Exposing a complex metabolic system: glycolysis in Saccharomyces cerevisiae
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5.1 Summary:

In this study, the control exerted by hexose transport on the frequency and average flux during glycolytic oscillations was experimentally determined. In a first approach, hexose transport activity was decreased by catabolite-inactivation, i.e. by incubation in the presence of glucose without additional growth nutrients. In a second approach, the hexose transport activity was modulated by varying the extracellular substrate concentration. Limitations to both methods are discussed. The control on the frequency was neither 0 nor 1, confirming the theoretical analysis of chapter 2 that control on glycolytic dynamics need not be confined to a single limiting step. The control on the flux depended on the activity of the transporter and increased from 0.3 at high transport activities to 1 at low activities. The results are compared with, and confirmed by, other studies by means of co-response analysis.

\[\text{In collaboration with Lena Gustafsson, Karel van Dam, Hans V. Westerhoff and Michael C. Walsh}\]
5.2 Introduction

The analysis of three mathematical models of glycolytic oscillations demonstrated that the control on limit-cycle characteristics was shared by all enzymes that constituted the system (chapter 2). In this chapter we set out to experimentally verify the theoretical results of chapter 2, by measuring the control of the hexose transport step on the frequency of glycolytic oscillations. The same methodology was employed to determine the control on the average glycolytic flux during the oscillations.

Our focus on the hexose transport step is based on the following considerations. It has been shown in yeast extracts that the rate at which glucose or fructose was added to the extract, strongly affected the period of the oscillations [152]. It was expected therefore, that hexose transport should have a significant control on the frequency of the oscillations in intact cells. Moreover, glucose transport has been implicated in many studies to have a high control on the glycolytic flux [36] (see also chapter 1). These suggestions were based on a supposedly low intracellular glucose concentration compared to that of extracellular glucose ([111], but see Chapter 4), on the fact that the control appears not to reside in the glycolytic enzymes themselves [319] and on mathematical models of glycolysis [77, 78, 108]. So far, no direct measurements of the control exerted by the glucose transporter on the glycolytic flux have been described, apart from a semi-quantitative attempt by ourselves [357].

To measure the control exerted by the sugar transport step, the activity of transport should be modulated in some way. The consequent effect on the flux and the frequency should be compared to the magnitude of the modulation. We will discuss three possible approaches to modulate the transport activity.

The control coefficient is generally defined as:

$$ C_{ij}^{X_j} = \left( \frac{\partial \ln v_i}{\partial \ln p} \right) \left( \frac{\partial \ln X_j}{\partial \ln p} \right)_X = R_p^{X_j} / \varepsilon_p^{v_i} \quad (5.1) $$

where $X_j$ is the controlled variable, $v_i$ is the activity of the controlling enzyme and $p$ is a parameter that specifically affects $v_i$ [331]. The information needed to calculate the control coefficient, is the extent to which enzyme $i$ is affected by the parameter $p$ (the elasticity $\varepsilon_p^{v_i}$, evaluated at fixed steady-state concentrations of the variables which may also affect $v_i$ [178, 180]), and the extent to which the system variable $X_j$ is affected by that change in the parameter (the response coefficient R [178, 180, 307]).

The most straightforward parameter to change is the amount of enzyme. In ideal metabolism [192] the elasticity of an enzyme with respect to its concentration is 1, as the
enzyme concentration is a multiplier in the rate equation. Changing the amount of hexose transporters in yeast in a controlled way via the genetic approach became only feasible very recently (see chapter 1). Considering the dependence of the dynamic behavior of yeast glycolysis on the history of the cells [283, 300], however, this approach may be difficult. Hierarchical (or pleiotropic) effects [389, 393] of changes in hexose transporter activity (e.g. via altered glucose signaling [292, 382]) may hamper clear interpretation of the results.

An alternative way to modulate the number of carriers may be glucose-induced inactivation of glucose transport [8, 56, 210]. The process of glucose inactivation, however, involves not only glucose transporter proteins, but also gluconeogenic enzymes, the best studied of which is fructose 1,6-bisphosphatase [110]. Although perhaps not specific, this approach was used with the assumption that the gluconeogenic enzymes should not affect the net forward pathway flux in the glycolytic direction to a significant extent.

Another approach is to modulate the activity of transport by varying the concentration of extracellular substrate. This substrate is an external parameter that is amenable to experimental manipulations [97, 178]. In this case the parameter \( p \) in Eq. 5.1 corresponds to the external substrate concentration. Thus, measurement of the response coefficient of the flux or frequency with respect to the extracellular substrate concentration and the elasticity of the transporter with respect to that same parameter should allow the calculation of the control coefficient. In this approach we have used glucose and mannose as glycolytic substrates. Mannose was used because the hexose transport step has a much higher \( K_m \) for mannose than it has for glucose (see Results).

The third approach is to use specific inhibitors of glucose transport. These were not available until very recently, when it was shown that maltose competitively inhibits glucose transport in *Saccharomyces cerevisiae* without being itself transported by the glucose carrier ([85], M. Walsh, personal communication). Details of the latter approach will be described elsewhere; here only the results are summarized to complement the conclusions drawn by the other experiments.

### 5.3 Materials and methods

**Strain and growth conditions**

All experiments were carried out with the diploid *Saccharomyces cerevisiae* strain X2180. Cells were grown at 30 °C on 1 % glucose supplemented with 0.67 % YNB (Difco), buffered by 100 mM phtalic acid/KOH pH 5.0, until glucose exhaustion as determined with glucose sticks (Boehringer). Cells were washed in 100 mM potassium phosphate buffer pH 6.8, starved in the same buffer for 2 h at 30 °C and kept on ice until further use (see also [299]). When cells were
grown on mannose, a growth curve was constructed to determine the optical density (OD) at which the mannose had been depleted from the medium.

For the glucose inactivation experiments, cells were harvested at glucose depletion, washed in phosphate buffer, resuspended in phosphate buffer containing 1% glucose and incubated for different periods at 30 °C. After this incubation in the presence of glucose, the cells were washed, resuspended in phosphate buffer and starved for 1 h. Cells were harvested and put on ice until further use.

**Measurement of oscillations**

Oscillations were induced by addition of sugar to various concentrations followed 4 minutes later by addition of cyanide (final concentration 4 mM). Oscillations were monitored by NAD(P)H fluorescence of a yeast suspension in a thermostated and magnetically stirred cuvette of a Shimadzu RF 5001PC spectrofluorimeter (352 nm excitation; 462 nm emission; 10 nm band width) at various temperatures. The frequency of the oscillations was determined as the reciprocal of the time interval between subsequent maxima.

![Figure 1. Eadie-Hofstee plot of the zero trans-influx kinetics of glucose transport after glucose inactivation. Cells were incubated for 0 h (●), 1 h (□), 3 h (▲) or 5 h (○) in phosphate buffer containing 1% glucose, starved in phosphate buffer for an additional 1 h, and assayed for glucose transport activity.](image)

**Measurement of fluxes**

Fluxes were measured by taking samples at regular time intervals and quenching them in trichloroacetic acid (TCA, 5% w/v final concentration). The samples were then vortexed and put on ice. After centrifugation the supernatant was diluted in 100 mM Pipes buffer pH 7 and analyzed by NADH-linked enzymatic assays on an automated analyzer (COBAS, Roche) according to Bergmeyer [25]. For the measurement of mannose, the glucose assay was modified by addition of phosphoglucose isomerase and phosphomannose isomerase.

Measurement of the glycolytic flux by means of microcalorimetry was performed as detailed in chapter 3.

**Measurement of sugar transport kinetics**

Glucose transport kinetics were measured by 14C-labeled sugar uptake over a period of 5 s according to [383]. It was checked that in the case of mannose uptake, washing with 0.5 M
unlabeled glucose gave the same results as washing with 0.5 M unlabeled mannose. Kinetic parameters were estimated by nonlinear regression analysis of the uptake rate as a function of the substrate concentration, using Grafit 3.0 software and a one-component zero trans influx rate equation (Chapter 4, [349]) to fit the data.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>K_m (mM)</th>
<th>V_max (nmol min⁻¹ mg protein⁻¹)</th>
<th>Glucose consumption rate (nmol min⁻¹ mg protein⁻¹)</th>
<th>Heat flux (µW mg protein⁻¹)</th>
<th>frequency (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.78 ± 0.20</td>
<td>236 ± 10</td>
<td>125 (100%)</td>
<td>76 (100%)</td>
<td>1.28 (100%)</td>
</tr>
<tr>
<td>1</td>
<td>2.28 ± 0.12</td>
<td>226 ± 3</td>
<td>117 (94%)</td>
<td>72 (95%)</td>
<td>1.11 (87%)</td>
</tr>
<tr>
<td>3</td>
<td>3.73 ± 0.33</td>
<td>140 ± 6</td>
<td>86 (67%)</td>
<td>50 (66%)</td>
<td>0.84 (66%)</td>
</tr>
<tr>
<td>5</td>
<td>4.73 ± 0.57</td>
<td>82 ± 6</td>
<td>61 (49%)</td>
<td>36 (48%)</td>
<td>0.72 (56%)</td>
</tr>
</tbody>
</table>

Table I. Effect of glucose inactivation at different incubation times on the kinetics of glucose transport, the glycolytic flux (as measured by glucose consumption and heat flux) and the frequency of oscillations. Numbers in brackets are percentages of the control (0h incubation). Cells were incubated for the indicated period in phosphate buffer containing 1% glucose, starved in phosphate buffer for an additional 1h, harvested and put on ice until further use.

5.4 Results

Glucose inactivation of the hexose transporter

When yeast cells are incubated in the presence of glucose, but without a complete growth medium, glucose transport activity rapidly declines. This has been observed by Busturia [56], who measured a half-life of glucose transport activity of about 6 h. We compared the effect of glucose inactivation on the glucose transport kinetics, on the glucose consumption rate and on the frequency of glycolytic oscillations. All experiments were performed at 20 °C. In Fig. 1, the glucose transport kinetics are shown for cells which have been incubated in the presence of 1% glucose for 0, 1, 3 and 5 hours and subsequently have been starved in phosphate buffer for an hour. A decrease in maximal rate and in affinity for glucose was observed, depending on the time of inactivation. The kinetic parameters as estimated by nonlinear regression analysis, are shown in Table I. In five hours the V_max of glucose transport dropped threefold from 236 nmol min⁻¹ mg protein⁻¹ to 82 nmol min⁻¹ mg protein⁻¹. From the data in Table I a half life of the V_max of
glucose transport can be estimated to be 3.1 h. In Table I also the effect of glucose inactivation on the glucose consumption rate and on the frequency of glycolytic oscillations is shown. Upon increasing duration of glucose inactivation the glucose consumption rate decreased with a half life of about 4.7 h. The flux measurements after glucose-inactivation were repeated in the calorimeter. Upon incubation with glucose, the rate of heat production decreased to the same extent as did the glucose consumption rate (Table I).

The incubation with glucose also decreased the frequency of the oscillations with increasing incubation time (Table I; half life 5.9 h). It was observed, however, that glucose inactivation led to strong damping of the oscillations and that the frequency during the oscillations was slightly decreasing during the time-course of the oscillations. Such an initial decrease in frequency is always observed (see below), but in cells not incubated with glucose, but with phosphate buffer alone, the oscillation settled into a sustained limit-cycle oscillation (see also Fig. 6). In glucose-inactivated cells, however, the oscillation was damped out before this limit-cycle was reached. It was therefore not possible to determine the frequency of a limit-cycle oscillation; rather, the average frequency over the oscillatory period was calculated (and shown in Table I). Control cells (i.e., cells incubated for the same periods in phosphate buffer containing no glucose) did show sustained oscillations with the same (high) frequency as cells incubated for 1 h in phosphate only. The reason for the damping in glucose-inactivated cells is not clear. Most likely the glucose inactivation also changed other parameters than glucose transport activity, which may lead to damped oscillations. Possibilities are that the activity of gluconeogenic enzymes did affect the dynamics of glycolysis to a larger extent than expected or that the lower flux led to decreased acetaldehyde concentrations, hampering the synchronization of the oscillations. The glucose inactivation approach therefore may not be specific enough for our purposes.

Other parameters that may affect the dynamics of glycolysis are the accumulation of glycogen and trehalose observed during the incubation with glucose (results not shown). The presence of increased levels of storage carbohydrates after incubation with glucose is reflected in the endogenous heat production rate of glucose-inactivated cells prior to glucose and cyanide addition: it was about twice the heat flux of cells not incubated with glucose (results not shown).
Interestingly, for the 5 h incubation, the heat flux after addition of external glucose did only marginally increase, indicating that the cells switched to glucose utilization (Table I), but at a low rate comparable to the rate when they were utilizing endogenous resources.

In view of the results of chapter 4, it should be interesting to compare the maximal rate of glucose transport (i.e., the zero trans influx rate) with the actual glucose consumption rate. This is done in Fig. 2, where the $V_{\text{max}}$ of glucose transport is plotted against the ratio of the zero trans influx rate and the glucose consumption rate. As the $V_{\text{max}}$ decreased, this ratio increased and approached 1 for the lowest $V_{\text{max}}$ (5 h incubation). This result suggests that during the gradual decrease of the transport activity, the transporter gained control over the glycolytic flux (see chapter 4). It cannot be ruled out, however, that the effects were partly caused by other glucose-induced parameter changes. The fact that the flux is only 60% of the zero trans influx rate exhibited by the cells not incubated with glucose, fits with the results of chapter 4.

When the flux is plotted against the $V_{\text{max}}$ of transport in ln-ln space, a slope of 0.7 was found (result not shown). This slope is an indication of the average flux control coefficient over the range of $V_{\text{max}}$'es. A similar exercise for the frequency gave a slope of 0.5, indicating the frequency control coefficient.

Varying the external glucose concentration

A more specific way to modulate the activity of the hexose transporter is to vary the extracellular substrate concentration. We have performed microcalorimetric measurements under very similar conditions as used for the glucose inactivation approach (i.e., for cells starved for 2 h in phosphate buffer, given 20 mM glucose and 4 mM cyanide at 21 °C). In this approach, the glucose concentration is varied by the system itself, simply by consumption of the glucose. The assumption is that the changing external glucose concentration is a much slower variable than the internal variables, and that the system reaches a new “steady state” at each glucose concentration. The effect of the external glucose concentration on the flux can then be asserted. Under the conditions used, oscillations occur. The analysis presented below will therefore be concerned with the control of the average flux during oscillatory conditions.

In Fig. 3 the flux, the glucose concentration and the zero trans influx rate are plotted. The flux (glucose consumption rate) was calculated from the heat flux by assuming that during the experiment the flux distribution does not alter to a great extent. The ethanol formed per glucose consumed, the largest contribution to the overall heat flux (chapter 3), does not change during these experiments (results not shown; see, e.g. flux determinations in chapters 3 and 8). The fact that the change in the heat flux and the glucose consumption rate in Table I agree very well, support the idea that the heat flux is a good measure of the glucose consumption rate. With this assumption the rate of sugar disappearance $\frac{d[Glc]}{dt}$ can be computed from the heat flux $\frac{dQ}{dt}$ by using the total heat produced $Q_{\text{total}}$ per total of glucose consumed $[\text{Glc}]_{\text{total}}$.
The concentration of glucose was then calculated backwards from the point of glucose depletion (t=43 min in Fig. 3) using the rate of sugar consumption. The reason that we did not start from 20 mM downwards is that during the initial 4 minutes the heat flux does not represent the rate of glycolysis: no cyanide was present in this period, leading to much higher heat fluxes caused by respiration [20, 379]. The glucose concentration reaches the starting level of 20 mM within 10% error. The zero trans influx rate was calculated by inserting the calculated glucose concentration into the rate equation for zero trans-glucose transport, using the kinetics from the control cells (0 h incubation time, Table I). For glucose concentrations above 10 mM the flux was about 70% of the zero trans influx. As the concentration of glucose declined, the difference between maximal uptake rate and flux decreased, until at around the K_m value of the transporter(s) for glucose (1.8 mM) the flux equaled the zero trans-influx rate. Although small quantitative differences exist between the glucose-inactivation approach and the calorimetry-based analysis, the conclusions are the same: the zero trans influx and the actual flux converged at decreasing transport activity. This implies that also the control of glucose transport over the flux increased with decreasing transport activity. We conclude that the control of the glycolytic flux exerted by the glucose transporter is dependent on the conditions and -importantly- was significantly lower than 1 at a high (20 mM) glucose concentration.
This conclusion can be made quantitative as the control coefficient can be calculated by using the definition of control as given by Eq. 5.1. The required quantities are the flux response coefficient (i.e. the change in the flux caused by a change in the glucose concentration) and the elasticity coefficient (i.e. the change in the activity of the transporter by a change in the glucose concentration). The former can be readily calculated from Fig. 3, the latter is more complicated. The complicating factor is that the sensitivity of a facilitated diffusion carrier for external glucose may depend on the internal glucose concentration. The elasticity of the hexose transporter for extracellular substrate (the ln-ln derivative of the rate equation for hexose transport of chapter 4) is:

$$
\varepsilon_{s}^{v_{transport}} = \left( \frac{\partial \ln v_{transport}}{\partial \ln s} \right)_{p} = \frac{1}{1 - \Gamma} - \frac{s}{K_{m}} + K_{i} \frac{sp}{K_{m}^{2}}
$$

(5.3)
where $s$ is the concentration of extracellular glucose, $p$ is the concentration of intracellular glucose and $\Gamma$ is the mass-action ratio, $p/s$. Clearly, the elasticity is also a function of $p$. The effect of intracellular glucose should therefore be evaluated.

For high affinity transport, one should be able to deduce the intracellular glucose concentration from the zero trans influx rate and the glucose consumption rate (chapter 4). This deduced concentration, as well as the elasticity of the carrier for external glucose with and without taking internal glucose into account, are shown in Fig. 4, as a function of the extracellular glucose concentration. The internal glucose concentration appeared to have varied between zero at low extracellular glucose concentrations and 0.6 mM at high extracellular glucose concentrations. The intracellular glucose concentration oscillated whereas the external glucose concentration was buffered by the large volume of the medium. The effect of internal glucose on the elasticity is largest at the high glucose concentrations (>10 mM), where the difference between the elasticities for external glucose (with and without taking internal glucose into account) is about a factor 1.5.

In Fig. 5 the flux response coefficient, the elasticity coefficient (taking internal glucose into account) and the resultant flux control coefficient are shown as a function of the activity of the hexose carrier. The response coefficient in each point of Fig. 4 was estimated by taking the slope over a range of 2 min (1 min on each side), thereby reducing the effects of noise and the oscillations. The flux control coefficient was calculated from the ratio of the response and elasticity coefficients, according to Eq. 5.1. At activities above $0.7 \cdot V_{\text{max}}$, the oscillation in the flux started to interfere with the analysis, leading to strongly oscillating control coefficients. The glucose transporter gained control at decreasing glucose transport activity: the control shifted from about 0.3 at $0.8 \cdot V_{\text{max}}$ to close to 1 at around $0.4 \cdot V_{\text{max}}$. The decrease in control at the very low activity ($<0.1 \cdot V_{\text{max}}$) is most likely an artifact caused by the low heat flux signal, making the calculation of the glucose consumption rate highly sensitive to noise and the exact position of the heat flux baseline.

**Varying the external mannose concentration**

The preceding calorimetric analysis was based on the second approach outlined in the introduction: modulating the transport activity by changing the external substrate concentration. In the experimental set up we have used, the concentration of substrate simply decreased due to consumption. The high affinity of the carrier for glucose, however, limited the concentration range in which large changes in transport activity could be achieved. The approach of manipulating the external substrate concentration to assess the control exerted on the frequency, therefore, was not possible.

To study the control of the frequency, a larger (and slower) variation in the uptake rate was required. Accordingly, mannose was used as a substrate, because the affinity of the hexose transporter(s) for mannose is much lower than for glucose. The elasticity for external mannose
was therefore expected to be much higher at millimolar concentrations of this sugar. This should result in a wider variation in enzyme activity and consequently in a larger response in frequency to changes in the mannose concentration. Mannose enters glycolysis in a very similar way as glucose: it is transported by the same carriers, phosphorylated by all three sugar kinases (hexokinase PI, PII and glucokinase) and converted into fructose 6-phosphate by phosphomannose isomerase [261] (as compared to phosphoglucone isomerase for glucose).

Unfortunately, cells grown on glucose exhibited strongly damped oscillations with mannose as a substrate. Cells grown on mannose and harvested at mannose depletion, however, did show limit-cycle oscillations. The cause of this intriguing result is unclear and may be strain dependent, as other Saccharomyces strains have been reported to exhibit mannose-induced oscillations when grown on glucose [205].

In Fig. 6 the frequency of the oscillation is shown during a train of oscillations induced by 40 mM mannose and 4 mM cyanide. The oscillations lasted for more than 2 hours. For comparison, we have included two frequency time-courses of glucose-induced oscillations in different batches of glucose-grown yeast cells. In the latter experiments 20 mM glucose was given at time zero, followed by 4 mM cyanide after 4 minutes. (The differences in duration of these experiments were due to differences in cell density.) An initial drop in frequency was observed that is characteristic for all oscillations observed so far, also with glucose-induced oscillations in cells grown on mannose. The oscillations reached a constant frequency after some
10 minutes. During the transient phase of most experiments the amplitude of the oscillations decreased as well. After a few cycles the amplitude recovered and the system settled in a limit-cycle, with a constant amplitude (not shown) and frequency (Fig. 6). Although the transient phase may not always be exactly reproducible (especially the time course of the amplitude may vary between batches of cells), the limit-cycle is. This is clearly illustrated for the two glucose experiments shown in Fig. 6. For the mannose oscillation, a constant frequency was observed between 10 and 60 minutes after mannose addition, indicating limit-cycle behavior.

Having established mannose-induced limit-cycle behavior, we followed the substrate-modulation approach by measuring the response of the frequency of the oscillations to a decrease in the mannose concentration, and by measuring the elasticity of the hexose transporter to extracellular mannose. To calculate the latter, we have measured the mannose uptake kinetics in mannose grown cells, at 20 °C, i.e. the temperature of the oscillation experiments (Fig. 7). For comparison, the glucose uptake kinetics under these conditions were also measured. For mannose transport, the $V_{\text{max}}$ was $174 \pm 8 \text{ nmol min}^{-1} \text{mg protein}^{-1}$ and the $K_m$ was $22.5 \pm 1.6 \text{ mM}$. For glucose transport, these parameters were $255 \pm 7 \text{ nmol min}^{-1} \text{mg protein}^{-1}$ and $1.9 \pm 0.1 \text{ mM}$, respectively. The latter values correspond well with the values found for glucose transport at 20 °C in glucose grown cells (Table I). Mannose obviously is a much poorer substrate for the hexose transport system than is glucose.

After establishing the kinetics of mannose transport in mannose-grown cells, we induced oscillations with 20 mM mannose and monitored the frequency and the mannose concentration in time (Fig. 8). Again a rapid initial decrease in frequency was observed after which the frequency slowly decreased as the mannose concentration in the medium was consumed. Assuming that the decrease in mannose concentration was slow compared to the adjustment of the glycolytic dynamics to this parameter change, the response of the frequency to the mannose concentration could be computed.

To calculate the frequency control coefficient of the hexose carrier from the frequency response coefficient towards extracellular mannose, the elasticity of the hexose carrier to the
extracellular mannose concentration is needed (Eq. 5.1). Again, this elasticity may depend on the product of mannose transport, the intracellular mannose concentration. To estimate this concentration, the zero trans influx rate was compared with the mannose consumption rate. The mannose consumption rate, however, is not constant, but rather, decreased with time (Fig. 8). To estimate the mannose consumption rate in each time point, the tangent in each point of the time course of the mannose concentration was computed by a cubic spline algorithm. The fitted mannose consumption rate was 2.5 times higher than the zero trans-influx rate (calculated with the mannose transport kinetics and the mannose concentration), irrespective of the mannose concentration. The reason for this discrepancy is unclear, but it resembles the problem encountered with low-affinity glucose transport in chapter 4. Also there the actual glucose consumption rate was higher than the zero trans-influx rate. It was suggested in chapter 4 that acceleration of glucose consumption may have occurred, perhaps by positive cooperativity with intracellular glucose, perhaps by other positive effectors that act on a longer time-scale than 5 seconds. In the case of mannose, a similar and even stronger activation of the transporter would be required to explain the higher mannose consumption rate compared to the uptake rate assayed over 5 s.

We appear to have observed such an activation process with microcalorimetry: when mannose-grown cells were given 20 mM mannose and 4 min later 4 mM cyanide, the following events were observed. First, the heat flux increased, followed within a minute by a rapid decrease. The rapid decrease was caused by the cells having consumed the oxygen: respiration is much more exothermic than anaerobic glycolysis ([20], see chapter 3). After the drop in heat flux, the heat production accelerated (the rate of which was unaffected by the addition of 4 mM cyanide) for some 10 min, then decreased again until a basal level was reached after 60 min. In Fig. 9, using the same calculation as done for Fig. 3, the mannose consumption rate was calculated as a function of time from the heat flux time-course as just described. (The spike at t=6 min is the air accompanied by the addition of cyanide; the second spike is air only.) The acceleration of the flux between t=3 min and t=11 min is about 2.5-fold. In the same figure the mannose concentration is needed (Eq. 5.1). Again, this elasticity may depend on the product of mannose transport, the intracellular mannose concentration. To estimate this concentration, the zero trans influx rate was compared with the mannose consumption rate. The mannose consumption rate, however, is not constant, but rather, decreased with time (Fig. 8). To estimate the mannose consumption rate in each time point, the tangent in each point of the time course of the mannose concentration was computed by a cubic spline algorithm. The fitted mannose consumption rate was 2.5 times higher than the zero trans-influx rate (calculated with the mannose transport kinetics and the mannose concentration), irrespective of the mannose concentration. The reason for this discrepancy is unclear, but it resembles the problem encountered with low-affinity glucose transport in chapter 4. Also there the actual glucose consumption rate was higher than the zero trans-influx rate. It was suggested in chapter 4 that acceleration of glucose consumption may have occurred, perhaps by positive cooperativity with intracellular glucose, perhaps by other positive effectors that act on a longer time-scale than 5 seconds. In the case of mannose, a similar and even stronger activation of the transporter would be required to explain the higher mannose consumption rate compared to the uptake rate assayed over 5 s.

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concentration was calculated from the mannose consumption rate, and the zero \textit{trans}-influx rate was computed from the mannose concentration. The failure of the zero \textit{trans}-influx kinetics to describe the flux properly is obvious: on average, the flux was about 3 times higher than the zero \textit{trans}-influx rate. Interestingly, however, the zero \textit{trans} influx rate was close to the flux prior to the acceleration at $t=3$ min. These results demonstrate the superiority of microcalorimetry to measure flux as compared to sampling of the sugar concentration in time: even though the acceleration is visible in the time course of the mannose concentration, the deviation is only small (Fig. 9). It is therefore not surprising that it was not detected in Fig. 8.

![Figure 9](image)

**Figure 9.** Mannose consumption rate, mannose concentration and zero \textit{trans} influx rate as computed from microcalorimetric data. See legend of Fig. 3 and text for explanation.

Importantly, the process that led to increased mannose transport activity may also affect the elasticity of the hexose transporter with respect to the extracellular mannose concentration. This complicates the calculation of the frequency control coefficient. On the assumption that this effect is small, however, the frequency control coefficient can be directly determined as the slope of a \textit{ln-ln} plot of the frequency against the zero \textit{trans} influx activity (Fig. 10). The resulting frequency control coefficient was found to be 0.7 on average (the highest points were part of the transient phase and were not taken into account when computing the control coefficient). A similar exercise was done for the mannose consumption rate, yielding a flux control coefficient of 0.9 (Fig. 10).
In this chapter two methods have been employed to modulate the activity of the hexose transporter and to study its effect on frequency and flux. The first method was based on glucose-inactivation of hexose transport. The second method was based on variation of the nature and concentration of the extracellular substrate. These approaches were only partially successful. Glucose inactivation has led to damping of the oscillations, possibly by pleiotropic effects of glucose-inactivation. The substrate modulation approach used with glucose and mannose as substrates, should be specific, but the analysis is complicated by the need to address internal regulation of the transporter when calculating the elasticity of the transporter towards the extracellular substrate. This complication made the approach less straightforward than initially realized. The results of both methods can be interpreted by means of co-response analysis, however, which will circumvent the above-mentioned difficulties (see below).

Glucose inactivation of glucose transport: real or only apparent?

The glucose inactivation approach appears to lack specificity. No limit-cycle behavior was observed after incubation with glucose. Gluconeogenic enzymes are known to be subject to glucose inactivation as well [107, 168, 247]. For fructose 1,6-bisphosphatase, it was shown that phosphorylation of the enzyme occurred, followed by ubiquitination and subsequent proteolytic degradation [327]. Also sugar transporters such as the maltose permease and the galactose carrier are subject to catabolite inactivation via proteolytic degradation [52, 169, 225, 296]. An important parameter that changed during the inactivation process, was the content of storage carbohydrates (see also [329] and references therein). With large amounts of glucose but no other nutrients for growth, it does seem a sensible thing to scavenge the glucose in the form of glycogen and / or trehalose. The amount of glucose that can be stored, however, is not unlimited. Reduction in the fermentation rate at long periods of glucose-incubation may be
caused by this limitation. One way of reducing the fermentation rate may be via inactivation of the transporter, as suggested by [329]. However, alternative ways of reducing the fermentation rate are quite feasible and cannot \textit{a priori} be excluded. One of these may be related to trehalose 6-phosphate synthase-mediated feedback inhibition on hexokinase ([363]; see also chapter 6). Importantly, such a mechanism of reducing the glycolytic rate may manifest itself in the initial uptake kinetics. The decrease in limiting rate and affinity of the carrier for glucose during the inactivation process may therefore only be apparent and caused by the transport assay failing to correspond to the true zero \textit{trans} influx kinetics. This serious complication of the glucose inactivation approach will be explored further in the next paragraphs.

Zero \textit{trans} influx measurements do only reflect the true kinetics of the transporter if the transporter is not feedback regulated to a significant extent. As discussed in chapter 4, for high-affinity transport, feedback is likely to be mediated by internal glucose. If this model is correct, the regulation of the transport rate is mediated by the effect of metabolism on the intracellular glucose concentration. This concentration should be low (i.e. much lower than the affinity of the carrier for internal glucose) during the 5 s of the uptake assay.

The fact that in chapter 4 the steady-state flux was found to be only half the rate of the zero \textit{trans} influx, indicates that some metabolic processes are active on a time scale longer than 5 s. They affect the internal glucose concentration (rising from low during the first 5 s to 1.5 mM at steady state) and hence the transport activity (which decreases from the zero \textit{trans} influx rate to the steady state flux). When cells have accumulated storage carbohydrates, however, the situation may be quite different. The cells incubated for 5 h with glucose showed a heat flux prior to glucose addition that was not much lower than after the addition of glucose. In these cells the mechanisms acting to reduce the transport rate may be fully active when the (labeled) glucose is added in a zero \textit{trans} uptake assay. The situation may therefore be comparable with energetically compromised cells [384] in that the activity of hexokinase may not be sufficient for adequate zero \textit{trans} influx measurements. Consequently, an alternative explanation for the fact that the zero \textit{trans} influx kinetics were equal to the steady state consumption rate for the 5h incubation (Fig 3), is that the measured transport kinetics did not correspond to the true zero \textit{trans} influx kinetics, rather that the transporter controlled the flux.

We have checked this possibility by computer simulation: is it possible to find the apparent decrease in \( V_{\text{max}} \) and affinity between control cells and 5 h incubated cells by a decrease in the activity of the hexokinase step only? We constructed a simple model with a glucose transporter followed by removal of the transported glucose by hexokinase (see Appendix). The rate equations were based on the glucose transport kinetics and the kinetics of hexokinase as detailed in chapter 8 (with a saturating ATP concentration and zero product concentrations). If the kinetics of transport were unaffected by glucose-inactivation, the intracellular glucose concentration should be 3.4 mM to accommodate the transporter to the observed flux of 61 nmol min\(^{-1}\) mg protein\(^{-1}\). Thus, the hexokinase step should have the same activity at that glucose
concentration. This allows any of a number of combinations of $V_{\text{max}}$ and $K_m$ values for the hexokinase step. The results were similar for either a high $V_{\text{max}}$ and low $K_m$ or vice versa: a decrease in apparent $V_{\text{max}}$ of some 40% and a decrease in apparent affinity of some 20% was calculated. Thus, even though reduced hexokinase activity does affect the apparent uptake kinetics (and in the right direction), it does not appear to be sufficient to describe the observed threefold decrease in $V_{\text{max}}$ and twofold change in affinity (Table I).

The extent of glucose inactivation of the carrier is therefore uncertain. The studies on catabolite inactivation of hexose transporters by the group of Lagunas [56, 210] were based on measurement of the uptake of xylose at 170 mM for 30 sec. It is unlikely that uptake of xylose at such long time intervals properly reflects true glucose transport kinetics. Xylose is not phosphorylated and in 30 s significant accumulation of intracellular xylose should occur. The intracellular xylose will inhibit the rate of xylose uptake (indeed, concave Eadie-Hofstee plots were observed for xylose kinetics when assayed for 5 s [Walsh, personal communication], similar to the kinetics of glucose uptake in a kinaseless mutant, cf. [343]). The extent to which this inhibition is significant depends on the rate of xylose uptake.

The results with xylose do show that the hexose transport activity is affected by catabolite-inactivation, but the extent to which this occurs is not certain. From this work and from the modeling described above, it appears that the hexose transporters are inactivated to some extent. The presence of (high concentrations of) trehalose and glycogen probably enhanced the apparent inactivation of glucose transport when measured with glucose as the substrate. Indeed, Busturia found a half life for the $V_{\text{max}}$ of hexose transport of about 6 h, whereas we found a half life of 3.1 h.

With so many unknowns, the extent to which hexose transport is really inactivated should be measured on the protein level, an approach that can be followed for an increasing number of HXT proteins [A. Kruckeberg, personal communication]. An estimation of the extent to which the kinetics have been altered, either genuinely by the glucose-inactivation process or only apparently by the limitations of the measurements of the uptake kinetic, may also be made by measuring the steady-state intracellular glucose concentration. If the true zero trans-influx kinetics had been measured in the 5 h incubated cells, the intracellular glucose concentration should be very low. If the kinetics were simply wrong, the intracellular glucose concentration should be around 3 mM. These experiments have not yet been done, however, as the original purpose of the glucose inactivation exercise was to measure the effect of altered transport activity on the flux and on the frequency of glycolytic oscillations. The strong damping of the oscillations already indicated that this approach was not specific enough and was abandoned. The analysis given above is therefore an invitation to workers in the field of regulation of glucose transport to reinvestigate to what extent the documented glucose-inactivation of glucose transporters is real or not. For example, the role of the cAMP-RAS pathway in catabolite-inactivation of the hexose transporters, shown by Ramos and Cirillo [285], may be complicated.
by the fact that this pathway also has profound effects on the metabolism of trehalose and glycogen [362].

It should be clear that the uncertainty in the extent to which the hexose transport activity has been inactivated makes the calculated flux and frequency control coefficients uncertain. This problem can be solved, however, by resorting to co-response analysis, as will be done shortly.

**Varying the external substrate concentration**

A substrate-modulation approach was used to determine the flux and frequency control coefficients of the hexose carrier. In this study, the substrate concentration was varied simply by consumption of the extracellular substrate. Our assumption, that the changing substrate concentration is a slow variable compared to the internal variables, is reasonable considering the fact that the flux through glycolysis is in the order of tens of millimoles min$^{-1}$ L cytosol$^{-1}$ whereas the rate of substrate disappearance from the medium is in the order of 0.5 millimoles min$^{-1}$ L$^{-1}$. The cytosolic flux is based on the following calculation: 1 mg protein corresponds to 3.75 μL cytosol (chapter 4), so that a rate of 100 nmol min$^{-1}$ mg protein$^{-1}$ amounts to 27 mmol min$^{-1}$ L cytosol$^{-1}$.

The important complication in the substrate-modulation approach is the assessment of the elasticity of the hexose carrier to the extracellular substrate concentration. For high affinity glucose transport, the symmetrical model of chapter 4 seemed appropriate for evaluating the effect of internal glucose on the elasticity of the carrier for external glucose. It yielded quantitative agreement between the kinetics, the concentration of glucose inside and outside, and the glucose consumption rate. For glucose therefore, we were able to correct for effects of intracellular glucose on the elasticity of the carrier for extracellular glucose.

For mannose, however, such a simple kinetic model failed to describe the mannose consumption and we had to resort to ignoring effects of internal regulation on the elasticity. In this case a definite analysis awaits further kinetic information about the apparent activation of mannose transport.

The failure of the zero trans influx kinetics to describe the rate of mannose consumption was also seen for glucose consumption in cells with low-affinity kinetics (chapter 4). It is interesting that in mannose-grown cells glucose transport exhibits high-affinity kinetics (Fig. 7). It appears therefore that high-affinity glucose carriers (such as HXT7 [292]) can also be subject to some activation process when faced with a poor substrate.

The observed activation of glycolysis during mannose consumption (Fig. 9) is very interesting. The fact that initially the zero trans-influx rate of mannose was equal to the mannose consumption rate is important, because it suggests that the discrepancy between transport rate and flux was not caused by a failure of the transport assay to measure true zero trans-influx kinetics, but was due to some unknown but slow activation process.
The extent to which the glucose transporter controls the glycolytic flux depends on the extracellular glucose concentration

With the knowledge obtained in chapter 4 we could analyze calorimetric data to calculate the control of the glucose transporter on the average flux during oscillations. It has been suggested that this control may be high. We found that the flux control of glucose transport increased with decreasing glucose concentration and hence, with decreasing transport activity. In general, a flux control coefficient of a particular enzyme is expected to increase from 0 at high activity to 1 at low activity [178, 180] (although not necessarily [188]). The extent to which control over the flux is exerted by the glucose transporter followed this trend: at high glucose concentrations the carrier has a high activity and little control over the flux, at low glucose concentrations it has low activity and becomes more flux-controlling. It is therefore not useful to discuss the control of the glucose transporter over the flux. Rather the flux control of the transporter should be evaluated for the conditions in which one is interested. Considering the different conditions used in this study as compared to the study of Schaaff et al [319], it is therefore not possible to conclude whether the elusive control in their study is in glucose transport or not. Other (and possibly more likely) candidates are steps outside glycolysis, such as ATP-consuming processes. It is notable, however, that in yeast the affinity of the carrier increases as the concentration of glucose decreases. One interpretation of this phenomenon is that this affinity modulation ensures a constant control exerted by the carrier over the glycolytic flux.

Control of frequency does not reside in a single step

Although exact quantification of the frequency control coefficient is hampered by the uncertainty in the elasticity of the hexose transporter for the extracellular mannose concentration, the experimental results indicate that the control is neither 0 nor 1. Results from maltose inhibition of the hexose transporter confirm this conclusion: the control of the frequency of glucose-induced oscillations in glucose-grown cells was found to be between 0.2 and 0.5 [C. Reijenga, personal communication].

These results are also in line with experimental data on cell-free extracts, where the period of the oscillation was found to depend on the substrate injection rate [152]. In such a system, the transport process has been substituted for by the addition of substrate at a fixed rate. The glycolytic flux is therefore completely controlled by the speed of the pump. Plotting Hess' original data in ln-ln space, the control of the injection rate on the frequency can be calculated to be 0.5.

These results may explain the change in frequency observed when other sugars such as fructose and mannose were used in intact cells [205]. The fact that, in extracts, injection of fructose at the same rate as glucose gave the same period [152], suggests that not the substrate
itself, but the rate at which it is transported, will affect the frequency. The decrease in frequency with other substrates than glucose can therefore be caused by the lower affinity and limiting rate of the transporter for those sugars (this study, [36]). More exotic explanations, focused on inhibition of the supposed oscillophore phosphofructokinase by sugar-specific metabolic products [203, 205], may not be required.

Comparing results: co-response analysis of flux and frequency

The problem of exact quantification of the extent to which a step is affected by a parameter (the elasticity \( \varepsilon_p \) in Eq. 5.1) is not new in the field of Control Analysis: inhibitor titrations can suffer from the same problem (see [13] for a good example). However, the results in this chapter can be compared and related to the results in cell-free extracts without exact quantification of the frequency control coefficient, by making use of co-response analysis [161]. A co-response is defined as the ratio of two response coefficients with respect to the same parameter change: it compares the relative sensitivity of one variable to a change in a parameter, with that of another variable to the same parameter change [162] (see also chapter 1). The relevant variables to compare in this study are the frequency and the flux. For extracts, the flux response coefficient with the substrate injection rate being the parameter is necessarily 1 (the injection rate completely controls the flux). The co-response coefficient is therefore the same as the frequency control coefficient: 0.5 (defining the co-response as the frequency response divided by the flux response). This value of the co-response coefficient can be compared to ours: the co-response coefficient for our data can be directly calculated from the slope of a ln-ln plot of the flux against the frequency [161]. This resulted in a co-response of 0.8 in the case of glucose-inactivation of the glucose transporter. For mannose-induced oscillations the co-response of flux and frequency was 0.7. The experiments with maltose as specific inhibitor of the glucose transporter gave a co-response of 0.6 [C. Reijenga, personal communication].

As the sum of flux and frequency control coefficients both sum to 1, a parameter change that would affect all enzymes to the same extent, should result in a co-response coefficient of 1. A comparison of the rate of ethanol production and the frequency of glycolytic oscillations at different temperatures (20, 25, 30 and 35 °C), yielded a co-response of 1.1 [M. Hemker, personal communication]. Temperature should affect all enzyme activities (although not necessarily to the same extent). A co-response close to 1 was also found when glucose-induced oscillations in Saccharomyces cerevisiae MC996 cells, grown on either glucose or galactose, were compared [294]. The parameter changed in this experiment, the carbon source during growth, is likely to have many effects on the enzymes in glycolysis. For one, glucose induction of many glycolytic enzymes will be absent in galactose grown cells [66].

The co-response coefficients with the hexose transporter being specifically affected, were 0.7 for mannose-modulation and 0.6 for maltose inhibition. These numbers are in line with the 0.5 calculated for the data in cell-free extracts. This co-response analysis enforces the conclusion
that control of frequency does not solely reside in the hexose transporter. If the frequency control coefficient of the hexose transporter were 1, the co-response of frequency and flux could only be smaller than 1 if the hexose transporter were to have a flux control coefficient larger than 1. This is extremely unlikely. Thus, even though the exact calculation of the individual frequency control coefficients was hampered by the uncertainty in $\epsilon_p^i$, from co-response analysis we can conclude that the frequency control should be significantly lower than 1. According to the frequency summation theorem (chapter 2), the sum of the frequency control coefficients should be 1. It necessarily follows that other enzymes in the cell should also control the frequency to some extent. This conclusion confirms the results of chapter 2, that the control of the frequency of glycolytic oscillations need not solely reside in a single step such as the "oscillophore", but that the control can be shared by the enzymes that constitute the pathway.

5.6 Appendix: simulating an uptake assay

We constructed a simple model for estimating the impact of hexokinase activity on the apparent kinetics of glucose transport as measured by labeled glucose incorporation. The model consisted of four differential equations for the variables (intracellular) glucose and glucose 6-phosphate and their labeled counterparts:

$$
\frac{d[Glc]_{in}}{dt} = v_{transport} - v_{HK} \\
\frac{d[Glc^*]_{in}}{dt} = v_{transport^*} - v_{HK^*} \tag{A5.1} \\
\frac{d[G6P]}{dt} = v_{HK} \\
\frac{d[G6P^*]}{dt} = v_{HK^*}
$$

where the asterix (*) indicates the labeled compounds and the activities of the transporter and hexokinase for these compounds. The following rate equations were used:
The rate equation for sugar transport with competition between unlabeled and labeled substrates and products was taken from [106]. It is identical to the one described in chapter 4 with the interactive constant \( K_i \) being 1 rather than 0.91. The kinetics for transport were taken from the control cells (Table I). The kinetics for hexokinase were adjusted within the constraints described in the main text. A ratio for intracellular to extracellular volume was taken as 0.02.

The differential equations were integrated for 5 s with the modeling software SCAMP [315]. Total label incorporation in glucose and glucose 6-phosphate over 5 s was taken as a measure of glucose transport activity. Kinetics measurements were simulated over a range of glucose concentrations, using the same specific activity of label as used in the experiments.