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Hexose Transport Regulation in Respiration-Deficient Fibroblast Cell Lines

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A Thesis

in

The Department

of

Biology

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

March 1990

ABSTRACT

Hexose Transport Regulation in Respiration-Deficient Fibroblasts

Susan Andrejchyshyn

Hexose transport regulation was studied in the (V79) parental and respiration-deficient (G14) i.e., NADH-Coenzyme Q reductase deficient, Chinese hamster lung fibroblast cell lines. Hexose transport, measured by 2-deoxy-D-glucose uptake, was 35% higher in glucose-fed G14 cells when compared to V79 cells. The G14 cells exhibited an elevated V_{max} with no differences in the K_m for 2-DG transport or K_I for D-glucose. In addition, hexose utilization was consistently higher in G14 cells. Factors which increase sugar transport (e.g., glucose deprivation, serum or insulin exposure) or decrease sugar transport (e.g., serum deprivation) in the V79 cell line had little effect on hexose transport in the G14 mutant cell line. Exposure to heat shock and/or glucose deprivation did not appear to result in an enhanced level of any specific peptides in either V79 or G14 cells.

A comparative study of transport regulation using a normal (MCH55) and a strain (WG750) of human diploid fibroblasts deficient in the same enzyme as the hamster cells was also performed. Hexose transport was strikingly higher in WG750 cells and this was expressed as an elevated V_{max} for 2-deoxy-D-glucose. Glucose-deprivation or short-term exposure to serum elevated transport in both cell strains. Finally, insulin stimulated hexose transport to a lesser degree in WG750 cells when compared to control cells.

The data demonstrate that the enzyme deficiency in G14 and WG750 cells has altered cellular response(s) to several factors affecting hexose transport. It is evident that studies using these cell lines will provide answers to questions concerning regulation of hexose transport in mammalian cells.

To my Parents

Lillian and Walter

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

CB: cytochalasin B

DME: Dulbecco's modified Eagle's medium

GRP: glucose-regulated protein

HSP: heat-shock proteins

P.D.: population doublings

PBS: phosphate buffered saline

TCA: trichloroacetic acid

2-DG: 2-deoxy-D-glucose

3-0-MG: 3-0-methyl glucose

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

Introduction

In this study the mechanism(s) of hexose transport regulation were investigated in a parental (V79) and a respiration deficient (G14) cell line of cultured Chinese hamster lung fibroblasts as well as in a normal (MCH55) and a respiration-deficient (WG750) cell strain of human diploid fibroblasts.

Hexose Transport in Mammalian Cell Systems: Effect of Culture Conditions and Serum.

Studies with animal cells in culture permit investigation of the regulation of transport systems under various conditions. For some time now cultured normal and transformed avian and mammalian cells have served as model systems for studying hexose transport regulation in the normal and malignant states. The use of cultural selection procedures has provided opportunities for the isolation of transport mutants which may help to identify regulatory components involved in hexose transport (Ullrey et al.,1982, Kalckar and Ullrey, 1984, D'Amore et al., 1986b).

It has been well established that the rate of hexose transport in mammalian cells is dependent upon many factors including specific nutrient deprivation, the rate of cell growth, cell density, the composition of the culture medium, etc (Martineau et al., 1972, Kletzien and Perdue, 1974a, Ullrey et al., 1975, Bader, 1976, Kalckar, 1976, Johnson and Schwartz, 1976, Christopher et al., 1977, Germinario et al., 1978, Kalckar et al., 1980, Ishibashi et al., 1982, Pessin et al., 1982, Tillotson et al., 1984).

Serum, in particular, contains a large number of peptide hormones or hormone-like growth factors which act to stimulate multiplication in many types of cells (Nishikawa et al., 1975, Johnson and Schwartz, 1976). Romano and Connell reported that serum-starved mouse 3T3 un-transformed cells enter the G_0 stage of the cell cycle and become growth arrested (Romano and Connell, 1982). Many metabolic changes have been associated with this quiescent state. Transfering actively growing untransformed mouse Balb^c/3T3 fibroblasts to serum-free media resulted in decreased incorporation of

leucine, thymidine, and uridine into macromolecules (Hershko *et al.*, 1971).

Many investigators have reported increased hexose uptake in actively growing cells when compared to quiescent cells (Bradley and Culp, 1974, Kletzien and Perdue, 1974a, 1974b, Lang and Weber, 1978, Dubrow *et al.*, 1978). Further, Kletzien and Perdue showed that the addition of serum to serum-starved cultures of chick embryo fibroblasts resulted in a rapid increase in hexose uptake characterized by an elevated V_{max} for sugar transport. The initial increase in transport was not affected by RNA or protein synthesis inhibitors suggesting that the initial effects of serum stimulation on hexose transport are regulated at both the post-transcriptional and post-translational level in these cells (Kletzien and Perdue, 1974a, 1974b). L6 myoblasts also displayed increased rates of hexose transport following prolonged exposure to serum (Klip *et al.*, 1984). In these cells, however, serum-deprivation-induced-increases in hexose transport are dependent on protein synthesis since 5μg/ml cycloheximide inhibited this depression by approximately 60% (Klip *et al.*, 1984).

Glucose Deprivation: Effects of Transporter Expression and Turnover

One of the most striking adaptations of mammalian cells is a greatly enhanced rate of hexose transport when maintained in media devoid of glucose. Tillotson reported that chick embryo fibroblasts grown in media without glucose developed a 4 to 10-fold increase in transport after several hours (Tillotson *et al.*, 1984). Others have demonstrated that L6 rat myoblasts expressed a 2-fold stimulation of 2-deoxy-D-glucose transport upon glucose starvation (D'Amore *et al.*, 1986a) and Christopher showed that hexose uptake by hamster NIL cells was increased 5 to 10-fold by either substituting fructose for D-glucose or by completely omitting D-glucose from the culture medium for 24 to 48 h (Christopher *et al.*, 1976b). Additionally, this response to glucose deprivation has been demonstrated in HeLa cells (Shaw and Amos, 1973), Chinese hamster lung fibroblasts (Franchi *et al.*, 1978), rat mammary adenocarcinoma cells (Gay and Hilf, 1980) and human fibroblasts (Salter and Cook, 1976, Germinario *et al.*, 1982).

The exact mechanism of up-regulation of hexose transport under conditions of glucose deprivation appears to differ from cell type to cell type. Yamada found that

chick embryos cultured for several hours displayed an increase of glucose carriers in the plasma membrane which was dependent on protein synthesis (Yamada *et al.*, 1983). This adaptive response in chick embryo fibroblasts could be blocked by the presence of inhibitors such as actinomycin D and cordycepin indicating a transcriptional level of control (Martineau *et al.*, 1972, Kletzien and Perdue, 1975b, Tillotson *et al.*, 1984). Others have demonstrated a post-translational level of control since protein synthesis was not required in Chinese hamster cells for the glucose-starvation-induced increase in sugar transport (Franchi *et al.*, 1978). Glucose-starvation-enhanced sugar transport was shown to be dependent on protein synthesis but not RNA synthesis in human skin fibroblasts indicating a translational level of control (Germinario *et al*, 1982).

Kletzien and Perdue reported that chick embryo fibroblasts which had been glucose starved for 40 h developed an increased V_{max} for sugar transport from 18 to 95 nmoles per milligram protein per minute with no change in the affinity or K_m for 2-DG (Kletzien and Perdue, 1975b). Similar kinetic data have been obtained for Chinese hamster cells (Franchi *et al.*, 1978) and human skin fibroblasts (Germinario *et al.*, 1982). The elevation in the V_{max} for sugar transport may be interpreted as representative of an increase in the number of functional carriers at the plasma membrane upon glucose starvation (Kletzien and Perdue, 1975b).

Glucose re-feeding of glucose-starved cultures initiates a gradual down-regulation of transport in many mammalian cell types termed the "glucose-mediated transport curb" (Martineau *et al.*, 1972, Musliner *et al.*, 1977, Germinario *et al.*, 1982). Concommitant addition of cycloheximide, however, maintains elevated hexose transport by preventing the decline of transport that normally occurs when glucose-starved cells are placed in media containing glucose (Yamada *et al.*,1983). These results suggest that regulation of inhibitory elements or of protein degradative enzymes may play a role in regulation of hexose transport (Hershko, 1973, Kalckar, 1976). Christopher, however, reported that when cycloheximide was added to glucose-fed hamster cells no changes in transport were observed until a putative precursor pool of transporters were used up (Christopher *et al.*, 1976b). At this point a decrease in hexose transport resulted since no new carriers could be synthesized. When cycloheximide was added to glucose-starved cells, there was an increase in transport suggesting a movement of preformed carriers from an inactive state to an active state. These results also demonstrated that glucose (or one of its metabolites) was required for inactivation/turnover of the carrier.

Glucose metabolites may alter carrier synthesis and/or inactivation at the transcriptional level by stimulating production of mRNA for the inactivation process although to date no evidence has been presented for this (Yamada *et al.*, 1983). It has been suggested that protein synthesis is necessary for carrier inactivation either by a mechanism whereby a specific protein directly inactivates the carrier or, alternatively, one or more non-specific inactivation mechanisms that require protein synthesis (Yamada *et al.*, 1983). One group found that glucose-deprived 3T3-C2 murine fibroblasts exhibited a 5-fold increase in hexose transport and cytochalasin B binding in plasma membranes (Haspel *et al.*, 1986). Immunoblots of plasma membranes showed that glucose-fed cells contained a 55,000 Da glucose transporter peptide as well as a smaller 42,000 Da polypeptide. Short-term (12 h) glucose deprivation resulted in accumulation of the 55,000 Da polypeptide whereas prolonged glucose deprivation caused the appearance and larger accumulation of the 42,000 Da polypeptide. Interestingly, it was shown that the appearance of the smaller polypeptide was dependent on protein synthesis while the 55,000 Da polypeptide was independent of protein synthesis. No changes in *in vitro* translatable glucose transporter mRNA or glucose transporter synthesis were found upon glucose deprivation. The author concluded that the increase in glucose transporters upon glucose deprivation resulted from a decrease in glucose transporter degradation (Haspel *et al.*, 1986).

Kalckar has proposed that specific requirements must be met in mammalian cells (such as NIL hamster cells, 3TC cells and Chinese hamster cells) in order to mediate the glucose transport curb including (1) the presence of glucose or one of its analogs; and (2) the presence of metabolites able to generate oxidative energy metabolism (Kalckar and Ullrey, 1984). Using inhibitors such as 2,4-dinitrophenol and malonate which are known to interfere with energy metabolism, several workers have shown that the addition of these inhibitors to glucose-fed hamster fibroblasts cells released the glucose-mediated transport curb (Kalckar *et al.*, 1979, Ullrey and Kalckar, 1981, Ullrey *et al.*, 1982). This indicates that regulation of the transport curb is coupled to oxidative energy metabolism (Ullrey *et al.*, 1982). Christopher and Morgan suggest that lysosomal cathepsin activity is involved as well since changes in the activity of several lysosomal cathepsins in NIL hamster cells were accompanied by exactly opposite changes in hexose transport (Christopher and Morgan, 1981).

Transport activity in hamster cells is believed to be regulated by a balance of activation and/or synthesis and turnover of hexose carriers (Christopher, 1977). Although the precise mechanism of carrier inactivation is not understood, it has been postulated that structural modification and subsequent degradation of the carrier may play a role (Tillotson *et al.*, 1984).

The Effect of Insulin on Hexose Transporter: Translocation and/or Intrinsic Activity

The biological effects of insulin are very diverse. One of the primary actions of insulin is to stimulate hexose transport in certain cell types (Crofford and Renold, 1965, Park *et al.*, 1968, Lawrence *et al.*, 1977, Olefsky, 1978, Germinario *et al.*, 1986). Insulin action has been studied in adipocytes (Olefsky, 1978, Germinario *et al.*, 1979, Cushman and Wardzala, 1980, Czech, 1980, Karnieli *et al.*, 1980, Ludvigsen and Jarett, 1980, Suzuki and Kono, 1980, Ciardaldi and Olefsky, 1982, James *et al.*, 1987, Marshall, 1989), muscle (Klip *et al.*, 1984, Kozka and Gould, 1984, Clausen and Flatman, 1987) rat diaphragm (Wardzala and Jeanrenaud, 1981), human red cell membranes (Jensen and Brahm, 1987), Xenopus oocytes (Janicot and Lane, 1989) and human skin fibroblasts (Fujimoto and Williams, 1974, Germinario *et al.*, 1979, Craig *et al.*, 1980, Ishibashi *et al.*, 1982, Germinario *et al.*, 1984, Cynober *et al.*, 1986).

The stimulatory effect of insulin in target tissues is rapid. Maximal activation by insulin of sugar uptake occurs in less than 5 min in adipocytes (Haring *et al.*, 1978, Whitesell and Gliemann, 1979, Frost and Lane, 1985, Kohanski *et al.*, 1986) whereas maximal effect occurs in 15-30 min in human fibroblasts (Germinario and Oliviera, 1979, Berhanu and Olefsky, 1981, Ishibashi *et al.*, 1981, Cynober *et al.*, 1985).

Insulin appears to enhance the V_{max} of hexose transport without influencing the K_m (Germinario and Oliviera, 1979, Ishibashi *et al.*, 1982, Cynober *et al.*, 1986). The increase in V_{max} ranges from 26-64% in fibroblasts to 80-800% in cardiocytes (Eckel *et al.*, 1983, Lindren *et al.*, 1982) and adipocytes (Olefsky, 1978, May and Mikulecky, 1983).

One of the widely accepted mechanisms to explain how insulin exerts its effect on hexose transport is the translocation hypothesis whereby exposure to insulin results in movement of glucose transporters from an intracellular storage site to the plasma

membrane, resulting in increased glucose uptake.

Cushman developed a method for determining the number of glucose transporters in subcellular fractions of isolated rat adipocyte cells (Cushman *et al.*, 1984). D-glucose-inhibitable cytochalasin B binding assays showed that the majority of basal glucose transporters were localized in a low-density microsomal membrane fraction associated with the Golgi fraction. When the cells were treated with insulin more than half of the intracellular transporters were translocated to the plasma membrane fraction. This translocation of transporters was both rapid and insulin concentration-dependent (Cushman, 1984).

In addition, Kono showed that translocation of glucose transporters in reconstituted rat adipocyte liposomes was reversible, dependent on ATP and metabolic energy, but independent of protein synthesis (Kono *et al.*, 1977, Suzuki and Kono, 1979, Kono *et al.*, 1981, Kono *et al.*, 1982, Kono, 1984). Treatment of fat cells with insulin prior to homogenization caused a 6 to 8-fold increase in glucose-transport-activity in the plasma membrane-rich fraction and a decrease in glucose-transport-activity in the Golgi-rich fraction. Treatment of plasma membrane and Golgi-rich fractions with inhibitors of metabolic energy such as 2,4-dinitrophenol or KCN blocked the insulin effect whereas incubation of cells with inhibitors of protein synthesis such as puromycin or cyclo-heximide did not alter the stimulatory or reversible effect of insulin. These results provided evidence for the hypothesis that the glucose transport mechanism is recycled between the plasma membrane and an intracellular storage pool by endocytotic and/or exocytotic reactions controlled by insulin (Kono, 1984)

Recently several investigators have suggested that in addition to translocation, a second mechanism may be involved in the stimulation of glucose transport by insulin (Baly and Horuk, 1987, Blok *et al.*, 1988, Joost *et al.*, 1988). One group employed a kinetic approach to examine the time course for stimulation of hexose transport, translocation of glucose transporters to the plasma membrane and the quantitative increase in cell surface transporters in 3T3-L1 adipocytes following insulin exposure (Gibbs *et al.*, 1988). Their results showed that the time required for the maximum stimulation of hexose transport was considerably longer than the time required for movement of transporters from intracellular locations, and for their subsequent appearance in the plasma membrane. Also, the 8-fold increase in transport by $1 \mu M$ insulin was 3 times larger than the 2.6-fold increase of surface transporters. From these results,

Gibbs suggested that the second mechanism of hexose transport regulation is an increase in the intrinsic activity of the transporters. This event is rate-limiting and operates after transporters translocate to the plasma membrane (Gibbs *et al.*, 1988). Another group demonstrated that incubation of rat adipose cells with 10 µg/ml cycloheximide resulted in a significant decrease in cytochalasin B binding sites in plasma membrane fractions derived from insulin-stimulated cells (i.e. 34 pmol/mg protein versus 14 pmol/mg protein with cycloheximide) to approximately basal levels (10 pmol/mg protein) (Baly and Horuk, 1987). This decrease in plasma membrane transporters upon exposure to cycloheximide was not associated with a reduction in insulin-stimulated hexose transport activity. Together, these data showed that insulin-stimulated hexose transport by a mechansim(s) other than translocation. The author suggested that glucose carriers in the plasma membrane when insulin is present must be more active than those which remain in the membrane under non-induced conditions (Baly and Horuk, 1987). At present, no mechanism is postulated for the putative changes in intrinsic activity of the transporter and this concept remains controversial.

The Hexose Transporter-A Family of Proteins

The facilitated transport of hexoses into mammalian cells is affected by multiple processes. A variety of mechanism(s) have been implicated including changes in the rate of transcription of the transporter gene, changes in the rate of degradation of the transporter protein (Haspel *et al.*, 1986, Flier *et al.*, 1987) and the insulin-induced translocation of the transporters from an intracellular site to the plasma membrane (Suzuki and Kono, 1980, Cushman and Wardzala, 1980). With respect to the latter mechanism, the traditional target tissues (e.g., muscle and fat) demonstrate the most dramatic effects (Simpson and Cushman, 1986). Recent evidence has indicated that there are two immunologically distinct translocatable glucose transporters in insulin-responsive tissues (Wang, 1987, Oka *et al.*, 1988). This has been further supported by the isolation and sequencing of several species of glucose transporters. These would include the erythrocyte/HepG2/rat brain transporter which has been detected in brain, heart, fibroblasts, and adipose cells (Mueckler *et al.*, 1985, Birnbaum *et al.*, 1986), a second distinct transporter expressed in normal liver, intestinal and renal cells (Thorens *et al.*, 1988, Fukumoto *et al.*, 1988) and a third transporter found in muscle and fat cells

(James *et al.*, 1988, James *et al.*, 1989). The latter transporter isoform appears to be the prominant insulin-translocatable species since induction of adipocyte differentation in the 3T3-L1 cell line resulted in increased expression of the fat/muscle cell transporter. Thus, it may be that this transporter is integral to the tissue-specific action of insulin on glucose transport. The data, however, do indicate that there is a family of glucose transporter/glucose transporter-like proteins expressed in mammalian tissues. These proteins have approximately 50% amino acid identity and contain 12 presumed membrane-spanning regions. It is interesting and exciting that the NH_2-terminal and COOH-terminal regions may be implicated in the differential expression and control of this family of transporters (Kayano *et al.*, 1988).

The Effect of Transformation on Hexose Transport

Viral transformation of chick, mouse and hamster fibroblasts also results in an increased rate of hexose transport (Hatanaka, 1976, Plagemann and Rickey, 1974). Although the exact mechanism responsible is not known it has been suggested that the increase in transport stems from an increased number of glucose transporters (Kalckar, 1976, Chrisopher, 1977, Weber *et al.*, 1984). This theory is consistent with cytochalasin B binding studies which demonstrated that the differences in cytochalasin B binding between non-transformed and transformed cells correlated with the differences in rates of hexose transport. Weber reported that chick embryo fibroblasts transformed with Rous sarcoma virus showed dramatic increases in hexose transport (Weber *et al.*, 1984). Interestingly, glucose starvation of these transformed cultures resulted in increased glucose-specific cytochalasin B binding and immunoprecipitable transporter indicating that transformed cells still had the capacity to respond to glucose deprivation. The rate of uptake of galactose was 2.5 to 3-fold greater in polyoma-transformed hamster cells ompared to non-transformed cells (Kalckar *et al.*, 1973). Studies performed by Kletzien and Perdue demonstrated that chick embryo fibroblasts transformed by a temperature-sensitive Rous sarcoma virus displayed increased 2-DG transport (Kletzien and Perdue, 1974b). Kinetic analysis revealed a greater than 2-fold increase in V_{max} for 2-DG while the K_m remained the same, consistent with the interpretation that increased glucose transport in transformed cells is a result of an increase in the number of transporters. Inhibitor data suggest that the increased synthesis of glucose trans-

porters is a result of regulation of translation or post-transcriptional processing of RNA (Kawai and Hanafusa, 1971, Kletzien and Perdue, 1975a, Beug *et al.*, 1978).

It has been suggested that elevation of hexose transport following transformation is a result of pre-existing carriers increasing their activity by allosteric alteration or changes in localization (Weber *et al.*, 1984). Christopher mentioned the possibility that regulation of hexose transport may be dependent on the action of a regulatory unit (Christopher, 1977). In the presence of glucose this labile factor would interact with the glucose carrier in such a way that movement of the carrier to the membrane would be inhibited. In the absence of glucose, activity of the regulatory unit would be lost resulting in higher levels of transport due to increased translocation of transporters to the plasma membrane. Transformed cells may possess a defective regulatory unit which only partially regulates transport under any conditions contibuting to the enhanced rate of hexose uptake characteristic of many transformed cells (Christopher, 1977). Others argue that increased glucose transport is a reflection of the growth state of the cell and not transformation (Romano and Cornell, 1982). Transport of 6-deoxy-D-glucose, a non-metabolizable D-glucose analog, was determined in growth-arrested (serum-starved) and actively growing (serum-fed) cultures of mouse 3T3 cells and SV40-transformed 3T3 cells. The data indicate that serum-fed and SV40-transformed cells have similar higher rates of transport when compared to serum-starved 3T3 cells.

More recently, one group has attributed the increase in hexose transport in *src*-transformed cells to decreased degradation of the glucose transporter (Shawver *et al.*, 1987). Pulse-chase experiments with ^{35}S-methionine were performed with normal and *src*-transformed chick embryo fibroblasts followed by immunoprecipitation of glucose transporter. Results showed that the half-life of the transporter in transformed cells was approximately 3 to 4-fold longer than in normal cells.

On the other hand another group, working with rat 3T3(FR3T3) fibroblasts transfected with *myc*, *ras* or *src* oncogenes, demonstrated increased 2-DG uptake (approximately 4-fold) as well as an increase in transporter protein as evidenced by immunoblots of membranes prepared from these cells using antibody directed against the human erythrocyte transporter (Flier *et al.*, 1987). Northern blot analysis using total cellular RNA from control and transfected cells probed with the human HepG2 transporter cDNA showed significant increases in the mRNA transcript encoding the human glucose transporter in transfected cells. From these results the authors

concluded that the increased rate of hexose transport in these transformed cells was due to increased expression of the glucose transporter gene (Flier et al., 1987). It is clear that controversy still exists regarding the mechanism by which transformation results in an accelerated rate of glucose transport as compared to untransformed cells.

Glucose-Regulated Proteins are Induced by Heat Shock and/or Glucose Deprivation

The exposure of cultured cells to stresses such as drugs, amino acid analogs, transition series metals or growth at elevated non-physiological temperatures induces the "stress response" (Welch and Feramisco, 1982). In particular, the cellular response to thermal stress has been demonstrated in a wide variety of organisms including bacteria, plants and animals, appears to be quite universal and has been conserved through evolution in very distant species (Burdon, 1982, Tissieres, 1982). The stress response has been described as the increased synthesis of mRNAs coding for a select group of proteins with concomittant reduced translation of pre-existing mRNAs coding for other cellular proteins (Welch and Feramisco, 1982). Although the exact location and role of heat-shock proteins (HSPs) have not been fully elucidated there has been considerable speculation regarding their physiological function. It has been postulated that they may play a part in the recovery of homeostasis following thermal stress although all stress proteins appear to be present and actively synthesized under normal conditions albeit at lower rates (Burdon, 1982, Welch and Feramisco, 1982, Rice et al., 1986). Alteratively, heat-shock proteins may be involved in the development of thermotolerance which allows cells to survive subsequent thermal stress that would normally be lethal. In fact it has been shown that a variety of organisms become resistant to lethal heat dose following prior exposure to heat shock although the correlation between the induction of heat-shock proteins and the development of thermotolerance has not been proven in mammalian cells (Burdon, 1982, Welch and Feramisco, 1982, Widelitz et al., 1986). Klevecz suggested that heat-shock proteins are phase-specific proteins which are preferentially translated by specific cellular signals in response to a variety of pertubations (Klevecz et al., 1982). It is important to note that induction of specific proteins may be dependent on particular stimuli which may ultimately help define their function(s).

A great deal of work has been done in recent years to help define the functions of these proteins since it is believed that stresses such as infectious diseases, acute inflammation, massive accidental damage to tissues, etc., trigger the heat-shock response in man (Tissieres, 1982).

Glucose-regulated proteins (GRPs) were originally detected in chick fibroblasts and have been studied extensively in mammalian cells (Pelham, 1986). They were first described as a subset of the heat-inducible stress proteins but recent evidence has demonstrated that they are also overproduced when cells are starved of glucose (Shiu et al., 1977, Zala et al., 1980, Pelham, 1986). The induction of GRPS upon glucose deprivation may be a specific defence mechanism important for cell survival. Two of the major GRPs of molecular weights of 78 and 98 KDa are found in many cell types and are particularly prevalent in secretory cells such as hepatocytes and myeloma cells suggesting that they may be an important factor for normal assembly of secreted proteins (Pelham, 1986). GRP 78 has been shown to be present in many fibroblast cell lines including hamster, rat, mouse, human and chicken as well as tissues such as brain, heart, lung and liver (Hightower and White, 1982, Resendez et al., 1985, Munro and Pelham, 1986). Shiu reported that GRP 78 had a slightly increased rate of synthesis in glucose-fed normal cell cultures (Shiu et al., 1979, Zala et al., 1980). Shiu postulated that GRP 78 may be an enzyme that is involved in hexose utilization or in energy-yielding reactions (Shiu et al., 1977). Zala observed an increased rate of synthesis of 75 and 95 KDa peptides in transformed cell cultures which had been glucose deprived whereas transformed cells maintained in media with high levels of glucose did not overproduce them (Zala et al., 1980). In addition, these peptides were stimulated in AD6 cells defective in glycoprotein synthesis and by normal Balb/3T3 or chick embryo fibroblast cells which had been treated with substances such as glucosamine and 2-DG which interfere with glycosylation (Pouyssegur et al., 1977, Zala et al., 1980). This suggested that the increased production of these peptides may be a result of cellular defect(s) in glycoprotein synthesis. Carlsson observed that the major ADP-ribose acceptor of in vivo avian and murine cells was identical to an 83 KDa protein which is stimulated by heat shock or glucose deprivation (Carlsson et al., 1983). Heat shock or glucose starvation resulted in reduced ADP-ribosylation of the peptide suggesting that this post-translational modification may play a part in regulating the function of HSP 83.

In addition to the synthesis of HSPs, heat shock also induces an increase in glucose uptake in cultured cells (Warren *et al.*, 1986, Warren and Pasternak, 1989). BHK cells kept at 45°C for 30 min expressed an approximately 2-fold increase in 2-DG uptake compared to control cells (Warren *et al.*, 1986). The stimulation of uptake was shown to be due to increased transport of the sugar and not due to increased rates of phosphorylation (Gray *et al.*, 1986).

Overproduction of GRPs has also been observed in a temperature-sensitive cell-cycle mutant hamster cell line, K12, whose mutation has resulted in a variety of cellular defects (Lee *et al.*, 1983, Attenello and Lee, 1984). K12 cells maintained at 40.5°C showed a 10 to 20-fold increase in synthesis of 78 KDa and 94 KDa polypeptides. These polypeptides were identified as being GRPs when glucose-starved cells cultured at 35°C also overproduced them. The use of 2 cDNA probes to follow the induction of the genes coding for these proteins revealed that the rapid accumulation of their corresponding mRNAs was due to transcriptional regulation. It was also determined that the expression of the two genes was strictly regulated by the level of glucose in the culture medium (Lee *et al.*, 1983). It remains to be determined whether the expression of these GRPs is related in some way to glucose transport regulation.

Objectives

The initial objective of this thesis involved a comparison of hexose transport regulation in two transformed cell lines of Chinese hamster lung fibroblasts, namely the parental (V79) and respiration-deficient mutant (G14). The G14 is one of a number of hamster cell mutants isolated and characterized (DeFrancesco *et al.*, 1976A, Whitfield *et al.*, 1981). These mutants were found to have a low rate of oxygen consumption, were auxotrophic for carbon dioxide and asparagine and required high concentrations of glucose (DeFrancesco *et al*, 1976B, Ditta *et al.*, 1976). Further studies demonstrated that many of these mutants were defective in Complex I of the electron-transport chain (DeFrancesco *et al.*, 1976A, Whitfield *et al.*, 1981, Day and Scheffler, 1982). Specifically, V79-G14 (i.e., G14) was found to have a defect in NADH- Coenzyme Q reductase resulting in an inability to generate energy via oxidative metabolism. The high requirement for glucose in G14 cells and the fact that NAD has been shown to play a role in the regulation of hexose transport under various conditions (Amos *et al.*, 1984)

led to the question of whether the enzyme defect in the G14 cells would affect the normal regulatory controls governing hexose transport. The approach taken to answer this question involved the use of several factors known to affect transport such as the presence or absence of serum or insulin which act as post-translational effectors of elevated hexose transport (Sefton and Rubin, 1971, Kletzien and Perdue, 1974a, Studt *et al.*, 1976, Diamond et al., 1978, Schneider *et al.*, 1878, Germinario and Oliveira, 1979, Howard *et al.*, 1979, Allen *et al.*, 1981).

To further characterize any differences that might exist in transport regulation, kinetic experiments were performed on the two cell types to determine the Michaelis-Menton constants K_m and V_{max} for 2-DG transport.

In addition, a comparative study of hexose transport regulation using a normal (MCH55) and a respiration-deficient (WG750) strain of human diploid fibroblasts was also carried out. WG750 is a cultured skin fibroblast strain taken from a patient with lactic acidemia (Darley-Usmar *et al.*, 1983, Frerman and Goodman, 1985, Robinson *et al.*, 1986). This strain was characterized as having low rates of 1-[^{14}C] pyruvate oxidation, lactate/pyruvate ratios 3-fold greater than control and low rates of ATP and oxygen consumption with NAD-linked substrates (Robinson *et al.*, 1986). Preliminary investigations indicated that WG750 had a defect in NADH-Coenzyme Q reductase (Robinson *et al.*, 1986). The objective of this study was to determine whether the enzyme defect in WG750 cells would manifest itself in non-transformed cells as compared to the hamster cell lines.

The final objective of this thesis was to determine whether there was a correlation between hexose transport and the enhanced level of glucose-regulated proteins induced by heat shock and/or glucose deprivation in V79 and G14 cells. It has been postulated that HSPs and/or GRPs may be involved in processes that regulate hexose transport and/or metabolism (Shiu *et al.*, 1977, Zala *et al.*, 1980, Burdon, 1982). It was questioned whether the stress caused by the enzyme defect in G14 cells would result in a higher constitutive expression of of one or more of these inducible proteins, and if so, whether expression of these proteins correlated with changes in hexose transport.

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Materials and Methods

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Cell Culture

Chinese Hamster Lung Fibroblasts

Wild type and respiration-deficient mutant Chinese hamster lung cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with essential and non-essential amino acids and 5% (v/v) fetal calf serum (Flow Laboratories, Inc.). The cells were incubated in a CO_2 plus air (7.5:92.5) atmosphere. Both cell types were supplied by Dr. Scheffler (Department of Biology, University of California, San Diego). When the cells had reached confluence they were harvested from appropriate culture vessels (75- cm^2 plastic flasks) (Falcon or Corning Co.) after incubation for 30 sec at 25°C with 0.04% (w/v) trypsin (Difco Labs, Detroit, Michigan) and 2 min at 25°C with 0.02 (w/v) EDTA. The cell suspension was centrifuged at 800xg for 4 min, the supernatant was discarded and the pellet resuspended in culture medium. The cells were then counted with a hemocytometer and subcultured into 75-cm^2 plastic culture flasks.

Human Diploid Fibroblasts

Normal and respiration-deficient mutant human skin fibroblasts were grown in Dulbecco's modified Eagle's medium (DME) supplemented with essential and non-essential amino acids and 10% (v/v) fetal calf serum (Flow Laboratories, Inc.). Both cell types were supplied by the Repository for Mutant Human Cell Strains (Montreal Children's Hospital, Montreal). The cells were incubated at 37 °C in a CO_2 plus air (5:95) atmosphere and the medium was changed three times weekly. When the cells had reached confluence they were harvested from appropriate culture vessels (175-cm^2 plastic flasks) (Falcon Co. or Corning Co.) after incubation for 2 min at 25 °C with 0.04% (w/v) trypsin (Difco Labs, Detroit, Michigan) and 5 min at 37 °C with 0.02% (w/v) EDTA. The cell suspension was centrifuged at 800xg for 4 min, the supernatant was discarded and the pellet resuspended in culture medium. The cells were then counted with a hemocytometer and subcultured into 175-cm^2 plastic tissue culture flasks (1:2 split ratio).

The cell number at confluence was used to calculate the number of population doublings (P.D.) accrued by the cells. Assuming that 50% of the cells will not attach and grow after subcultivation (Good, 1972), the number of P.D. was calculated from the equation P.D.= log (final number of cells)-log (initial/2)/log 2. In all experiments, the cells employed had not attained more than 35 P.D. (determined by cell counts at every passage).

General Experimental Procedure

Chinese Hamster Lung Fibroblasts

Cells were plated at a density of approximately 4×10^5 cells per 35-mm plastic Petri dishes (Corning Co.) and allowed to incubate for 24 h. The cell monolayers were then rinsed once with serum-free MEM (0% MEM) containing 1 mg per ml bovine serum albumin and 4 mg per ml glucose and incubated in 0% MEM for 24 h, unless otherwise stated. For some experiments the cells were then exposed to glucose-free conditions (glucose-free 0% MEM) for 4 h or continued to be glucose fed. Prior to change to glucose-free conditions the cell monolayers were rinsed once with glucose-free 0% MEM.

Human Diploid Fibroblasts

Cells were plated at a density of approximately 1×10^5 cells per 35 mm plastic Petri dishes (Corning Co.) and grown to confluence (usually 1 week). The cell monolayers were rinsed once with 0% MEM containing 1 mg per ml bovine serum albumin and 4 mg per ml glucose and incubated in 0% MEM for 24 h, unless otherwise stated. For some experiments the cells were then exposed to glucose-free conditions (glucose-free 0% MEM) for 4 or 8 h or continued to be glucose fed. Prior to change to glucose-free conditions the cell monolayers were rinsed once with glucose-free 0% MEM.

2-deoxy-D-Glucose Uptake Procedure

Hexose transport was assessed by measuring accumulation of ^{3}H-2-deoxy-D- glucose (2-DG) after short incubation times (0.5-5 min). Under the conditions employed hexose transport is the rate-limiting step in the accumulation of ^{3}H-2-DG (Germinario *et al.*, 1978, Germinario *et al.*, 1989). Furthermore, accumulation during the experimental period reflects the initial rate of hexose uptake (Germinario *et al.*, 1978). In all experiments sugar-uptake determinations were performed in triplicate and zero-time controls were subtracted in order to correct for non-specific adsorption. The cell monolayers were first rinsed twice with 2 ml glucose-free phosphate buffered saline (PBS) at pH 7.4 (37°C). Then 0.8 ml of PBS containing the ^{3}H-labelled 2-DG was added to the cells for 0.5, 2 or 5 min at 37°C. The specific activity of 1 mM labelled 2-DG was 4.5 μCi/ μmole. Following the incubation the radioactive medium was removed and the cell monolayers were washed four times (2 ml each time) with cold PBS (4°C) at pH 7.4. Zero-time control plates were rinsed twice with 2 ml cold PBS (4°C) at pH 7.4 and then 0.8 ml of PBS containing ^{3}H-labelled 2-DG was added. The radioactive medium was rapidly removed and the cell monolayers were washed 4 times (2 ml each time) with cold PBS (4°C) at pH 7.4. The monolayers were dissolved in 1N NaOH and aliquots were taken for liquid scintillation counting and protein determination (Lowry *et al.*, 1951). For more information on probes and inhibitors used for sugar transport studies, refer to Appendix I.

3-O-Methyl Glucose Uptake and Efflux Procedure

Hexose transport was assessed by measuring the transport of ^{3}H-3-O-methyl-glucose (3-O-MG) after varying incubation times (15 sec-5 min). In all experiments sugar-uptake determinations were performed in triplicate. At the time of the assay for sugar uptake the cell monolayers were rinsed twice with 2 ml of glucose-free PBS at pH 7.4 (23°C). Then 0.8 ml of PBS containing the ^{3}H-labelled 3-O-MG was added to the cells for appropriate time intervals at 23°C. The specific activity of 1 mM labelled 3-O-MG was 4.5 μCi/μmole. After incubation the radioactive medium was removed and the cell monolayers were washed 4 times (2 ml each time) with cold PBS (4°C) at pH 7.4 containing 0.3 mM phloretin to prevent the efflux of free sugar. Zero- time control plates

were rinsed twice with 2 ml cold PBS containing phloretin (4°C) at pH 7.4 then 0.8 ml of PBS containing ^{3}H-labelled 3-0-MG was added. The radioactive medium was rapidly removed and the cell monolayers were washed 4 times (2 ml each time) with cold PBS containing phloretin (4°C) at pH 7.4. At the time of the assay for sugar efflux the cell monolayers were rinsed twice with 2 ml of glucose-free PBS at pH 7.4 (23°C). Then 0.8 ml of PBS containing the ^{3}H-labelled 3-O-MG was added to the cells and the cells were incubated at 23°C for 30 min. The radioactive medium was then removed and replaced with 1 ml PBS (23°C) at pH 7.4 for varying periods of time. The cell monolayers were then washed 4 times (2 ml each time) with cold PBS containing phloretin (4°C) at pH 7.4. For all experiments the monolayers were dissolved in 1N NaOH and aliquots were taken for liquid scintillation counting and protein determination (Lowry *et al.*, 1951). For more information on probes and inhibitors used for sugar transport studies, refer to Appendix I.

Hexokinase Assay Procedure

Cells were plated at a density of approximately 1×10^6 cells per 100-mm Petri dishes (Lux Scientific Corporation, Calif) and grown to confluence. The cell monolayers were then rinsed once with 0% MEM containing 1 mg per ml bovine serum albumin and 4 mg per ml glucose and incubated in 0% MEM for 24 h. For some experiments the cells were then exposed to glucose-free conditions (glucose-free 0% MEM) for 4 h or continued to be glucose fed. Prior to change to glucose-fed conditions the cell monolayers were rinsed once with glucose-free 0% MEM. To harvest the cells the plates were rinsed twice with 5 ml of cold glucose-free PBS at pH 7.4 (4°C). 3 ml of cold 0.02 M Tris-HCl in 0.32 M sucrose was added to the plates and the plates were scraped, pooled into centrifuge tubes and centrifuged at 200xg for 5 min. The cell pellets were resuspended in 3 ml of homogenizing buffer containing 0.4M KCl, 0.02M Tris-HCl, 1.5 mM EDTA and 2mM dithiothreitol and homogenized in the cold (4°C) with a cold-equilibrated hand glass homogenizer. The homogenates were then cleared by centrifugation at 500xg for 5 min and the supernatants assayed for hexokinase activity. The procedure for the determination of hexokinase activity employed the selective precipitation of phosphorylated ^{3}H-labelled 2-DG by barium sulphate (Germinario *et al*, 1978).

The hexokinase reaction mixture (1.0 ml) contained 10mM HEPES buffer (pH 7.4), 10 mM ATP, 10mM $MgCl_2$, 10 mM KCl, 0.5 mM ^{3}H-labelled 2-DG (4.5 μCi/μmole 2-DG) and a 0.2 ml aliquot of cellular homogenate (2 mg/ml). After a 5-min incubation (37°C) the phosphorylation of 2-DG was stopped by the addition of 0.5 ml of 0.3N $Ba(OH)_2$ and 0.3 ml of 5% (w/v) $ZnSO_4$. Fresh stock solutions of these reagents were prepared and titrated on the day used. The free 2-DG remaining in the supernatant was determined by liquid scintillation counting. The amount of phosphorylated sugar was determined by subtraction of the amount of free 2-DG in the control samples. Furthermore, paper chromatography indicated that the metabolism of 2-DG by homogenates of cultured fibroblasts goes no further than 2-deoxy-D-glucose-6-phosphate (Germinario *et al*, 1978). In addition, when the reaction mixture was treated with the barium-sulphate reagents or with alkaline phosphatase, all the soluble radioactivity migrated under the free 2-DG peak (Germinario *et al*, 1978).

Measurement of Leucine Incorporation

To assay for leucine incorporation, 0.1 ml of 4,5-^{3}[H]-leucine (5 Ci/mmole) in 0% MEM containing 5.55 mM glucose was added per 35-mm Petri plate containing 1 ml of culture medium. Following a 30-min incubation at 37°C, the cell monolayers were washed 4 times (2 ml each time) with cold (4°C) PBS at pH 7.4. The cell monolayers were then incubated for 1 h at 4°C with 1 ml of cold 10% (w/v) trichloroacetic acid (TCA). The supernatant was then removed and the cell monolayers were washed 2 times (2 ml each time) with cold 10% TCA. The cell monolayers were dissolved in 1 ml of 1 N NaOH and aliquots were taken for protein determination and liquid scintillation counting. In all experiments, triplicate determinations were employed.

Heat Shock Induction Procedure

Chinese hamster lung fibroblasts were grown in DME supplemented with 5% fetal calf serum on 35-mm plastic petri dishes. Upon reaching near confluence the cells were stressed by the addition of fresh culture medium (0% MEM containing 1 mg per ml bovine serum albumin and 4 mg per ml glucose) prewarmed to 46°C and containing 2% dialyzed fetal calf serum (heat shock). Normal cells were maintained at 37°C and the

heat-shocked cells were kept at 46°C. Following a stress period of 12 min the culture medium was removed, and the cells were washed once with glucose-free 0% MEM (37°C). The cells were then incubated at 37°C under glucose-free or glucose-fed conditions (supplemented with 2% dialyzed fetal calf serum) for 4 or 8 h. The medium was removed and the cells were washed 2 times (2 ml each time) with warm PBS (37°C) at pH 7.4. The cells were then labelled for 45 min under the appropriate conditions (with or without glucose) with ^{35}S methionine (ICN Biochemicals, Inc. specific activity > 1000 Ci/mmol) in 0.6-0.8 ml of methionine-free DME supplemented with 2% dialyzed fetal calf serum. After labelling, the medium was removed and the cells were washed 4 times (2 ml each time) with cold PBS (4°C) at pH 7.4. For analysis by one-dimensional polyacrylamide gel eletrophoresis, the cells were solubolized by the addition of 0.45-0.6 ml of SDS-gel electrophoresis sample buffer containing 1%SDS, 50mM dithiothreitol, 40mM tris-HCl (pH 6.8), 7.5% glycerol and bromphenol blue and the samples were boiled for 3 min (Welch et al., 1983).

SDS-Polyacrylamide Gel Electrophoresis

The proteins were analyzed on 6% polyacrylamide gels according to the procedure of Laemmli (1970). 36 μg of total cell protein were added to each lane. Electrophoresis sample buffer and running buffer were as described by Laemmli (1970). Molecular mass markers used included myosin (200,000 Da), B-galactosidase (116,000 Da), phosphorylase b (97,400 Da), bovine serum albumin (68,000 Da), and ovalbumin (42,699 Da) (all from Bio-Rad, Ca). After staining gels with coomassie brilliant blue and destaining the ^{35}S-labelled gels were dried and exposed to Kodak X-OMAT-AR film at -85°C for 2 weeks.

Radioactive Materials

Glucose-L-[1-^{3}H(N)]- (50 Ci/mmole), 2-D-[2,6-^{3}H] glucose (50 Ci/mmole), 3-0-MG, [methyl-^{3}H]- (50 Ci/mmole), L-leucine, [4,5-^{3}H] (5 Ci/mmole) and Trans ^{35}S-Label (1259 Ci/mole) were obtained from ICN Radiochemicals, Irvine, California. Trans ^{35}S label is produced from a cellular hydrolysate of E. coli grown in the presence of carrier-

free $^{35}SO4^-$. Product contains 70% L-methionine, $[^{35}S]$, 15% L-cysteine, $[^{35}S]$, and various non-labelled amino acids.

Chemicals

Insulin (bovine pancreas crystalline) and 2-deoxy-D-glucose were obtained from Sigma Chemical Co., (St Louis, Mo.). Cytochalasin B was obtained from Aldrich Chemical Co (Milwaukee, Wi). Phloretin was obtained from ICN Biomedicals Inc. (Plainview, NY). Bovine Serum Albumin and L-glucose were obtained from ICN Pharmaceuticals Inc (Cleveland, Ohio).

Scintillation Counting

Samples were added to 5 ml of counting solution (Formula 963, New England Nuclear Corp, Boston, Massachussetts) in 7-ml vials. Vials were counted in a liquid scintillation spectrophotometer (LKB Rackbeta) with a counting efficiency of 28-30% for ^3H. A "calibration curve" was constructed for ^3H using a series of samples of ^3H-toluene with known DPMs (disintegrations per minute) and quenched to varying degrees with diluted saturated pycric acid, 1N NaOH, H_2O, and cells solubolized in 1N NaOH. Counting efficiency method was employed to generate the standard Quench correction curve.

Statistics

Statistical analysis was performed using the Student's t-test for paired and unpaired data, 1-way and 2-way Analysis of Variance (ANOVA), 5% being the level being chosen for significance.

Results

Respiration Deficient and Parental Chinese Hamster Fibroblasts

It has been shown that glucose utilization in the respiration-deficient Chinese hamster cell line, G14, is substantially greater than that of the parental cell line, V79 (DeFrancesco *et al.*, 1976). The data in Table I clearly indicate that glucose utilization is indeed significantly elevated in glucose-fed G14 cells (p<0.02, n=5). Cell monolayers were incubated in serum-free glucose-containing media for 40-48 h before samples from the media were analyzed for glucose content. The average increase was 74% over the control cells.

The high requirement for glucose in G14 cells led to the question of whether the defect in NADH-Coenzyme Q reductase affected the normal regulatory controls on glucose transport. The data in Table II show that basal 2-DG transport in glucose-fed G14 cells was significantly higher than the parental cell line (p<0.001, n=15). Cell monolayers were incubated in serum-free glucose-containing media for 24 h prior to assay for ^{3}H-2-DG uptake. The uptake of ^{3}H-2-DG ranged from 13.8 to 22.1 nmoles/mg protein/5 min in V79 cells and from 19.6 to 36.5 nmoles/mg protein/5 min in G14 cells. The average transport ratio for G14 versus V79 cells was 1.37 ± 0.28, ranging from 1.05 to 2.17. The reason for these differences is not clear but is thought to be due to experimental variability.

It has been widely reported that Cytochalasin B (CB) is a specific inhibitor of the glucose carrier, inhibiting the saturable facilitated component of glucose transport without affecting the non-saturable component (Lin *et al.*, 1974, Dolberg *et al.*, 1975, Jung and Rampal, 1977, Germinario *et al.*, 1978). To further characterize the differences observed regarding 2-DG transport in G14 vs V79 cells uptake experiments were performed in the presence and absence of CB. As shown in Table III, the addition of 10 μM CB inhibited 2-DG transport significantly (approximately 90%) in both cell lines (2-way ANOVA, p< 0.01, n=3). The data also reconfirm that 2-DG transport in glucose-fed G14 cells is significantly higher than in V79 cells (2-way ANOVA, p< 0.01, n=3). The uptake of L-glucose was compared with 2-DG uptake to determine the contribution of diffusion to total sugar uptake. The data indicate that L-glucose uptake could account for less than 1% of 2-DG uptake in both cell lines. Although CB virtually eliminates the facilitated uptake of sugar

without affecting simple diffusion the data show a discrepancy between the uptake of L-glucose compared with 2-DG uptake performed in the presence of 10 μM CB. This is likely due to the fact that 2-DG and CB compete with each other for the same binding site so that the inhibition of saturable uptake by CB is not complete when 2-DG is present.

Experiments utilizing 3-0-methyl glucose (3-O-MG), a glucose analog which is transported by the glucose carrier but not phosphorylated, were carried out to determine whether the enhanced uptake of ^{3}H-2-DG observed in G14 cells can indeed be attributed to an increase in transport and not phosphorylation. Because of the swiftness of 3-O-MG transport at 37°C, these experiments were performed at 22°C. Confluent cell monolayers were incubated in serum-free glucose-containing media for 24 h prior to assay for sugar uptake. The data in Figure 1 show the time course of ^{3}H-3-O-MG uptake in V79 and G14 cells. It is apparent that the G14 cells transport 3-O-MG at an elevated rate when compared to V79 cells. The rate of 3-0-MG uptake was approximately 60% greater in G14 versus V79 cells at the 30-sec time-point (i.e., 0.135 ± 0.029 nmoles 3-0-MG/mg protein versus 0.086 ± 0.016 nmoles 3-0-MG/mg protein). This result is reasonably close to the data in Table II which described the uptake of 2-DG in G14 and V79 cells. These data indicated a 40% greater uptake of 2-DG in G14 versus V79 cells (i.e., 2.56 nmoles 2-DG/mg protein/30 sec versus 1.9 nmoles 2-DG/mg protein/30 sec). It might be argued that the facilitated mode of 2-DG uptake is more complex than 3-O-MG transport and could conceiveably account for the difference. Experimental variability is also a factor since the data in Figure 1 showing the time course of 3-0-MG transport represents 3 paired experiments whereas the data in Table II showing 2-DG uptake represents a total of 15 paired experiments. Most importantly, together, these data suggest that hexose transport is up-regulated in G14 cells. The data in Figure 2 show the rate of efflux of ^{3}H-3-O-MG (22°C) with cells which had been preincubated to equilibrium (30 min) with ^{3}H-3-O-MG. The data show that the initial transport of free sugar out of the cells is more rapid in G14 cells although the differences are small. Part of the reason for this may be that phloretin is more effective in inhibiting hexose efflux than hexose uptake (Wildbrandt, 1954). This result is in agreement with the previous data showing a higher rate of influx of ^{3}H-3-O-MG in G14 cells since cells which have the highest uptake activity should also have the greatest efflux activity.

Next, a kinetic analysis of 2-DG transport was performed on V79 and G14 cells. Table IV indicates the results of measurements of initial rates of 2-DG uptake at substrate

concentrations between 0.025mM and 10.0mM corrected for diffusional uptake. Analysis by Lineweaver-Burk plots (Lineweaver and Burk, 1934) revealed that the increase in transport for G14 cells was due to an increase (2.8-fold) in the V_{max} for transport (Figure 3). The nearly 3-fold increase in V_{max} for 2-DG in G14 cells does not appear to agree with the approximately 40% increase in 2-DG uptake in G14 cells shown in Table II. In Table II, 2-DG transport was determined after the cells had been incubated with ^{3}H-2-DG for 5 min. At this time point 2-DG transport may no longer be on the linear portion of the uptake curve so that the data may not be truly representatibe of rate-limiting transport experiments. The K_m, or affinity for 2-DG was essentially the same in the two cell types. Together, these results suggest an increase in the number of functional carriers at the plasma membrane in G14 cells. As shown in Table V, the dissociation constant for the enzyme-inhibitor complex K_i, was determined for D-glucose, a potent inhibitor of 2-DG transport (Germinario et al., 1985, Germinario et al., 1989). Serum-starved monolayers were monitored for uptake using either 0.5 or 3.0mM ^{3}H-2-DG and 5 different concentrations of inhibitor (0.5mM to 10.0mM). Dixon plot analysis (Dixon and Webb, 1964) showed that the K_i's for D-glucose were essentially no different (i.e., 0.75 ± 0.25 vs 0.80 ± 0.50) (Figure 4). This is in agreement with the K_m data showing that the affinity for 2-DG is the same in the two cell lines.

The Effect of Glucose Starvation on V79 and G14 Cells

To further characterize any differences that might exist in transport regulation, both cell types were exposed to a variety of conditions known to affect transport such as the presence or absence of glucose or serum or the addition of insulin. The adaptive response of V79 cells to glucose deprivation is shown in Figure 5. Basal transport of glucose-fed G14 cells was greater than that of V79 cells (i.e., 24.8 ± 2.6 nmoles/mg protein/5 min versus 19.0 ± 2.1 nmoles/mg protein/5 min respectively). The V79 cells nearly doubled their hexose transport (78% increase) after 2 h in glucose-free media while in the G14 cells hexose transport was only 13% elevated. After 4 h, the V79 cells were elevated 90% while hexose transport in the G14 cells was only increased 26%.

The Effect of Serum on Hexose Transport in V79 and G14 Cells

Since all the experiments discussed were performed with cells which had been serum starved for 24 h, it was of interest to determine whether the elevation in hexose transport observed in glucose-fed cells would be expressed in the presence of serum. The data in Table VI demonstrate that hexose transport in serum-fed cultures of V79 and G14 cells was essentially the same (31.2 ± 6.0 nmoles 2-DG/mg protein/5 min vs 33.0± 6.5 nmoles 2-DG/mg protein/5 min) and that the hexose transport differences in the two cell lines were observed only after 24 h of serum deprivation. Under conditions of serum deprivation, the V79 cells expressed a significant 33% decrease in hexose transport (1-way ANOVA, $p<0.01$, n=3) while the G14 cells expressed no significant difference (1-way ANOVA, $p>0.20$, n=3) (i.e., 33.0 ± 6.5 nmoles 2-DG/mg protein/5 min with serum vs 36.1 ± 1.6 nmoles 2-DG/mg protein/5 min without serum). This is interesting since, when cultured mammalian cells are deprived of the required growth-promoting substances in serum, they generally respond by decreasing their rate of hexose transport (Germinario et al., 1982, Klip, 1982). Furthermore, short-term readdition of serum to serum-starved V79 and G14 cells stimulated hexose transport in V79 cells by 35% over control within 1 h and by 75% over control within 2 h but had no effect on G14 cells (Figure 6). Although exposure of the V79 cell line to 5% (v/v) fetal calf serum led to an increase in the serum:control transport ratio within 30 min in other experiments (Germinario et al., 1989) there appeared to be a lag of 1 h before serum re-feeding led to increased hexose transport in V79 cells in these experiments.

The Effect of Insulin on Hexose Transport in V79 and G14 Cells

Table VII provides further evidence of the deregulation of hexose transport in G14 cells. Although the data represent a limited number of experiments (n=2), the data reconfirm results obtained in earlier experiments (n=8) performed in this laboratory. Basal 2-DG transport for unstimulated (control) cells was 15.0 ± 2.6 nmoles/mg ptn/5 min versus 21.0 ± 3.5 nmoles/mg ptn/5 min for V79 and G14 cells respectively. When V79 and G14 cells were exposed to insulin (100 mU/ml) the V79 cells expressed a hormone-dependent significant increase in transport activity of 92% over control following a 2 h incubation (1-way ANOVA, $p<0.01$, n=2) whereas transport activity was essentially unchanged in G14 cells (1-way ANOVA, $p>0.75$, n=2). Supraphysiological concentrations of insulin act to stimulate growth via cellular receptors for the insulin-like growth

factors (IGFs) (Van Obberghen-Schilling and Pouyssegur, 1983). It has been reported that the IGF-I receptor closely resembles the insulin receptor and is insulin-sensitive (Kasuga *et al.*, 1982). It has been shown that an insulin concentration of 100 mU/ml will result in 100% saturation of insulin receptors as well as 50% displacement of IGF-I from its receptor (Leckett, B., and Germinario, R.J., personal communication). Even so, the data still indicate that the G14 cells maintain a larger number of carriers in the plasma membrane such that the cells are no longer sensitive to the stimulatory effects of a peptide hormone.

Hexose Phosphorylation by Parental and Mutant Cell Homogenates

Additional evidence to suggest that differences seen in hexose transport regulation are due to carrier-mediated transport is further supported by the data obtained from experiments which measured hexokinase activity in V79 and G14 cellular homogenates. These studies demonstrated that the level of hexokinase activity in homogenates prepared from cells incubated in glucose-free medium or glucose-containing medium was essentially the same (Figure 7). However, the data did show that hexokinase activity was higher in glucose-starved V79 and G14 cells at the 5, 10, and 15 min time points (2-way ANOVA, $p<0.01$, n=2). No difference was found in the level of hexokinase activity in homogenates prepared from V79 and G14 cells incubated in serum-free or serum-fed medium (Figure 8). These experiments also show that the hexokinase activity in V79 cells is equivalent to the hexokinase activity in G14 cells.

Respiration Deficient and Normal Human Skin Fibroblasts

Having obtained human diploid fibroblasts with the same enzyme defect as the hamster cells it was questioned whether these cells could be used to further examine hexose transport regulation in respiration-deficient cells.

The data in Table VIII show that basal 2-DG transport in glucose-fed WG750 cells was strikingly higher (6-fold) than in the parental MCH55 ($p<0.01$, n=11). Cell monolayers were incubated in glucose-containing serum-free media for 24 h prior to assay for ^{3}H-2-DG uptake.

A comparative kinetic analysis of 2-DG transport was performed on MCH55 and WG750 cells. Table IX indicates the results of measurements of initial rates of 2-DG

uptake at substrate concentrations between 0.025 mM and 3.0 mM corrected for diffusional uptake. Lineweaver-Burk plots were used to calculate the kinetic constants for 2-DG (Lineweaver and Burk, 1934) (Figure 9). The mutant WG750 displayed a dramatic 9.5-fold increase in the V_{max} for transport when compared to the control strain (i.e. 17.7 $\pm$ 1.1 vs 1.8 $\pm$ 0.6). The K_m for 2-DG was essentially the same in the two cell types (1.09 $\pm$ 0.5 vs 1.0 $\pm$ 0.0). In addition, the K_I was determined for D-glucose using Dixon plot analysis (Dixon and Webb, 1964) (Figure 10). Determination of the K_I is important since K_I values can be thought of as a measure of the affinity (or K_m) of the inhibitor for the transport system. Serum-starved monolayers were monitored for uptake using either 1.0 or 3.0 mM ^{3}H-2-DG and 4 different concentrations of inhibitor (1.0 mM to 10.0 mM). As was the case with the hamster cells, the K_I's for D-glucose in the normal and mutant human cells were the same (i.e. 1.7 $\pm$ 0.0 vs 1.7 $\pm$ 0.07) (Table X). Again, this would indicate that hexose carrier affinity for the hexose is the same in the two cell lines.

The data in Figure 11 show the time course of glucose deprivation-enhanced hexose transport in the parental MCH55 cell line and respiration-deficient WG750. Serum-starved confluent cell monolayers were exposed to glucose-free conditions for the indicated time intervals. The data show that both cell strains respond to short-term glucose deprivation by increasing their rate of glucose transport. Transport was increased by 33% in the control cell line following 4 h in glucose-free medium and by 47% following 8 h of glucose deprivation. The mutant responded to 4 h of glucose starvation by increasing its transport by 20% and by 53% after 8 h of incubation in medium devoid of glucose.

Figure 12 depicts the response of serum-starved MCH55 and WG750 cells to short-term re-addition of serum. The data demonstrate that serum stimulated 2-DG transport in MCH55 cells to a greater degree than in WG750 cells. Serum addition resulted in a 241% increase in 2-DG transport after a 2 h incubation in serum-containing medium whereas a 2 h incubation in serum-containing medium resulted in a 69% increase in transport in the mutant cells. WG750 cells required 60 min of exposure to serum before an increase in hexose transport was detected. This observation parallels results obtained with the hamster cells (Figure 6).

The data in Table XI show the stimulatory effects of 10 and 100 mU/ml insulin on ^{3}H-2-DG transport in MCH55 and WG750 cells. Confluent cell monolayers were serum starved for 24 h and then exposed to the hormone for 2 h. The data show a concentration-dependent increase in insulin stimulated 2-DG transport activity in MCH55 and WG750

cells. Insulin stimulated 2-DG transport significantly in the MCH55 cells (1-way ANOVA, p<0.01, n=3) but not in WG750 cells (1-way ANOVA, p>0.25, n=3). MCH55 cells increased their transport by 70% when incubated with 10 mU/ml insulin while in the WG750 cells transport was only 20% elevated. Upon administration of 100 mU/ml insulin ^{3}H-2-DG transport increased 260% in the control cells but only 30% in the mutant. Basal 2-DG transport for unstimulated (control) cells was 3.2 ± 0.5 nmoles/mg ptn/5 min versus 13.8 ± 3.8 nmoles/mg ptn/5 min for MCH55 and WG750 cells respectively. The results agree with the previous data which showed that WG750 cells respond to glucose deprivation and serum stimulation, apparently to a lesser degree than their "wild-type" counterparts. However, although the magnitude of response of WG750 cells to the presence or absence of serum, glucose or insulin appears to be smaller in comparison to MCH55 cells in reality this is not the case. Keeping in mind that basal transport is 6-fold higher in the mutant cells, quantitatively more transporters are responding to these effectors of transport than in the normal cells. For more information on the net effects of insulin and other stimuli on transporter translocation *vis-a-vis* 2-DG transport measurements in MCH55 and WG750 cells, refer to Appendix II.

The Effect of Heat Shock on V79 and G14 Cells

The data in Figure 13 show the effect of heat shock on ^{3}H-leucine incorporation in V79 and G14 cells. Confluent glucose and serum-fed cell monolayers were stressed by incubation at 46°C for 12 min. The cells were then incubated at 37°C for various lengths of time (0-7.5 h) prior to determination of ^{3}H-leucine incorporation. The data show that both V79 and G14 cells expressed a drastic (93%) inhibition of protein synthesis directly after heat shock (0 Time in Figure 13). Both cell types gradually recovered their ability to synthesize proteins during the 7.5 h incubation period as evidenced by a greater incorporation of label at later time periods. It was apparent, however, that the G14 cells recovered from the stress more rapidly than the parental V79 cells. ^{3}H-leucine incorporation was found to be 93% of control in G14 cells 5 hours post-heat shock whereas incorporation in V79 cells was only 65% of control. By 7.5 h post-heat shock, protein synthesis was 98% of control in G14 cells but only 73% of control in V79 cells. These results suggest a mechanism(s) in G14 cells that may act to accelerate recovery from heat shock. In addition, heat-shocked cells that generate HSPs recover more readily from

heat shock (Subject and Sciandra, 1982).

The rapid recovery of protein synthesis in G14 cells following heat shock prompted the question of whether heat-shocked G14 cells would display differences in rates of 2-DG uptake when compared to V79 cells. Confluent glucose and serum-fed cell monolayers were stressed by incubation at 46°C for 12 min. The cells were then incubated at 37°C for various lengths of time (0-7.5 h) prior to determination of 2-DG uptake. The data in Figure 14 show that the heat-shock treatment resulted in a transient increase in 2-DG uptake in both cell lines. The elevation peaked 2 h post-heat shock and then transport returned to near normal levels after 7.5 h incubation at 37°C. This fluctuation in 2-DG transport may be part of a survival mechanism induced in V79 and G14 cells to help combat the environmental stress.

The data in Figure 15 show a one-dimensional gel of control and heat-shocked V79 and G14 cells. The effect of glucose starvation in the V79 cell line after 4 or 8 h (Lanes B,C versus H,I, glucose-fed versus glucose-starved, respectively) did not demonstrate any expression of GRP's. This same condition for the glucose-fed G14 (Lane A) versus the glucose-starved G14 (Lane G) showed no differences in the ^{35}S-labelled bands. Heat shock, however, did result in the appearance of a heat-inducible peptide of approximately 110 KDa molecular weight for the glucose-fed G14 (Lanes A versus D) or V79 (Lanes B,C versus E,F) cells. Glucose-deprived cells of both types i.e., G14 and V79 exhibited this same up-regulation of expression of the 110 KDa molecular weight species (Lanes G versus J, for the G14 cell line and Lanes H,I versus K,L for the V79 cell line).

Table I. Glucose Utilization in V79 and G14 Cells.

Cell Line	Glucose Utilized (μmoles/mg protein/h)	n[a]
V79	69.2 ± 4.1	5
G14	120.3 ± 35.2	5

Cell monolayers were incubated in serum-free MEM containing 4 mg per ml glucose for 40 - 48 h. The samples from the media were analyzed at this time for glucose content using Glu-cinet (Sclavo Inc., Wayne, N.J.). Data represent average $\pm$ SEM of the indicated number of experiments (triplicate plates in each experiment).

a Number of experiments

b $P < 0.02$

Table II. Basal 2-DG Transport in Glucose-fed V79 and G14 Cells.

Cell Line	2-DG Transport (nmoles/mg protein/5 min)	n^b
V79	19.0 ± 2.3	15
G14	25.6 ± 4.7	15

Cell monolayers were serum-starved (0% MEM + 4 mg/ml glucose) for 24 h. The concentration of 2-DG employed was 0.5 mM. Data represent average ± SEM of the indicated number of experiments (triplicate plates in each experiment).

a $P < 0.001$

b Number of experiments

Table III. The Effect of the Presence and Absence of CB on 2-DG Uptake by V79 and G14 Cells.

Cell Line	n	2-DG Transport (nmoles/mg protein/5 min)		L-glucose Uptake
		-CB	+CB	
V79	3	18.9 ± 1.7	1.5 ± 0.4	0.02 ± 0.01
G14	3	23.6 ± 1.6	2.2 ± 0.5	0.19 ± 0.03

Cell monolayers were serum-starved (0% MEM + 4 mg/ml glucose) for 24 h. Sugar uptake was determined for 2-DG in the presence of CB (10 µM) and for 2-DG and L-Glucose in the absence of CB. Ethanol (0.25%) was present in the control since it was the solvent used to dissolve CB. The concentration of 2-DG employed was 0.5 mM. Data represent average ± SEM of the indicated number of experiments (triplicate plates in each experiment).

n Number of experiments.

Table IV. K_m and V_{max} of Hexose Transport for V79 and G14 Cells.

Cell Line	K_m	V_{max}	n
	(mM)	(nmoles 2-DG/mg protein/30 sec)	
V79	3.0 ± 0.2	9.5 ± 0.6	3
G14	3.9 ± 0.6	26.9 ± 4.2	3

Cell monolayers were serum-starved (0% MEM + 4 mg/ml glucose) for 24 h and then monitored for 2-DG uptake employing 6 concentrations of cold 2-DG (0.025 mM to 10.0 mM) corrected for L-glucose uptake. Data represent the average $\pm$ SEM of the indicated number of experiments (triplicate plates in each experiment).

n Number of experiments.

Table V. D-Glucose as an Inhibitor of Hexose Transport for V79 and G14 Cells.

Cell Line	K_I (mM)	n
V79	0.75 ± 0.25	2
G14	0.80 ± 0.50	2

Cell monolayers were exposed to serum-free conditions (0% MEM + 4 mg/ml glucose) for 24 h. The experiments employed 2 concentrations of 2-DG (0.5 mM and 3.0 mM) and 5 concentrations of D-glucose (0.5 mM to 10.0 mM). Uptake time was 2 min. Data represent the average ± SEM of the indicated number of experiments (triplicate plates in each experiment).

n Number of experiments.

Table VI. The Effect of Serum on Hexose Transport in V79 and G14 Cells.

Cell Line	2-DG Transport (nmoles/mg protein/5 min)		n
	+ serum	- serum	
V79	31.2 ± 6.0	20.8 ± 1.2	3
G14	33.0 ± 6.5	36.1 ± 1.6	3

Hexose transport was measured in both cell types in the presence of serum (5 days in culture) and after 24 h serum deprivation of these serum-fed cultures. The concentration of 2-DG employed was 0.5 mM. Data represent average $\pm$ SEM of the indicated number of experiments (triplicate plates in each experiment).

n Number of experiments.

Table VII. The Effect of Insulin on Hexose Transport in Serum-Deprived V79 and G14 Cells.

Cell Line	Insulin : Control Ratio	n
V79	1.9 ± 0.4	2
G14	1.1 ± 0.1	2

Cell monolayers were serum-starved (0% MEM + 4 mg/ml glucose) for 24 h and then incubated with or without 100 mU/ml insulin for 2 h. The concentration of 2-DG employed was 0.5mM. Data represent average $\pm$ SEM of the indicated number of experiments (triplicate plates in each experiment). Data were normalized to unity.

n   Number of experiments.

Table VIII. Basal 2-DG Transport In Glucose-Fed Human Diploid Fibroblasts

Cell Line	2-DG Transport (nmoles/mg protein/2 min)	n[b]
MCH55	3.6 ± 1.8	11
WG750	21.6 ± 10.5	11

Cell monolayers were serum-starved (0% MEM + 4 mg/ml glucose) for 24 h. The concentration of 2-DG employed was 1.0 mM. Data represent average ± SEM of the indicated number of experiments (triplicate plates in each experiment).

a $P < 0.01$

b Number of experiments

Table IX. K_m and V_{max} of Hexose Transport for MCH55 and WG750 Cells

Cell Line	K_m	V_{max} (nmoles 2-DG/mg protein/2 min)	n
MCH55	1.1 ± 0.5	1.8 ± 0.6	2
WG750	1.0 ± 0.0	17.7 ± 1.1	2

Cell monolayers were serum-starved (0% MEM + 4 mg/ml glucose) for 24 h and then monitored for 2-DG uptake employing 7 concentrations of 2-DG (0.025 mM to 3.0 mM) corrected for L-glucose uptake. Data represent average ± SEM of the indicated number of experiments.

n Number of experiments

Table X. D-Glucose as an Inhibitor of Hexose Transport for MCH55 and WG750 Cells

Cell Line	K_I (mM)	n
MCH55	1.7 ± 0.0	2
WG750	1.7 ± 0.1	2

Cell monolayers were exposed to serum-free conditions (0% MEM + 4 mg/ml glucose) for 24 h. The experiments employed 2 concentrations of 2-DG (1.0 mM and 3.0 mM) and 4 concentrations of D-glucose (1.0 mM and 10 mM). Uptake time was 2 min. Data represent average ± SEM of the indicated number of experiments (triplicate plates in each experiment).

n Number of experiments

Table XI. The Effect of Increasing Concentrations of Insulin on 2-DG Transport in Cultured Human Skin Fibroblasts

Treatment	Insulin : Control Ratio		n
	MCH55	WG750	
Control	1.0	1.0	2
+ 10 mU/ml	1.7 ± 0.4	1.2 ± 0.3	2
+ 100 mU/ml	2.6 ± 0.1	1.3 ± 0.4	2

Cell monolayers were serum-starved (0% MEM + 4 mg/ml gluc) for 24 h and then incubated for 2 h with 10 mU/ml or 100 mU/ml insulin or without insulin. The concentration of 2-DG employed was 1.0 mM, uptake time 5 min. Data represent average ± SEM of the indicated number of experiments (triplicate plates in each experiment). Data were normalized to unity.

n   Number of experiments.
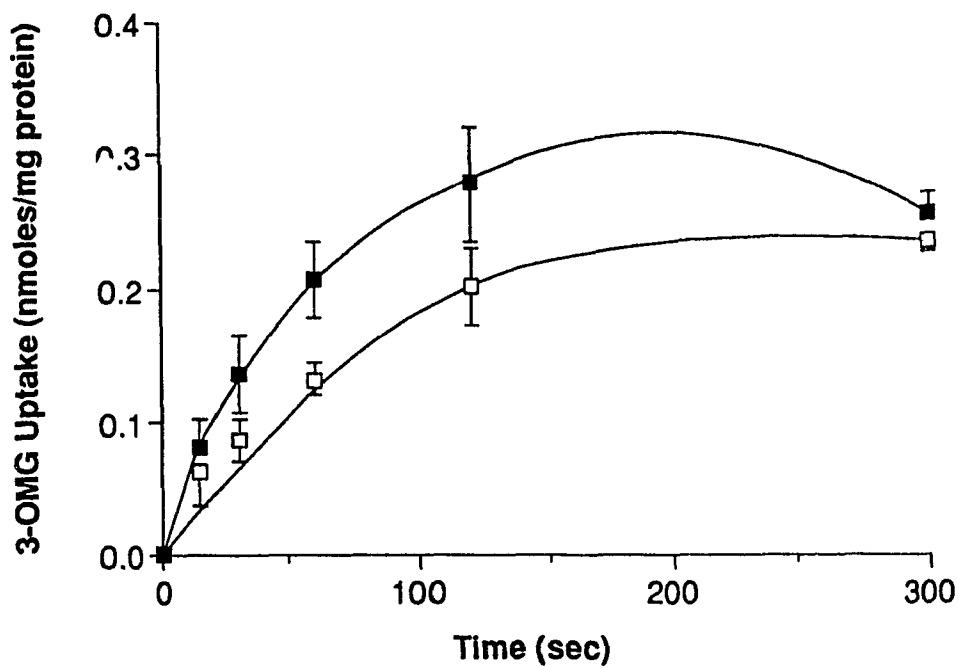
Figure 1. Time course of ^{3}H-3-0-MG uptake in V79 and (□) and G14

(■) cells. Confluent cell monolayers were serum starved (0% MEM) for

24 h prior to assay for sugar uptake. Data represent average ± SEM of 2

different experiments (triplicate plates in each experiment).
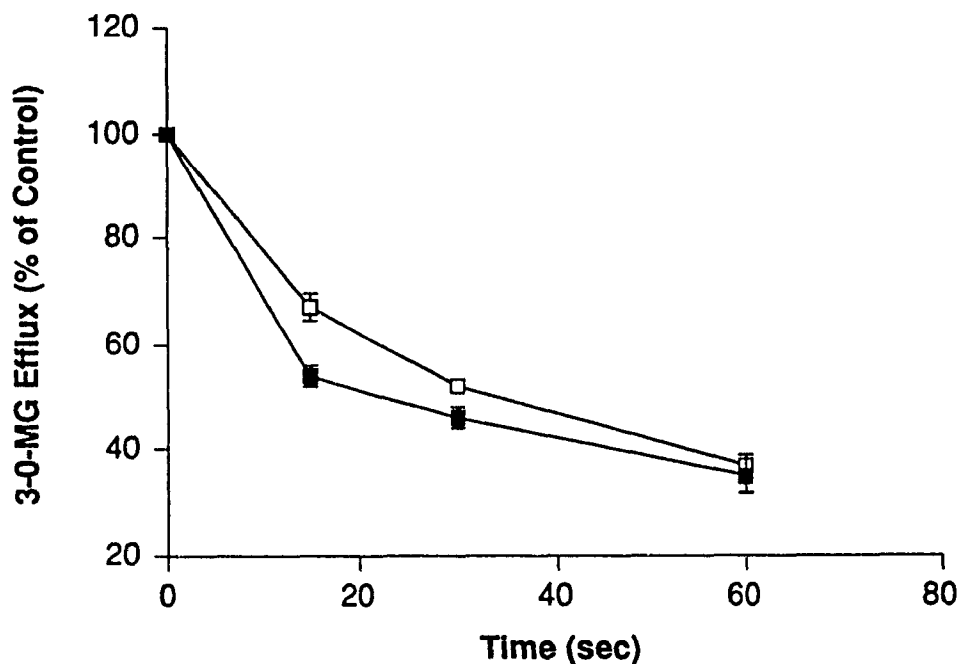
Figure 2. Time course of ^{3}H-3-0-MG efflux in V79 (□) and G14 (■)

cells. Confluent cell monolayers were serum starved (0% MEM) for 24 h

and then preincubated with ^{3}H-3-0-MG for 30 min prior to assay for sugar

efflux. Data represent average ± SEM of 3 different experiments (triplicate

plates in each experiment).

Figure 3. Lineweaver-Burk double reciprocal plot of 2-DG uptake

by confluent serum starved (0% MEM, 24 h) V79 (□) and G14 (■)

cells. Points represent average of triplicate determinations.

Figure 4. Dixon plot analysis of D-glucose as an inhibitor of hexose transport for confluent serum starved (0% MEM, 24 h) V79 and G14 cells. (□) refers to 0.5 mM ^{3}H-2-DG, (■) refers to 3.0 mM ^{3}H-2-DG. Points represent average of triplicate determinations.
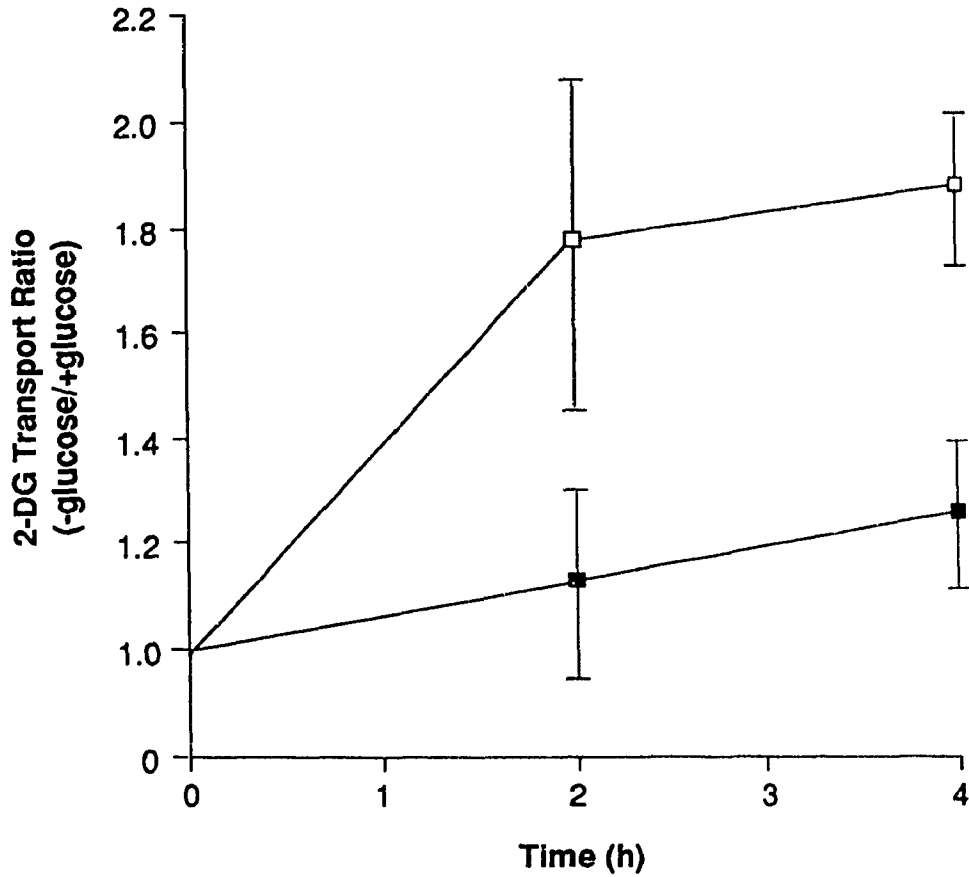
Figure 5. The effect of glucose starvation on hexose transport in V79 and G14 cells. Serum-starved (0% MEM, 24 h) confluent V79 (□) and G14 (■) cell monolayers were exposed to glucose-starved conditions for 2 or 4 h prior to assay for sugar uptake. Points represent average ± SEM of 4-6 separate experiments (triplicate plates in each experiment).

Figure 6. The effect of serum re-feeding on hexose transport in V79 and

G14 cells. Hexose transport was measured in serum deprived (0% MEM, 24

h) V79 (□) and G14 (■) cells after short term re-exposure to serum. %

Control determined as the ratio of increase of 2-DG transport in serum-fed

cells versus (baseline) serum-starved cells. Data represent the average of 2

determinations ± SEM (triplicate plates in each experiment).
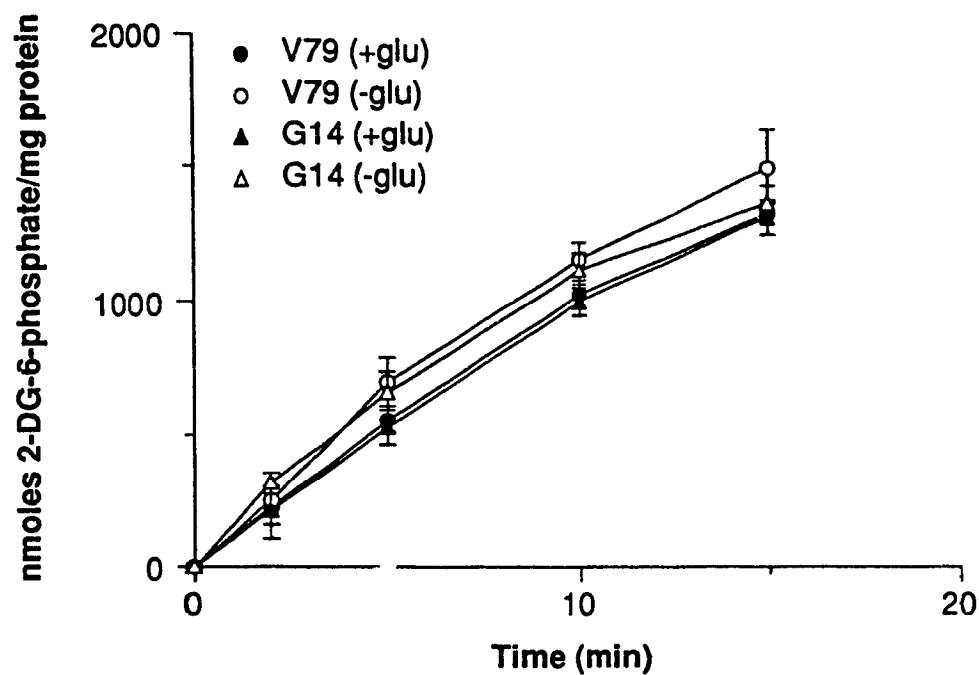
Figure 7. The phosphorylation of 2-DG by glucose-starved and glucose-fed V79

and G14 cell homogenates. Phosphorylation of glucose-starved (4 h) (○) or glucose-

fed (●) V79 cells and glucose-starved (4 h) (△) or glucose-fed (▲) G14 cells as a

function of time. Points represent the average ± SEM of 2 separate experiments

(triplicate plates in each experiment).
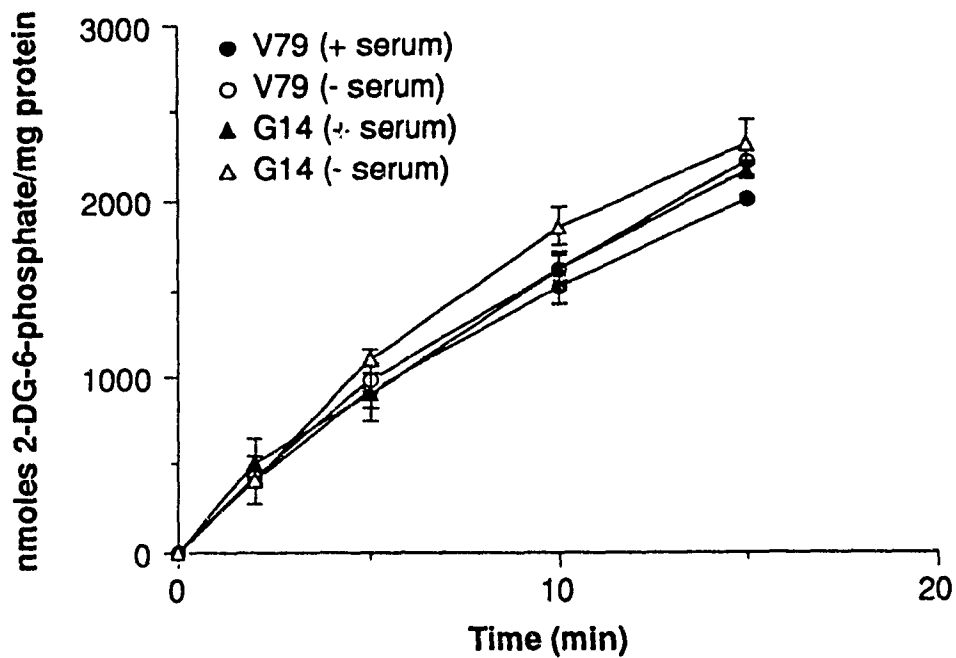
Figure 8. The phosphorylation of 2-DG by serum-starved and serum-fed V79 and G14 cell homogenates. Phosphorylation of serum-starved (0% MEM, 24 h) (O) or serum-fed (●) V79 cells and serum-starved (0% MEM, 24 h) (Δ) or serum-fed (▲) G14 cells as a function of time. Points represent the average ± SEM of 2 separate experiments (triplicate plates in each experiment).
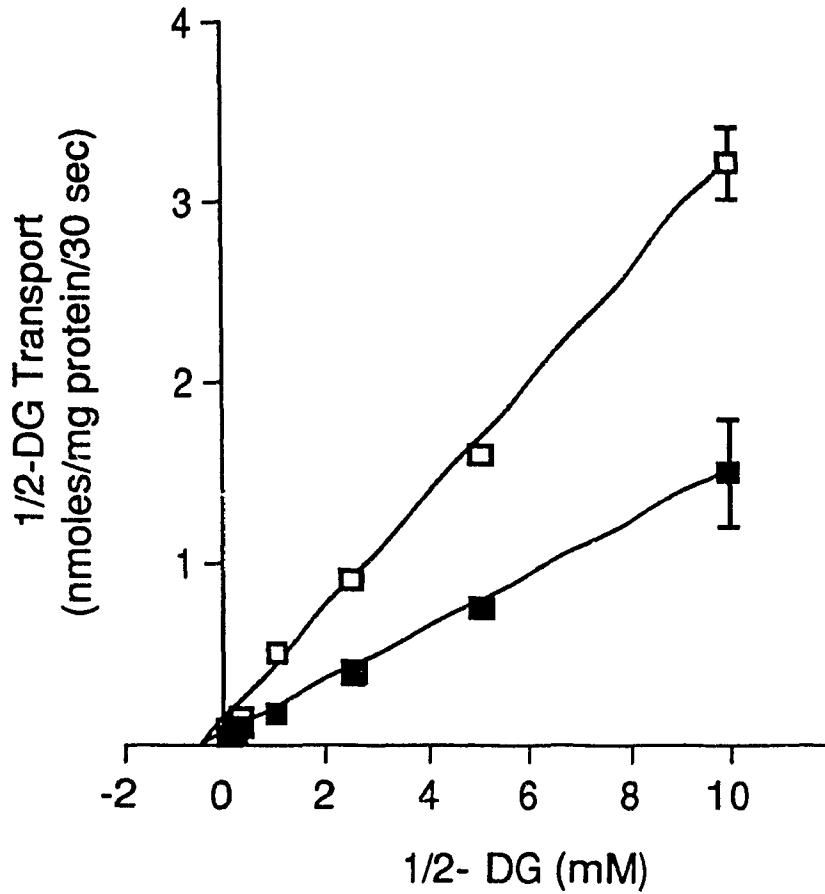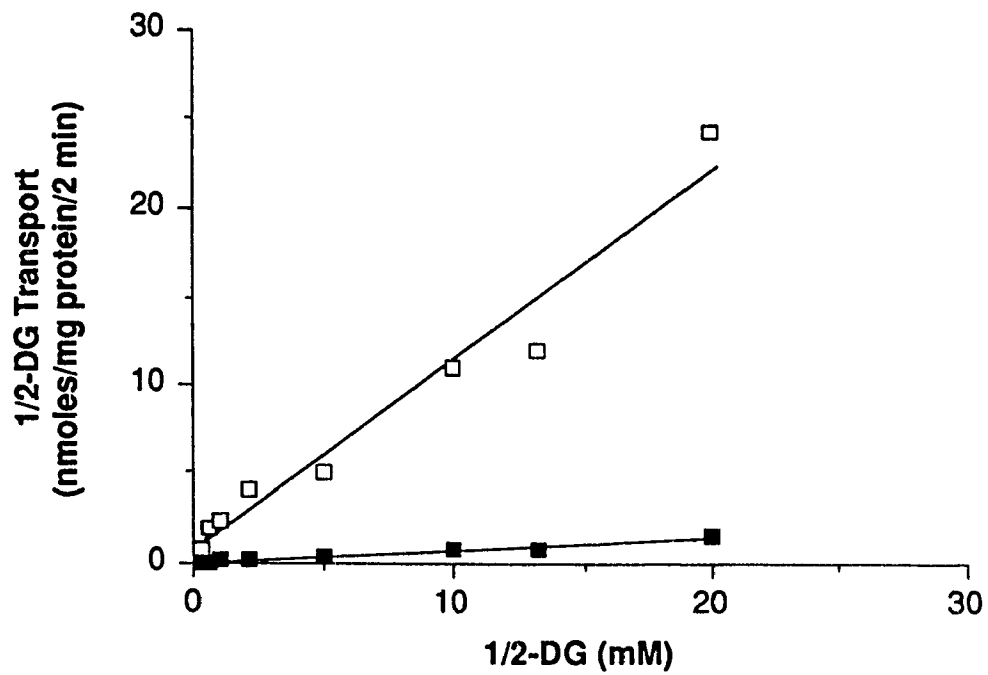
Figure 9. Lineweaver-Burk double reciprocal plot of 2-DG uptake by confluent serum starved (0% MEM, 24 h) MCH55 (□) and WG750 (■) cells. Points represent the average of triplicate determinations.
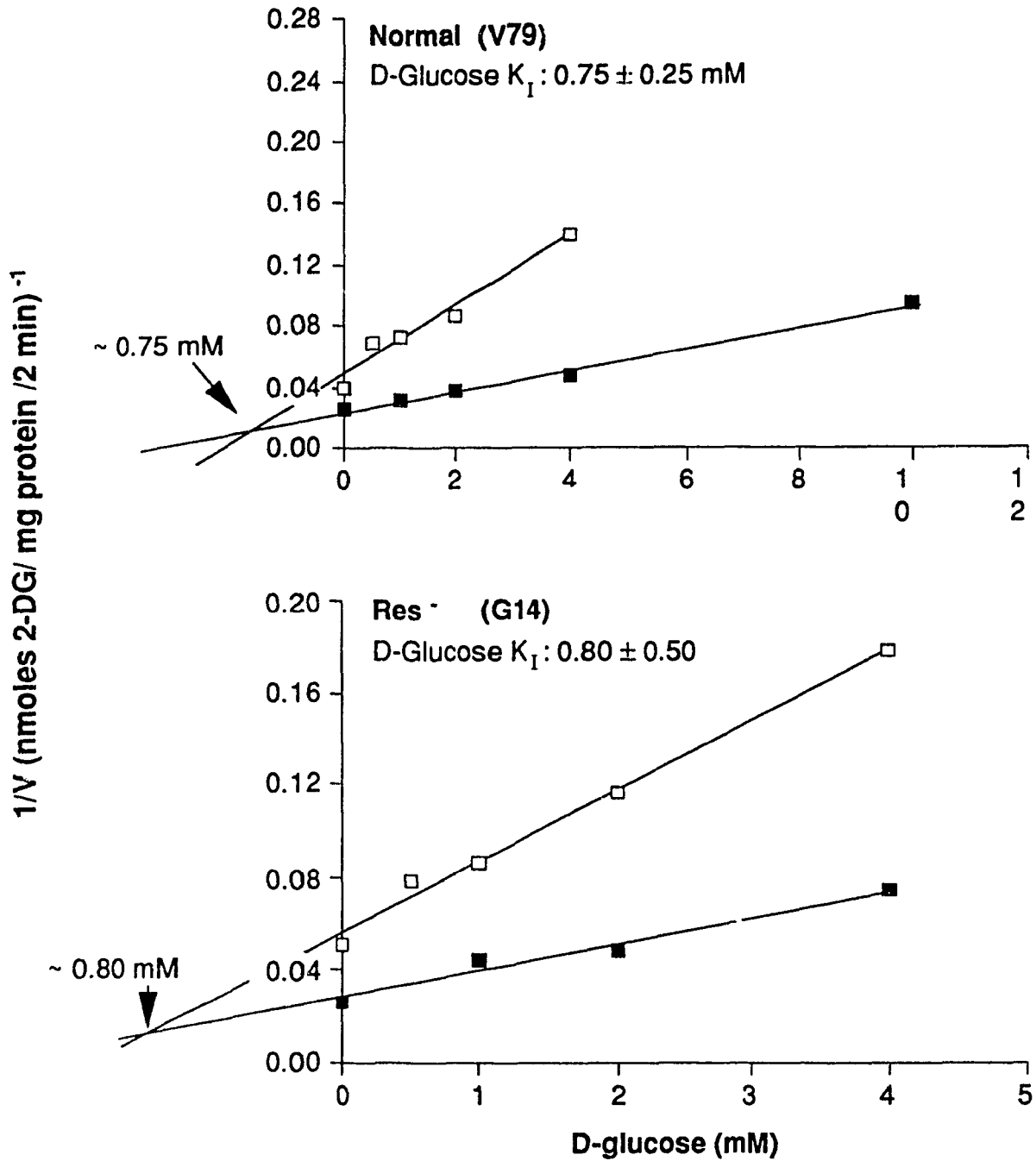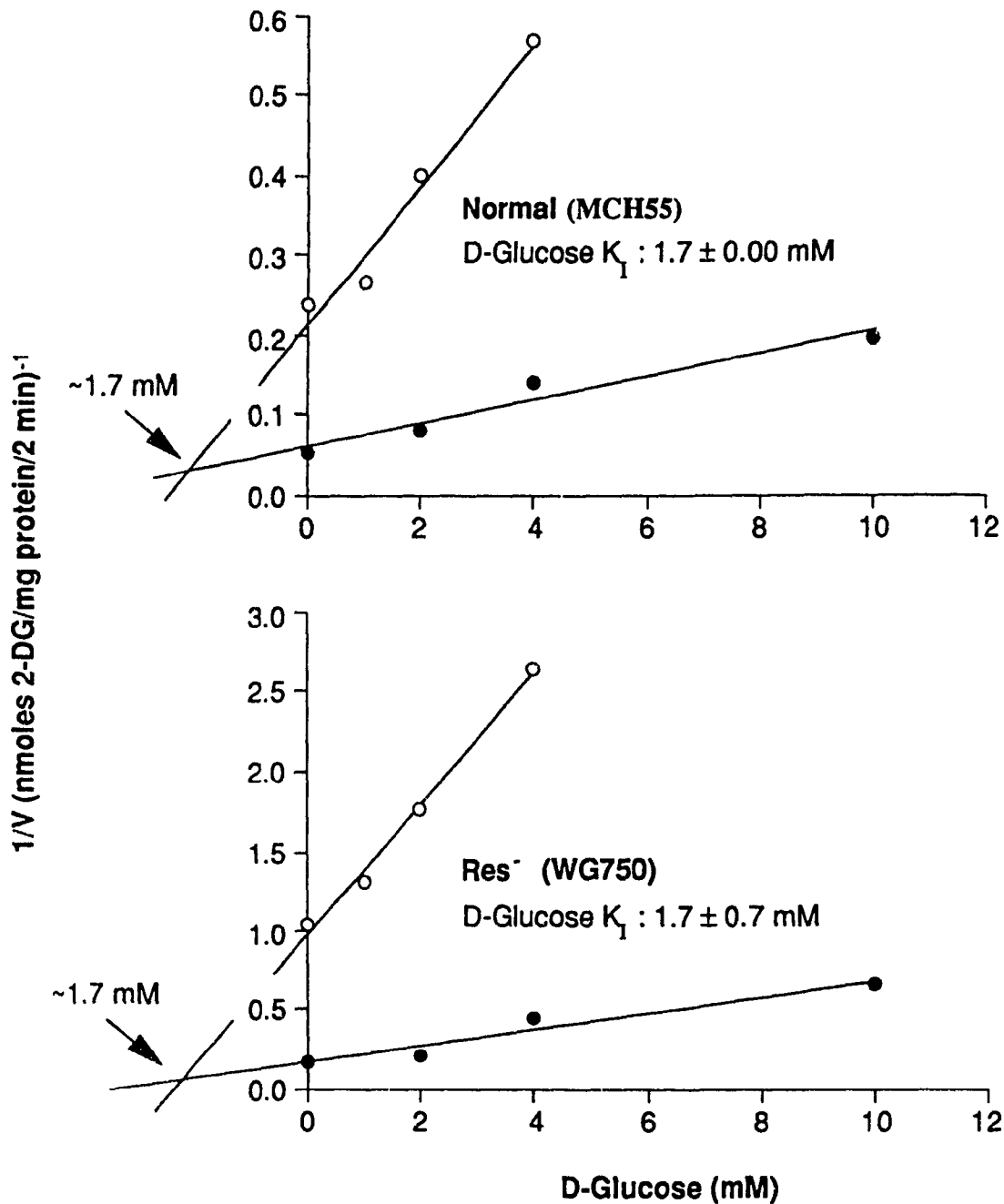
Figure 10. Dixon plot analysis of D-glucose as an inhibitor of hexose

transport for confluent serum starved (0% MEM, 24 h) MCH55 and

WG750 cells. (O) refers to 1.0 mM ^{3}H-2-DG, (●) refers to 3.0 mM

^{3}H-2-DG. Points represent the average of triplicate determinations.
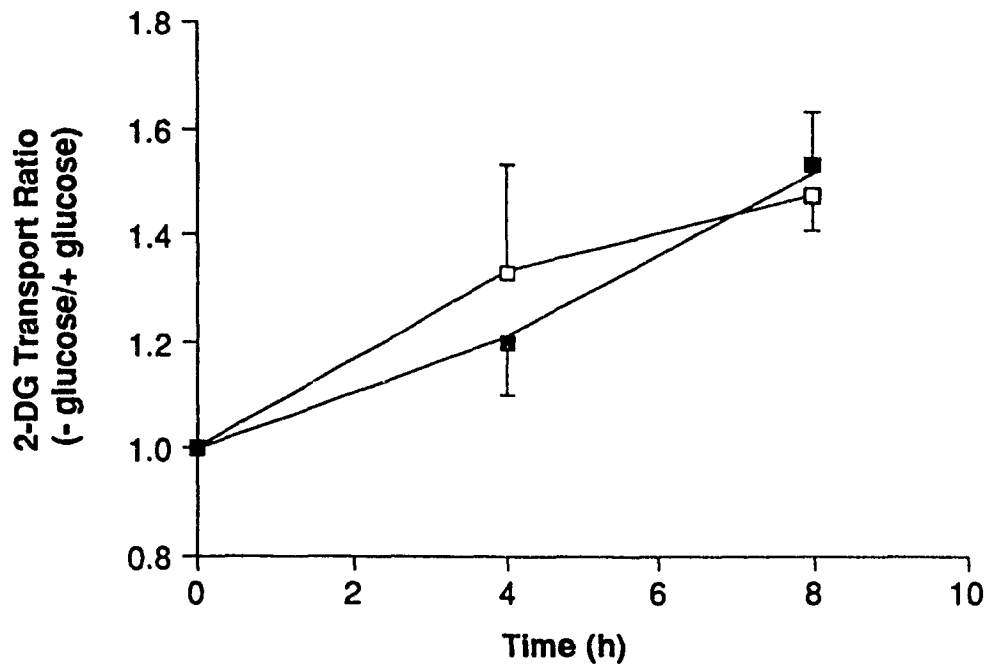
Figure 11.   The effect of glucose starvation on hexose transport in

MCH55 and WG750 cells.   Serum starved (0% MEM, 24 h)   MCH55 (□)

and WG750 (■) cell monolayers were exposed to glucose starved

conditions for 4 or 8 h prior to assay for sugar uptake. Points represent

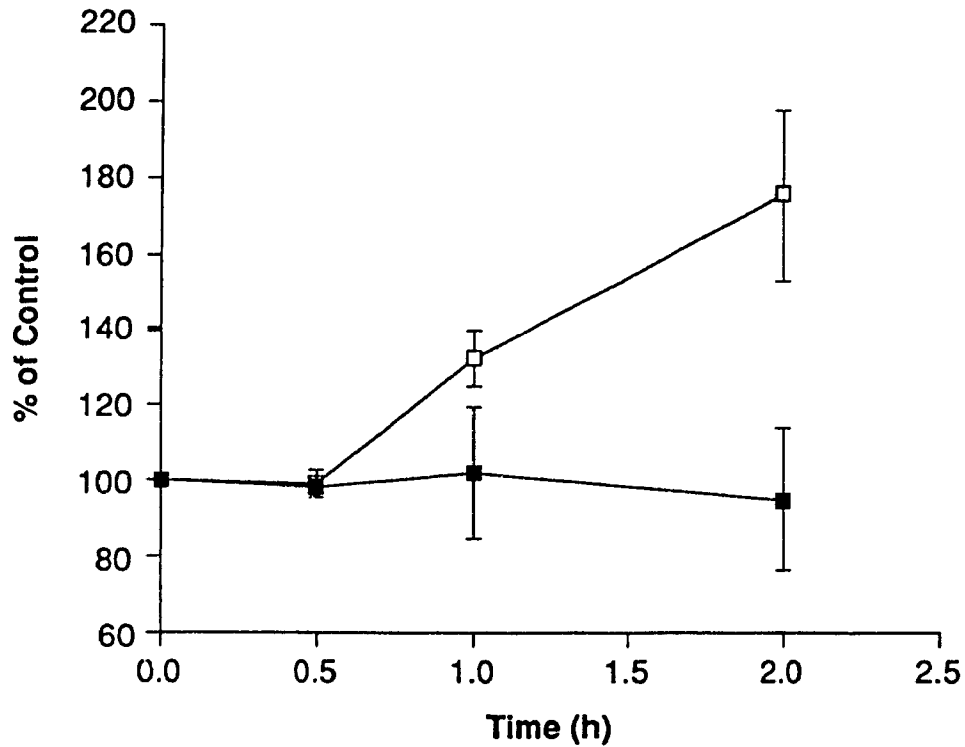average ± SEM of 3 separate experiments (triplicate plates in each

experiment).
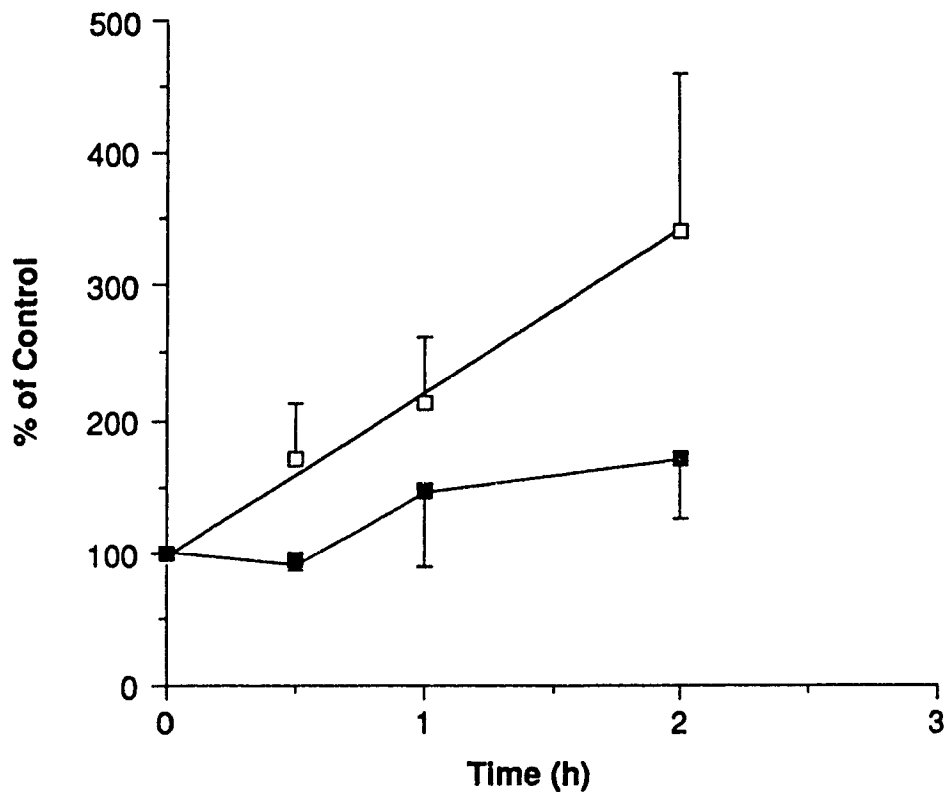
Figure 12. The effect of serum re-feeding on hexose transport in MCH55 and WG750 cells. Hexose transport was measured in MCH55 (□) and WG750 (■) cells after 24 h serum deprivation of serum-fed cultures and after short-term re-exposure to serum. % Control determined as the ratio of increase of 2-DG transport in serum-fed cells versus (baseline) serum-starved cells. Data represent average of 2 determinations ± SEM (triplicate plates in each experiment).
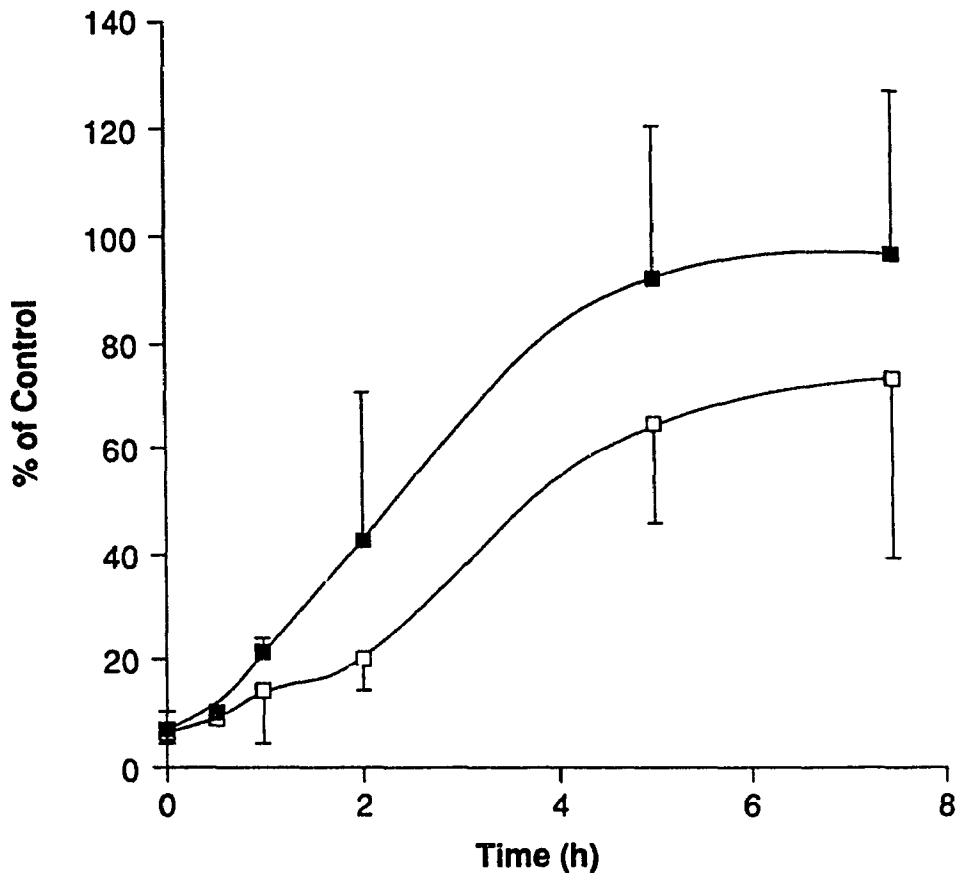
Figure 13. The effect of heat shock on ^{3}H-leucine incorporation in V79 and G14 cells. Prior to heat shock (12 minute incubation at 46°C) confluent cell monolayers of V79 (□) and G14 (■) cells were incubated at 37°C for 1 h in medium containing 2% (v/v) fetal calf serum. After heat shock cells were exposed to 37°C medium with 2% (v/v) fetal calf serum and protein synthesis was monitored by measuring ^{3}H-leucine incorporation into TCA precipitable material. % Control determined as the ratio of increase of leucine incorporation in cells cultured at 37°C after heat-shock treatment versus the leucine incorporation determined directly following heat-shock treatment.
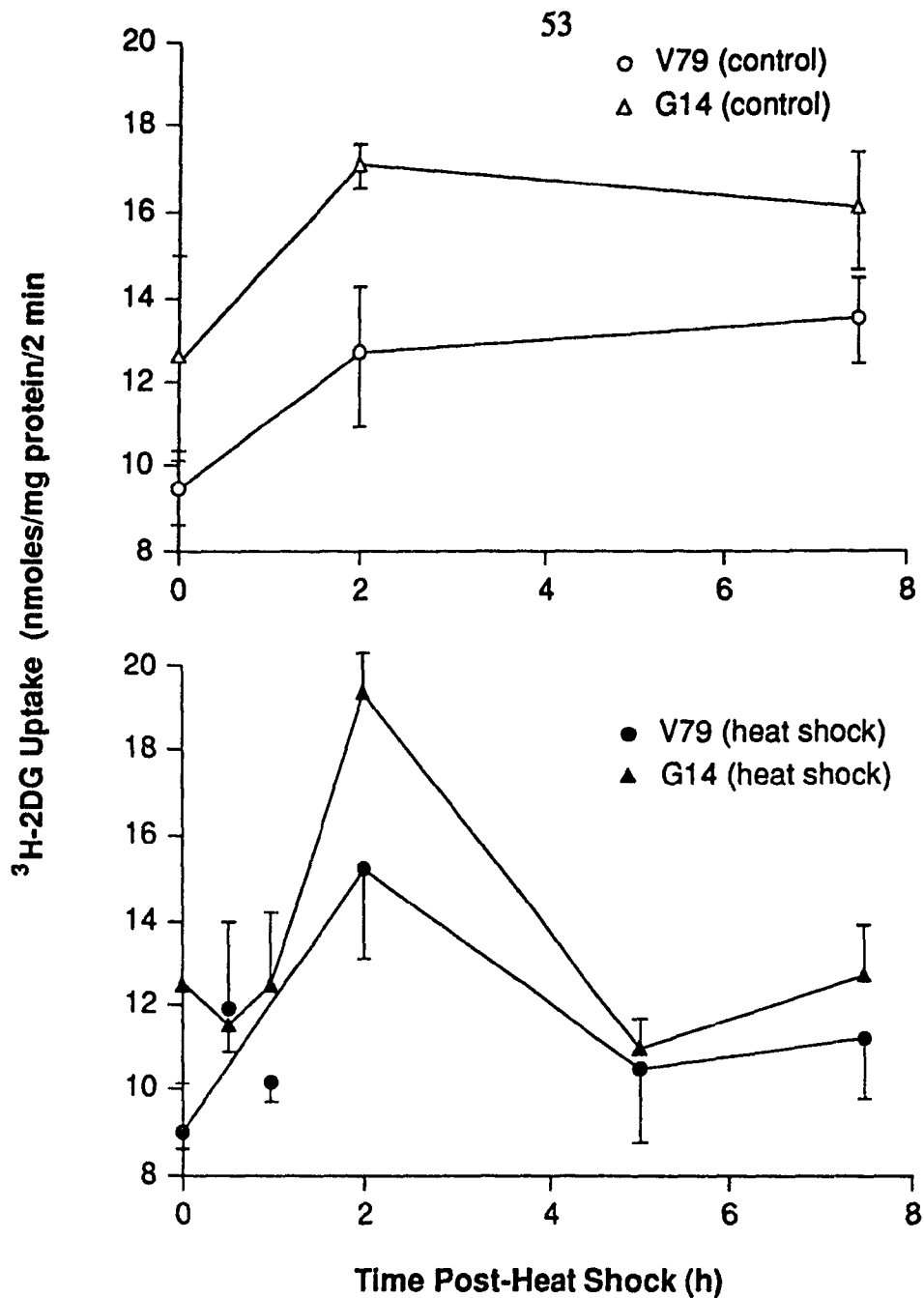
Figure 14. The effect of heat shock on^{3}H-2-DG uptake in V79 and G14

cells. Confluent cell monolayers of glucose-fed V79 and G14 cells were

incubated at 37°C or were stressed by incubation at 46°C for 12 min. The

cells were then incubated at 37°C with glucose for the indicated time

intervals prior to assay for^{3}H-2-DG uptake.
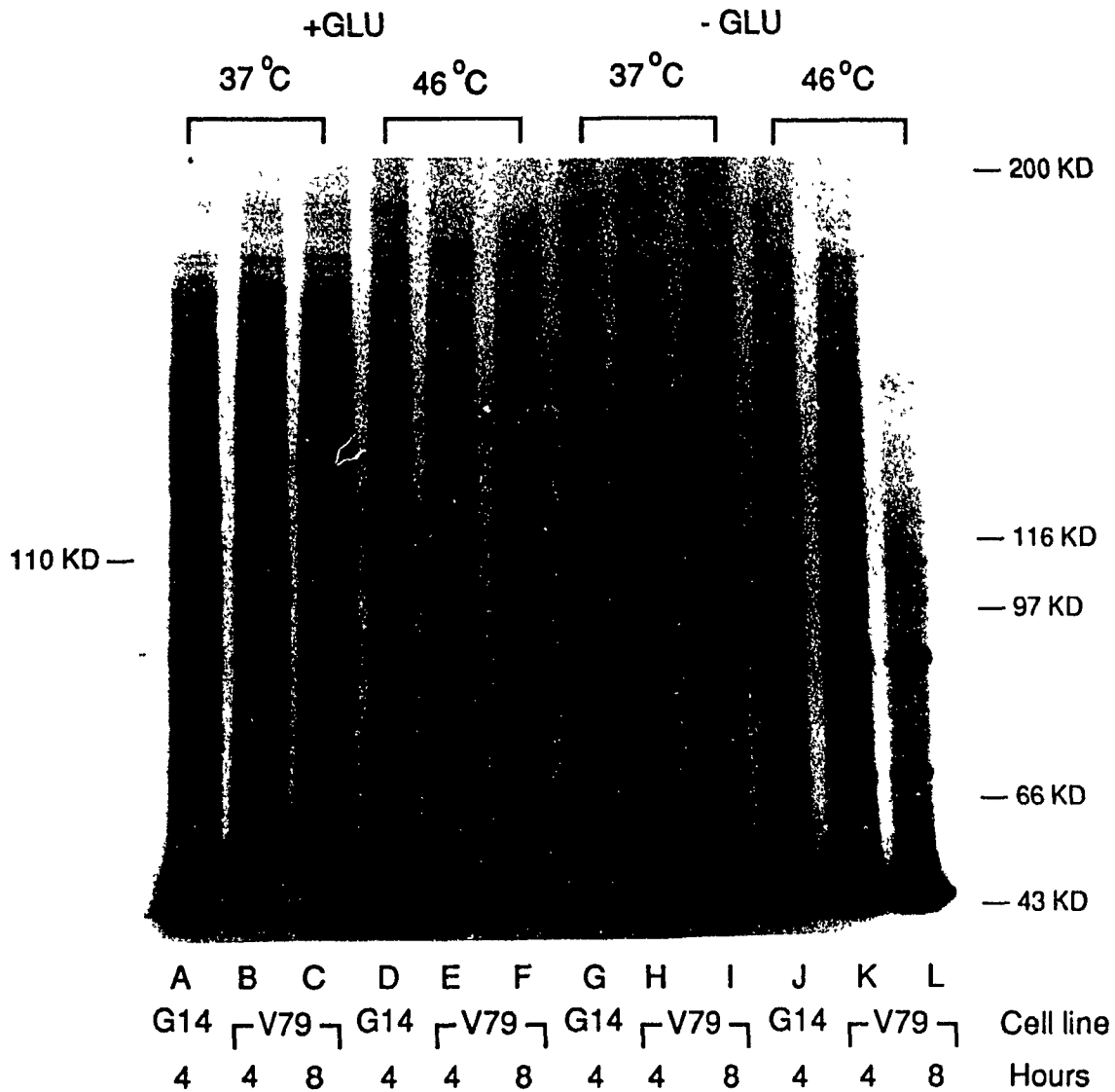
Figure 15. SDS polyacrylamide gel electrophoresis of control and heat shocked V79 and G14 cells. Control or heat shocked V79 and G14 cells were cultured for 4 or 8 h in glucose containing or glucose free medium containing 2% (v/v) dialyzed fetal calf serum. Cells were then pulse labelled with ^{35}[S] methionine for 45 min in order to determine general protein and HSP synthesis. Group 1 refers to glucose fed control cells cultured for 4 h (Lane A, G14, Lane B, V79) or 8 h (Lane C, V79). Group 2 refers to glucose fed and heat shocked cells cultured for 4 h (Lane D, G14, Lane E, V79) or 8 h (Lane F, V79). Group 3 refers to control cells cultured without glucose for 4 h (Lane G, G14, Lane H, V79) or 8 h (Lane I). Group 4 refers to heat shocked cells cultured without glucose for 4 h (Lane J, G14, Lane K, V79) or 8 h (Lane L). The left vertical axis indicates the molecular weight of the HSP as determined from the indicated molecular weight standards. 36 µg of protein were loaded onto each lane using the Lowry assay for protein determination.

Discussion

Respiration Deficient and Parental Chinese Hamster Fibroblasts

The data presented herein clearly indicate that the respiration-deficient G14 transformed cell line exhibits several differences in hexose transport regulation when compared to the parental V79 cell line.

The initial observation that G14 cells had a significantly higher rate of glucose utilization when compared to control cells (Table I) was expected since this mutant is strictly dependent on glycolytic energy for survival and growth (Ditta *et al.*, 1976). DeFrancesco, in 1976 demonstrated that wild-type Chinese hamster lung fibroblasts produce 40% of their total energy requirement from electron transport and 60% from glycolysis (DeFrancesco *et al.*, 1976). Since G14 cells are unable to oxidize mitochondrial NADH and generate energy via the electron transport chain it was questioned whether the rate of glycolysis would be increased in the mutant to compensate for the defect in respiration.

Experiments measuring basal 2-DG uptake in glucose-fed cultures demonstrated that G14 cells do transport hexose at an elevated rate when compared to control cells (Table II). Under glucose-fed conditions the cell lines also displayed slightly different doubling times 10.3 ± 0.7 h and 12.1 ± 0.6 h for V79 and G14 cells respectively (data not shown) indicating that these cells double and grow well in the presence of glucose. Characterization and studies on the regulation of transport by these highly glycolytic cultures was of obvious interest; however, it was important to establish whether differences observed in 2-DG uptake in G14 versus V79 cells were carrier-mediated and not simply due to differences in non-saturable uptake. Experiments were therefore performed employing the fungal metabolite cytochalasin B (CB). CB is a potent inhibitor of glucose transport in many cells (Kletzien *et al.*, 1972, Mizel and Wilson, 1972, Estensen and Plagemann, 1972) binding to the glucose transporter in erythrocytes more tightly than D-glucose (Baldwin and Leinhard, 1981). The addition of 10 µM CB to glucose-fed cultures inhibited 2-DG transport by 90% in both cell lines (Table III). In addition, experiments using the non-metabolized sugar L-glucose revealed that diffusional uptake could account for less than 1% of total uptake in both cell lines (Table

III) although non-saturable uptake was enhanced in G14 cells. Weber has defined diffusional uptake of a hexose as "simple diffusion into the cell, trapping in the intracellular space, binding to the surface of the cell, and transport by other carriers which have a low affinity for the transported species" (Weber, 1973). These data indicate that the observed differences in transport between the two cell lines stem from differences in expression of the hexose transport carriers at the cell membrane.

Studies were performed to resolve the question of whether the increased rate of uptake in G14 cells was due to increased transport or alternatively to changes in rates of phosphorylation. Sugar transport experiments using 3-O-methyl-glucose (3-O-MG) a glucose analog that is transported but not phosphorylated, demonstrated that the increase in transport of this sugar (60% greater than control cells) could account for the increased rate of uptake of 2-DG in G14 cells (40% greater than control cells). The rate of influx and efflux of 3-O-MG was tested with both V79 and G14 cells (Figure 1 and Figure 2). These data show that the transport of free sugar out of the cells is as rapid as the transport into the cells and that the rate of transport is higher in G14 cells. Competition studies have shown that 3-O-MG uses the same carrier system as glucose in chick fibroblasts, in mammalian cells (Venuta and Rubin, 1973, Cuatrecasas and Tell, 1973, Kletzien and Perdue, 1973, Kletzien and Perdue, 1974a), and Chinese hamster lung fibroblasts, (Germinario et al., 1989). In addition, Christopher found that 3-O-MG was shown to be transported in hamster cells at the same rate as the metabolizable sugar galactose and could inhibit galactose transport as well as glucose or 2-DG (Christopher, 1977). These studies provide evidence that 3-O-MG , a non-metabolizable sugar, enables measurement of the transport step independent of phosphorylation.

The phosphorylation of 2-DG results in the formation of an anionic product that does not exchange with free sugar (Romano, 1976). This intracellular trapping of the phosphorylated product may drive the equilibrium of the reaction toward phosphorylation. Thus, an increase in the level or activity of hexokinase could lead to an increased accumulation of 2-DG in the cell that is not related to transport at all. It was important then to determine whether there were significant differences in hexokinase activity in G14 versus V79 cells.

In this investigation hexokinase activity was measured in cell-free homogenates of V79 and G14 cells. No significant differences were found between hexokinase activity

in V79 versus G14 cultures which had been glucose fed and starved or serum fed and starved (Figure 7 and Figure 8) although some differences in hexokinase activity were discovered between glucose-starved versus glucose-fed cells (Figure 7). However, others have shown no differences in hexokinase activity in cellular homogenates from chick embryo fibroblasts or hamster cells cultured with or without glucose (Kletzien et al., 1974a, Musliner et al., 1976). No significant differences in hexokinase activity were found between glucose starved or glucose-fed cells. It is unlikely then, that the margin of difference in uptake rates between V79 and G14 cells can be due to anything other than differences in transport rates.

The increase in initial rates of hexose uptake by G14 cells reflects a modification of the normal cellular transport system. The kinetic mechanism by which the alteration of sugar uptake occurs in G14 cells was investigated. The interpretation of kinetic data involving transport systems; however, is often difficult. A critical point in studying a carrier system is the use of initial rate measurements which are also rate-limiting (Kletzien and Perdue, 1974a, Kletzien and Perdue, 1975b, Christopher et al., 1976a, Germinario et al., 1978).

It is important to note that in this study incubation times of 2 min or less were used to measure sugar transport to satisfy th' criteria. Also, the intracellular accumulation of free 2-DG was ~ 13-15% of the total uptake at 10 mM 2-DG and ~ 5-7% at 0.1 mM 2-DG (Germinario et al., 1990). It has also been stressed that accuracy in kinetic analyses of the facilitated mode of transport must include corrections for sugar entry through diffusional processes (Weber, 1973, Kletzien and Perdue, 1974b, Dolberg et al., 1975, Germinario et al., 1978). For this reason the uptake of L-glucose was used to measure non-saturable uptake. 2-DG was used to monitor sugar uptake since it remains a largely unmodified substrate due to the fact that it is non-metabolizable past 2-DG-6-phosphate. A kinetic analysis of the initial rates of 2-DG transport by Lineweaver-Burk plots showed that the increased rate of sugar transport in G14 cells was associated with a 55% greater V_{max} with no significant changes in apparent K_m (Table IV and Figure 3). In theory, this increase in V_{max} could result from an increase in the number of functional transporters in the plasma membrane, or from an increase in the rate at which already functional transport systems operate.

The apparent K_m values for the transport of 2-DG by V79 and G14 cells were similar to those obtained in various types of un-transformed and transformed rat (Hatanaka and

Gilden, 1970, Renner *et al.*, 1972, Plagemann, 1973, D'Amore *et al.*, 1986b, Naftalin and Rist, 1989), mouse (Hatanaka, 1970, Bose and Zlotnick, 1973, Romano and Colby, 1973, Plagemann and Richey, 1974), hamster (Isselbacher, 1972) and chicken (Hatanaka and Hanafusa, 1970, Harris and Whitmore, 1974, Kletzien and Perdue, 1975b) cells whereby experimental values for K_m ranged between 0.3 and 5mM.

The data showing that there is no difference in K_m in V79 versus G14 cells was further substantiated by the data shown in Table V and Figure 4. The data show the calculated K_I values for the competitive inhibition of 2-DG uptake by D-glucose in intact V79 and G14 cells. The dissociation constant for the enzyme-inhibitor complex was nearly identical for V79 and G14 cells. These results support other work which indicated that D-glucose and 2-DG are transported by the same transporter in these cells (Germinario *et al.*, 1989). In addition, these K_I values are within the range reported for other cell culture systems (Renner *et al.*, 1972, Weber, 1973).

The data in Figure 5 indicate the adaptive response of V79 and G14 cells to glucose deprivation. The V79 cells increased their rate of transport by 78% after 2 h in glucose-free media while in the G14 hexose transport was only elevated 13%. After 4 h of glucose starvation hexose transport increased 90% in V79 cells but only 26% in G14 cells. These results show that while V79 cells respond to glucose deprivation by increased hexose transport, the G14 cells do not have the same capability. The data suggests that regulatory factors controlling the number or availability of intracellular glucose carriers which normally translocate to the plasma membrane upon glucose starvation may be already surface located in G14 cells.

Franchi showed that the transport of 2-DG, glucose and 3-O-MG in Chinese hamster cells is regulated by the level of glucose in the medium (Franchi *et al.*, 1978). Glucose uptake was elevated 8-fold after 16 h of glucose starvation while the uptake of 2-DG and glucosamine increased 3 and 4 fold respectively. Glucose-starved cells took up 3-O-MG at least 4 times more rapidly than glucose-fed cells indicating that glucose starvation affects transport and not phosphorylation. Furthermore, in these same studies it was shown that the induction of glucose uptake was rapid and not prevented by the addition of 10 μg/ml cycloheximide. This suggested that the synthesis of new carrier protein was not necessary for depression of glucose uptake in glucose-starved hamster cells. Kinetic data showed that glucose-fed and glucose-starved hamster cells had the same K_m value for glucose but a different V_{max} (4-5 versus 20-25 nmoles/min/

mg protein). From these results it was suggested that regulation of glucose uptake in hamster cells is a post-translational process whereby glucose or one of its "close metabolites" would exert an inhibitory effect on the "activation" of the glucose-carrier protein. An alternate mechanism whereby activation of the glucose carrier would be altered by a glucose-activated protease was also suggested (Franchi *et al*, 1978). Others (Kletzien and Perdue, 1975b, Christopher, 1977, Gay and Hilf, 1980, Paris *et al.*, 1980, Yamana *et al.*, 1983) working with other mammalian cell culture systems believe that glucose starvation causes an increase in the number of functional transporters in the plasma membrane due to their redistribution in the cells.

The literature regarding the effects of serum on hexose transport is fairly extensive. Generally speaking serum deprivation leads to decreased transport activity (Sefton and Rubin, 1971, Schimke, 1973, Kletzien and Perdue, 1974a, Germinario *et al.*, 1982) whereas readdition of chicken serum or fetal calf serum to serum-deprived cultures results in elevated hexose transport (Perdue, 1976, Gammon and Isselbacher. 1976). In general then, serum deprivation of cell cultures leads to a state of quiescence (i.e., a decrease in the activity of biochemical systems such as sugar transport). These quiescent cells are then employed in transport experiments resulting in an amplified response to the addition of factors such as insulin or other growth factors.

Hexose transport in serum-fed cultures of V79 and G14 cells was essentially the same (31.2 ± 6.0 nmoles 2-DG/mg ptn/5 min versus 33.0 ± 6.5 nmoles 2-DG/ mg ptn/ 5 min); however, following 24 h of serum deprivation hexose transport differences were uncovered (Table VI). Under conditions of serum deprivation the V79 cells expressed a 33% decrease in hexose transport while the G14 cells showed no difference in transport (i.e. 33.0 ± 6.5 nmoles 2-DG/mg ptn/5 min versus 36.1 ± 1.6 nmoles 2-DG/ mg ptn/5 min without serum). Furthermore, short-term re-addition of serum to serum-starved V79 and G14 cells stimulated hexose transport in V79 cells by 35% over control within 2 h but had little if any effect on G14 cells (Figure 6).

The fact that V79 cells respond to serum addition or deprivation by regulating their hexose transport contrasts with Hershko's claim that transformed cells are fairly insensitive to growth limitations imposed by serum (Hershko *et al.*, 1971). 3T3 cells transformed by Simian virus 40 showed no change in rates of RNA and protein synthesis, uridine uptake and protein degradation. Also, the re-addition of serum to serum-deprived transformed cells had little or no effect on processes affected by the

presence of serum. Perdue also reported that the addition of serum to virus-transformed chick embryo cells previously deprived of serum did not result in stimulation in the rate of transport (Perdue, 1976). However, the inability of G14 cells to modulate hexose transport under conditions of serum deprivation or serum addition is not surprising. Energy is required for many cellular processes e.g., biosynthetic reactions and the synthesis and turnover of cellular constituents. G14 cells obtain all their energy from substrate level phosphorylation by converting glucose to lactate while in wild-type cells 60-65% of required ATP is derived from glycolysis and the remainder from oxidative phosphorylation (Soderberg et al., 1980). Since transformed cells have high demands for ATP to permit rapid and density-independent growth it seems logical for G14 cells to render themselves unresponsive to the normal effects of serum deprivation on sugar transport. Serum-treated Swiss mouse 3T3 cells exhibited a 5-6 fold increase in V_{max} for 2-DG uptake with an increased level of mRNA for transporter following 1 h of exposure (Kitigawa et al., 1989). The increased level of mRNA was totally inhibited by actinomycin D but unaffected by cycloheximide suggesting that the increase in mRNA was due to enhanced gene transcription. Kitigawa has suggested that growth factors present in serum may stimulate glucose transport in mouse cells in two ways: First, short-term exposure (within 1 h) may involve translocation of glucose carriers to the plasma membrane whereas longer-term exposure (1-3 h) would involve biosynthesis of the transporter. Perhaps G14 cells are unresponsive to short-term re-exposure to serum due to the fact that more carriers are already surface located so that the capacity to mobilize stored carriers is low.

Insulin has been known to replace the serum requirement for growth of chick embryo fibroblasts and has been shown to stimulate hexose transport in cultured human fibroblasts (Germinario and Oliviera, 1979, Howard et al., 1979, Berhanu and Olefsky, 1981, Germinario et al., 1984). Kosaki showed that insulin is capable of regulating glucose transporter gene expression since insulin stimulation resulted in a 1.6-fold stimulation of glucose transporter mRNA levels in human fibroblasts (Kosaki et al., 1988). Although fibroblasts possess an insulin receptor which is regulated by supra-physiological levels of insulin (as well as insulin-sensitive IGF-I receptors), fibroblasts are markedly less sensitive to insulin than adipocytes in the stimulation of sugar transport (Hollenberg and Cuatrecasas, 1975, Rechler and Podskalny, 1976, Germinario and Oliveira, 1979, Mott et al., 1979). This is largely due to a smaller number of

insulin receptors present in fibroblasts (Baldwin *et al.*, 1981, Kadle *et al.*, 1984). Garcia de Herros showed that the limiting factor in the pathway regulating glucose transporter mRNA levels in fibroblasts by insulin is the low number of receptors. The introduction of the insulin receptor cDNA by DNA-mediated gene transfer in NIH/3T3 HIR3.5 cells resulted in the ability to express 3×10^6 insulin receptors per cell (Garcia de Herros and Birnbaum, 1989). Insulin activated glucose transporter mRNA levels in these cells to the same extent and with the same insulin concentrations as in adipocytes. The response of fibroblasts to insulin is variable and is largely influenced by culture conditions such as confluence, serum or glucose availability. Maximal stimulation is obtained with serum-deprived, glucose-fed cultures after 30-60 min preincubation with insulin (Germinario and Oliveira, 1979, Berhanu and Olefsky, 1981, Ishibashi *et al.*, 1982, Germinario *et al.*, 1984, Cynober *et al.*, 1985).

The data in Table VII show that insulin stimulated hexose transport in serum-starved V79 cells by 92% following a 2 h incubation whereas transport activity was unchanged in G14 cells. The inability of G14 cells to respond to insulin or serum demonstrate that regulation of hexose transport has been necessarily altered to compensate for the adaption to the inherent genetic defect in these cells.

There appear to be at least three different mechanisms, and probably more, responsible for regulating glucose transport in mammalian cells: 1) alteration of the half-life of the glucose transporter i.e., by altered degradation (Haspel *et al.*, 1986, Shawver *et al.*, 1987) 2) activation of existing glucose transporters (e.g., by redistribution of carriers to the plasma membrane (Cushmann and Wardzala, 1980, Suzuki and Kono, 1980, 3) induction of glucose transporter biosynthesis (e.g., by altering levels of mRNA (Birnbaum *et al.*, 1987, Flier *et al.*, 1987, White and Weber, 1988).

Further studies in the laboratory will be directed at elucidating the mechanism(s) responsible for the compensatory adaptations of hexose transport regulation in G14 cells. The enhancement of glucose transport in G14 cells may be due to an increased number of hexose transporters at the cell membrane. This view is supported by kinetic evidence which indicated that the V_{max} for transport is increased in G14 cells. One approach to examine this possibility would involve isolation of subcellular membrane fractions from V79 and G14 cells grown under various conditions (e.g., in the presence or absence of serum or insulin). Low and high density microsomes and plasma membrane would be isolated by the modified methods of Buchanan and Horner

(Buchanan *et al.*, 1985, Horner *et al.*, 1987). The number of glucose transporters in each of the membrane fractions would be assessed by D-glucose- inhibitable cytochalasin B binding assays. This quantitation of the number of glucose transporters in the subcellular fractions would indicate whether there is an intracellular redistribution of hexose transporters in G14 cells.

Alternatively, there may be altered rates of synthesis or degradation of glucose transporters in G14 cells since total RNA of serum-deprived cultured V79 and G14 cells analyzed by the laboratory of Dr. Jeffrey Flier using the HepG2 glucose transporter cDNA, demonstrated a 1.6-fold elevation of glucose transporter mRNA in these cells. The rates of biosynthesis of the glucose transporter protein in the two cell types could be determined by labelling cells with ^{35}S methionine for increasing periods of time followed by lysing of the cells and immunoprecipitation of the transporter (White and Weber, 1988). Audioradiographs of SDS gels of immunoprecipitates would be scanned with a video densitometer to determine the relative intensity of each band. If incorporation of label into the transporter was greater in G14 cells than in V79 cells this would be indicative of increased biosynthesis of glucose transporter protein in G14 cells. The rates of degradation or turnover of the glucose transporter protein in V79 and G14 cells could also be tested. Cells would be labelled with ^{35}S methionine for extended periods of time (e.g., 16 h) and then chased with cold methionine for varying periods of time (e.g.,) 0,3,6,12,24 h (White and Weber, 1988). Audioradiographs of SDS gels of immunoprecipitates would be analyzed by densitometry to determine the half-lives for transporter turnover in the two cell types. If incorporation of label at the time points tested is greater in G14 cells compared to V79 cells this would indicate a decreased rate of carrier turnover in G14 cells.

The Effect of Heat Shock on V79 and G14 Cells

One of the questions asked in this work regarded the relationship of hexose transport in V79 and G14 cells and the reported enhanced level of GRPs induced by heat shock and/or glucose deprivation. Specifically, it was of interest to determine whether the stress caused by the enzyme defect in G14 cells would result in preferential synthesis of a polypeptide(s) recognized as a HSP and/or GRP, and if so, whether this protein(s) was associated with the change in hexose transport and therefore may be involved in

hexose transport regulation.

The data showing the effect of heat shock on ^{3}H-leucine incorporation in V79 and G14 cells was encouraging (Figure 13). The data indicated that G14 cells recovered from the imposed thermal stress more rapidly than the parental cell line. The reason for this is unknown. Perhaps the swift recovery of G14 cells is contingent upon the prior synthesis of a stress protein or a greater level of synthesis of this protein compared to V79 cells. Heat-shock proteins are known to be present at relatively high levels under non-inducing conditions (Welch et al., 1982). It is possible that G14 cells constitutively synthesize enhanced levels of a peptide as a means of combating the stress already imposed by the enzyme defect. This would confer a degree of protection against subsequent stress situations such as heat shock.

Heat-shock treatment also resulted in a transient increase in 2-DG uptake in both cell lines (Figure 14). The elevation peaked 2 h post-heat shock and then transport returned to near levels after 7.5 h incubation at 37^{0}C. Warren also showed that BHK-21 cells heat shocked at 45^{0}C for 30 min increased their hexose uptake less than 20 min after exposure after which the heat-induced increase was stable for over 2 h (Warren et al., 1986). Experiments employing 3-O-MG demonstrated that hexose transport, not phosphorylation, was affected by the stress.

The physiological significance of this increase in hexose uptake is not understood. It has been suggested that increased hexose uptake is a general response of cells to various environmentally induced stresses (Warren et al., 1986). The data indicating that V79 and G14 cells increase hexose uptake equally in response to heat shock might support this view. It has also been suggested that the increase in glucose transport is probably not associated with HSPs since the addition of cycloheximide to inhibit protein synthesis does not affect the increase in hexose transport induced by stress (Henle and Leeper, 1982, Lee and Dewey, 1987, Warren et al., 1986). However, the increase in glucose uptake upon glucose starvation (which may also be considered a stress) is, in Chinese hamster cells, independent of protein synthesis (Franchi et al., 1978). Therefore de novo synthesis of a protein may not be necessary for the enhancement of glucose transport. Perhaps heat shock acts to conformationally change a preformed glucose- regulated protein directly or translationally, altering its activity and increasing glucose transport.

Analysis by SDS polyacrylamide gel electrophoresis of heat-shocked glucose-fed or

starved cells indicated that heat shock induced preferential synthesis of one peptide corresponding to 110 KDa in both V79 and G14 cells. However heat shock and/or glucose deprivation did not appear to result in an enhanced level of any specific peptide(s) in G14 cells compared to V79 cells (Figure 15).

It would appear that the conditions chosen for heat shock are not responsible since this treatment was previously shown to increase hexose uptake (Figure 14). It is also known that maximum induction of HSPs in HeLa cells is achieved if cells are placed at 45-46°C for between 5-10 min (Burdon *et al.*, 1982). Proteins induced by stress are more clearly defined; however, when analyzed by isoelectric focusing followed by SDS-polyacryamide gel electrophoresis so that in future, experiments should employ this method.

Pelham has reported that GRPs are in fact not heat-shock inducible but are overproduced when cells are starved of glucose (Pelham, 1986). Others have also reported that the synthesis of a number of cellular proteins is glucose- dependent (Pouysseger *et al.*, 1977, Kasambalides and Lanks, 1978, 1979, Kasambalides, 1981, Lade-D'avila, 1979). Glucose-starved L6 rat myoblast cells displayed an increased V_{max} for 2-DG and 3-0-MG transport whereas this treatment had no effect on an isolated hexose-transport mutant D1/S4 (D'Amore and Lo, 1988). Electrophoresis and immunoblotting studies showed that the mutant cells had a significantly diminished amount of a 112 KDa protein. The authors postulated that this 112 KDa protein may be a regulatory component involved in triggering the activation of hexose transport brought on by glucose starvation (D'Amore and Lo, 1988). If this is true, one might expect differential induction of a glucose- regulated protein in glucose starved V79 and/or G14 cells which had been cultured at 37°C . It is possible that 4 or 8 h of glucose starvation is not sufficient to induce a glucose regulated protein. However conditions necessary for maximum induction which allow maximum expression are not always feasible. DeFrancesco has reported that a respiration-deficient Chinese hamster cell line CCL16-B2 res- dies within 6 h when deprived of glucose (DeFrancesco, 1976). Since G14 cells are also strictly dependent on glucose for their survival, conditions were chosen to minimize cellular lethality.

Kasambalides showed that glucocorticoids may alter the response of glucose-deprived cultures by eliciting a heat-shock-like response (Kasambalides and Lanks, 1983). The addition of dexamethasone to glucose-starved L929 cells stimulated the

synthesis of 82 KDa and 85 KDa proteins both of which are known to be glucose regulated. Kim showed that Chinese hamster cells exposed to insulin for 48 h expressed a 2.5 to 3-fold increase in GRP transcript level (Kim *et al.*, 1987). Also, Warren showed that the extent of stimulation of insulin on hexose transport in BHK-21 cells was similar to the increase in transport as a result of heat shock (Warren *et al.*, 1986). He suggested that heat-induced alterations in hexose transport reflect an alteration in plasma membrane function similar to those induced by insulin. Glucose-regulated proteins have also been shown to be induced by substances such as tunicamycin, glucosamine, and calcium ionophores (Olden *et al.*, 1979, Welch *et al.*, 1983, Resendez *et al.*, 1985). Since different types of stress may result in induction of different specific HSPs and/or GRPs, future experiments may employ some of these substances to examine expression of GRPs in V79 and G14 cells.

Respiration Deficient and Normal Human Skin Fibroblasts

The results of the comparative study of transport regulation using a normal (MCH55) and a respiration-deficient (WG750) strain of human fibroblasts were most exciting. The data indicated that WG750 cells displayed altered responses to several factors affecting glucose transport. However, unlike the hamster respiration mutant, the human mutant was able to upregulate its transport to some degree in response to these factors. The data suggest that the mechanism(s) leading to elevated hexose transport in human cells differ from those which control hexose transport in hamster cells.

The data in Table VIII show that basal 2-DG transport in glucose-fed WG750 cells was 6-fold higher than in the parental cell strain ($p<0.01$, n=11). This result was not unexpected since in addition to displaying significantly increased lactic acid production, cellular death occurs very rapidly in WG750 cells after the glucose in the media has been used up (Robinson *et al.*, 1986). It is important to note that confluent cell cultures were used in these and all experiments since cells which are less confluent display significantly higher rates of hexose uptake and utilization when compared to confluent cultures (Dubrow *et al.*, 1978, Germinario *et al.*, 1978, Wohlheuter *et al.*, 1980, McKay *et al.*, 1983).

Kinetic analysis of the initial rates of 2-DG transport by Lineweaver-Burk plots showed that the increased rate of transport in WG750 cells reflected a 9.5-fold increase in the V_{max}

when compared to age-matched MCH55 cells while the K_m was essentially the same in the two cell types (Table IX and Figure 11). The K_i for D-glucose inhibition of 2-DG uptake in MCH55 and WG750 cells was also determined. Dixon plot analysis indicated that the K_i's for D-glucose were nearly identical (Table X and Figure 10) (Dixon and Webb, 1964). These data suggest that the human respiration-deficient mutant recruits more glucose transporters to the plasma membrane when compared to control cells. Similar results were obtained with the hamster cells (Table IV, Table V, Figure 3, Figure 4) whereby G14 cells also expressed an elevated V_{max} for 2-DG transport compared to V79 cells. Together, these results suggest that mammalian cells which are respiration-deficient will counteract this defect by modulating cellular controls governing hexose transport at the level of the plasma membrane.

Both MCH55 and WG750 cells maintained in glucose-deprived culture medium for 8 h had elevated levels of hexose transport activity compared with that of normally fed cells (Figure 11). Serum re-addition of serum-starved cultures led to increased 2-DG transport in MCH55 cells and a time delayed increase in transport in WG750 cells (Figure 12). Normal cells responded to the effects of serum by showing increased hexose transport within 30 min whereas the mutant took 60 min before an increase in hexose transport was detected. Serum addition resulted in a 2.4-fold increase in 2-DG transport in MCH55 cells after a 2 h incubation in serum-containing medium whereas the same treatment elevated 2-DG transport 1.7-fold in WG750 cells. These results contrast the unresponsiveness of G14 cells tested under similar conditions (Figure 5, Figure 6).

The action of insulin on hexose uptake in the present study appeared to differ from that observed with the hamster cells. V79 cells expressed a hormone-dependent increase in transport activity when exposed to insulin whereas transport activity was unchanged in G14 cells (Table VII). 10 mU/ml and 100 mU/ml insulin stimulated 2-DG transport in MCH55 cells 1.7 and 2.6-fold respectively whereas decreased stimulation was obtained with WG750 cells (1.2 and 1.3-fold respectively) (Table XI). These results, together with the kinetic data showing a dramatically increased V_{max} for 2-DG in WG750 cells, may indicate the presence of large compartmentalized intracellular pools of carriers. These carriers would respond to stimuli by translocation from these intracellular storage sites to the plasma membrane. Alternatively, there may be a faster recycling of glucose transporters between the intracellular pools and plasma

membrane in WG750 cells than in MCH55 cells. Preliminary data from Dr. Germinario's laboratory indicates a clear increase in human transporter mass in WG750 cells in the crude cell pellet, in nucleotidase enriched plasma membrane preps, and in the microsomal pellet (data not shown). This result is not surprising since basal 2-DG transport was shown to be 6-fold higher in these cells, perhaps reflective of an increased rate of transporter synthesis.

Additional information concerning transport regulation was obtained when total RNA of serum-deprived cultures of MCH55 and WG750 cells analyzed by the laboratory of Dr. Jeffrey Flier using the HepG2 glucose transporter cDNA indicated a greater than 10-fold elevation of glucose transporter in WG750 cells (data not shown). This result may indicate that the predominant mechanism for transport regulation in WG750 cells resides at the level of transporter mRNA. The increase in transport activity in these cells may reflect an increased rate of gene transcription or increased stability of the gene product.

This is not the first report to describe an adaptive enhancement of hexose uptake in mammalian cells whose respiration is somehow inhibited. One group studied regulation of glucose transport in rat liver Clone 9 cells which have been characterized as possessing a low intracellular glucose concentration and limited glycogen stores (Mercado et al., 1989). Preincubation of these cells with 0.5 mM cyanide for 1 h resulted in a 7-fold increase in ^{3}H-3-0-MG uptake compared to control cells. This increase in 3-0-MG uptake persisted for several hours after removal of the cyanide and was attributed to an increase in V_{max} while the K_m for the sugar remained unaltered. NIL hamster cultures maintained in medium containing 0.2 mM 2,4-dinitrophenol showed an approximately 2-fold greater uptake of ^{14}C galactose compared to control cells (Kalckar et al., 1979). Other uncouplers of oxidative phosphorylation, namely oligomycin and carbonyl cyanide p-trifluromethoxyphenylhydrazone caused similar increases in hexose uptake. It has also been shown that rat thymocytes display dramatic increases in 3-0-MG transport in the presence of uncouplers such as 2,4-dinitrophenol or when intracellular ATP levels are depleted by anoxia (Reeves, 1975). Cells incubated in the absence of oxygen for 1 h displayed a 3-4-fold increase in the initial rate of 3-0-MG uptake. The ability of anoxia to stimulate glucose transport has also been investigated in other tissues. Membrane fractions from rat hearts perfused in the presence and absence of oxygen were prepared in order to quantitate the number of

glucose transporters in the membrane fractions (Wheeler, 1988). Results revealed that anoxia resulted in a 20-70% increase in glucose transporter in the plasma membrane fraction and a 20-30% decrease in glucose transporter in high-speed pellet fractions. The extent of translocation of transporters due to anoxia coupled with an approximately 2-fold stimulatory effect on glucose transport was similar to that produced when rat heart was exposed to insulin. Based on the similarity in results obtained with these 2 effectors of transport, it was concluded that translocation of glucose transporters plays an important role in the response of rat heart to anoxia. However, a better approach to determine the precise mechanisms of hexose transport regulation is through the use of mutants isolated for defects related to hexose transport. The fact that the cells described in this study have been biochemically and genetically characterized offers a distinct advantage over the other systems discussed wherein the effects were artificially induced.

Although the data in this study suggest that there are quantitative differences in glucose transporter or in the distribution of the transporter within subcellular fractions of WG750 versus MCH55 cells, and in V79 versus G14 cells, the comments and conclusions given in this work are partially speculative. However, the observations that emerge from this report provide a framework for future experiments directed towards an understanding of the molecular mechanisms of transport regulation in human and mammalian cell systems. The results presented herein indicate that the hamster and human respiration-deficient fibroblast cell systems provide excellent tools for this purpose.

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Appendix I. Information on Probes and Inhibitors used for Sugar Transport Studies.

1. 2-DG: Only phosphorylated and accumulated as 2-DG-6-PO$_4$. Under conditions of determined time and concentration of 2-DG, total uptake is representative of transport as long as phosphorylation is not rate limiting.

2. 3-0-MG: Non-metabolizable, rapid equilibrium-reached in short time. Enters via sugar carrier.

3. L-glucose: Measure of diffusional or non-saturable uptake.

4. D-glucose: Competitive inhibitor for sugar uptake. Shares same carrier as 2-DG and 3-0-MG.

5. Phloretin: Specific inhibitor of sugar transport, inhibiting efflux better thar influx. Used to allow cells to retain accumulated 3-0-MG during wash procedure.

6. CB: Specific inhibitor of carrier mediated sugar transport. Binds at cytoplasmic surface of glucose carrier. Used at saturating concentrations to obliterate carrier-mediated transport.

Appendix II. Relative change in the number of transporters
"translocated" by various treatments in MCH55
and WG750 Cells

Treatment	n[a]	Mean difference (stimulated-basal) (nmoles 2-DG/mg protein/5 min[b])	
		MCH55	WG750
+ 5% serum (2 h)	2	7.4	15.7
+ 100 mU/ml Insulin (2 h)	2	11.5	17.9
- Glucose (8 h)	3	10.0	33.3

a. Number of experiments

b. Values obtained by calculating the difference in ^{3}H-2-DG uptake

obtained with cells incubated with the treatment versus the

^{3}H-2-DG uptake obtained in the basal (unstimulated) state.