Controlling the Growth Rate of *Saccharomyces cerevisiae* Cells Using the Glucose Analogue D-Glucosamine

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By using competition between glucose and its analogue D-glucosamine, we have produced a system in which it is possible to vary the steady-state growth rate of populations of *Saccharomyces cerevisiae* cells without otherwise altering the composition of the medium or significantly affecting catabolite repression. We demonstrate that D-glucosamine inhibits the accumulation of glucose derived label and the phosphorylation of glucose by hexokinase (EC 2.7.1.1).

**INTRODUCTION**

Being able to control the growth rate of populations of cells at constant temperature is a useful facility both for industrial purposes and as an investigative tool for studies on cell growth and division. Several methods are in wide use (Pirt, 1975).

Batch culture methods rely on altering the overall nutritional composition of the medium or by changing the carbon source. This method of control suffers from the drawback that general cellular metabolism may change and cells may be relieved of carbon catabolite repression on non-fermentable carbon sources (Magasanik, 1961; Polakis & Bartley, 1965).

The chemostat supplies carbon sources, such as glucose, at rates that become limiting for growth. Although the supply can be adjusted to ensure continued population growth, the metabolic profile of cells growing with significantly reduced growth rates is typical of cells that are not catabolite repressed (Beck & von Meyenburg, 1968).

Diffusion capsules (Pirt, 1971) reduce growth by allowing a limited supply of substrate to diffuse into the medium along a concentration gradient. However, since the requirement for substrate increases with the exponentially growing population, the supply/demand curve is constantly changing, making steady growth difficult to maintain.

It has been demonstrated that certain non-metabolizable glucose analogues are capable of inhibiting the growth of yeast via effects on fermentation (Woodward & Hudson, 1953). We report here a method of modulating the continuous exponential growth of *Saccharomyces cerevisiae* in batch culture using the competition between glucose and its analogue D-glucosamine to limit the metabolism of glucose without significantly altering the repression status of the cell. The effect of glucosamine on the uptake and initial metabolism of glucose were also examined.

**METHODS**

*Strains, media and culture conditions.* S. cerevisiae strain SR665-1 (MATa met2 tyr1 cyh2 cdc39 gal1) was obtained from S. Reed (Research Institute of Scripps Clinic, La Jolla, CA, USA), and strain D273-lla (Matα adel his1 trp2) from L. Johnston (National Institute for Medical Research, Mill Hill, UK). Complex media (YEP) contained 1% (w/v) yeast extract, 2% (w/v) mycological peptone and carbon source as indicated. Agar (2%, w/v) was used to solidify the media. For liquid cultures, cells were shaken in conical flasks filled to less than 30% total volume. All growth experiments were done at 23°C unless stated.

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**Invertase assay.** Samples (1 ml) were incubated at 31 °C for 10 min. Sucrose (0.5%, w/v) was added to start the reaction which was stopped by addition of 1.5 ml dinitrosalicylic acid reagent (Miller, 1959) after an appropriate time. Colour was developed by boiling for 15 min, the absorbance read at 640 nm and compared with a standard curve prepared with glucose.

**Hexokinase assay.** Cells were grown to 1 × 10⁷ cells ml⁻¹ in YEP plus 2% (w/v) glucose at 23 °C. They were centrifuged, washed twice in sterile distilled water and resuspended in 0.1 M-potassium phosphate buffer (pH 7.0) containing 10 mM-magnesium chloride. A sample (0.7 ml) of this slurry was mixed with sufficient (450-500 µg) glass beads to give a thick paste in a 1.5 ml Eppendorf tube. The tube was vortexed three times for 20 s with periods on ice between each treatment. The supernatant was centrifuged from the tubes by puncturing the base and standing the tube inside a second decapped 1.5 ml tube. This 'piggyback' arrangement was centrifuged in a 50 ml tube. The crude cell-free extract (1 ml) was passed through a Sephadex G50 column, and eluted with 0.1 M-potassium phosphate buffer (pH 7.0) containing 10 mM-magnesium chloride. Hexokinase was then assayed using the method of Lobo & Maitra (1977). Protein content was determined using the Bio-Rad protein assay.

**Glucose uptake.** Cells were grown to 1 × 10⁷ cells ml⁻¹ in YEP plus 2% (w/v) glucose at 23 °C, harvested by centrifugation (6000 r.p.m. for 5 min in a Sorvall RC5B centrifuge, GSA rotor), washed twice in distilled water and resuspended at 2 × 10⁸ cells ml⁻¹ in 20 mM-Tris/HCl buffer (pH 6.7). Cell suspension (2.5 ml) was added to Universal bottles containing appropriate concentrations of D-[l-14C]glucose (220 MBq mmol⁻¹; Amersham) and glucosamine, made to a final volume of 2.5 ml with Tris/HCl buffer. This solution was equilibrated at 30 °C prior to addition of cells. Samples (1 ml) taken at 20 s intervals were transferred to a 0.45 µm Millipore filter and washed with 5 ml Tris/HCl buffer. Filters were transferred to 7.5 ml Optiphase Safe (Fisons) scintillation fluid and counted in an LKB Rackbeta 1217 liquid scintillation spectrometer.

**Cell number.** This was measured using an Electrozone Celloscope electronic particle counter.

### RESULTS

D-Glucosamine is not metabolized at an appreciable rate and does not support growth of *S. cerevisiae* (Burger & Hejmová, 1961). Moreover, glucosamine prevents growth of yeast on glycerol or ethanol (Furst & Michels, 1977). We have extended these observations by failing to observe growth of *S. cerevisiae* D273-11a on YEP containing 1% (w/v) glucosamine and any of the following substrates added at 2% (w/v); raffinose, cellobiose, mannitol, sorbitol, sodium succinate, sodium acetate, lactate, sucrose, galactose or glycerol. Concentrations of glucosamine greater than 0.1% completely inhibited growth on 2% (w/v) sodium pyruvate but allowed, with reductions in rate, continued growth on 0.05% (w/v) glucose.

The relationship between glucose and glucosamine with respect to effects on growth rate was further quantified by growing cells at 23 °C in YEP medium supplemented with various concentrations of glucose and glucosamine (Fig. 1). It was found that for a fixed concentration of glucose, the glucose:glucosamine ratio would extend the population doubling time in a predictable manner, and that this effect was greatest at low concentrations of glucose. These results suggested that a glucose/glucosamine competition may have been reducing the flux of glucose available for growth.

D-Glucosamine is capable of producing a repressed state similar to glucose repression (Furst & Michels, 1977) and has been used in the selection of carbon catabolite repression resistant mutants (Michels & Romanski, 1980). Invertase (EC 3.2.1.26) catalyses the hydrolysis of sucrose to fructose and glucose and is subject to glucose repression (Dodyk & Rothstein, 1984). It has frequently been used as an indicator of the repression status of cells (Matsumoto et al., 1983; Bailey & Woodward, 1984). Table 1 depicts the relationship between carbon source, growth rate and invertase activity. Although the addition of 1% (w/v) glucosamine significantly reduces the growth rate, the average invertase activity in these cells is more typical of the fast-growing, repressed, glucose-grown cells than of derepressed cells metabolizing pyruvate.

Glucose accumulation and the effect of glucosamine upon it were examined further. In the absence of glucosamine intact cells were incubated with radiolabelled glucose, and its intracellular accumulation followed over 20 s intervals. A Lineweaver–Burk (1934) plot of glucose accumulation against extracellular glucose concentration gave apparent V and Kₘ values of 21 nmol per 10⁸ cells min⁻¹ and 1 mM respectively. Increasing concentrations of
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D-Glucosamine concn (%. w/v)

Fig. 1. Effect of varying the glucose:glucosamine ratio on the growth rate of S. cerevisiae SR665-1. Cells were grown at 23 °C on YEP medium with various concentrations of glucose and glucosamine. O, 0.5% glucose; △, 1.0% glucose; □, 2.0% glucose. Data are the means of at least two separate experiments.

Table 1. Growth rate and invertase activity of S. cerevisiae grown on various carbon sources

S. cerevisiae SR665-1 was grown at 23 °C on YEP plus various carbon sources. Invertase activity (mean ± se of four separate experiments) was determined as described in Methods.

<table>
<thead>
<tr>
<th>Carbon source (%, w/v)</th>
<th>Population doubling time (min)</th>
<th>Invertase activity [μmol glucose formed (μg protein)]^-1 min^-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (2)</td>
<td>120</td>
<td>0.063 ± 0.01</td>
</tr>
<tr>
<td>Glucose (2) + glucosamine (1)</td>
<td>420</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Sodium pyruvate (2)</td>
<td>210</td>
<td>0.71 ± 0.06</td>
</tr>
</tbody>
</table>

Glucosamine had increasingly inhibitory effects on glucose accumulation (Fig. 2) but the data failed to fit standard inhibitor kinetic plots. It is possible that glucosamine affects the various elements of the multi-event glucose uptake system differently.

Since facilitated diffusion is proposed to be involved in glucose uptake, hexokinase activity plays an essential role in uptake by maintaining the necessary concentration gradient. As an element of uptake, hexokinase can be examined independently of membrane transport by using partially purified cell-free extracts. A Lineweaver–Burk plot of hexokinase activity relative to glucose concentration gives V and K_m values of hexokinase for glucose as 4.4 μmol NADPH formed mg^-1 min^-1 and 0.19 mM respectively. The ability of D-glucosamine to inhibit hexokinase activity was assayed at glucose concentrations around the K_m value. In this case the results generated fit the Dixon plot characteristic of competitive inhibitors (Dixon, 1953) (Fig. 3). This plot gave a K_i value for the effect of D-glucosamine on the ability of hexokinase to phosphorylate glucose of 1.7 mM.

DISCUSSION

The results demonstrate that glucosamine can reduce the growth rate of yeast cells metabolizing glucose. Glucosamine can act as a competitive inhibitor of hexokinase in vitro and inhibit the accumulation of labelled glucose in vivo; but the complex nature of the glucose uptake
system, in which transport may be linked to phosphorylation, makes it difficult to state, from these data, the point(s) at which glucosamine disrupts glucose transport. However, the role of hexokinase in the establishment of catabolite repression in yeast has been demonstrated (Entian et al., 1984) and glucosamine induced catabolite repression is well-documented (Furst & Michels, 1977; Hockney & Freeman, 1980). Consequently, glucosamine must cross the membrane to bind with intracellular hexokinase in vivo and may use the glucose transport system to gain entry. If so, glucosamine is likely to inhibit both transport and phosphorylation of glucose.

There are two systems for the transport of glucose into yeast cells (Bisson & Fraenkel, 1983a; Lang & Cirillo, 1987). The low affinity uptake system (apparent $K_m$ 20 mM) is mediated by constitutive carrier-mediated facilitated diffusion. The high-affinity system (apparent $K_m$ 2 mM) depends on any one of three kinases (hexokinase I and II and glucokinase) and is regulated by catabolite repression and inactivation (Bisson, 1988). There is a reciprocal relationship between the levels of high- and low-affinity processes (Ramos et al., 1988). Transport of a non-metabolizable glucose analogue, 6-deoxy-D-glucose, is kinase-dependent in the same way as glucose suggesting that the role of the kinases is not merely through phosphorylation and metabolism of the sugar itself. The glucose analogue is transported with biphasic kinetics with high and low $K_m$ components (Bisson & Fraenkel, 1983b).

The uptake results reported here referred to the intracellular accumulation of glucose-derived label and were therefore a measure of the total system rather than its individual components. The non-standard inhibition kinetics shown in Fig. 2 suggested that both high- and low-affinity components were being affected. At the higher glucose concentrations (17.5 mM) the low-affinity system was being used and was subject to standard competitive inhibition from D-glucosamine. However, the reduced effect of the inhibitor at lower glucose concentrations

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**Fig. 2.** Effect of glucosamine on the accumulation of labelled glucose by intact cells of *S. cerevisiae* SR665-1. The incubation solution contained various concentrations of labelled glucose and glucosamine. Accumulation was deemed to begin upon addition of cells. Inhibition is expressed relative to the maximum observed accumulation of 16.7 nmol glucose per 10⁸ cells min⁻¹ which is assigned a value of 100%. ○, 17.5 mM-glucose; △, 12.5 mM-glucose; □, 10.0 mM-glucose; ●, 7.5 mM-glucose. Data are the means of at least two replicates.

**Fig. 3.** Inhibition of hexokinase by D-glucosamine. Hexokinase was assayed in partially purified cell free extracts of *S. cerevisiae* SR665-1 in the presence of various concentrations of glucose and glucosamine. ○, 0.05 mM-glucose; △, 0.10 mM-glucose; □, 0.20 mM-glucose.
(7.5 mM) suggested that the high-affinity system was now becoming more important and this system had a lower affinity for the inhibitor. Indeed, the ability to study hexokinase independent of transport allowed stronger conclusions to be reached. The $K_m$ and $K_i$ values of 0.19 mM and 1.7 mM were sufficiently similar to support the observation that glucosamine was acting as a competitive inhibitor of hexokinase.

Glucosamine provides the same trigger for repression as glucose. However, since catabolism proceeds no further, its energetic contribution is zero, there is a reduction in the energy available from glycolysis and a consequential reduction in growth rate. Although certain strains of yeast show a resistance to glucosamine, the principle of competition by a non-metabolizable catabolite repressor can be extended to alternative glucose analogues (Woodward et al., 1953). The controlled reduction of growth rate allowed by this system may bring the convenience of the shake flask to experiments that previously demanded a glucose-limiting chemostat. Furthermore, it will now be possible to vary the growth rate over a wide range without altering the catabolite-repression status of the cells or the composition of the medium.

REFERENCES


