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High-affinity Glucose Uptake in *Saccharomyces cerevisiae* is not Dependent on the Presence of Glucose-Phosphorylating Enzymes

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Glucose uptake in *Saccharomyces cerevisiae* is believed to consist of two kinetically distinguishable components, the affinity of which is modulated during growth on glucose. It has been reported that triple hexose-kinase deletion mutants do not exhibit high-affinity glucose uptake. This raises the question of whether and how high-affinity glucose uptake is related to the presence of glucose-phosphorylating enzymes. In this study the kinetics of glucose uptake in both wild-type cells and cells of hexose-kinase deletion mutants, grown on either glycerol or galactose, were determined using a rapid-uptake method. In wild-type cells glucose uptake measured over either 5 s or 200 ms exhibited high affinity. In contrast, in cells of hexose-kinase deletion mutants the apparent affinity of glucose uptake was dependent on the time scale during which uptake was measured. Measurements on the 5-s scale showed apparent low-affinity uptake whereas measurements on the 200-ms scale showed high-affinity uptake. The affinity and maximal rate of the latter were comparable to those in wild-type cells.

Using a simple model for a symmetrical facilitator, it was possible to simulate the experimentally determined relation between apparent affinity and the time scale used.

The results suggest that high-affinity glucose transport is not necessarily dependent on the presence of glucose-phosphorylating enzymes. Apparent low-affinity uptake kinetics can arise as a consequence of an insufficient rate of removal of intracellular free glucose by phosphorylation.

This study underlines the need to differentiate between influences of the translocator and of metabolism on the apparent kinetics of sugar uptake in yeast.

KEY WORDS — transport; glucose uptake; *Saccharomyces cerevisiae*; yeast; rapid kinetics

INTRODUCTION

It is generally believed that glucose uptake in yeast proceeds via a facilitated diffusion process (Heredia *et al.*, 1968). As a consequence, it is to be expected that the internal concentration of glucose exerts an effect on the net transport rate. This could lead to an underestimation of the transport rate if measurements are not truly initial. However, subsequent metabolism usually tends to keep the internal glucose concentration very low. It can be calculated that within 5 s (the time scale of most uptake experiments) most of the internalized sugar has been converted into other metabolites. Indeed, under steady-state conditions in cells exposed to a glucose

concentration of 100 mM, the intracellular glucose concentration did not exceed 0.4 mM (Becker and Betz, 1972). Alternatively, it has been suggested that glucose uptake in yeast is mediated by an active phospho-transferase system, analogous to that in many prokaryotes (van Steveninck, 1968, 1969; van Steveninck and Rothstein, 1965). This view has now been abandoned since it was shown that the non-phosphorylatable glucose analogue 6-deoxy-glucose could be transported by *Saccharomyces cerevisiae*, albeit with an affinity that was at least ten-fold lower with respect to glucose uptake (Bisson and Fraenkel, 1983b). In addition, it has been claimed that free sugar appeared first in the intracellular sugar pool (Romano, 1982; Kotyk and Michaljanicova, 1974).

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To determine the true initial kinetics of the transport step, it is necessary to study glucose transport separately from metabolism (Serrano and Delafuente, 1974). For this reason, in earlier glucose-uptake experiments non-metabolizable glucose analogues like 6-deoxy-glucose were used as a substrate (Bisson and Fraenkel, 1983b; Franzusoff and Cirillo, 1982). One disadvantage of using this glucose analogue is that the results may not be fully representative for glucose transport because of the structural difference between these substrates. The difference in affinity of the uptake system for the two substrates is probably a reflection of this problem (Bisson and Fraenkel, 1983b).

An alternative method is to use mutants in which glucose metabolism is affected. Indeed, it has been shown that in mutants which lack the possibility to phosphorylate glucose by deletion of the three hexose-kinase enzymes (hexokinase PI, hexokinase PII and glucokinase), a change in apparent uptake kinetics as compared to wild type occurs (Bisson and Fraenkel, 1983a; Lang and Cirillo, 1987). The absence of these hexose kinases led to a decrease both in maximal transport rate (V_{\max}) and affinity (increase in K_m). Therefore it was concluded that high-affinity glucose transport was dependent on the presence of hexose kinases. A similar idea was also proposed for high-affinity galactose transport (Ramos *et al.*, 1989).

Studies on glucose uptake in yeast cells which were depleted of energy have also demonstrated similar effects. When measured on a 5-s time scale, an apparent reduction in V_{\max} and a decrease in K_m was observed, as compared to energy-sufficient cells (Walsh *et al.*, 1994b). This effect has been attributed to a decrease in phosphorylation capacity which led to a build-up of internal free glucose in the cells, resulting in reduction of net glucose uptake. On a 200-ms time scale, however, the kinetics of glucose uptake in energy-depleted cells were similar to those in energy-sufficient cells.

Based on the reasoning above, it is likely that the apparent kinetics, measured after 5 s, in the hexose kinase-less mutants are obscured by the fact that the internal sugar significantly inhibits net transport. To measure transport on such a short time scale that the effects of either subsequent metabolism in the wild type or build-up of internal free sugar in the triple-kinase deletion mutant should be minimal, we used the method that we

developed previously (Walsh *et al.*, 1994b). We measured glucose uptake after 200 ms both in triple-kinase mutants and in wild-type yeast cells. To be able to compare our results with the data in the literature, we also measured uptake after 5 s. Furthermore, the kinetics of equilibration of glucose over a long time in triple-kinase deletion mutants have been examined.

We show that it is possible to simulate the experimental results using a model for a simple translocator that is often used to describe glucose transport in erythrocytes (Stein, 1986; Walmsley, 1988).

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources; D-[U- 14 C]glucose was from Amersham International. Media constituents were from Difco, other chemicals were from Sigma Chemical Co. and were of reagent grade.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951).

Strains and growth conditions

In this study the following *S. cerevisiae* strains were used: DFY1 (*MATa lys1-1*; Bisson and Fraenkel, 1983a) and DFY570 (*MATa lys1-1 leu2-1 hxx1::LEU2 hxx2::LEU2 glk1::LEU2*; Walsh *et al.*, 1991). These strains were constructed by D. G. Fraenkel and collaborators, Harvard Medical School, Boston (Bisson and Fraenkel, 1983a; Walsh *et al.*, 1991) and kindly donated by J. Thevelein.

Cells were grown at 30°C in a rotary shaker in medium containing either 2% glycerol or 2% galactose, 6.7 g/l yeast nitrogen base, 100 mM-potassium phthalate at pH 5.0, supplemented with 30 µg/ml lysine and 60 µg/ml leucine. Cells were grown for at least five doubling times, up to an OD_{600} of between 0.5 and 1.0. Cells were harvested by centrifugation (10 min, 1200 g, 4°C), resuspended and washed three times with 100 mM-potassium phosphate buffer pH 6.5 at a temperature of 4°C, and finally made up in the same buffer to a cell concentration of 10% (w/v) for fast-uptake experiments and 5% (w/v) for 5-s uptake experiments. Cells were kept on ice until they were subjected to experiments.

Glucose uptake assay

The rate of glucose uptake over 5 s was measured using a modification (Walsh *et al.*, 1994a) of the method of Bisson and Fraenkel (1983a). Cells were resuspended to a concentration of 5% (w/v). Uptake was measured at glucose concentrations ranging from 0.1 mM to 250 mM (specific radioactivity 740 to 6 Bq nmol⁻¹). Yeast cells (50 µl) and five-times concentrated radiolabelled D-[U-¹⁴C]glucose (12.5 µl) were pre-incubated separately at the assay temperature, then mixed and incubated for 5 s. Uptake was terminated by quenching in 10 ml of 100 mM-potassium phosphate buffer pH 6.5, containing 500 mM unlabelled glucose kept at -5°C on salt-ice mixtures. Cells were then collected rapidly on glass-fibre filters (Whatman GF/C) and washed with 20 ml of ice-cold quenching solution. Filters were transferred immediately to scintillation vials containing 5 ml of scintillant. The control blank in each experiment consisted of labelled glucose added to the quenching solution immediately before the yeast cells. These procedures were performed as quickly as possible to prevent any fermentative loss of incorporated radiolabel. The time between mixing of cells and glucose and transfer of filter to scintillant was less than 1 min.

Rapid glucose-uptake assay

The initial rate of glucose uptake on a sub-second time scale was measured using the quench-flow technique described previously (Walsh *et al.*, 1994b; de Koning and van Dam, 1992). The two 2.5 ml syringes were filled, one with two-times concentrated radiolabelled D-[U-¹⁴C]glucose (0.6 mM to 200 mM, specific activity 62 to 0.4 Bq nmol⁻¹; final concentration in the assay 0.3 mM to 100 mM) and one with two-times concentrated cell suspension (10%, w/v; final concentration in the assay 5%, w/v). The control blank in each experiment consisted of a shot with the quench-flow machine using a very short incubation tube, resulting in an incubation time of 4 ms. Radioactivity was measured with a Beckman liquid scintillation counter.

Data analysis

Data were analysed by computer-assisted non-linear regression with proportional weighting using Enzfitter software.

RESULTS

Glucose uptake, measured over 5 s, in glycerol-grown cells of the triple-kinase deletion mutant showed low affinity, which could be described by Michaelis-Menten kinetics (Figure 1A) with K_m of 14 ± 1 mM and V_{max} of 85 ± 5 nmol min⁻¹ mg protein⁻¹, which was in agreement with previous reports (Bisson and Fraenkel, 1983a; Lang and Cirillo, 1987).

The corresponding wild type showed high-affinity uptake with a K_m of 2.7 ± 0.1 mM and a V_{max} of 162 ± 3 nmol min⁻¹ mg protein⁻¹. This confirmed the effect of deletion of the three kinases on glucose transport measured over 5 s (Bisson and Fraenkel, 1983a; Lang and Cirillo, 1987).

Figure 1B shows the kinetics of glucose uptake in the triple-kinase mutant, grown on glycerol, and measured after 200 ms. These kinetics differed greatly from the kinetics measured over 5 s. Glucose uptake now exhibited high-affinity kinetics with a K_m of 2.1 ± 0.2 mM and a V_{max} of 167 ± 11 nmol min⁻¹ mg protein⁻¹. These uptake characteristics were identical to those of wild-type cells measured on either time scale (Figure 1A,B). Similar results were found for glucose uptake in galactose-grown cells (Figure 2). These results suggested that reduction in phosphorylation capacity only affected high-affinity uptake when measured over longer periods.

The most straightforward explanation for this observation is that, in 5-s glucose-uptake measurements in the triple-kinase deletion mutant, the effect of internal glucose on net glucose transport is already large. Indeed, on this time scale the intracellular glucose can accumulate up to 2.0 mM and therefore the assumption of zero-*trans* influx conditions is not valid. The expected magnitude of this effect will be discussed below.

To estimate the contribution of intracellular glucose on influx, we performed a time-course experiment in the triple-kinase deletion mutant, in which the equilibration of glucose was followed during 1 min (Figure 3). In this experiment, labelled glucose at a concentration of either 1 mM or 10 mM was used. Measurements over the first second were performed using the quench-flow technique. For longer time-scale measurements (2 s up to 1 min) the 5-s protocol was employed (see Materials and Methods).

Incorporation of D-[U-¹⁴C]glucose with either 1 mM or 10 mM glucose was approximately linear during the first 200 ms (Figure 3A); after that time,

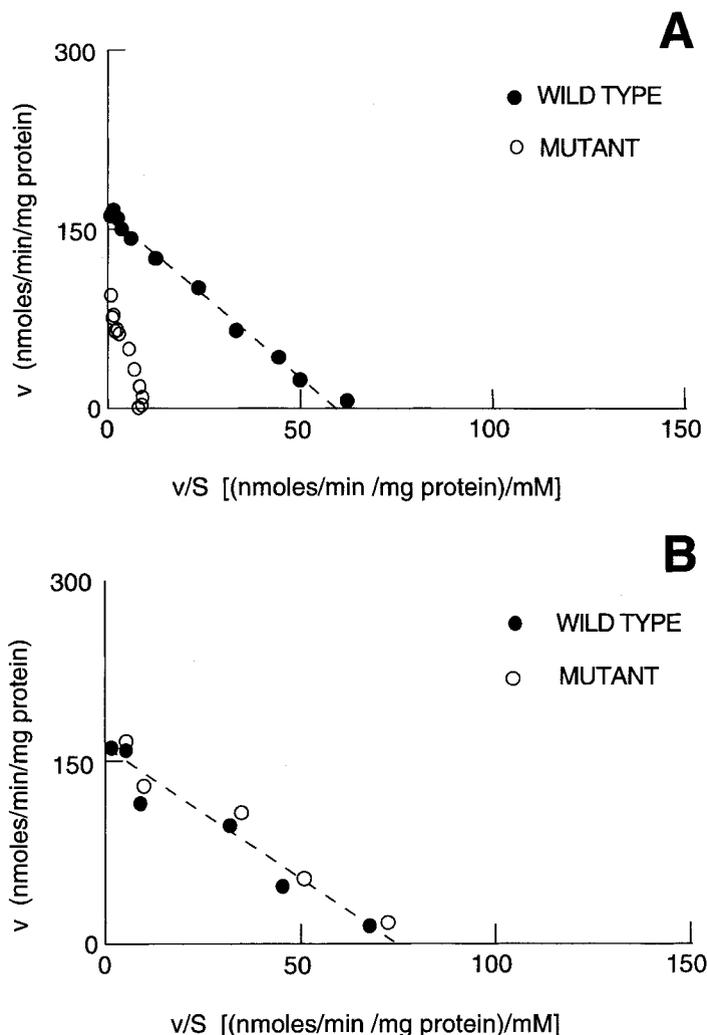


Figure 1. (A) 5 s kinetics of glucose uptake by wild type and a triple-kinase deletion mutant of *S. cerevisiae* grown on glycerol. Uptake was measured after 5 s, as described in Materials and Methods, in either wild type or triple-kinase deletion mutant. Each point is the mean of three determinations. (B) 200 ms kinetics of glucose uptake by wild type and a triple-kinase deletion mutant of *S. cerevisiae* grown on glycerol. Uptake was measured after 200 ms, as described in Materials and Methods, in either wild type or triple-kinase deletion mutant. Each point is the mean of duplicate experiments.

the rate of glucose incorporation started to bend off significantly. At an external concentration of 1 mM, the internal glucose concentration equilibrated to 84% of the external concentration, assuming that 1 mg protein corresponds to 3.75 μ l cell water (de Koning and van Dam, 1992), whereas at a concentration of 10 mM, glucose equilibrated only to 43% during this period (Figure 3B).

In an effort to simulate the apparent glucose transport kinetics on the two different time scales, we adopted the four-state model (Figure 4). This model is the simplest description of a transport process and it is often used to describe the kinetics of glucose transport, for example in erythrocytes (Stein, 1986; Walmsley, 1988).

In this simulation the rate constants governing inward and outward reorientation of the loaded

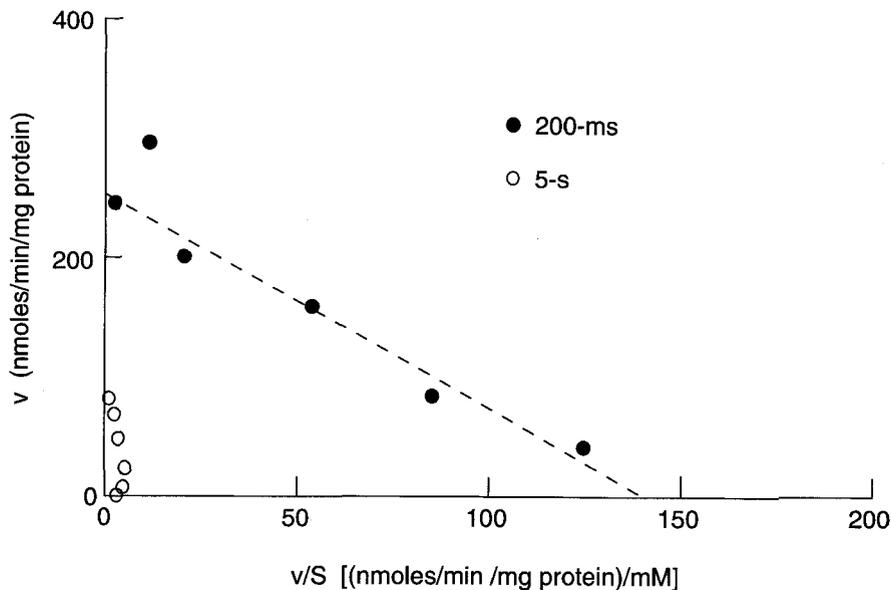


Figure 2. 200 ms and 5 s kinetics of glucose uptake by triple-kinase deletion mutant of *S. cerevisiae* grown on galactose. Uptake was measured after 200 ms or 5 s, as described in Materials and Methods. In the 5-s experiments, each point is the mean of three determinations. In the 200-ms experiments, each point is the mean of two determinations.

and the unloaded carrier were assumed to be the same. The same assumption has been made for the dissociation constants of the two carrier-substrate complexes, K_{di} and K_{do} . The incorporation of glucose was simulated over 5 s using external glucose concentrations ranging from 0.3 mM to 100 mM. The results at 200 ms and 5 s are presented in an Eadie-Hofstee graph (Figure 5).

The simulation corresponded reasonably well with the experimental data, suggesting that the four-state model gives an adequate description of glucose transport in *S. cerevisiae*.

DISCUSSION

Our experimental data and their simulation support the hypothesis that the apparent low-affinity glucose uptake observed in the triple-kinase deletion mutants, when measured after 5 s, is a consequence of build-up of the internal free glucose.

The simulation of our experimental results with the simple four-state model shows that it is possible to approximate the trends in glucose uptake in triple-kinase deletion mutants. No attempt was made to refine the simulation so as to reflect precisely the full time course of glucose

uptake. It was concluded, however, that during the first 200 ms the uptake is approximately linear, justifying the use of this period in the experimental analysis. Since the measurements of rate constants were beyond the aim of this study, the simulation relies on an assumption of the values of these rate constants and the symmetry of the transport process. At a later stage, when these values are known, it will be possible to approximate the transport process in *S. cerevisiae* even better than with this simulation and address such questions as the symmetrical nature of the transport process.

An important implication of the results presented in this paper is that a 5-s transport experiment does not, under some circumstances, adequately report the true kinetics of the glucose transporter in *S. cerevisiae*. Indeed, based on modelling studies, it has been demonstrated that apparent changes in kinetics of a transport process can be induced by changes in the subsequent metabolism (ter Kuile *et al.*, 1994; Fuhrmann and Völker, 1992). Thus, relatively long-term (5 s) transport experiments may lead to erroneous conclusions concerning the regulation of this process, especially in mutants affected in metabolism. The possible problems with these long-term transport experiments have already been discussed by other

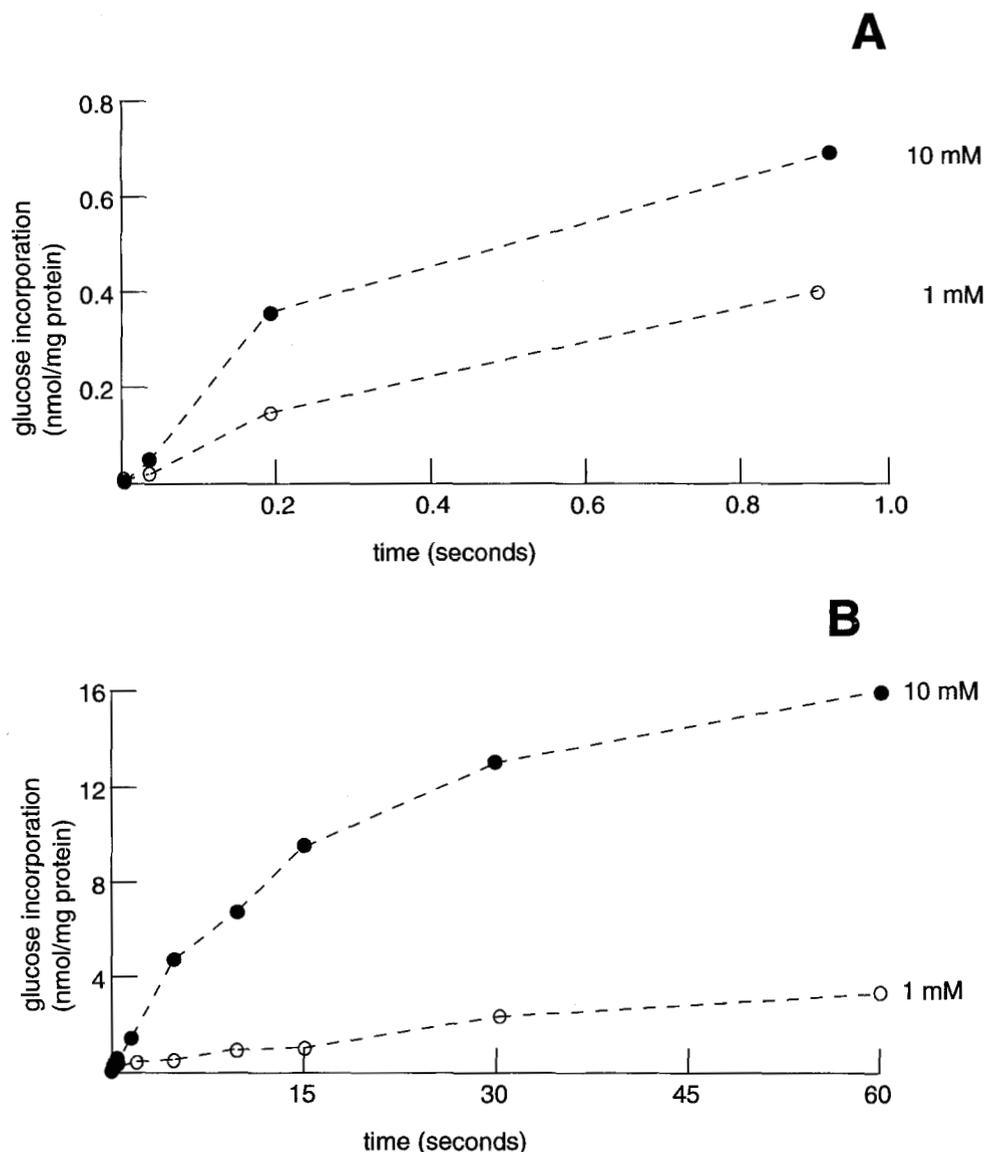


Figure 3. (A) Equilibration of glucose during 1 s after addition of 1 or 10 mM glucose to a triple-kinase deletion mutant of *S. cerevisiae* grown on glycerol. Uptake of either 10 or 1 mM glucose over a period of 1 s was measured according to the rapid glucose-uptake assay described in Materials and Methods. Each point is the mean of duplicate measurements. (B) Equilibration of glucose during 1 min after addition of 1 or 10 mM glucose to a triple-kinase deletion mutant of *S. cerevisiae*. Uptake at points later than 1 s was measured according to the method used for the 5-s uptake assay, described in Materials and Methods. Each point is the mean of triplicate measurements.

authors (Serrano and Delafuente, 1974; Fuhrmann and Völker, 1992). The conclusion that a triple-kinase deletion mutant has a high affinity for glucose has already been drawn by Fuhrmann *et al.* (1989) on the basis of experiments and simulations with plasma membrane vesicles. These

authors also concluded that in these mutants the 5 s uptake assay is too slow to be considered relevant for initial kinetics. Only with our new technique of quench-flow measurements of glucose uptake has it become possible to actually demonstrate the true consequences of this problem.

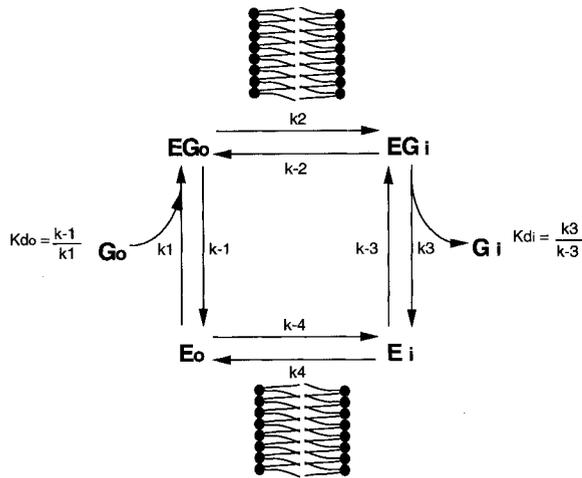


Figure 4. Four-state carrier model. According to this model the transporter (E) interconverts between a conformation in which there is an outward-facing substrate binding site (Eo) and one in which there is an inward-facing substrate binding site (Ei). When the substrate binding site is occupied (EGi or EGo), a conformational change results in the translocation of the bound substrate across the membrane.

The data of the 5-s glucose-uptake experiment in the triple-kinase deletion mutant show some deviations from a model of a single-uptake system obeying Michaelis-Menten kinetics, since the decrease in uptake rate at glucose concentrations below 1 mM is proportionally greater than the

decrease in substrate concentration. The diagnostic feature for this behaviour in the Eadie-Hofstee transformation is an inwardly curved line in the lower part of the graph (Figure 1A).

Sometimes such curvatures appear in Eadie-Hofstee graphs of other workers. For instance Thevelein and co-workers show Eadie-Hofstee graphs of the results of 5-s glucose-uptake experiments with mutants lacking one or two putative glucose transporters, HXT1 and SNF3, and the gene encoding a subunit of the trehalose synthase complex, GGS1 (Neves *et al.*, 1995). These graphs also are inwardly curved at the lower part. Possibly in this mutant, which is affected in glucose metabolism, there is build-up of relatively high intracellular glucose concentrations resulting in reduced net uptake rates, especially at low glucose concentrations. It is not unlikely that in other mutants, affected in metabolism, similar effects may appear on glucose transport measured over 5 s.

Furthermore, it is plausible that these effects also apply to other facilitated diffusion processes, such as the galactose transport system in *S. cerevisiae*. According to Ramos and co-workers, the inducible high-affinity galactose-uptake process is lost in the absence of a functional galactokinase (Ramos *et al.*, 1989). This conclusion, however, was based on galactose-uptake measurements with an incubation time of 1 min. It is likely, under these conditions, that the build-up of internal

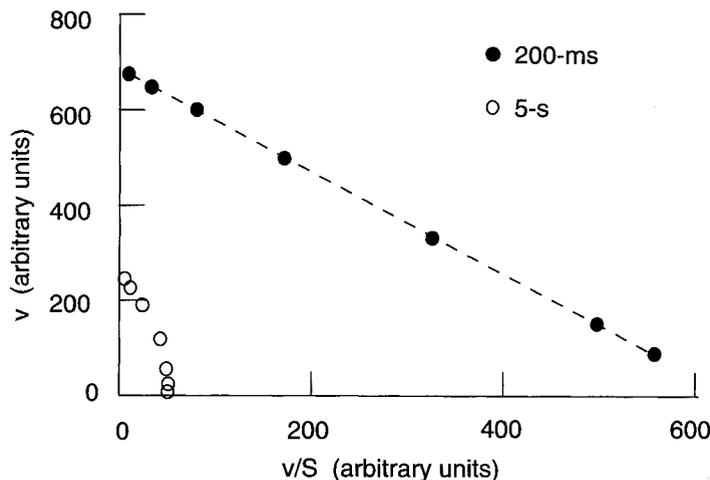


Figure 5. Eadie-Hofstee representation of a simulation of glucose transport according to the four-state carrier model. The data were generated with the modeling program SCAMP (Sauro, 1993). External glucose concentrations (G_o) ranging from 0.3 to 100 mM were used. The following set of parameters was used: $k_1=k_{-3}=0.7 \text{ ms}^{-1} \text{ mM}^{-1}$; $k_{-1}=k_3=0.05 \text{ ms}^{-1}$; $k_2=k_{-2}=k_4=k_{-4}=1 \text{ ms}^{-1}$; $E_i=E_o=0.007 \text{ mM}$.

galactose on this time-scale is high and, analogous to glucose uptake in the triple-kinase deletion mutant, results in reduced net galactose-uptake rates. Such effects on net galactose uptake may then obscure high-affinity galactose uptake in galactokinase-less yeast cells.

Of course some changes in the kinetic properties of the glucose transporter are true and not time-scale dependent. The apparent modulation of the kinetic properties of glucose uptake as a result of deletion of the hexose kinases, differs from the affinity modulation in wild-type cells during batch growth on glucose. In the latter case the changes in kinetic properties, i.e. the decrease in K_m but constant V_{max} , measured using the 5-s assay (Walsh *et al.*, 1994a), have been confirmed using the 200-ms assay (Smits and Walsh, unpublished results).

It should be noted that our results weaken the conclusion of Bisson and Fraenkel (1983a), who proposed that high-affinity glucose transport was dependent on an interaction between transporter and kinases. The latter explanation was supported by several authors, who reported that the kinetics of 6-deoxy-glucose uptake, a sugar that cannot be phosphorylated, was dependent on the presence of the hexose-kinases. It was shown that, in derepressed wild-type cells, 6-deoxy-glucose uptake, measured over 5 s, showed high-affinity kinetics, whereas in triple-kinase deletion mutants, measured on the same time scale, no high-affinity kinetics for glucose uptake could be detected (Romano, 1982; Bisson and Fraenkel, 1983). One would expect that in both situations the intracellular free sugar concentration builds up due to a lack of subsequent phosphorylation. An explanation for this could be that as a result of the relatively low affinity of 6-deoxy-glucose uptake, the effect of the build-up of internal 6-deoxy-glucose will be small after 5 s. This would reduce the inhibitory effect of the free internal sugar, i.e. 6-deoxy-glucose, on net transport, such that high-affinity glucose uptake is not obscured. Another possibility is, as was suggested earlier (Entian and Fröhlich, 1984), that the three hexose kinases have a secondary regulatory effect other than maintaining a low level of free internal glucose.

Interestingly, the main conclusion of this paper, namely that high-affinity transport is not dependent on the presence of the hexose kinases, is supported by data from the earlier study of 6-deoxy-glucose uptake in the triple-kinase deletion mutant (Bisson and Fraenkel, 1983a).

Examination of the results of these authors show that their data on the inhibition of 6-deoxy-glucose uptake by glucose are consistent with a K_i of 2 mM for glucose. The K_i for glucose inhibition of 6-deoxy-glucose uptake approximates the K_m for glucose uptake, assuming that glucose is a competitive inhibitor of 6-deoxy-glucose. Since such inhibition would take place externally and 6-deoxy-glucose is transported with a much reduced rate as compared to glucose, this K_i estimation would be less sensitive to the error we have demonstrated for the measurement of zero-*trans* glucose uptake. Therefore, it could also be concluded from this earlier study that galactose-grown cells of the triple-kinase deletion mutant do exhibit high-affinity glucose uptake.

In summary, a triple-kinase deletion mutant is a useful strain to study the mechanism of glucose transport, since subsequent removal of the intracellular sugar by metabolism is absent. Recently it has been proposed that at least ten putative transporters exist in *S. cerevisiae*, all presumably having their individual kinetic properties (Bisson *et al.*, 1987; Ko *et al.*, 1993; Kruckeberg and Bisson, 1990; Lewis and Bisson, 1991; Reifenberger *et al.*, 1995). It is important to realize that glucose transport has always been described in terms of apparent kinetic parameters. In our view, studying the initial kinetics of each putative glucose transporter separately (in a triple-kinase deletion background) is an important first step towards a full understanding of glucose transport in *S. cerevisiae*.

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