Adaptation of the Kinetics of Glucose Transport to Environmental Conditions in the Yeast Candida utilis CBS 621: a Continuous-culture Study

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The relation between the kinetic parameters of glucose transport and the physiology of Candida utilis CBS 621 was studied in chemostat cultures. In glucose-limited cultures the transport parameters were dependent on the growth rate of the yeast. Three different transport systems were found which differed by an order of magnitude in their affinity constants, namely a high-affinity \( (K_m, 25 \text{ \mu M}) \), a medium-affinity \( (K_m, 190 \text{ \mu M}) \), and a low-affinity uptake system \( (K_m, 2000 \text{ \mu M}) \). Cells growing at a dilution rate of 0.45 h\(^{-1}\) or less had the high- and medium-affinity uptake systems. At a dilution rate of 0.52 h\(^{-1}\) the high-affinity system was absent and both the medium- and low-affinity systems were present. At a dilution rate close to \( \mu_{\text{max}} \) (0.57 h\(^{-1}\)) only the low-affinity system was detected. The \textit{in situ} contribution of each of the transport systems to glucose consumption in glucose-limited cultures was estimated on the basis of their kinetic parameters \( (K_m, V_{\text{max}}) \) and the residual glucose concentration in these cultures. The sum of the calculated rates of transport corresponded to the \textit{in situ} rate of glucose consumption by the cultures as determined from the yield constant and the dilution rate. The dependence of the transport parameters on the growth rate and hence on the environmental sugar concentration was also evident in cells grown under nitrogen limitation. In contrast to carbon-limited cells, nitrogen-limited cultures growing at \( D = 0.15 \text{ h}^{-1} \) did not exhibit the high-affinity glucose uptake system, whereas the medium- and low-affinity systems were present.

INTRODUCTION

Glucose can be transported into yeasts via three different uptake mechanisms: by facilitated diffusion (Romano, 1982); by active transport through a proton–glucose symporter as shown for species of Candida (Spencer-Martins & van Uden 1985a) and Rhodosporidium (Hauer & Höfer, 1982; Höfer & Misra, 1978); or by a group translocation process in which the glucose is phosphorylated during transport (van Steveninck \textit{et al.}, 1985). In a variety of cases the dependence of the rate of transport on the assay sugar concentration yielded a biphasic kinetic plot indicating the presence of two uptake systems (Bisson & Fraenkel, 1983; Spencer-Martins & van Uden, 1985a; van den Broek \textit{et al.}, 1986).

A comparison between the results of different studies in quantitative terms is complicated by the fact that in nearly all cases cells from batch cultures have been studied. Frequently even shake-flask cultures have been used. In such cultures growth is intrinsically oxygen-limited and leads to ethanol production (Rieger \textit{et al.}, 1983; van Dijken & Scheffers, 1986). As a result, at the time of harvesting, cells to be used for assays of glucose transport may even be growing on ethanol.

Apart from the oxygen concentration, the glucose concentration is also a decisive parameter for sugar metabolism in yeasts. Yeasts which exhibit a Crabtree effect, such as Saccharomyces cerevisiae, perform alcoholic fermentation in the presence of excess sugar despite a sufficient
supply of oxygen (van Dijken & Scheffers, 1986). Under these conditions many respiratory enzymes, and transport systems as well, are subject to catabolite repression (Busturia & Lagunas, 1985, 1986; Spencer-Martins & van Uden, 1985a, b). It is thus not surprising that different results have been obtained in transport studies with yeast species grown in batch culture.

The advantages of using continuous cultures rather than batch cultures are several: not only are the conditions well defined and easily reproduced, but cells can also be grown at low sugar concentrations, thus avoiding catabolite repression. In this study we have quantified the kinetic parameters of glucose transport in the yeast Candida utilis grown in a chemostat. The results reveal that three different transport systems, all under environmental control, may be present in this yeast.

**METHODS**

*Micro-organism and growth conditions. Candida utilis CBS 621 was maintained on malt-agar slopes. The organism was grown at 30°C in a laboratory fermenter with a 1 litre working volume. The dissolved-oxygen tension was recorded with a steam-sterilizable Clark-type oxygen electrode, and was kept above 50% air saturation. The pH was controlled by automatic addition of 2 m-KOH. The medium was prepared according to Bruinenberg et al. (1983). The organism was grown under glucose limitation (S, 5 g l⁻¹) with ammonium as the nitrogen source, or under ammonium limitation (S, 0.4 g l⁻¹) with glucose as the carbon source (S, 5 g l⁻¹), or under glutamate limitation (carbon limitation without ammonium) (S, 5 g l⁻¹).

Measurements of residual free-glucose concentration in continuous culture. Cells were rapidly (within 3 s) transferred from the culture into liquid nitrogen. The frozen cell suspension was thawed to 0°C at room temperature, and then centrifuged in an Eppendorf Microcentaur centrifuge at 4°C (2 min, 13000 r.p.m.). The glucose concentration in the supernatant was determined with a Boehringer glucose kit (enzymic hexokinase/glucose-6-phosphate dehydrogenase/UV method).

*Binding of glucose to cells and filters.* In order to estimate substrate binding to cells, the cells were killed by heating. This procedure was adopted since dilution of untreated cells at zero-time (Konings & Freese, 1972), followed by addition of labelled substrate, gave higher binding values, probably due to high metabolic activity. Also blocking metabolic activity by fixation in 2 m-trichloracetic acid (TCA) resulted in increased binding.

A cell suspension (10 ml) was cooled on ice for 5 min and put in a boiling water-bath for 1 min, or 2 min for nitrogen-limited cultures, and rapidly cooled on ice. After this procedure no endogenous oxygen consumption could be observed in a biological oxygen monitor, nor was glucose-dependent oxygen uptake detectable. This procedure yielded cells without apparent changes in the structure of cell wall or plasmalemma as observed by light and electron microscopy. Furthermore, no protein was released from the cells as a result of this treatment. Binding was determined at the desired glucose concentration in duplicate; in all cases it was a linear function of the glucose concentration.

*Transport assay.* Cells were harvested from the continuous culture and directly used for transport experiments. At high dilution rates (D > 0.4 h⁻¹) the cells were washed (1 min, 3000 r.p.m.; bench centrifuge), and resuspended in mineral salts solution [5 g (NH₄)₂SO₄ l⁻¹, 3 gKH₂PO₄ l⁻¹, 5 g MgSO₄, 7H₂O l⁻¹, pH 5] to give the same optical density as the original culture. Nitrogen-limited cultures were washed twice following the same procedure. Uptake experiments were done within 30 min of harvesting (washing) of the cells. During the experiments the cells were aerated by rapid magnetic stirring at room temperature. Cell suspension (100 μl; 2.5 g dry weight l⁻¹) was added to flat-bottom glass tubes (diameter 1 cm, height 3 cm), and placed in a thermostated metal block with a magnetic stirrer (30°C). During the uptake experiments the cell suspension was rapidly stirred by 7 mm long Teflon stirrer bars. After temperature equilibration for 2 min the reaction was started by addition of the labelled substrate ([U-¹⁴C]glucose; 3 mCi mmol⁻¹ [111 MBq mmol⁻¹]). The reaction was stopped by dilution of the suspension with 2 ml 0.1 M-LiCl at 4°C, and the mixture was filtered (nitraccellose; 0.45 μm pore size, 35 mm diameter) within 5 s. The filters were washed with 2 ml 0.1 M-LiCl at 4°C, and put in glass scintillation vials with 5 ml scintillation fluid (Emulsifier Scintillator 299). After 30 min (clearing of filters) the radioactivity was measured in a Beckman LS 3801 bench-top scintillation counter. In all experiments the mean of three 5 s replicate analyses was determined. The statistical variation in the determination of each point is approximately 20%. Hanes plots were based on the mean of three independent measurements at each single substrate concentration (Hanes, 1932). In all cases the rate of uptake was proportional to the cell density between 0.4 and 2.5 g dry wt l⁻¹.

*Hexokinase assay.* Cells were harvested from continuous culture, washed once with buffer (10 mM-potassium phosphate, pH 7.5, 2 mM-EDTA), concentrated 5-fold in sonication buffer [100 mM-potassium phosphate, pH 7.5, 2 mM-MgCl₂, 2 mM-dithiothreitol (DTT)], and sonicated at 0°C in an MSE 150 W sonicator. Cell debris was removed by centrifugation (15 min, 20000 r.p.m.; Sorvall RC-5B centrifuge, SS-34 rotor). The assay mixture contained (in a final volume of 1 ml): hexokinase (EC 2.7.1.1); imidazole/HCl buffer, 50 mM, pH 7.6; NADP⁺,
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1 mM; MgCl₂, 10 mM; and glucose-6-phosphate dehydrogenase, 50 U. Absorption was measured at 340 nm on a Hitachi model 100-60 spectrophotometer.

Dry weight of cultures. This was determined by weighing appropriate samples after filtration on membrane filters (Millipore; 0.45 µm pore size), and drying at 80 °C.

Protein determination. This was done by the method of Lowry, using bovine serum albumin as standard.

Electron microscopy of cells. This was done as described by Smith & Batenburg-van der Vegte (1985).

Chemicals. [U-¹⁴C]Glucose [3 mCi mmol⁻¹ (111 MBq mmol⁻¹)] was obtained from Amersham. All other chemicals were obtained from commercial sources at the highest purity available.

RESULTS

Residual glucose concentration in glucose-limited cultures

Since in glucose-limited chemostat cultures the rate of sugar consumption, and hence the rate of sugar transport, is a function of the residual glucose concentration in the culture, measuring this parameter was a prerequisite for an evaluation of transport kinetics. This required rapid sampling and rapid fixation in order to avoid interference by the high metabolic activity of the cells. Fixation of samples in liquid nitrogen followed by thawing at 0 °C and centrifugation at 4 °C proved to be an adequate method. This was evident from the fact that, in accordance with continuous-culture kinetics, the residual glucose concentration at a particular dilution rate was independent of the reservoir concentration in the culture (results not shown). This latter parameter should have affected the residual glucose concentration if the sampling and/or fixation procedures had been inadequate.

The residual glucose concentration in glucose-limited chemostat cultures of C. utilis followed classical Monod kinetics (Herbert et al., 1956; Table 1). The apparent affinity constant for glucose and the μ_max of the organism were determined from a plot of s versus s/D⁻¹ (Hanes, 1932). From the slope of the line a K_s of 15 µM and, from the intercept with the x-axis, a μ_max of 0.595 h⁻¹ were calculated. From wash-out experiments a μ_max of 0.585 h⁻¹ was determined, in good agreement with the calculated value and showing the reliability of the method used to measure the residual glucose concentration.

Measurement of kinetic parameters of glucose transport

Inherent to in vivo studies of transport is the interference caused by subsequent metabolism of the substrate. In order to avoid this problem substrate analogues have frequently been used to obtain information on the kinetics of transport in vivo (Kotyk & Michaljaničová, 1978; Jaspers & van Steveninck, 1975; Barnett & Sims, 1976b; Meredith & Romano, 1977; Franzusoff & Cirillo, 1982; van den Broek et al., 1986). Apart from the fact that such studies can only yield qualitative information with respect to the uptake of the real substrate, it must be emphasized that the results obtained with substrate analogues do not always mimic changes in the transport parameters of the real substrate (Barnett & Sims, 1976b). It was therefore decided to use glucose itself in assays of transport kinetics. The interference of metabolism with transport kinetics was studied by following the time-dependent accumulation of radioactivity in cell suspensions incubated with [U-¹⁴C]glucose. Incubation times of less than 10 s yielded transport rates that were linearly proportional to time. After 10 s the uptake rate decreased, probably due to metabolic activity of cells and decreasing external glucose concentration (Fig. 1). Therefore in all experiments a 5 s incubation time was used for the determination of the initial rate of uptake.

Cells grown at low dilution rate could be used for the assays without further treatment, since at the time of the assay (approximately 5 min after harvesting), the residual glucose concentration was below detection level, and hence did not interfere. However, with cultures grown at high dilution rates washing of cells was required due to the interference of high residual substrate concentrations. In order to assess the effect of washing of cells on transport kinetics, glucose uptake by cells grown at low dilution rate and assayed after or without washing steps was studied. It appeared (results not shown) that this pretreatment had no effect on the kinetic parameters of glucose uptake. It was therefore assumed that this also held for cells grown at high dilution rates.
Fig. 1. Uptake of [U-14C]glucose. Cells from a glucose-limited continuous culture growing at a dilution rate of 0.3 h⁻¹ were incubated with 175 μM-glucose.

Fig. 2. Hanes plot of the kinetics of [U-14C]glucose transport by cells from a glucose-limited culture growing at D = 0.1 h⁻¹ (○) and D = 0.52 h⁻¹ (●). The intercept with the x-axis gives the substrate affinity constant (Kₘ) and the slope equals 1/Vₘₐₓ.

Table 1. Apparent affinities of glucose uptake by cells grown at different dilution rates in glucose-limited chemostat cultures

<table>
<thead>
<tr>
<th>Dilution rate h⁻¹</th>
<th>Glucose concn (μM)</th>
<th>High-affinity carrier</th>
<th>Medium-affinity carrier</th>
<th>Low-affinity carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>3</td>
<td>25</td>
<td>195</td>
<td>-</td>
</tr>
<tr>
<td>0.2</td>
<td>8</td>
<td>23</td>
<td>195</td>
<td>-</td>
</tr>
<tr>
<td>0.3</td>
<td>13</td>
<td>23</td>
<td>190</td>
<td>-</td>
</tr>
<tr>
<td>0.35</td>
<td>18</td>
<td>25</td>
<td>160</td>
<td>-</td>
</tr>
<tr>
<td>0.38</td>
<td>29</td>
<td>27</td>
<td>165</td>
<td>-</td>
</tr>
<tr>
<td>0.40</td>
<td>40</td>
<td>25</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>0.43</td>
<td>70</td>
<td>20</td>
<td>180</td>
<td>-</td>
</tr>
<tr>
<td>0.45</td>
<td>95</td>
<td>25</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>0.52</td>
<td>180</td>
<td>-</td>
<td>200</td>
<td>2100</td>
</tr>
<tr>
<td>0.57</td>
<td>330</td>
<td>-</td>
<td>-</td>
<td>1950</td>
</tr>
</tbody>
</table>

- Transport system not detectable.

Kinetic parameters of glucose transport in glucose-limited cultures

Considerable differences were noted between the kinetic constants of glucose uptake in cells growing at low dilution rate (D = 0.1 h⁻¹), and those of cells growing at high dilution rate (D = 0.52 h⁻¹). In both cases Hanes plots were biphasic, indicating the presence of two transport systems with different affinities (Fig. 2). At D = 0.1 h⁻¹ the affinity constants were 25 and 190 μM whereas at the high dilution rate values of 190 and 2000 μM were calculated (Fig. 2). This prompted a more detailed study of the relation between the kinetics of glucose uptake and the dilution rate (Table 1, Fig. 3). At dilution rates below 0.52 h⁻¹ Hanes plots revealed two affinity constants of 25 and 190 μM. Above this dilution rate the high-affinity system was absent, but instead a low-affinity system was detectable together with the medium-affinity system. This latter system was absent at a dilution rate of 0.57 h⁻¹ and only one low-affinity constant was estimated from a Hanes plot (Table 1).
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The capacity, that is the $V_{\text{max}}$ of each of the three uptake systems as a function of the dilution rate is presented in Fig. 3. Both the high- and the medium-affinity system increased in capacity with the increase in dilution rate up to approximately 0.3–0.35 h$^{-1}$. Above this dilution rate the capacity of the high-affinity system decreased and was absent at $D = 0.52$ h$^{-1}$. The medium-affinity system also decreased above $D = 0.3$ h$^{-1}$ but increased at dilution rates between 0.4 and 0.5 h$^{-1}$. Above $D = 0.45$ h$^{-1}$ the capacity of the medium-affinity system decreased and a low-affinity system became apparent (Fig. 3).

Estimation of the kinetics of glucose uptake in the chemostat

From the results presented above it is evident that more than one uptake system may be present in cells from steady-state cultures that are glucose-limited. The capacity of each of these systems is apparently correlated to the dilution rate and hence the glucose concentration. In order to evaluate this correlation, the contribution of each of the carrier systems to the in situ glucose uptake rate was calculated from the kinetic parameters and the residual substrate concentration at a particular dilution rate according to

$$v_{(\text{in situ})} = \frac{V_{\text{max}} \cdot S_{(\text{in situ})}}{K_m + S_{(\text{in situ})}}$$

The in situ rate of transport by each of the three systems is shown in Fig. 4. It is apparent that the high-affinity system is the most prominent at low dilution rates. At higher dilution rates, where the activity of the high-affinity system is decreasing, the medium-affinity system becomes dominant (0.45–0.52 h$^{-1}$), whereas at dilution rates above 0.52 h$^{-1}$ the low-affinity system accounts for more than 50% of the total glucose transported. The sum of the rates of the individual systems is shown in Fig. 5. The calculated overall rate of glucose transport is close to the estimated rate based on the overall growth kinetics:

Specific rate of glucose consumption = maintenance rate + dilution rate·cell yield$^{-1}$

Possible effects of metabolism on transport parameters

Previous studies have shown that hexokinases may play a role in glucose uptake in yeasts, and particularly in Saccharomyces cerevisiae (Meredith & Romano, 1977; Bisson & Fraenkel, 1983;
Fig. 5. Sum of the individual fluxes of glucose via the three carriers as a function of the dilution rate (h⁻¹). The solid line represents the glucose uptake rate calculated from a maintenance energy of 2.3 μmol g⁻¹ min⁻¹ (Atkinson & Mavituna, 1983) and a mean growth yield of 0.5 g cells (g glucose)⁻¹.

Fig. 6. Hexokinase activity in extracts of cells of C. utilis as a function of the dilution rate in glucose-limited cultures.

Table 2. Apparent substrate affinities (Kₘ) and transport capacities (Vₘₐₓ) of nitrogen- and glutamate-limited cultures at different dilution rates in continuous culture

Nitrogen limitation was at an (NH₄)₂SO₄:glucose ratio of 8:100 (w/w). The statistical variation is specified in Methods.

<table>
<thead>
<tr>
<th>Carrier*</th>
<th>Kₘ (μM)</th>
<th>Vₘₐₓ (μmol g cells⁻¹ min⁻¹)</th>
<th>D (h⁻¹)</th>
<th>Glucose concn (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen-limited</td>
<td>175</td>
<td>1300</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>Glucose-limited</td>
<td>25</td>
<td>195</td>
<td>140</td>
<td>90</td>
</tr>
<tr>
<td>Glutamate-limited</td>
<td>30</td>
<td>195</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Glucose-limited</td>
<td>23</td>
<td>195</td>
<td>150</td>
<td>130</td>
</tr>
</tbody>
</table>

*1, High-affinity carrier; 2, medium-affinity carrier; 3, low-affinity carrier.

Franzusoff & Cirillo, 1982; van Steveninck et al., 1985). The possibility that the observed changes in the kinetics of glucose transport may be explained by changes in hexokinase activities was investigated (Fig. 6). At low dilution rates the total activity of hexokinases was relatively constant but decreased at higher dilution rates. At all dilution rates tested the Kₘ for either glucose or ATP did not change: Kₘ (glucose) = 180 ± 10 μM and Kₘ (ATP) = 110 ± 10 μM. Although it is intrinsically impossible to exclude the effect of subsequent metabolism on transport kinetics, the above results make it unlikely that our results can be explained at the level of sugar phosphorylation.

Transport parameters of nitrogen- and glutamate-limited cultures

The results clearly show that the kinetic parameters of glucose transport in C. utilis are a function of the residual substrate concentration in the culture. This is also evident from a comparison of the transport parameters of a glucose-limited culture and those of a nitrogen-limited culture at the same dilution rate (Table 2). The higher residual glucose concentration in the nitrogen-limited culture apparently triggered the synthesis of the low-affinity system and simultaneously the synthesis of the high-affinity system was repressed. In glutamate-limited cultures the 25 μM and 190 μM systems could be observed. It can thus be concluded that the transport systems are regulated by a repression/derepression mechanism.
Glucose transport in Candida utilis

Table 3. Kinetic parameters of glucose transport in different yeasts as obtained from the literature

<table>
<thead>
<tr>
<th>Organism</th>
<th>Affinity ($K_m$) (mM)</th>
<th>Capacity ($V_{max}$) $[\mu$mol (g cells)$^{-1}$ min$^{-1}$]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>7.6</td>
<td>100</td>
<td>Barnett &amp; Sims (1976b)</td>
</tr>
<tr>
<td></td>
<td>1.9*</td>
<td>2*</td>
<td>Romano (1982)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>75†</td>
<td>Bisson &amp; Fraenkel (1983)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>75‡</td>
<td>Meredith &amp; Romano (1977)</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>2.1*</td>
<td>17*</td>
<td>Kotyk &amp; Michaljaničová (1978)</td>
</tr>
<tr>
<td>Candida utilis</td>
<td>10</td>
<td>40</td>
<td>Barnett &amp; Sims (1976a, b)</td>
</tr>
<tr>
<td></td>
<td>0.17</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Candida wickerhamii</td>
<td>1.7</td>
<td>27†</td>
<td>Spencer-Martins &amp; van Uden (1985a)</td>
</tr>
<tr>
<td></td>
<td>0.18</td>
<td>32‡</td>
<td></td>
</tr>
<tr>
<td>Rhodosporidium toruloides</td>
<td>0.55</td>
<td>17</td>
<td>Barnett &amp; Sims (1976b)</td>
</tr>
</tbody>
</table>

- Value not presented in reference.
* Measured with 6-deoxy-D-glucose as the substrate.
† Recalculated from data.
‡ Recalculated from data on the assumption that 5 g wet wt = 1 g dry wt.

As well as the glucose-limited cultures, the nitrogen-limited culture also had an in situ rate of glucose consumption which matched the calculated rate of glucose consumption.

**DISCUSSION**

The results of our chemostat studies with *C. utilis* reveal that in this organism the kinetics of glucose transport are adapted to the residual concentration via a well-balanced synthesis of three transport systems (Table 1, Fig. 3) which are characterized by their affinity constants. The capacity, i.e. the $V_{max}$ of these systems, reached high values, namely 130, 160 and 300 $\mu$mol (g cells)$^{-1}$ min$^{-1}$, for the high-, medium- and low-affinity transport systems respectively. These values are much higher than the $V_{max}$ values obtained with *Candida* species so far (Table 3). This can be explained by the cultivation conditions. In batch cultures, which have been used in most transport studies, the glucose concentration is saturating for the glucose transport systems during exponential growth. In glucose-limited chemostat cultures, on the other hand, cells are grown at glucose concentrations which are limiting for these systems.

The medium- and low-affinity uptake systems have been reported previously for *Candida* species (Table 3), but the occurrence of a transport system with a $K_m$ of 25 $\mu$M has so far not been described. This uptake system allows growth of *C. utilis* at very low glucose concentrations corresponding to low dilution rates.

It is evident that the glucose concentration in the culture is a decisive parameter in the regulation of the synthesis of the three transport systems. However, not only the extracellular glucose concentration but also the physiological status of the cells is of importance for the capacity ($V_{max}$) of the three transport systems. This is evident from a comparison of the results obtained with cells growing at high dilution rate under glucose limitation and those obtained with cells growing under nitrogen limitation at a low dilution rate. In both cases the high external glucose concentrations resulted in low-affinity constants but the low growth rate of the nitrogen-limited culture caused low $V_{max}$ values as compared to those of glucose-limited cultures. This specific induction of carriers in response to glucose concentration has been found previously with stationary phase cultures of yeasts (no glucose present). In that case also carriers with higher affinity for glucose were induced (Spencer-Martins & van Uden, 1985a; van den Broek et al., 1986) (Table 3). The physiological rationale of introducing different carriers at different external glucose concentrations remains as yet unclear. Cells probably would easily...
survive with only a high-affinity system by adjusting its capacity. The answer probably is that the different carriers have different energy requirements (e.g. H⁺-stoichiometries) that make a system with a lower affinity preferable at higher external glucose concentrations.

REFERENCES


