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**LA THÈSE A ÉTÉ  
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**Studies on Regulation of Insulin Receptors in  
Cultured Human Diploid Fibroblasts**

**Alice Michaelidou**

**A Thesis  
in  
The Department  
of  
Biology**

**Presented in Partial Fulfillment of the Requirements  
for the Degree of Master of Science at  
Concordia University  
Montréal, Québec, Canada**

**July 1985**

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Studies on Regulation of Insulin Receptors in  
Cultured Human Diploid Fibroblasts

Alice Michaelidou

Cultured human diploid fibroblasts bind insulin via specific receptors which are susceptible to temperature, pH and nutritional changes. Glucose, the major energy source for living cells, has a regulatory effect on specific insulin binding to these receptors.

Cells placed in medium containing high concentrations of glucose express higher trace binding capacity than cells placed in media containing lower concentrations of sugar.

Complete glucose starvation decreases insulin binding significantly over a time period of 12 to 18 hours and this effect is reversible within 6 hours by glucose refeeding. The reversibility of the glucose starvation effect on insulin binding by glucose refeeding is blocked by cycloheximide an inhibitor of protein synthesis, and tunicamycin, an inhibitor of glycoprotein synthesis, but not by the RNA synthesis inhibitor Actinomycin D. This suggests that the macromolecular events involved in the glucoregulatory effect on insulin binding involve protein synthesis and glycoprotein synthesis but not RNA synthesis.

Additionally, the action of insulin on sugar transport in normal human fibroblasts, after the six hours of glucose refeeding, has been studied.

It was observed that glucose deprivation as opposed to glucose feeding increased sugar transport. After 12 hours of glucose starvation, glucose-refed cells expressed increased transport when insulin-stimulated. Cycloheximide inhibited depression of transport in continuously fed and reversed cells, and it also inhibited insulin induced increase in sugar transport.

When tunicamycin was added, transport remained at the controls' level in all groups, (i.e. continuously fed, reversed and reversed and insulin-stimulated cells). This indicated that glycoprotein synthesis is not necessary for glucose induced depression of sugar transport in fed or reversed cells. Also, glycoprotein synthesis is not required for insulin stimulation of sugar transport.

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To my Parents

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## INTRODUCTION

Glucose concentration (Howard et al., 1979; Ishibashi et al., 1982; Germinario et al., 1984) and serum concentration in the culture medium for human fibroblasts (HF) (Germinario et al., 1984) have been shown to influence the activation of the cell's sugar transport mechanism by insulin.

The magnitude of the sugar transport stimulation is related to the amount of insulin given to the cells (Germinario et al., 1984). The amount of insulin transported to the intracellular compartment is measured by means of specific binding of  $^{125}\text{I}$ -insulin to the cells. Specific insulin binding, in turn, is directly related to the amount of glucose present in the medium. In a glucose concentration range from 5.55 mM to 22.2 mM in Chinese hamster kidney cells (Wyse and Chang, 1981) and from 0 mM to 22.2 mM in HF (Germinario et al., 1984) binding was elevated in the fed vs starved or poorly fed cells. This glucose effect was reversible within 6 hours after glucose refeeding (Germinario et al., 1984).

Additionally, glucose concentration has an inverse effect on hexose transport, increasing the transport when the concentration is decreasing. This glucose-deprivation effect is also reversible within 6 hours after changing the medium conditions by glucose readdition. Furthermore, regulation of insulin binding to its receptor has been studied under conditions of serum starvation, glucose starvation and reversal from the glucose-starved to the glucose-fed state.

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Additionally, the effect of inhibitors for protein (i.e. cycloheximide), RNA (i.e. actinomycin D), and glycoprotein (i.e. tunicamycin) synthesis on insulin binding and transport changes during this reversal of glucose starvation was investigated.

### Structure and Chemical Composition of the Receptor

The first step for peptide hormones to exert their actions on target cells is to bind to specific receptor sites located on the cell membrane.

The receptor for insulin (Cuatrecasas, 1972) is an intrinsic membrane glycoprotein complex (Rosen et al., 1979; Reed et al., 1981; Ronnet and Lane, 1981; Hedo et al., 1983; Fujita-Yamaguchi, 1984) of approximately 350,000 M.W. (Cuatrecasas, 1972a; Massague et al., 1981; Pollet et al., 1981) and its structure has been studied by a variety of techniques including immunoprecipitation by antireceptor antibody (Kasuga et al., 1982a; Roth et al., 1982; Hedo et al., 1983) two dimensional and SDS-polyacrylamide gel electrophoresis (Jacobs et al., 1980; Massague et al., 1980; Pollet et al., 1982; Fujita-Yamaguchi, 1984) photoaffinity labeling (Berhanu et al., 1982; Fehlmann et al., 1982; Roth et al., 1982), isoelectric focusing, peptide mapping and amino acid analysis (Fujita-Yamaguchi, 1984). Various tissues and cell systems have been employed for these studies such as human placental membranes (Jacobs et al., 1979; Massague et al., 1980; Fujita-Yamaguchi, 1984), hepatocytes (Fehlman et al., 1982) IM-9 cultured human lymphocytes (Kasuga et al., 1982; Pollet et al., 1982;

Hede et al., 1983), rat adipocytes (Massague et al., 1980; Berhanu et al., 1982), rat kidney and lung membranes (Massague et al., 1980) and liver plasma membranes (Jacobs et al., 1979; Jacobs et al., 1980; Massague et al., 1980; Fehlmann et al., 1982).

Subunits of various molecular weights have been isolated; Jacobs et al. (1979, 1980) isolated two subunits (135,000 and 45,000 M.W.) from rat liver and human placental membranes; Kasuga et al. (1982) reported three subunits of 135,000, 95,000 and 210,000 M.W. in IM-9 lymphocytes. Pollet et al. (1982) observed, in the same tissue, a 310,000 M.W. multimeric unit which segregates and produces two components of 170,000 M.W. Each of these latter components, yields, after reduction, subunits of 120,000 and 50,000 M.W. respectively. Pilch and Czech (1980) isolated two subunits of 125,000 and 300,000 M.W. from fat cells and liver plasma membranes, whereas Massague et al. (1980) found, in a variety of tissues, three major complexes of 350,000 320,000 and 220,000 M.W. under non-reducing conditions. These complexes exhibited a combination of three subunits of 125,000-135,000 M.W.  $\alpha$ , 90,000 M.W.  $\beta$  and 45,000-49,000  $\beta_1$ . In accordance with the previous findings, is Fujita-Yamaguchi's observation (1984) of three, 125,000  $\alpha$ , 90,000  $\beta$ , and 50,000  $\beta_1$  M.W. subunits.

During affinity labelling studies (Jacobs et al., 1979; Fujita-Yamaguchi, 1984), the  $\alpha$  subunit is the predominantly labelled one and for that reason it has been suggested that this is the one necessary for binding the insulin component. Both  $\beta$  and  $\beta_1$  subunits however, are also occasionally labelled (Fujita-Yamaguchi,

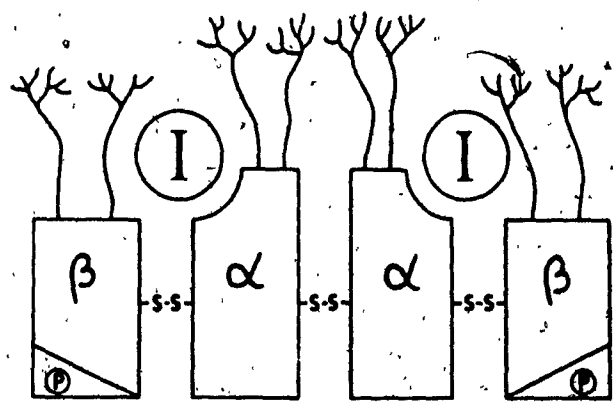
1984) and they may also have binding activity. It has been proposed that  $\beta_1$  is a derivative of  $\beta$ , because after proteolytic digestion,  $\beta$  yields a peptide similar to the  $\beta_1$  subunit (Massague et al., 1981; Fujita-Yamaguchi, 1984). Furthermore, peptide mapping (Massague et al., 1981; Fujita-Yamaguchi, 1984) showed a considerable homology between  $\beta$  and  $\beta_1$  and digestion of  $\beta$  with elastase generates a peptide similar to  $\beta_1$  (Massague et al., 1981). In all studies concerning the structure of the receptor, it has been shown that the peptide subunits are linked with disulfide bonds covalently at a predominant state (Massague et al., 1981; Fujita-Yamaguchi, 1984). In a less frequent, potentially reduced state, the  $\alpha$ - $\beta$  bonds are reduced (Jacobs et al., 1980).

The intact receptor has been shown to be a tetramer consisting of two  $\alpha$  and two  $\beta$  subunits (Jacobs, et al., 1980; Massague et al., 1980; Pilch and Czech, 1980a; Massague et al., 1981) which when treated with reducing agents, generates the individual subunits. It has been proposed that the basic structure of the native receptor is  $\alpha_2\beta_2$  (Jacobs et al., 1980; Fujita-Yamaguchi, 1984) and that it undergoes conformational changes when insulin binds to it (Ginsberg et al., 1976; Pilch and Czech, 1980) (Fig. 1). The dimeric  $[\beta-\alpha]-[\alpha-\beta]$  structure of the receptor gives it a bilateral symmetry. This, along with indirect evidence (Flier et al., 1976; Harrison et al., 1979) provided by antireceptor antibody techniques suggest a more-than-one molecule binding structure. In order to elucidate the biochemical nature of the receptor reactions with various substances such as

Fig. 1 Model of Insulin Receptor Structure (Gammeltoft, 1984).

The four subunits of the receptors are connected with disulfide bonds (-S-S-): The  $\alpha$  subunits contain the insulin (I) binding sites; the  $\beta$  subunits contain the ATP binding sites. (Roth et al., 1983) for phosphorylation (P) of a tyrosine residue.

FIGURE 1





trypsin, phospholipases, organic solvents (Cuatrecasas, P., 1972), neuroaminidases (Cuatrecasas and Illiano, 1971), lectins such as wheat germ agglutinin (Cuatrecasas and Tell, 1973; Marsh et al., 1984) and concanavalin A (Cuatrecasas and Tell, 1973), inhibitors such as tunicamycin (Rosen et al., 1979; Reed et al., 1981; Ronnet and Lane, 1981) and amino acid analyses (Fujita-Yamaguchi, 1984) have shown clearly that the insulin receptor is a glycoprotein. Further, this is supported by the observation of the incorporation of galactose, glucosamine, mannose and fucose into both subunits (Hedo et al., 1983).

The major role of the receptor is transmission of information from the extracellular to the intracellular environment. The question raised was whether it is a transmembrane protein.

Since carbohydrate moieties of cell membrane glycoproteins are located on the external surface, all receptor subunits are most likely exposed to the extracellular environment; affinity-labelling studies with insulin under conditions in which the hormone was unable to have access to the interior of the cell supported this view (Massague et al., 1980, 1981), since all subunits were labelled with the hormone. On the other hand, it was found that only the ( $\beta$ ) subunit is exposed to the interior of the cell whereas the ( $\alpha$ ) subunit does not appear to be a transmembrane component (Hedo et al., 1982).

Insulins of various species, or chemically altered insulins vary widely in their biological potentials in vitro. These differences are due to their differences in affinity for binding to the receptor

(Kosmakos and Roth, 1980). The insulin receptors of different species, including those of cultured human IM-9 lymphocytes, rat fat cells, mouse liver membranes, RPMI 6237 human lymphocytes, turkey erythrocytes in vitro and human fat cells and monocytes in vivo, are identical with respect to binding affinity and specificity to insulin. They are also identical in terms of temperature and pH effects, and kinetic properties (Ginsberg, 1977).

#### Aggregation and Internalization of the Insulin-Receptor Complex

After binding, aggregation of the occupied receptors and internalization of the insulin receptor complex takes place (Goldfine et al., 1978; Goldfine et al., 1982; Kahn et al., 1978; Maxfield et al., 1978; Bergeron et al., 1979; Carpentier et al., 1979; Berhanu et al., 1983). The aggregates form sometimes  $\nabla$  clathrin-coated pits (Pearse, 1976). The formation of these pits has been observed in 3T3-4 mouse fibroblasts (Maxfield et al., 1978), IM-9 lymphocytes (Carpentier et al., 1981), and in 3T3-L<sub>1</sub> mouse adipocytes (Carpentier et al., 1982). This has not been observed in rat hepatocytes (Bergeron et al., 1979). Coated pits are invaginations of the cell membrane which form small vesicles enclosing receptor bound insulin that are subsequently pinched off (Pearse, 1976). In this way insulin is thought to be transported to the interior of the cell.

### Fate of Insulin

Although the hormone-receptor complex enters the cell as a unit, it seems that the two molecules follow different pathways after entry (Maxfield et al., 1978; Carpentier et al., 1981; Carpentier et al., 1982). All lines of evidence support the idea that insulin's fate after entering the cell, is receptor-mediated (Gliemann and Sonne, 1978; Baldwin et al., 1980) or non-receptor-mediated degradation (Gliemann and Sonne, 1978; Baldwin et al., 1980; Sonne and Gliemann, 1980).

Lysosomes, are generally accepted as the receptor-mediated degradation site, because degradation is inhibited when lysosomotropic agents such as chloroquine (Carpentier et al., 1979; Marshall and Olefsky, 1979; Suzuki and Kono, 1979; Heidenreich et al., 1984), dibucaine and tetracaine (Suzuki and Kono, 1979) or ammonium chloride (Marshall and Olefsky, 1979) are used. Subsequent accumulation of intracellular insulin has been observed.

Following internalization, the insulin-containing vesicles start moving towards the Golgi apparatus and this takes 10 minutes in rat liver at 37°C (Bergeron et al., 1979). The origin of these vesicles as well as their nature is still quite obscure. Some of them resemble lysosomes with respect to morphology (Bergeron et al., 1979; Carpentier et al., 1981) and the presence of lysosomal enzyme markers (Bergeron et al., 1979). They, however, do not precipitate within the band of the lysosomal fraction (Khan et al., 1982). It has been proposed that these vesicles originate from the golgi region (Bergeron

et al., 1979) because they sediment along with this fraction and because of their low-density lipoprotein-like markers (Carpentier et al., 1981; Khan et al., 1982). Their sedimentation characteristic though, is under question because the golgi suspension used was not pure, and further fractionation revealed a small number of vesicles carrying lysosomal enzyme markers, and also containing some of the internalized insulin (Khan et al., 1982). In addition, no insulin or only traces of it have been found in the golgi apparatus per se (Bergeron et al., 1979).

Also, insulin has been associated with other cell organelles, such as the nuclear envelope (Goldfine et al., 1982) and endoplasmic reticulum (Horvat et al., 1975; Goldfine et al., 1978) in IM-9 lymphocytes and rat liver-membrane.

The degree of intracellular insulin degradation varies depending on the tissue. Suzuki et al. (1979) studied receptor mediated insulin degradation on cell-free and whole-cell systems. They reported that only a small percentage of the internalized insulin in fat cells is degraded intracellularly; the rest is recycled back into the incubation medium through an ATP-dependent process. In human fibroblasts (Balwin et al., 1980) and in rat hepatocytes (Carpentier et al., 1979) insulin receptor-mediated degradation has been observed and 30-40% of the radioactivity applied ( $^{125}\text{I}$ -insulin) is located intracellularly during the steady-state stage of binding at 37°C (Carpentier et al., 1979). In IM-9 lymphocytes receptor-mediated degradation is almost non-existent (Balwin et al., 1980) even under

conditions close to physiological, e.g., at 37°C and pH 7.4 (Sonne and Gliemann, 1980).

### Fate of the Receptor

Although insulin is degraded when transported to the interior of the cell after binding to its receptor, the receptor itself does not seem to share the same fate. Evidence (Terris and Steiner, 1980; Prince et al., 1981; Marshall and Olefsky, 1983; Heidenreich et al., 1984) suggests that insulin and receptor are internalized together but while insulin follows a degradative pathway, the receptor at some point, dissociates from the hormone, and is recycled to the cell membrane. Studies with chloroquine have indicated that a fraction of the internalized receptors may be degraded (Green and Olefsky, 1982; Heidenreich et al., 1984) in adipocytes. In adipocytes (Berhanu et al., 1982; Heidenreich et al., 1984) photoaffinity-labelling studies suggest that the receptor is internalized and a fraction of it is proteolytically degraded. On the other hand, lactoperoxidase-labelling of the receptor with  $^{125}\text{I}$  and immunoprecipitation with antireceptor antibody had shown that no proteolytic degradation occurs in fat cells (Hedo et al., 1982). This is an indication that photoaffinity-labelling might alter the physiological pathway the receptor follows after internalization. It is also interesting to note that photoaffinity labelled receptors on IM-9 lymphocytes are not internalized; instead, they are shed into the surrounding medium (Berhanu and Olefsky, 1982).

The evidence, so far, concerning the fate of the insulin-receptor complex suggests that after binding, in most cells, the hormone is internalized along with the receptor. At some point there is sequestration of the receptor which, recycles back to the cell membrane (Marshall et al., 1981); the small fraction which does not recycle is intracellularly degraded. The duration of this recycling has been estimated to be different among cells of various systems. In cultured human lymphocytes, insulin receptors exhibit a half-life ( $t_{1/2}$ ) of 30-40 hours after treatment with cycloheximide (Khan, 1976). In 3T3-L<sub>1</sub> mouse adipocytes after treatment with tunicamycin, they expressed a  $t_{1/2}$  of 9 hours (Rosen et al., 1979) and 15-16 hours (Reed et al., 1981) while cycloheximide lengthened insulin receptor turnover rate to 24-25 hours (Rosen et al., 1979; Reed et al., 1981). On the other hand, in the same cell type, the heavy isotope density-shift technique without the use of inhibitors, revealed a half-life of approximately 7.5 hours (Reed et al., 1981) with a range of 6.7 to 9.4 hours.

Potent Postbinding Events.

Since after binding, the two molecules follow a different pathway in the cell (insulin is degraded, receptor is recycled) there are three alternatives to speculate for the role of the receptor in insulin action. It either 1) acts as a simple carrier for the insulin molecule; 2) the insulin receptor complex participates in a series of biochemical reactions that result in an insulin effect on the cells

or 3) the receptor itself acts as a major factor for some of the cell's functions.

The first possibility can be ruled out because it has been shown that the receptor undergoes biochemical changes after interaction with insulin. Kasuga et al. (1982) reported that after binding, insulin stimulates phosphorylation of its own receptor, in particular, the  $\beta$  subunit in cultured IM-9 human lymphocytes and in H-35 hepatoma cells. The phosphorylation has been found on a tyrosine residue (Kasuga et al., 1982; Cobb and Rosen, 1984). In the latter study in a cell-free system, phosphorylation of the  $\alpha$  subunit was not only demonstrated but was greater than that of the  $\beta$  subunit. It has been proposed that phosphorylation of the receptor might be the beginning of the biochemical events leading to the expression of insulin's action, and this raised the question whether the receptor is a protein kinase. Evidence for this is given by Roth et al. (1983) in IM-9 lymphocytes. They concluded that the receptor is a protein kinase which, upon stimulation by insulin, becomes autophosphorylated and phosphorylates other proteins as well. The two kinds of the subunits serve two purposes; the  $\alpha$  subunit contains the insulin binding site; the  $\beta$  subunit expresses the kinase activity and has an ATP binding site. Simpson et al. (1984) using an antireceptor antiserum could mimic insulin's acute effects such as stimulation of glucose transport, phosphorylation of integral membrane proteins and internalization of the receptor. Yet, there was no stimulation of phosphorylation of the  $\beta$  subunit. They suggested that, receptor phosphorylation may not be

a requirement for acute insulin action, although they did not exclude that the "longer term growth-promoting effects of insulin may require receptor phosphorylation." The preceding information supports the last two alternatives: 1) That the receptor participates along with insulin for the various expressions of the hormone's action, and 2) that the receptor per se is important for certain of those actions without insulin being present. After binding and activation of the receptor, an unknown mechanism is turned on leading to the final effects characteristic of the action of the hormone.

Although  $H_2O_2$  (Jarrett and Seals, 1979), calcium and cyclic nucleotides (Czech et al., 1984) may be involved in a general cellular response to insulin they do not appear to be the actual mediators. Seals and Jarrett (1980) reported that insulin addition to a plasma membrane-mitochondria system would activate pyruvate dehydrogenase. This would not happen if only mitochondria were used; the presence of membranes was necessary. This suggests that binding to the membrane and interaction with the receptor is necessary and this interaction generates a mediator which activates the enzyme. The mediator has been isolated from various cell systems (Jarrett and Seals, 1979; Larner et al., 1979; Kiechle et al., 1981; Saltiel et al., 1981; Seals and Czech, 1981), it has a molecular weight of 1000-2000 (Larner et al., 1979; Kiechle et al., 1981; Seals and Czech, 1981) and it is a peptide (Larner et al., 1979; Seals and Czech, 1981; Czech et al., 1984) whose amino acid sequence has not yet been completely identified (Larner et al., 1982).



### Factors Regulating Insulin-Receptor Interactions

Two factors governing the interaction between the insulin molecule and the receptor are a) the affinity with which the receptor holds the hormone and b) the concentration of the ambient hormone. Evidence suggests that the receptors exist in multiple affinity states (Gammeltoft, 1984), high, intermediate and low, either as separate classes or as isomers of the same unit. Each state is regulated by the occupancy of the receptor by the hormone. The higher the number of occupied sites, the higher the dissociation rate of the insulin-receptor complex. This was ascribed to negatively cooperative interactions between the filled and empty receptors, (De Meyts et al., 1976). There is a lot of controversy on this subject, and the molecular mechanisms regulating this phenomenon are still under investigation (Gammeltoft, 1984).

The concentration of the ambient hormone is critical for the response of the cell to it. When cells are exposed to excessive amounts of insulin, binding decreases as a time and concentration-dependent event (Gavin et al., 1974; Baldwin et al., 1980); this decrease is due to either rapid internalization of the receptor (Mott et al., 1979; Knipp and Lane, 1981) or receptor recycling slowing down (Knipp and Lane, 1981) or receptor degradation (Kosmakos and Roth, 1980; Berhanu and Olefsky, 1982; Green and Olefsky, 1982). This latter phenomenon of hormone concentration-dependent decrease in binding is known as "down regulation" of the receptors and it exists in pathological conditions in vivo.

### Human Fibroblasts as a Model for Insulin Action

As it has been discussed, a large number of cell systems, animal and human, have been employed in order to clarify the stages preceding and following internalization of the insulin molecule. This is partly due to the various effects insulin exerts on the cell. Protein synthesis, stimulation of RNA and DNA synthesis, ATP formation, regulation of sugar transport, decrease of glycogenolysis, lipolysis, gluconeogenesis, ureogenesis, ketogenesis, protein degradation, increase of glucose oxidation and lipogenesis, and enhancement of cell growth have been shown to be affected by insulin (Porte and Halter, 1981).

Among the cellular systems used for demonstration of insulin's biological potentials are the cultured diploid human fibroblasts. Although, these cells are not an actual primary target for insulin, they represent a very good system for studying the insulin-receptor complex actions.

Because of their undifferentiated nature, human fibroblasts represent a "neutral" tissue, untouched by specific requirements that might affect the number and/or the functional expression of the receptors. Additionally, these cells are diploid, not transformed. Thus, the regulatory mechanism for the receptors under given conditions is less likely to be disturbed by unknown genetic factors (e.g. transformation). Although the number of the receptors is not as great as in other cell systems, those that exist are of high affinity for the insulin molecule (Prince et al., 1981).

The demonstrated effects of insulin on human fibroblasts (HF) include stimulation of  $\alpha$ -aminoisobutyric acid transport (Rechler et al., 1982; Hollenberg and Cuatrecasas, 1985), DNA (Rechler et al., 1972; Hollenberg and Cuatrecasas, 1975), RNA and protein synthesis (Fujimoto and Williams, 1974), glucose oxidation (Goldstein and Littlefield, 1969) and uptake (Fujimoto and Williams, 1974; Germinario and Oliveira, 1979; Berhanu and Olefsky, 1971; Germinario et al., 1984). Stimulation of sugar uptake in the cells is the primary physiological role of insulin. The environmental conditions and regulatory mechanisms controlling this function have been of great interest and intensively investigated. Serum and glucose are two major nutritional components for cells in culture, and they have been shown to influence insulin's action on the cells (Wyse and Chang, 1981, 1982; Germinario et al., 1984). The effect of these two factors on human fibroblasts has been investigated in the present study.

#### Serum Effect

Normal, untransformed cells express controlled growth which is essential for the development of normal living organisms. These cells express "density-dependent regulation" of growth, that is, they proliferate to a certain "saturation density" and after reaching this state, they stop growing and become quiescent (Holley, 1975).

Evidence indicates that this arrest is not due to contact between cells which suppresses any further proliferation, but to "limitation

of any one of a variety of materials, in the medium surrounding the cells" (Holley, 1975). Those materials may be macromolecular components (Todaro et al., 1965) in the serum, or, low molecular weight substances (Holley et al., 1974) in the serum or in the nutrient medium and their removal arrests the cells at the same phase regardless of the regulating factor (Pardee, 1974). Experiments performed on 3T3 mouse fibroblasts (Holley and Kiernan, 1974) indicated that their growth is controlled by serum macromolecules. Components such as insulin (Hershko et al., 1971), fibroblast growth factor and dexamethasone (Holley and Kiernan, 1974), phosphate ions, glucose and amino acids (Holley and Kiernan, 1974) and others have been shown to be involved in the control of cell growth.

By altering the experimental conditions, substances acting as proliferation and growth controlling factors have been identified as being serum constituents (Holley, 1975). By depleting the medium of serum and therefore withholding the growth factors, quiescent cultures are produced. When this occurs, cells are found in the  $G_1$  or  $G_2$  phase of the cell cycle within 12 hours from the moment of serum withdrawal (Holley and Kiernan, 1974). The quiescent cells can remain, at these postmitotic ( $G_1$ ), stationary ( $G_2$ ) phases, healthy over a period of a few days and the effect of serum-deprivation is reversible upon serum readdition (Todaro et al., 1965).

## OBJECTIVES

The binding of insulin to its specific receptors triggers a chain of biochemical reactions resulting in various biological responses:

The culture conditions such as serum and glucose concentration greatly influence the insulin receptor concentration and the response of human fibroblasts to insulin (Germinario et al., 1984).

Since insulin is an important hormone (Porte and Halter, 1981), it was of particular interest to study the macromolecular processes involved in the regulation of its receptors by serum and glucose.

In order to pursue this task the following questions were raised:

- 1) What are the optimal conditions for insulin to bind to its receptors under the influence of serum and/or glucose feeding or starving conditions?
- 2) Is glucose an indispensable component for insulin receptor binding ability or can it be unaffectedly replaced by other sugars?
- 3) What is the role of RNA, protein and glycoprotein synthesis in the regulation of insulin receptors by glucose?
- 4) How is insulin action (in terms of sugar transport stimulation) influenced by the same conditions that regulated its binding to its receptor?
- 5) How does stimulation of protein and glycoprotein synthesis by insulin affect insulin stimulation of sugar transport?

## MATERIALS AND METHODS

Cell Culture

All studies were performed with human diploid fibroblasts obtained from deltoid or foreskin biopsies.

Cells were cultured in 175-cm<sup>2</sup> plastic flasks in antibiotic-free Eagle's minimal essential medium (Eagle, 1959) supplemented with 1 mM pyruvate and 10% (v/v) fetal bovine serum (10% MEM) (Microbiological Association, Bethesda, Maryland). Cells were incubated at 37°C in an atmosphere of 95% air and 5% CO<sub>2</sub>, the feeding medium being changed three times weekly.

Confluent cell monolayers were harvested from the culture vessels after being treated with 0.04 (w/v) trypsin for 2 minutes at room temperature, followed by treatment with 0.2% (w/v) EDTA for 5 minutes at 37°C. The cells were counted with a haemocytometer and were plated at a density of approximately  $1.0 \times 10^5$  cells per 35 mm diameter dish (Corning Co.) to reach confluence (Seven to ten days).

In all experiments, the cells had not completed more than 50% of their in vitro lifespan. The number of population doublings accrued by the cells at the time of the experiment was divided by the total number of population doublings expected at senescence; this number times 100 equals the percentage lifespan completed (Germinario et al., 1980).

Experimental Procedure

When cells reached confluence, the medium was removed and the

monolayers were rinsed with 1 ml serum-free medium (0% MEM) containing 1 mg/ml bovine serum albumin (BSA, Sigma Chemical Co.) and 4 mg/ml (22.2 mM) glucose (0% MEM + Glu), or for experiments involving serum and glucose-free conditions, rinsing was performed with 1 ml serum and glucose-free MEM (0% MEM - Glu).

At this point cells were exposed to various experimental conditions according to protocols employed, described in the Results section for each case.

#### Insulin Binding Procedure

Binding experiments were performed with  $^{125}\text{I}$ -insulin-labelled according to a modification of the Chloramine T method to a specific activity of 180-200  $\mu\text{Ci}/\mu\text{g}$ . (Brenner, B., personal communication).

For labelling,  $\text{Na}^{125}\text{I}$  was purchased from Amersham, Chloramine T from BDH and Crystalline Porcine Monocomponent Insulin from Eli Lilly and Co.

#### 1) Iodination of Insulin

The iodination was performed at room temperature. One mCi of  $\text{Na}^{125}\text{I}$  (Sp. Act. 14-16 mCi/ $\mu\text{g}$ ) was mixed with 5  $\mu\text{l}$  of 1 mg/ml insulin and 50  $\mu\text{l}$  of 0.5 M Phosphate Buffer pH 7.5; ten microliters of 0.5 mg/ml of Chloramine T solution was then added and incubated for 30 seconds. At the end of this time, 100  $\mu\text{l}$  of saturated solution of Tyrosine (0.4 mg/ml) was added and the whole was incubated for 60 seconds. At the end of this time the reaction mixture was eluted

in 0.5 ml fractions through a Sephadex G-25M column (Pharmacia), which was pre-equilibrated with 1% BSA. Ten microliters of each fraction were then counted to define the peak fractions. The two peaks with the highest counts were pooled and used in binding studies.

## 2) Binding Procedure

After aspiration of the culture medium, the cell monolayers were rinsed twice with 2 ml Hank's-HEPES (20 mM) Buffer supplemented with 2% (w/v) BSA (Sigma), pH 7.4. One milliliter of  $^{125}\text{I}$ -insulin (1 ng/ml) incubation media was added to the cells, alone or together with 40  $\mu\text{g/ml}$  unlabelled insulin (Insulin-Toronto, 100 U/cc, Connaught Laboratories) to determine total and non-specific binding. Plates were incubated at 22°C for 120 minutes on a shaking tray (45 shakes/min).

At the end of the incubation period, the cells were rinsed three times with 3 ml ice-cold Hank's Buffer. Then the cell monolayers were dissolved in 1.2 ml in NaOH for 1 hour. One-ml aliquots were transferred to 5 ml glass tubes, covered, placed in plastic carriers and counted for determination of cell-associated radioactivity in an automatic gamma-counter (Nuclear-Chicago, Model 1085) with a 72% efficiency.

Specific binding was calculated by subtracting the non-specific from the total binding per mg protein.



### Procedure for Determination of Protein and RNA Synthesis

Cell monolayers were rinsed twice with 2 ml warm (37°C) phosphate-buffered saline (PBS) pH:7.4. One milliliter warm PBS containing 5  $\mu$ Ci of L-Leucine-[4:5- $^3$ H] (58 Ci/mmol, ICN)  $\pm$  20  $\mu$ g/ml cycloheximide (Sigma) or uridine [5- $^3$ H] (25 Ci/mmol, New England Nuclear Corp. Boston, Mass.)  $\pm$  0.03  $\mu$ g/ml actinomycin D (Sigma) and 5.55  $\mu$ moles/ml, D-glucose (Sigma Chem. Co.) was added and the cells were incubated for 30 minutes and 60 minutes respectively at 37°C.

At the end of this time period, the radioactive solution was removed and the cell monolayers were rinsed four times with 2 ml ice-cold PBS. One milliliter of 10% cold TCA was added for 1 hour at 4°C. After rinsing twice with 3 ml cold TCA, the monolayers were dissolved in 1 ml 1N NaOH for 1 hour and 0.1 ml aliquots were mixed with 5 ml counting solution (Formula 963, Du Pont) and counted in a liquid scintillation counter (LKB, Wallac, Model 1217 Rackbeta) with a 32% efficiency for  $^3$ H. Aliquots of 0.2 ml were assayed for protein determination (Lowry et al., 1951).

### Procedure for Determination of Glycoprotein Synthesis Inhibition by

#### Tunicamycin

Preliminary experiments concerning concentration and time-course were performed according to two different protocols.

The first protocol involved a total serum starvation time of 52 hours. The cells were rinsed and placed in serum-free media containing 4 mg/ml glucose (0% MEM + Glu). At the end of the first 24

hours of this time period, the cells were given 1 ml of 0% MEM + Glu containing 0.1, 0.3, 0.5, 1.0, 1.5, and 2.0  $\mu\text{g/ml}$  tunicamycin (Boehringer-Mannheim) and were incubated at 37°C for various time intervals.

Control plates received only 1 ml 0% MEM + Glu. Four, 12 and 24 hours after the change, the cell monolayers were rinsed with 1 ml 0% MEM containing 0.1 ml glucose and were given 1 ml of the same medium containing 0.1, 0.3, 0.5, 1.0, 1.5 and 2.0  $\mu\text{g/ml}$  tunicamycin respectively and 2.5  $\mu\text{Ci/ml}$  Mannose-D-[2- $^3\text{H}$  (n)] (S.A. 27.2 Ci/mMole New England Nuclear).

The plates were incubated for 4 more hours at 37°C. At the end of this time they were rinsed three times with 2 ml cold PBS. One ml of 10% cold TCA was added for 1 hour at 4°C. Then, the cell monolayers were rinsed twice with 3 ml cold TCA and solubilized in 1 ml 1N NaOH for 1 hour. Aliquots of 0.2 ml were taken for scintillation counting and protein determination.

The second protocol involved a concomitant incubation of the antibiotic and the labelling precursor. After the first 24 hours of serum starvation, the cell monolayers were given 1 ml 0%/MEM containing 1 mg/ml glucose and the same tunicamycin concentrations as in the first protocol, along with 2.5  $\mu\text{Ci/ml}$   $^3\text{H}$ -Mannose, and incubated at 37°C.

Four, 12 and 24 hours after the change, the medium was removed, the cells were rinsed three times with 3 ml cold PBS and precipitated in 1 ml 10% cold TCA for 1 hr at 4°C. Solubilization and counting were performed as in the first protocol.

In both protocols, one half hour before the end of each incubation with tunicamycin time-period, separate cell monolayers were assayed for protein synthesis inhibition determination as described in the preceding section.

In both protocols zero-time controls equalled the background radioactivity.

#### Procedure for Glucose Transport

Cell monolayers were rinsed twice with warm (37°C) PBS, pH 7.4 and they were given 0.8 ml warm glucose-free PBS containing 1.0 mM (S.A. 4.5  $\mu\text{Ci}/\mu\text{mole}$ )  $^3\text{H}$ -Deoxy-D-Glucose (New England Nuclear).

Incubation was performed at 37°C and the sugar uptake time was 2 minutes.

At the end of each time-interval, the labelled medium was removed, the cell monolayers were rinsed four times with 2 ml cold PBS and solubilized in 1 ml 1N NaOH for 1 hour.

Aliquots of 0.2 ml were used for liquid scintillation counting and protein determination.

In all experiments zero-time controls were subtracted. Sugar transport was rate-limiting and linear under the conditions used (Germinario and Oliveira, 1979). An Apple II<sup>+</sup> computer was used for calculation of the liquid scintillation data.

For experiments involving insulin stimulation of sugar transport, 100 mU/ml of Crystalline Bovine Pancreas Insulin (Sigma) was added to appropriate plates for 2 hours prior to the sugar uptake time.

## RESULTS

The data in Figure 2 show the effect of pH on insulin binding. It is clearly shown that the optimum pH for insulin binding is 7.4.

At this pH, for the optimum temperature for binding, two temperatures were used: 37°C, the physiologic for the human body temperature and 22°C, room temperature.

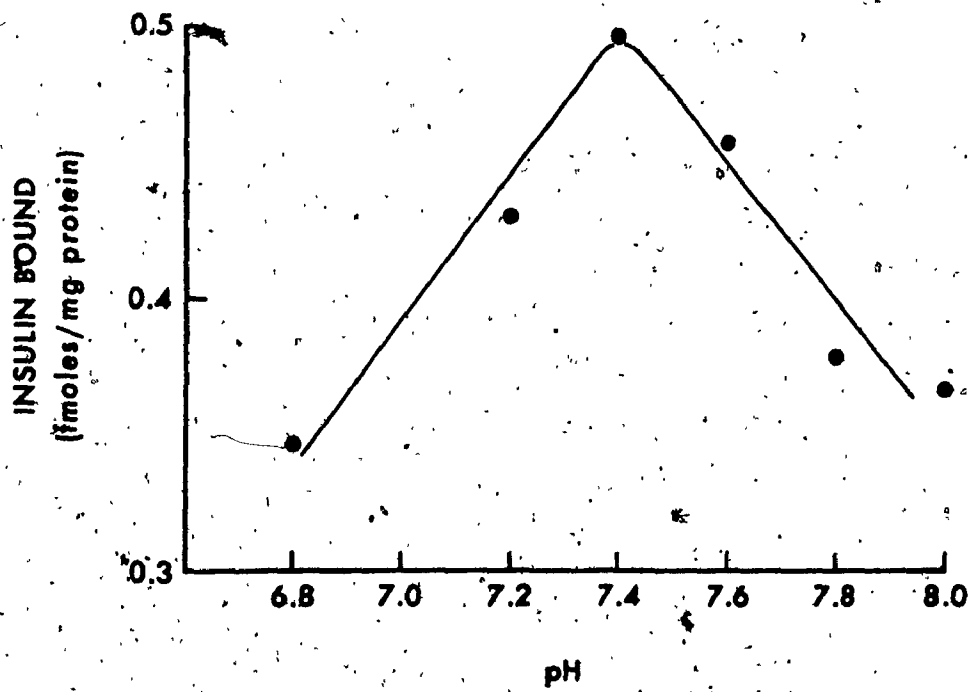
The data in Figure 3 illustrate the specific insulin binding and the percent insulin degradation as % TCA-soluble DPM's. The  $^{125}\text{I}$ -insulin is > 95% precipitable by TCA and total or partial degradation of the labelled insulin leads to TCA-soluble counts. Specific binding reached equilibrium after 60 minutes of incubation and remained at steady state for an additional 60 minutes at 22°C. Insulin degradation was not altered throughout the time course at this temperature. At 37°C specific binding was continuously increasing, reaching the 22°C binding level at 90 minutes. At 120 minutes it was higher than at 22°C, but insulin degradation was increased as well. During the first 90 minutes at 37°C, degradation was not altered.

After defining the two major conditions for binding, the effect of serum concentration concomitantly with glucose concentrations on insulin binding was studied (Table I). The data indicate that although 0% MEM + 4 mg/ml glucose increases binding in comparison with 0% MEM + 1 mg/ml glucose, it does not express any differences when compared with the 10% MEM-treated groups. These two groups do not express any differences between them either.

**Fig. 2 Effect of pH on Insulin Binding.**

Cell monolayers were treated with serum-free MEM for 24 hours, at the end of which they were exposed to insulin binding buffer containing 1 ng/ml  $^{125}\text{I}$ -insulin of various pH's at 22°C. Each point represents averages of triplicate plates.

FIGURE 2



**Fig. 3 Effect of Selected Temperatures at Various Time Intervals on Insulin Binding.**

Cell monolayers were serum-starved for 24 hours. At the end of this time period they were assayed for insulin binding at 22°C and 37°C for 30, 60, 90 and 120 minutes. After binding, aliquots of the binding buffer were TCA-precipitated for determination of insulin degradation at each temperature expressed as % TCA soluble DPM's (A). Each point represents means of triplicate plates.

FIGURE 3

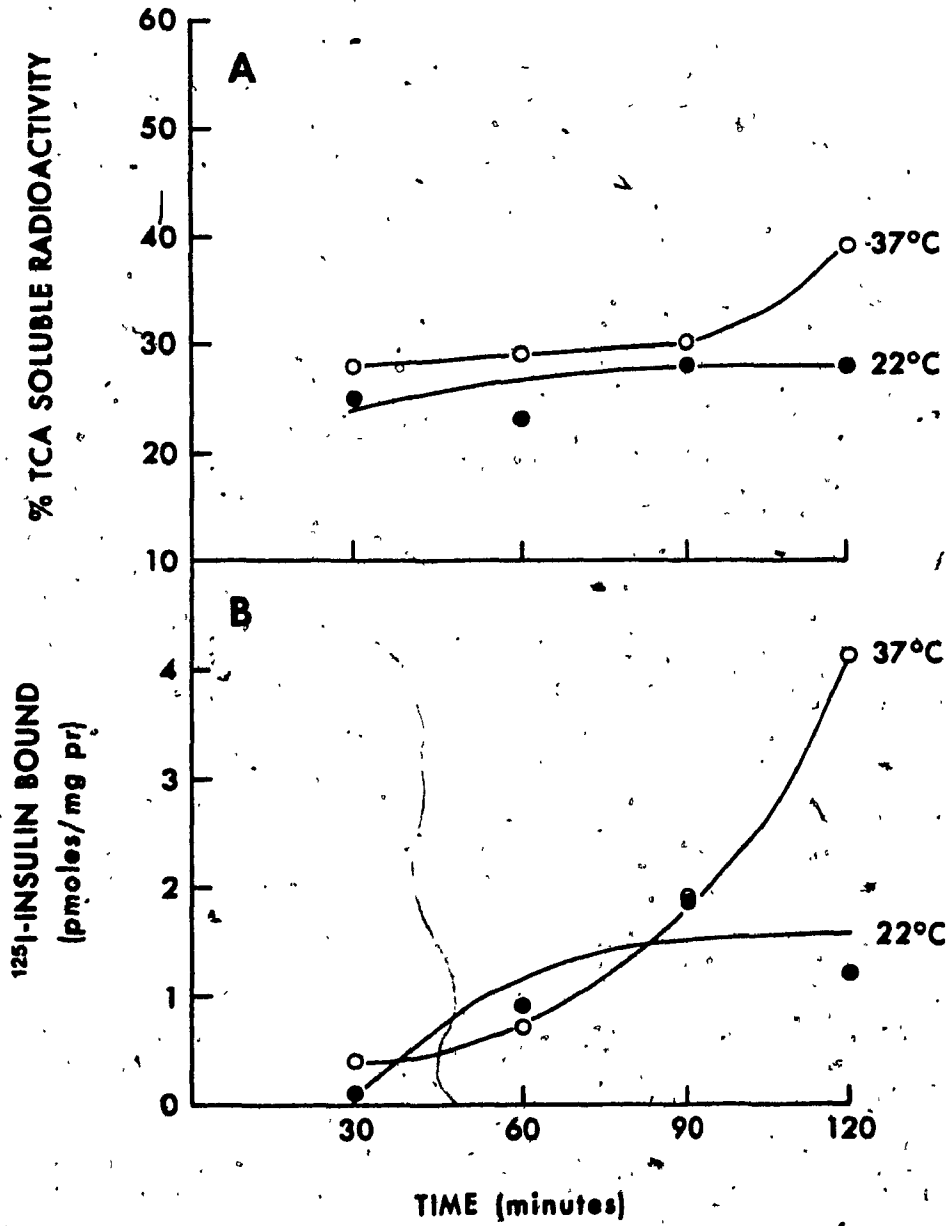




Table I. Effect of Serum and Glucose Concentration on Insulin Binding.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
0% MEM + 1 mg/ml Glu <sup>a</sup>	1.465 ± 0.335
0% MEM + 4 mg/ml Glu <sup>b</sup>	2.450 ± 0.650
10% MEM + 1 mg/ml Glu <sup>c</sup>	2.480 ± 1.050
10% MEM + 4 mg/ml Glu <sup>d</sup>	2.560 ± 0.940

Confluent cells grown in 10% serum-supplemented MEM containing 1 mg/ml glucose were rinsed with 1 ml 0% glucose-free MEM and divided in two groups. One group received, after rinsing, 1 ml 0% MEM containing 1 mg/ml glucose<sup>a</sup> or 4 mg/ml glucose<sup>b</sup>; the other group, after rinsing, was given 1 ml regular 10% serum-containing MEM supplemented with 1 mg/ml<sup>c</sup> or 4 mg/ml glucose<sup>d</sup>. Twenty-four hours after the change the cells were assayed for insulin binding.

Data represent averages of two experiments ± SEM (Standard Error of the Mean); triplicate plates were used in each experiment.

The effect of glucose replacement by two other sugars on insulin binding was investigated in another series of experiments (Table II). The results clearly show that over a period of 24 hours in the absence of glucose, binding was reduced. When glucose was replaced by galactose (a metabolizable sugar) binding was not altered. Interestingly, in the presence of 3-O-MG, which is a synthetic non-metabolizable sugar, binding was elevated.

The preceding results are in accordance with those of others (Wyse and Change, 1981, 1982; Germinario et al., 1984), indicating that glucose concentration in the feeding medium is an important factor which affects the binding of the insulin molecule to its own receptor.

The glucose deprivation effect is reversible in as little as 6 hours from the moment of glucose readdition (Germinario et al., 1984). The effect of inhibitors of protein, RNA and glycoprotein synthesis (as well as the regulatory role of glucose) on this reversible phenomenon have been consequently studied herein.

The data in Table III show the effect of glucose deprivation on insulin binding over a period of 12-18 hours. The glucose-starved cells expressed only 60% of the binding of the glucose-fed controls and this is in agreement with previous reports (Germinario et al., 1984).

When, at the end of the glucose starvation time-period, the cells were re-fed for 6 hours (reversal phase) with serum-free medium containing 4 mg/ml glucose, insulin binding approached the levels seen

Table II. Effect of Various Sugars During 24 Hours of Serum Starvation on Insulin Binding.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
+Glu <sup>a</sup>	1.558 ± 0.242
-Glu <sup>b</sup>	0.960 ± 0.290
+Gal <sup>c</sup>	1.593 ± 0.387
+3-O-MG <sup>d</sup>	3.215 ± 0.385

Cells were rinsed with 0% MEM free of glucose and given 1 ml of % MEM containing no glucose<sup>b</sup>, or 4 mg/ml. of glucose<sup>a</sup>, galactose<sup>c</sup>, 3-O-MG<sup>d</sup> over a period of 24 hours. At the end of this period insulin binding was measured.

Data represent average of two experiments ± SEM; triplicate plates were used in each experiment.

Table III. Effect of 12-18 Hour Glucose Starvation on Insulin Binding.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
+Glu <sup>a</sup>	2.34 ± 0.096
-Glu <sup>b</sup>	1.42 ± 0.24 <sup>c</sup>

After 24-30 hr serum starvation, cells were glucose-starved for 12-18 hours. At the end of this time period, insulin binding performed was shown to be decreased in glucose-deprived cells<sup>b</sup> as opposed to glucose-fed cells<sup>a</sup>.

Data present means of three representative experiments ± SEM; triplicate plates were used in each experiment.

<sup>c</sup> Significant difference (t-test) from glucose fed control (P < 0.05).

in the continuously fed cells. The restoration of binding within 6 hours is statistically significant (t-test,  $P < 0.05$ ) (Table IV).

At the end of the glucose starvation time, the cells were changed to glucose-fed conditions. It is at this point that the inhibitors were added in order to determine the effect they exert on any occurring changes. The data in Table V show the effect of 0.03  $\mu\text{g/ml}$  actinomycin D (Act D) on insulin binding in glucose-deprived cells after glucose refeeding. The cells show no differences in binding when compared with the untreated controls. In these experiments RNA synthesis inhibition was approximately 80% in both, continuously starved and reversed cells. Concomitant protein synthesis inhibition was less than 30% in reversed cells and there was no inhibition in the starved cells (Table VI). Concomitant sugar transport measurement was in agreement with a previous report (Germinario *et al.*, 1982) demonstrating that sugar transport was enhanced in glucose-starved vs. glucose-fed cells and the reversal was not blocked by Act D (Table VII).

Cycloheximide (CHX) a protein synthesis inhibitor, first tested in a concentration series of experiments for the reversal. The data in Figure 4 illustrate the effect of various CHX concentrations on insulin binding and  $^3\text{H}$ -Leucine incorporation into TCA-precipitable material. It can be seen that there is a parallel inhibition on both,  $^{125}\text{I}$ -Insulin binding and protein synthesis, reaching saturation levels at similar concentrations (10-20  $\mu\text{g/ml}$ ). Experiments, using 20  $\mu\text{g/ml}$  CHX were done to elucidate further the effect of CHX on insulin binding in glucose-starved and

Table IV. Effect of Glucose Refeeding for 6 Hours on Insulin Binding after 12-18 Hours of Glucose Starvation.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
-Glu <sup>a</sup>	0.485 ± 0.04
-Glu → +Glu <sup>b</sup>	0.628 ± 0.03
+Glu → +Glu <sup>c</sup>	0.735 ± 0.07

The general protocol for this study involved a total of 48-hr serum-starvation period. During the first 24-36 hours, the cells were given 1 ml 0% MEM containing 4 mg/ml glucose. At this point, after rinsing with 0% MEM -Glu, they received 1 ml of the same free of glucose 0% MEM for an additional 12-18 hours. At the end of this time insulin binding was measured in one group and the rest were changed to serum-free, glucose-fed state for the reversal phase for 6 more hours. The various inhibitors were added at this phase and at the end of this time period binding was performed.

a Glucose-starved cells for 12-18 hours.

b Glucose-starved cells for 12-18 hours were refed (reversed) for 6 hours.

c Continuously-fed cells.

When glucose-starved cells were fed with 0% MEM + Glu, binding was restored to statistically significant values (t-test,  $P < 0.05$ ).

Data represent means of four experiments ± SEM; triplicate plates were used in each experiment.

Table V. Effect of Actinomycin D During 6 Hours of Glucose Derepression of Insulin Binding.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
+Glu → +Glu	2.33 ± 0.22
-Glu → -Glu <sup>a</sup>	1.44 ± 0.05 <sup>d</sup>
-Glu → -Glu + Act D <sup>b</sup>	1.29 ± 0.24 <sup>d</sup>
-Glu → +Glu	1.80 ± 0.43
-Glu → +Glu + Act D <sup>c</sup>	1.84 ± 0.44

<sup>a</sup> Continuously-starved cells.

<sup>b</sup> Continuously-starved, Act D treated cells.

<sup>c</sup> Reversed, Act D treated cells.

<sup>d</sup> The results represent mean of two experiments ± SEM; the rest represent means of three experiments; triplicate plates were used in each experiment.

Table VI. Effect of Actinomycin D on RNA and Protein Synthesis on HF During 6 Hours of Reversal to Fed State.

Treatment	% RNA Synthesis Inhibition	% Protein Synthesis Inhibition
-Glu <sup>a</sup> → -Glu	80.13 ± 5.22	No Inhibition
-Glu → +Glu	77.8 ± 3.38	27.65 ± 4.9



Table VII. Effect of Actinomycin D on Sugar Transport.

Treatment	2-DG <sup>c</sup> Transport (nmoles/mg Protein/2 min)
-Glu → -Glu <sup>a</sup>	8.89
-Glu → -Glu + Act D <sup>a</sup>	8.84
-Glu → +Glu <sup>b</sup>	3.14
-Glu → +Glu + Act D <sup>b</sup>	3.57

Cells were serum-starved for a total of 48 hours. The last 18 hours, they were either continuously glucose-starved<sup>a</sup>, or glucose-starved for 12 hours and then refed for 6 more hours<sup>b</sup>. Actinomycin D, at a concentration of 0.03 µg/ml, was added to cells of both groups during these last 6 hours; sugar transport was measured at the end of this time period.

Data represent one experiment with triplicate plates in each group.

<sup>c</sup> Deoxy-D-Glucose

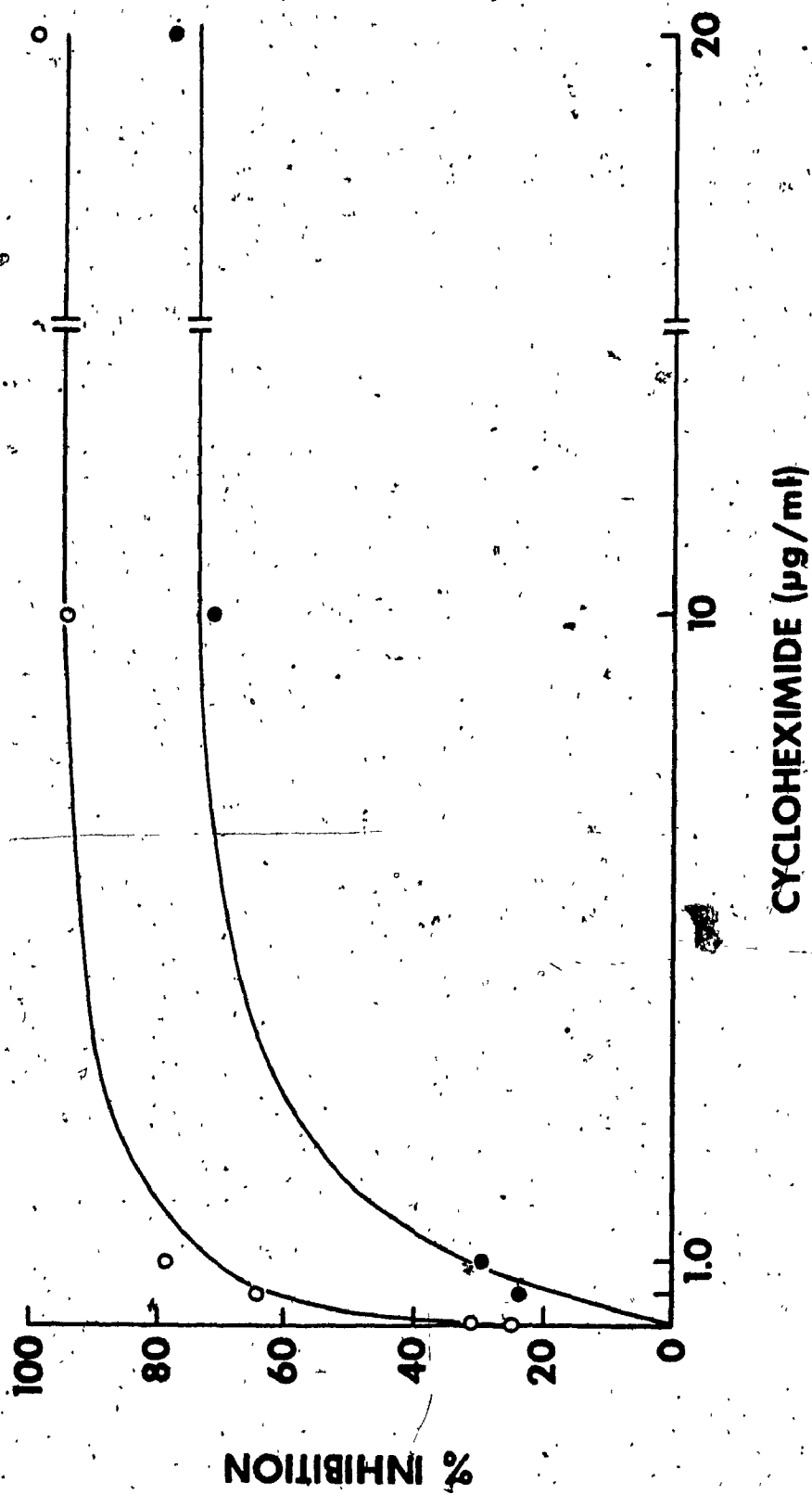
Fig. 4 Effect of Various Concentrations of CHX on Insulin Binding and  $^3\text{H}$ -Leucine Incorporation into TCA-Precipitable Material.

Cell monolayers were glucose-starved for 12 hours. Then they were fed with 1 ml 0% MEM containing 4 mg/ml glucose along with 0.005, 0.05, 1.0, 10., or 20  $\mu\text{g/ml}$  cyclohexamide. Six hours later insulin binding was measured as well as  $^3\text{H}$ -leucine incorporation into synthesized protein. Points represent means of two experiments (duplicate plates) in the  $^3\text{H}$ -leucine incorporation inhibition curve and means of triplicate plates in the insulin binding inhibition curve.

●—●  $^{125}\text{I}$ -insulin

○—○  $^3\text{H}$ -leucine

FIGURE 4



cells. The data in Table VIII show this effect on continuously starved cells (12-18 hours) as well as on reversed monolayers. It is obvious that for binding restoration from glucose-free to glucose-fed state protein synthesis was necessary.  $^3\text{H}$ -Leucine incorporation was reduced by 88%. The increase in insulin binding after glucose refeeding approaches the continuously fed controls but in the presence of CHX the binding was significantly lower ( $P < 0.05$ ). The data in Table IX show the effect of the same CHX concentration on insulin binding in cells continuously fed for 48-hrs. In those cells the  $^3\text{H}$ -leucine incorporation was inhibited by 84%.

The effect of glycoprotein synthesis inhibition on the return of insulin binding was subsequently studied. Preliminary experiments with tunicamycin involving various concentrations and exposure time intervals were done according to two protocols. (For details see Materials and Methods). The data in Figure 5 show the effect of various tunicamycin concentrations on the incorporation of  $^3\text{H}$ -mannose in TCA-precipitable material during a concomitant incubation of tunicamycin with the labelled sugar. In all three time intervals a plateau is observed in the percent inhibition of  $^3\text{H}$ -mannose incorporation for concentrations 1.5-2.0  $\mu\text{g/ml}$ . The maximal inhibition for the 12-hr time-course was 82-85%, and for the 24-hr time course, 87-90%. In the 4-hr time course, glycoprotein synthesis was inhibited by approximately 53-57%. Protein inhibition measured by  $^3\text{H}$ -Leucine incorporation in TCA-precipitable material was 15-17%, 21-23% and approximately 25% for the above concentrations and the

Table VIII. Effect of Cycloheximide on Insulin Binding in HF after 12-18 Hours of Glucose Starvation and 6 Hours of Refeeding.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
+Glu <sup>a</sup> → +Glu	0.72 ± 0.1
-Glu → -Glu <sup>a</sup>	0.38 ± 0.08
-Glu → -Glu + CHX <sup>a</sup>	0.41 ± 0.04
-Glu → +Glu	0.59 ± 0.007
-Glu → +Glu + CHX <sup>b</sup>	0.48 ± 0.009 <sup>c</sup>

<sup>a</sup> Continuously starved, cycloheximide-treated cells.

<sup>b</sup> Reversed, cycloheximide-treated cells.

<sup>c</sup> Significant difference (t-test) from glucose-reversed control (P < 0.05).

After being glucose-starved for 12-18 hours, the cells were refeed for 6 hours. At the end of this period insulin binding was performed. Cycloheximide (20 µg/ml)-treated cells expressed a decrease in binding statistically significant compared to non-treated controls.

Data represent means of three experiments ± SEM; triplicate plates were used in each experiment. Protein synthesis inhibition in the reversed group was 88% ± 3.6.

Table IX. Effect of Cycloheximide on Insulin Binding in Serum-Starved, Glucose-Fed Human Fibroblasts.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
+Glu <sup>a</sup> → +Glu	0.72 ± 0.1
+Glu → +Glu + CHX <sup>a</sup>	0.56 ± 0.09 <sup>b</sup>

<sup>a</sup> Continuously fed, cycloheximide-treated cells.

<sup>b</sup> Significant difference (t-test) from glucose-reversed controls (P < 0.05).

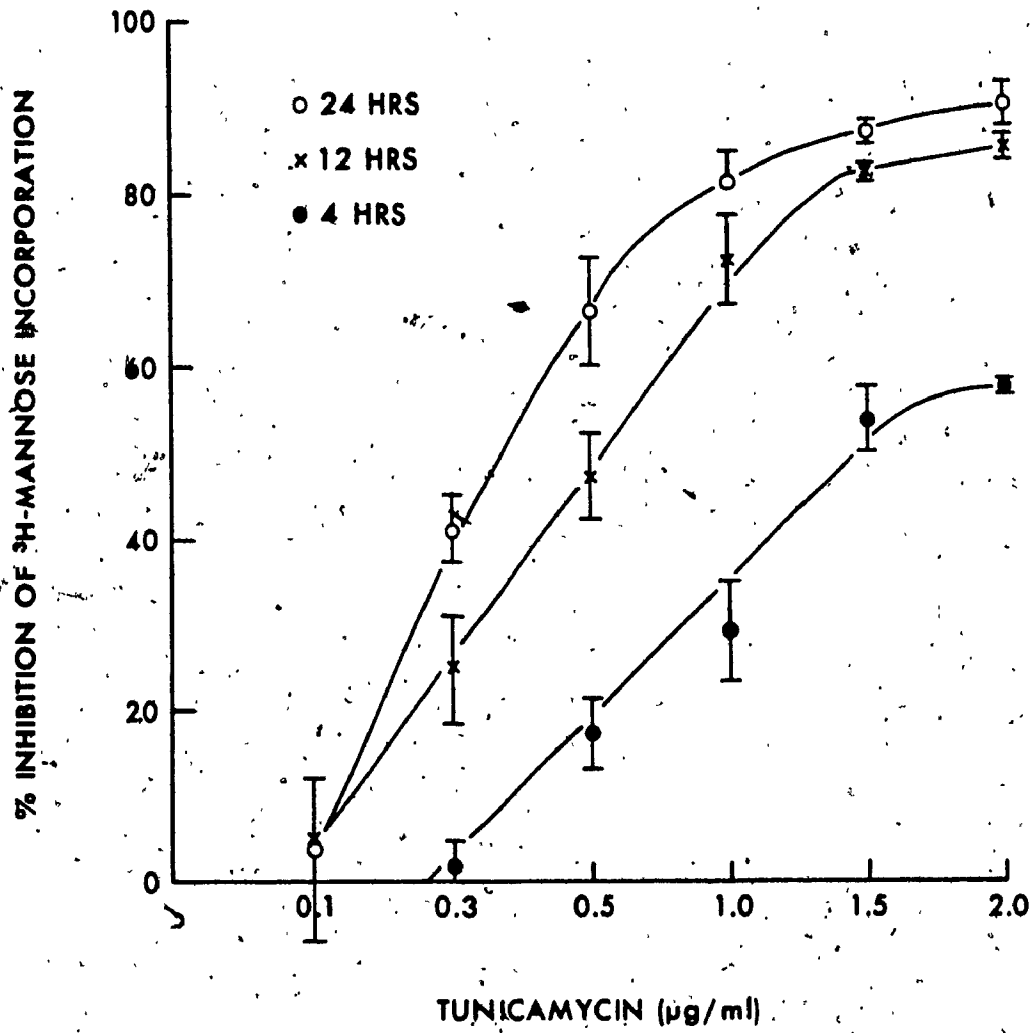
Cell monolayers were serum-starved, but glucose-fed (4 mg/ml) for 42 hours. At this point they were given 20 µg/ml CHX for 6 hours. Protein synthesis was reduced by 84% ± 5.5

Data represent the mean of three experiments ± SEM; triplicate plates were used in each experiment.

Fig. 5 Effect of Various Concentrations of Tunicamycin  
on  $^3\text{H}$ -Mannose Incorporation into TCA-Precipitable  
Material.

Inhibitor and labeled precursor were incubated together for 4, 12 and 24 hours. At the end of each time-interval the cells were TCA-precipitated and glycoprotein associated radioactivity was measured. Each point represents an average of two experiments with triplicate plates in each experiment.

FIGURE 5





respective times of 4, 12 and 24 hours.

The data in Figure 6 show the concentration and time-course effect of tunicamycin in the pulse-chasing protocol. In this series of experiments the plateau is much more defined between 1.0-2.0  $\mu\text{g/ml}$ ; its midpoint corresponds to 1.5  $\mu\text{g/ml}$  tunicamycin concentration and this is consistent at all three time-intervals. The percent inhibition of glycoprotein synthesis for the plateau concentrations ranges from 94-99, 98-99 and 95-99 for the 4, 12 and 24-hr time-intervals respectively. Protein synthesis inhibition, measured as in the first protocol, was 23-38%, 32-55% and 43-49% for the plateau concentrations and time-courses.

The pulse-chasing protocol and the 1.5  $\mu\text{g/ml}$  tunicamycin concentration were chosen to detect the role of glycoprotein synthesis on the 6-hr glucose reversal of insulin binding since the midpoint did not change at all three time courses. The data in Table X show this effect; in the reversed cells, binding was elevated as compared with the continuously starved cells; but when tunicamycin was added, the binding values remained significantly lower than those of the controls. This suggested that for glucose-induced restoration of insulin binding, glycosylation is required.

The effect of 1.5  $\mu\text{g/ml}$  of tunicamycin was also studied on cells continuously fed for 48 hours. The results (Table XI) show that the antibiotic decreased binding with a concomitant inhibition in glycoprotein formation of approximately 96%.

One of the major biological effects insulin exerts on cells is to stimulate overall glucose metabolism. As it has been mentioned the

Fig. 6. Effect of Various Concentrations of Tunicamycin on Glycoprotein Formation; Percent Inhibition of  $^3\text{H}$ -Mannose Incorporation into TCA-Precipitable Material.

After 4, 12 and 24 hour preincubation with 0.1-2.0  $\mu\text{g}/\text{ml}$  tunicamycin, cells were incubated with 2.5  $\mu\text{Ci}/\text{ml}$   $^3\text{H}$ -mannose for 4 more hours.

Each point represents the mean of two experiments with triplicate plates in each experiment.

FIGURE 6

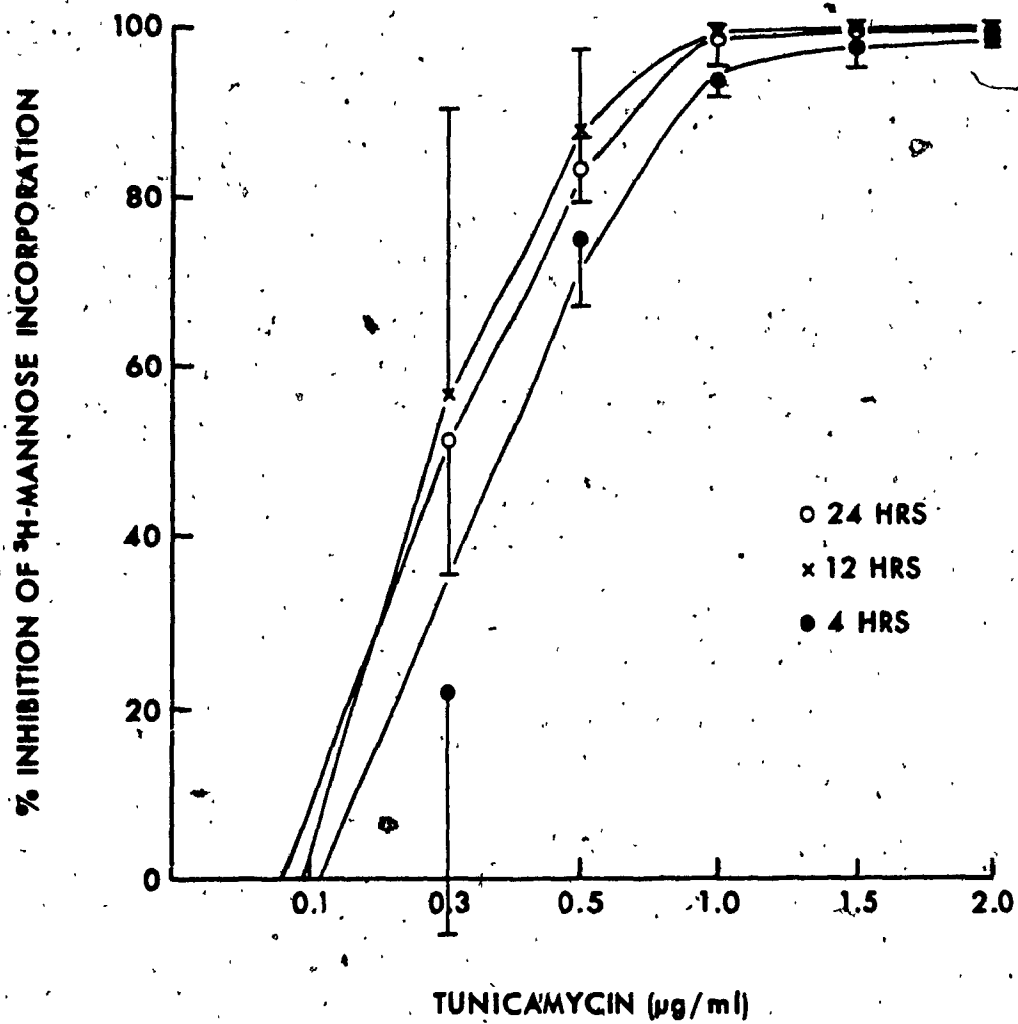


Table X. Effect of Tunicamycin on Insulin Binding During 6 Hours of Reversal from Glucose-Free to Glucose-Fed State.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
-Glu → -Glu	0.90 ± 0.17
-Glu → -Glu + T <sup>a</sup>	1.27 ± 0.30
-Glu → +Glu	1.32 ± 0.09
-Glu → +Glu + T <sup>b</sup>	0.85 ± 0.04 <sup>c</sup>

<sup>a</sup> Continuously starved, tunicamycin-treated cells.

<sup>b</sup> Reversed, tunicamycin-treated cells.

<sup>c</sup> Statistically significant difference from the untreated controls (P < 0.05).

Cells were serum-starved for 30 hours. At this point, they were rinsed with 1 ml of serum and glucose-free MEM, and given 1 ml from the same medium for 12 more hours. At the end of this time period they were either glucose-refed (4 mg/ml) for 6 additional hours or continued being glucose-starved. Tunicamycin (1.5 µg/ml) was added at the beginning of this time phase in cells of both groups. Insulin binding was performed at the end of the 6-hr reversal time.

Data represent the mean of four experiments ± SEM in the -Glu groups and mean of five experiments ± SEM in the reversed groups; triplicate plates were used in each experiment.

Table XI. Effect of Tunicamycin on Insulin Binding in Glucose-Fed Groups.

Treatment	Specific Insulin Bound (fmoles/mg Protein)
+Glu → +Glu	1.38 ± 0.38
+Glu → +Glu + Ta <sup>a</sup>	0.83 ± 0.37 <sup>b</sup>

<sup>a</sup> Continuously fed, tunicamycin-treated cells.

<sup>b</sup> Significant difference (t-test) from the untreated controls.  
(P < 0.05).

Cells were given 0% MEM + Glu for a total of 48 hours. During the last 6 hours, fresh medium was added with or without 1.5 µg/ml of tunicamycin.

<sup>3</sup>H-mannose incorporation was inhibited by 95.67% ± 1.45.

Data represent the mean of three experiments ± SEM; triplicate plates were used in each experiment.

concentration of glucose in the culture medium is a regulatory factor for the magnitude of the cells' response to insulin. Glucose concentration also has an inverse effect on sugar transport, which is reversible within 6 hours.

The data in Table XII show that human fibroblasts which were serum-starved but glucose-fed for 42 hours expressed lower transport values than their counterparts which were serum-starved but glucose-starved for the last 12 hours of this time period. This is in agreement with previous reports (Salter and Cooke, 1976; Germinario 1981, Germinario *et al.*, 1982). In cells which were glucose-fed for 42 hours and continued in the same medium for an additional 6 hours, 2-DG transport was slightly decreased (Table XIII). The addition of insulin (100 mU/ml) increased transport in these cells. Continuously fed cells treated with CHX (20 µg/ml) for 6 hours expressed an increase in transport and still responded to addition of insulin.

The data in Table XIV show the effect of tunicamycin (1.5 µg/ml) on 2-DG transport in continuously fed cells. It is obvious that tunicamycin does not affect sugar transport. However, under the same experimental conditions glycoprotein synthesis was inhibited by 85% (Fig. 7).

Insulin addition (100 mU/ml) to controls and tunicamycin-treated cells elevated the transport to the same levels, indicating that for insulin stimulation of sugar transport in continuously fed cells, glycoprotein synthesis is not necessary. The respective glycoprotein incorporation into TCA precipitable material was inhibited by 85% (Fig. 7).

Table XII. Effect of Glucose Starvation (12 Hours) on 2 DG-Transport.

Treatment	2-DG Transport (nmoles/mg Protein/2 min)
+Glu <sup>a</sup>	7.078 ± 2.05
-Glu <sup>b</sup>	17.071 ± 3.73

<sup>a</sup> The average of two experiments ± SEM; triplicate plates were used in each experiment.

<sup>b</sup> The average of four experiments ± SEM; triplicate plates were used in each experiment.

Cells were serum-starved for a total of 42 hours. During the last 12 hours, they were rinsed with serum- and glucose-free MEM and they were given 1 ml of serum-free, glucose-containing (4 mg/ml)<sup>a</sup>, or glucose-free MEM<sup>b</sup>. At the end of this time, sugar transport was measured.

Table XIII. Effect of Cycloheximide on Insulin-Stimulation of Sugar Transport in Fed Cells.

Treatment	2-DG Transport (nmoles/mg Protein/2 min)	Treatment	2-DG Transport (nmoles/mg Protein/2 min)
+Glu <sup>a</sup>	7.08 ± 2.05		
+Glu → +Glu	5.63 ± 2.21	+Glu → +Glu + Ins	9.56 ± 2.6
+Glu → +Glu + CHX <sup>b</sup>	8.67 ± 1.85	+Glu → +Glu + CHX + Ins <sup>b</sup>	10.18 ± 1.9

<sup>a</sup> Glucose transport was measured after 42 hours of serum-starvation.

<sup>b</sup> During the last 6 hours, 20 µg/ml CHX were added, and two hours prior to the end of this time period, 100 mU of insulin were added to the appropriate groups.

Cells were glucose-fed continuously throughout the course of the experiments for 48 hours.

In all groups, values represent the average of two experiments ± SEM; triplicate plates were used in each group.



Table XIV. Effect of Tunicamycin on Insulin-Stimulation of Sugar Transport in Fed Cells.

Treatment	2-DG Transport (nmoles/mg Protein/2 min)	Treatment	2-DG Transport (nmoles/mg Protein/2 min)
+Glu	7.08 ± 2.05		
+Glu <sup>b</sup> → +Glu	5.63 ± 2.21	+Glu → +Glu + Ins	9.56 ± 2.6
+Glu → +Glu + T <sup>a</sup>	5.75 ± 1.57	+Glu → +Glu + T + Ins <sup>a</sup>	9.55 ± 2.4

<sup>a</sup> During the last 6 hours of the experiments (reversal phase), cells were given 1.5 µg/ml tunicamycin; 100 mU/ml of insulin were added during the last 2 hours to the appropriate groups for insulin stimulation of sugar transport.

Control cells treated as in Table XIII.

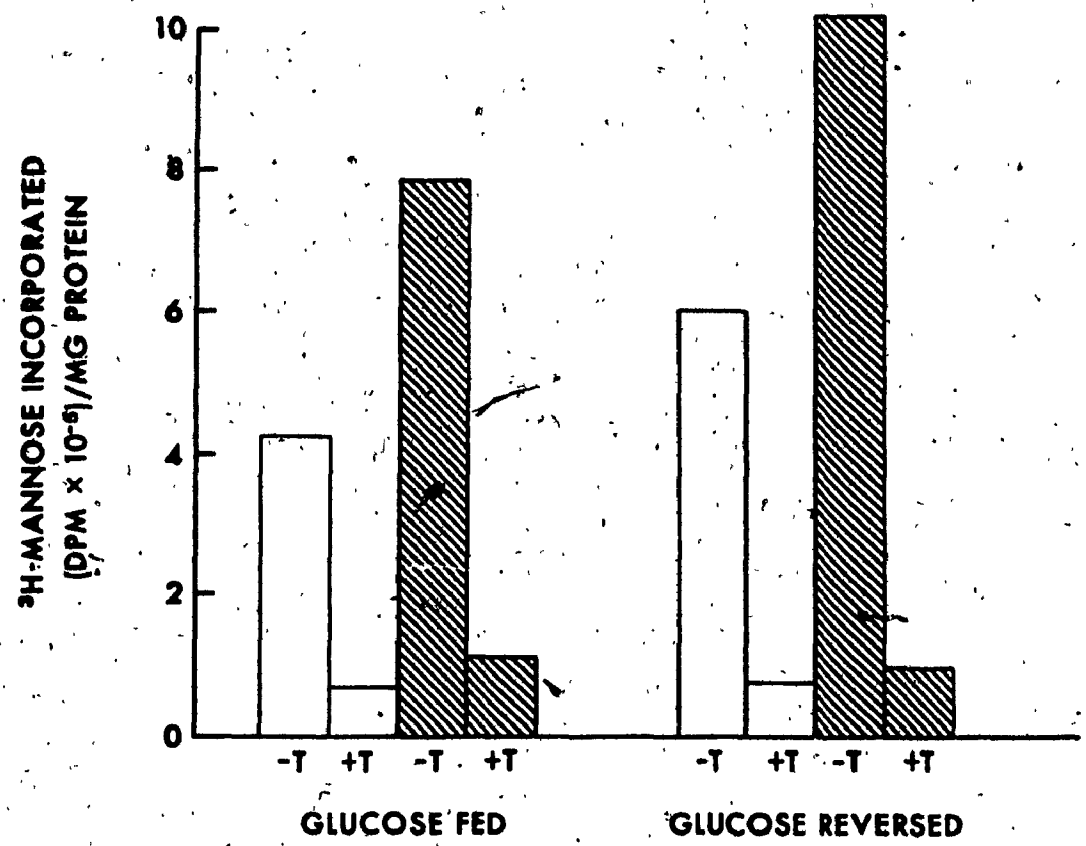
Data represent averages of two experiments ± SEM; triplicate plates were used in each experiment.

Fig. 7 Effect of 100 mU of Insulin on  $^3\text{H}$ -Mannose Incorporation in TCA-Precipitable Material in Cells 1) Continuously Glucose-Fed and 2) Reversed from the Glucose-Starved to the Glucose-Fed State.

Cell monolayers were serum- and glucose-starved or glucose-fed according to the general protocol; at the end of the glucose starvation time, the glucose-starved cells were refed with 1 ml 0% MEM + Glu  $\pm$  tunicamycin (1.5  $\mu\text{g}/\text{ml}$ ). Cells continuously fed were also exposed to  $\pm$  tunicamycin. Two hours prior to the end of the 6-hour reversal time period, 100 mU/ml of insulin was added. At the end of the 6 hours, 2.5  $\mu\text{Ci}/\text{ml}$  of  $^3\text{H}$ -mannose were added in a pulse chasing experiment.

Open squares represent control cells untreated, whereas hatched squares represent cells treated with insulin. The data illustrate one representative experiment of four, with triplicate plates.

FIGURE 7



As was shown earlier, glucose-deprived cells express enhanced transport. The repression of sugar transport due to reversing the cells from glucose-starved to glucose-fed state is demonstrated in Table XV. Within 6 hours the decrease in transport in refeed cells was approximately 53% (17.07 vs 7.97 nmoles/mg Protein/2 min.). As in the continuously fed group, protein synthesis is required because CHX inhibited the glucose-induced repression by almost 59% (7.97 vs 12.68 nmoles/mg Protein/2 min.). Reversed cells treated with insulin (100 mU/ml) 2 hours prior to the end of the 6-hr time-period expressed an elevation in transport by 45% (11.57 vs 7.97 nmoles/mg Protein/2 min.). Reversed cells treated with CHX showed no increase in transport when treated with insulin (12.68 vs 12.11 nmoles/mg Protein/2 min.).

Under the same experimental conditions the effect of tunicamycin was tested on the reversal phase (Table XVI). Tunicamycin had no effect on the reversed cells, treated or non-treated with insulin; that is, it did not inhibit the repression of 2-DG transport during the refeeding phase, nor did it inhibit insulin stimulation of it.

This observation suggests that glycoprotein synthesis is not necessary for the reversal of sugar transport; however, glycoprotein formation under the same experimental conditions was inhibited by tunicamycin by approximately 95% in the reversed cells treated and untreated with insulin (Fig. 7). The effect of insulin stimulation on glycoprotein synthesis is shown in Figure 7. Continuously fed and reversed cells expressed an increase of approximately 187% and 176%

Table XV. Effect of Cycloheximide on Insulin-Stimulation of Sugar Transport in Reversed Cells.

Treatment	2-DG Transport (nmoles/mg Protein/2 min)	Treatment	2-DG Transport (nmoles/mg Protein/2 min)
-Glu <sup>a</sup>	17.07 ± 3.73		
-Glu <sup>a</sup> → +Glu <sup>b</sup>	7.97 ± 1.69	-Glu → +Glu + Ins <sup>c</sup>	11.57 ± 2.99
-Glu → +Glu + CHX <sup>b</sup>	12.68 ± 2.09	-Glu → +Glu + CHX + Ins <sup>c</sup>	12.11 ± 3.14

Cells were serum-starved for 48 hours. Eighteen hours before the end of this time, they were glucose-starved; twelve hours later<sup>a</sup>, sugar transport assay was performed; the remaining cells were given 1 ml of serum-free but glucose-containing medium, with or without 20 µg/ml CHX. Four hours later, 100 mU/ml of insulin were added to appropriate groups.

Data represent means of four experiments<sup>b</sup> ± SEM, or three experiments<sup>c</sup> ± SEM; triplicate plates were used in each experiment.

Table XVI. Effect of Tunicamycin on Insulin-Stimulation of Sugar Transport in Reversed Cells.

Treatment	2-DG Transport (nmoles/mg Protein/ 2 min)	Treatment	2-DG Transport (nmoles/mg Protein/ 2 min)
-Glu <sup>a</sup>	17.07 ± 3.73		
-Glu <sup>b</sup> → +Glu <sup>a</sup>	7.97 ± 1.69	-Glu → +Glu + Ins <sup>b</sup>	11.57 ± 2.99
-Glu → +Glu + T <sup>a</sup>	7.25 ± 2.46	-Glu → +Glu + T + Ins <sup>b</sup>	11.53 ± 2.02

Cells were cultured as in Table XV. During the 6 hours of reversal, they were treated with 1.5 µg/ml tunicamycin. Insulin (100 mU/ml) was added during the last two hours of the reversal time.

Data represent means of four experiments<sup>a</sup> and three experiments<sup>b</sup> ± SEM; triplicate plates were used in each experiment.

respectively after treatment with insulin. Under both conditions, tunicamycin inhibited glycoprotein synthesis whether or not insulin was present.

## DISCUSSION

The aim of this work was to characterize the regulatory factors affecting specific insulin binding to the insulin receptor in cultured diploid human fibroblasts (HF) (e.g. under conditions of serum and glucose starvation).

Normal human fibroblasts represent a very good cell system for these studies since they are human cells, are not transformed and their response to the various culture and experimental conditions is not likely to be disturbed by unknown genetic aberrations.

pH and Temperature

Insulin binding to cultured human diploid fibroblasts was characterized by examining pH, temperature and time-dependence, and degradation of  $^{125}$ I-insulin by the cells.

Reports on the effect of pH vary, depending on the tissue used and the culture conditions. For Chinese hamster kidney cells (Wyse and Chang, 1981), and rat hepatoma membranes (Freychet et al., 1971), optimum binding was achieved at pH 8.0; for rat adipocytes (Cuatrecasas, 1971), mouse fibroblasts (Thomopoulos et al., 1976) and human lymphocytes (Gavin et al., 1973) pH 7.8 was the optimum. For human fibroblasts in suspension, pH 8.0 has been used, or pH 7.4 for human fibroblasts in monolayer (Baldwin et al., 1981). The results presented herein, showing an optimum pH of 7.4 for monolayers of human fibroblasts (Fig. 2) are in agreement with the latter report.



Binding can also be affected by temperature. The two temperatures tested in this study were 22°C which is the normal room temperature and 37°C which is the physiologic body temperature of humans. The time-course experiments for these two temperatures showed that a steady state was formed between 80-120 minutes at 22°C; on the contrary, at 37°C for 2 hours no steady state was achieved (Fig. 3). At 22°C, insulin degradation during the 2-hr binding course was almost unchanged showing a negligible overall increase of approximately 3%, whereas at 37°C insulin degradation was abruptly elevated during the 90-120 minutes of binding procedure without a steady state being attained (Fig. 3).


In an earlier study, binding on human fibroblasts in suspension at 15°C showed a steady state after one hour and up to three hours (Rechler and Podskalny, 1976). In the same report, in agreement with our results, binding performed at 37°C for 2 hours did not attain a steady state and hormone degradation was extensive. Time-course studies at 16°C and 30°C on human fibroblast monolayers expressed a steady state that was achieved after approximately 3 hours of binding at 16°C (Baldwin et al., 1981), but it was 100% greater than that observed at 30°C; insulin degradation was 8% and 45% respectively. The results of these reports indicate that experiments using fibroblast suspensions differ from those of fibroblast monolayers. In the present study the experiments were carried out at 22°C for 120 minutes; at this temperature, steady state was rapidly attained, insulin degradation was low and plate handling minimal.

Specific binding averaged approximately 50% of the total binding (data not shown).

### Cell Growth

It has been shown (Thomopoulos et al., 1976, 1977) that mouse fibroblasts bind more insulin when they are at a stationary phase than when they are growing. This happens regardless of the factors (i.e. contact inhibition or serum starvation) involved in the growth arrest. Further, the responsiveness of confluent cells to insulin is greater compared to that of growing cells (Ishibashi et al., 1982). The various phases of the cell cycle also seem to affect the cellular concentration of the receptors as it has been demonstrated in mouse fibroblasts (Thomopoulos et al., 1977). This cell system expressed higher insulin binding during their G<sub>1</sub> or G<sub>2</sub> phases than during the mitotic period and this increase was solely due to a higher receptor number, without the affinity being altered.

In agreement to that is Wyse and Chang's (1982) report on Chinese hamster kidney cells (CHK). Binding increased with increasing cell density and remained relatively stable when cells reached confluence. The CHK cells grown in medium supplemented with serum from different animal species showed differences in binding. Insulin binding was inversely related to the growth obtained with the different sera. The above results suggest that decreased cell growth is associated with high binding in mouse fibroblasts and Chinese hamster kidney cells.



### Glucose Concentration

The data reported herein on normal human fibroblasts (HF) show the effect of cell density and cell cycle controlling factors on insulin binding in association with the glucose concentration in the feeding medium. Confluent cells, sustained 24 hours of serum deprivation in medium containing 1 mg/ml glucose. During this period quiescent, non-proliferating cultures were obtained which expressed lower binding than control cells continuously remaining in medium supplemented with 10% fetal calf serum and 1 mg/ml (5.55 mM) glucose. If cells were placed in serum-free or serum-containing medium with 4 mg/ml (22.2 mM) glucose, binding levels were close to those of the serum-fed cells of the first group (Table I). Similar results were reported by Wyse and Chang (1981) in CHK cells and by Germinario *et al.*, (1984) in HF. In the first study, cells cultured for 18 passages in medium containing 5.55 mM glucose expressed lower binding than their counterparts grown in an environment of 22.2 mM glucose. In the second study it was shown that higher concentrations of glucose (0-22.2 mM) had a positive effect on insulin binding in serum-starved fibroblasts.

Serum contains many growth and other factors which can influence insulin binding and action (Hershko *et al.*, 1971; Germinario *et al.*, 1984). In the present study, within the first 12 hours, the cells of the serum-deprived groups were had ceased proliferating and became quiescent (Holley and Kiernan, 1974). At this stage the only factor influencing the insulin binding is the concentration of glucose in the

feeding medium. In the continuously serum-fed groups, although the amount of added glucose varies, it does not affect insulin binding. It has been shown that serum contains factors which decrease protein degradation (Hershko et al., 1971); this might account for the similarity in the binding values of the serum-fed cells regardless of their glucose content. Of possible importance is that serum contains glucose and serum glycoproteins contain heteropolysaccharide units which can be reutilized as sugar sources. Due to these two factors, the serum-fed groups' glucose supply is higher than 1 or 4 mg/ml. In these latter groups, however, binding is at the same level as that of the serum starved but high glucose-fed group (22.2 mM). This could be an indication that when HF reach confluence, the serum factors might not be as influential on the insulin receptor regulation (Thomopoulos et al., 1976) as the amount of glucose in the medium.

#### Effect of Other Sugars

Replacement of glucose by galactose showed that the latter can substitute for glucose over a period of 24 hours as far as insulin binding is concerned. This sugar which is metabolizable and can actually be converted to UDP-glucose and enter the glucose metabolic pathways (Lehninger, 1981), has been shown to substitute almost mole to mole for glucose in the feeding medium for mouse L-fibroblasts and human HeLa Cells (Eagle, 1955). It has been shown (Germinario et al., 1982) that when D-galactose is substituted for D-glucose in human

fibroblasts, 2-DG transport is increased to levels similar to those of glucose-starved cells. Also, it has been shown (Germinario et al., 1984) that 2-DG transport and insulin binding are inversely correlated; that is, glucose starvation increases 2-DG transport but decreases insulin binding and vice versa. Although, in the present work, 2-DG transport was not studied under these conditions, insulin binding was similar in D-galactose-fed and glucose-fed cells. This might be an indication that, even though galactose can replace glucose nutritionally, it acts in a different fashion from glucose as far as the inverse relationship between hexose transport and insulin binding are concerned. Replacement of glucose by 3-O-MG, a nonmetabolizable sugar, led to binding levels similar to those expressed by glucose-fed cells. With regard to the inverse correlation between glucose transport and insulin binding, the effect of 3-O-MG on binding is what one might expect based on the effect of this sugar on 2-DG transport (Germinario et al., 1982). Since this sugar is non-metabolizable, this effect might be due to an indirect (because of its stereochemistry) activation of factors which can affect insulin binding and glucose transport.

#### Macromolecular Control

Glucose is the major carbon source used by the cells, in order to survive and carry out their functions. Complete glucose deprivation had various effects on the cells. During the course of this study, depending on the cell strain, some cells could not tolerate more than

18 hours of glucose starvation; others tolerated as long as 66 hours of glucose deprivation. The reasons for this variability are unknown. In our experiments glucose starved cells looked viable and healthy. Cells deprived of glucose for 12-18 hours expressed approximately half the specific insulin binding of those fed with glucose. This effect was reversible; that is, if the starved cells were placed in a serum-free medium containing glucose (4 mg/ml), the binding was gradually restored within 6 hours. This phenomenon was first described by Germinario et al. (1984). The question raised at this point was as to what molecular events control this decrease and reversibility mechanism. The decrease in binding due to glucose starvation does not involve changes in the affinity of the receptors (Wyse and Chang, 1981; Germinario et al., 1984) but it is caused by a reduction in the number of the receptor sites expressed on the cell surface (Germinario et al., 1984).

It has been shown in mouse myeloma cells (Stark and Heath, 1979) that glucose deprivation induces production of proteins not completely glycosylated; yet glycosylation does not entirely cease and when glucose is added, glycosylation is completed. Additionally, it has also been shown that the receptor subunits contain carbohydrates (Hedo et al., 1983). Further, receptors which have not been glycosylated (aglycoinsulin) are not functional (Ronnett and Lane, 1981).

The above observations could account for the speculation that the decrease in the number of the receptors in the glucose-starved (-Glu) state might be due to production of defective, poorly glycosylated

receptors incapable of binding insulin and not to absence of receptors.

Since the insulin receptor is glycoprotein in nature, inhibitors of protein, glycoprotein and RNA synthesis were used in an attempt to clarify the mechanism(s) involved in the receptor decrease due to glucose deprivation and restoration due to subsequent glucose refeeding.

Actinomycin D, the RNA synthesis inhibitor (Germinario et al., 1982) had no effect on binding during the reversal phase (Table V) even though RNA synthesis was inhibited by approximately 80% (Table VI). This suggests that, although the specific insulin binding was reduced in glucose-starved cells, for its restoration, synthesis of new message was not necessary within the time-frame of the experiments. This does not agree with a previous report (Prince et al., 1981) where it was found that, in HF, restoration of receptors after insulin induced loss of binding was completely inhibited by Act D. This might be suggestive of different mechanisms controlling the loss of receptors due to excess hormone vs glucose starvation. On the other hand, since the concentration of Act D was almost 70 times higher than that used in the present study, protein synthesis might have been disturbed (Germinario et al., 1982) accounting for the observed inhibition of the binding return.

The next factor considered likely to be involved in the glucose starvation-induced loss of binding and its reversal was protein synthesis. The data (Table VIII) showed that protein synthesis was

required for the return of insulin binding. This may involve synthesis of receptor structural proteins, synthesis of proteins participating in biochemical reactions (i.e. proteins involved in receptor expression), or both. The affinity of the receptor has been found to be undisturbed by CHX (Reed et al., 1981).

The results herein (Table VIII) are indicative of a structural rather than expressional protein synthesis inhibition. The binding data of the three groups 1) -Glu  $\rightarrow$  -Glu (continuously starved), 2) -Glu  $\rightarrow$  +Glu (reversed), and, 3) -Glu  $\rightarrow$  +Glu + CHX (reversed and treated with CHX) are suggestive of this view. In the first group, the cells have been starved for a total of 24 hours. In the second group, the number of receptors increases by 55% when they are placed in glucose-containing medium for the last six hours. If CHX is added at the beginning of this glucose reversal phase, the receptor number increases by 26% when compared to the starved group. Since the cells (-Glu  $\rightarrow$  -Glu), which have already stopped proliferating due to the serum starvation, are also deprived of glucose which is a major energy source, their functional activities will have become minimal. Therefore the number of the functional receptors in those starved cells is greatly reduced (Tables III, VIII). This could be due to either 1) structural deformities e.g. incomplete glycosylation (Stark and Heath, 1979) which prevents them from being functional (Ronnett and Lane, 1981), 2) a decrease or 3) a complete arrest in their production. In the latter case, receptors expressed in the -Glu stages must have been retrieved from the receptor pool (Deutsch



et al., 1982) formed during the previous glucose-fed state, or by those hidden in the cell membrane bilayer (Cuatrecasas, 1972).

When the cells, after 12 or 18 hours, are refed with glucose they resume their functions and start producing (more?) receptors. When CHX is added, synthesis of new receptors is inhibited; and, because of ~~the~~ availability of the energy source, expression of the preformed receptors takes place reaching the untreated glucose-starved (-Glu → -Glu) group's levels, and even higher, because, due to glucose, glycosylation of incomplete receptors takes place.

CHX could act at three levels: 1) inhibition at the level of receptor synthesis, 2) inhibition at the level of the synthesis of proteins controlling the exit of the receptors to the cell surface, and 3) inhibition at the level of the synthesis of proteins regulating the entry of the receptors for degradation. If CHX inhibited proteins regulating the exit of the receptors from the intracellular compartment to the surface, the binding expressed by the -Glu → +Glu + CHX group should be lower than that of the -Glu → -Glu group; since, even though, in response to glucose, new receptors were formed they would be prevented from emerging to the surface along with the pre-existing ones.

If CHX acted at the entry of the receptors for degradation, there should be an accumulation of receptors on the surface due to constant expression of the old and the newly formed ones whereas internalization is inhibited. Therefore the binding value of these cells should be even higher than those of the -Glu → +Glu group, in which the turnover of the receptors has become normal. Yet, CHX

blocks binding restoration in reversed cells and this is suggestive of a translational (structural) block, indicating that recovery from glucose starvation requires synthesis of new receptors.

Use of CHX has had various effects on binding in different tissues under various experimental conditions. The results of this report are in agreement with Kosmakos' and Roth's (1980) report on receptor restoration following insulin-induced receptor decrease in IM-9 lymphocytes, which was blocked by CHX. However, in 3T3-L<sub>1</sub> fibroblasts (Kadle et al., 1983) and 3T3-L<sub>1</sub> adipocytes (Deutsch et al., 1982) CHX treatment resulted in an accumulation of insulin receptors at the cell surface in the first case, and, it did not affect externalization of intracellular pre-existing receptors from the interior of the cell in the second.

The receptor for insulin is a glycoprotein (Reed et al., 1981; Ronnet and Lane, 1981; Hedo et al., 1983; Caro et al., 1984) and glycosylation is one of the crucial steps in its synthesis. Further, it been shown to occur at a post-translational stage in 3T3-L<sub>1</sub> adipocytes (Ronnet and Lane, 1981). Tunicamycin, which inhibits dolichol-mediated protein glycosylation decreased specific binding to various tissues such as 3T3-L<sub>1</sub> adipocytes (Rosen et al., 1979, Ronnet and Lane 1981, Reed et al., 1981) as well as IM-9 lymphocytes (Keefer and DeMeyts, 1981).

In the present study, tunicamycin decreased binding by 40% in serum-starved, glucose-fed confluent human fibroblasts within 6 hours (Table XI).

The glucose-starved and -reversed cells expressed an inhibition in binding of approximately 35% within 6 hours (Table X). This indicated that protein glycosylation was necessary for glucose-refeeding restoration of binding in HF (Table X). This suggests that glycosylation of the receptors themselves or other proteins, or both, is essential for the recovery of the receptors from the cytosol to the cell surface after glucose refeeding. In the non-fed groups there is no inhibition of glycoprotein formation, and the binding values of the tunicamycin-treated cells are higher than those of the untreated controls.

This generally indicates that tunicamycin did not have any effect on binding in continuously starved cells as if glycosylation did not take place at this stage and hence there was no inhibition. This finding though was persistent in all four experiments performed and it might be indicative of an event whose interpretation is more complex than the above general statement.

At the -Glu  $\rightarrow$  -Glu stage the cells, which "operate on the principle of maximum economy of parts and processes" (Lehninger, 1981), decrease their functional processes to a minimum due to lack of energy source. One could speculate that low energy requiring protein factors are produced to control this slowing down of the functions including the expression of the receptors. When tunicamycin is added, this protein is inhibited and the expression of the pool receptors is accelerated and thus binding levels are higher in this group in comparison with the controls. When glucose is added, the cells which

have no need for producing this factor any more, resume their functions, the production and turnover of the receptors returns to normal and binding is restored.

When tunicamycin is concomitantly added with glucose, the retarding factor is removed, the turnover of the preformed receptors goes back to normal but the synthesis of new ones is arrested at the glycosylation stage. The values of the  $-Glu \rightarrow -Glu$  and  $-Glu \rightarrow +Glu + T$  groups are almost equal and this might account for this interpretation. That is, since glycosylation is arrested, no more production of complete new receptors takes place and the binding remains at the same levels as in the starved group with only an expression of the receptors completed at a preceding stage. There is a dissimilarity in the binding expressed by the reversed cells treated with CHX compared with those treated with tunicamycin. According to the interpretation given, they both express only preformed receptors and therefore they should express similar binding in regard to their respective  $-Glu \rightarrow -Glu$  groups. The binding expressed by the CHX-treated group ( $-Glu \rightarrow +Glu + CHX$ ) is higher than the respective  $-Glu \rightarrow -Glu$  group, whereas, the binding of the tunicamycin-treated group ( $-Glu \rightarrow +Glu + T$ ) is almost equal to that of their respective  $-Glu \rightarrow -Glu$  group. This difference might be explained by the fact that in the CHX + glucose group, glycosylation is completed in the poorly glycosylated receptors produced during the starvation state. This lead to the observed increase over the  $-Glu \rightarrow -Glu$  group. In the  $-Glu \rightarrow +Glu + T$  cells, glycosylation of those receptors poorly glycosylated at the  $-Glu$  state and of those newly synthesized, was

completely arrested. This lead to no change over the -Glu → -Glu group.

Recovery of insulin-induced down regulated receptors was also found to be inhibited by tunicamycin (Kadle et al., 1984) in mouse fibroblasts. This indicated that glycoprotein synthesis is necessary for reappearance of the receptors, but cycloheximide treatment demonstrated that protein synthesis is not required for their recovery in this tissue. In both cases, insulin-induced and glucose-starvation-induced decrease in binding is due to a decrease in the number of the receptors and not to changes in their affinity (Kadle et al., 1984, Germinario et al., 1984). It seems though that the mechanism for their recovery to the surface is not the same.

In human fibroblasts, receptor recovery due to glucose refeeding requires both, protein as well as glycoprotein synthesis. In 3T3-L<sub>1</sub> fibroblasts, recovery of receptor loss due to hormonal induction requires glycoprotein but not protein synthesis. In IM-9 lymphocytes, receptor restoration, lost through insulin induction, requires protein synthesis (Kosmakos and Roth, 1980). There is no data about glycoprotein requirement in this report. These inconsistent results suggest that not all tissues are regulated the same way as far as insulin receptors are concerned, the regulation being also dependent on the factors causing the receptor loss.

The data presented on the effect of the three inhibitors on the reversal phase of the glucose starvation scheme could be suggestive of the fact that for restoration of glucose-starvation-loss of binding:

- 1) no new message synthesis is required,
- 2) protein synthesis is

necessary, 3) protein glycosylation is also required. It could also to be speculated that this receptor loss is due to an arrest in their completion, induced by lack of glucose.

#### Relation to Insulin Action

Binding of the insulin molecule to its receptor represents the first step in a series of biochemical reactions through which insulin exerts its effect on the cells. One of the major actions of insulin is to promote glucose metabolism in the cells. This effect has been extensively studied in various tissues including chick fibroblasts (Shaw and Amos, 1973) rat adipocytes (Cushman et al., 1984), human fibroblasts (Germinario and Oliveira, 1979; Berhanu and Olefsky, 1981; Ishibashi et al., 1982) and also cell-free systems (Kono 1984). Besides insulin, another factor of considerable interest because of the regulatory effect it exerts on glucose transport in the cells, is the glucose concentration in the culture medium. Complete glucose deprivation causes a striking elevation in the amount of glucose transported into the cells (Shaw and Amos, 1973; Salter and Cook, 1976; Germinario et al., 1982; Yamada et al., 1983) whereas glucose feeding decreases it.

As it has been shown (Germinario et al., 1984) the two phenomena of glucose transport and insulin binding are inversely related, the relation being controlled by the concentration of glucose in the feeding medium; that is, factors elevating sugar transport depress

insulin binding and vice versa, and this phenomenon is reversible within 6 hours, the reversibility also being dependent on the concentration of glucose in the medium.

The utilization of CHX in the present study during the glucose transport reversal phase (Table XV) basically demonstrated that protein synthesis was necessary for glucose-induced repression of sugar transport and for the insulin-induced stimulation of this function. The first of these two observations is in agreement with previous reports on CEF (Yamada et al., 1983) and on HF (Germinario et al., 1982).

Various hypotheses have been developed concerning the mechanism of the glucose-starvation-induced increase in the glucose transport. Some investigators have proposed that sugar carrier's regulation is due to a balance between their synthesis and their inactivation (Christopher, 1977; Yamada et al., 1983) and, that metabolites of glucose may stimulate carrier inactivation (Christopher, 1977), through a process where protein-degrading systems are involved (Germinario et al., 1982). In the present study, both glucose-induced depression of sugar transport and the return of insulin-stimulated sugar transport were inhibited by CHX. This indicated that protein synthesis is necessary for both functions.

According to the hypothesis of translocation of pre-existing intracellular glucose transporters (Cushman et al., 1984; Kono, 1984) insulin only exposes them (-Glu. → +Glu + Ins) counteracting somehow the glucose metabolite effect (Christopher, 1977; Germinario et al., 1982) in a process that requires protein synthesis

(-Glu  $\rightarrow$  +Glu + CHX + Ins). It is interesting to note that CHX blocks the glucose-induced decrease in glucose transport in reversed cells and, it also blocks the glucose-induced return of insulin binding (Table VIII); this blockage results in the inverse phenomenon (Germinario et al., 1984) of increasing sugar transport, while concomitantly decreasing insulin binding.

According to the data (Table VIII) in the present report at the -Glu  $\rightarrow$  +Glu + CHX state, binding is decreased due to a decrease in the number of the receptor sites. When these cells are treated with insulin (-Glu  $\rightarrow$  +Glu + CHX + Ins) the amount of hormone bound will be lower than that of the controls (-Glu  $\rightarrow$  +Glu + Ins). At this point one could consider three possibilities for the fact that insulin does not appear to have any effect on transport in CHX-treated cells. The first possibility is that insulin at that low binding concentration (Table VIII) is not sufficient to stimulate transport in these cells (Table XV). The requirement of protein synthesis for insulin action (which is inhibited by CHX) in the -Glu  $\rightarrow$  +Glu + CHX + Ins group may be referring to the production of insulin receptors. Since CHX inhibits this production (Table VIII) it prevents insulin from binding at sufficient amounts and therefore acting. Thus CHX acts as an inhibitor of insulin receptor synthesis and subsequently as a mobilizer of intracellular sugar carriers at the same time.

The second possibility is that, although due to CHX the number of the active binding receptors is lower, insulin action occurs; in this case the effect in the -Glu  $\rightarrow$  +Glu + CHX + Ins group is not additive



as one might expect, because CHX and insulin may act through different pathways to expose the same pool of intracellular transporters (compare  $-Glu \rightarrow +Glu + CHX$  with  $-Glu \rightarrow +Glu + Ins$ ). Therefore if one of them (CHX) exposes the carriers ( $-Glu \rightarrow +Glu + CHX$ ), the addition of the other (insulin) does not have any further effect ( $-Glu \rightarrow +Glu + CHX + Ins$ ). The third possibility is that CHX inhibits the insulin action at the level of translocation of the carriers. That is, although insulin binding is low in the CHX-treated group, insulin could cause translocation of the carriers to the surface through a protein synthesis requiring process: CHX inhibits protein synthesis at this level and therefore insulin action is prevented.

The decrease in the number of insulin receptors due to CHX does not affect either of the last two explanations under these conditions. Tunicamycin on the contrary did not have any effect on the repression of sugar transport in any of the fed or reversed groups, or even in insulin-stimulated cells (Tables XIV and XVI). This indicates that glycoprotein synthesis is not involved in the glucose-induced depression of sugar transport. Further, insulin stimulation of this function does not require glycoprotein formation either, although insulin does stimulate glycoprotein synthesis (Fig. 7).

This is in disagreement with previous reports, where, use of tunicamycin resulted in defective glucose transport in chick embryo fibroblasts (Olden et al., 1979), and in impaired insulin sensitivity in 3T3-L<sub>1</sub> adipocytes (Rosen et al., 1979). However, the different

cell type or experimental conditions employed in both reports might account for this disagreement.

It has been postulated that glucose starvation-induced increase in sugar transport in chick fibroblasts (Kletzien and Perdue, 1975) is associated with the synthesis of two non-glycosylated membrane proteins (GRP's) (Shiu et al., 1977). It has been shown that during glucose deprivation, glycosylation is impaired (Stark and Heath, 1979; Turco, 1980). If these two GRP's are effective in raising transport in the non-glycosylated form, they should be ineffective in a glucose-containing medium; because, due to the presence of glucose, they would become glycosylated (Stark and Heath, 1979; Turco, 1980). Therefore, when tunicamycin is added and glycosylation is inhibited, these two proteins are not inactivated any longer; thus, transport should be elevated in these cells as in the -Glu group. In the present study such a phenomenon is not observed and this indicates that in normal human fibroblasts the regulatory mechanism of sugar transport might be different. Either these proteins are not being produced in the -Glu state; or they are completely eliminated upon addition of glucose; or, even if they are synthesized and active in the reversed, tunicamycin-treated cells (-Glu  $\rightarrow$  +Glu + T), glucose metabolites act through a different pathway to decrease transport counteracting the effect of any other component.

Insulin does stimulate sugar transport in tunicamycin-treated fed and reversed cells; one could speculate that somehow insulin counteracts the effect of glucose and activates these two proteins

which increase transport. However, the data from the  $-Glu \rightarrow +Glu + T$  group do not support this speculation.

If the aforementioned translocation theory is valid, the latter observation demonstrates that for insulin-induced expression of glucose carriers on the cell surface, glycoprotein synthesis is not required. Under the same conditions, insulin stimulated glycoprotein synthesis by 176% of the controls.

According to the insulin binding data, at the  $\pm Glu \rightarrow +Glu + T$  state, the receptor number decreases. One would expect that, due to this, the response of the cells to insulin would be decreased and hence, sugar transport would be decreased. However, tunicamycin-treated cells upon stimulation with insulin ( $\pm Glu \rightarrow +Glu + T + Ins$ ) express the same transport as the untreated controls ( $\pm Glu \rightarrow +Glu + Ins$ ). This might lead to the speculation that, within a certain range, the concentration of the receptors may not be critical for a certain magnitude of the response to insulin within the 6 hours of reversal.

It is interesting to note that, the inverse correlation governing sugar transport and insulin binding, as far as glucose concentration and CHX treatment are involved, breaks down here. As it was shown before, treatment with galactose involved the same breakdown in this inverse correlation, but the incubation conditions were different.

Agents which decrease binding do not increase transport. This latter observation indicates that these agents could be related to proteins (Germinario et al., 1982) but not to glycoproteins.

In conclusion, the data in the present work confirm that glucose concentration is an important factor for both insulin binding and action (Germinario et al., 1984).

They also show that the presence of glucose enhances both insulin binding and action. For insulin binding, both protein and glycoprotein synthesis are required, whereas, for glucose-induced insulin action, glycoprotein formation is not necessary but protein formation is required.

However, glucose alone exerts an inverse effect on insulin binding-sugar transport (Germinario et al., 1984). Under the same environmental conditions glucose increases insulin binding while decreasing sugar transport. In both phenomena, protein synthesis is necessary, whereas glycoprotein synthesis is required only for the increase in insulin binding, and not for the decrease in sugar transport.

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