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Hexose Transport Regulation in
Cultured Human Diploid Fibroblasts:
Hormonal and Non-hormonal

Fotini Vlachopoulou

A Thesis

in

The Department

of

Biology

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ABSTRACT

Hexose Transport Regulation in
Cultured Human Diploid Fibroblasts:
Hormonal and Non-hormonal

Fotini Vlachopoulou

Hormonal (i.e. insulin) and non-hormonal (i.e. glucose-deprivation) aspects of hexose transport regulation were studied. Insulin (100 nU/ml) was shown to stimulate 2-DG transport in glucose-fed fibroblasts (38% increase over control), but was ineffective in stimulating transport in glucose-starved cultures. Further, the oxidant H_2O_2 (2 mM) had no effect on basal transport activity, while it markedly inhibited elevated transport due to glucose-starvation, or insulin- and serum-stimulation ("activated" transport). The reducing agent dithiothreitol (8-20 mM) was able to protect from and reverse H_2O_2 -induced inhibition of 2-DG transport in glucose-starved human fibroblasts. The permeant sulfhydryl reagent N-ethylmaleimide (0.5 mM) inhibited both insulin-stimulated and glucose-deprivation enhanced transport activity. The relatively impermeant thiol reagents, dithio-bis-nitrobenzoic acid (3 mM) and N-Iodoacetyl-N'-(5-sulfo-1-naphthyl) ethylenediamine (10 mM), had no effect on either insulin-activated or glucose-starvation elevated hexose transport. None of the

sulfhydryl reagents tested had a significant effect on 2-DG transport in glucose-fed cells. In addition, NEM pre-treatment prevented insulin from stimulating hexose transport in glucose-fed fibroblasts. Both dithio-bis-nitrobenzoic acid and N-Iodoacetyl-N'-(5-sulfo-1'-naphthyl) ethylenediamine failed to block the coupling process.

Glucose-mediated reversal of derepressed transport in the presence of cycloheximide (50 µg/ml) induced a marked decline (43%) in transport activity. However, following 1 h of incubation in glucose starvation medium with or without cycloheximide a significant increase in 2-DG transport was observed.

The data indicate that the "activated" state of the hexose transport system is sensitive to intramembrane and/or intracellular thiol modification. Insulin action on hexose transport does not appear to involve exofacial thiol groups. However, blockage of intramembrane and/or intracellular sulfhydryl groups may block insulin-induced translocation of glucose carriers to the plasma membrane. Finally, glucose-mediated down-regulation of derepressed transport appears to involve glucose-induced internalization of transporters which are then subjected to degradation.

To my Parents °
Eleni and Philippos

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INTRODUCTION

A primary action of insulin, upon binding to its receptors on the plasma membrane of responsive cells, is to stimulate glucose transport. This effect of insulin has been extensively studied in adipocytes and muscle cells which are the hormone's major target cells (Klip, 1982, review). Certain agents, such as oxidants, have been shown to mimic insulin action on hexose transport (Czech et al., 1974a, 1974b; Czech, 1976a, 1980; Ciaraldi and Olefsky, 1982; Forsayeth and Gould, 1983; Kozka and Gould, 1984). In contrast, the sulfhydryl reagents N-ethylmaleimide (NEM) and dithio(bis)nitrobenzoic acid (DTNB) prevented both insulin and oxidants from stimulating hexose transport in rat adipocytes (Czech, 1976a, 1976b, 1976c). On the basis of these observations, Czech (1976c, 1980) developed the "Thiol-Redox" model for insulin action. According to this model the stimulatory effect of insulin and the oxidants is mediated through the oxidation of certain essential membrane sulfhydryl groups to the disulfide form. However, more recent evidence provided new insight into the process by which insulin stimulates glucose transport. Insulin acts by inducing translocation of transporters from an intracellular pool to the plasma membrane, thus increasing the number of functional transporters, rather than altering their

intrinsic activity (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Gorga and Lienhard, 1984; Oka and Czech, 1984). Furthermore, upon dissociation of insulin from its receptor, glucose transporters recycle, by a process similar to endocytosis, into the intracellular pool (Cushman et al., 1984; Kono, 1984). None the less sulfhydryl groups appear to be involved in the coupling between insulin binding and activation of hexose transport. Supportive evidence comes from the recent observation that exofacial (May, 1985) and vicinal (Frost and Lane, 1985) sulfhydryl groups are important for the regulation of hexose transport by insulin.

It is well established that animal cells deprived of glucose develop greatly enhanced hexose uptake rates. Kinetic studies indicated that this response to glucose starvation is due to an increase in the number of functioning carriers in the plasma membrane (Kletzien and Perdue, 1975; Christopher et al., 1976a; Franchi et al., 1978; Germinario et al., 1982). Furthermore, glucose refeeding of derepressed cells results in return to the basal level of hexose transport activity. Both the development of glucose-starvation enhancement of hexose transport and the reversal by glucose addition require protein synthesis since cycloheximide inhibited both processes (Christopher et al., 1976b; Gay and Wilf, 1980; Germinario et al., 1982). Interestingly, cycloheximide was

shown to differentially affect the hexose transport process in glucose-fed cells, such that, low concentrations of the inhibitor decreased hexose transport activity, while high concentrations of cycloheximide enhanced transport activity. When glucose-starved cells were treated with low concentrations of cycloheximide there was no loss of transport activity. In light of these observations Christopher (1977) and Yamada et al. (1983) proposed that hexose transport activity depends on the number of functional carriers in the plasma membrane, which reflects a balance between carrier synthesis and inactivation. Furthermore, it was recently suggested that the glucose-mediated down-regulation of the hexose transport system may involve internalization of the carrier-glucose complex, followed by carrier degradation (Kalckar, 1983; Kalckar and Ullrey, 1984a).

In this study, cultured human skin fibroblasts were used in order to investigate the mechanism of hexose transport regulation in a cell system of human origin. The advantage of this system is that the cells are easily established from skin biopsies, can be grown in large quantities and stored effectively in liquid nitrogen without loss in proliferative capacity. Moreover, in certain studies, such as insulin action, human skin fibroblasts provide a system many generations removed from the hormonal

influences of the donor.

In this thesis, two aspects of hexose transport regulation were studied, hormonal (i.e. insulin) and non-hormonal (i.e. glucose-deprivation), in an attempt to identify any common steps in the two regulatory mechanisms. Major differences exist between the two types of transport regulation. However, in both cases elevated transport activity reflects an increase in the number of glucose transporters in the plasma membrane. Additionally, return of hexose transport to the basal level appears to be mediated by internalization of the glucose carriers. Throughout this study the term "activated" transport is often used in order to collectively describe elevated transport due to glucose-deprivation, or insulin- and serum-stimulation.

The initial objective, in this study, was to compare the action of insulin on hexose transport activity to that of hydrogen peroxide, an insulinomimetic agent, under the two states of the hexose transport system: "repressed" (presence of glucose) and "derepressed" (absence of glucose). Additionally, since H_2O_2 is a non-specific oxidant, the effect of dithiothreitol (DTT), a specific sulfhydryl reducing agent (Cleland, 1964), on hexose transport was also examined. As an additional objective, the effects of permeant (i.e. NEM) (Abbott and

Schachter, 1976; May, 1985). and impermeant (i.e. DTNB and 1,5-I-AEDANS) (Czech, 1976a; Hudson and Weber, 1973; Pulido-Cejudo, 1985) sulfhydryl reagents, on basal and insulin-stimulated hexose transport were compared. Further, the action of the sulfhydryl reagents on glucose-deprivation enhanced hexose transport was also examined. Finally, I studied the role of protein synthesis and degradation in non-hormonal hexose transport regulation.

LITERATURE REVIEW

Hexose uptake and its regulation in mammalian and avian cells has received considerable attention. It is well established that glucose is transported into cells by carrier-mediated facilitated diffusion independent of energy-generating processes (Crofford, 1967; Illiano and Cuatrecasas, 1971).

Hexose transport across the plasma membrane is sensitive to various stimuli or conditions in cultured animal cells. Serum, growth factors, and viral transformation are known to increase transport activity (Kletzien and Perdue, 1974b; Barnes and Collowick, 1976; Kletzien and Perdue, 1974a). Additionally, insulin, sugar deprivation, and oxidative energy metabolism have also been shown to affect the hexose transport process.

Insulin Action on Hexose Transport

Insulin action has been studied in a variety of cells such as erythrocytes (Gambhir et al., 1977), lymphocytes (Gavin et al., 1973; Olefsky and Reaven, 1974), monocytes (Olefsky and Reaven, 1976), adipocytes (Czech, 1976b; Olefsky, 1978; Czech, 1980; Ludvigsen and Jarrett, 1980; Suzuki and Kono, 1980; Cushman and Wardzala, 1980; Ciaraldi and Olefsky, 1982), rat soleus muscle (Yu and Gould, 1979;

Kozka and Gould, 1984), muscle cells (Klip et al., 1984a, 1984b), chicken embryo fibroblasts (Smith-Johannsen et al., 1977), and human skin fibroblasts (Germinario and Oliveira, 1979; Berhanu and Olefsky, 1981; Germinario et al., 1984). One of the hormone's major actions is to promote overall glucose metabolism which is largely, but not exclusively, due to increased glucose transport into target cells (Olefsky, 1975, 1976; Lawrence et al., 1977; Czech, 1980).

Time course studies on the stimulatory effect on hexose transport indicated that this effect is rapid. Maximal stimulation in human fibroblasts occurred within 30-60 min of incubation (Germinario and Oliveira, 1979; Berhanu and Olefsky, 1981), while in chicken embryo fibroblasts (7.5 min) (Smith-Johannsen et al., 1977) and adipocytes (60-100 sec) (Haring et al., 1978; Whitesell and Gliemann, 1979) this effect was more acute. Furthermore, examination of the effect of protein synthesis inhibitors, such as cycloheximide and puromycin, on insulin action indicated that protein synthesis is not a requirement for insulin-induced enhancement of hexose transport (Germinario and Oliveira, 1979; Yu and Gould, 1979). On the contrary, stimulation of hexose transport by insulin depends upon metabolic energy. 2,4-dinitrophenol (DNP) and cyanide (KCN), typical inhibitors of oxidative phosphorylation, blocked the action of insulin on glucose transport in intact

cells (Kono et al., 1977b; Chandramouli et al., 1977; Siegel and Olefsky, 1980) with a concomitant decrease in the intracellular ATP level to less than 10% of normal (Kono et al., 1977b). Further, removal of the hormone resulted in a rapid decrease of glucose transport to the basal level (Crofford, 1975; Vega and Kono, 1979). This reversal of the insulin effect was blocked by agents which lower ATP concentration (Kono et al., 1977a; Vega et al., 1980).

Kinetic studies on the transport of various hexoses indicated that the stimulatory effect of insulin is due to an increase of the apparent V_{max} with no effect on the apparent K_m (Olefsky, 1978; Whitesell and Gliemann, 1979; Germinario and Oliveira, 1979).

The interesting observation that oxidants mimicked insulin action on hexose transport by adipocytes, led Czech to propose the "Thiol-Redox" model for the regulation of glucose transport by insulin (Czech, 1976c, 1980).

According to this model the hexose transport system or a regulatory component of the system is considered to exist in two forms; one characterized by a low V_{max} transport activity and the other by a high V_{max} activity. The chemical difference between these two forms may be the redox state of key membrane sulfhydryls. Insulin as well as oxidants are viewed as converting the reduced inactive form of the transport system (low V_{max}) to the active oxidized

state (high V_{max}) by directly oxidizing these key membrane sulfhydryls to the disulfide form. Evidence in support of this hypothesis derives from the observation that the oxidants H_2O_2 , vitamin K_5 , methylene blue and diamide mimicked the stimulatory effect of insulin on transport in adipocytes (Czech et al., 1974a, 1974b; Czech, 1976a), while reductants such as dithiothreitol inhibited hexose transport (Czech et al., 1974a). Furthermore, the sulfhydryl reagents N-ethylmaleimide (NEM) and dithio(bis)nitrobenzoic acid (DTNB) prevented the ability of insulin or oxidants in stimulating hexose transport, while addition of insulin or oxidants prior to incubation with either NEM or DTNB completely protected against the inhibitory effect of the sulfhydryl reagents (Czech, 1976a, 1976b, 1976c). Basal transport activity was not significantly affected by these agents. Moreover, it was found that NEM prevented the elevated transport activity of insulin treated fat cells from returning to control levels upon trypsinization of the cells which destroys insulin receptors and terminates the hormone's action (Czech, 1976a). The observation that insulin stimulates intracellular H_2O_2 accumulation in fat cells supported the "Thiol-Redox" model (May and de Haen, 1979a, 1979b). Furthermore, Mukherjee and Lynn (1977) reported an enzyme (NADPH) activity associated with the plasma membrane

which is stimulated by exposure of adipocytes either to insulin or sulfhydryl reagents. A product of this enzymatic reaction is H_2O_2 formation.

Recently, three groups working independently each using a different approach to study the mechanism of insulin-stimulated hexose transport reached the same conclusion: Insulin increases glucose transport activity by inducing translocation of glucose transporters to the plasma membrane from a large intracellular pool, possibly the Golgi apparatus.

Gorga and Lienhard (1987) developed the "one transporter per vesicle" method (1T/V) in order to assess whether the insulin-induced glucose transport enhancement is due to a change in the intrinsic activity of a fixed number of carriers in the plasma membrane or to an increase in the number of functional transporters in the plasma membrane. Consequently, they reconstituted transporter molecules into phospholipid vesicles in such a way so that there is one or no transporter per vesicle. The rationale for the 1T/V method is that if insulin increases the activity of a fixed number of carriers without altering the total number of carriers, then the half-time for equilibration of D-glucose with vesicles prepared from insulin treated membranes will be less than that for vesicles from basal membranes. On the contrary, if insulin increases the number of functional

transporters without altering their intrinsic activity, then the equilibrium level of D-glucose uptake will be higher for the vesicles derived from insulin treated membranes, while the half-time for equilibration will remain constant. The experimental evidence presented favoured the second alternative. Vesicles prepared from insulin treated plasma membranes exhibited a higher equilibrium level of D-glucose uptake relative to that for vesicles from basal membranes. Furthermore, the equilibrium level for vesicles from insulin-treated microsomes was lower when compared to that for vesicles from basal microsomes. These observations supported the hypothesis that insulin causes translocation of transporters from an intracellular location to the plasma membrane.

Cushman and Wardzala (Cushman and Wardzala, 1980; Cushman et al., 1984) observed that plasma membranes prepared from adipocytes exposed to insulin exhibited a 4- to 5-fold increase in the number of D-glucose-inhibitable cytochalasin B-binding sites relative to plasma membranes prepared from basal cells. Furthermore, this change of the D-glucose-inhibitable cytochalasin B-binding sites in the plasma membranes in response to insulin was accompanied by a quantitatively identical decrease in the number of cytochalasin B-binding sites in the low-density microsomal membrane fraction associated with the Golgi apparatus.

These insulin-induced changes in the number of cytochalasin B-binding sites were rapid, reversible and insulin concentration dependent (Karnieli et al., 1981). On the basis of the results obtained for the K_d of cytochalasin B binding to the glucose transporters it was further proposed that, upon dissociation of insulin from its receptor, glucose transporters recycle back, by a process similar to endocytosis, into the intracellular pool (Cushman et al., 1984).

Kono and his group (Suzuki and Kono, 1980; Kono et al., 1982; Kono, 1984; Smith et al., 1984) used a different approach to the problem of insulin action on hexose transport regulation by measuring glucose transport activity in a cell-free system following reconstitution into liposomes. They reported that when fat cells were exposed to insulin before homogenization, the glucose transport activity in the plasma membrane-rich fraction increased whereas that in the Golgi-rich fraction decreased. The observed increase in the plasma membrane-rich fraction from insulin stimulated cells was 5- to 8-fold relative to that from basal cells (Kono et al., 1982). Moreover, the effect of insulin on glucose transport activity associated with the plasma membrane-rich fraction (activation) and the Golgi-rich fraction (deactivation) was blocked by inhibitors of metabolic energy such as 2,4-dinitrophenol and cyanide.

Furthermore, the reversal of insulin effect on both plasma membrane-rich and Golgi-rich fractions, following treatment of cells with collagenase which terminates insulin action, was also inhibited by 2,4-dinitrophenol and cyanide.

Neither the development nor the reversal of insulin effects on the glucose transport activities associated with the two subcellular fractions was affected by cycloheximide or puromycin (Kono et al., 1981). In light of these observations Kono (1984) proposed that the glucose transport mechanism is recycled between the plasma membrane and an intracellular storage pool by endocytotic and exocytotic reactions in response to insulin.

Oka and Czech (1984) presented more recent evidence in favour of the translocation hypothesis. By the use of a photoaffinity labelling technique of the hexose transporters in intact adipocytes by ^3H -cytochalasin B, they were able to demonstrate that insulin action results in exposure to the extracellular medium of previously sequestered hexose transporters.

Sugar Deprivation-induced Adaptive Regulation of Hexose Transport Activity

Cultured animal cells deprived of glucose were shown to manifest a striking adaptive response by developing greatly enhanced hexose uptake. This glucose-starvation induced

enhancement of hexose transport has been observed in chicken embryo fibroblasts (Martineau et al., 1972; Kletzien and Perdue, 1975; Christopher et al., 1976a; Amos et al., 1977), hamster NIL fibroblasts (Kalckar and Ullrey, 1973; Christopher et al., 1976b), Chinese hamster lung fibroblasts (Franchi et al., 1978), rat mammary adenocarcinoma cells (RMAC) (Gay and Hilf, 1980) and human skin fibroblasts (Salter and Cook, 1976; Germinario et al., 1982). This adaptive response was also elicited by maintaining the cell cultures in "non-glycolytic" sugars, such as D-fructose, D-xylose, 3-O-methyl-D-glucose, D-galactose, or L-glucose (Musliner et al., 1977; Amos et al., 1977; Gay and Hilf, 1980). However, in human skin fibroblasts 3-O-methyl-D-glucose substitution for D-glucose did not derepress hexose transport (Germinario et al., 1982).

Kinetic studies of the transport of various hexoses by intact cells indicated that the enhanced transport rate following glucose deprivation is due to an increase in the apparent V_{max} with the K_m remaining constant (Kletzien and Perdue, 1975; Christopher et al., 1976a; Franchi et al., 1978; Germinario et al., 1982). This observation was interpreted to reflect an increase in the number of functioning carriers in the plasma membrane. This view gains support from more recent data deriving from the use of plasma membranes from glucose-starved and glucose-fed

chicken embryo fibroblasts. Photoaffinity labelling of the D-glucose transport system with ^3H -cytochalasin B indicated a 12-fold increase of the total D-glucose-sensitive labelling in the starved cell plasma membranes (Pessin et al., 1982, 1984).

Chicken embryo fibroblasts appeared to possess two transport systems for D-glucose: A constitutive low-affinity system which has a K_m of 1 mM and a high-affinity with a K_m of 40-50 μM (Christopher et al., 1976a; Amos et al., 1977). Glucose starvation derepressed the high-affinity carrier and increased the V_{max} for the low-affinity carrier as well. The high-affinity system appeared to be inhibited by thiol reagents. Starved cells treated with N-ethylmaleimide lost virtually all of the high-affinity system transport activity (Christopher et al., 1976a). Human skin fibroblasts were also shown to possess a high- and a low-affinity transport system; however, both were expressed under either glucose-fed or glucose-starved conditions. Glucose deprivation induced an increase in the V_{max} of both systems without affecting their K_m (Germinario et al., 1982).

The events involved in the development of glucose-starvation induced enhancement of hexose transport appear to be slightly different among the various cell cultures studied. The presence of inhibitors of protein

(e.g. cycloheximide and puromycin) and RNA synthesis (e.g. actinomycin D and cordycepin) blocked the adaptive response to glucose starvation in chicken embryo fibroblasts (Kletzien and Perdue, 1975; Musliner et al., 1977; Amos et al., 1977). Furthermore, both protein and RNA synthesis appeared to be required in RMAC cells (Gay and Hilf, 1980), while in Chinese hamster lung fibroblasts protein synthesis was not a requirement (Franchi et al., 1978). In hamster NIL cells and human fibroblasts the enhanced hexose transport following sugar deprivation was dependent only on protein synthesis (Christopher et al., 1976b, 1977; Germinario et al., 1982).

Glucose refeeding of derepressed cells resulted in return to the basal level of hexose transport activity (Martineau et al., 1972; Kletzien and Perdue, 1975; Salter and Cook, 1976; Musliner et al., 1977; Gay and Hilf, 1980; Germinario et al., 1982). Furthermore, the presence of inhibitors of protein synthesis, such as cycloheximide, during glucose refeeding of derepressed cells, blocked the glucose-induced reversal of hexose transport.

In light of these observations Christopher (Christopher et al., 1976b, 1976c; Christopher, 1977) proposed that hexose transport regulation is dependent upon two opposing forces, synthesis of carriers vs inactivation of carriers and that protein synthesis is required, at least in part,

for both processes. During growth in glucose-containing medium a balance between synthesis and turnover is achieved. Upon glucose removal from the medium the turnover system either ceases to be active or it is itself inactivated.

The nature of the turnover mechanism has not been clarified yet. However, the observation that high concentrations of cycloheximide stimulate hexose transport by glucose-fed cells implied that the turnover mechanism may be a degradative process, perhaps a proteolytic one, since it is documented that inhibitors of protein synthesis are also good inhibitors of endogenous degradation (Christopher et al., 1976b, 1976c). Further evidence for the involvement of lysosomal proteases in the mechanism of carrier inactivation derives from the observation that inhibition of thiol cathepsins blocked the loss of transport activity in glucose-fed NIL hamster fibroblasts (Christopher and Morgan, 1981).

In observing the effects of cycloheximide on glucose-fed NIL hamster fibroblasts Christopher et al. noted that low concentrations of cycloheximide (0.1-10 µg/ml) greatly decreased transport activity, while high concentrations of the inhibitor (100 µg/ml) increased transport activity by these cells (Christopher et al., 1976b, 1976c). However, when starved cells were treated with low concentrations of cycloheximide there was no loss

of transport activity (Christopher et al., 1976b, 1976c). These initial observations were extended and confirmed by examining the effects of cycloheximide and emetine on hexose transport in intact chicken embryo fibroblasts or plasma membrane vesicles prepared from these cells (Yamada et al., 1983; Tillotson et al., 1984). These investigators proposed that hexose transport activity depends on the number of functional carriers in the plasma membrane, which reflects the balance between carrier synthesis and inactivation. Both processes require protein synthesis, but are differentially sensitive to cycloheximide or emetine. Low concentrations of the inhibitors block synthesis, while high concentrations block both synthesis and inactivation. Glucose starvation-induced enhanced hexose transport activity appears to be a result of decreased carrier inactivation.

However, the nature of the carrier inactivation mechanism still remains obscure. It has been hypothesized that a particular product of glucose metabolism may play a role in promoting carrier inactivation (Martineau et al., 1972; Musliner et al., 1977; Amos et al., 1977; Christopher, 1977; Gay and Hilf, 1980; Germinario et al., 1982).

Pouyssegur et al. (1980) isolated a mutant strain of Chinese hamster fibroblasts defective in phosphoglucose isomerase, which was as responsive to glucose as the

parental wild-type cell. However, neither glucosamine nor mannose, both being effective in the parental strain, were capable of mediating down regulation of the hexose transport system in the mutant strain (Ullrey et al., 1982). The hexose monophosphate shunt was implicated as a possible regulator of hexose transport, since, in the mutant, glucose flows predominantly through this pathway while mannose and glucosamine feed primarily into the glycolysis and the tricarboxylic acid cycle. Further studies on the mutant strain revealed that glucose alone was incapable of repressing transport when the cells were grown in medium devoid of L-glutamine; however, a combination of mannose and glucose reversed hexose transport (Kalckar and Ullrey, 1984a, 1984b). These observations suggested that glucose-mediated repression of transport requires two metabolic pathways: 1) the pyruvate-tricarboxylate cycle, 2) glucose-6-phosphate metabolism, either the hexose monophosphate shunt or through glucose-1-phosphate, UDP-glucose and UDP-galactose (Kalckar and Ullrey, 1984a).

The potential role of the hexose monophosphate shunt as a regulator of transport was studied with respect to the intracellular phosphoribosyl diphosphate (PPRibP) concentrations in chick embryo fibroblasts (Gay and Amos, 1983). Since PPRibP is critical for nucleic acid synthesis it was speculated it may act as a feedback signal in the

repressive regulation of hexose transport. Under certain conditions the rate of glucose transport and the PPRibP concentrations were inversely related. Addition of guanine or hypoxanthine decreased transport in glucose-fed cultures but did not affect transport derepression following substitution of D-xylose for D-glucose. However, guanosine and analogues of guanosine sharply decreased transport in glucose-fed cells and blocked the enhancement of transport due to replacement of D-glucose by D-fructose or D-xylose. These data indicated a potential role of guanine nucleotides in repression of glucose transport (Gay and Amos, 1983).

Since the flow of glucose through the hexose monophosphate shunt depends upon the availability of NADP^+ , the effect of altering the levels of pyridine nucleotides on transport regulation was examined by several investigators. Glucose-fed chick embryo fibroblasts or NIL hamster fibroblasts depleted of NAD(H) , following prolonged growth in medium devoid of nicotinamide, exhibited derepressed hexose transport. Addition of NAD^+ , but not nicotinamide, repressed transport (Amos et al., 1984; Mandel and Amos, 1984). D-glucose alone failed to reverse transport in NAD(H) -depleted cells grown in the absence of glucose. However, simultaneous addition of NAD^+ reversed transport (Amos et al., 1984). It was suggested that an ADP-ribosylation of the hexose carrier protein may

be a normal step in the turnover of carriers. Furthermore, the repressive effect of purines on transport in cells grown on glucose under normal conditions (Gay and Amos, 1983) was abolished in cells depleted of NAD(H) (Mandel and Amos, 1984). These data indicated that the restriction of the hexose monophosphate shunt due to NADP(H) depletion may be involved in the observed refractoriness to purines.

Oxidative Energy Metabolism as a Regulator of Hexose Transport

Hexose transport in animal cells is considered to be partially controlled by the metabolic state of the cell. Whitfield and Morgan (1973) and Reeves (1975) demonstrated that certain conditions or agents, such as anoxia or uncouplers of oxidative phosphorylation which greatly deplete intracellular ATP concentrations, enhanced 3-O-methyl-D-glucose transport in avian erythrocytes and rat thymocytes respectively. Likewise, 2,4-dinitrophenol (DNP) and oligomycin were shown to derepress 3-O-methyl-D-glucose and D-galactose uptake in NIL hamster fibroblasts grown in D-glucose or D-glucosamine. However, DNP did not affect the enhanced hexose transport of cells maintained in medium containing D-fructose in place of D-glucose (Kalckar et al., 1979). In addition, when malonate, which interferes with the tricarboxylic acid cycle, was incubated with glucose-fed

cultures, increased hexose transport activity (Ullrey and Kalckar, 1981). Recently, the antibiotic valinomycin, a K^+ -specific ionophore, was shown to stimulate 2-deoxy-D-glucose and 3-O-methyl-D-glucose uptake in Swiss 3T3 fibroblasts. The enhanced transport followed a rapid decrease in intracellular ATP content induced by valinomycin (Yamanishi, 1984).

The mechanism by which intracellular ATP level affects hexose transport activity remains unknown. Kalckar et al. (1979) speculated that the oxidative phosphorylation requirement for glucose-mediated down-regulation of the hexose transport system may be related to the ATP requirement for protein degradation. On the other hand, in light of the recent findings on insulin action in adipocytes, it was also suggested that the ATP requirement may indicate endocytosis or translocation of hexose transporters as part of the regulation system (Kalckar and Ullrey, 1984a).

MATERIALS AND METHODS

Cell Culture

Human skin fibroblast cultures were established from deltoid skin or foreskin biopsies. The cells were grown in antibiotic-free Eagle's minimal essential medium (MEM) (Eagle, 1959) (Flow Laboratories, Inc.) supplemented with 1mM pyruvate and 10% (v/v) fetal calf serum (Microbiological Associates, Bethesda, Maryland). The cultures were incubated at 37°C in a CO₂ 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 (150 cm² or 175 cm² plastic flasks; Costar 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 1000 rpm 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 150 cm² or 175 cm² plastic tissue culture flasks (1:3 split ratio). The total number of cells obtained 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: $P.D. = \{\log(\text{final number of cells}) - \log(\text{initial}/2)\} / \log 2$. In all experiments cells had not completed more than 50% of their in vitro lifespan. The latter was calculated as the ratio of the number of population doublings accrued by the cells at the time of the experiment over the total number of population doublings expected at senescence, times 100.

General Experimental Procedure

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 one week). The cell monolayers were 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 hours, unless otherwise stated. Depending on the design of the experiment the cells were either exposed to glucose-free conditions (glucose-free 0% MEM) or they continued to be glucose-fed (0% MEM) for a period of time ranging from 6 to 18 hours. Prior to change to glucose-free conditions the cell monolayers were rinsed once with 0% MEM devoid of glucose.

2-deoxy-D-glucose Uptake Procedure

2-deoxy-D-glucose (2-DG) uptake was assessed by measuring intracellular ^3H -labelled 2-DG after a short

incubation period (2 min). Under the conditions employed hexose transport is the rate limiting-step in the accumulation of ^3H -2-DG (Germinario et al., 1978). 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 absorption. At the time of the assay for sugar uptake the cell monolayers were 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 ^3H -labelled 2-deoxy-D-glucose (2-DG) was added to the cells for 2 min at 37°C . The specific activity of 1 mM labelled 2-DG was $4.5 \mu\text{Ci}/\mu\text{mole}$. After 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 ^3H -labelled 2-DG was added. The radioactive medium was rapidly removed and the cell monolayers were washed four times (2 ml each time) with cold PBS (4°C) at pH 7.4. The monolayers were dissolved in 1 N NaOH and aliquots were taken for liquid scintillation counting and protein determination (Lowry et al., 1951).

Measurement of Leucine and Uridine Incorporation

At the time of the assay for leucine or uridine incorporation, the culture medium was removed and the cell monolayers were washed two times (2 ml each time) with PBS at pH 7.4 (37°C). Then 1 ml of PBS containing 5.55 mM glucose and 5 µCi/ml of 5-³H-uridine (25 Ci/mmol) or 4,5-³H-leucine (5 Ci/mmol) was added per 35 mm Petri plate. Cell monolayers treated with cycloheximide or actinomycin D prior to assay for leucine or uridine incorporation, were exposed to isotope solution containing the corresponding concentration of the inhibitors. Following a 30 min or 60 min incubation at 37°C, the cell monolayers were washed four times (2 ml each time) with cold (4°C) PBS at pH 7.4. The cells were then exposed to cold 10% (w/v) trichloroacetic acid (TCA) for one hour at 4°C, the supernatant was removed and the monolayers washed two times with cold 10% TCA. The cell monolayers were dissolved in 1 N NaOH and aliquots were taken for liquid scintillation counting and protein determination (Lowry et al., 1951). In all experiments, triplicate determinations were employed.

Radioactive Materials

³H-labelled 2-DG (5 Ci/mmol), ³H-uridine (25

Ci/mole) and ^3H -leucine (58 Ci/mole) were obtained from New England Nuclear Corporation, Boston, Massachusetts.

Chemicals

Dithiothreitol (DTT), cycloheximide, actinomycin D (grade I), insulin (bovine pancreas crystalline), and 2-deoxy-D-glucose were obtained from Sigma Chemical Co., (St. Louis, Mo.). D-glucose was purchased from Fisher Scientific Ltd., (Montreal, Que.) and L-glucose from Calbiochem-Behring, (San Diego, Ca.). Hydrogen peroxide was obtained from BDH, (Montreal, Que.) and N-ethylmaleimide (NEM) from Baker, (Montreal, Que.).

N-Iodoacetyl-N'-(5-sulfo-1-naphthyl) ethylenediamine (1,5-I-AEDANS) was from Aldrich Chemical Co., (Milwaukee, Wi.) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) from Boehringer Mannheim, (Dorval, Que.). Bovine serum albumin was purchased from ICN Pharmaceuticals Inc., (Cleveland, O.).

Scintillation Counting

All samples were counted for radioactivity in 5 ml of a counting solution (Formula 963, New England Nuclear Corporation, Boston, Massachusetts) in 7 ml vials. Radioisotope counting was done in a liquid scintillation

counter (LKB RackBeta) with a counting efficiency of 29-30%
for ^3H .

Statistics

The data were statistically analyzed on an Apple II+
computer. The test employed was the Student's t-test for
paired or grouped data, 5% being the significance level,
unless otherwise stated.

RESULTS

Comparison of the Effects of Sulfhydryl Modifying Agents on Basal and "Activated" Hexose Transport.

The data in Table I show clearly that insulin (100 mU/ml) stimulated 2-DG transport in glucose-fed fibroblasts ($P < 0.001$, $n=12$). Transport activity was assessed after 2 h exposure to insulin, since it has been reported that maximal stimulation of hexose transport in human fibroblasts is observed after 30 min incubation with the hormone and remains fairly constant up to 4 h (Germinario and Oliveira, 1979). The average increase over control was 38%. On the other hand, insulin had no effect on cells starved of glucose (Table I). In addition to the two experiments described here this observation has been made consistently by others (Germinario et al., 1984).

It has been shown that oxidants mimic the stimulatory action of insulin on hexose transport in rat adipocytes (Czech et al., 1974a; Czech, 1976a). The data in Figure 1 show the effect of H_2O_2 (4 mM), as a function of time, on 2-DG transport in glucose-fed human skin fibroblasts. Although H_2O_2 appeared to have a slight stimulatory effect on 2-DG transport after 45 min exposure, this increase was not significant ($P > 0.05$, $n=2$). The data in Figure 2 illustrate the effect of 45 min

exposure to varying concentrations of H_2O_2 on 2-DG transport by glucose-fed fibroblasts. Hexose transport activity remained virtually unaltered within the concentration range tested (0-4 mM). When fibroblast cultures deprived of glucose were exposed to 4 mM H_2O_2 for varying time intervals a rapid decrease in 2-DG transport was observed (Figure 3). As early as 15 min after the addition of H_2O_2 transport activity declined by 22% while after 30 min the decrease was 37%. Longer incubation with H_2O_2 did not appear to further inhibit 2-DG transport. The data in Figure 4 demonstrate the action of H_2O_2 on transport activity by glucose-starved cultures as a function of concentration. The inhibitory effect of H_2O_2 on 2-DG transport was already evident at a concentration of 0.5 mM (45 min incubation). Maximum inhibition was observed at a concentration of 1-2 mM H_2O_2 (51-55% inhibition) beyond which transport activity levelled off. In all subsequent experiments 30 min and 2 mM were chosen as the exposure time and concentration, respectively, for H_2O_2 . Table II shows the cumulative data of several experiments (n=4-19) on H_2O_2 action on 2-DG transport by glucose-fed and glucose-starved human skin fibroblasts. It is evident that the oxidant markedly inhibited transport activity in cultures deprived of glucose

at both concentrations employed (2 mM and 4 mM). After 30 min of H_2O_2 addition 2-DG transport decreased by 34-36% (2 mM H_2O_2 , $P < 0.001$, $n=19$; 4 mM H_2O_2 , $P < 0.005$, $n=4$). On the other hand, the slight stimulatory effect of 4 mM H_2O_2 on transport activity by glucose-fed cells was not significant ($P > 0.05$, $n=4$).

Since basal transport activity remained virtually unaltered following exposure to H_2O_2 , while the activated hexose transport mechanism, due to glucose deprivation, appeared to be sensitive to the oxidizing agent, it was of interest to examine whether glucose-fed cells stimulated by insulin would respond to H_2O_2 addition by decreasing transport activity as well. After 42 h incubation in serum-free medium containing 22.2 mM D-glucose, cell monolayers were exposed to 2 mM H_2O_2 for 30 min or 100 μ U/ml insulin for 2 h. Some of the insulin-treated cells were additionally incubated with 2 mM H_2O_2 for 30 min. The data in Figure 5 indicate that H_2O_2 did, in fact, cause a decrease in the insulin-stimulated transport activity, even less than that of the basal level. The increase of 2-DG transport due to insulin treatment was 37% over control ($P < 0.005$, $n=5$), while the H_2O_2 -induced inhibition of insulin-activated 2-DG transport was 45% ($P < 0.05$, $n=5$). Insulin-activated 2-DG transport in the absence of H_2O_2 following a 30

min withdrawal of the hormone declined by 5% (i.e. 8.10 ± 1.16 nmoles 2-DG/mg protein/2 min with insulin vs 7.63 ± 0.55 nmoles 2-DG/mg protein/2 min without insulin).

The similarity of the sugar transport responses of fibroblasts in the "activated" state (either due to sugar deprivation or insulin stimulation) to H_2O_2 addition led to the examination of the effect of the oxidizing agent on serum-stimulated hexose transport as well. Cell monolayers were incubated in serum-free medium (0% MEM) for 48 h. Then the cells had a change of medium such that some were exposed to the same conditions (0% MEM) or to 0% MEM containing 2 mM H_2O_2 (30 min), while others received medium supplemented with 10% (v/v) serum for 2 h. Some of the serum-treated cells were additionally exposed to 2 mM H_2O_2 for 30 min. As shown in Figure 6 serum stimulated transport activity by 50% over control. Interestingly, H_2O_2 addition did not significantly affect 2-DG transport by serum-starved cells, while it inhibited transport activity in serum-stimulated cells by 46%. Serum-stimulated 2-DG transport in the absence of H_2O_2 following a 30 min removal of serum declined by 12% (i.e. 10.91 ± 0.26 nmoles 2-DG/mg protein/2 min with serum vs 9.53 ± 0.77 nmoles 2-DG/mg protein/2 min without serum). Since serum-starved glucose-fed cells at some point were actively growing in serum, it was questioned how long after

serum starvation would this effect of H_2O_2 on sugar transport appear. Glucose-fed fibroblasts, exposed for 24 h to serum-free conditions expressed H_2O_2 sensitivity of 2-DG transport (Table III). After 30 min incubation with 2 mM H_2O_2 transport activity was inhibited by 59% ($P < 0.025$, $n=4$). On the other hand, when the cells were deprived of serum for 42 h transport activity was no longer sensitive to the oxidizing agent ($P > 0.05$, $n=15$).

Next, the effect of dithiothreitol (DTT), a specific sulfhydryl reducing agent (Cleland, 1964), on hexose transport was examined. The data in Figure 7 indicate that the reducing agent did not affect 2-DG transport, in either glucose-fed or glucose-starved fibroblasts, in the concentration range tested (0-20 mM). At 4 mM of DTT transport activity was slightly inhibited in the former, while slightly elevated in the latter, but the differences were not significant ($P > 0.05$, $n=3,2$). Beyond 8 mM of the reductant 2-DG transport levelled off in both glucose-fed and glucose-starved cells. Since DTT is a specific sulfhydryl reducing agent it was questioned whether it was able to protect against H_2O_2 -induced inhibition of transport in glucose-starved fibroblasts, which would suggest involvement of sulfhydryl oxidation in the observed inhibition. Cell monolayers were incubated in medium devoid of glucose for 18 h and then exposed to 2 mM H_2O_2 .

for 30 min in the absence or presence of varying concentrations of DTT (0-20 mM) (Figure 8). At 4 mM of the reducing agent hexose transport was restored to 89% of the control value, while beyond 8 mM of DTT the protection attained was complete. However, the possibility arose that the presence of DTT along with H_2O_2 in solution might have resulted in "neutralization" of H_2O_2 and therefore the observed protection by DTT was an artifact. Consequently, it was questioned whether DTT was able to reverse the H_2O_2 effect on 2-DG transport in glucose-starved fibroblasts. Cell monolayers were deprived of glucose for 18 h and then exposed to 2 mM H_2O_2 . Following a 30 min incubation, some cells received 20 mM DTT for 30 min while H_2O_2 was removed from others (30 min). As shown in Figure 9 DTT treatment brought transport to the level of the untreated control; thus indicating involvement of sulfhydryl oxidation in H_2O_2 action.

Since H_2O_2 , a membrane permeant oxidant, did not affect basal transport but inhibited glucose-starvation enhanced transport by oxidizing sulfhydryl groups, it was reasoned that permeant sulfhydryl reagents could have the same effect as H_2O_2 . Therefore, the effects of permeant (NEM) (Abbott and Schachter, 1976; May, 1985) and impermeant (DTNB and 1,5-I-AEDANS) (Czech, 1976a; Hudson and Weber, 1973; Pulido-Cejudo, 1985) sulfhydryl agents on basal

and derepressed transport were compared. Table IV presents the reagents used, their properties and structure. The data in Table V are expressed as the ratio of 2-DG transport in the presence of the sulfhydryl reagent relative to that in its absence. It is evident that none of the sulfhydryl reagents tested affected 2-DG transport in glucose-fed fibroblasts. The slight inhibition observed by NEM (0.5 mM, 5 min) treatment was not significant ($P > 0.05$, $n=3$). However, when glucose-starved cultures were exposed to NEM, transport activity declined by 44% ($P < 0.025$, $n=4$). Furthermore glucose-deprivation enhanced transport was not sensitive to either DTNB (3 mM, 5 min) or 1,5-I-AEDANS (10 mM, 15 min) treatment.

Since only elevated transport due to glucose-deprivation, as compared to basal, appeared to be sensitive to permeant (i.e. NEM) but refractory to impermeant (i.e. DTNB or 1,5-I-AEDANS) sulfhydryl reagents, it was questioned whether insulin-stimulated transport activity would exhibit a similar sensitivity to the sulfhydryl agents tested. The data in Table VI show that following a 5 min treatment of human fibroblasts with 0.5 mM NEM basal 2-DG transport declined slightly but not significantly ($P > 0.05$, $n=3$). On the other hand, when insulin-stimulated cells, which exhibited a 35% increase over control in transport activity, were exposed to NEM

hexose transport declined to that of the basal level. Interestingly, when cell monolayers were pre-incubated with the sulfhydryl reagent and then treated with insulin they failed to increase their transport activity.

The data in Table VII show that a 5 min treatment of human fibroblasts with 3 mM DTNB, did not affect basal 2-DG transport. Interestingly, when cell monolayers were pre-incubated with insulin and then exposed to DTNB transport activity remained at the elevated state, as opposed to the data obtained for NEM (Table VI) and H_2O_2 (Figure 5). Moreover, prior incubation of human fibroblasts with the sulfhydryl reagent did not prevent insulin from stimulating hexose transport. On the contrary, the observed stimulation (52%) was higher than that induced by insulin alone (27%).

Next, the effect of 1,5-I-AEDANS, on basal and insulin-activated transport was studied. Preliminary experiments indicated that 1,5-I-AEDANS at a concentration range of 0.5-10 mM did not affect 2-DG transport in either glucose-fed or glucose-starved fibroblasts (data not shown). Due to the high photosensitivity of the reagent, resulting in degradation, the concentration used for all subsequent experiments was 10⁻⁶ mM, while all preparations and reactions were carried out in dark. As shown in Table VIII a 15 min treatment of human fibroblasts with 10⁻⁶ mM 1,5-I-AEDANS did

not significantly affect basal 2-DG transport ($P > 0.05$, $n=3$). Similarly, insulin-stimulated hexose transport was not sensitive to addition of the sulfhydryl reagent. When cell monolayers were pre-incubated with 1,5-I-AEDANS and then exposed to insulin the hormone's ability to stimulate transport was not prevented. Furthermore the observed stimulation (52%) was higher than that afforded by insulin alone (33%). These data are in agreement with those presented for DTNB (Table VII).

The data presented so far, with respect to the effect of sulfhydryl modification on basal and "activated" transport, are summarized in Table IX. Briefly, basal transport activity appeared to be refractory to all sulfhydryl modifying agents tested, while "activated" transport was sensitive only to those able to penetrate the plasma membrane, i.e. H_2O_2 and NEM. Since the effect of NEM was similar to that of H_2O_2 , although their reaction with sulfhydryl groups differs, it could be deduced that the site of H_2O_2 action is intracellular and/or intramembrane as well. However, it was decided to verify this conclusion by exposing cell monolayers to 1,5-I-AEDANS or DTNB in order to block exofacial sulfhydryl groups, prior to incubation with H_2O_2 . The rationale was that if H_2O_2 inhibited transport activity, despite the blockage of

exofacial sulfhydryls by 1,5-I-AEDANS or DTNB, the site of action of the former must be intracellular and/or intramembrane.

As shown in Table X, when glucose-starved fibroblasts were exposed to 10 mM 1,5-I-AEDANS for 15 min prior to H_2O_2 addition hexose transport declined to the same extent as with H_2O_2 treatment alone (50% average inhibition over control). Similar results were obtained when DTNB was used as the sulfhydryl blocking agent (Table XI).

Role of Protein Synthesis and Degradation in Hexose

Transport Regulation

The adaptive response of human fibroblasts to glucose deprivation is demonstrated in Table XII. Glucose starvation induced an over 2.5-fold increase in 2-DG uptake ($P < 0.001$, $n=15$).

The data in Figure 10 show that following a 4 h incubation in starvation medium containing 50 $\mu\text{g/ml}$ cycloheximide, the rate of 2-DG transport remained constant. Interestingly, when the cells were exposed to the inhibitor in the presence of glucose, transport activity was greatly decreased (43% inhibition) (Figure 11). When cells preincubated with cycloheximide under glucose-free conditions had a change to glucose refeeding medium with or

without 0.03 µg/ml actinomycin D 2-DG transport declined rapidly (Figure 10). Furthermore, incubation in glucose starvation medium or glucose starvation medium containing 50 µg/ml cycloheximide resulted, after 1 h, in a slight decrease of 2-DG uptake after which transport activity levelled off. As shown in Figure 11 cell monolayers pretreated with cycloheximide in the presence of glucose, had a different hexose transport response following change of the incubation conditions. Interestingly, cells exposed to glucose-free medium containing cycloheximide markedly increased 2-DG transport following 1 h of incubation, after which transport activity levelled off. Fibroblasts treated with starvation medium only, expressed, although to a lesser degree, the same enhancement of hexose transport which was followed by a decrease and then a plateau. Additionally, when cell monolayers received glucose refeeding medium with or without 0.03 µg/ml actinomycin D, transport activity appeared slightly elevated in the first hour of incubation after which it declined rapidly.

Table XIII presents the cumulative data of several experiments (n=3-8) on the effect of cycloheximide on the primary and secondary reversal of hexose transport.

Glucose-starved cultures were treated with 50 µg/ml cycloheximide in the presence of glucose for 4 h (1^o reversal) and then exposed to glucose-free medium,

glucose-free medium containing 50 µg/ml cycloheximide, glucose-containing medium, or glucose-containing medium with 50 µg/ml cycloheximide for 1 h (2^o reversal). The data are expressed as percent increase over the primary reversal. As shown in Table XIII following 1 h of incubation in glucose-free medium with or without cycloheximide 2-DG transport increased by 47% (P<0.001) and 22% (P<0.05) over the primary reversal respectively. Furthermore fibroblast cultures exposed to glucose-free medium in the presence of cycloheximide for 1 h exhibited significantly elevated hexose transport activity as compared to those treated with glucose-starvation medium (P<0.05) or glucose-containing medium with or without 50 µg/ml cycloheximide (P<0.005).

In all experiments cycloheximide or actinomycin D inhibition of leucine or uridine incorporation into TCA precipitable material was greater than 90% and 80% respectively.

Table I. Insulin action on 2-DG transport in glucose-fed and glucose-starved human skin fibroblasts.

Treatment	2-DG transport (nmoles/mg protein/2 min)			
	Glucose-fed ^a	n ^c	Glucose-starved ^b	n ^c
Control	5.78±0.48	12	12.5±1.09	2
Insulin	7.97±0.61	12	14.8±2.59	2

Cell monolayers were serum-starved (0% MEM) for 24 h and then exposed to glucose-fed or glucose-starved conditions for 18 h. Then the cells were incubated for 2 h in the corresponding medium with or without 100 mU/ml insulin. Data represent average±SEM of the indicated number of experiments (triplicate plates in each experiment).

a P<0.001

b P>0.05

c Number of experiments.

Table II. H_2O_2 action on 2-DG transport in glucose-fed and glucose-starved human skin fibroblasts.

Treatment	2-DG transport (nmoles/mg protein/2 min)			
	Glucose-fed ^a	n ^c	Glucose-starved ^b	n ^c
Control	5.92±0.56	15	13.99±0.89	19
2 mM H_2O_2	5.70±0.61	15	9.22±0.76	19
Control	5.0±1.01	4	11.4±2.37	4
4 mM H_2O_2	6.2±1.12	4	7.2±1.34	4

Serum-starved (0% MEM, 24 h) fibroblast cultures were exposed to glucose-fed or glucose-starved conditions for 18 h. Then the cells were incubated, where indicated, in the corresponding medium containing 2 mM or 4 mM H_2O_2 for 30 min. Data represent average±SEM of the indicated number of experiments (triplicate plates in each experiment).

a H_2O_2 vs control $P > 0.05$

b 2 mM H_2O_2 vs control $P < 0.001$

4 mM H_2O_2 vs control $P < 0.05$

c Number of experiments.

Table III. The differential effect of H_2O_2 on 2-DG transport as a function of serum-starvation time.

Serum-starvation time	Treatment	2-DG transport (nmoles/mg protein/2 min)	n ^c
24 h ^a	Control	9±0.92	4
	H_2O_2	3.73±0.202	4
42 h ^b	Control	5.92±0.56	15
	H_2O_2	5.7±0.61	15

Cell monolayers were serum-starved (0% MEM) for 24 h or 42 h and then exposed for 30 min to 0% MEM containing 2 mM H_2O_2 . Data represent average±SEM of the indicated number of experiments (triplicate plates in each experiment).

a H_2O_2 vs control $P < 0.025$

b H_2O_2 vs control $P > 0.05$

c Number of experiments.

Table IV. Sulfhydryl modifying agents.

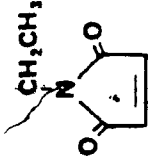
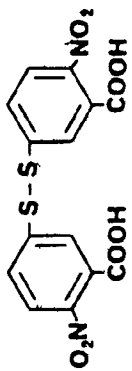
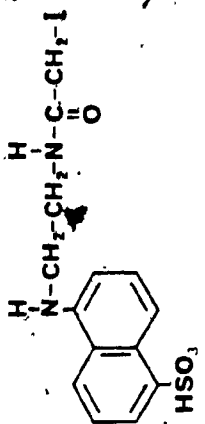
Name	Abbreviation	SH reaction	SH specificity	Membrane permeability	Structure
Hydrogen peroxide	—	oxidation	non-specific	permeant	H_2O_2
Dithiothreitol	DTT	reduction	specific	permeant	$HS-CH_2(CHOH)_2CH_2-SH$
N-ethylmaleimide	NEM	blockage	specific	permeant	
5,5'-dithio-bis(-2-nitrobenzoic acid).	DTNB	blockage	specific	impermeant	
N-Iodoacetyl-N'-1-naphthyl ethylenediamine	1,5-I-AEDANS	blockage	specific	impermeant	

Table V. Effect of permeant and impermeant sulfhydryl reagents on 2-DG transport in glucose-fed and glucose-starved human skin fibroblasts.

Treatment	2-DG transport (sulfhydryl reagent:control ratio)			
	Glucose-fed	n ^a	Glucose-starved	n ^a
NEM	0.87±0.12	3	0.56±0.05 ^b	4
DTNB	1.02±0.07	5	0.96±0.05	5
1,5-I-AEDANS	1.04±0.07	5	0.92±0.04	4

Cell monolayers were exposed to serum-free conditions (0% MEM) for 24 h and then incubated with or without glucose (22.2 mM), where indicated, for 18 h. The cells were, then, treated with or without 0.5 mM NEM (5 min), 3 mM DTNB (5 min) or 10 mM 1,5-I-AEDANS (15 min) in PBS. Data represent average±SEM of the indicated number of experiments (triplicate plates in each experiment).

a Number of experiments.

b P<0.025

Table VI. Effect of NEM on basal and insulin-stimulated hexose transport.

Pre-incubation	Incubation	2-DG transport (nmoles/mg protein 2 min)
None	None	5.31±0.65
None	NEM	4.56±0.48
None	Insulin	7.19±0.53
NEM	Insulin	4.63±0.17
Insulin	NEM	5.56±0.52

Cell monolayers were exposed to serum-free conditions (0* MEM) for 42 h and then pre-incubated, where indicated, with 0.5 mM NEM in PBS or 100 μ M insulin for 5 min or 2 h respectively. The cells were, then, incubated with or without the corresponding concentrations of NEM (5 min) or insulin (2 h), as indicated. NEM treatment was followed by 5 min incubation with equimolar DTT solution to inactivate unreacted NEM. Data represent average \pm SEM of three separate experiments (triplicate plates in each experiment).

Table VII. Effect of DTNB on basal and insulin-stimulated hexose transport.

Pre-incubation	Incubation	2-DG transport (nmoles mg protein 2 min)
None	None	5.31±0.18
None	DTNB	5.81±0.18
None	Insulin	6.73±0.29
DTNB	Insulin	8.06±0.09
Insulin	DTNB	7.18±0.07

Cell monolayers were exposed to serum-free conditions (0% MEM) for 42 h and then pre-incubated, where indicated, with 3 mM DTNB in PBS or 100 µU/ml insulin for 5 min or 2 h respectively. The cells were then washed with 0% MEM and incubated with or without the corresponding concentrations of DTNB (5 min) or insulin (2 h), as indicated. Data represent average±SEM of three determinations.

Table VIII. Effect of 1,5-I-AEDANS on basal and insulin-stimulated hexose transport.

Pre-incubation	Incubation	2-DG transport (nmoles / mg protein / 2 min)
None	None	6.81±0.22
None	1,5-I-AEDANS	7.66±0.68
None	Insulin	9.07±1.06
1,5-I-AEDANS	Insulin	10.34±0.51
Insulin	1,5-I-AEDANS	9.10±0.91

Cell monolayers were exposed to serum-free conditions (0% MEM) for 42 h and then pre-incubated, where indicated, with 10 mM 1,5-I-AEDANS in PBS or 100 mU/ml insulin for 15 min or 2 h respectively. The cells were then washed with 0% MEM and incubated with or without the corresponding concentrations of 1,5-I-AEDANS (15 min) or insulin (2 h), as indicated. Data represent average±SEM of three separate experiments (triplicate plates in each experiment).

Table IX. Effect of permeant and impermeant sulhydryl modifying reagents on basal and "activated" hexose transport.

Abbreviation	Reagent	SH reaction	Membrane permeability	2-DG transport (% of control)		
				Basal	-Glucose	"Activated" Insulin Serum
DTP	Reduction	permeant	87±20 (2)	116±13 (2)	ND ^a	ND
H ₂ O ₂	oxidation	permeant	102±10 (15)	67±5 (19)	60±7 (6)	53±12 (3)
NEM	blockage	permeant	87±12 (3)	56±5 (4)	77±2 (3)	ND
DTNB	blockage	impermeant	102±7 (5)	96±5 (5)	103±3 (2)	ND
1,5-I-AEDANS	blockage	impermeant	104±7 (5)	92±4 (4)	101±2 (3)	ND

^a Not done. Numbers in parentheses represent number of experiments (triplicate plates in each experiment).

Table X. Effect of 1,5-I-AEDANS on H₂O₂-induced inhibition of hexose transport in glucose-starved human skin fibroblasts.

Pre-incubation	Incubation	2-DG transport (nmoles/mg protein/2 min)
None	None	20.09±0.54
None	1,5-I-AEDANS	19.33±2.3
None	H ₂ O ₂	9.96±1.89
1,5-I-AEDANS	H ₂ O ₂	9.20±0.63

Cell cultures were exposed to serum-free conditions (0% MEM) for 24 h and then glucose-starved for 18 h. Following a 15 min pre-incubation with 10 mM 1,5-I-AEDANS in PBS, where indicated, the cells were incubated with or without 10 mM 1,5-I-AEDANS (15 min) or 2 mM H₂O₂ (30 min). Data represent average±SEM of two separate experiments (triplicate plates in each experiment).

Table XI. Effect of DTNB on H₂O₂-induced inhibition of hexose transport in glucose-starved human skin fibroblasts.

Pre-incubation	Incubation	2-DG transport (nmoles/mg protein/2 min)
None	None	6.65±0.37
None	DTNB	6.07±0.13
None	H ₂ O ₂	4.75±0.18
DTNB	H ₂ O ₂	4.91±0.29

Cell monolayers were exposed to serum-free conditions (0% MEM) for 24 h and then glucose-starved for 18 h. Following a 5 min pre-incubation with 3 mM DTNB in PBS, where indicated, the cells were treated with or without 3 mM DTNB (5 min) or 2 mM H₂O₂ (30 min), as indicated. Results are of one representative experiment (average±SEM of three determinations) of three different experiments.

Table XII. The effect of glucose-deprivation on 2-DG transport.

Cellular environment	2-DG transport (nmoles/mg protein/2 min)
No glucose	11.6±0.87 ^a
5.55 mM glucose	5.2±0.047

Confluent cell monolayers were serum-starved (0% MEM) for 24 h and then incubated with or without glucose for 18 h. Results represent average±SEM of 15 separate experiments (triplicate plates in each experiment).

^a P<0.001

Table XIII.- Effect of cycloheximide on hexose transport reversal of glucose-deprived human skin fibroblasts.

Treatment (2 ^o reversal)	2-DG transport (% increase over 1 ^o reversal)	P ^b	n ^a
Starved	122±8	<0.05	8
Starved+CHX ^c	147±7	<0.001	8
Fed	95±12	>0.05	6
Fed+CHX	94±9	>0.05	3

Serum-starved (0% MEM, 24 h) cell cultures were glucose-starved for 18 h and then incubated with 50 µg/ml cycloheximide (CHX) in the presence of glucose for 4 h (1^o reversal). Then the cells were exposed to glucose-free medium, glucose-free medium+CHX, glucose-containing medium, or glucose-containing medium+CHX for 1 h (2^o reversal). Data represent average±SEM of the indicated number of experiments (triplicate plates in each experiment).

a Number of experiments.

b Treatment (2^o reversal vs 1^o reversal).

c Starved+CHX vs starved P<0.05

Starved+CHX vs fed P<0.005

Starved+CHX vs fed+CHX P<0.005.

Fig. 1. Time course of H_2O_2 action on 2-DG transport in glucose-fed human skin fibroblasts. Confluent cell monolayers were incubated in 0% MEM for 42 h and then exposed to 4 mM H_2O_2 for the indicated time intervals. Points represent average \pm SEM of 2-4 separate experiments (triplicate plates in each experiment).

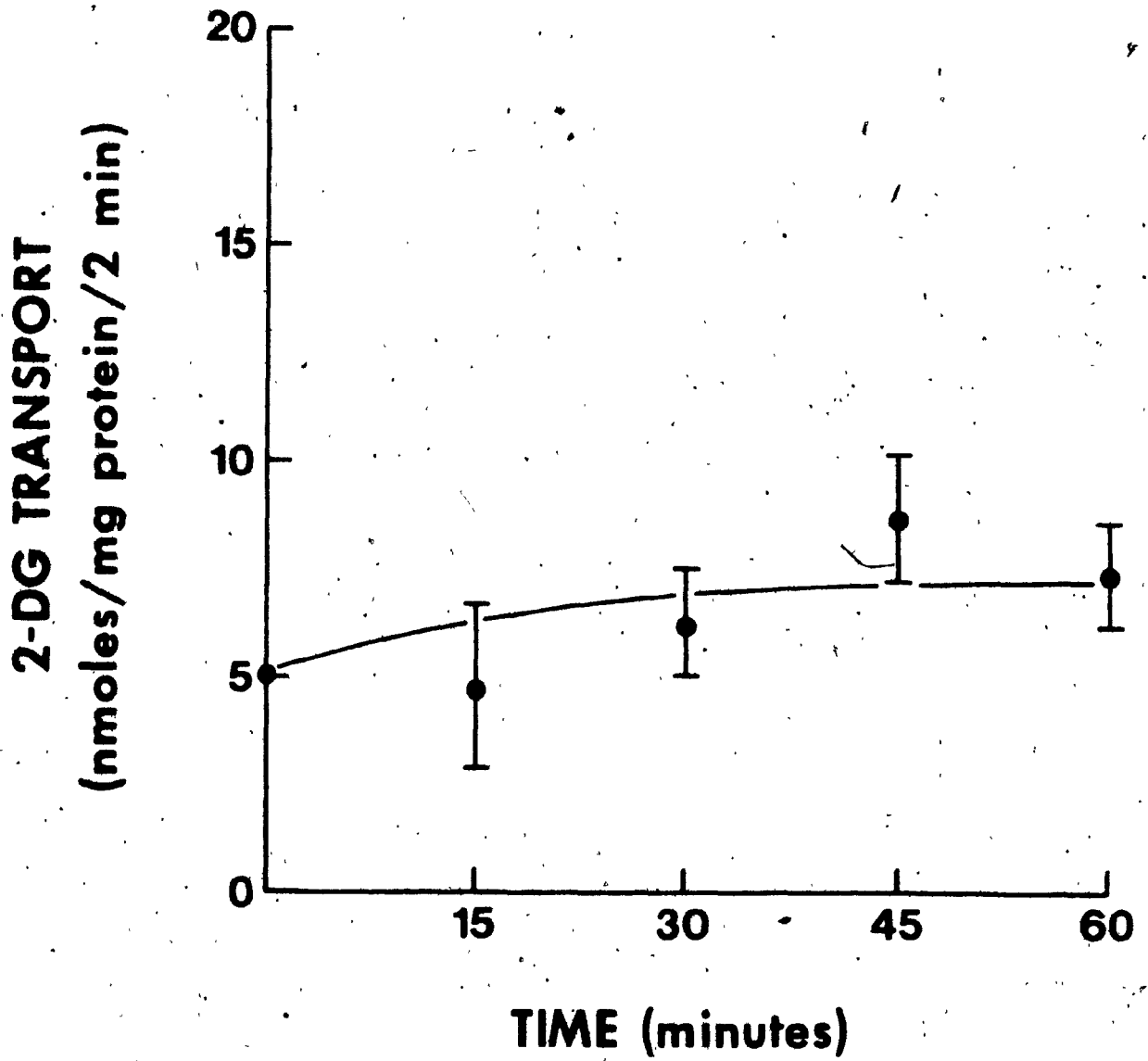


Fig., 2. H_2O_2 action on 2-DG transport in glucose-fed human skin fibroblasts as a function of concentration. Confluent cell monolayers were incubated in 0% MEM for 42 h and then exposed to varying concentrations of H_2O_2 for 45 min. Points represent average \pm SEM of two separate experiments (triplicate plates in each experiment).

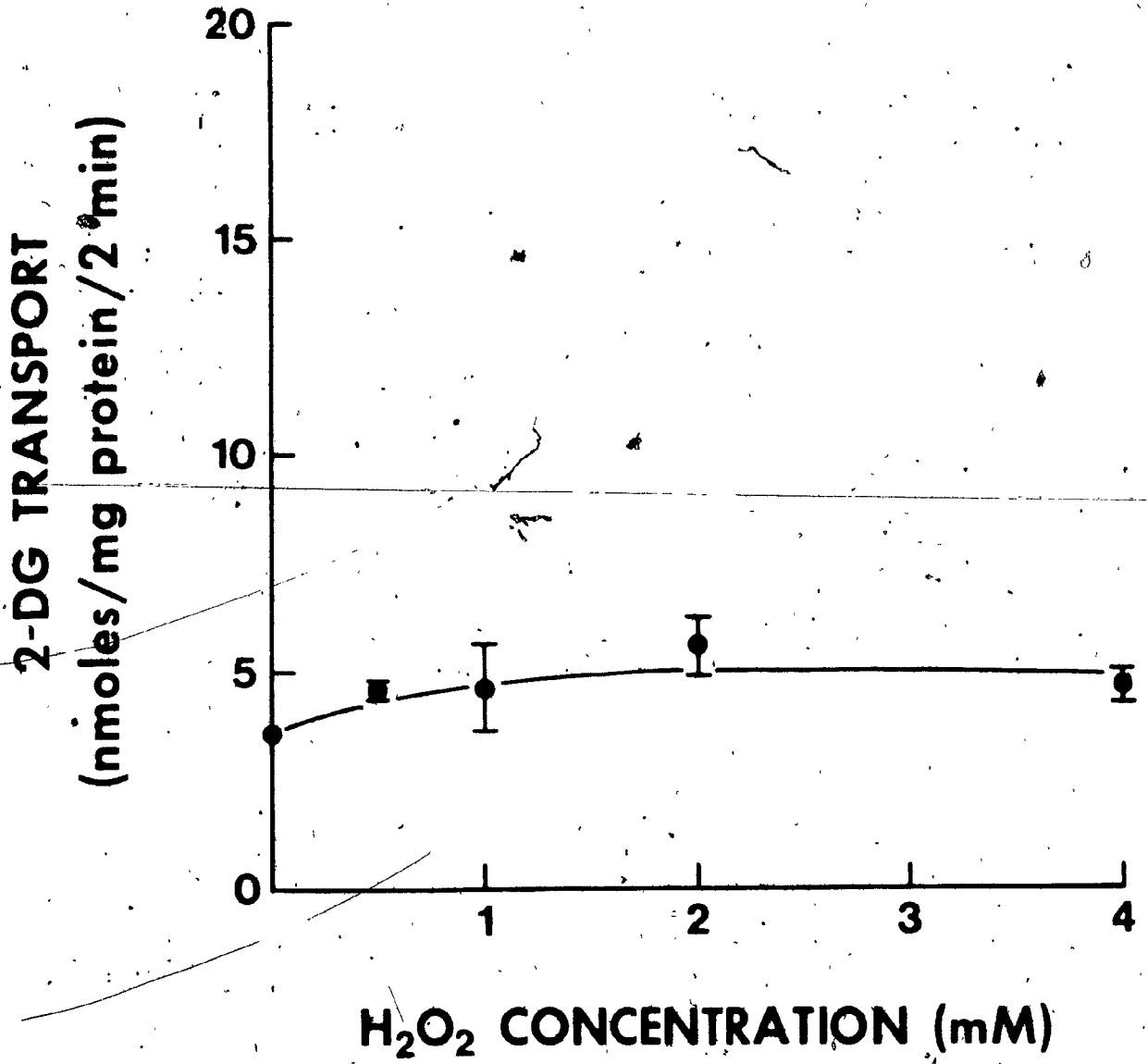


Fig. 3. Time course of H_2O_2 action on 2-DG transport in glucose-starved human skin fibroblasts. Serum-starved (0% MEM, 24 h) confluent cell monolayers were exposed to glucose-free conditions for 18 h. The cells, then, were incubated in glucose-free medium containing 4 mM H_2O_2 for varying time intervals. Points represent average \pm SEM of 2-4 separate experiments (triplicate plates in each experiment).

2-DG TRANSPORT
(\bar{n} moles/mg protein/2 min)

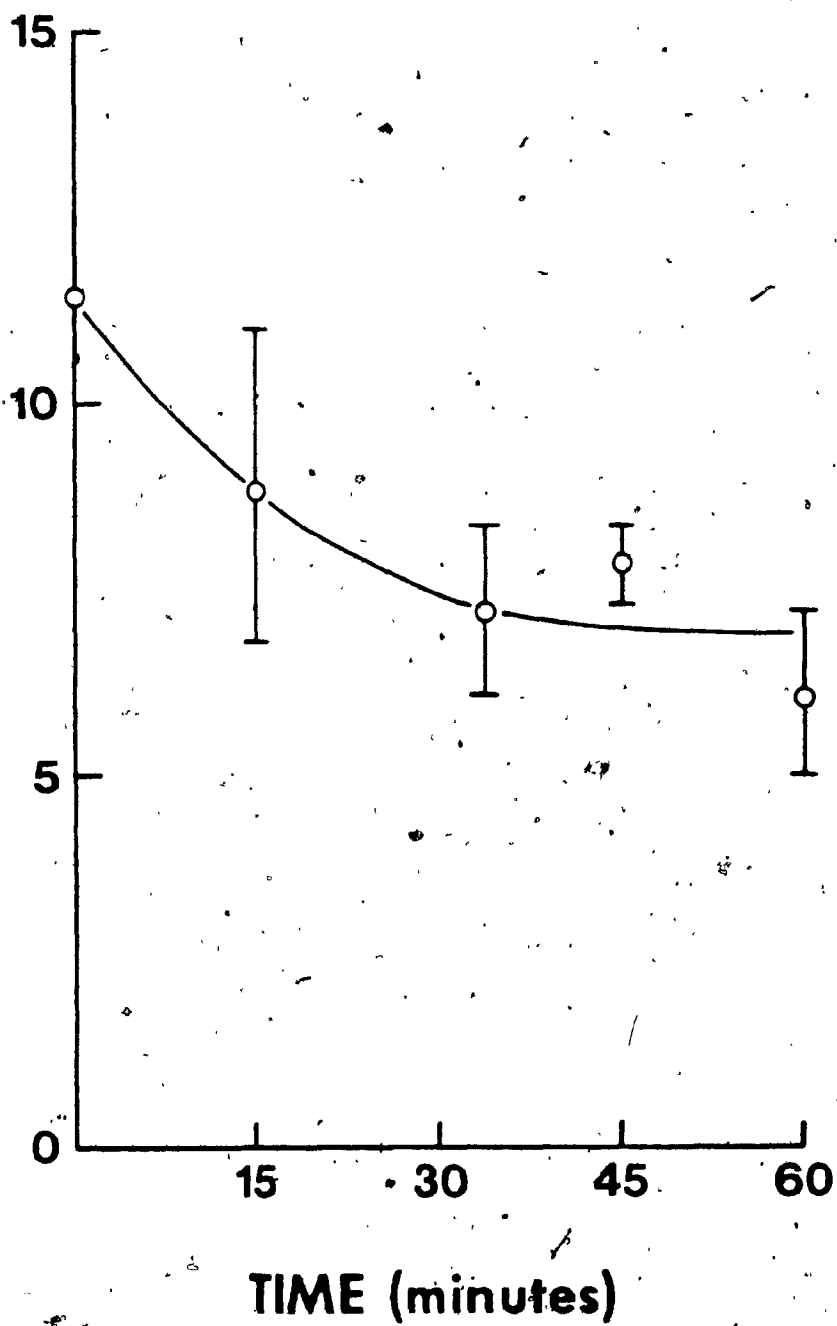


Fig. 4. H_2O_2 action on L-DG transport in
diploco-started human skin fibroblasts as a function of
concentration. Serum-starved MEM, 24 h confluent
cell monolayers were exposed to glucose-free conditions
for 4 h. Then, glucose-free media containing various
concentrations of H_2O_2 was added 45 min prior to
assay for sugar uptake. Points represent a average SEM of
two separate experiments (triplicate plates in each
experiment).

2-DG TRANSPORT
(nmoles/mg protein/2 min)

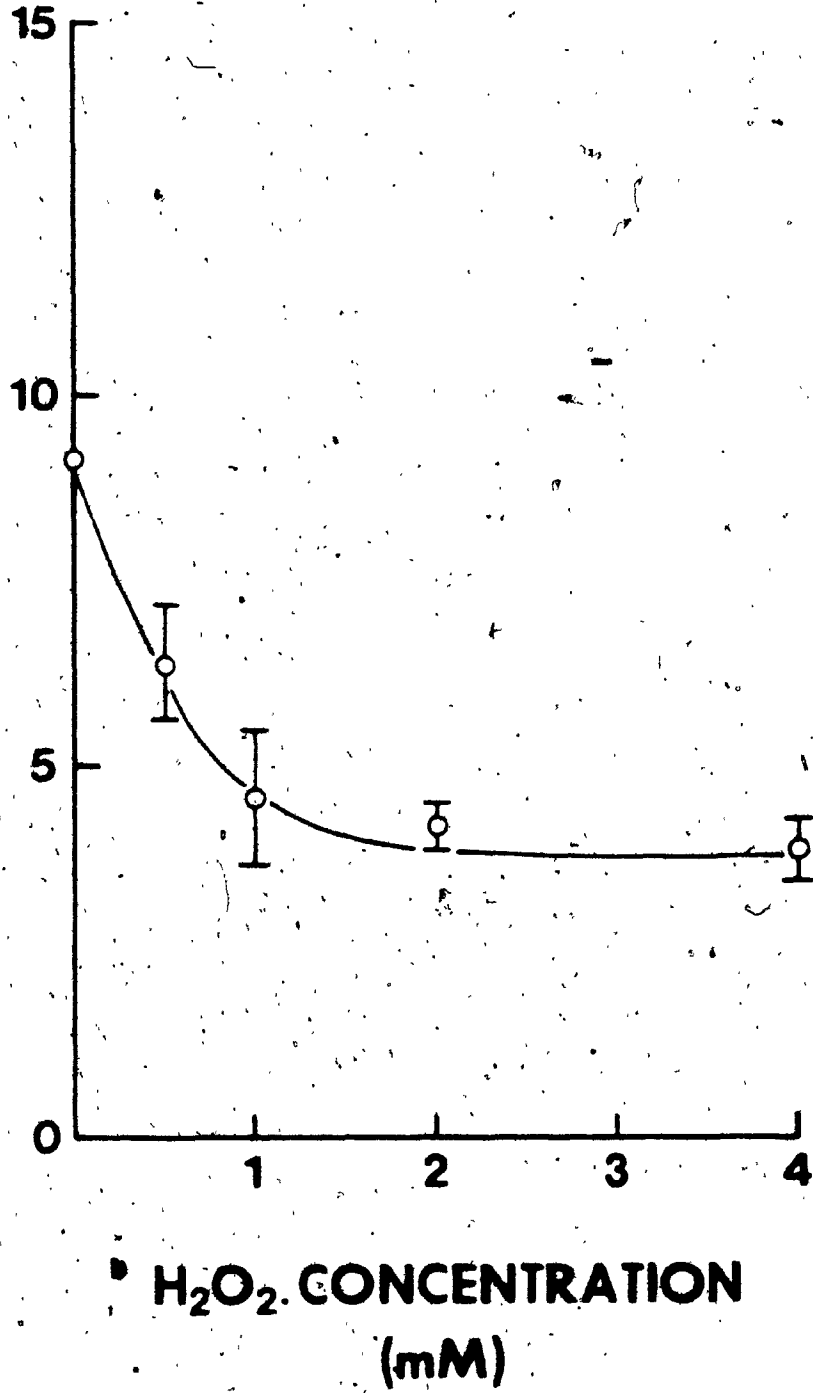


Fig. 5. H_2O_2 action on insulin-stimulated hexose transport activity. Confluent cell monolayers were serum-starved (0% MEM) for 42 h and then exposed to 2 mM H_2O_2 or 100 mU/ml insulin for 30 min or 2 h respectively. Some of the insulin-treated cells were additionally incubated for 30 min with 2 mM H_2O_2 . Data represent average \pm SEM of five different experiments (triplicate plates in each experiment).

2-DG TRANSPORT
(nmoles/mg protein/2 min)

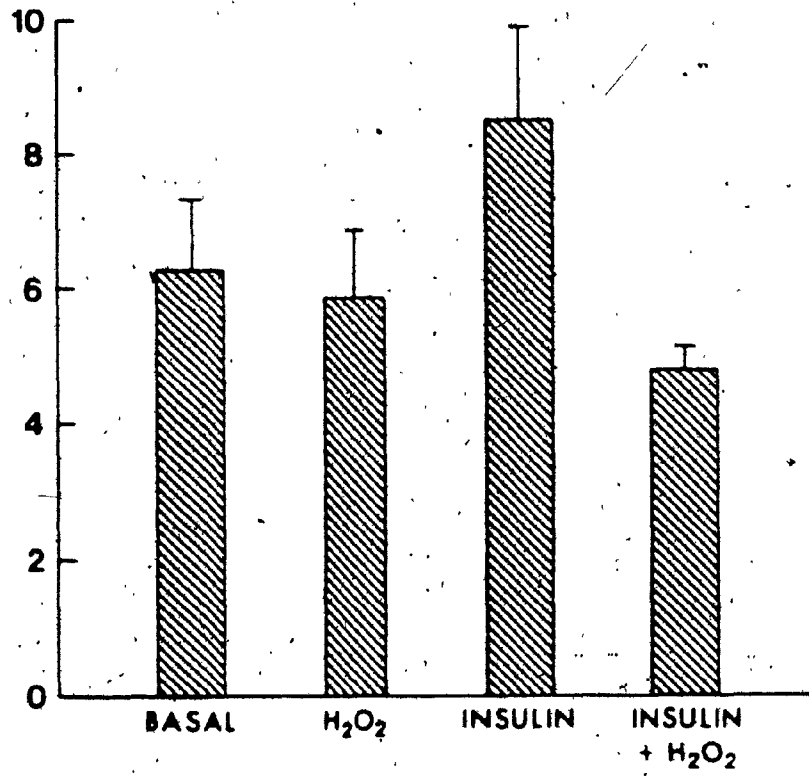


Fig. 6. H_2O_2 action on serum-stimulated hexose transport activity. Confluent fibroblast cultures were serum-starved (0% MEM) for 48 h and then exposed to 2 mM H_2O_2 or 10% (v/v) serum for 30 min or 2 h, respectively. Some of the serum-treated cells were additionally incubated for 30 min with 2 mM H_2O_2 . Data represent average \pm SEM of three separate experiments (triplicate plates in each experiment).

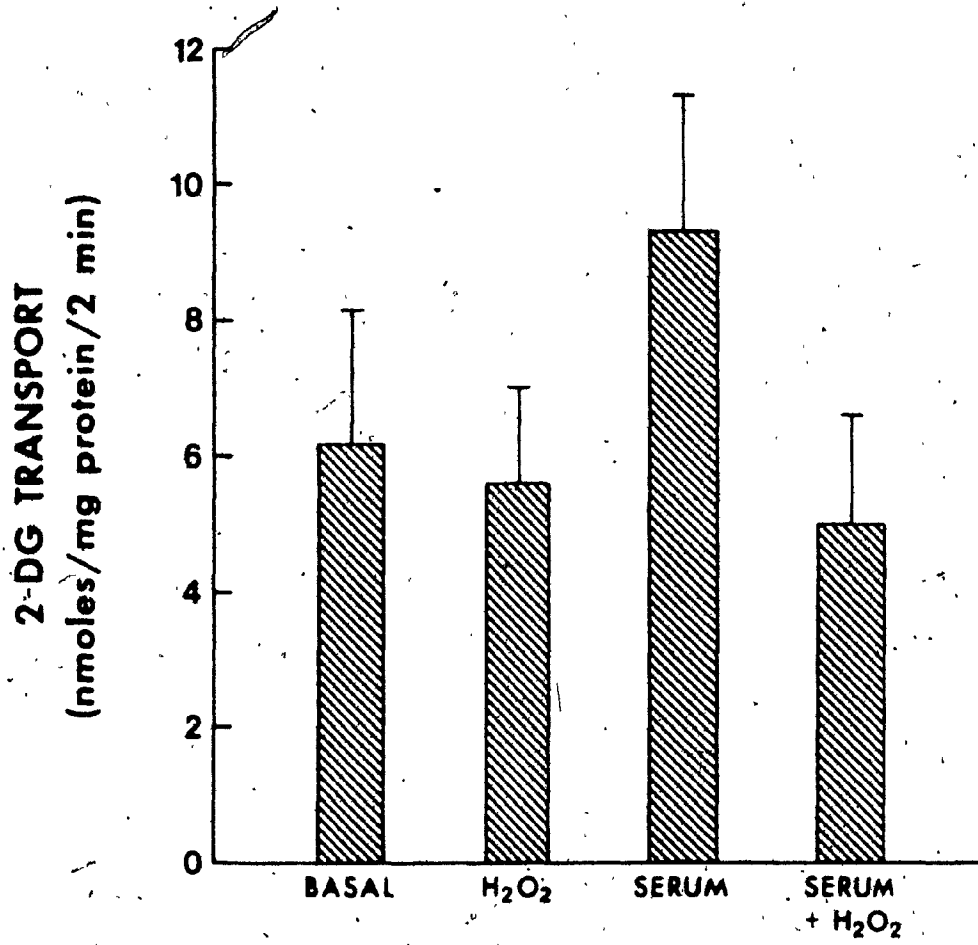
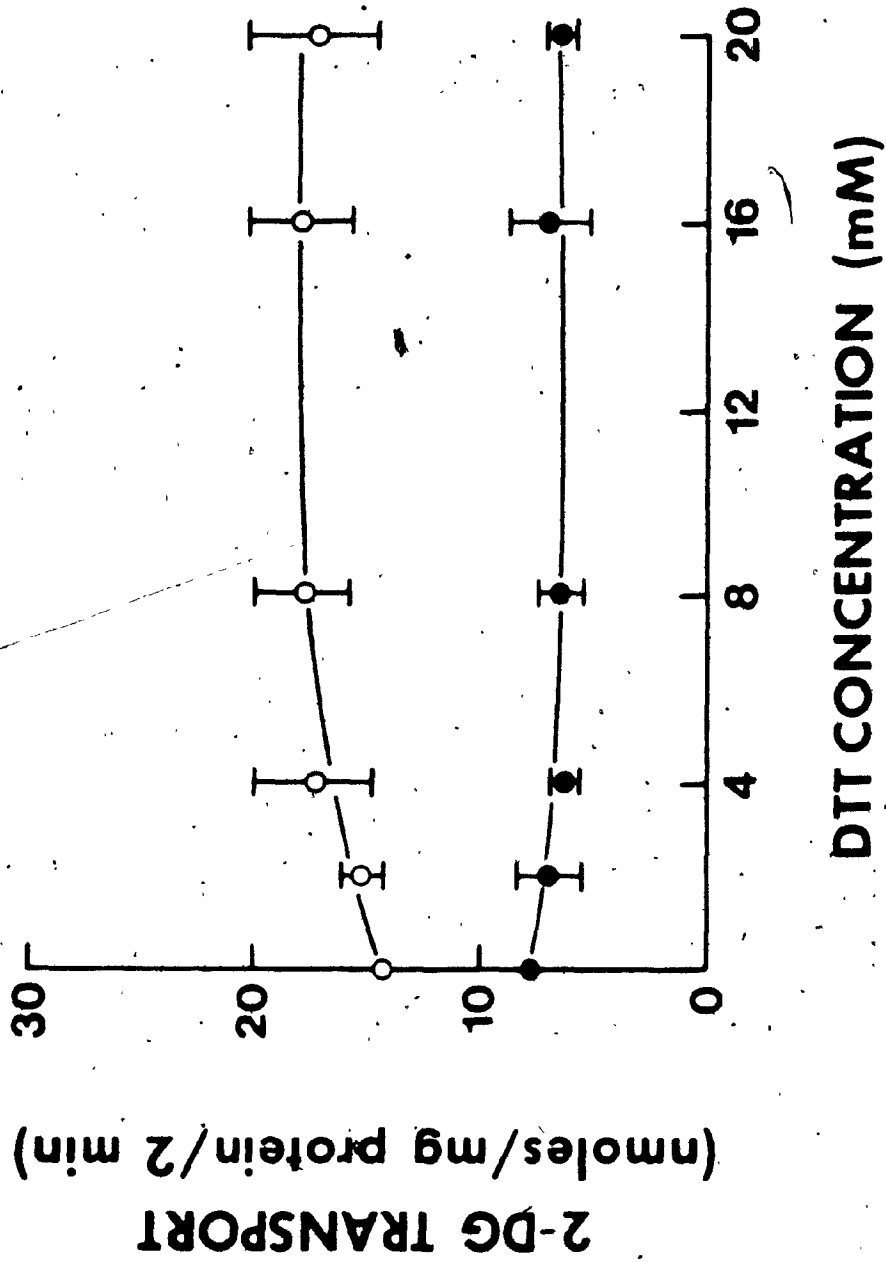


Fig. 7. Effect of DTT concentration on 2-DG transport in cultured human skin fibroblasts. Confluent cell monolayers were serum-starved (0% MEM) for 24 h and then incubated in the presence (●) or absence (○) of glucose for 18 h. Then the corresponding medium containing varying concentrations of DTT was added for 30 min. Points represent average \pm SEM of 2-4 separate experiments (triplicate plates in each experiment).



2-DG TRANSPORT

(nmoles/mg protein/2 min)

DTT CONCENTRATION (mM)

Fig. 8. DTT protection against H_2O_2 -induced inhibition of 2-DG transport in glucose-starved human skin fibroblasts. Serum-starved MEM, 24 hr cell monolayers were glucose-starved for 18 hr and then incubated for 30 min with (○) or without (●) 2 mM H_2O_2 in the presence of the indicated concentrations of DTT. Points represent average \pm SEM of two separate experiments (triplicate plates in each experiment).

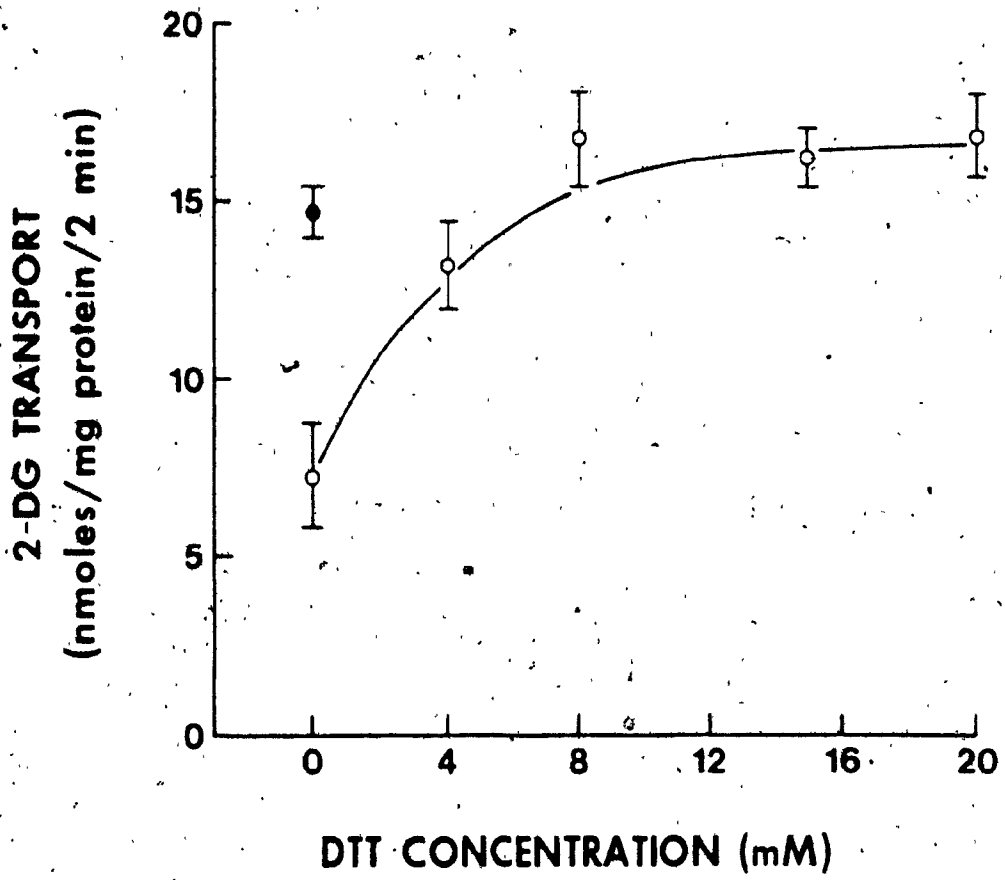


Fig. 4. DTT reversal of H_2O_2 -induced
inhibition of 2-DG transport in glucose-starved human skin
fibroblasts. Serum-starved (0 MEM, 24 h) fibroblast
cultures were glucose-starved for 18 h and then incubated
with 10% or without (●) 2 mM H_2O_2 for 30 min. Some
of the H_2O_2 -treated cells were exposed to 2 mM
DTT (▲) for 30 min, while H_2O_2 was removed from
others (■). Points represent a single SEM of three
determinations.

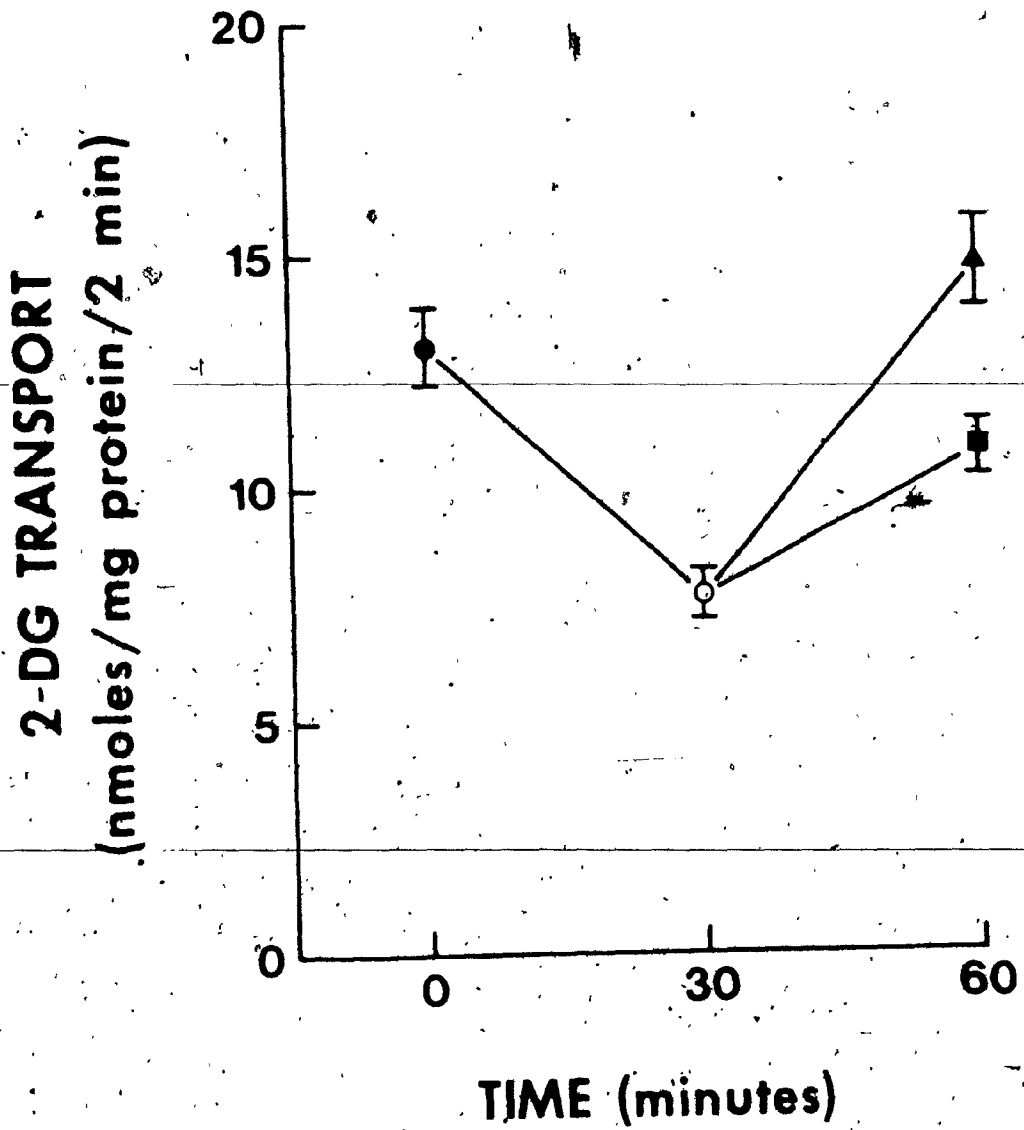
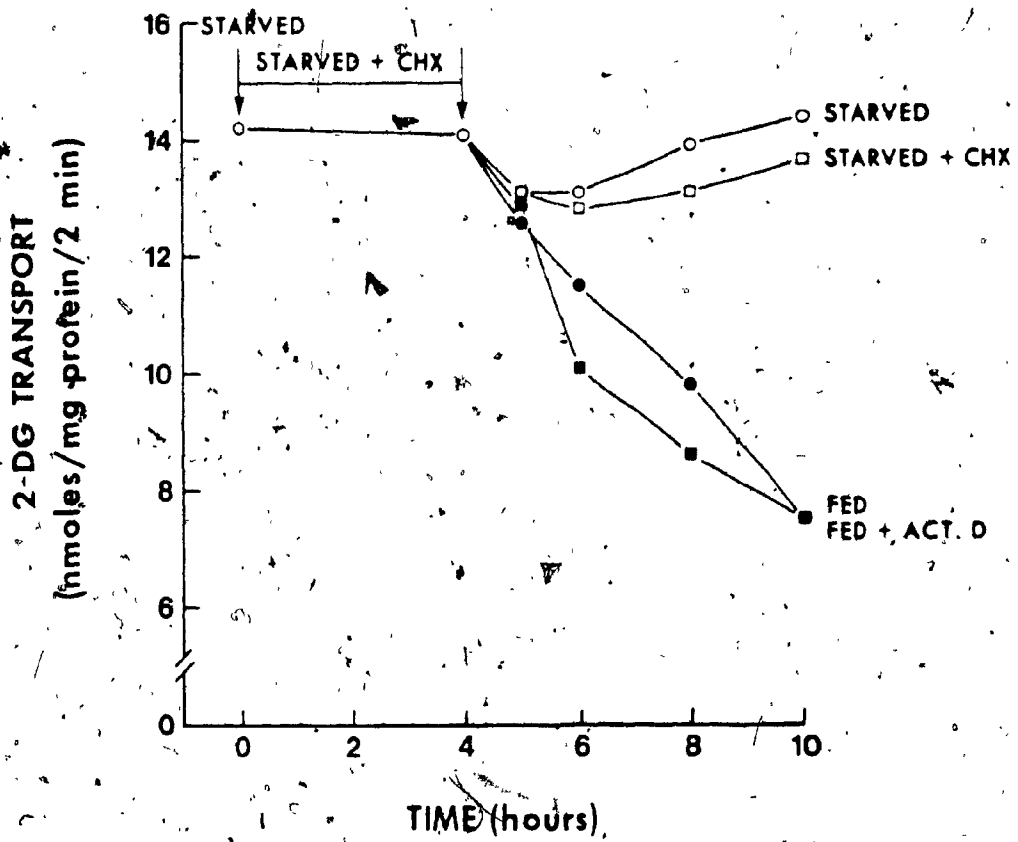
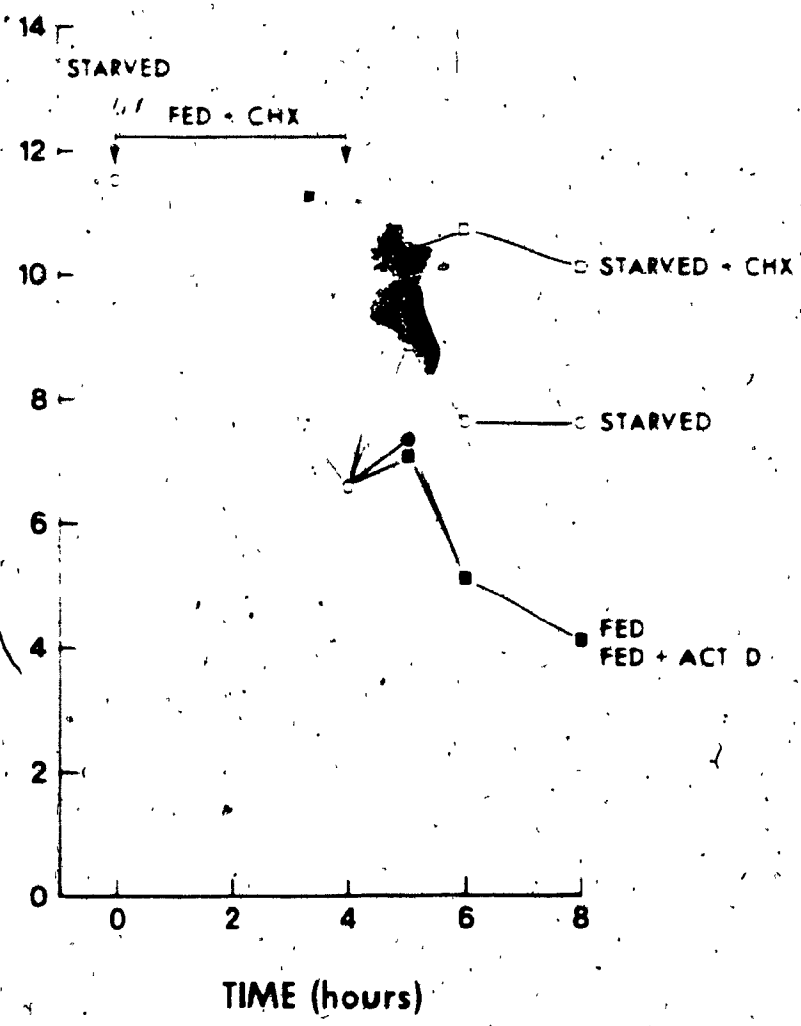


Fig. 10. Effect of cycloheximide on reversal of derepressed transport in human skin fibroblasts. Confluent cell monolayers were serum-starved (0% MEM) for 24 h and then exposed to glucose-free conditions for 12-18 h. The glucose-starvation period was followed by a 4 h incubation in 0% MEM (glucose-free) containing 50 μ g/ml cycloheximide (CHX) (1^o reversal). Then the cells were exposed to glucose-free medium with (□) or without 50 μ g/ml CHX (○), or glucose-containing medium with (■) or without 0.03 μ g/ml actinomycin D (●) for varying time intervals (2^o reversal). Points represent average of two different experiments (triplicate plates in each experiment). SEM was less than 10%.



... with \square ...
... without ...
... intervals ...
... Points represent average of three separate
experiments (triplicate plates in each experiment). SEM
was less than 30%.

2-DG TRANSPORT
(nmoles/mg. protein/2 min)



5.

insulin binding, stimulate 2-DG transport and obliterate the insulin-induced activation of hexose transport in human fibroblasts. There is disagreement, however, with other investigators (Berhanu and Olefsky, 1981) who indicated

transportation response to glucose-stimulation. As a consequence, insulin treatment would induce no further increase in hexose transport.

The data showing a lack of response of glucose-fed cultures to H_2O_2 treatment (Figures 1 and 2, Table II) are in contrast to the reported insulin-like effect of H_2O_2 in rat adipocytes (Czech et al., 1974a, 1974b;

Brown, 1968; Claforn and Sotek, 1972; Paterson and
Paterson and Gould, 1976; Kozka and Gould, 1984, and
Whicker et al., Fibroblasts (CHI - Smith-Gunnarsson et al.,
1977). The different tissue origins of the cell systems
used may have contributed to the apparent disagreement.
Further, the different experimental procedures followed
in the experiments of the present study may have influenced
the results, which are discussed below. The cells were
grown in the presence of glucose, and the effect of glucose
on the transport of hexose was tested. Smith-Gunnarsson
et al. (1977) found that the transport of hexose was
stimulated by the presence of glucose. It was
found that the transport of hexose was stimulated by
the presence of glucose. The results of the present
study are similar to those of Smith-Gunnarsson et al.
(1977) in that the transport of hexose was stimulated
by the presence of glucose. However, the effect of
the oxidant was tested in glucose-fed fibroblasts,
and it was found that the oxidant produced no
stimulation of hexose transport (Table II).
Considering the data on H₂O₂ action in
cultures deprived of glucose it is shown that the oxidant

insulin transport, actually, in a time- and concentration-dependent manner (Figures 3 and 4, Table II). Similar results have been reported by Ishikawa and Howard (1977) for human fibroblasts, although in this study H_2O_2 was shown to initially inhibit hexose transport. In the present study, which is 1/3 of the control, a lower concentration of oxidant will account for the observed effect, which is the reason for the use of H_2O_2 in the present study. H_2O_2 was applied at a concentration of 1 mM.

The sensitivity of insulin-stimulated transport to H_2O_2 in the present study is similar to that reported by Ishikawa and Howard (1977) for rat adipocytes, where insulin transport with H_2O_2 resulted in better response than with H_2O_2 alone (Ishikawa et al., 1974). This disagreement is not surprising, since from the data presented so far and to be presented later it is apparent that human fibroblasts do not exhibit the same response as rat adipocytes to H_2O_2 treatment.

Serum-stimulated hexose transport is shown to be sensitive to the oxidizing agent as well (Figure 6). Furthermore, the observed sensitivity to H_2O_2 appears to be a function of the serum-deprivation time, such that, cultures exposed to serum-free conditions for 24 h are sensitive to the oxidizing agent, while after a 42 h period

of serum-stimulated hexose transport is refractory to H₂O₂ treatment (Table III). These data may be interpreted to reflect a delayed effect of serum deprivation, time, which maintains the cells in the activated state, thus rendering transport activity sensitive to oxidation.

Since H₂O₂ is a non-specific oxidant it was of interest to determine whether its action on hexose transport is mediated through sulfhydryl oxidation, which has been implicated in insulin- and oxidant-stimulated transport in rat adipocytes (Czech et al., 1974a; 1974b; Czeck, 1976b) and rat skeletal muscle (Barnett and Gould, 1984; Kozka and Gould, 1984). The ability of PBT, a specific sulfhydryl reducing agent (Liliana, 1964), to protect from Figure 8) and reverse Figure 9) action indicates that the H₂O₂-induced inhibition of transport activity in glucose-starved cultures is mediated through thiol oxidation. PBT treatment alone had no effect on hexose transport in either glucose-fed or glucose-starved fibroblasts (Figure 7). This observation is in agreement with the results obtained in chicken embryo fibroblasts (Christopher et al., 1976a) but differs from the reported inhibition of transport activity by the reducing agent in rat adipocytes (Czech et al., 1974a). So far it appears that the "activated" hexose transport system, due to glucose-starvation, insulin- or serum-stimulation, is

sensitive to oxidant, while basal transport activity is
resistant to the oxidizing agent. Since H₂O₂
action is mediated through sulfhydryl modification and
oxidation in the insulin-stimulated cultures, it is likely
that the H₂O₂ effect on insulin-stimulated
glucose transport is due to the oxidation of the
sulfhydryl groups of the insulin receptor. The results
of the present study are in agreement with the previous
findings that the effect of sulfhydryl reagents on
"activated" transport is to show that NEM treatment
affects insulin-stimulated transport. The results
also indicate that the effect of NEM on insulin-stimulated
transport (Tables II and IV) while basal transport activity
was resistant to NEM exposure. The differential effect of
NEM on hexose transport in glucose-fed and insulin-stimulated
cultures has been reported earlier in chicken embryo
fibroblasts (Christopher et al., 1976a) and Chinese hamster
fibroblasts (Franchi et al., 1978). Furthermore, it was
shown that NEM treatment did not affect hexokinase activity,
thus indicating that the observed inhibition occurred at the
glucose transport step (Christopher et al., 1976a). NEM has
also been reported to be ineffective on basal transport in
rat adipocytes (Czech et al., 1974a; Czech, 1976a, 1976c)
and rat soleus muscle (Kozka and Gould, 1984). The results
on the sensitivity of insulin-stimulated transport to the

Table II. It is shown that only the permeant NEM is able to inhibit "activated" transport, while treatment with either TNB or 1,3-I-AEDANS is ineffective. The results show that H₂S and NEM block "activated" transport in fibroblasts. However, it could be suggested that the inactivation of transport molecules per se or its binding to the transport intramembrane and/or extramembrane sites in the basal state, but essential for activation, can result in inactivation. The above findings appear to be essential in insulin-mediated transport in rat adipocytes (Czech, 1985; Czech, 1986; Czech, 1987; Fogka and Gould, 1987). It is not clear whether they are involved in the relationship between insulin binding and activation of hexose transport in human fibroblasts. It is shown that neither TNB nor 1,3-I-AEDANS (Table III) prevented insulin-stimulated elevating glucose transport. Since both of the reagents employed are relatively impermeant, due to their hydrophobic nature and large size, it could be suggested that exofacial sulfhydryl groups are not involved in insulin action on hexose transport in human fibroblasts. This conclusion is in contrast to the reported importance of exofacial thiol groups for insulin action in rat adipocytes (May, 1985). In the latter, exofacial sulfhydryl blockage was shown to be

essential for basal transport. It is well known that in this study, basal transport appears to be related to the relationship with important sulfur amino acids (Table VI). The use of a different important sulfur amino acid, methionine, instead of methionine, is also important. Furthermore, the relationship between the amino acid and fibroblasts and rat adipocytes with respect to the effects of NEM and basal transport is also important. The discrepancy in the requirements between insulin and hexose transport act. which is not very clear. However, since rat adipocytes are a specialized tissue and will give a different response than fibroblasts or other cells. Therefore, it is known that certain amino acids, such as methionine, are known to be part of the monolayer. The possibility cannot be excluded that extracellular sulfhydryl groups may be involved in insulin action, but are inaccessible to the bulky molecules of ITNB and 1,5-I-AEDANS. Interestingly, NEM treatment blocked insulin action on hexose transport (Table VI), which is in agreement with similar reports in rat adipocytes (Czech et al., 1974a; Czech, 1976c; May, 1985) and rat soleus muscle (Kozka and Gould, 1984). There are three possible explanations to account for the observed inhibition by NEM: 1) the sulfhydryl reagent substantially depleted intracellular ATP which is known to prevent insulin action on hexose transport.

Chandrasekhar et al., 1977, in NEM
treated cells, insulin binding with certain
antibodies to insulin receptor, and NEM
treated cells had two insulin binding and
antibodies to insulin receptor. In addition with
certain antibodies to insulin receptor (p. 17)
antibodies to insulin receptor was not inhibited
by NEM treatment. It is concluded that the
antibodies to insulin receptor were not inhibited
by NEM treatment. NEM's effect on insulin
binding to insulin receptor was not assessed
in this study. However, there is evidence indicating that
NEM did not block insulin binding in rat hepatocytes (Morgan
et al., 1987). Additionally, Fraboni and Stentz (1985)
reported that treatment of human fibroblasts with the thiol
reagent completely inhibited cell-mediated insulin
degradation, but had no effect on intracellular intact
insulin. Thus, it appears that NEM did not prevent the
hormone from binding to its receptor. Furthermore, although
the sulfhydryl reagent was shown to inhibit binding of the
hormone to its receptor in rat adipocytes (Czech et al.,
1974a) the reported inhibition was 50%. Since NEM

pre-treatment completely abolished the hormone's action on hexose transport, it appears that the sulfhydryl reagent had, at least, a partial effect on the hexose transport system per se. On the other hand, Kozka and Gould (1984) observed a parallel inhibition of insulin binding and stimulation of transport activity following NEM treatment of rat soleus muscle. Considering the contradictory reports on the effect of NEM on insulin binding and action the possibility, that the thiol reagent may have a partial effect on the coupling process itself cannot be excluded. Consequently, it is reasonable to suggest that the inhibitor effect of NEM on insulin action in human fibroblasts may be, at least partially, mediated by blockage of intracellular and/or intramembrane sulfhydryl groups crucial for the insulin-induced translocation of glucose transporters to the plasma membrane.

Role of Glucose in Hexose Transport Regulation

Hexose transport regulation is considered to reflect a balance between carrier synthesis and inactivation (Christopher et al., 1976b; Christopher, 1977). Under glucose-deprivation conditions the activity of the carrier turnover mechanism appears to decline, while upon glucose refeeding of derepressed cells the carrier inactivation process appears to predominate (Yamada et al., 1983;

Tillotson et al., 1984): Recently, it has been suggested that the glucose-mediated down-regulation of the hexose transport system may involve internalization of the carrier-glucose complex, followed by carrier degradation (Kalckar, 1983; Kalckar and Ullrey, 1984a). The data presented in Figure 11 and Table XIII appear to support this hypothesis. When cells deprived of glucose, thus exhibiting elevated transport rates, were exposed to glucose in the presence of high concentrations of cycloheximide (50 µg/ml) hexose transport activity declined by 43% (Figure 11). However, this transport activity did not appear to be lost, since it was recovered as soon as one hour after removal of glucose from the medium. Several facts suggest that it is the same carriers translocated to the plasma membrane, rather than de novo synthesis of transporter molecules. First, the drastic decline in transport activity observed following a 4 h incubation with 50 µg/ml cycloheximide in the presence of glucose most likely cannot be explained in terms of carrier inactivation, since this high concentration of the inhibitor is known to prevent both protein synthesis and intracellular degradation in chicken embryo fibroblasts (Yamada et al., 1983; Tillotson et al., 1984). Additionally, cycloheximide at a concentration of 5 µg/ml was shown to inhibit protein degradation by 30%, following a 4 h incubation, in HeLa cells and diploid human

fibroblasts (Ceccarini and Eagle, 1976). Second, cells exposed to glucose-free medium containing 50 µg ml⁻¹ cycloheximide, which inhibited protein synthesis by 90%, exhibited a rapid increase in 2-DG transport which after one hour, levelled off. Furthermore, these cells (i.e. glucose-starved plus 50 µg ml⁻¹ CHX) exhibited the highest hexose transport activity as compared to cells exposed to glucose-free or glucose-containing medium with or without actinomycin D. The observed elevation would be in agreement with the low turnover activity of glucose-starved cells and inhibition of intracellular degradation by high concentrations of cycloheximide (Ceccarini and Eagle 1976; Yamada et al., 1983; Tillotson et al., 1984). Third, carrier synthesis cannot account for the observed elevation of hexose transport following one hour incubation in glucose-free medium, since it is well established that glucose starvation-induced enhancement of hexose transport takes several hours to develop (Kletzien and Perdue, 1975; Amos et al., 1977; Germinario et al., 1982). Moreover, this initial increase in 2-DG transport was followed by a slight decline suggesting a limited activity of the turnover mechanism, which is consistent with the concept of decreased carrier inactivation under glucose-deprivation conditions (Yamada et al., 1983; Tillotson et al., 1984). Fourth, when the cells were incubated in glucose-containing medium, a

slight initial increase was observed which was followed by a gradual decline in hexose transport activity. These data are in agreement with the reported increased rate of degradation under glucose-fed conditions (Yamada et al., 1983; Tillotson et al., 1984). The initial increase in hexose transport rate can be explained in terms of translocation of carriers which had not been inactivated yet. This lag may be due to the protein synthesis requirement for activation of the carrier degradation mechanism (Christopher et al., 1976b; Germinario et al., 1982).

When glucose-starved cells were exposed to glucose-free conditions in the presence of 50 µg/ml cycloheximide, hexose transport activity remained at the derepressed state following a 4-h incubation (Figure 10). Furthermore, upon change of the incubation conditions transport activity declined slightly in cells exposed to starvation medium or starvation medium plus 50 µg/ml cycloheximide, while a rapid decrease was observed in cells incubated in glucose-refeeding medium with or without actinomycin D. These data, which are in sharp contrast to those presented in Figure 11 and Table XIII offer further support to the notion of glucose-mediated internalization of transporters, which are then subjected to inactivation or possibly recycle upon escape of degradation.

The findings presented herein appear to be in disagreement with those reported for chicken embryo fibroblasts (Yamada et al., 1983). The response of the latter, when subjected to a similar treatment, was markedly different from the one reported in this study for human fibroblasts, suggesting a possible transcriptional regulation of the hexose transport system. However the data in Figures 10 and 11 indicate that RNA synthesis is not involved in hexose transport reversal of derepressed fibroblasts. This disagreement may be due to the well established fact that both protein and RNA synthesis are required for the adaptive response to glucose deprivation to develop in chicken embryo fibroblasts (Kletzien and Perdue, 1975; Musliner et al., 1977; Amos et al., 1977), while only protein synthesis appears to be a requirement for either the development or the reversal of glucose starvation-induced enhancement of hexose transport in human fibroblasts (Germiario et al., 1982).

CONCLUSION

The evidence presented in this study is suggestive of several interesting points with respect to the two activated states of the hexose transport mechanism in human diploid fibroblasts, i.e., insulin-stimulation and glucose-deprivation. First, glucose-starvation may be viewed to induce translocation of insulin translocatable transporters, to the plasma membrane. Second, intramembrane and or intracellular thiol perturbation of the "activated" transport system may result in carrier inactivation. Third, exofacial sulphhydryl groups do not appear to be involved in insulin action on hexose transport in human fibroblasts. However, intramembrane and/or intracellular thiol modification may block the signal that triggers insulin-induced translocation of glucose transporters to the plasma membrane. Fourth, the glucose-mediated down-regulation of the hexose transport mechanism may involve internalization of the carrier-glucose complex, followed by carrier degradation.

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