. Elution was by 20 mM Tris, 1 M sodium chloride, pH 7.4. 100 µl fractions were assayed for prolactin receptor activity. 84% of the initial activity was recovered in the load and wash fraction of the C_4-diamine-Sepharose column. 15% of the initial activity was recovered in the load and wash fractions of the C_6-diamine-Sepharose column. Essentially no activity was recovered upon elution with 20 mM Tris, 1 M sodium chloride, pH 7.4. Also, 2 M sodium chloride failed to elute activity from the C_6-diamine-Sepharose column.

buffer may have resulted in precipitation of the receptor upon elution.

Since the C_5-diamine-Sepharose column bound the prolactin receptor and activity could be recovered by elution, a larger sample (20 ml of octyl-glucoside solubilized receptor diluted 1:1 with 40 mM Tris, pH 7.4) was applied to the column. Elution was by an increasing gradient of 500 ml 20 mM Tris, pH 7.4, and 500 ml Tris 0.5 M sodium chloride, pH 7.4. Figure 35 shows the column profile. While not proven, speculation was that octyl-glucoside co-elutes with the receptor activity thus maintaining the solubility of the prolactin receptor. All chromatography steps were performed at 4°.

p-Chlormercuribenzoate-Sepharose

Materials

p-Chloromercuribenzoate and N,N-dimethylformamide were purchased from the Sigma Chemical Company. 1,4-Diaminobutane was purchased from the Aldrich Chemical Company. Sepharose was purchased from Pharmacia Fine Chemicals. 1-Ethyl-3-(dimethylaminopropyl)carbodiimide was purchased from Bio-Rad or the Pierce Chemical Company.

Methods

p-Chloromercuribenzoate-Sepharose was synthesized as described by Ruiz-Carrillo (115). Sepharose 4B, 250 ml, was activated and then reacted with 250 ml of 12%

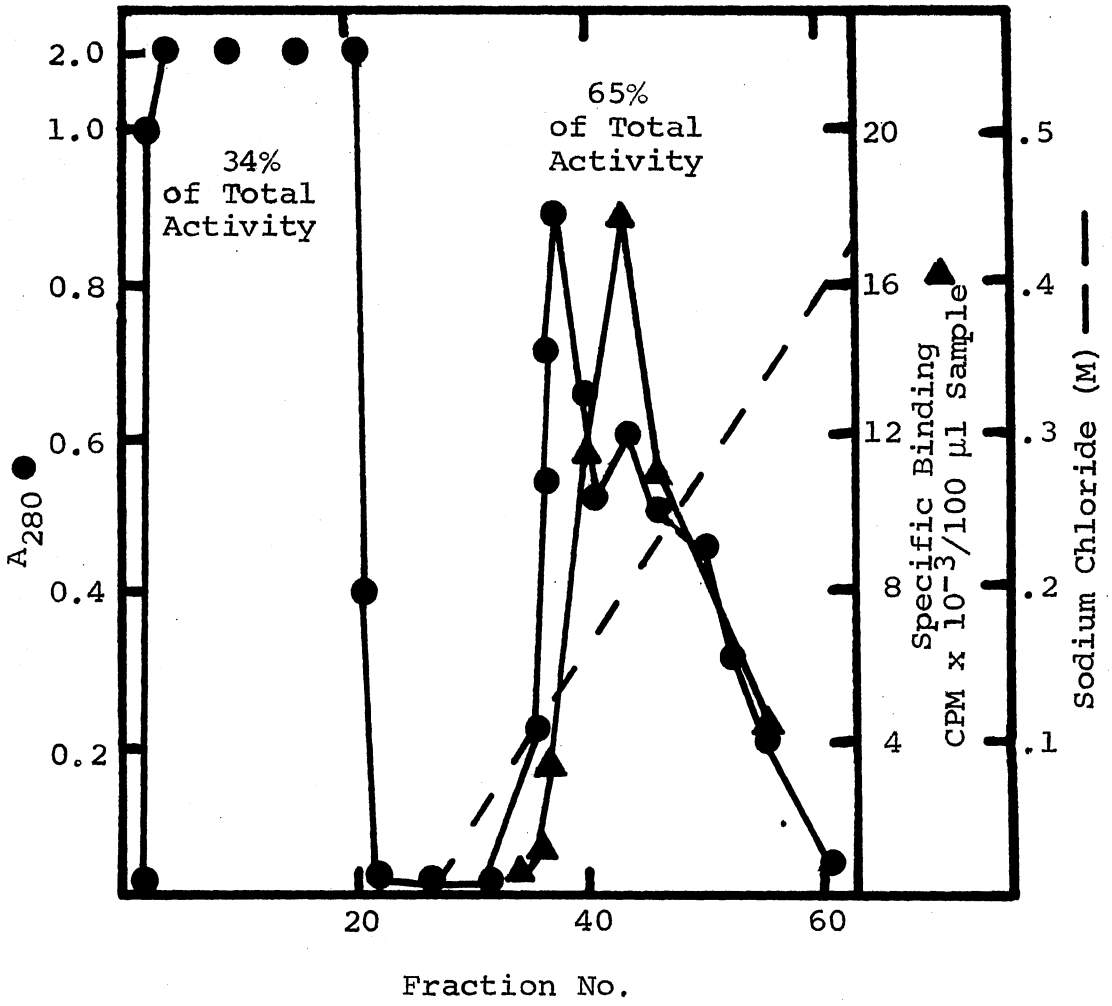

Figure 35. Chromatography of Octyl-Glucoside Solublilized Receptor on C_5-Diamine-Sepharose

Twenty ml of octyl-glucoside solubilized prolactin receptor were diluted 1:1 with 40 mM Tris, pH 7.4 buffer and applied to a 2 cm x 20 cm C_5-diamine-Sepharose column that was equilibrated in 20 mM Tris, pH 7.4. Five ml fractions were collected. Elution was by an increasing gradient of 500 ml of 20 mM Tris, pH 7.4 and 500 ml of 20 mM Tris, 0.5 M sodium chloride, pH 7.4. The wash and load fractions were pooled and assayed; 100 µl aliquots of the elution fractions were assayed. The prolactin receptor was purified five fold.

1,4-diaminobutane at pH 9.5 with gentle stirring for 24 hours at 4°. The substituted gel was then washed with 4 liters of deionized water; 4 liters of 0.1 M sodium acetate, 1 M sodium chloride pH 5.0; 4 liters of 0.1 M sodium bicarbonate, 1 M sodium chloride pH 9.5; and another 4 liters of deionized water. Qualitatively, the Sepharose and wash were checked for free amines by the 2,4,6-trinitrobenzenesulfonate test (111). To the washed amino-Sepharose, 200 ml of 40% (v/v) N,N-dimethylformamide and 6.25 gm of sodium p-chloromercuribenzoate were added with stirring. The pH was adjusted to 4.8 with concentrated hydrochloric acid, and 7.7 gm of 1-ethyl-3-(dimethylaminopropyl)carbodiimide were added. p-Chloromercuribenzoate is not readily soluble at this pH. The mixture was stirred for 24 hours at room temperature. The pH of the mixture was then raised with concentrated sodium hydroxide until all the p-chloromercuribenzoate was in solution. The solution was quickly filtered through a Buchner funnel and washed with 4 liters of deionized water, 4 liters of 0.1 M sodium bicarbonate, 1 M sodium chloride pH 9.0, and 4 liters of deionized water. The resultant derivative no longer gave an orange color by the 2,4,6-trinitrobenzenesulfonate test, indicating that no free amino groups were left. The reported affinity of p-cloromercuribenzoate-Sepharose for cysteine is 1.2-2.5 moles per ml of packed resin (115).

The resin was prepared for chromatography by pouring a 2 cm x 25 cm column and equilibrating with 500 ml of 50

mM sodium acetate, 20 mM EDTA, 10 mM mercuric chloride, pH 4.8, 4°. Excess mercuric chloride was removed by washing with 500 ml of 0.1 M sodium phosphate, 0.2 M sodium chloride, 1 mM EDTA ph 6.0. The column was then equilibrated with approximately 1 liter of 20 mM Tris, 0.1% Triton X-100, pH 7.4 overnight.

Soluble receptor (either Triton X-100 solubilized or octyl-glucoside solubilized) or partially purified receptor off C_5-diamine was then applied to the column. The column was washed with 20 mM Tris until the absorbance at 280 mM was zero. The PCMB column was eluted by 20mM Tris, 0.1 M cysteine, 0.1% Triton X-100, pH 7.4 buffer.

Results

Chromatography of the soluble receptor on p-chloromercuribenzoate-Sepharose resulted in a 10-fold purification over soluble receptor or a 3-4 fold purification compared to C_5-diamine-Sepharose purified receptor when material off the C_5-diamine was chromatographed (Figure 36). Recovery of activity ranged from a low of 40% to a high of 90% with the average recovery of about 65-70%.

Ovine Prolactin-Sepharose

Materials

Cyanogen bromide, 1,4-diaminobutane, and 3,3-dithiopropionic acid were purchased from Aldrich Chemical Company. Sodium meta-periodate, sodium borohydride, and diaminodi-

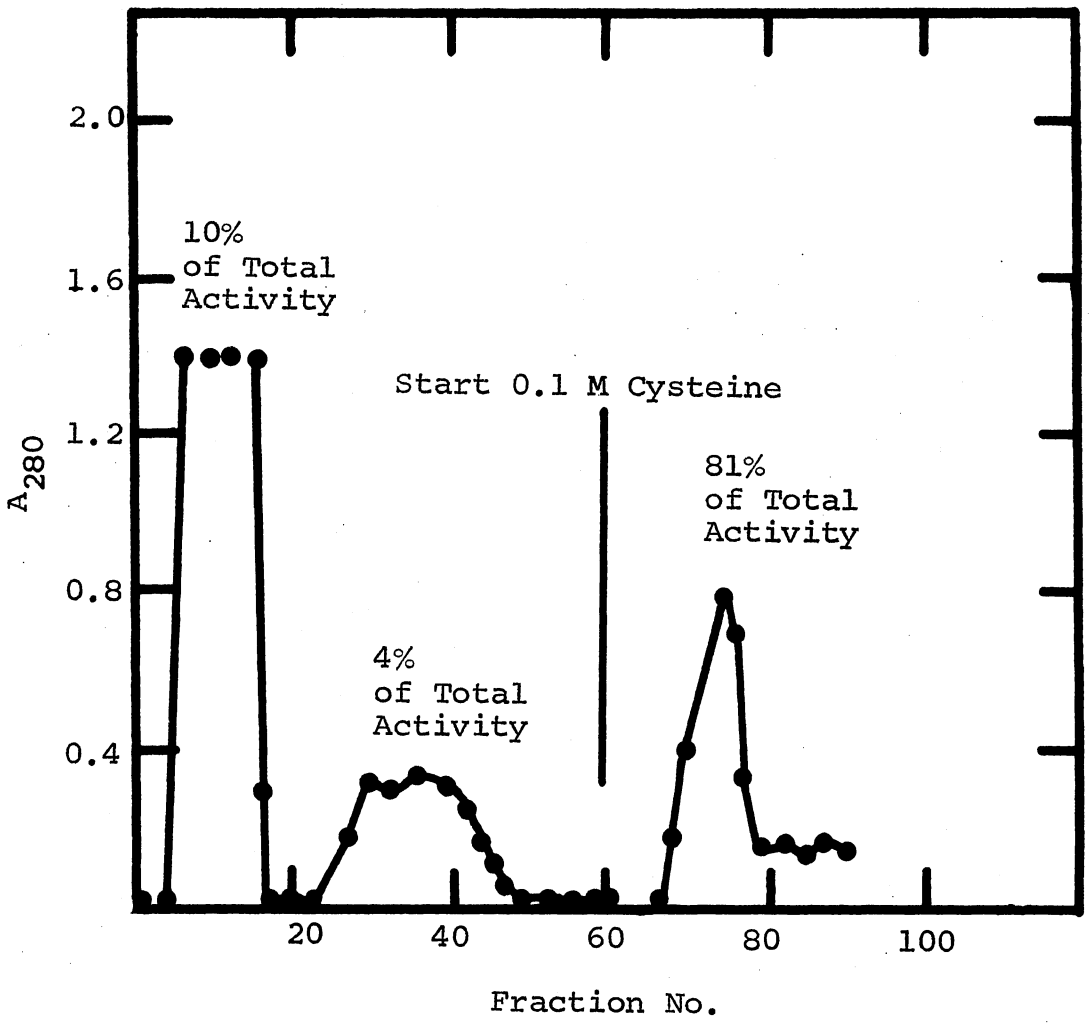

Figure 36. Chromatography of Triton X-100 Solubilized Receptor on p-Chloromercuribenzoate-Sepharose

Twenty ml of Triton X-100 solubilized receptor were diluted 1:1 with 40 mM Tris, pH 7.6 buffer and applied to a 1.5 cm x 25 cm PCMB-Sepharose column. The column was washed with 20 mM Tris, 0.1% Triton X-100, pH 7.6 buffer. Elution was by buffer plus 0.1 M cysteine. The two wash peaks were pooled and assayed and the elution peak was also pooled and assayed. Eight ml fractions were collected through fraction number 60 followed by five ml fractions.

propylamine were purchased from Tridona Chemical Company. N-Hydroxysuccimide was purchased from the Pierce Chemical Company. Succinic anhydride was purchased from Fisher Scientific Company. Activated alumina gel (Wn-3) was from the Sigma Chemical Company. Ovine prolactin, NIH-P-S12, was supplied by the National Institute of Arthritis and Metabolic Diseases Pituitary Hormone Distribution Program.

Methods

Two types of activated Sepharose 4B were used to synthesize prolactin affinity columns. Cyanogen bromide activated Sepharose 4B was prepared as described previously (111).

Sepharose 4B was also activated by oxidation using sodium meta-peroxidate (111). To a suspension of 100 ml of Sepharose 4B in 80 ml of water, 20 ml of 1 M sodium meta-periodate were added. The suspension was placed in a tightly closed 500 ml polyethylene bottle and gently shaken for 2 hours at room temperature. The oxidized Sepharose was then filtered on a Buchner funnel and washed with 2 liters of deionized water. The washed, oxidized Sepharose was then reacted with 100 ml of 2 M diaminodipropylamine at pH 5.0. After 24 hours of shaking at room temperature, solid sodium carbonate was added to raise the pH to 9.0. The suspension was then moved to a shaker bath at 4° and 10 ml of freshly prepared 5 M sodium borohydride were added over a 12 hour period. The reduced derivative was washed with 4 liters of

1 M sodium chloride. Washing was continued until no free diamine could be detected in the wash by the 2,4,6-trinitrobenzenesulfonic acid test (111).

The extension arms were lengthened by reaction with succinic anhydride (111). The aminoalkyl-Sepharose was diluted 1:1 with deionized water and cooled to 4^{o} in an ice-bath. Succinic anhydride was added (1 millimole per ml of packed gel) and the pH was quickly raised to 6.0 with 5 N sodium hydroxide. The suspension was magnetically stirred and the pH was maintained at 6.0 with sodium hydroxide until no further pH change was observed. The solution was then left for 4 more hours at 4^{o}. The succinylaminoalkyl-Sepharose was washed with deionized water and then reacted with 0.1 N sodium hydroxide for 40 minutes at room temperature to cleave labile carboxylic groups. The gel was washed extensively with deionized water. The gel was dried by washing with ten volumes of anhydrous dioxane (dioxane was dried by passage through a neutral alumina column) and the suspended in an equal volume of dioxane. Solid N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were added to achieve final concentrations of 0.1 M each. The suspension was gently stirred for 90 minutes in a closed container at room temperature. The N-hydroxysuccimide ester was washed with 10 volumes of anhydrous dioxane and 2 volumes of anhydrous methanol. The gel may be stored as a 1:1 suspension in dioxane over molecular sieves but more commonly was lypholized and stored

at 4° under Dririte.

To couple prolactin to the N-Hydroxysuccinimide ester of agarose, the lypholized gel was suspended in a small volume of 50 mM acetate, pH 6.0 at 4°. The gel was then quickly filtered and washed with 2 volumes of the acetate buffer. This step is necessary to remove all the free, water-soluble carbodiimide or N-hydroxysuccinimide present. The N-hydroxysuccinimide ester of agarose is reasonably resistant to pH 6.0, and loss of coupling sites on the resin is minimal. After the resin was washed, it was immediately added to a solution of prolactin in 0.1 M sodium phosphate pH 8.0. The concentration of prolactin was between 0.5 mg and 1 mg per ml of swollen gel. To determine coupling efficiency, 50 μCi of ^{125}I-labeled prolactin were included in the reaction mixture. The suspension was incubated for 24 hours at 4° with gentle shaking. The resin was then poured into a column and washed with 0.1 M Tris, 1 M sodium chloride pH 8.5, until prolactin levels in the elution stablized. The column was washed with 0.1 M Tris, 4 M urea, pH 8.0 for 24 hours at room temperature. Coupling efficiency was between 70-80%.

Another prolactin-Sepharose derivative was synthesized starting from the diaminodipropylamine Sepharose (periodate oxidized) which, instead of succinic acid, terminated in 3,3'-dithiopropionic acid. Diaminodipropylamino-Sepharose was diluted 1:1 with water and 0.2 gm of 3,3'-dithiopropionic acid were added per 10 ml of Sepharose; the pH was

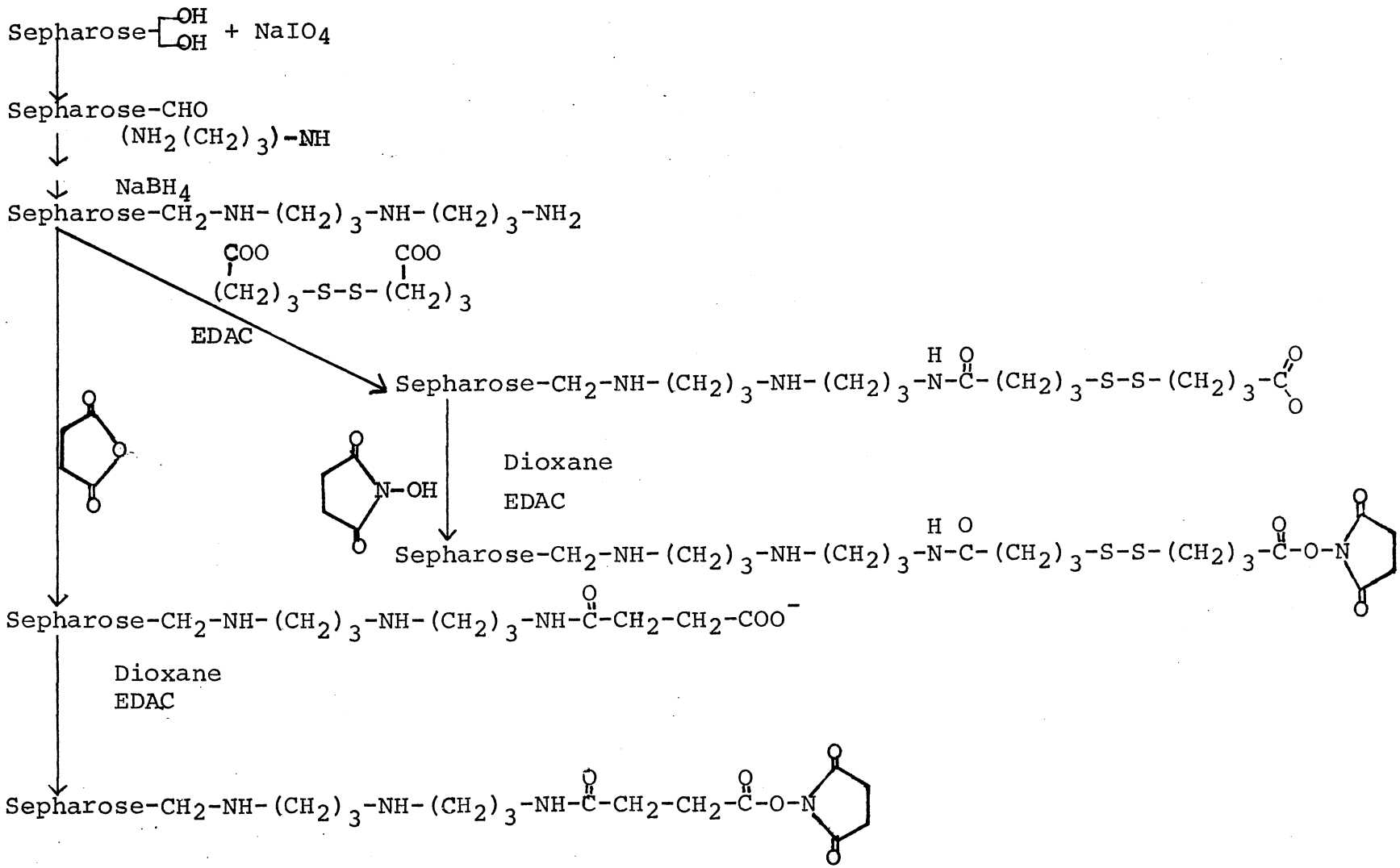

Figure 37. Synthesis of Affinity Column Resins

adjusted to 4.8, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added to a final concentration of 0.1 M. The pH was maintained at 4.8 by titration with sodium hydroxide until it stabilized and then the solution was stirred gently for an additional 4 hours at 4°. The gel was then washed extensively with deionized water. Anhydrous conditions were obtained by washing with 10 volumes of anhydrous dioxane. The gel was then suspended in an equal volume of anhydrous dioxane. This suspension was made 0.1 M in N-hydroxysuccinimide and 0.1 M in 1-ethyl-3-(dimethyl-aminopropyl)carbodiimide and allowed to react for 90 minutes at room temperature. The gel was then washed with 10 volumes of dioxane and 10 volumes of methanol and lyophilized. Coupling was as described for the other derivative. Coupling efficiency was 50-60%.

The synthesis of prolactin-Sepharose derivatives is summarized in Figure 37.

Results

Derivatives synthesized from periodate-oxidized Sepharose were more stable than those synthesized from cyanogen bromide-activated Sepharose. The cyanogen bromide-activated Sepharose derivative with 0.5 mg prolactin incorporated per ml of gel released 1 μg of prolactin per hour. The column size was approximately 1.5 ml. The elution buffer was 20 mM Tris, 0.1% Triton X-100, pH 7.6 and the columns had a flow rate of 2 ml per hour. When a

prolactin affinity column, synthesized from periodate oxidized Sepharose was monitored for ligand release under the same condition, the rate of release was only 10 ng per hour. When the dissociation constant of the ligand-receptor complex is extrememly low and the receptor concentration is low, instability of the hormone-Sepharose linkage may lead to release of hormone which effectively competes with the matrix-bound material (111). For this reason, the cyanogen bromide-activated Sepharose derivatives were unsatisfactory but the periodate-activated Sepharose derivatives were useful.

The prolactin-Sepharose resins were stored in 20 mM Tris, 4 M urea, pH 7.5 at 4^{o}. Chromatographic columns were poured using a 1:5 and a 1:10 dilution of prolactin-Sepharose to Sepharose 4B. The 1 ml columns were washed for 2-4 hours with 20 mM Tris, 10 mM magnesium chloride, 0.1% Triton X-100, pH 7.6 before the sample was applied. Two types of receptor preparations were applied to the columns, either receptor off the p-chloromercuribenzoate-Sepharose column (dialized against 20 mM Tris, 10 mM magnesium chloride, 0.1% Triton X-100) or receptor solubilized with 1% Triton X-100 or 50 mM octyl-glucoside (diluted 1:1 with 40 mM Tris, 20 mM magnesium chloride).

When 2 ml of partially purified prolactin receptor or 4 ml of a 1:1 dilution of soluble receptor were applied to the columns, 5-30% of the activity washed through without binding to either the 1:5 or 1:10 diluted prolactin-

Sepharose resin. Elution was attempted using a number of agents dissolved in 20 mM Tris, 0.1% Triton X-100, 0.1% BSA, pH 7.6. Table V summarizes the results. One to 6 M urea, 0.5 M lithium chloride, 2 M lithium chloride, 2 M magnesium chloride, 4 M magnesium chloride, 5 M magnesium chloride, 0.1 M mercaptoethanol, 1 M mercaptoethanol, 0.1% sodium dodecyl sulfate, pH 12, pH 3, and elution at 37^{o} resulted in only a 2-10% recovery of activity. In only one case, at 37^{o}, was elution higher and this resulted in a 33% recovery. Also, elution with 10 mM ethylenediaminetetraacetic acid in 20 mM Tris, 0.1% BSA, pH 7.6 gave the same results. However, elution with 0.25 M EDTA resulted in a 60-98% recovery of activity in the elution. These results may be suspect since total recovery in the wash and eluate varied from 140 % to 200 % and activity was lost upon dialysis against 20 mM Tris, 10 mM magnesium chloride, 0.1% Triton X-100, pH 7.6 buffer. The purity was not determined due to the presence of BSA in the eluting buffer.

Another approach was used to attempt to purify prolactin receptor. The receptor was chromatographed on a Sepharose-diaminodipropylamine-3,3-dithiopropionate-prolactin column. A 1 ml column containing approximately 0.1 mg of bound prolactin was equilbrated in 20 mM Tris, 10 mM magnesium chloride, 0.1% Triton X-100, pH 7.6 for 4 hours at room temperature. Two ml of receptor, partially purified on p-chloromecuribenzoate-Sepharose (dialyzed against the column buffer) were applied to the column over a 2 hour

TABLE V

SUMMARY OF THE EFFECTIVENESS OF VARIOUS DISSOCIATION AGENTS ON ELUTION OF PROLACTIN RECEPTOR FROM A PROLACTIN-SEPHAROSE COLUMN

Activity in Wash %	Elutate	Activity in Elution
12%	1 M Urea	8%
20%	2 M Urea	8%
15%	4 M Urea	10%
16%	6 M Urea	9%
38%	20 mM PCMB	2%
	50 mM Sodium Acetate	
27%	pH 3	3%
34%	pH 12	5%
33%	37°	12%
28%	0.5 M LiCl	9%
26%	2 M LiCl	11%
38%	0.1 M Mercaptoethanol	8%
41%	1 M Mercaptoethanol	9%
29%	2 M Magnesium Chloride	5%
32%	4 M Magnesium Chloride	8%
31%	5 M Magnesium Chloride	8%
38%	0.2% SDS	0%
12%	10 mM EDTA	8%
44%	20 mM EDTA	98%

One to two ml ovine prolactin-succinyldiaminopropylamine-Sepharose columns were poured and equilibrated in 20 mM Tris, 10 mM magnesium chloride, 0.1% Triton X-100, pH 7.6. Each column contained 0.1 to 0.2 mg of bound prolactin per ml of Sepharose. Two to four ml of octyl-glucoside or Triton X-100 solubilized receptor (diluted 1:1 with 40 mM Tris, 20 mM magnesium chloride) were applied to each column. Elution was by the agent indicated dissolved in 20 mM Tris, 0.1% BSA, 0.1% Triton X-100, pH 7.6. Wash and elution fractions were pooled separately and assayed for prolactin activity.

period. Twenty percent of the activity did not bind to the column. Elution was by buffer containing 1 **M** mercaptoethanol. The mercaptoethanol was incorporated into the buffer to reduce the disulphide bond in the extension arm of the column. Thus, in the elution, free prolactin and prolactin-receptor complex should be present. The elution tubes were dialyzed against 20 mM Tris, 0.1% Triton X-100, pH 7.6 and lypholized (total volume 5 ml). The lypholized material was redissolved in 0.5 ml of deionized water and iodinated using choramine-T and 1 mCi of sodium 125-iodide (61). The iodinated material was then applied to a Bio-Rad A 1.5m, 1 cm x 120 cm chromatography column equilibrated in 20 mM Tris, 10 mM magnesium chloride 0.1% Triton X-100, pH 7.6 at 4^{o}. Three peaks were present in the elution profile, one in the salt peak corresponding to free 125-iodide, one marking as free prolactin, and one nearer the void volume which may be the receptor-hormone complex (Figure 38).

Table VI is a summary of the purification of receptor solubilized by octyl-glucoside. There was a 26-fold purification upon chromatography on C_5-diamine-Sepharose followed by chromatography on p-Chloromercuribenzoate-Sepharose. Purity was not determined for chromatography on prolactin-succinyldiaminodipropyl-Sepharose. However, if the same degree of purification was obtained as that obtained by Shiu and Friesen (78), the overall fold purification would be 39,000. If the prolactin receptor

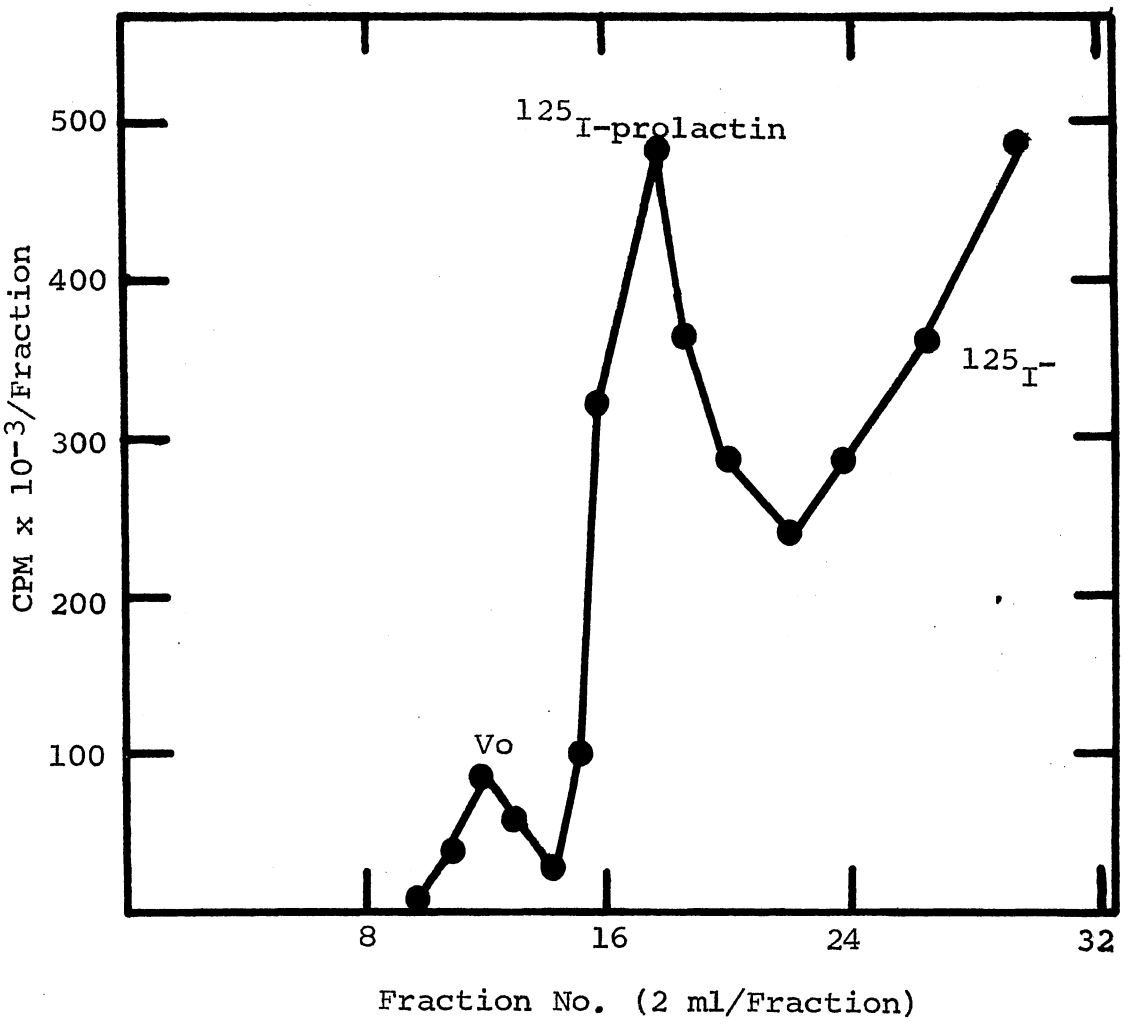

Figure 38. Chromatography of the Iodinated Elution of Prolactin-3,3'Dithioproprionatediaminodipropylamine-Sepharose on a Bio-Rad 0.5m Column

Two ml of partially purified (off PCMB-Sepharose) prolactin receptor was dialzyed against 20 mM Tris, 10 mM magnesium chloride, 0.1% Triton X-100, pH 7.6 buffer and applied to a prolactin-3,3'dithioproprionatediaminodipropylamine-Sepharose column. Elution was by buffer plus 1 M mercaptoethanol. The elution was labeled with 125-iodine as described in Methods and applied to a 1 cm x 120 cm Bio-Rad 0.5m column equilibrated in 20 mM Tris, 0.1% Triton X-100, pH 7.6. Two ml fractions were collected and counted.

TABLE VI

PURIFICATION OF RABBIT MAMMARY GLAND PROLACTIN RECEPTOR

Column	Load (mg)	Elution (mg)	% Activity Remaining	Fold Purification (total)
C_5 diamine	560	63	65	4.4
PCMB-Sepharose	63	11	46	26.4
Prolactin-Sepharose	11	-	6	39,000*

*Fold purification not determined-Shiu and Friesen (75) purified prolactin receptor 1500 fold on a Affi-Gel-10-prolactin affinity column. Assuming this degree of purification, the prolactin receptor was purified 39,000 fold.

is assumed to require the same degree of purification as the insulin receptor (200,000-400,000 fold purification), then the prolactin receptor is approximately 10% pure.

CHAPTER VII

Discussion and Summary

To date, only one polypeptide hormone receptor has been purified to near homogeneity. The insulin receptor was purified by ion-exchange chromatography on DEAE-cellulose, chromatography on Concanavalin-A-Sepharose (50). Perhaps the reasons other protein hormone receptors have not been purified are the difficulties encountered due to the extremely low levels of receptor found in tissues and the limited number of techniques available for purifying a protein that is only soluble in detergents. Also, due to the high affinities of the receptor-hormone complex, dissociation of a receptor from a hormone-Agarose affinity column, except in the presence of strong dissociation agents, is prohibited. Before purification may even be attempted, an assay for the receptor must be developed. This requires detecting the binding of 10^{-10} to 10^{-11} moles of the receptor and hormone. To date, the only successful method used has been radioactive labeling of the hormone with 125-iodide or 3-tritium. High specific activities of the order of 3000 Ci/mmole are required. Therefore, a method of incorporating a radioactive molecule in the protein hormone must be found that does not interfere with the

binding activity of the hormone. Iodination using the chloramine-T method (25) or the lactoperoxidase method (28) have been the most successful.

Since most, if not all, polypeptide hormone receptors are located on the plasma membrane, they must be solubilized from their lipid environment into an aqueous solution. The nonionic detergents, such as Triton X-100, Tergitol NPX, and octyl-glucoside, have proven to be the most effective. Once in apparently soluble form, conditions must be determined which stablize the receptor through the required purification process.

The prolactin receptor from the rabbit lactating mammary gland was isolated from a crude particulate membrane-associated microsomal fraction as described by Shiu et al. (85). Yields of the prolactin receptor in the 100,000 x g microsomal pellet were 50-80% of the activity found in the original mammary gland homogenate.

Iodination of ovine prolactin was attempted by a variety of methods, including chloramine-T iodination, lactoperoxidase iodination, and labeling by using the ^{125}I-labeled Bolton-Hunter reagent. Choramine-T iodination yielded a highly labeled product which possessed no binding activity. This could possibly be due to oxidation of a histidine residue or a methionine residue. Few, if any, high molecular weight aggregates were found as shown by low backgrounds in the filter assay. Upon iodination by the method of Turkington (99), using

lactoperoxidase, high molecular weight aggregates formed which were essentially impervious to dissociation agents. Only treatment with 6 M urea caused disaggregation to a significant percentage. Neither the aggragated form nor those disaggregated by 6 M urea were active in binding to the prolactin receptor. Iodination by the Thorell and Johansson lactoperoxidase method (100) yielded active ^{125}I-labeled prolactin. The specific activities ranged from 30 μCi/μg to 120 μCi/μg and averaged 60 μCi/μg. The iodinated product was purified by successive chromatography on a Bio-Gel P-6 column (0.5 cm x 40 cm) and a Bio-Gel P-100 or P-150 column (1 cm x 120 cm). Few aggregates were detected in the elution from the Bio-Gel P-150 column and those present were inactive in the binding assay. The purified ^{125}I-labeled prolactin was 80-90% active and was stable for up to two months when stored in the presence of bovine serum albumin at 4°. The use of commercially available lactoperoxidase resulted in a lower incorporation of 125-iodide into prolactin than lactoperoxidase isolated from skim milk.

The receptor could be solubilized using 0.5% or 1% Triton X-100, 0.5 to 1% Tergitol NPX, 30 mM cholic acid, or 50 mM octyl-glucoside. It seems likely that "buried" prolactin receptors exist since a 2-3 fold enhancement of activity was observed upon solubilization. The soluble receptor was stable for at least 1 month at 4° when stored under sterile conditions.

The receptor appears to be a protein since it is susceptible to inactiviation by chymotrypsin and trypsin but it may not be a glycoprotein since treatment with α-mannosidase, neuraminidase, or β-galactosidase did not significantly affect binding activity. Also, it failed to bind the lectins, Concanavalin-A and wheat germ agglutinin, which are specific for mannosyl and N-acetylglucosyl residues, respectively. While this does not prove the absence of a carbohydrate moiety, it does suggest that the prolactin receptor is a protein but not a glycoprotein. Further studies using other lectins specific for other carbohydrates or purification of sufficient quantities of the receptor to allow carbohydrate analysis are needed to resolve this question.

Treatment of soluble receptor with phospholipase A, C, and D failed to destroy its ability to bind prolactin. It appears therefore, that the receptor does not have a phospholipid moiety that is essential for activity; or, if present, the lipid is protected from enzymatic degradation.

Unlike the insulin receptor (52), the rabbit mammary gland prolactin receptor did not display negative cooperativity. Dissociation of the prolactin-receptor complex in the presence of 10^{-6} M prolactin did not increase over the rate of dissociation in the presence of no prolactin when measured by the dilution method of DeMeyts, et al. (52). Noncurvilinear Scatchard analysis also supports the

conclusion the cooperativity is not present.

A variety of compounds were tested as to their effect on dissociation. Only the salts magnesium chloride and sodium chloride, and urea increased the rate of dissociation, and then only at relatively high concentrations (2 M sodium chloride, 4-5 M magnesium chloride, 2 M urea). The organic solvents acetone, ethanol, and dioxane had no effect on the rate of dissociation at concentrations as high as 30%. Glycerol at 50% (v/v) detectably slowed the rate of dissociation and stabilized the receptor-prolactin complex. The rate of dissociation increased with temperature. p-Chloromercuribenzoate had no effect on the rate of dissociation, suggesting that the sulfhydryl groups necessary for activity in the receptor is protected by bound prolactin. It appears, from the results, that the predominant forces responsible for the binding of prolactin to its receptor are ionic in nature since salts increased the rate of dissociation; and not hydrophobic in nature since organic solvents had no effect on the rate of dissociation.

Magnesium chloride was found to be required for the binding reaction but only over a very narrow concentration range, 10 mM to 30 mM. When magnesium chloride concentrations were above 0.1 M, there was a decrease in binding. When the concentration was in the optimal range, binding increased four-fold.

The receptor was shown to have a sulfhydryl group critical for binding activity. p-Chloromercuribenzoate

or N-ethylmaleimide treatment inactivated the prolactin receptor. 2-Mercaptoethanol was not required in buffers to insure stability. It is possible that bovine serum albumin was serving to protect the receptor since it contains sulfhydryl groups.

Purification of the Triton X-100 solubilized receptor was first attempted by amino acid-Sepharose hydrophobic chromatography. The receptor, however, was not soluble in the high concentrations of phosphate or sulfate necessary for effective chromatography on these columns. The receptor would adsorb to a C_5-diamine-Sepharose chromatography column and could be eluted by an increasing gradient of sodium chloride. Octyl-glucoside-solubilized receptor performed better than Triton X-100 solubilized receptor on this column. It appeared that Triton X-100 bound extremely tightly to the diamine-Sepharose columns; and, thus, the columns would lose capacity. No detergent was required in the elution buffer on this column. It seems likely that octyl-glucoside co-elutes with the receptor and thus maintains its solubility. A four to six-fold purification was typical upon chromatography of the receptor on this column. Recovery of activity averaged 60-70%.

The receptor would also bind to and could be eluted from a PCMB-Sepharose affinity column. Elution was by 0.1 M cysteine in the chromatography buffer. The purification was approximately 10-fold over soluble receptor and the recovery average 70-80%.

The final step in any purification procedure for a hormonal receptor must be successful binding to and elution from a hormone-agarose affinity column. For the prolactin receptor-prolactin interaction, the dissociation constant for the complex was shown to be 2×10^{-10} M by Scatchard analysis. Thus, dissociation of the receptor-prolactin-Sepharose complex could be expected to be difficult.

Ovine prolactin affinity columns were synthesized from cyanogen bromide activated Sepharose and sodium periodate oxidized Sepharose. The most stable derivative was periodate oxidized and reduced Sepharose-diaminodipropylaminosuccinyl-ovine prolactin. Leakage of prolactin from the column was minimal and the column was stable for at least one month when stored in buffer containing 4 M urea. Either octyl-glucoside or Triton X-100 solubilized receptor would bind to this column, but elution was difficult. Elution with 5 M magnesium chloride (75) gave only 8-10% recovery of activity. Other salts in high concentration (up to 6 M urea), 1 M mercaptoethanol, or increased temperature did not improve recoveries. Elution with ethylenediaminetetraacetic acid, 0.25 M, resulted in very high recoveries; but the activity was lost upon dialysis. It seems likely that these high recoveries might be an artifact of the assay procedure even though 0.25 M EDTA did not give apparent activity in the assay blanks.

Thus, upon attempted elution from hormone-Sepharose affinity columns, the prolactin receptor did not behave

as the insulin receptor. The insulin receptor could be eluted by 4 M urea in good yields. Perhaps the prolactin receptor was successfully eluted but was irreversibly denatured upon treatment with strong dissociation agents. Another possibility is the loss of an essential cofactor such as a phospholipid or other small molecule essential for activation.

Another method used to purify prolactin receptor was chromatography on a prolactin-Sepharose derivative which had a disulfide bond incorporated in the extension arm. The entire complex could be eluted by cleaving this bond with 2-mercaptoethanol. Purification using this procedure might allow a large enough quantity of the receptor to be isolated to characterize its components and thus determine a successful elution method from a prolactin-Sepharose affinity column.

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