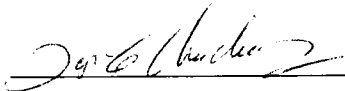


To the Graduate Council:

I am submitting herewith a thesis written by Lajwanti S. Khemlani entitled "Insulin-Receptor Interaction in the Rat Hepatoma H-35 Cell Line." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

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John W. Koontz, Major Professor

We have read this thesis  
and recommend its acceptance:

  
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Accepted for the Council:

  
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Vice Provost

and Dean of the Graduate School

INSULIN-RECEPTOR INTERACTION  
IN THE RAT HEPATOMA H-35 CELL LINE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Lajwanti S. Khemlani

December 1989

## DEDICATION

This thesis is dedicated to my beloved mother Mrs. Lachmibai  
S. Khemlani and father Mr. Sobhrajmal Chandiram.

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## ABSTRACT

Binding of insulin to the cell surface insulin receptor is the first step in insulin action. Upon binding, the insulin-receptor complex is internalized and the receptor depending on the cell line is either recycled or is degraded.

In KRC-7 cells, a subclone of the H-35 rat hepatoma cell line, insulin binding parameters such as the binding affinity ( $K_d$ ) and the receptor number per cell have been determined for the cell surface, particulate cell membranes and solubilized receptor. Competition inhibition studies have been performed with control cells and cells incubated with a high concentration of insulin. Results from cell surface insulin binding to control cells have indicate a single, high affinity insulin binding site on the cell surface. Cells incubated with insulin demonstrate a 75% reduction in insulin binding. Scatchard analysis of this data indicate that the decrease in insulin binding is due to the decrease in cell surface receptor number per cell and not due to decrease in the binding affinity. To calculate the total receptor number insulin binding to the particulate cell membrane and solubilized receptor has been performed. And, Scatchard analysis indicate the possibility of the presence of an intracellular low affinity binding site. In KRC-7 cells insulin binding to the solubilized receptor from cells incubated with insulin demonstrate that the internalized receptor does not remain functional in terms of insulin binding.

Insulin-receptor interaction studied in the presence of wheat germ agglutinin (WGA), a plant protein, has indicated that WGA increases insulin binding by two fold. Preliminary insulin binding in the presence of WGA performed in our laboratory has indicated the WGA increases the insulin binding affinity and not the insulin receptor number. Insulin receptor aggregation has been postulated to be the reason for the increase in insulin binding affinity in the presence of WGA. Therefore, to determine whether WGA induces receptor aggregation, cross-linking studies have been performed in the presence and in the absence of WGA. SDS electrophoresis and autoradiography have been utilized to visualize insulin receptor subunits. Results indicate that WGA does not induce receptor aggregation in KRC-7 cells.

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*The woods are lovely, dark, and deep,  
But I have promises to keep,  
And miles to go before I sleep,  
And miles to go before I sleep.*

ROBERT FROST

## CHAPTER I

### INTRODUCTION

#### Receptor Criteria:

There is general agreement in the literature that the term "receptor" refers to a macromolecule capable of recognizing and selectively binding with a ligand. After binding the ligand, the receptor is capable of generating a signal that initiates a chain of events leading to the biological response.

In order to avoid possible semantic confusion, Ronald Kahn has suggested that a hormone receptor be defined as a "cellular component which has the ability to selectively recognize and bind the hormone and which has binding characteristics consistent with a potential for signal generation. Most importantly, the binding should show specificity for hormonal analogues which parallels known biological effects of these hormones. In addition, the kinetics of hormone-receptor interaction and the affinity of the receptor for the hormone should be consistent with the kinetics and dose response of the biological action of the hormone" (1).

Thus, in keeping with the above definition, an insulin receptor can be defined as a macromolecule capable of both recognizing an insulin molecule and transducing a signal which in turn initiates a cascade of biological events. The signal transduced from the cell exterior to the interior can be attributable to insulin either by activation or induction of some factor(s) known as mediator or second messenger. That the effector unit resides within the receptor structure, however, is not necessary.

### History of the Insulin Receptor:

The existence of insulin receptors was first suggested by Stadie et al. in 1949 (2). Hechter (3) and Reiser (4) proposed that binding of insulin to its receptor is essential for the biological activity of insulin. Early studies by Cuatrecasas using chemically attached insulin on sepharose beads showed, for the first time, the presence of insulin receptors on isolated fat cells and liver membranes (5,6). This was later confirmed by Cuatrecasas using radioactively iodinated insulin and measuring displacement of its binding by native insulin (7). With minor modifications (8), this technique is currently used for the study of insulin-receptor and other peptide hormone-receptor interactions.

The presence of insulin receptors, thereafter, has been demonstrated on primary as well as established cell lines from various tissues grown in culture. Relatively large numbers of insulin receptors are present in human placenta, rat liver and rat adipocytes, and in cultured cell lines, such as 3T3-L1 adipocytes and IM-9 human lymphocytes (43).

### Insulin Receptor Gene:

The existence of a single insulin receptor gene in the haploid human genome has been demonstrated. The chromosomal location of the insulin receptor gene was determined and mapped to chromosome 19 (11). Further studies conducted on the insulin receptor gene using the Southern blot technique have revealed a

coding region of more than 45 kb (11). Recently, the human insulin receptor gene, INSR, and its promoter region has been isolated and characterized by Seino et al. (9). They determined the exon-intron organization, and also demonstrated that the gene spans more than 120 kilobasepairs. The coding region of the human INSR consists of 22 exons, and all the introns are located in the protein coding region of the gene.

There is considerable evidence from several studies to show that the insulin receptor belongs to a large family of tyrosine specific protein kinases which consists of a number of growth factor receptors and oncogene products (10). Comparison of the tyrosine kinase domain of the oncogenes src, ras and v-erb-B with the human insulin receptor gene indicates that the exon-intron organization of this region has not been well conserved through evolution (9). Some introns, however, appear in similar positions between pairs of genes. Sequence analysis of the INSR promoter has shown that it is G+C rich, but lacks a TATA and a CAAT box (9).

#### Insulin Receptor Structure:

Since a full length cDNA for the insulin receptor has been sequenced, the discussion of the receptor structure will begin with information obtained by analysis of the putative protein sequence extrapolated from the DNA sequence. Ullrich et al. (10) and Ebina et al. (11) have elucidated the entire 1370-1383 amino-acid sequence of the human insulin receptor precursor. Their

studies have shown that a 27 amino-acid signal sequence precedes an  $\alpha$  subunit which consists of 719 amino acid residues (10,11). The other subunit, known as the  $\beta$  subunit, is preceded by four basic amino acids, (Arg,Lys,Arg,Arg) and consists of 620 amino acid residues (10,11). The basic amino acid stretch is typical of processing signals for precursor cleavage. The signal sequence is removed from the precursor during the synthesis of the mature insulin receptor.

Further studies employing the insulin receptor cDNA have indicated that the 719-residue-long  $\alpha$  subunit sequence is largely hydrophilic and exists exclusively on the extracellular face of the plasma membrane. The  $\alpha$  subunit is also rich in cysteine and contains fifteen evenly distributed consensus sequences for asparagine-linked glycosylation (10). On the other hand, the 620 amino acid long  $\beta$  subunit does not exist exclusively on the extracellular face of the plasma membrane and it contains only nine cysteine residues. Based on its amino acid sequence the  $\beta$  can be divided into three domains (10,11); an amino-terminal domain of 194 amino acid residues consisting of four potential asparagine-linked glycosylation sites and four cysteine residues; an adjacent domain consisting of 23-26 highly hydrophobic residues representing a single transmembrane domain which anchors the insulin receptor in the membrane; and, the carboxy-terminal domain consisting of 403 amino acid residues. The cytoplasmic or the carboxy terminus is characterized by a high content of hydrophilic residues (58%, mostly acidic) and a high content of

proline and glycine (16%) (11). The carboxy terminal domain contains three basic amino acids (Arg-Lys-Arg) which flank the C-terminus of the transmembrane domain and two potential N-linked glycosylation sites (10,11). Located in this domain is the "consensus" ATP-binding site that is involved in the tyrosine kinase activity of the insulin receptor (10,11).

Recent interest in exploring the relationship of the insulin receptor to other growth factor receptors possessing an extracellular ligand binding domain and a ligand dependent tyrosine kinase activity has indicated similarities between insulin receptor and the products of the src family of oncogenes and the receptor for EGF. The availability of the complete amino acid sequence of the human insulin receptor and the EGF receptor has enabled the study and comparison of several structure-function relationships in these two receptors. It is now well documented that, although the receptor for EGF functions in the plasma membrane as a single polypeptide (as compared to the heterotetrameric structure of the insulin receptor), it exhibits similarities in the primary sequence and overall organization of the insulin receptor. Other points of homology between the two receptors are: the presence of the extracellular cysteine rich domain; a 23 amino acid long transmembrane domain; three basic amino acids (Arg,Lys,Arg) in the cytoplasmic side of the membrane; a Pro-Ser sequence flanking the extracellular domain of

the membrane and the ATP binding site on the cytoplasmic domain (10,11).

The cytoplasmic domain of these two receptors is also homologous to the products of the src family of oncogenes. An ATP binding site is located 49 amino acid residues from the membrane in the insulin receptor, the EGF receptor, as well as in the sequence of the v fms oncogene (10). Furthermore, in all cases, the lysine residue essential for kinase activity is present 25 amino acids from the ATP binding site (10,20). One other point of interest is that, although the structure-function relationship of the EGF-I receptor is similar to the insulin receptor; the EGF receptor does not trigger cellular events similar to that of the insulin receptor (10,11).

Characterizing the insulin receptor on the basis of the putative protein sequence as described above supports earlier work done by other researchers. It is, therefore, now well established that the insulin receptor is an integral membrane glycoprotein of apparent molecular weight 350,000-460,000, and is composed of two  $\alpha$  and two  $\beta$  subunits (12,13,14,23). Each  $\alpha$  subunit has an apparent molecular weight of 125,000-135,000 and each  $\beta$  subunit an apparent molecular weight of 95,000 (14,15,16). A third subunit called the  $\beta'$  with an apparent molecular weight of 45,000 has been observed (14,15,16). This subunit is considered to be a proteolytic fragment of the  $\beta$  subunit. These molecular weight estimates have been confirmed by subsequent cross-linking and photoaffinity labeling techniques (14,15,16).

The insulin receptor thus is a heterotetramer, and is made up of two heterodimers linked by disulfide bridges in the form of  $\beta$ - $\alpha$ - $\alpha$ - $\beta$  (14,15,16).

The disulfide linkages found in the insulin receptor structure have been classified as Class I and Class II according to their susceptibility to reducing agents (15). Class I disulfide bonds link a  $\beta$   $\alpha$  dimer to another  $\beta$   $\alpha$  dimer and Class II disulfide bonds link an  $\alpha$  subunit to a  $\beta$  subunit. According to the literature (18,19) Class I disulfide bonds are more susceptible to reductants, the reduction being partly reversible. Class II disulfide bonds, however, are much stronger and can be reduced only after denaturation of the insulin receptor (19). Partially reduced receptor halves have been reported in rat liver and other tissues (18). The physiological significance of these disulfide bonded structures however, remains obscure. In vitro studies using Class I disulfide reduced insulin receptor halves have shown that the two halves remain closely linked by strong noncovalent interactions.

#### Functional Aspects of the $\beta$ Subunit of the Insulin Receptor:

Since the early 1980's the tyrosine kinase activity of the insulin receptor has been a subject of several investigations (22). It has been demonstrated that binding of insulin to the insulin receptor activates the tyrosine specific kinase. The activated kinase, after autophosphorylation of the insulin receptor  $\beta$  subunit, phosphorylates several exogenous substrates (23,

24). Recently, endogenous protein substrates for this kinase activity have been identified (25,26). However, the significance of the endogenous substrates in insulin action is, as yet, not clear.

Consequently, to better understand the role of the insulin receptor tyrosine kinase activity, it is important to determine which of insulin's pleiotropic actions depend upon the kinase activity of the receptor. Evidence from other studies indicates that a defect in the tyrosine kinase activity results in failure to stimulate several metabolic processes (27,28,29).

Autophosphorylation of the insulin receptor on its  $\beta$  subunit has been considered as a prerequisite for stimulation of the protein kinase activation towards exogenous substrates. However, the exact correlation between these two events has not been clear. Most recently, Ponzio et al. (30) have shown that the autophosphorylation of the beta subunit is not always correlated with the stimulation of the exogenous protein kinase activity. His study also demonstrates that not under all conditions can biological effects of insulin cannot always be linked to the receptor autophosphorylation. Presence of cryptic tyrosine kinase activity not associated with the insulin receptor has been suggested.

The above studies, thus suggest a need for better understanding the role of the insulin receptor autophosphorylation.

### Role of Glycosylation in the Insulin Receptor Structure:

Glycoproteins are ubiquitous macromolecules. The precise role(s) of the carbohydrate moiety of glycoproteins has not been completely understood, however, there is evidence to show that carbohydrate residues function as important cell surface component (31,32). Several studies have indicated that the carbohydrate residues: (a) serve as recognition markers for the cell surface as well as for the ligands; (b) are involved in targeting of macromolecules, e.g., some enzymes, to intracellular organelles; (c) play a role in conformational stabilization of the protein; (d) are involved in assembly of receptor oligomeric structures; and (e) are essential for biological activity (31,32).

Glycosylation appears to be an important processing step in the synthesis of the insulin receptor. The carbohydrate moiety is an essential structural component of the insulin receptor for ligand binding.

Reed et al. (33) have used inhibitors of protein glycosylation to determine the role of the carbohydrate moiety in the insulin receptor function. Using the heavy isotope density-shift technique they showed that addition of tunicamycin, (a potent inhibitor of core oligosaccharide addition step in the synthesis of insulin receptor) caused the depletion of cell-surface and total cellular insulin receptors in 3T3-L1 adipocyte. The inhibitor prevented the formation of functional newly synthesized insulin receptors without affecting the receptor degradation rate. The effects of inhibitors of terminal glycosylation have

also been studied (31,32). For example, monensin inhibits several Golgi processes including terminal glycosylation, proteolytic processing, and transport of proteins. In the presence of monensin the insulin receptor is synthesized as a 340,000 dalton disulfide-linked, oligomeric protein capable of binding insulin in intact cells (31,32). Upon reduction, 180,000 dalton subunits of the receptor are obtained, indicating that the proteolytic cleavage of the proreceptor to  $\alpha$  and  $\beta$  subunits has not occurred. Treatment of cells with neuraminidase results in loss of terminal sialic acid residues from the carbohydrate moiety of the insulin receptor. This leads to insulin receptors composed of  $\alpha$  and  $\beta$  subunits of lower molecular weight than subunits of receptors from untreated cells (34). These studies therefore indicate that glycosylation is essential for the production of a functional insulin receptor.

#### Insulin:

Insulin is a 5.8 kd polypeptide hormone synthesized in the beta cells of the pancreas, and released into the circulation by exocytosis (35). The mature insulin molecule consists of two chains, an A chain of 21 residues and a B chain of 30 residues, covalently linked by two disulfide bonds (35).

The binding of insulin to the cell surface receptor elicits a complex response, that ultimately affects carbohydrate, lipid and protein metabolism (36).

Based on X-ray crystallography structure along with studies utilizing anti-insulin antisera, Weitzel et al. (37) have proposed that, beginning with arginine 22, the COOH terminus of the B chain, in itself is the "active center" of the insulin molecule in terms of receptor binding and biological activity. Other residues of importance in this "active center" include the COOH-terminal region of the A chain beginning with and including A13 leucine and A14 tyrosine, and the amino terminus of the A chain (38).

#### Insulin-Receptor Interaction:

Studies with a wide variety of tissues have shown that the insulin-receptor interaction is rapid, reversible, and saturable (1,39). The initial step in the insulin-receptor interaction involves binding of the ligand insulin to the  $\alpha$  subunit of the receptor (12,13). Upon binding of insulin, the occupied receptors are internalized, via the coated pit-dependent or independent processes. Internalized receptor-ligand complexes dissociate in the endosomes probably due to acidification of the vesicle (40,41). Most of internalized insulin is degraded, whereas a small portion of the internalized hormone remains undegraded and is released by a pathway termed retroendocytosis (42). The fate of the internalized receptor depends on the type of tissue involved (39,43). The internalized receptor route is still incompletely understood as the specific biochemical signals that control the intracellular routing of receptors and the bio-

logical function of the itinerary is not clear. The main function of endocytosis could be the mediation of hormone degradation which in turn could serve as a signal for the termination of a message induced by binding of insulin to its receptor (44). An alternative possibility has been proposed where the internalization process could be a means of delivering the hormone or a proteolytic fragment of insulin (or both) to intracellular sites, where it (they) could mediate the biological effects of insulin (44). Therefore, although the initial events in cellular processing of insulin and its receptor are similar, their ultimate fates are very different.

Evidence from morphological studies using lymphocytes have indicated that the process of internalization can result in a reduction of cell surface receptors for insulin. Several researchers have also demonstrated that the concentration of insulin receptors per cell is inversely related to the level of insulin to which the cell is exposed (45). This process is termed "down regulation" and has been shown to occur in several tissues (46). The purpose of this phenomenon, as suggested by investigators (45), could be the regulation of plasma insulin concentration.

#### Kinetics of Insulin Binding:

The binding of insulin to target tissues such as adipose, muscle, and liver has been extensively investigated. Unfortunately, the controversial nature of the binding data has made its

interpretation difficult. It is generally expected that insulin will have a higher affinity for its receptor in tissues where its effects are greater than in tissues where its effect is only marginal. The quantitative aspects of the insulin-receptor interaction are complex, however, in calculating binding parameters it is assumed that a steady state of binding has been achieved. Kinetic binding parameters characterizing the binding of insulin to its receptors can be derived from insulin-receptor competition (between  $^{125}\text{I}$ -insulin and excess unlabeled insulin) experiments. Scatchard plots are used to obtain the affinity constant and the receptor number (46).

Earlier studies done by Cuatrecasas and Kahn on binding of insulin to fat cells showed that the binding could be described by a simple reversible bimolecular interaction between cell surface receptors and insulin (1, 7). Other studies (50), however, strongly suggested that insulin binding is complex and this complex behavior manifests itself in a curvilinear Scatchard plot obtained for insulin-binding. Several hypotheses have been proposed to explain this nonclassical observation. Initially, a nonlinear Scatchard plot was attributed to the presence of two or more classes of insulin binding sites that have different affinities for insulin (47). However, De Meyts et al. suggested the existence of negative cooperativity through site-site interactions where insulin already bound to a receptor reduces the affinity of other as yet unoccupied receptors (48, 49). Researchers supporting the theory of negative cooperativity also

demonstrated that this property is intrinsic to the insulin receptor (50). Deger et al. (51) put forth a theory to explain negative cooperativity on the structural basis of the insulin receptor and it was demonstrated that only the tetrameric insulin receptor could exhibit negative cooperativity. But, this theory has been challenged because it is generally considered that increased dissociation of  $^{125}\text{I}$ -insulin from its receptor is only observed under conditions where total receptor occupancy is decreasing and is not due to changes in insulin receptor conformation.

#### Lectins:

Lectins are sugar-binding plant proteins that agglutinate a variety of normal and neoplastic animal cells (52). Lectins have also been demonstrated to mimic insulin action (53). Each lectin binds specifically to a certain sugar sequence in oligosaccharides or glycoconjugates (54). Insulin receptor has been shown to contain carbohydrate moieties (55), thus specific lectins bind to the insulin receptor with high specificity. Very low concentrations of plant lectins have been shown by Cuatrecasas (54) to have a profound insulin-like effects on metabolic processes of isolated fat cells. Since in some cell line lectins have also shown the ability to interact directly with insulin cell surface receptors (53) they have been used as molecular probes for investigating the architecture of the cell surface and to provide a better understanding of the mechanism of insulin action. Studies done by Cuatrecasas et al. have demon-

strated that multivalent lectins such as concanavalin A (Con A molecular weight: 100,000) (56) and wheat germ agglutinin (WGA, molecular weight: 36,000) (57) alter insulin binding to intact cells, membranes, and partially purified receptor preparations (54). This binding has been shown to be reversible. Low concentrations of WGA have been shown to enhance the specific binding of insulin to insulin receptors in isolated fat cells and liver membranes (55). Higher concentrations of this lectin inhibit the binding of insulin to these tissues. However, higher concentrations of Con A do not inhibit insulin binding (53).

Preliminary insulin binding studies carried out in our laboratory in the presence of WGA have shown similar results: i.e., enhanced insulin binding with low concentrations of WGA, and a decrease in specific insulin binding below the control level with higher levels of WGA. However, Con A, in the H-35 cell line produces enhanced insulin binding as the concentration of the lectin is increased. Although to date there has been no direct evidence to explain the effect of WGA and Con A on insulin binding, cross-linking or aggregation of the insulin receptors, in the presence of these lectins, has been suggested as a possibility (56, 57).

Therefore, although the mechanism of insulin action is still not clear, several achievements made in recent years with respect to insulin receptor structure and function have aided in better understanding of the insulin receptor. Rapid progress has been made towards quantitation of hormone-receptor interaction by

radiolabeled ligand binding (1). The biochemical basis of internalization of hormone receptor complexes, degradation of insulin and fate of insulin receptors (43) has been studied in several tissues that are targets for insulin action. Identification and characterization of basic subunit structures, and elucidation of protein kinase activity of the insulin receptor has further aided this study.

#### Reuber H-35 Rat Hepatoma Cell Line as a Model for the Study of Insulin Action:

Rat hepatoma cell lines have been extensively used for the study of gene expression and induction of several enzymes. The ease with which large quantities of these cells can be cloned and maintained along with very little variation from the observed liver-like characteristics has made the rat hepatoma cell line useful in several studies. The Reuber H-35 (H4-II-E-C3) "minimal deviation" hepatoma cell line is one such system which has proved to be extremely valuable in the study of mechanism of insulin action. This cell line has been identified as a well differentiated cell line (58) i.e., it retains most of the adult rat liver morphology and functions. Some of the important functions of this cell line are: production of the gluconeogenic enzymes (phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase and triosekinase), production of four liver specific enzymes, constitutive secretion of serum albumin, induction of tyrosine aminotransferase (TAT) by glucorticoids, and

induction of glycogen synthase and TAT by insulin.

Fao and KRC-7 cell lines are two subclones of the Reuber H-35 cell line. The Fao cell line, classified as a well differentiated cell line, exhibits all the above characteristics of the Reuber H-35 rat hepatoma cells. Insulin binding studies conducted with the Fao cells by Crettaz and Kahn (62) have indicated increased binding due to increased number of insulin receptors as compared to the dedifferentiated or partially differentiated cell lines. The KRC-7 cell line, although descended from the well differentiated Reuber H-35 hepatoma cell line does not exhibit all the characteristics of an adult rat liver cell line. Studies have indicated that the KRC-7 cells do not secrete serum albumin, a characteristic of an adult rat liver (59).

Hormones like glucagon that are connected to the adenylate cyclase system do not seem to induce TAT or PEPCK in this cell line. This, however, does not necessarily indicate the absence of adenylate cyclase in the KRC-7 cell. Studies with cholera toxin in the KRC-7 cells have shown the induction of TAT and PEPCK, concomitant with the activation of protein kinase (63). This study also indicates the presence of low, but detectable level of adenylate cyclase. Further studies have shown that the adenylate cyclase activating hormones do not induce TAT and PEPCK enzymes in the KRC-7 cells, because the receptors for these hormones are absent on the plasma membrane of this cell line. The KRC-7 cells, however, respond to insulin even at very low concentrations (64). Therefore, this suggests the presence of

high affinity insulin binding receptors on the KRC-7 cell surface. Studies with KRC-7 cells in the presence of insulin have demonstrated an increase in the TAT enzyme activity without an increase in the transcript level (62).

Some other unique results of insulin action in the KRC-7 cells come from studies done by Koontz (65) and Taub et al. (66). Koontz has unmistakably demonstrated that insulin acts through the insulin receptors to affect cell growth. This study has thus resolved several previous controversies over whether insulin's growth effects occur through the insulin receptor. Furthermore, Taub et al. (66) have demonstrated that in the quiescent KRC-7 cell, insulin is able increase the transcript levels of c-myc and c-fos. Furthermore, this effect was not observed with other growth factors like EGF or PGDF in the KRC-7 cells. Phorbol myristic acetate has been shown to increase the c-fos and c-myc levels in this cell line, but without stimulating DNA synthesis (66). Evidence from the above work thus suggests the KRC7 cell line to be a possible model of a regenerative liver or a fetal liver even though it is a clone of a well differentiated cell line. The unique characteristics of the KRC-7 cell line thus present a suitable model system to carry out insulin binding and receptor structure studies.

### Significance of this Research:

Preliminary insulin binding studies carried out in our laboratory have indicated the presence of high affinity insulin receptors on the cell surface of the KRC-7 cells. Insulin binding studies done in the presence of lectins, WGA, and Con A have demonstrated that in the presence of low concentrations of WGA, specific insulin binding increases and at high concentrations, specific binding diminishes to below control levels. Con A, on the other hand, increases insulin binding at increasing concentrations. Con A has been characterized as a mitogenic lectin and WGA as a nonmitogenic lectin (19). Preliminary studies done in our laboratory have indicated that WGA can mimic the insulin mitogenic response in the KRC-7 cells. To date, the precise reason for the insulin mimetic effect by lectins is unknown, although, the receptor aggregation has been suggested as circumstantial evidence.

These preliminary studies have served as a basis for developing my project. This project should enable our laboratory to further characterize the insulin receptor and the insulin receptor associated actions in the KRC7 cells.

### Specific Aims:

The specific aims of this research project are:

1) To determine insulin binding parameters such as the value for the dissociation constant ( $K_d$ ) and the receptor number for the KRC-7 cell line. The  $K_d$  and the receptor number per cell will be estimated for the cell surface, cell particulate and solubilized receptor.

2) To measure these same parameters,  $K_d$  and receptor number per cell, in cells which have been incubated with high concentrations of insulin to stimulate down-regulation of the receptors. By comparing these values with those obtained with untreated cells it will be possible to determine: the extent of receptor loss from the cell surface, and whether the receptors have been internalized and are still capable of binding insulin or have been lost from the pool of functional receptors, i.e. degraded.

3) To demonstrate the effect of WGA on insulin receptor. This will be accomplished by confirming the effect of WGA on insulin binding. This work will be extended by cross-linking of radiolabeled insulin to its receptor in the presence of WGA to determine whether WGA induced aggregation of the insulin receptor can be detected.

### Experimental Design:

I and II. a) Insulin Binding to Intact Cells:

In order to determine the  $K_d$  and the cell surface receptor number, competition binding studies will be conducted using

intact cells. Similar binding studies will also be done with cells incubated with high concentration of insulin prior to measuring binding. Binding data will be analyzed using Scatchard analysis. Indicated binding parameters for insulin treated and untreated cells will determine the effect of incubation of KRC-7 cells with a high concentration of insulin.

b) Insulin Binding to Homogenized Cells:

Cell particulate matter will be prepared by disrupting the KRC-7 cells. Competition insulin binding studies will be performed using the crude membrane extract. Scatchard analysis of these data will indicate the total (cell surface plus intracellular), membrane-associated insulin receptors and the dissociation constant of insulin binding to these membranes.

c) Insulin Binding to Solubilized Receptor:

Insulin treated and untreated cells will be used to prepare solubilized receptor. A nonionic detergent will be used to solubilize: (a) cell particulate matter, and (b) whole cells. Competition binding studies will be done with solubilized receptor prepared from insulin treated and untreated cells. Scatchard analysis of the binding data will be performed to determine the values for the dissociation constant and the receptor number for control and treated cells. Comparison of the binding parameters for insulin treated cells with the control cells will also demonstrate the fate of insulin receptors, in the the KRC-7 cell line, in terms of insulin binding.

III. a) Effect of Insulin Binding to its Receptor in the Presence of WGA:

Total and nonspecific insulin binding (i.e. in the absence and presence of excess cold insulin) in presence and absence of WGA will demonstrate the effect of WGA on insulin binding.

b) Cross-linking of  $^{125}\text{I}$ -insulin to the Insulin Receptor and Visualization of the cross-linked Insulin Receptor:

Total and nonspecific (in presence of excess unlabelled insulin) insulin binding will be conducted in presence and in absence of WGA. Covalent affinity cross-linking of bound iodinated insulin to its receptor will be performed. The insulin receptor oligomeric structure will be demonstrated by running SDS polyacrylamide gel electrophoresis of the radiolabeled, cross-linked receptor and comparing this to iodinated high molecular weight protein standards. This will provide evidence as to whether WGA causes aggregation of receptors to a form detectable as a higher molecular weight form.

## CHAPTER II

### MATERIALS AND METHODS

#### Materials:

Porcine insulin was obtained from Elanco Products Co., Indianapolis, IN. Cell culture medium, Dulbecco's modified Eagle's medium (DMEM), was obtained from Gibco, Grand Island, NY. Calf serum and fetal bovine serum were products of M.A. Bioproducts, Walkersville, Ma. Iron-supplemented calf serum was obtained from Hyclone Laboratories, Inc., Logan, Utah. Cell culture dishes were from Corning Glass Works, Corning, NY; Costar, Cambridge, MA; or from Falcon, Becton Dickinson labware, Lincoln Park, New Jersey. Polyethylene glycol, M.W. 8000 and bovine gamma-globulin, fraction V were obtained from Sigma Chemical Company, St. Louis, Missouri. Dimethyl sulfoxide (DMSO) was from Fisher Scientific Co. Fair, New Jersey. Disuccinimydyl suberate was bought from Pierce Chemical Co., Rockford, IL. High molecular weights were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. and  $^{125}\text{I}$  was obtained from Dupont-New England Nuclear.

#### Methods:

##### Cell Culture:

The KRC-7 cells were maintained in DMEM supplemented with either 5% fetal calf serum and 5% calf serum or 10% Iron-supplemented calf serum. HEPES buffer was added to a final concentra-

tion of 25 mM, pH 7.4. Cells were maintained in a humidified atmosphere of 10% CO<sub>2</sub>, 90% air. Cells were maintained on serum free media for at least 24 hours before binding assays.

#### Iodination of Insulin:

Iodination of insulin was carried out by a minor modification of the method of Freychet et al (67) as described by Terris and Steiner (68). A Sephadex G-50 (fine) column was used for separation of the iodinated hormone from free iodine. Elution was carried out using 0.1M acetic acid containing 0.1% bovine serum albumin. The fractions containing insulin were assayed for acid precipitability and those fractions exhibiting greater than 95% precipitability were pooled, lyophilized and stored frozen at -20°C. Before using in assay, the lyophilized powder was dissolved in Ca<sup>2+</sup> and Mg<sup>2+</sup> free phosphate-buffered saline containing 1% bovine serum albumin and rechecked for acid precipitability. Specific activity immediately following iodination varied from 90-140  $\mu\text{Ci}/\mu\text{g}$ .

#### Cell Surface Insulin Binding:

Binding of radioiodinated insulin in the presence of increasing concentrations of radioinert insulin (competition binding) to insulin receptors was assayed with cells in monolayer culture. Cells were grown in cluster dishes of 24 x 15 mm wells. When 75 to 80% cell confluency was attained, the medium was changed to serum free DMEM for at least 24 hours. Cells were then washed

twice with ice-cold phosphate-buffered saline and a binding assay mixture containing serum-free DMEM, 1% BSA, 25 mM HEPES, pH 7.4, 0.4 ng/ml  $^{125}\text{I}$ -insulin and unlabeled insulin (varying from 0 to 1  $\mu\text{g/ml}$ ) was added to the wells. The binding experiments were conducted at 4°C to minimize degradation of iodinated ligand and to inhibit endocytosis. After a period of 16 hours, the binding reaction was terminated by aspirating the mixture from the wells, washing the cells four times with ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin and then dissolving the cell monolayer in 0.1 N NaOH. This solution was then transferred to a 12 x 75 mm test tube and radioactivity was determined in a Beckman 4000 gamma counter.

#### Down Regulation:

To achieve down regulation, cells were maintained under conditions identical to those of the control cells, except in this case cells were incubated with 10  $\mu\text{g/ml}$  unlabeled insulin. Control cells and cells treated with insulin were incubated at 37°C. After an initial incubation period of 24 hours both sets of cells were removed from the incubator, but only the down regulated cells were washed with serum free DMEM to remove unbound insulin. Cells were further incubated at 37°C for 15 minutes. The above wash procedure was repeated once more to ensure the further removal of unbound ligand. The binding assay was next performed as mentioned above on both sets of cells.

#### Efficiency of the Wash Procedure:

Cells cultured in 12 x 24 mm dishes were used for this purpose. To achieve down regulation, cells were incubated with 5  $\mu$ g/ml unlabeled insulin and 100,000 counts of  $^{125}$ I-insulin. After an initial period of 24 hour incubation, at 37°C, cells were washed with serum free DMEM media to remove exogenously added insulin. Cells were further incubated at 37°C for 15 minutes and the wash step was repeated for a total of five times to check whether the washing steps used previously were sufficient to remove the exogenously added insulin. In between every wash step cells were incubated at 37°C degrees for 15 minutes. Cells were solubilized with 0.1 M NaOH and counted in a Beckmann 4000 gamma counter.

#### Preparation of Particulate Cell Membranes:

Confluent 100 mm dishes were used to obtain total cell membranes. Cells were washed once with ice cold phosphate-buffered saline, pH 7.4 prior to scraping them off the plate with a rubber spatula. Cells in 1 mM NaHCO<sub>3</sub> were homogenized using a dounce homogenizer (8 strokes with pestle "A") and then centrifuged at a 15,000 rpm for 15 minutes. The pellet consisting of the cell particulate material was resuspended in a small volume of 1mM NaHCO<sub>3</sub> and centrifugated as described above. The pellet obtained was resuspended in 50 mM potassium phosphate buffer containing 4 mM magnesium sulfate, pH 7.4. The total cell membranes thus

obtained were used for binding assays. Cell homogenization as well as the centrifugation was carried out at 4°C.

#### Solubilization of the Insulin Receptor

##### (a) Solubilized Membrane Extract:

Cell particulate matter prepared by the above procedure was solubilized essentially by the method of Kohanski and Lane (84) at 4°C for 60 minutes using 0.67% Triton X-100. Solubilized membranes were centrifuged at 100,000 x g for 2 hours at 4°C. The supernatant thus obtained was used for insulin binding assays.

##### (b) Solubilization of Whole Cells:

Confluent cells in 100 mm dishes were used for these experiments. Control and down regulated cells were washed twice with ice cold phosphate-buffered saline, pH 7.4, and harvested in the same buffer using a rubber spatula. The cell suspension thus obtained was centrifuged at 4°C, 15,000 rpm. The cell pellet was solubilized by incubation (with stirring) in 50 mM HEPES buffer containing 1% Triton X-100 for 1 hour at 4°C. The solubilized cell membranes were then centrifuged at a speed of 100,000 x g at 4°C for 2 hours. The supernatant thus obtained contained the soluble insulin receptor and was used for insulin binding assays.

## Insulin Binding Assay for Particulate Membranes and Solubilized Receptors:

### Total and Nonspecific Binding:

Protein concentration was determined by the method of Lowry (69). The binding mixture for insulin binding studies consisted of 0.4 ng/ml  $^{125}\text{I}$ -insulin, phosphate-buffered saline pH 7.4, 1% bovine serum albumin, and for the membrane fraction varying concentrations of membrane protein in 50 mM potassium phosphate buffer containing 4 mM magnesium sulfate. For the solubilized receptors, the assay contained varying concentrations of solubilized receptor proteins. The total assay volume was 700  $\mu\text{l}$ . Nonspecific binding was measured in the presence of 1  $\mu\text{g/ml}$  unlabeled insulin.

### Competition Insulin Binding:

The binding assay included either the total cellular membrane preparations or the solubilized receptor protein, 0.4 ng/ml  $^{125}\text{I}$ -insulin, varying concentrations of unlabeled insulin (0 to 1  $\mu\text{g/ml}$ ) and an appropriate buffer (mentioned above) to bring the total volume of the assay to 700  $\mu\text{l}$ .

Binding experiments were conducted at 4°C for 16 hours with gentle shaking or as indicated in figure legends. To terminate the binding reaction, the 700  $\mu\text{l}$  binding assay mixture was first divided into triplicates of 200  $\mu\text{l}$ . Then for particulate matter bound and free  $^{125}\text{I}$ -insulin were separated by addition of 12.5% polyethylene glycol followed by centrifugation at 6000 rpm. The

pellet was washed with an aqueous solution of 12.5% PEG and bound radioactivity in the pellet was determined using a Beckmann 4000 gamma counter. Separation of bound and free iodinated insulin for solubilized receptors was carried out essentially by the method of Cuatrecasas (71) in the presence of 12.5% polyethylene glycol and 0.14% gamma globulin prepared in 5 mM HEPES, pH 7.4.

#### Iodination of Protein Standards:

Iodination of high molecular weight protein standards was carried out by a minor modification of the method of Freychet et al (67) as described by Terris and Steiner (68). The iodinated protein standards were separated from free iodine by passing through a Sephadex G-50 (fine) spun column saturated with 0.1% bovine serum albumin prepared in 0.1 M acetic acid. The fractions containing iodinated protein standard were assayed for acid precipitability. The sample was lyophilized and stored frozen at -20°C. The lyophilized powder was dissolved in 0.2 M sodium phosphate, pH 7.4 for use in SDS-polyacrylamide gel electrophoresis.

#### Cross-linking of $^{125}\text{I}$ -insulin:

Radiolabeled insulin was cross-linked to its receptor as described by Crettaz and Kahn (70). Confluent cells in 100 mM dishes, maintained on serum free DMEM for at least 24 hours were incubated with 40 ng/ml  $^{125}\text{I}$ -insulin in HEPES binding buffer (100 mM HEPES, 120 mM NaCl, 5 mM KCl, 12 mM  $\text{MgSO}_4$ , 8 mM glucose

and 1% bovine serum albumin) in the presence and absence of unlabeled hormone or 2.5  $\mu\text{g/ml}$  WGA at 4°C for 16 hours with gentle shaking. Thereafter, the binding medium was aspirated and the cells washed twice with ice cold PBS. Freshly prepared disuccinimidyl suberate, at a concentration of 100 mM in dimethyl sulfoxide, was added to cells in albumin free HEPES binding buffer, to give a final concentration of 1 mM. After incubating for 15 minutes at 4°C, the reaction was quenched with 4 volumes of ice-cold 10 mM Tris-HCL, pH 7.4, containing 1 mM EDTA. After 10 minutes, the Tris-EDTA solution was aspirated, the cells were washed twice with cold PBS and solubilized in 2% NaDodSO<sub>4</sub>, 10mM sodium phosphate, pH 7.0. The cell lysate was transferred to 12 x 75 tubes, radioactivity determined using a Beckmann 4000 gamma counter and stored at -20°C until further analysis by SDS-polyacrylamide gel electrophoresis.

#### Electrophoresis:

Cross-linked samples were heated for 2-3 minutes in the presence of 10% glycerol, approximately 0.01% bromophenol blue, and supplemented where indicated with 5% beta-mercaptoethanol. Electrophoresis was performed according to Laemmli (72). The resolving gel was either 4 or 7.5%, according to the experiment, while the stacking gel was either 3 or 4%. Iodinated or pre-stained protein standards were used to calculate molecular weights as described previously (73). Iodinated high molecular weight standards used were: Thyroglobulin (330,000), ferritin

(220,000), albumin (67,000), catalase (60,000), lactate dehydrogenase (36,000) and ferritin subunit (18,500). The gels were dried and autoradiographed with Kodak X-OMAT RP film.

Data Analysis:

Binding data were analyzed using the computer program "LIGAND" (74).

## CHAPTER III

### RESULTS

#### Internalization of Cell Surface Insulin Receptors:

In vivo studies have indicated that plasma insulin concentration and the insulin receptor number are inversely related. Similar results have been obtained in vitro by several investigators (45,47,58). Previous studies have also demonstrated that the requirements for receptor internalization are: exposure of cell surface receptors to an excess of exogenously added insulin and an incubation temperature of 37°C (45). Based on previous studies in the other cell lines cell surface receptor internalization was induced in the KRC-7 cells at 37°C by the addition of 10 µg/ml insulin.

Because the cells are incubated with a high concentration of insulin to induce down-regulation, it is important to wash away all of the unbound insulin from this incubation before attempting to measure residual receptors with radioiodinated insulin. Results indicated that the wash procedure used removed more than 99% of the added <sup>125</sup>I-insulin (Figure 1).<sup>\*</sup> This, therefore, confirms that the wash procedure is adequate and that any decrease in binding is due to internalization of receptors and not due to the presence of bound insulin. Calculations demonstrate that 0.6 ng

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<sup>\*</sup>All Tables and Figures may be found in the Appendix.

of insulin is present in cells after two washes. However, it should be kept in mind that this amount also includes insulin that has been internalized.

A time course of the loss of insulin receptor during incubation of cells with  $1.67 \mu\text{M}$  insulin was conducted. Figure 2 shows that cell surface insulin binding decreases as a function of time. It can be seen that specific insulin binding rapidly decreases until a steady state is achieved. This plateau in insulin binding is attained after approximately 20 hours.

#### Cell Surface Insulin Binding:

This result demonstrates a decrease in insulin binding upon prolonged incubation of the cells in the presence of insulin. To determine whether this is a result of a decrease in receptor number or binding affinity measurements of the  $K_d$  and receptor number were performed. To measure cell surface insulin binding parameters displacement of  $^{125}\text{I}$ -insulin using increasing concentrations of unlabeled insulin was performed. This allowed simultaneous measurement of the binding affinity ( $K_d$ ) and the number of cell surface binding sites ( $B_{\text{max}}$ ). Binding was conducted using cells treated with or without (control)  $10 \mu\text{g/ml}$  insulin. Unlabeled insulin concentrations for a competition binding study ranged from 0 to  $1 \mu\text{g/ml}$  and  $^{125}\text{I}$ -insulin concentration was held constant at  $0.4 \text{ ng/ml}$ .

A representative displacement curve for insulin binding to control cells and cells treated with insulin is demonstrated in

Figure 3. It can be seen that compared to control cells the amount of insulin bound was much lower in cells treated with 10  $\mu\text{g/ml}$  insulin. However, the value of  $\text{IC}_{50}$  (12.6 nM for control and 12.6 nM for treated cells) remained constant. Saturation of the receptor is observed at an insulin concentration of 1  $\mu\text{g/ml}$ .

The above data were treated more quantitatively by Scatchard analysis. "LIGAND" indicated that this data will only fit a one site model (Figure 4). Therefore, straight lines are fitted to the data. These lines are parallel indicating that the  $K_d$  remains the same in both the cases;  $11.4 \pm 1.7$  nM for control cells and  $11.1 \pm 2.2$  nM for cells incubated with insulin. This implies that the decrease in binding observed in the displacement plot is caused by a decrease in the receptor number and not a change in the binding affinity. From the intercept of the two lines on the abscissa it can be seen that the  $B_{\text{max}}$  decreases from  $0.3 \pm 0.04$  nM in the control cells to  $0.11 \pm 0.02$  nM in the insulin treated cells indicating a 66% decrease in the binding sites. The decrease in  $B_{\text{max}}$  values constitutes a decrease in the receptor number per cell from  $73,000 \pm 9,000$  for control cells to a receptor number of  $24,000 \pm 4,800$  for cells treated with insulin. These experiments were repeated several times, the results of which are summarized in Table I. From the compilation of all this type of experiment the results indicate an average  $K_d$  of 10 nM and receptor number of 67,000/cell. For the insulin treated

cells the averages are 11.5 nM and receptor number of 16,000/cell.

To provide a different view of these data a Hill plot was generated for a representative experiment of the control data (Figure 5). A value of 0.92 for the Hill coefficient (nH) was obtained from the slope of the line indicating a single noninteracting species of the receptor.

#### Insulin Binding to Cell Membrane Fractions:

As determined in the previous section, there are  $67,000 \pm 8,000$  cell surface receptors per cell. The next step was to determine the total (cell surface and intracellular) number of receptors. This was done using whole cell particulate matter. Figure 6 shows the displacement of  $^{125}\text{I}$ -insulin with increasing concentrations of unlabeled ligand. These data were transformed to a Scatchard plot and it fit a one site model (Figure 7). A  $K_d$  of  $2.4 \pm 0.98$  nM and a  $B_{\text{max}}$  of  $0.13 \pm 0.04$  nM were obtained. The average value of  $B_{\text{max}}$  yields a total receptor number of  $62,000 \pm 19,000$  receptors per cell. These experiments were done for a total of three times with results summarized in Table IV.

It should be noted that these results are compromised because the binding reaction was carried out for 3 hours at  $4^\circ\text{C}$ . Subsequent experiments demonstrated that binding had not achieved equilibrium within three hours. Therefore, these results are likely to be an underestimate of the actual values. However, they do indicate that the procedures used for the assay are valid.

### Insulin Binding to Solubilized Receptor:

As noted earlier in Table I, cell surface insulin binding decreases in cells treated with 10  $\mu\text{g/ml}$  insulin. A 76% decrease in receptor number is shown to be the cause of this decrease. In order to determine if the internalized receptor remains functional in terms of insulin binding, solubilized receptors were prepared to conduct further binding studies. Cell particulate matter and whole cells were used for this purpose. Binding parameters were determined for cells treated with or without 10  $\mu\text{g/ml}$  insulin. If the receptors are internalized but not degraded then solubilization should expose these receptors and binding analyses would reveal them.

Figure 8 demonstrates a representative displacement curve for solubilized receptor prepared from insulin treated and untreated cells. It can be seen that compared to control cells, cells treated with insulin yield a decreased amount of insulin binding. Furthermore the data points from the cells treated with insulin are more scattered. Transformation of the data to a Scatchard plot (Figure 9) indicates a decreased slope for the down regulation experiment. This implies an increased  $K_d$ . Concurrent with this increase in the  $K_d$  value ( $1.62 \pm 0.70$  nM to  $66.30 \pm 80.22$  nM) an increase in the  $B_{\text{max}}$  from  $0.05 \pm 0.02$  nM to  $0.46 \pm 0.63$  nM is observed. In terms of receptor number, the experiment with control membranes yielded an average total number of  $94,000 \pm 20,000$  receptors per cell.

Binding parameters obtained for insulin binding to solubilized receptor prepared from cell particulate matter are summarized in Table V. Upon comparison of the insulin binding parameters indicated in Table V to those of control cell particulate matter (Table IV) it can be seen that the average  $K_d$  for insulin binding to cell particulate matter ranges from 2.0 to 4.2 nM and from 1.2 to 4.5 nM for solubilized receptor from control cells. This indicates that solubilization does not alter insulin binding affinity to the receptor.

These results indicate that the total number of membrane associated receptors (94,000/cell) is about 40% greater than cell surface receptors alone (67,000). However, the  $K_d$  for binding is approximately 2.9 nM which is about 4 fold higher affinity than binding to the cell surface. Examining the data for down-regulated cells suggests that the high affinity receptors have been lost and the cells are left with a low affinity - high capacity form. The problem with this is that this form was not observed in the control cells. Examination of the Scatchard plot in Figure 9 hints at such a form in control cells. But data analysis doesn't reveal it. Perhaps it is by only causing such a substantial loss of the high affinity form is the lower affinity form revealed.

Solubilized receptor was also prepared from whole cells to conduct competition binding studies. These experiments were performed as another approach to answer the same question; whether

insulin receptor is capable of binding insulin once the receptor is internalized. Figure 10 shows a displacement curve from membranes from control and down regulated cells. As seen in Figure 10, compared to the control cells the amount of insulin bound to the solubilized receptor from down regulated cells was lower. A Scatchard plot (Figure 11) of these data, shows an increase in the  $K_d$  ( $5.0 \pm 1.7$  nM for control cells to  $19.3 \pm 12.35$  nM for down regulated cells).  $B_{max}$  changes from  $0.062 \pm 0.02$  for control cells to  $0.102 \pm 0.07$  for down regulated cells. The binding parameters for insulin binding to solubilized receptor prepared from insulin treated and untreated cells are summarized in Table VIII.

#### Effect of WGA on Insulin Binding:

WGA, a bifunctional plant lectin has been shown to alter insulin binding in intact cells, membranes and partially purified receptors (53). In the KRC-7 cells, WGA has a biphasic effect on insulin binding. As seen from Figure 12, low concentrations of WGA increase insulin binding; and a peak in WGA induced insulin binding is observed at  $2.5 \mu\text{g/ml}$ . As the concentration of WGA is increased to higher than  $2.5 \mu\text{g/ml}$ , insulin binding decreases. Preliminary insulin binding studies carried out in our laboratory in the presence and absence of WGA, have indicated that the increase in insulin binding is due to an increase in the binding affinity and not due to an increase in the receptor number.

### Affinity Cross-linking Studies:

$^{125}\text{I}$ -insulin has been covalently cross linked to the insulin receptor using dissucinimidyl suberate (DSS). DSS is a bifunctional affinity cross-linking chemical reagent. Cross linking studies were conducted to ask whether WGA causes receptor aggregation to a form detectable as a higher molecular weight form.  $^{125}\text{I}$ -insulin was cross-linked to the insulin receptor after steady state insulin binding in the presence and the absence of WGA had been achieved. Highly porous 4% polyacrylamide gel has been used for electrophoresis to obtain good resolution of the high molecular oligomeric weight protein. Electrophoresis of the cross-linked insulin receptor was run under reducing and nonreducing conditions. When the cross-linked samples were treated with  $\beta$ -mercaptoethanol prior to SDS-polyacrylamide electrophoresis, a predominant band of apparent Mw 135,000 is observed (Figure 13). This is expected from previous reports (14). It indicates that the  $\alpha$  subunit is involved in insulin binding. Figure 14 shows the mobility of the oligomeric protein under nonreducing conditions. Specificity for cross-linking of insulin to the insulin receptor is indicated (Figures 13 and 14) by the presence and absence of saturating concentration of (1  $\mu\text{g/ml}$ ) unlabeled insulin. A representative nonreducing gel demonstrates the presence of three high molecular weight forms of the insulin receptor in the presence and in the absence of WGA (Figure 14). The presence of these multiple forms indicate

$\alpha_2\beta_2$ ,  $\alpha_2\beta\beta'$  and  $\alpha_2\beta'2$  heterotetramers. The other three bands which are comparatively lighter could represent an  $\alpha\beta$  subunit and a  $\alpha\beta'$  and an  $\beta$  subunit. Therefore, our results indicate, that similar receptor forms are obtained in the presence and absence of WGA. The presence of an aggregate induced by WGA was not observed in this autoradiograph. This experiment was repeated 5 times with virtually identical results in each experiment.

## CHAPTER IV

### DISCUSSION

The present study has been divided into two parts. The first part involves characterization of insulin-receptor interaction in terms of insulin binding to the insulin receptor in KRC-7 cells. The second part involved cross-linking of  $^{125}\text{I}$ -insulin to the insulin receptor in the presence and absence of WGA. This part of the research demonstrated that WGA did not induce receptor aggregation which can be detected by affinity cross-linking studies.

The insulin binding studies have been performed employing cell surface, cell particulate and solubilized receptor. As noted earlier, binding of insulin has been performed as with cells treated with or without  $10\ \mu\text{g/ml}$  insulin. Binding to the cell surface has indicated the type of binding site present on the cell surface and the number of cell surface insulin receptors in cells treated and untreated with insulin. Insulin binding to homogenized cells yields the total number of membrane associated insulin receptors. Binding of insulin to the solubilized receptor indicates the total number of solubilized receptors from cells treated or untreated with a high concentration of insulin.

Cell surface insulin binding in the KRC-7 cells shows the presence of a single, high affinity binding site. Scatchard analysis for insulin binding to receptors on the surface of KRC-7

cells indicates a  $K_d$  of  $10 \pm 2.4$  nM (Table IX). The receptor number per cell is  $67,000 \pm 8,000$  (Table IX). Preincubation of cells with  $10 \mu\text{g/ml}$  insulin reduces the cell surface insulin binding by 76% (Table IX). This decrease in binding can be accounted for solely by a decrease in the cell surface receptor number. The affinity of insulin binding to the reduced number of cell surface receptors remains essentially unchanged at  $11.5 \pm 5.0$  nM. Thus, from the above mentioned binding parameters it can be said that incubation of the KRC-7 cells with an excess of insulin induces "down regulation" without changing the binding affinity.

Binding parameters in Table IX show that a decrease in the receptor number from insulin-treated cells is accompanied by a large standard error in the value of receptor number per cell. This large variance reflects the difficulty in measuring insulin binding due to the presence of fewer cell surface receptors in cells incubated with insulin.

A second type of analysis used for the binding to the cell surface was the Hill plot. The reason for using this is that the Scatchard plot was linear suggesting a single class of binding sites. These results are unusual for insulin binding in that normally a curvilinear Scatchard plot is obtained. This result has been interpreted as being due to negative cooperativity between insulin receptors. We should be able to get an index of cooperativity from the slope of the Hill plot. The results of the Hill plot give a slope of about 1 indicating non-interacting

receptors. These results confirm the results of the Scatchard analysis and are further evidence that binding to the cell surface insulin receptor in the KRC-7 cells is to a single class of non-interacting receptors. These results are evidence of the unique nature of the interaction of insulin with the insulin receptor on the KRC-7 cells.

Estimation of the total number of membrane associated insulin receptors indicated a value of  $41,000 \pm 18,000$  per cell (Table IX). The  $K_d$  for binding to cell particulate matter is  $3.1 \pm 1.1$  nM. These results also indicate that the affinity of insulin binding to the receptor is not destroyed due to disruption of cells. However, compared to the control cell surface binding studies, binding conducted with cell particulate matter indicates a decrease in receptor number per cell. This is because insulin binding to cell particulate matter was not conducted under optimal conditions, that is, the binding reaction did not reach a steady state equilibrium. Previous studies have indicated that at  $4^\circ\text{C}$ , binding requires 14-16 hours to achieve equilibrium (1,7). Because of this the value for  $K_d$  and receptor number per cell are both inaccurate. But, these results were an indication that the PEG precipitation technique for separation of bound and free hormone was suitable for use with particulate membranes.

As noted earlier, preincubation of KRC-7 cells with an excess of insulin induces insulin-receptor internalization which in turn decreases the cell surface receptor number. To determine

whether the internalized receptors remain capable of binding insulin, solubilized receptor prepared from cells treated with insulin was employed for conducting insulin binding studies. Solubilized receptor from cells untreated with insulin was used as a control.

The results of insulin binding to solubilized receptors prepared by employing cell particulate matter indicate a  $K_d$  of  $2.9 \pm 1.7$  nM and a total receptor number of  $94,000 \pm 20,000$  per cell (Table IX).

Upon comparison (Table IX) of binding parameters determined for solubilized receptor from control cell particulate extract, to solubilized receptor from cells treated with insulin, the low affinity receptors become apparent. It is also observed that the receptor number in down regulated cells is greater when measuring solubilized cells than when only measuring cell surface receptors (Table IX). That difference in the receptor number constitutes receptors that did not internalize plus the intracellular receptors. However as noted earlier, the affinity of insulin binding to the solubilized receptor from insulin treated cells is lower. This indicates that upon internalization binding of insulin to the high affinity receptors is affected. This further implies that internalized receptors undergo a conformational change such that they do not remain functional in terms of high affinity insulin binding. Or the decrease in high affinity receptor number per cell could also indicate the degradation of those receptors. Binding of insulin to solubilized receptors

from insulin treated whole cells shows a trend consistent with the results mentioned above (Table IX). That is, compared to control solubilized receptors, a larger  $K_d$  and a larger receptor number per cell for insulin treated cells is observed. Upon comparison of the results for insulin binding to solubilized receptor from homogenized cells to those for solubilized receptor from whole cells, it can be observed that the low affinity binding site becomes more apparent only when the high affinity binding sites are degraded.

An important point to make here is that even though the solubilized receptor from insulin treated cells shows an increase in binding parameters compared to the solubilized receptor from control cells, the values from the treated cells are not as reproducible. The large uncertainty in the  $K_d$  and the receptor number per cell is reflected in the high standard deviation (Table IX). This uncertainty implies that even if a second binding site is present it has very low binding affinity. The presence of the low affinity binding site becomes apparent as the number of high affinity receptors diminishes.

Binding data from all experiments fit to a one site model. However, in two out of six experiments, control cell surface insulin binding data also fit the classical two site model with  $P < 0.05$  (Table II). This model indicates the presence of two heterogenous binding sites: one with a high binding affinity and low capacity and the other with a low binding affinity and high

capacity. For example, the  $K_d$  for the high affinity binding site in experiment number 5 (Table II) is  $4.6 \pm 1.2$  nM and for low affinity binding site is  $489 \pm 489$  nM. As indicated from the P value, a two site model fits the data in experiment number 5 significantly better.

Binding data from solubilized receptor preparations of control cell particulate extract fit a two site model three out of seven times (Table VI). Statistical analysis (Table VII) shows that when the binding data is fitted to a two site model, the mean squares decreases. However, even though the two site model gives a closer fit to the data, the calculated P value is  $> 0.05$  implying that this fit is not statistically significant. However, as shown in Table VII, although it cannot be definitely said that a second binding site exists, an approximately 80% possibility of this is indicated.

Comparison of the  $K_d$  values determined from the two site model for the control, cell surface binding and binding to the solubilized receptor model shows as noted earlier, a high affinity binding site with a  $K_d$  of  $4.6 \pm 1.2$  nM and a low affinity binding site with a  $K_d$  of  $489 \pm 489$  nM (Table II). For the solubilized receptor, the two  $K_d$  values were found to be  $0.6 \pm 0.8$  nM and  $83.0 \pm 176.8$  nM (Table VI). It can be seen from the above results that the  $K_d$  for the cell surface low affinity binding site ranges from 0 to 978 nM, whereas with solubilized receptor, the  $K_d$  for the low affinity binding site ranges from 0 to 259.8 nM. This underscores the previously mentioned uncer-

tainity in determining the low affinity binding site.

Comparison of the Scatchard plots for solubilized receptor from control and insulin treated cells (Figures 9 and 11) supports the notion of the presence of a low affinity binding site. As can be seen from these plots and the displacement curve in Figure 10 the scatter of data points is largely around low insulin concentrations. This implies that upon internalization it is the high affinity insulin receptor number that decreases. The Scatchard plot for this data (Figure 11) shows that when the high affinity receptors are decreased, the low affinity receptors become more apparent.

As noted earlier, since the insulin binding data could fit a two site model only occasionally, a one site model was used for Scatchard analysis throughout the insulin binding investigation. In summarizing the insulin binding studies it can be said that in the KRC-7 cells the high affinity insulin binding sites are located only on the cell surface, and treatment of cells with high concentrations of insulin leads to "down regulation." The receptors internalized by down-regulation in this cell line do not remain capable of binding insulin. As mentioned earlier, the presence of a low affinity binding site has been indicated but nothing definite can be concluded.

Therefore, to confirm the existence of the low affinity binding site, further experiments would have to be performed. These experiments would consist of preparation of solubilized

receptor from cells treated and untreated with trypsin. This should destroy the high affinity cell surface receptors. Then the cells could be solubilized and binding to the solubilized receptors carried out. Scatchard analysis of this data would thus indicate if another insulin binding site is present in the KRC-7 cells. A preliminary experiment to show that the cell surface receptors have been cleaved would involve cell surface insulin binding done to cells treated and not treated with trypsin.

The anticipated results for the preliminary experiment would be decreased cell surface binding for cells treated with trypsin. The decrease in this binding would be proportional to the cell surface receptor loss due to trypsinization. On attaining a decrease in cell surface binding one would expect with solubilized receptor a decrease in high affinity binding in cells treated with trypsin. These results would demonstrate if indeed a low affinity insulin binding site is present.

Table II, as noted earlier, indicates that two out of six times binding data fit a two site model, whereas for solubilized receptor the data fit a two site model three out of seven times. However, the  $K_d$  value of  $489 \pm 489$  for the low affinity binding site on the cell surface is much higher than the  $83.0 \pm 176.8$  for the solubilized receptor. But, as shown from Table III the cell surface binding data for this experiment fits a two site model with a  $P < 0.05$ . This indicates that there is certainly a second site present on the cell surface but binding to this site is not always observed because it is not always easily detect-

able. This second site as mentioned earlier is a low affinity binding site. Solubilization exposes these masked receptors and insulin binding increases. In the KRC-7 cells, the results suggest that it is the low affinity binding site that is exposed upon solubilization.

So, to confirm the presence of a low affinity binding site on the plasma membranes as opposed to intracellular organelles, insulin binding would have to be conducted with intracellular and plasma membrane. Membrane fractions from different cell organelles would have to be prepared using differential centrifugation and sucrose gradient techniques.

Insulin induced receptor loss has been demonstrated in several other cell lines such as: cultured human lymphocytes (45,79), rat adipocytes (82), cultured fibroblasts (78) and cultured hepatocytes (80). In a study by Prince et al using cultured human fibroblasts, it was observed that decreased insulin binding was entirely due to a decrease in the number of insulin receptor sites per cell (78). Similar results were obtained by Gavin et al using human lymphocytes the IM-9 cell line (45,79). However, the Fao cell line, another clone obtained from the chemically transformed H-35 cell line, does not show identical results. In the Fao cell line, down regulation experiments show not only a reduced receptor number, but also an increase in binding affinity (70). The Fao cells show a 75-80% decrease in receptor number (70), similar to results obtained with the KRC-7.

Insulin binding studies with the Fao cells have shown the presence of a heterogenous species of insulin binding sites (70). Scatchard analysis of the data in their study has indicated the presence of high affinity, low capacity and low affinity, high capacity receptor sites. The same investigators have also shown that during down regulation, it is the low affinity receptors that are internalized (70). In the KRC-7 cells it is the high affinity receptors that are degraded.

Upon internalization the fate of the receptor depends on the cell line. In rat hepatocytes, internalized insulin receptors are recycled to the cell surface (83). In cultured chick liver cells the internalized receptor is degraded (81). In mouse adipocytes (82) and human IM-9 lymphocytes (79), insulin receptors get degraded upon internalization. Green and Olefsky have showed that incubation of adipocytes with insulin prior to insulin binding leads to a greater loss of cell cell-surface than total receptors. Their study indicated a 62% loss of cell-surface insulin binding. They observed an accumulation of intracellular receptors. However, a loss of 46% in total insulin binding was observed (82). They have suggested that subsequent to internalization, some of the receptors are degraded or processed to a form that does not bind insulin.

In order to determine whether the change in binding affinity in the Fao down regulation experiments was a "stable modification," Crettaz et al (70) measured insulin binding to the cell surface and plasma membrane fractions. Results were similar in

both cases indicating that the increase in binding affinity along with the change in cell surface receptor number is an intrinsic property of the Fao cell line. In the KRC-7 cells binding characteristics as seen from the  $K_d$  values (Table IX) are not affected to a great extent by solubilization. However, there is a decrease in binding affinity in solubilized membrane fractions isolated from down regulated cells. The decrease in high affinity insulin binding can be explained by the reduction in the high affinity receptor number. This would indicate that upon internalization the high affinity insulin receptors do not remain functional in terms of insulin binding. A possible explanation for this could be an alteration in the conformation to a form that is less capable of binding insulin. An alternative explanation could be that the receptors get degraded.

In its native state the insulin receptor in cultured rat hepatoma cells exists in several forms (14,15,16). In the Fao cell line immunoprecipitation studies followed by autoradiography have indicated the presence of insulin receptor forms ranging from 520 kilodaltons to 95 kilodaltons (75). Cells in which down regulation has been achieved have shown not only decreased intensity in the observed bands, but also the disappearance of the 520 kilodalton receptor form (75). Previous work done by the same investigators demonstrated that down regulation increases the insulin binding affinity (70). On comparison of the binding study with the receptor organization the investigators came to the conclu-

sion that that the low affinity receptors are the the high oligomeric receptor forms and these forms get degraded upon down regulation. Cross-linking studies with the same cell line has indicated the presence of four oligomeric forms of receptor (75). This demonstrates that most of the forms of the receptor form in this cell line can bind insulin. As noted earlier, IM-9 lymphocytes, do not show a change in receptor affinity upon down regulation. To study the receptor organization in this cell line immunoprecipitation studies were conducted simultaneously with those for the Fao cell line. The results from this study indicated that down regulation reduces the intensity of the 520 kilodalton band to a greater extent than the other bands. The intensity of all other bands is equally reduced but there is no disappearance of any band due to receptor internalization. So, from this result it can be concluded that in down regulation experiments in which cell surface insulin binding affinity increases, the low affinity receptors are internalized.

In the KRC-7 cells cross-linking experiments have shown the presence of three high oligomeric forms that can bind insulin. In the Fao cells these three high oligomeric forms have been considered to bind insulin with high affinity (75). In KRC-7 cells therefore one could consider these three oligomeric forms to be involved in the high affinity insulin binding sites on the cell surface. In fact, this is exactly what is observed when the competition insulin binding is conducted with the cell surface insulin receptors. Cross-linking has only been conducted with

the control cells, because after down regulation crosslinking the remaining few receptors on the cell surface would be difficult. However, to find the changes that occur in receptor forms upon down regulation one could conduct experiments similar to those done by Crettaz et al (75). The result expected from this down regulation experiment would be the presence of a higher oligomeric receptor form and bands of other oligomeric forms with decreased intensity. This would be due to the decreased high affinity receptor number.

WGA, a plant lectin, increases insulin binding in the KRC-7 cells by two fold (Figure 12). As mentioned earlier, preliminary experiments done in our laboratory have indicated that this increased insulin binding is due to increased binding affinity and not increased receptor number. Similar results have been obtained by several other investigators. Livingston et al (76) using column chromatography and polyacrylamide gel electrophoresis have tried to determine if insulin receptor aggregates are responsible for the increased insulin binding affinity. Insulin receptor clusters were not observed in this study. The authors, concluded that a simple interaction between WGA and the receptor is essential for the binding effect observed in the presence of WGA. A recent report by Wilden et al (77) confirms this observation.

In KRC-7 cells cross-linking experiments done in the presence of WGA show the absence of aggregates of insulin receptor. In keeping with the increase in insulin binding in presence of WGA,

increased intensity in the bands was observed. In one experiment there was an indication of a higher molecular weight band in the presence of WGA (data not presented). In order to confirm if the higher molecular band is real or an experimental artifact, further studies would have to be conducted. Purification of insulin receptor would be the first step in this study. Cross-linking studies done with purified receptor will indicate that the aggregate, if observed, is solely due to the interaction of WGA with the insulin receptor. The purified receptor would be used for insulin binding in presence and in absence of WGA.  $^{125}\text{I}$ -insulin would be cross-linked to the insulin receptor and the cross-linked samples electrophoresed on an SDS polyacrylamide gel to check for the presence of aggregates. If an aggregate is observed, gel filtration chromatography could be used to obtain the various oligomeric forms of insulin receptor. The work would be repeated for each form of the insulin receptor. The results from this experiment would show if receptor aggregation was due to a specific receptor form found in the presence of WGA.

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## APPENDIX

TABLE I. A One Site Model Scatchard Analysis of Cell Surface Insulin Binding to Control Cells and Cells Incubated with Insulin.

| Expt#                                          | - INSULIN   |              |                                                 | + INSULIN   |               |                                  |
|------------------------------------------------|-------------|--------------|-------------------------------------------------|-------------|---------------|----------------------------------|
|                                                | Kd (nM)     | Bmax (nM)    | molecules/mg<br>$\times 10^{11}$                | Kd (nM)     | Bmax (nM)     | molecules/mg<br>$\times 10^{10}$ |
| 1                                              | 7.6 (0.99)  | 0.310 (0.03) | 1.86                                            |             |               |                                  |
| 2                                              | 9.6 (0.77)  | 0.276 (0.02) | 1.66                                            |             |               |                                  |
| 3                                              | 12.7 (2.20) | 0.252 (0.04) | 1.50                                            |             |               |                                  |
| 4                                              | 11.4 (1.71) | 0.315 (0.04) | 1.89                                            | 11.1 (2.20) | 0.106 (0.021) | 6.36                             |
| 5                                              | 11.8 (2.40) | 0.326 (0.05) | 1.95                                            | 16.7 (13.6) | 0.041 (0.032) | 2.46                             |
| 6                                              | 6.7 (0.94)  | 0.249 (0.25) | 1.49                                            | 6.8 (2.72)  | 0.066 (0.022) | 3.96                             |
|                                                |             |              |                                                 |             |               |                                  |
| Avg Kd (nM) = 10 (2.4)                         |             |              | Avg Kd (nM) = 11.5 (4.96)                       |             |               |                                  |
| Avg molecules/mg = $1.73 \times 10^{11}$ (0.2) |             |              | Avg molecules/mg = $4.26 \times 10^{10}$ (1.97) |             |               |                                  |
| Avg receptors/cell = 67,000 (8,000)            |             |              | Avg receptors/cell = 16,000 (7,600)             |             |               |                                  |

TABLE II. A Two Site Model Scatchard Analysis of Cell Surface Insulin Binding to Control Cells.

| <u>- INSULIN</u>     |                     |                       |                     |                       |
|----------------------|---------------------|-----------------------|---------------------|-----------------------|
| <u>High Affinity</u> |                     |                       | <u>Low affinity</u> |                       |
| Expt #               | K <sub>d</sub> (nM) | B <sub>max</sub> (nM) | K <sub>d</sub> (nM) | B <sub>max</sub> (nM) |
| 5                    | 4.6 (1.2)           | 0.13 (0.03)           | 489 (489)           | 3.15 (3.65)           |
| 6                    | 4.6 (1.1)           | 0.17 (0.05)           | 263 (479)           | 0.73 (1.21)           |

TABLE III. Statistical Analysis of Cell Surface Insulin Binding to Control Cells.

| Expt# | Model    | Sum of Squares | D.F. | Mean Square | P     |
|-------|----------|----------------|------|-------------|-------|
| 5     | One Site | 1907           | 11   | 173         | 0.002 |
|       | Two Site | 474            | 9    | 53          |       |
| 6     | One Site | 1008           | 11   | 92          | 0.038 |
|       | Two Site | 489            | 9    | 54          |       |

TABLE IV. A One Site Model Scatchard Analysis of Insulin Binding to Cell Membranes.

| Expt # | <u>- INSULIN</u>    |                       |                                   |
|--------|---------------------|-----------------------|-----------------------------------|
|        | K <sub>d</sub> (nM) | B <sub>max</sub> (nM) | molecules/mg<br>x10 <sup>11</sup> |
| 1      | 4.4 (4.30)          | 0.071 (0.06)          | 0.88                              |
| 2      | 2.4 (0.98)          | 0.129 (0.04)          | 1.60                              |
| 3      | 2.6 (1.36)          | 0.058 (0.02)          | 0.72                              |

Avg K<sub>d</sub> (nM) = 3.1 (1.1)

Avg molecules/mg = 1.06x10<sup>11</sup> (0.47)

Avg receptors/cell = 41,000 (18,000)

TABLE V. A One Site Model Scatchard Analysis of Insulin Binding to Solubilized Receptors from Membrane Extracts; Control Cells and Cells Incubated with Insulin.

| Expt # | - INSULIN           |                       |                                   | + INSULIN           |                       |                                   |
|--------|---------------------|-----------------------|-----------------------------------|---------------------|-----------------------|-----------------------------------|
|        | K <sub>d</sub> (nM) | B <sub>max</sub> (nM) | molecules/mg<br>x10 <sup>11</sup> | K <sub>d</sub> (nM) | B <sub>max</sub> (nM) | molecules/mg<br>x10 <sup>11</sup> |
| 1      | 1.71 (0.86)         | 0.068 (0.02)          | 3.02                              |                     |                       |                                   |
| 2      | 1.40 (0.66)         | 0.065 (0.02)          | 2.89                              |                     |                       |                                   |
| 3      | 4.50 (1.58)         | 0.061 (0.02)          | 2.04                              |                     |                       |                                   |
| 4      | 3.11 (1.59)         | 0.040 (0.02)          | 1.60                              |                     |                       |                                   |
| 5      | 5.74 (3.78)         | 0.074 (0.04)          | 2.96                              |                     |                       |                                   |
| 6      | 1.62 (0.70)         | 0.053 (0.02)          | 2.36                              | 66.30 (80.22)       | 0.46 (0.63)           | 18.4                              |
| 7      | 2.04 (1.37)         | 0.057 (0.03)          | 2.28                              | 7.65 (6.27)         | 0.06 (0.05)           | 2.4                               |

Avg K<sub>d</sub> (nM) = 2.87 (1.67)  
 Avg molecules/mg = 2.45x10<sup>11</sup> (0.53)  
 Avg receptors/cell = 94,000 (20,000)

TABLE VI. A Two Site Model Scatchard Analysis of Insulin Binding to Solubilized Receptors from Membrane Extracts using Control Cells.

| <u>- INSULIN</u> |                      |                       |                     |                       |
|------------------|----------------------|-----------------------|---------------------|-----------------------|
| Expt#            | <u>High Affinity</u> |                       | <u>Low Affinity</u> |                       |
|                  | K <sub>d</sub> (nM)  | B <sub>max</sub> (nM) | K <sub>d</sub> (nM) | B <sub>max</sub> (nM) |
| 1                | 0.64 (0.77)          | 0.03 (0.02)           | 83.0 (176.8)        | 0.67 (0.58)           |
| 2                | 0.30 (0.44)          | 0.02 (0.02)           | 19.6 ( 31.2)        | 0.23 (0.24)           |
| 4                | 1.50 (1.14)          | 0.02 (0.01)           | 2150 (14427)        | 22.0 ****             |

TABLE VII. Statistical Analysis of Insulin Binding to Solubilized Receptors from Membrane Extracts using Control Cells.

| Expt # | Model    | Sum of Squares | D.F. | Mean Squares | P    |
|--------|----------|----------------|------|--------------|------|
| 1      | One Site | 924            | 11   | 84           | 0.22 |
|        | Two Site | 664            | 9    | 74           |      |
| 2      | One Site | 1951           | 11   | 177          | 0.14 |
|        | Two Site | 1254           | 9    | 139          |      |
| 4      | One Site | 430            | 11   | 39           | 0.17 |
|        | Two Site | 291            | 9    | 32           |      |

TABLE VIII.

| Concentration       | Avg Kd (nM) | Avg molecules/mg           | Avg receptors/cell | Avg Kd (nM) | Avg molecules/mg             | Avg receptors/cell |
|---------------------|-------------|----------------------------|--------------------|-------------|------------------------------|--------------------|
| 10 <sup>-10</sup> M | 7.66 (1.95) | 1.6x10 <sup>10</sup> (0.3) | 62,000 (12,000)    | 43.7 (35)   | 3.73x10 <sup>11</sup> (3.64) | 144,000 (140,000)  |

TABLE IX. Summary of Scatchard Analyses of Insulin Binding to Cell Surface, Particulate Cell Membranes and Solubilized receptors; Control Cells and Cells Incubated with Insulin.

|                              | <u>- INSULIN</u>    |                                    | <u>+ INSULIN</u>    |                                    |
|------------------------------|---------------------|------------------------------------|---------------------|------------------------------------|
|                              | K <sub>d</sub> (nM) | receptors/cell<br>x10 <sup>3</sup> | K <sub>d</sub> (nM) | receptors/cell<br>x10 <sup>3</sup> |
| Cell Surface                 | 10.0 (2.4)          | 67 (8)                             | 11.5 (5.0)          | 16 (7.6)                           |
| Particulate<br>Membranes     | 3.1 (1.1)           | 41 (18)                            |                     |                                    |
| Solubilized (a)<br>Receptors | 2.9 (1.7)           | 94 (20)                            |                     |                                    |
|                              | (b) 7.7 (2.0)       | 62 (12)                            | 43.7 (35)           | 144 (140)                          |

(a) = Plasma membrane prep.

(b) = Whole cells.

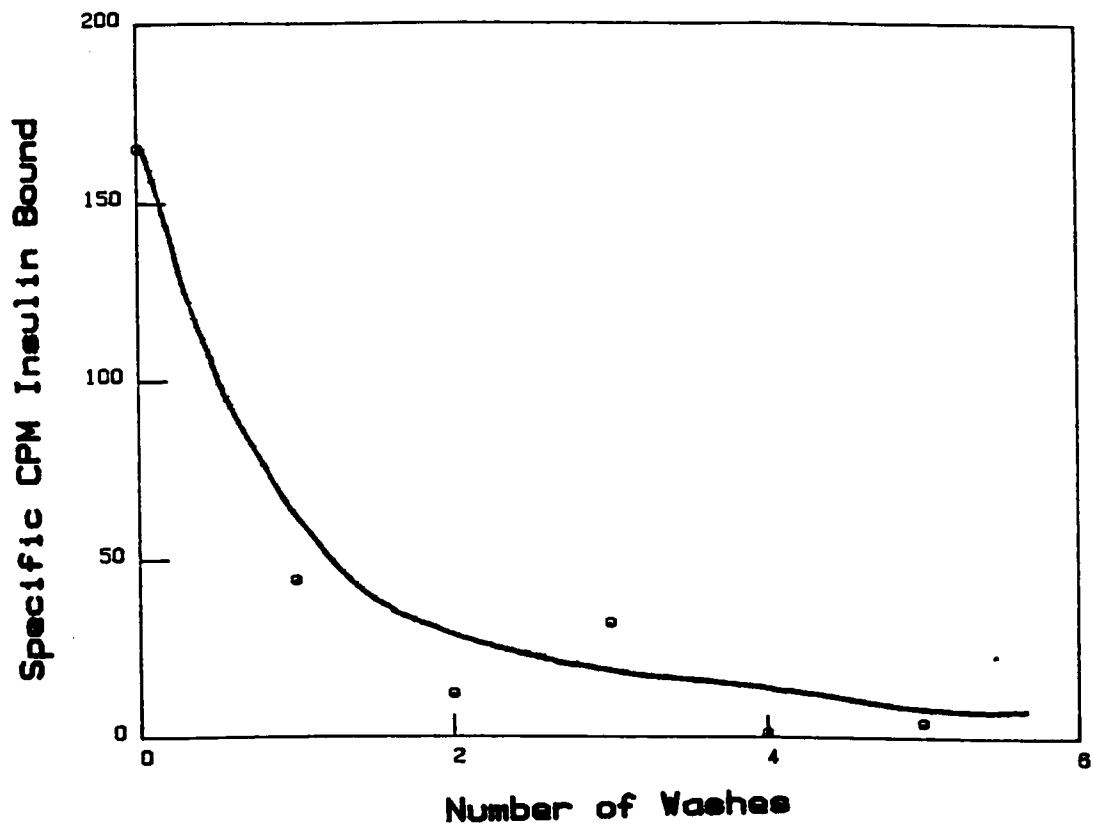


FIG. 1. Efficiency of Wash Procedure. Specific  $^{125}\text{I}$ -insulin bound is plotted as a function of number of washes. Confluent monolayers of KRC-7 cells in 12x24 mm dishes were incubated with insulin at  $37^{\circ}$  for 24 hours as mentioned under "Materials and Methods." As indicated cells were washed at different times prior to performing insulin binding studies.

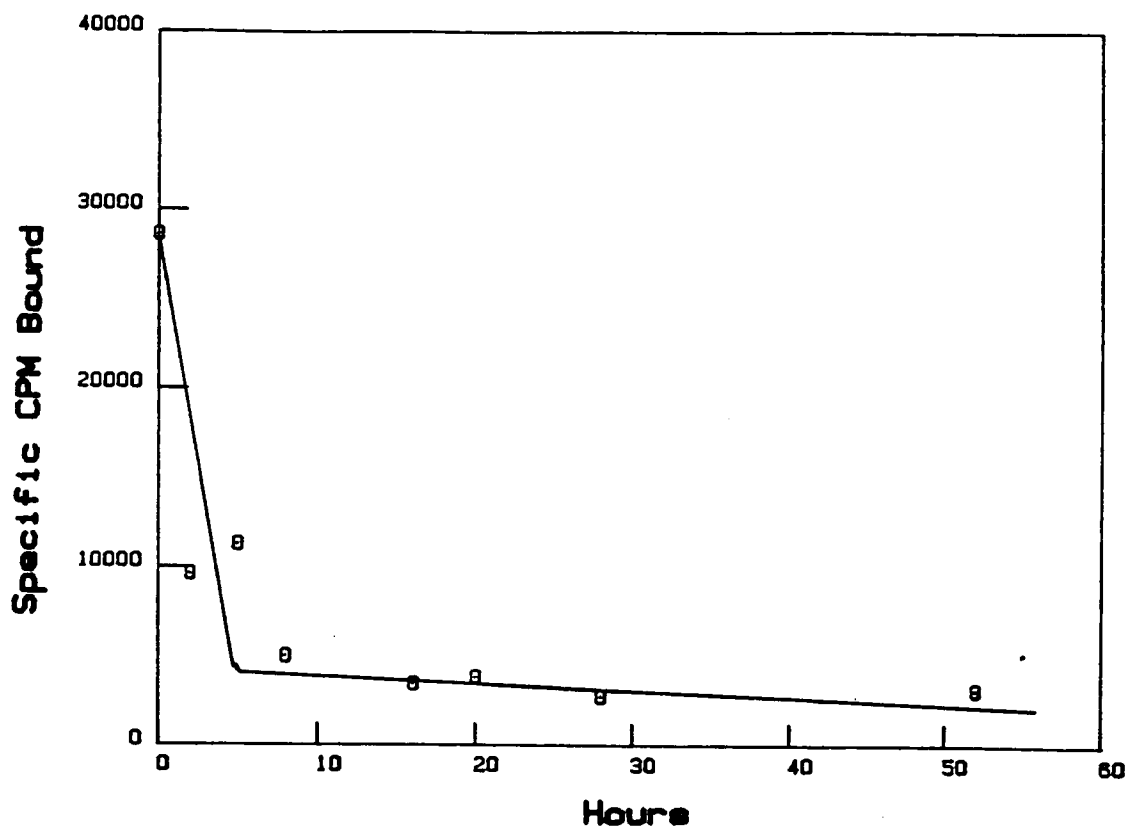


FIG. 2. Time Course for Insulin Induced Down-Regulation in KRC-7 Cells. 12x24 mm dishes with confluent monolayers of cells were incubated with 10  $\mu\text{g/ml}$  unlabeled insulin for the indicated time periods at 37°C. Insulin binding assay was performed as described under materials and methods.

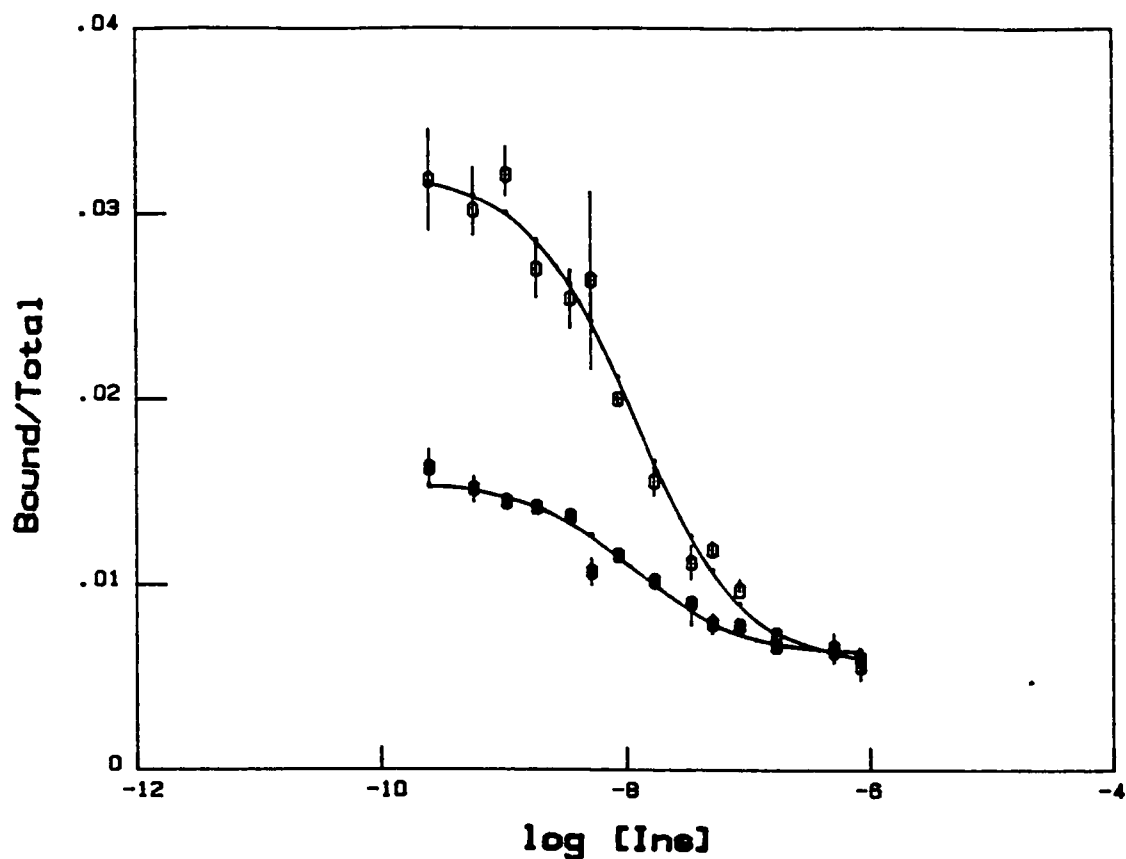


FIG. 3. Displacement of  $^{125}\text{I}$ -insulin by Unlabeled Insulin from KRC-7 Cell Surface. Competitive inhibition insulin binding assay for control and down regulated cells was at performed  $^{\circ}\text{C}$  for at least 16 hours. Bound and free  $^{125}\text{I}$ -insulin was separated as mentioned under materials and methods. Open circles (o) represent data points for control cells and solid circles (●) for down regulated cells. Each point represents an average of triplicate data points. Error bars indicate standard deviation for each point.

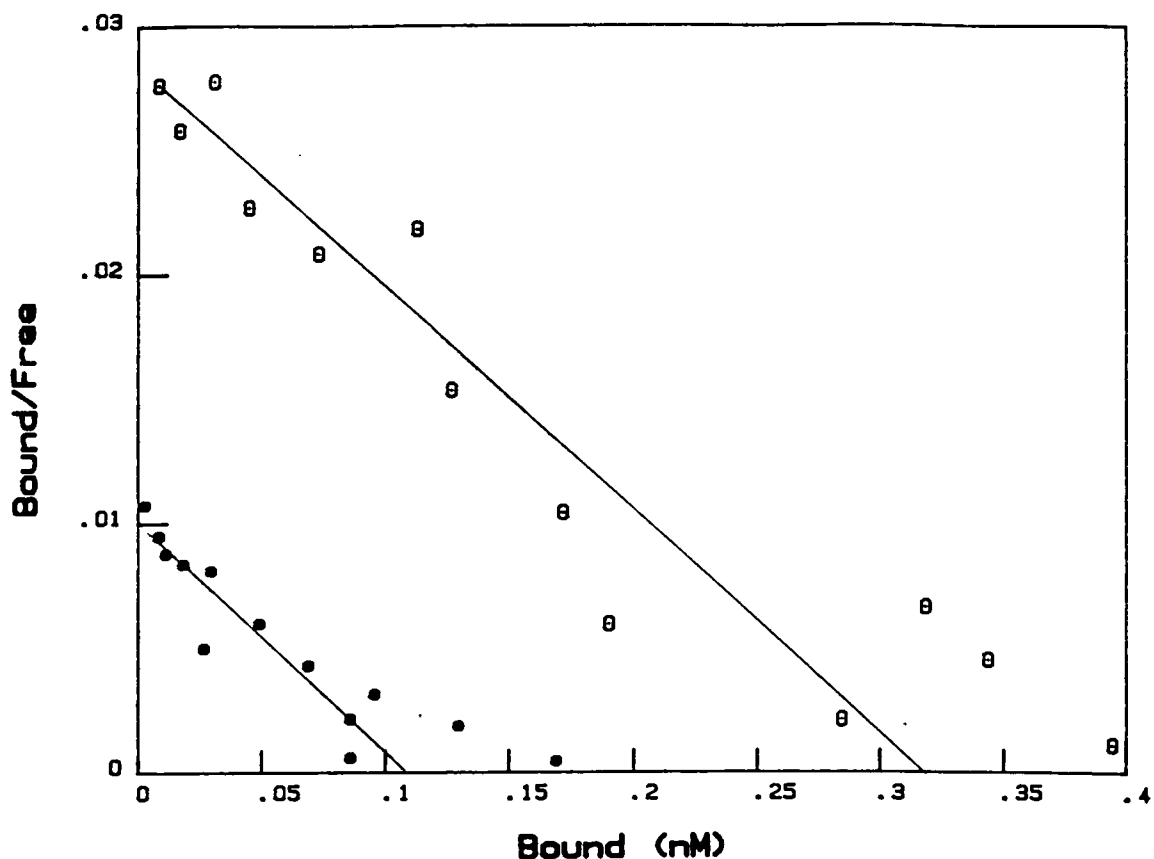


FIG. 4. Scatchard Plot for Cell Surface Insulin Binding to Control and Down-Regulated Cells. Open circles (o) represent control cells and solid circles (●) down regulated cells. Bound/Free  $^{125}\text{I}$ -insulin plotted as a function of Bound  $^{125}\text{I}$ -insulin. For control cells,  $K_d = 11.4 \pm 1.71$  nM and  $B_{\text{max}} = 0.315 \pm 0.04$  nM and for down-regulated cells,  $K_d = 11.1 \pm 2.20$  and  $B_{\text{max}} = 0.106 \pm 0.021$ . Receptor number per cell for control cells =  $73,000 \pm 9,000$ . For cells incubated with insulin, receptor number per cell =  $24,000 \pm 4,800$ .

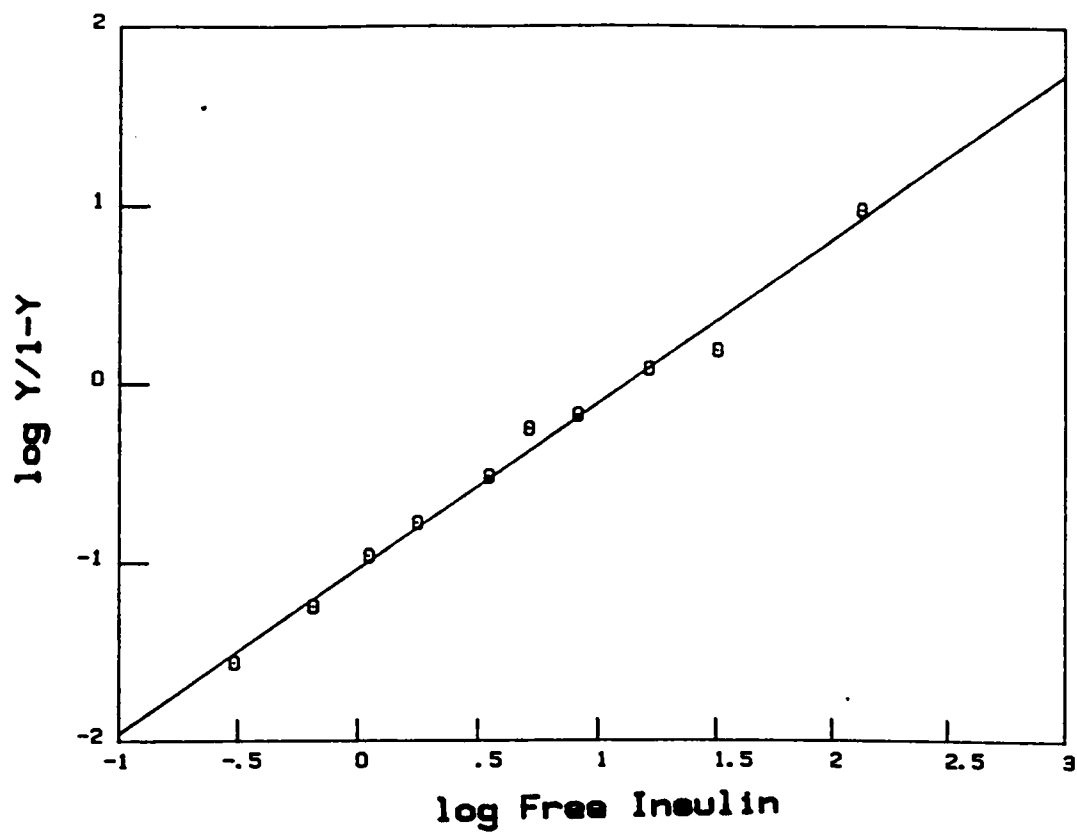


FIG. 5. Hill Plot derived from control cell surface insulin binding data of Figure 4.  $n_H = 0.92$ .

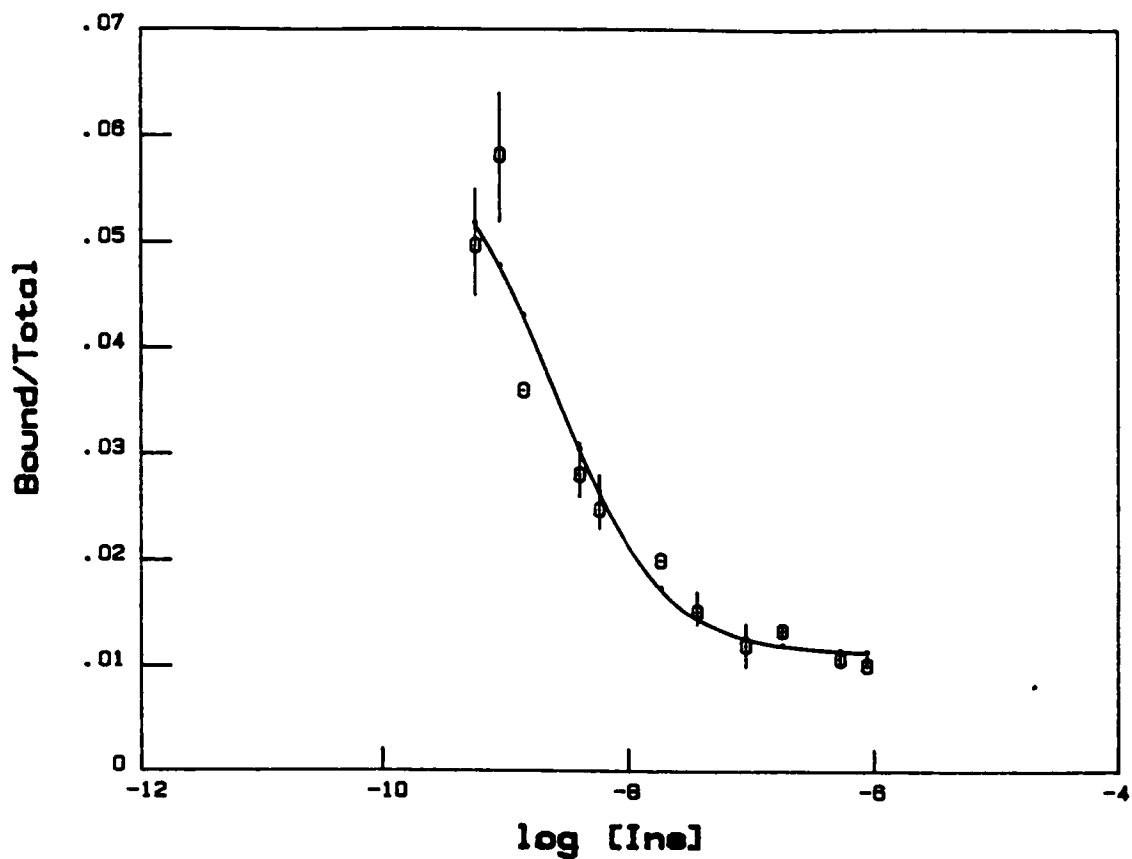


FIG. 6. Displacement of  $^{125}\text{I}$ -insulin as a Function of Increasing Concentrations of Unlabeled Insulin from the Particulate Cell Membranes. Cell membranes were prepared and insulin binding was performed as mentioned under "Materials and Methods." Each data point is an average of triplicates and error bars represent standard deviation of each point.

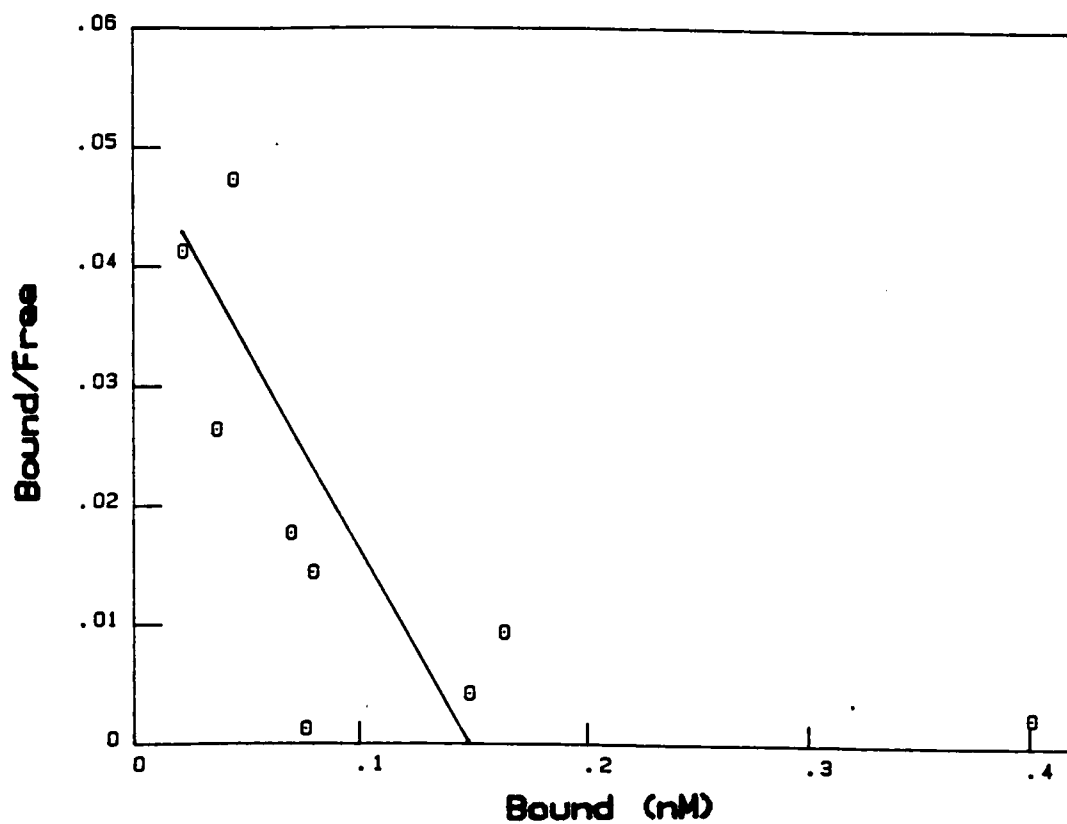


FIG. 7. Scatchard Plot for Insulin binding to the KRC-7 Particulate Cell Membrane. Bound/Free  $^{125}\text{I}$ -insulin plotted as a function of Bound  $^{125}\text{I}$ -insulin.  $K_d = 2.4 \pm 0.98$ ,  $B_{\text{max}} = 0.129 \pm 0.04$  nM. Receptor number per cell =  $62,000 \pm 19,000$ .

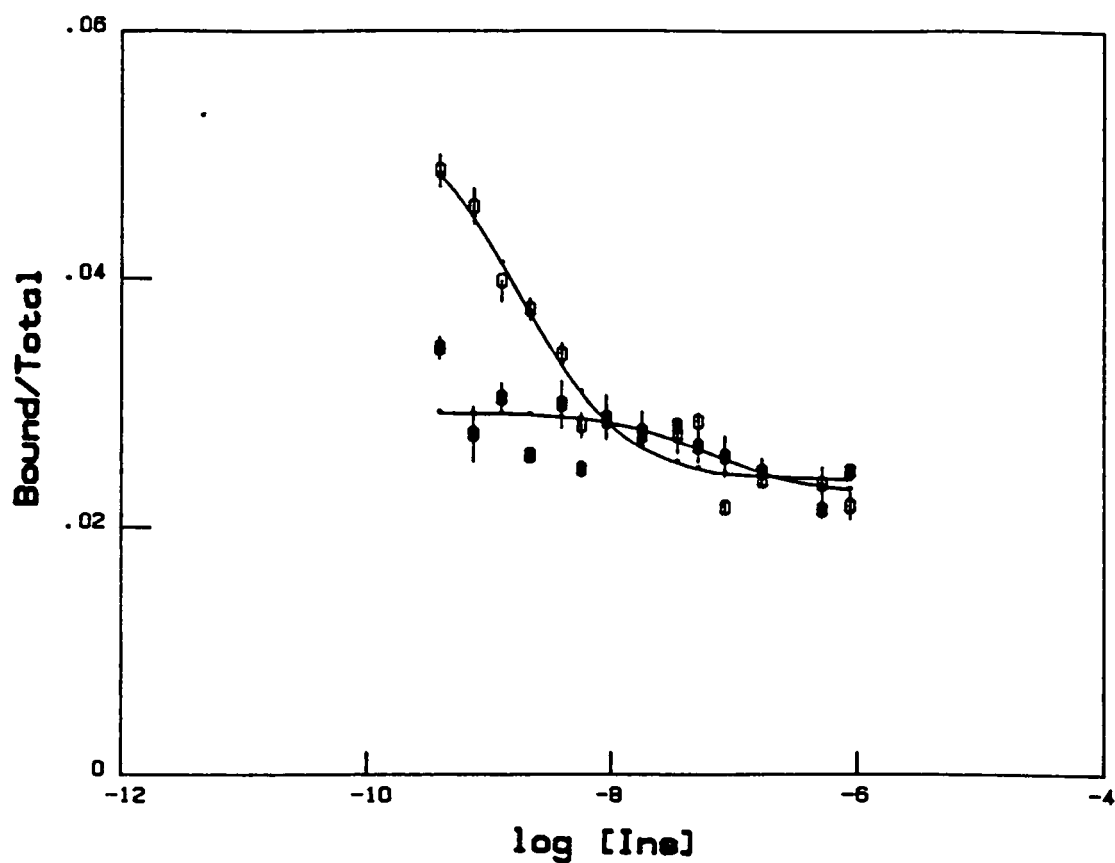


FIG. 8. Displacement of  $^{125}\text{I}$ -insulin from the Solubilized Receptor (membrane extracts) as a Function of Concentrations of Unlabeled Insulin. Open circles (○) represent solubilized receptor from control cells and solid circles (●) from down-regulated cells. Each point is an average of triplicate data points and the error bars represent standard deviation of the data points.

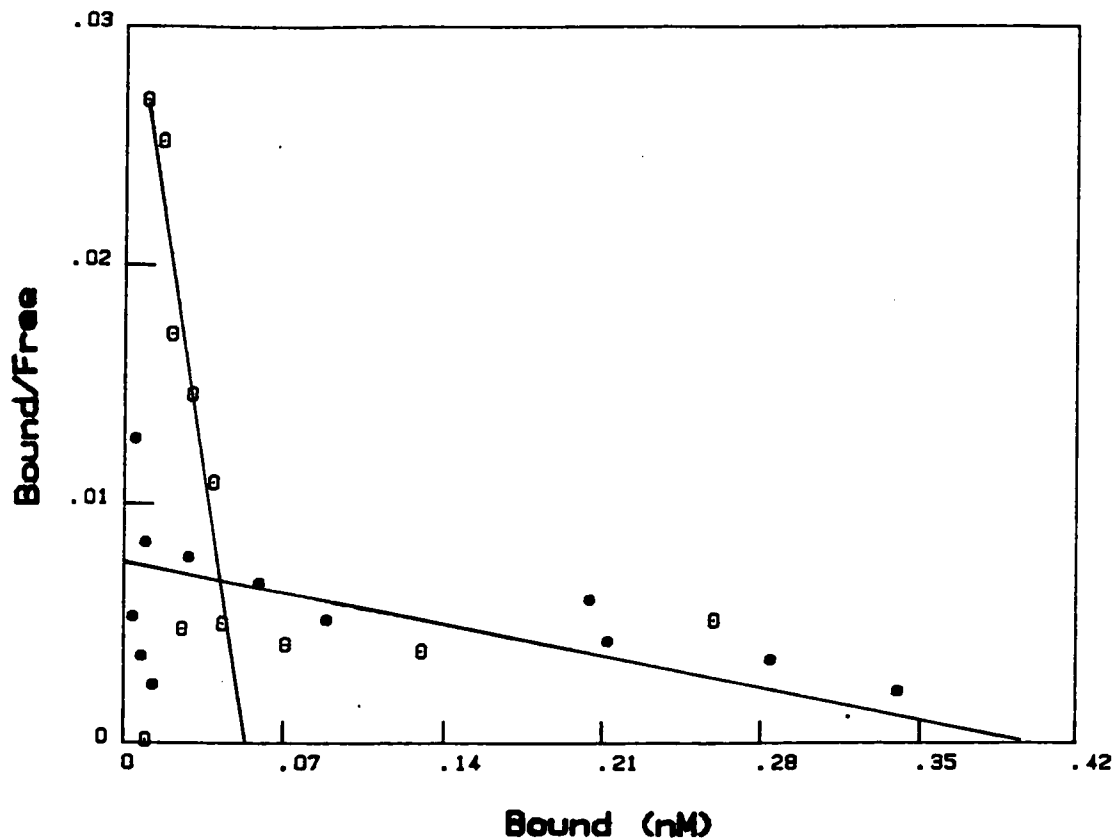


FIG. 9. Scatchard Plot for Insulin Binding to Solubilized Receptor from Membrane Extracts. Open circles (o) represent control and solid circles (●) downregulated cells. From the control experiment,  $K_d = 1.62 \pm 0.70$  nM,  $B_{max} = 0.053 \pm 0.02$  nM and from the down regulation experiment,  $K_d = 66.30 \pm 80.22$  nM and  $B_{max} = 0.46 \pm 0.63$  nM. Receptor number per cell for control cells =  $91,000 \pm 34,000$ . For cells incubated with insulin, receptor number per cell =  $71,000 \pm 97,000$ .

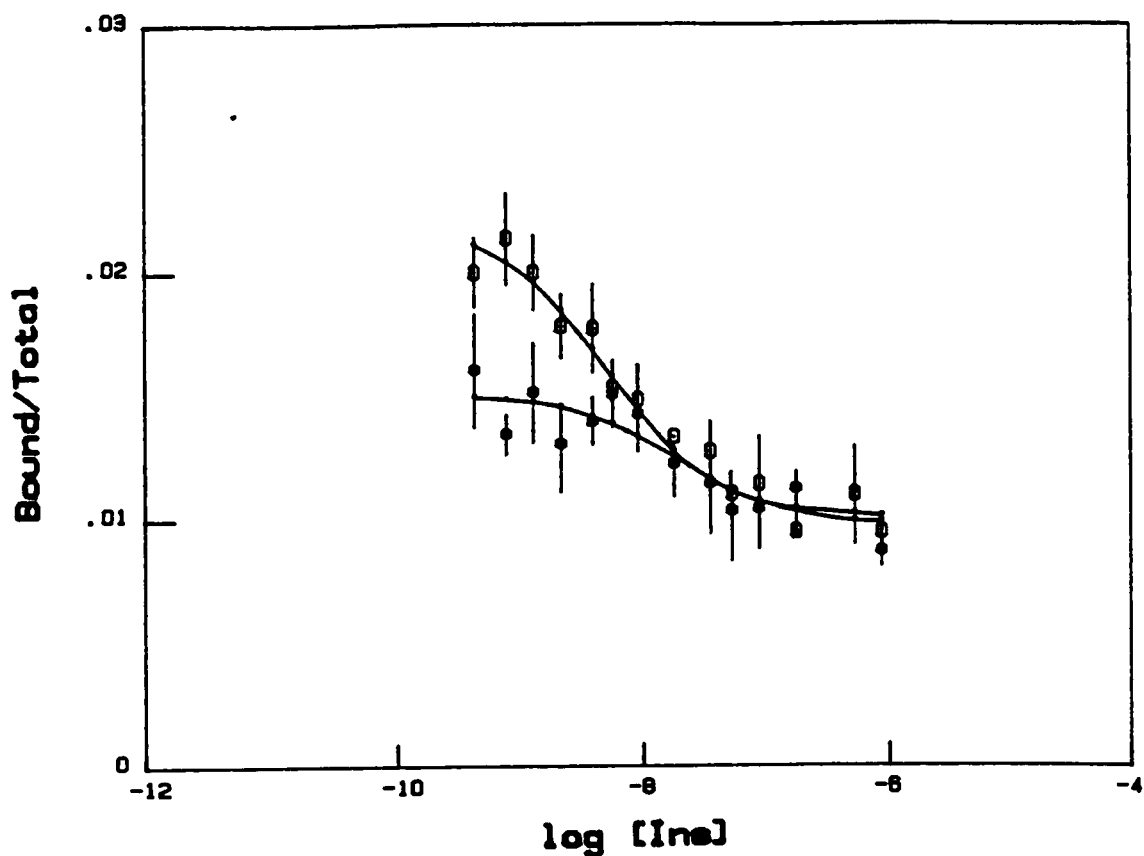


FIG. 10. Displacement of  $^{125}\text{I}$ -insulin by Unlabeled Insulin from Solubilized Receptor (Whole Cells). Open circles (o) represent control cells and solid circles (●) down regulated cells. Each point represents an average of triplicate data points and error bars represent standard deviation of each data point.

Solubilized receptor preparation and competition inhibition insulin binding assay was performed as mentioned under "Materials and Methods."

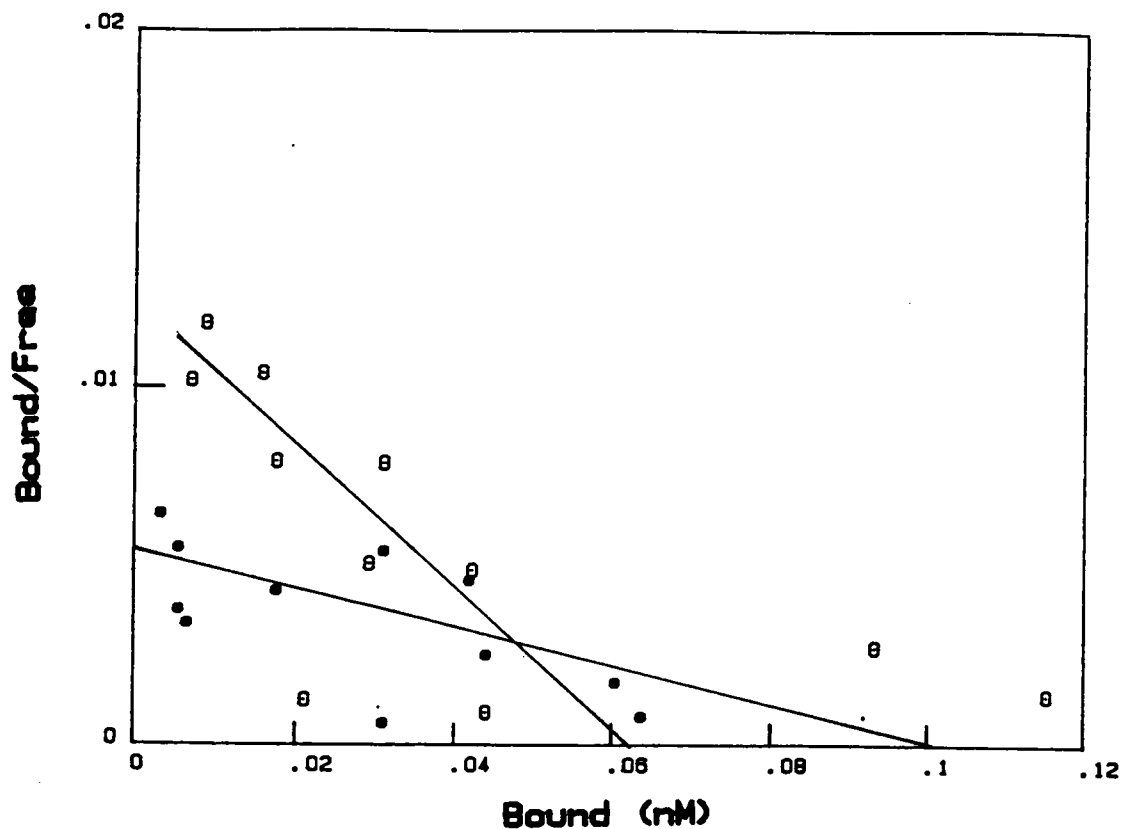


FIG. 11. Scatchard Plot for Insulin Binding to Solubilized Receptor from Intact Cells. Cells were incubated without, open circles (o) and with insulin, solid circles (●). For control cells,  $K_d = 5.00 \pm 1.70$  nM and  $B_{max} = 0.062 \pm 0.02$  nM. For cells incubated with insulin,  $K_d = 19.00 \pm 12.35$  nM, and  $B_{max} = 0.102 \pm 0.07$  nM. Receptor number per cell for control cells =  $50,000 \pm 16,000$ . For cells incubated with insulin, receptor number per cell =  $83,000 \pm 30,000$ .

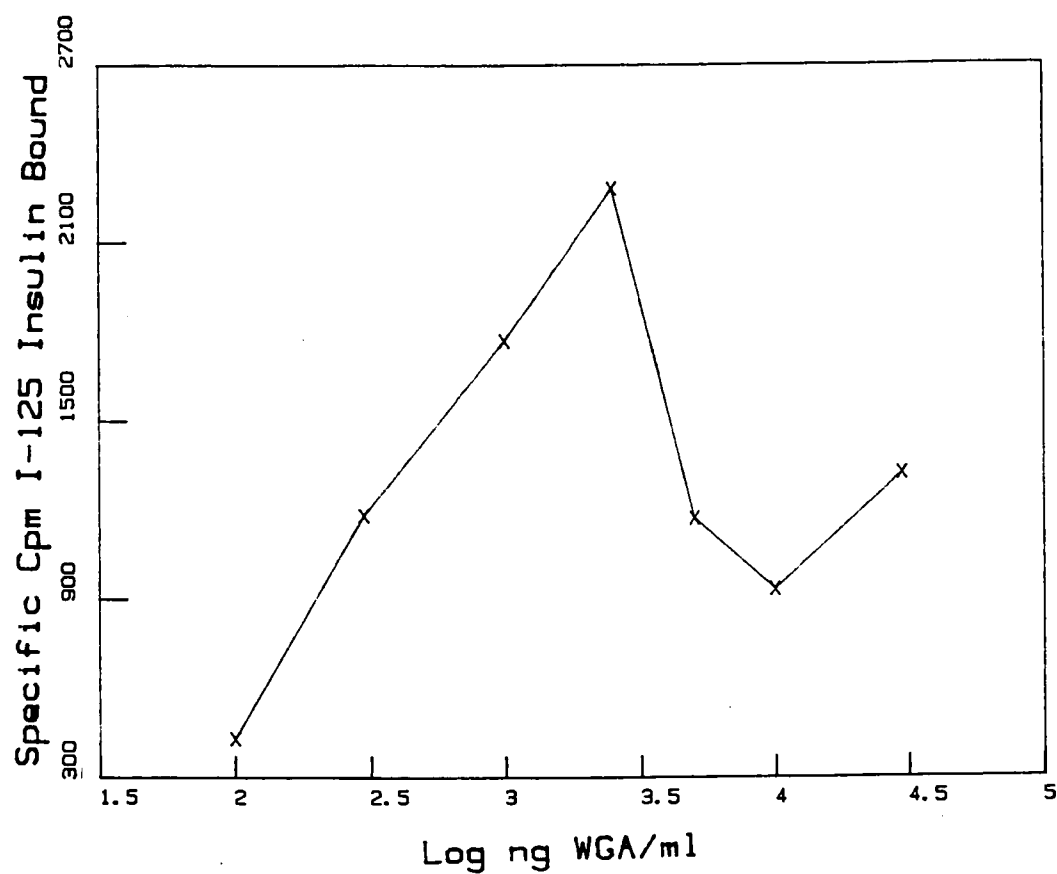


FIG. 12. Effect of WGA on the Specific Binding of  $^{125}\text{I}$ -insulin to KRC-7 Cell Surface Insulin Receptor. Specific insulin bound in the presence of increasing concentrations of WGA .

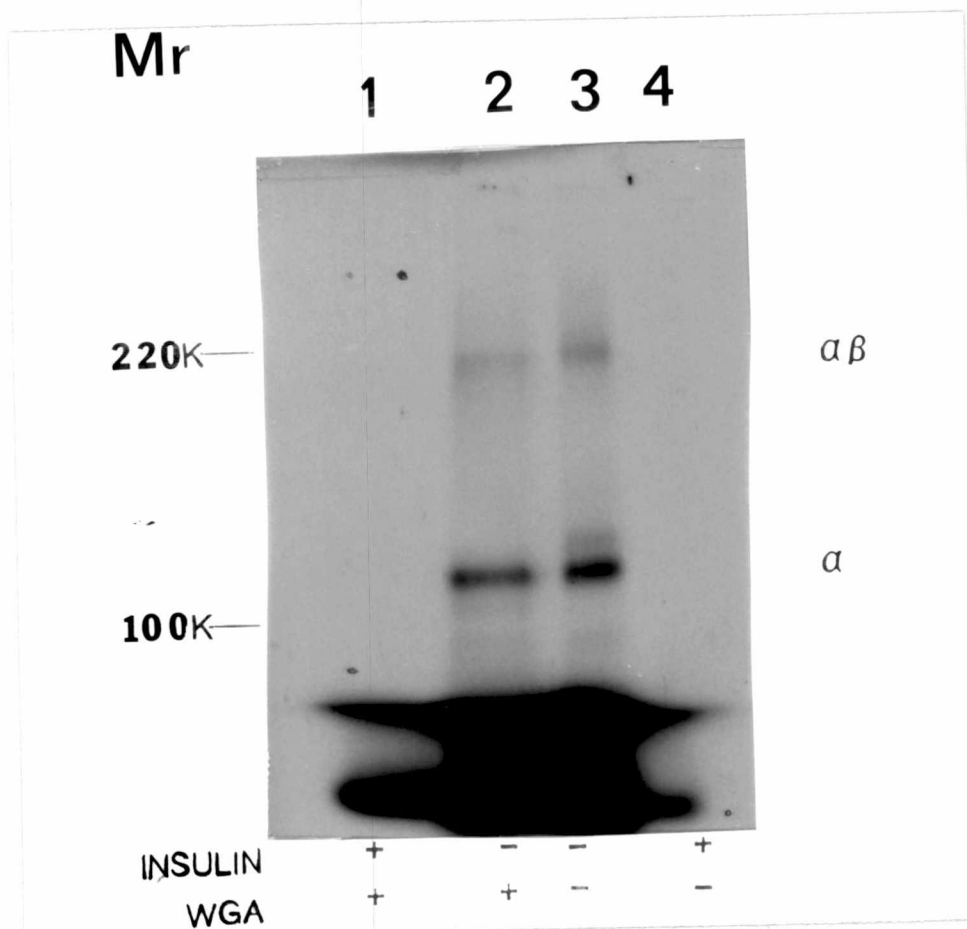


FIG. 13. Migration of the Reduced Receptor in the Presence and Absence of WGA. Cross-linked insulin receptor was incubated with 5% of  $\beta$  mercaptoethanol, and electrophoresed in the reduced state in a 4 stacking gel and 7.5% running SDS-polyacrylamide gel as described under "Materials and Methods." The receptor bands were visualized by autoradiography.

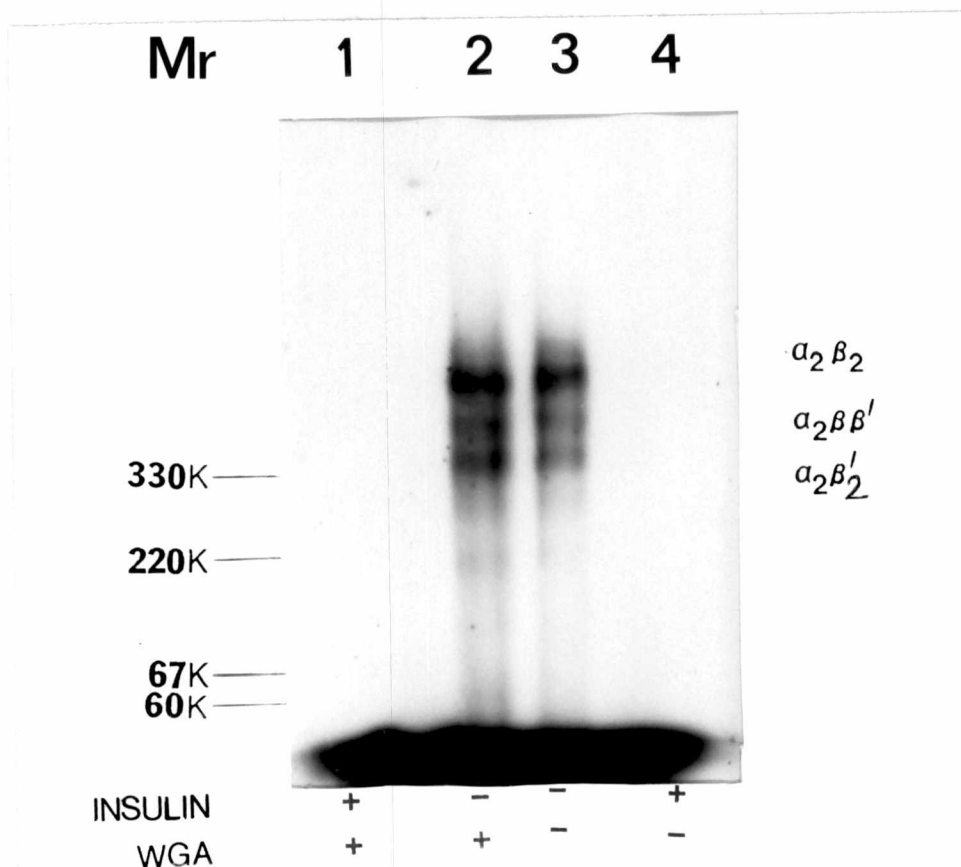


FIG. 14. Comparison of the  $^{125}\text{I}$ -insulin Affinity Cross-linked KRC-7 Cell Surface Receptors in the Presence and Absence of WGA. Cell surface insulin receptors were cross-linked with 40 ng/ml  $^{125}\text{I}$ -insulin using 1 mM disuccinimidyl suberate as described under "Materials and Methods."  $^{125}\text{I}$ -insulin affinity cross-linking to the insulin receptors was performed after insulin binding was conducted at  $4^{\circ}\text{C}$  for 16 hours in the presence of 1  $\mu\text{g}/\text{ml}$  unlabeled insulin and 2.5  $\mu\text{g}/\text{ml}$  WGA (lane 1), in the absence of insulin and presence WGA (lane 2), in the absence of insulin and WGA (lane 3) and in the presence of unlabeled insulin but absence of WGA (lane 4). The receptor bands were visualized by nonreducing SDS-polyacrylamide gel electrophoresis and autoradiography.

## VITA

Lajwanti Sobhrajmal Khemlani was born in April 1960 in Poona, in the state of Maharashtra, India. She was brought up and received her original education in that city.

Her secondary education was at Rosary High School and her higher secondary education at Saint Annes School and Nowrosjee Wadia college. She did her undergraduate studies at Wadia College and Ferguson college receiving a Bachelors degree in Chemistry from Poona University in 1982.

The author arrived in the United States on December 24, 1984 and was awarded a Bachelor's degree with high honors in Biology (Organismal & Systems) by the University of Tennessee in the summer of 1986.

She started her graduate studies in the Biochemistry department in the fall of 1986 and has been working on the research presented in this thesis, in the laboratory of Dr. John Koontz, since the summer of 1987.

She intends to work towards a Ph.D. in Dr. J. E. Churchich's laboratory.