Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*

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mRNA steady-state levels and activities of enzymes of intermediary carbon metabolism (hexokinase, phosphoglucosomerase, phosphofructokinase, glucose-6-phosphate dehydrogenase, phosphoglucosomutase) and glucose-regulated enzymes (pyruvate decarboxylase, pyruvate dehydrogenase, invertase, alcohol dehydrogenase) were determined in glucose-limited continuous cultures of an industrial strain of *Saccharomyces cerevisiae* at different dilution rates (D) ranging from 0.05 to 0.315 h⁻¹. The activity of most enzymes measured remained constant over this range except for alcohol dehydrogenase I/II which decreased proportionally with increasing dilution rate. A decrease in phosphoglucosomutase activity occurred with increasing dilution rate but reached a minimum at D 0.2 h⁻¹ and from thereon remained constant. A decrease in pyruvate decarboxylase activity and a slight decrease in phosphoglucosomerase activity was observed. At D 0.29/0.315 h⁻¹, at the onset of the Crabtree effect, most glycolytic enzymes remained constant except for pyruvate decarboxylase and glucose-6-phosphate dehydrogenase which increased at D 0.315 h⁻¹ and alcohol dehydrogenase I/II which decreased. The *ADHI/II* and *PDC1* mRNA levels obtained at the different dilution rates were in accordance with the activity measurements. The mRNA level of *HXK1* decreased with increasing dilution rates, whereas the transcription of *HXK2* increased. Pyruvate dehydrogenase (*PDA1*) and *PGII* mRNA fluctuated but no significant change could be detected. These results indicate that there is no transcriptional or translational regulation of glycolytic flux between D 0.05 h⁻¹ and 0.315 h⁻¹ except at the branch point between oxidative and fermentative metabolism (pyruvate decarboxylase/pyruvate dehydrogenase) at D 0.315 h⁻¹. Surprisingly regulation of the Crabtree effect does not seem to involve transcriptional regulation of *PDA1*. The concentrations of ATP and cAMP decreased slightly during the increase in dilution rate, but increased again at D 0.315 h⁻¹. The concentrations of glucose 6-phosphate and fructose 6-phosphate did not increase when the dilution rate increased as expected from the activities of hexokinase, phosphoglucosomerase and phosphofructokinase. Instead, a decrease in the glucose 6-phosphate and fructose 6-phosphate concentrations was observed. The concentration of glucose 1-phosphate also decreased with increasing dilution rate but increased again at D 0.29 and 0.315 h⁻¹, whereas the fructose 1,6-diphosphate concentration increased from 0.05 h⁻¹ to 0.315 h⁻¹. These data indicate that glycolytic flux in *S. cerevisiae* is regulated mainly by allosteric regulation of glycolysis when growth rate is increased. Invertase was present (mRNA and activity) at every dilution rate which indicates that glucose-specific repression of enzyme systems is not present in glucose-limited continuous cultures, not even when the yeast produces ethanol. This also indicates that the Crabtree effect is not related to glucose repression.

Introduction

When *Saccharomyces cerevisiae* is grown on glucose the synthesis of many enzymes is repressed at the transcriptional level. The enzymes whose synthesis is repressed

include enzymes involved in the use of carbon sources other than glucose, e.g. invertase, alcohol dehydrogenase, and the enzymes of the Leloir pathway. Gluconeogenic (fructose-1,6-bisphosphatase) and TCA cycle (malate dehydrogenase) enzymes are also repressed. This phenomenon is known as carbon catabolite repression (Gancedo & Gancedo, 1986; Entian, 1986). Catabolite inactivation or activation is the term assigned to the rapid loss or rise of activity of the enzymes fructose-

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1,6-bisphosphatase (Gancedo & Gancedo, 1971) and trehalase, respectively (van der Plaats, 1974; Ortiz *et al.*, 1983; Uno *et al.*, 1983) upon addition of glucose to cells growing on non-fermentable carbon sources such as glycerol. This not only causes changes at the transcriptional level which results in long-term repression of enzyme synthesis, but also causes momentary decreases or increases in activity of certain enzymes. It is not known if the effects of glucose are mediated via one signal transduction pathway or whether more than one pathway is involved.

Most probably the RAS/cAMP pathway (Broach, 1991) is also involved in glucose signalling. Upon addition of glucose a transient increase in cAMP is observed, which seems to coincide with phosphorylation of the transcriptional activator ADRI (Cherry *et al.*, 1989) and phosphorylation of proteins (e.g. fructose-1,6-bisphosphatase; Rittenhouse *et al.*, 1987) at the cAMP-dependent phosphorylation site. However, the RAS/cAMP pathway is a signal transduction pathway for general responses, because mutants in this pathway show pleiotropic phenotypes (e.g. Toda *et al.*, 1987). The exact signal which activates the RAS/cAMP pathway is not known but Beullens *et al.* (1988) found evidence that the metabolism of glucose beyond glucose 6-phosphate is not necessary for the increase in cAMP.

Most data on the mechanism of glucose repression come from experiments using mutants with pleiotropic phenotypes under non-defined physiological conditions in shake-flask batch cultures. Continuous cultures offer an excellent tool to study glucose repression under defined physiological conditions (Fiechter *et al.*, 1981) because the growth rate of the organism can be regulated, allowing reproducible sampling of cells. At low growth rates [for strain SU32 a dilution rate (D) $< 0.275 \text{ h}^{-1}$] in glucose-limited continuous cultures all glucose is consumed oxidatively resulting in a respiratory quotient ($RQ = r\text{CO}_2/q\text{O}_2$) of 1.04. In this case the residual glucose concentration is approximately 0.1 mM (Postma *et al.*, 1989b; this study). However, at higher dilution rates (for SU32 $> 0.275 \text{ h}^{-1}$) an increase in RQ and residual glucose concentration as well as ethanol production is observed. This ethanol production in the presence of oxygen and in the absence of an excess of glucose is known as the Crabtree effect (Crabtree, 1929). The occurrence of the Crabtree effect is often linked to glucose repression (Dunlop & Ye, 1990; Postma *et al.*, 1989a). The dilution rate at which ethanol is produced seems to be strain-dependent (von Meyenburg, 1969; Postma *et al.*, 1989a; Rieger *et al.*, 1983; this study).

To investigate glucose repression by means of continuous culture it is essential first to describe precisely the exact physiological state of the yeast. Important parameters are mRNA levels and activities of glycolytic

enzymes (from hexokinase to aldolase), of glucose-regulated enzymes (invertase, alcohol dehydrogenase II, pyruvate decarboxylase) and of enzymes at the branch point of fermentative and oxidative metabolism (pyruvate decarboxylase and pyruvate dehydrogenase), and the concentrations of glycolytic intermediates of the first part of glycolysis (glucose 6-phosphate to fructose 1,6-bisphosphate), ATP and cAMP. This study was carried out first to investigate if there is glucose-specific repression of enzymes (e.g. alcohol dehydrogenase II; Postma *et al.*, 1989a) at higher dilution rates and to analyse if glucose repression is indeed related to aerobic ethanol production (e.g. Dunlop & Ye, 1990). Second, it is important to examine the regulation of glycolytic flux because the signal transmitting glucose repression may depend upon high concentrations of extra- or intracellular glucose or of a glucose metabolite.

Methods

Strain and growth conditions. Commercial bakers' yeast strain SU32 was grown in medium containing (g l^{-1}) NH_4Cl (7.63), KH_2PO_4 (2.81), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.59), 10 ml trace elements l^{-1} [a $100\times$ concentrate containing (g l^{-1}) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (5.5), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (3.75), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1.4), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (2.2), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.4), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.45), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.26), H_3BO_3 (0.4), KI (0.26) and Na_2EDTA (30)] and 1.5 ml vitamin solution l^{-1} [a $1000\times$ concentrate containing (g l^{-1}) biotin (0.05), thiamin (5), myo-inositol (47), pyridoxin (1.2) and pantothenic acid (23)] per glucose (20) in the feed. *S. cerevisiae* SU32 was grown at 30°C under glucose limitation, with 10 or 30 g glucose l^{-1} in the feed, in a fermenter with a 2 l working volume connected to an Applikon ADI1020 controller unit. All results shown are from the experiments with 10 g glucose l^{-1} in the continuous feed. The pH was automatically maintained at 5.0 by the addition of 2 M- NH_4OH . The air flow rate was 2 l min^{-1} and the dissolved oxygen tension was kept above 20% by regulating the stirrer speed. CO_2 production [$r\text{CO}_2$ in mmol h^{-1} per gram dry weight (g)], oxygen consumption ($q\text{O}_2$ in $\text{mmol h}^{-1} \text{g}^{-1}$) and ethanol formation were measured continuously on line by connecting the fermenter head-space to a VG MM8-80 gas analysis mass spectrometer.

Preparation of samples. Samples were taken in duplicate every day for three successive days only when a steady-state had been reached.

For the preparation of cell free extracts, used in enzyme assays, 1.5 ml culture liquid was transferred from the fermenter to an Eppendorf tube and put on ice. The cells were pelleted for 30 s by centrifugation and resuspended in 1 ml 10 mM-potassium phosphate buffer (pH 7.5) containing 2 mM-EDTA. The cells were again pelleted and resuspended in 1 ml 100 mM-potassium phosphate buffer (pH 7.5) containing 2 mM- MgCl_2 and 2 mM-dithiothreitol. Cell-free extracts were prepared by adding an equal volume of glass beads and the cells were lysed by shaking the suspension four times at maximum speed on a vortex mixer for 30 s with 1 min intervals on ice. Lysed cells were separated from the glass beads after which the suspension was centrifuged to remove cell debris. The clear supernatant was frozen quickly in liquid nitrogen and kept at -80°C until it was used for enzyme assays. The protein concentration of cell-free extracts was determined by the method of Bradford (1976) with the Bio-Rad dye-reagent using bovine serum albumin (Sigma) as a standard. Protein concentrations were always between 1–6 mg (ml extract) $^{-1}$.

Table 1. Oligonucleotides used in this study

mRNA detected	Oligonucleotide sequence
<i>HXK1</i>	5' TAAGGTGTCCTTGGTGTTCAGCAATTC 3'
<i>HXK2</i>	5' AATTGGCTCAGAGATACCTTGTGGGAA 3'
<i>PGI1</i>	5' ACTTCGGTTTCGTTAGTGGACAAAGCAGCG 3'
<i>ADHI/II</i>	5' CGTTACCAATTGACAGTATTCACAGGCCA 3'
<i>PDA1</i>	5' CACTGTGTCCGAGCCCTCGATGTCCTC 3'
<i>PDC1</i>	5' AGACATTCTGTGGAAAACAGTGAAGTCACC 3'
<i>SUC2</i>	5' TGGGTCAGTGTGAAGAAAGTTTGCAAGGC 3'
<i>ACT1</i>	5' TGTCTTGGTCTACCGACGATAGATGGGAAG 3'
<i>H2A/H2B</i>	5' GACTGGAGCACCAGAACCAATTCCTTGGGC 3'

mRNA extraction was as described by Sierkstra *et al.* (1991).

Samples for determination of glycolytic intermediates ATP and cAMP were taken by transferring 3 ml of culture liquid from the fermenter to a tube in liquid nitrogen (within 10 s). These were stored at -80°C . The frozen culture liquid was heated quickly to 80°C in boiling ethanol for 1 min to inactivate enzymes as quickly as possible. This liquid mixture was removed by vacuum drying. The resulting pellet was dissolved in 1 ml of H_2O and stored at -80°C until assayed. All enzymes were inactivated by this extraction procedure.

Sampling of culture liquid for the determination of extracellular metabolites (acetate, acetaldehyde, glycerol, pyruvate, ethanol and the residual glucose concentration) was performed as described by Postma *et al.* (1988). However, this method overestimates the extracellular concentrations of metabolites because lysis occurs due to freezing of the cells.

For the determination of dry weight 20 ml culture was collected from the fermenter's outlet. The cells were pelleted, washed with distilled water and transferred to pre-weighed glass vials in which they were dried overnight at 120°C .

Measurement of metabolites, ATP and cAMP. Glycolytic intermediates were measured enzymically as described by Bergmeyer (1974). ATP was assayed by measuring glucose consumption by hexokinase. Extracellular metabolites (ethanol, acetate, acetaldehyde, pyruvate and glycerol) were determined enzymically or by HPLC. Total cAMP was determined with the Amersham cAMP-kit based on competition between labelled and unlabelled cAMP with a cAMP-binding protein.

Enzyme assays. Enzyme activities were measured at maximal velocity as described by Bergmeyer (1974) in 50 mM-imidazole/HCl buffer (pH 7.0) containing 5 mM- MgCl_2 . Reaction velocities were proportional to the amount of enzyme added. Activities are in U (mg total cellular soluble protein) $^{-1}$ in which 1 U is defined as the conversion of 1 μmol of substrate min^{-1} at 30°C at pH 7.0. All enzyme assays were performed on the Cobas Mira autoanalyser of Hoffmann Laroche.

Labelling of oligonucleotides. For Northern analysis the oligonucleotides listed in Table 1 were used. *HXK1*, *HXK2*, *PGI1*, *ADHI/II*, *PDA1*, *PDC1*, *SUC2*, *ACT1* and *H2A/H2B* are the structural genes for hexokinase isoenzyme 1 and 2, phosphoglucosomerase, alcohol dehydrogenase I and II, pyruvate dehydrogenase (E1 α -subunit), pyruvate decarboxylase, invertase, actin and histones 2A and 2B, respectively. Oligonucleotides were labelled by incubating 25 pmol of oligonucleotide with 1 unit of T4 polynucleotide kinase and 1.85 MBq [^{32}P]ATP.

Northern blot analysis. RNA samples (containing 3 μg total RNA) were separated on a denaturing formamide/formaldehyde gel. RNA

Fig. 1. The specific oxygen consumption ($q\text{O}_2$; $\circ$), CO_2 production ($r\text{CO}_2$; $\blacksquare$), RQ ($\square$) and yield ($\bullet$) as a function of the dilution rate of *S. cerevisiae* SU32. RQ is defined as the quotient of the CO_2 produced ($\text{mmol g}^{-1} \text{h}^{-1}$) and the O_2 consumed ($\text{mmol g}^{-1} \text{h}^{-1}$). The yield is defined as the amount of biomass (g) per gram carbon source.

was blotted onto Hybond paper using the vacuene system of Pharmacia or by capillary blotting. The RNA was crosslinked to Hybond by exposure to UV light. Blots were prehybridized at least 2 h in hybridization mix [50 mM-Tris/HCl, pH 7.5, 10 mM-EDTA, 1 M-NaCl, 0.1% SDS, 0.1% sodium pyrophosphate, 0.2% Ficoll, 0.2% BSA, 0.2% PVP and a denatured mixture of 0.1 mg ssDNA ml^{-1} and 0.01 mg poly(rA) ml^{-1}] at 42°C . Hybridization was performed overnight at 42°C in hybridization mix with the denatured oligonucleotide (5 min, 100°C). The filters were washed once for 20 min with $5\times$ SSC, once in $2\times$ SSC and twice in $1\times$ SSC, all at 42°C . Thereafter the filters were exposed to X-ray film. Histone or actin mRNAs were used as internal controls. The amount of specific mRNA was measured on a LKB Ultrosan XL densitometer. Different exposure times were used in order to obtain reliable scanning data.

Biochemicals. All enzymes, ATP, NADP, NAD, NADH and metabolites were purchased from Boehringer, except glycerol-3-phosphate dehydrogenase, triose-phosphate isomerase and fructose-2,6-bisphosphate which were purchased from Sigma.

Results

Physiological parameters

S. cerevisiae strain SU32 was grown under glucose limitation at different dilution rates in a chemostat. The specific oxygen uptake and carbon dioxide production increased linearly with increasing dilution rate up to 0.275 h^{-1} (Fig. 1). The residual glucose concentration at this dilution rate was 0.1 mmol l^{-1} . At the onset of the Crabtree effect between 0.275 and 0.29 h^{-1} , the maximal specific oxygen uptake of SU32 was observed ($6.5 \text{ mmol O}_2 \text{ g}^{-1} \text{h}^{-1}$). At $D 0.29 \text{ h}^{-1}$, $0.3 \text{ g ethanol l}^{-1}$, $0.14 \text{ g acetate l}^{-1}$ and $0.01 \text{ g pyruvate l}^{-1}$ was observed in the medium. This secretion of metabolites resulted in a decrease in growth yield from 0.45 to $0.42 \text{ [g biomass (g glucose)}^{-1}]$. An increase in RQ to 1.15 was observed at this dilution rate. A further increase in dilution rate to 0.315 h^{-1} resulted in a decrease of the specific oxygen uptake to $4.4 \text{ mmol O}_2 \text{ g}^{-1} \text{h}^{-1}$ and an increase in CO_2 production from 7.3 to $12.0 \text{ mmol CO}_2 \text{ g}^{-1} \text{h}^{-1}$, and

Fig. 2. Northern blots of mRNA isolated at different dilution rates from 0.05 to 0.315 h^{-1} are shown. The Northern blots were hybridized with probes specific to the mRNA of (a) *HXK1*, (b) *HXK2*, (c) *PGII*, (g) *SUC2* and (d) *PDC1* with actin as an internal control. Blots (e) and (f) were hybridized with probes specific to the mRNA of (e) *PDA1* and (f) *ADHI/II* with histone as internal control. The blots were quantified by densitometry. The specific amount of mRNA (as corrected for the total amount of RNA on the gel by the scanned value for the internal control, histone or actin) is shown beneath each lane. The amount of specific mRNA at D 0.05 h^{-1} is taken as the 100% value. ND, Not determined.

consequently in an increase in RQ to 2.8. As a result of this shift to fermentative metabolism ethanol production by the yeast increased to 2.3 g l^{-1} . Also, 0.11 g acetate l^{-1} , 0.25 $\text{g acetaldehyde l}^{-1}$ and 0.02 g pyruvate l^{-1} were observed in the culture liquid. The residual glucose concentration increased to 0.27 mmol l^{-1} .

S. cerevisiae SU32 produced a constant amount of 9 $\text{mg glycerol g}^{-1}$ at low dilution rates ($< 0.275 \text{ h}^{-1}$). This increased to 50 mg g^{-1} when the yeast produced ethanol

(D 0.315 h^{-1}) as observed by others (Postma *et al.*, 1989a). The experiments were also carried out with a glucose concentration of 30 g l^{-1} in the feed: no differences were observed in the physiological parameters (results not shown).

mRNA levels at increasing dilution rates

To investigate transcriptional regulation of *HXK1*, *HXK2*, *PGII*, *PDC1*, *PDA1*, *ADHI/II* and *SUC2*,

mRNAs were isolated at different dilution rates and Northern analysis was performed. Due to extensive sequence identity between the two ADH genes no oligonucleotide could be designed which could discriminate between the two different mRNAs. Therefore, the signal on the Northern blots comes from both mRNAs. This is also the case for both histone genes H2A and H2B.

The transcription of *HXK1* (panel A) was constant at D 0.05 and 0.1 h^{-1} , but then decreased with increasing dilution rate to about 10% of the initial value at 0.29 h^{-1} (Fig. 2a). The *HXK2* mRNA (Fig. 2b) increased slightly at 0.1 h^{-1} and then decreased again to the initial value at 0.2 h^{-1} . From 0.2 to 0.315 h^{-1} , a threefold increase in the *HXK2* mRNA could be seen. Transcription of *PGI1* (Fig. 2c) fluctuated between 88 and 46% of the initial value at D 0.05 h^{-1} .

PDC1 mRNA decreased with increasing dilution rate but remained constant at D 0.2 h^{-1} and higher; however, at 0.315 h^{-1} an induction of the *PDC1* mRNA occurred (Fig. 2d). The amount of specific *PDA1* mRNA fluctuated between 108 and 46% of the initial value (Fig. 2e).

A decrease in the mRNA level of *ADHI/II* occurred with increasing dilution rate (Fig. 2f); at 0.315 h^{-1} the mRNA amount was only 0.3% of the initial value at 0.05 h^{-1} . *SUC2* mRNA was present at all dilution rates, even when the yeast produced ethanol (Fig. 2g) but the highest level of *SUC2* mRNA was observed at low dilution rates.

Enzyme levels at increasing dilution rates

Increasing the dilution rate of *S. cerevisiae* in a glucose-limited continuous culture leads to an increase in glycolytic flux, e.g. from 0.1 to 0.2 h^{-1} the sugar consumption g^{-1} increases twofold. To determine whether *S. cerevisiae* responds to this increase in glycolytic flux by changing the amount of its glycolytic enzymes, total soluble protein was isolated at each dilution rate and the activity of different enzymes was measured in this crude extract.

From D 0.05 to 0.315 h^{-1} the activity of hexokinase remained constant at about 2 U (mg protein) $^{-1}$, whereas the activity of phosphoglucosomerase decreased slightly. The activity of phosphoglucomutase decreased from 1.2 U (mg protein) $^{-1}$ at 0.05 h^{-1} to 0.37 U (mg protein) $^{-1}$ at 0.275 h^{-1} and remained constant at 0.29 and 0.315 h^{-1} (Fig. 3a).

The activity of glucose-6-phosphate dehydrogenase remained constant during the increase in dilution rate at about 0.25 U (mg protein) $^{-1}$ but an increase in activity to 0.45 U (mg protein) $^{-1}$ was observed at 0.315 h^{-1} . A slight decrease in phosphofructokinase activity was observed.

Fig. 3. Specific activities of the following enzymes as a function of the dilution rate: (a) HXK (hexokinase: ●), PGI (phosphoglucosomerase: ○) and GAL5 (phosphoglucomutase: ■); (b) G-6-PDH (glucose-6-phosphate dehydrogenase: ○), PFK (phosphofructokinase: □) and PDC (pyruvate decarboxylase: ●); (c) SUC2 (invertase: □), ADHI (alcohol dehydrogenase I: ●) and ADHII (alcohol dehydrogenase II: ○). Results shown are the means of three independently isolated samples assayed in duplicate.

Table 2. The concentration of glucose 6-phosphate (G-6-p), fructose 6-phosphate (F-6-p), glucose 1-phosphate (G-1-p), fructose 1,6-bisphosphate (F-1,6-bp) and ATP ($\mu\text{mol g}^{-1}$) as a function of the dilution rate (D)

The concentration of cAMP (intra- and extracellular) as a function of the dilution rate is given in nmol g^{-1} . The values represent determinations of at least four independent isolations. ND, Not determined.

D (h^{-1})	G-6-p	F-6-p	G-1-p	F-1,6-bp	ATP	cAMP
0.05	1.92 ± 0.02	0.29 ± 0.1	7.3 ± 1.2	1.3 ± 0.22	5.4 ± 0.8	9.3 ± 1.5
0.1	0.98 ± 0.23	0.2 ± 0.09	5.0 ± 0.5	1.6 ± 0.2	5.3 ± 0.7	7.4 ± 1.3
0.15	1.22 ± 0.3	0.2 ± 0.05	2.9 ± 0.7	2.8 ± 0.4	4.9 ± 0.8	5.4 ± 0.9
0.2	0.63 ± 0.1	0.06 ± 0.02	0.6 ± 0.02	3.2 ± 0.5	4.2 ± 0.5	6.0 ± 1.6
0.275	0.56 ± 0.14	0.04 ± 0.01	0.38 ± 0.05	ND	4.3 ± 0.1	5.7 ± 0.1
0.29	0.24 ± 0.02	0.02 ± 0.01	0.63 ± 0.05	ND	3.2 ± 0.6	3.5 ± 0.1
0.315	1.6 ± 0.4	0.18 ± 0.01	1.1 ± 0.15	6.3 ± 1.1	5.5 ± 0.6	6.0 ± 2.1

Pyruvate decarboxylase activity decreased during the increase in dilution rate but increased again at 0.315 h^{-1} (Fig. 3b).

The activity of alcohol dehydrogenase I (acetaldehyde as substrate) and alcohol dehydrogenase II (ethanol as substrate) decreased from 45 and $8.1 \text{ U (mg protein)}^{-1}$, respectively, at D 0.05 h^{-1} to 1.2 and $0.29 \text{ U (mg protein)}^{-1}$, respectively, at D 0.315 h^{-1} (Fig. 3c). Invertase activity was present at every dilution rate, even when the yeast produced ethanol [maximum activity $6 \text{ U (mg protein)}^{-1}$ at D $0.1 \text{ h}^{-1}/0.15 \text{ h}^{-1}$; minimum activity $4.2 \text{ U (mg protein)}^{-1}$ at D $0.05 \text{ h}^{-1}/0.315 \text{ h}^{-1}$]. The yeast strain used is not constitutive for invertase expression (results not shown).

Metabolite levels at increasing dilution rates

Metabolite levels in continuous cultures are dependent on the influx of glucose and the *in vivo* V_{max} and K_m of the enzymes which convert the substrates into the metabolites measured and the concentration of the cofactors.

The glucose 6-phosphate and fructose 6-phosphate concentrations decreased considerably from D 0.05 to 0.29 h^{-1} from 1.92 and $0.29 \mu\text{mol g}^{-1}$ to 0.24 and $0.02 \mu\text{mol g}^{-1}$, respectively. However, at D 0.315 h^{-1} an increase in the glucose 6-phosphate and fructose 6-phosphate concentrations up to 1.6 and $0.18 \mu\text{mol g}^{-1}$, respectively, occurred (Table 2). Glucose 1-phosphate decreased from $7.3 \mu\text{mol g}^{-1}$ at D 0.05 h^{-1} to $0.38 \mu\text{mol g}^{-1}$ at D 0.275 h^{-1} and then increased again (to $1.1 \mu\text{mol g}^{-1}$). The concentrations of ATP and cAMP decreased from D 0.05 to 0.29 h^{-1} but at 0.315 h^{-1} there was a rise in both the cAMP and ATP concentrations. The concentration of fructose 1,6-bisphosphate increased from $1.3 \mu\text{mol g}^{-1}$ at D 0.05 h^{-1} to $6.3 \mu\text{mol g}^{-1}$ at 0.315 h^{-1} (Table 2).

Because of the fast sampling method used there was no separation of medium from cells, so the amount of cAMP

given in Table 1 is the sum of intra- and extracellular cAMP. To investigate if there was any extracellular cAMP, as reported by Smith *et al.* (1990), culture liquid was quickly ($<10 \text{ s}$) taken from the fermenter at D 0.1 h^{-1} and the medium was separated from the cells by filtration through a $0.22 \mu\text{m}$ Millipore filter. The filtrate was used in a cAMP assay and cAMP was indeed found in the medium (30 to 50%). Additional experiments confirmed that this phenomenon is not restricted to continuous growth (results not shown).

Discussion

The addition of glucose to cells growing on non-repressing carbon sources causes repression of gene transcription (e.g. *ADHII*; Cherry *et al.*, 1989) and the phosphorylation of enzymes, e.g. fructose-1,6-bisphosphatase (Rittenhouse *et al.*, 1987). These phosphorylation events seem to be cAMP-dependent and therefore it was proposed that the signal mediating this glucose-induced phosphorylation is transferred by the RAS/cAMP pathway. However, it is not known if all the effects of glucose are mediated by this pathway or whether other signal transduction pathways are involved. The exact signal which triggers these events is not known either, but the signal must be located in the first part of the glycolytic pathway as shown by Beullens *et al.* (1988).

No repression of enzyme systems was observed in glucose-limited continuous cultures (see below) which indicates that the presence of glucose and its glycolytic intermediates is not sufficient to cause glucose repression. This implicates that a threshold concentration of extra- or intracellular glucose or a glucose metabolite (e.g. glucose 6-phosphate) is responsible for the signal mediating glucose repression. To test this hypothesis it is essential to investigate regulation of glycolytic flux

because the flux determines the concentrations of metabolites *in vivo* which could eventually lead to the signal for glucose excess.

In this study we investigated whether regulation of glycolytic flux occurs by measuring mRNA levels and enzyme activities *in vitro* at different dilution rates in continuous cultures. The analysis of the enzyme activity and mRNA level for phosphoglucosomerase and the enzyme activity of phosphofructokinase determined at different dilution rates demonstrates that there is no clear correlation between the activity or mRNA level of these enzymes and the dilution rate, although the *PGII* mRNA fluctuated and a slight decrease in phosphoglucosomerase activity was observed when the dilution rate increased. Total glucose-phosphorylating activity (glucokinase and hexokinase I and II) remained constant during the increase in dilution rate but a decrease in the amount of *HXX1* mRNA and a threefold increase in the *HXX2* transcript was observed at higher dilution rates. These results indicate that glycolytic flux is not regulated at either the transcriptional or translational levels. However, the *in vivo* activities of the measured enzymes could change with increasing dilution rate due to allosteric regulation, phosphorylation or the amount of cofactor(s) present. It is known, for example, that ATP is a very potent regulator of glycolytic flux and that at high concentrations it decreases the *in vivo* activity of hexokinase (Kopetzki & Entian, 1985), phosphoglucosomerase (Barman, 1969) and phosphofructokinase (Bar *et al.*, 1990). The latter has been shown to be subject to a number of allosteric regulators, e.g. fructose 2,6-bisphosphate, P_i and AMP (Reibstein *et al.*, 1986).

Recent results from Schaaff *et al.* (1989) also indicate that glycolytic flux is strictly regulated. In order to increase the rate of fermentation they cloned the genes of glycolytic enzymes into a multi-copy vector and transformed the plasmids into yeast. Although increased amounts of enzyme were found, ethanol production was not increased and the levels of glycolytic intermediates were not reduced. Our data, which show decreased or constant amounts of glycolytic intermediates and constant enzyme activities during the increase in dilution rate, also strongly suggest that glycolytic flux is regulated through allosteric activation of the glycolytic enzymes. Studies are currently being carried out to investigate if glycolytic flux can be modelled with the data obtained by this study and additional data from the literature.

S. cerevisiae SU32, a commercial bakers' yeast strain, grown under glucose limitation reaches its maximum specific oxygen uptake of $6.5 \text{ mmol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ between D 0.275 and 0.29 h^{-1} . At this dilution rate the yeast starts producing ethanol. This aerobic production of ethanol by *S. cerevisiae* under conditions where there is no glucose excess is known as the long-term Crabtree effect, as

opposed to the short-term Crabtree effect, which involves glucose excess (Van Urk, 1990). A further increase in dilution rate to 0.315 h^{-1} results in a decrease of the specific oxygen uptake to $4.4 \text{ mmol O}_2 \text{ g}^{-1} \text{ h}^{-1}$. Rieger *et al.* (1983) reported a constant specific oxygen uptake of the yeast during the Crabtree effect and they ascribed the decrease in specific oxygen consumption observed by von Meyenburg (1969) to a limitation in the feed. To exclude the possibility that a limitation in the feed was the cause of the decrease in specific oxygen uptake in our experiment, the experiment was performed with two different glucose concentrations (10 and 30 g l^{-1}). No differences in the physiological parameters were observed. In addition, Postma *et al.* (1989a) reported a slight decrease in specific oxygen uptake in the Crabtree effect. Therefore, it seems likely that the oxygen uptake at the onset of the Crabtree effect is strain-specific.

Our results indicate that not only at the onset of the Crabtree effect but also when the yeast produces ethanol there seems to be no transcriptional regulation of *PDA1* (the $E1\alpha$ -subunit of the pyruvate dehydrogenase complex), although a fluctuation in the mRNA level between 108 and 46% of the initial value was observed at different dilution rates. In contrast, *PDC1* is regulated at the transcriptional level, as reported by Schmitt *et al.* (1983). At D 0.315 h^{-1} , when the yeast produces $2.3 \text{ g ethanol l}^{-1}$, an increase of the *PDC1* mRNA and activity was observed. However, this increase can not be due to an excess of glucose because the concentration of glucose was only 0.27 mmol l^{-1} . This indicates that a mechanism of regulation, other than induction by glucose, is operative on *PDC1* at the onset of the Crabtree effect. The transcriptional inducer of *PDC1* could be one of the overflow metabolites at the branch point of the oxidative and the fermentative pathways (e.g. pyruvate, acetate, acetaldehyde or NADH). At the same dilution rate at which pyruvate decarboxylase increases, the activity of glucose-6-phosphate dehydrogenase also increases and the specific oxygen uptake decreases. Therefore, it seems likely that these changes are mediated by the overflow metabolism and are not specifically due to glucose.

When the dilution rate increased, *ADHI/II* mRNA decreased and the activity of alcohol dehydrogenases I and II also decreased. It has been proposed that the decrease in activity of alcohol dehydrogenase II (but not of alcohol dehydrogenase I) and acetyl-CoA-synthetase is due to glucose repression of these enzymes at higher dilution rates in continuous cultures (Postma *et al.*, 1989a). *ADHII* is known to be regulated by glucose via the transcriptional activator ADRI (Cherry *et al.*, 1989). Invertase expression seems to be regulated only by glucose repression and derepression (Entian, 1986; Carlson, 1987), whereas regulation of *ADHII* is more

complex (Denis, 1984). In contrast with the mRNA level and activity of alcohol dehydrogenase II, the mRNA level and activity of invertase hardly changed. Therefore, the decrease of alcohol dehydrogenase II cannot be glucose-specific or else the mechanism of glucose repression for invertase is different from the mechanism by which glucose controls the expression of *ADHII*. A carbon-limited continuous culture with a non-repressing carbon source (galactose) is currently under investigation in our laboratory to distinguish between these two possibilities. The fact that invertase was present even when the yeast produced ethanol suggests there is no glucose-specific repression and that there is no link between glucose repression and the Crabtree effect in *S. cerevisiae*.

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