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Metabolic Responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon Transition from Glucose Limitation to Glucose Excess

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When chemostat cultures of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621, grown under glucose limitation, were pulsed with excess glucose, both organisms initially exhibited similar rates of glucose and oxygen consumption. However, striking differences were apparent between the two yeasts with respect to the production of cell mass in the culture and metabolite excretion. Upon transition from glucose limitation to glucose excess, *S. cerevisiae* produced much ethanol but the growth rate remained close to that under glucose limitation. *C. utilis*, on the other hand, produced little ethanol and immediately started to accumulate cell mass at a high rate. This high production rate of cell mass was probably due to synthesis of reserve material and not caused by a high rate of protein synthesis.

Upon a glucose pulse both yeasts excreted pyruvate. In contrast to *C. utilis*, *S. cerevisiae* also excreted various tricarboxylic acid cycle intermediates, both under steady-state conditions and after exposure to glucose excess. These results and those of theoretical calculations on ATP flows support the hypothesis that the ethanol production as a consequence of pyruvate accumulation in *S. cerevisiae*, occurring upon transition from glucose limitation to glucose excess, is caused by a limited capacity of assimilatory pathways.

KEY WORDS—Crabtree effect; respiration; fermentation; *Saccharomyces; Candida.*

INTRODUCTION

Since the early studies by Warburg (1926) and Crabtree (1929) on the effect of glycolysis on respiration in tumour cells, many papers have appeared on the interrelation between respiration and fermentation in eukaryotic cells. In particular, the Crabtree effect in yeasts has been extensively studied (Polakis and Bartley, 1965; De Deken, 1966; Barford *et al.*, 1981; Fiechter *et al.*, 1981). The Crabtree effect has been defined by Fiechter *et al.* (1981) as: 'The repression of respiratory activity by glucose under aerobic conditions and subsequent deregulation of glycolysis with formation of ethanol'. Indeed, many investigators have observed repression of the synthesis of enzymes of the citric acid cycle and the respiratory chain (Polakis and Bartley, 1965; Beck and von Meyenburg, 1968; Knöpfel, 1972; Haarasilta and Oura, 1975; Petrik *et al.*, 1983) and repression of the synthesis of mitochondria (Neal *et al.*, 1971; Jayaraman *et al.*, 1966) under conditions of glucose excess. However, the tendency of Crabtree-positive yeasts, such as *Saccharomyces* species, for alcoholic fermentation of glucose under strict aerobic conditions does not necessarily involve a long-term adaptation via repression of the synthesis of respiratory enzymes. It is well known from the manufacturing of bakers' yeast that under glucose limitation alcoholic fermentation does not occur in aerobically grown *S. cerevisiae*. If, however, glucose is suddenly added in excess, alcoholic fermentation sets in immediately. This so-called short-term Crabtree effect (Fiechter *et al.*, 1981; Petrik *et al.*, 1983) does not involve repression or inactivation of the existing respiratory potential, at least not initially.

The immediate onset of alcoholic fermentation upon transition of *S. cerevisiae* from glucose limitation to glucose excess has been ascribed to a limited respiratory capacity of this yeast. Alcoholic fermentation becomes apparent when the rate of sugar uptake exceeds the capacity of the respiratory pathways (Petrik *et al.*, 1983; Rieger *et al.*, 1983).

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The results described in this paper indicate, however, that the occurrence of alcoholic fermentation in *S. cerevisiae* upon relief from glucose limitation does not necessarily result from a bottleneck in the respiratory metabolism but may be caused by a restricted assimilatory capacity.

METHODS

Micro-organisms and growth conditions

Saccharomyces cerevisiae CBS 8066 and *Candida utilis* CBS 621 were maintained on malt agar slopes. The yeasts were grown under glucose limitation at 30°C in laboratory fermenters with a working volume of 1 l. The dissolved-oxygen tension was kept above 20% of air saturation; pH was controlled automatically at 5·0 by addition of 2 M-KOH. The mineral medium was prepared according to Bruinenberg *et al.* (1983). The reservoir glucose concentration was 12·5 g/l. The dilution rate was either 0·1 or 0·2 h^{-1}.

Glucose-pulse experiments

After non-oscillating steady-state cultures had been obtained, the medium flow was stopped and glucose was added to the culture to an initial concentration of approximately 50 mM. Samples were taken aseptically and immediately (within 20 s) centrifuged for 2 min at 13 000 rpm in an MSE microcentrifuge; it was determined that all cells were sedimented within 15 s. Supernatants were frozen until analysed for metabolites or protein.

Determination of dry weight

For dry-weight measurements nitrocellulose filters (Gelman, pore width 0·45 μm) were used. After removal of the medium by suction, the filter with the pellet was washed with demineralized water. The filter was then dried in a Sharp R-7400 magnetron oven for 15 min. Cell mass production rate in the cultures was determined via linear regression of the logarithm of dry weight values at 2, 10, 20 and 30 min (phase I) and 30, 40, 50 and 60 min (phase II).

Determination of C and N content

For the determination of C and N contents of biomass, cells were washed twice with distilled water and dried at 70°C. Analyses were performed using a Perkin Elmer Elemental Analyser 240 B.

Analysis of oxygen consumption and CO_2 production

Oxygen and CO_2 were analysed in the dried off-gass (Permapure dryer, Inacom Instruments) from the fermenter by using a Servomex OA-184 oxygen analyser and a Beckman CO_2 analyser. The gas flow rate (as measured in the exhaust line) was determined with a water-filled precision gas flow meter (Schlumberger, Holland).

The CO_2 production and oxygen consumption in the fermenters was calculated according to equations (1), (2) and (3):

$$Q_{CO_2} = (Q_{gas,out} \times pCO_{2,out} - Q_{gas,in} \times pCO_{2,in})/V_m \quad (1)$$

$$Q_{O_2} = (Q_{gas,in} \times pO_{2,in} - Q_{gas,out} \times pO_{2,out})/V_m \quad (2)$$

$$Q_{gas,in} = (1 - pCO_{2,out} - pO_{2,out}) \times Q_{gas,out}/0{\cdot}79 \quad (3)$$

In which Q_{CO_2} and Q_{O_2} represent the CO_2 production and oxygen consumption rates (mol/h); Q_{gas} represents the gas flow rate (l/h); V_m is the molar volume at atmospheric pressure and room temperature (l); pO_2 and pCO_2 stand for the volume fraction of O_2 and CO_2.

Analysis of metabolites

Spectrophotometric assays were performed at 30°C with a Hitachi 100-60 spectrophotometer. Acetate was assayed enzymatically with the Boehringer test kit no. 148261. Citrate and oxaloacetate were determined in an assay mixture containing 163 mM-glycylglycine buffer, pH 7·8; 0·15 mM-NADH; 0·2 mM-$ZnCl_2$; and sample. The extinction was read at 340 nm. The reaction for oxaloacetate determination was then started by adding 6 U/ml of malate dehydrogenase and the second extinction was read. Oxaloacetate was never present at detectable levels. Citrate could then be determined by measuring the increase in extinction following the addition of 0·24 U/ml citrate lyase.

Ethanol was assayed according to the colorimetric method of Verduyn *et al.* (1984). Glucose was determined with the GOD-PAP method of Boehringer. Glycerol was assayed enzymatically with the Boehringer test kit no. 148270.

The assay mixture for malate determination consisted of 250 mM-glycylglycine-glutamate buffer, pH 9; 2·4 mM-NAD^+; 1·6 U/ml glutamate oxaloacetate transaminase; and sample. The malate concentration was calculated from the increase in extinction at 340 nm as a result of the addition of 6 U/ml malate dehydrogenase.

2-Oxoglutarate was assayed with the following mixture: 240 mM-triethanolamine–HCl buffer,

pH 7·6; 1 mM-ADP; 0·15 mM-NADH; 32 mM-ammonium acetate; and sample. The concentration of 2-oxoglutarate was calculated from the increase in extinction at 340 nm following the addition of 12 U/ml glutamate dehydrogenase.

The assay mixture for the determination of pyruvate consisted of 124 mM-triethanolamine–HCl buffer, pH 7·6; 0·30 mM-NADH; 1·5 mM-EDTA; and sample. The concentration of pyruvate was calculated from the increase in extinction at 340 nm following the addition of 2 U/ml lactate dehydrogenase.

Succinate was determined with an assay mixture consisting of 108 mM-glycylglycine buffer, pH 8·4; 0·015 mM-$MgSO_4.7\,H_2O$; 0·15 mM-NADH; 0·04 mM-CoA; 0·054 mM-inosin triphosphate; 0·076 mM-phosphoenolpyruvate; 2 U/ml lactate dehydrogenase; 1·67 U/ml pyruvate kinase; and sample. The concentration of succinate was calculated from the increase in extinction at 340 nm following the addition of 0·5 U/ml succinyl-CoA synthetase.

Calculation of ATP production

ATP production needed for growth can be calculated from the equation:

$$q_{ATP,needed} = \frac{\mu + Y_{ATP} \times q_{ATP,m}}{Y_{ATP}} \quad (4)$$

in which $q_{ATP,needed}$ [mmol ATP × (g cells)$^{-1}$ × h^{-1}] represents the amount of ATP that is required for growth at a given specific rate μ (h^{-1}). The values for specific rate of ATP consumption in maintenance processes ($q_{ATP,m}$) and Y_{ATP} were taken from von Meyenburg (1969) as 1·572 mmol ATP × (g cells)$^{-1}$ × h^{-1} and 0·012 g cells/mmol ATP, respectively.

In non-fermenting aerobic steady-state cultures the rate of ATP production, q_{ATP}, is linearly proportional to the specific rate of oxygen consumption:

$$q_{ATP} = k \times q_{O_2} \quad (5)$$

It is assumed that the ATP consumption rate from equation (4) and the ATP production rate from equation (5) are equal. It is relevant to note that k in this equation does not only depend on the P/O ratio but also on ATP produced via substrate-level phosphorylation in glycolysis and the tricarboxylic acid (TCA) cycle. On the basis of the above-mentioned values for Y_{ATP} and $q_{ATP,m}$, and the measured oxygen consumption rates, q_{O_2}, under steady-state conditions (3·0 ± 0·5 and 5·6 ± 0·5 mmol × (g cells)$^{-1}$ × h^{-1} for $D = 0{\cdot}1$ and 0·2, respectively), k was calculated to be 3 ± 0·4 for both *S. cerevisiae* and *C. utilis*.

In the calculation of ATP production during the glucose pulse experiments, equation (5) should be corrected because of additional substrate-level phosphorylation caused by ethanol and acetate formation, and ATP consumption due to glycerol production. Substrate-level phosphorylation yields 1 mol of ATP for 1 mol of ethanol or acetate produced, whereas 1 mol of ATP is consumed for every mol of glycerol formed. Moreover, the corrections should take into account that production of 1 mol of acetate causes consumption of 1 mol of O_2 and that in production of 1 mol of glycerol, O_2 consumption is diminished by 0·5 mol. The rate of ATP production from the fraction of glucose that is completely oxidized to CO_2 is:

$$q_{ATP,complete\ oxidation} = k \times (q_{O_2} - q_{acetate} + 0{\cdot}5 \times q_{glycerol}) \quad (6)$$

The rate of ATP production via oxidative phosphorylation gained or lost due to acetate and glycerol formation can be given as:

$$q_{ATP,acetate/glycerol} = P/O \times (2 \times q_{acetate} - q_{glycerol}) \quad (7)$$

Excluding ATP production via substrate-level phosphorylation due to ethanol and acetate production and ATP consumption due to glycerol production, we define:

$$q_{ATP,respiration} = q_{ATP,complete\ oxidation} + q_{ATP,acetate/glycerol} \quad (8)$$

The total rate of ATP production, including ATP produced via substrate-level phosphorylation during ethanol and acetate production and ATP consumption during glycerol production, can be calculated with the following equation:

$$q_{ATP,total} = q_{ATP,respiration} + q_{ethanol} + q_{acetate} - q_{glycerol} \quad (9)$$

In our calculations a P/O ratio of 1·1 was used according to von Meyenburg (1969).

Protein determination

Protein concentrations in culture supernatants were determined by the Lowry method. Bovine serum albumin (Sigma, fatty acid-free) served as a standard. The protein content of whole cells was determined as follows. Cells were harvested and washed once with demineralized water. Cell suspensions (about 6 mg/ml) were then boiled in 1 mM-KOH for 10 min. After cooling, $CuSO_4.5H_2O$

was added to a final concentration of 25 mM. After 5 min this mixture was centrifuged in an MSE microcentrifuge and the absorbance at 555 nm was determined with a Hitachi 100-60 spectrophotometer.

Glycogen determination

Glycogen content of cells was determined by method 4 from Quain (1981). The glucose released at the end of this procedure was determined as mentioned above.

Trehalose determination

Trehalose was determined according to Stewart (1975) with the following modifications. After the extraction procedure, to 200 µl of the combined supernatants containing the extracted trehalose, 17·5 µl 4% (v/v) hydroxyl amine was added, and with 1 M-sodium acetate buffer (pH 5·9) the final volume was brought to 1 ml, leading to a pH of approximately 5·7. Then 0·2 U of trehalase (Sigma T8778) was added and the mixture was incubated for 2 h at 37°C. It was established by using a standard trehalose solution that all the trehalose added was indeed hydrolysed to glucose by this procedure. The glucose released at the end of this procedure was determined as mentioned above.

Biochemicals and enzymes

These were obtained from Boehringer Mannheim unless stated otherwise.

RESULTS

Anabolic reactivity

In order to elucidate the mechanism of the rapid alcoholic fermentation known to occur in *S. cerevisiae* after relief of glucose limitation, a comparative study was made of the transient behaviour of *S. cerevisiae* and *C. utilis* following the addition of a glucose pulse to glucose-limited chemostat cultures. The results presented in Figure 1 and Table 1 illustrate the marked differences in anabolic reactivity between the two yeasts under these conditions.

When *S. cerevisiae* was pulsed with glucose, the rate of biomass production (μ_{DW}) during the first 30 min, designated as phase I, was equal to the dilution rate at which the organism was pregrown (Table 1). *Candida utilis*, on the other hand, immediately accumulated biomass at a higher rate. In order to investigate if this 'growth rate', as determined by dry weight measurements, was related to production of protein (μ_{PROT}), the protein and N-contents were measured (Table 2). In contrast to *S. cerevisiae*, *C. utilis* showed a sharp decrease of protein and N-content during phase I. This indicates that *C. utilis* accumulates endogenous reserves in this phase. Indeed, during phase I the glycogen content increased considerably (Table 2). Trehalose remained virtually absent (not shown). The growth rate in phase I, based on protein production, μ_{PROT}, is related to the biomass production rate based on the measurement of dry weights, μ_{DW}, according to the equation:

$$\mu_{PROT} = \mu_{DW} + 2 \times (\ln P_{0.5} - P_{SS}) \qquad (10)$$

in which P_{SS} and $P_{0.5}$ are the protein content of the cells (g/g) during steady state and 0·5 h after addition of glucose. If the errors in the protein determinations are taken into account (Table 2), it can be estimated that also in *C. utilis* protein synthesis continued at approximately the same rate at which it was precultured.

During the second phase of the experiment (30–60 min after the glucose pulse, Tables 1 and 2), the biomass production rate of both organisms was approximately the same. Moreover, the protein content of the cells remained constant.

Catabolic reactivity

The specific rates of glucose and oxygen consumption by both yeasts, immediately after transition to glucose excess (phase I), were approximately the same (Figure 1 and Table 1), although large differences in metabolic behaviour were apparent. In *S. cerevisiae* an immediate alcoholic fermentation occurred whereas only little ethanol was produced by *C. utilis* (Figure 1 and Table 1). This ethanol production by *C. utilis* normally became apparent after 20 min. It was not caused by oxygen limitation in the culture fluid because the oxygen concentration was kept above 20% of air saturation. Even if pure oxygen was blown through the fermenter, ethanol could be detected.

Both yeasts excreted acetate after a glucose pulse. As with ethanol, metabolite production was more pronounced in *S. cerevisiae*. Glycerol production could only be detected in *S. cerevisiae* cultures (Table 1).

Theoretical analysis of ATP consumption and production in S. cerevisiae

In order to evaluate whether the alcoholic fermentation in *S. cerevisiae* is a necessity in energetic terms, a calculation was made on the kinetics of

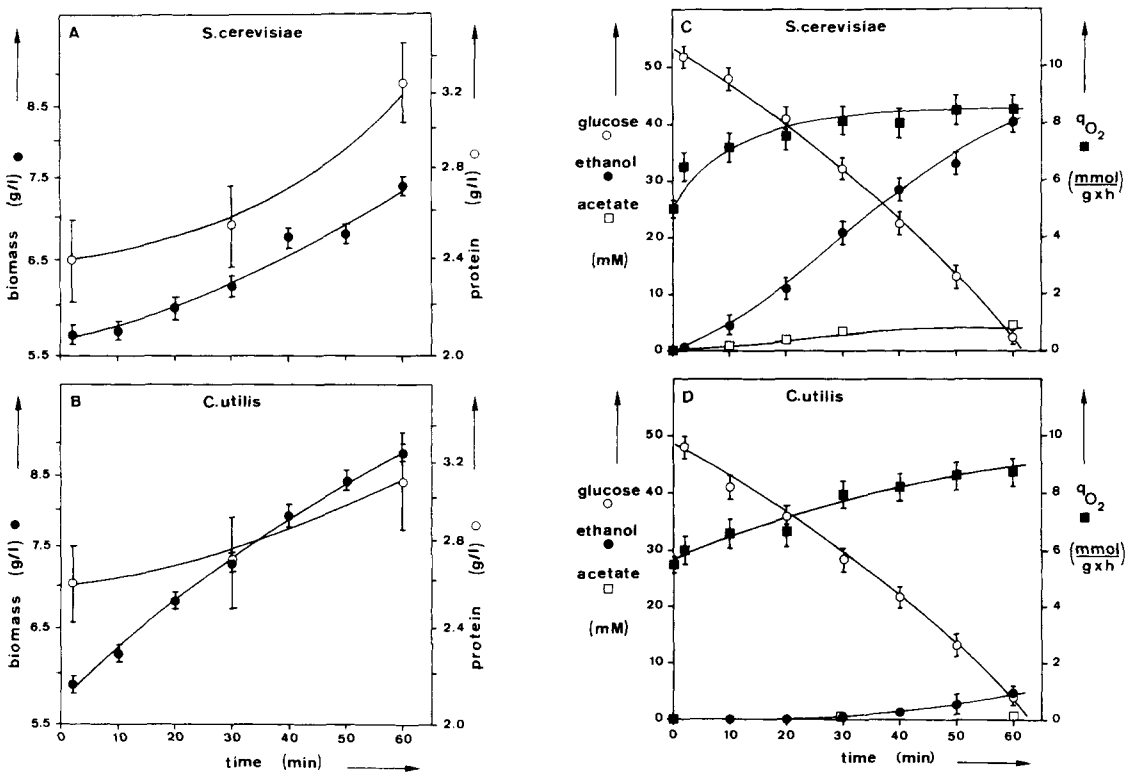

Figure 1. Typical plots of glucose pulse experiments with cultures of *S. cerevisiae* and *C. utilis* pre-grown under glucose limitation at $D = 0{\cdot}2\ h^{-1}$. Estimated error intervals are indicated by bars. A, B: Semi-log plots of biomass concentration and protein concentration during the experiment. C, D: Oxygen consumption rates and ethanol, acetate and glucose concentrations during the experiment.

Table 1. Fluxes, q [mmol × (g cells)$^{-1}$ × h^{-1}], during the first and second phase (see text) of glucose pulse experiments after pre-cultivation under glucose limitation at $D = 0{\cdot}1\ h^{-1}$ or $D = 0{\cdot}2\ h^{-1}$. Standard deviation values are given for two to four separate experiments. μ_{DW} is the production rate of biomass, determined by dry weight measurements.

	S. cerevisiae				*C. utilis*			
	$D = 0{\cdot}1\ h^{-1}$		$D = 0{\cdot}2\ h^{-1}$		$D = 0{\cdot}1\ h^{-1}$		$D = 0{\cdot}2\ h^{-1}$	
	Phase I	Phase II	Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
μ_{DW} (h^{-1})	0·09±0·03	0·31±0·03	0·19±0·02	0·32±0·02	0·59±0·02	0·37±0·04	0·53±0·03	0·33±0·02
$q_{glucose}$	5·4±0·5	9·0±1·0	6·7±0·7	9·5±1·0	5·2±0·8	5·2±0·3	5·5±1·2	5·6±0·8
$q_{ethanol}$	5·6±0·8	7·7±0·7	6·7±1·3	8·6±1·3	0·1±0·1	0·2±0·1	0·1±0·05	0·6±0·3
$q_{acetate}$	1·3±0·5	1·3±0·6	1·3±0·4	0·8±0·5	0·2±0·2	0·3±0·3	0	0·1±0·1
$q_{glycerol}$	0·5	0·5	0·4	0·1	0	0	0	0
q_{O_2}	6·6±0·3	7·5±0·3	7·7±0·3	8·1±0·3	7·3±0·3	8·4±0·3	7·0±0·3	8·4±0·3
q_{CO_2}	12·0±0·9	15·7±1·4	12·0±0·3	15·5±0·5	6·7±0·3	7·9±0·3	6·3±0·3	7·3±0·3

ATP turnover in this yeast. If it is assumed that the efficiency of mitochondrial respiration, i.e. the P/O ratio, does not change after a glucose pulse and that growth proceeds at a fixed value for Y_{ATP}, the rates of ATP production and consumption can be calculated (see Methods).

Table 2. Protein, N, C and glycogen contents [(g/g cells) × 100] of cells during steady state (SS) and after the addition of excess glucose. The glycogen content is expressed as glucose equivalents. Cultures were grown at $D = 0{\cdot}1$ or $0{\cdot}2\ h^{-1}$. The estimated error in all protein determinations was ±3.

	$D = 0{\cdot}1\ h^{-1}$								$D = 0{\cdot}2\ h^{-1}$	
	S. cerevisiae				*C. utilis*				*S. cerevisiae*	*C. utilis*
Time (min)	C	N	Protein	Glycogen	C	N	Protein	Glycogen	Protein	Protein
SS	43·7	7·7	42	6	45·9	9·6	53	3	42	44
15	44·8	8·0	—	—	45·8	8·7	45	—	—	41
30	45·1	8·1	43	2	45·7	8·2	40	17	41	37
60	—	8·6	44	—	45·6	7·8	39	14	44	35

Table 3. Theoretical calculation of ATP consumption and production [q_{ATP}, mmol × (g cells)$^{-1}$ × h^{-1}] after transition of *S. cerevisiae* from glucose limitation to glucose excess. Cells were pre-cultured at $D = 0{\cdot}1$ or $0{\cdot}2\ h^{-1}$.

	$D = 0{\cdot}1\ h^{-1}$		$D = 0{\cdot}2\ h^{-1}$	
	Phase I	Phase II	Phase I	Phase II
$q_{ATP,needed}$	9·1	27·4	17·4	28·2
$q_{ATP,total}$	25·1	30·2	31·8	33·0
$\frac{q_{ATP,total}}{q_{ATP,needed}}$	2·8	1·1	1·8	1·2
$q_{ATP,respiration}$	18·5	20·7	21·4	23·5
$\frac{q_{ATP,respiration}}{q_{ATP,needed}}$	2·0	0·8	1·2	0·8

On the basis of these assumptions the rate of ATP production in *S. cerevisiae* after a glucose pulse is much higher than that needed for growth in phase I (Table 3). Even if ATP production via substrate-level phosphorylation during ethanol and acetate production is neglected, the specific rate of ATP production in this phase exceeds that needed for sustaining growth at the observed rates. Possibly, the excess ATP produced will be dissipated by the action of ATPase. No attempts were made to calculate the ATP turnover for *C. utilis* during the transition in view of the marked changes in cellular composition.

Excretion of tricarboxylic acid cycle intermediates

Both yeasts rapidly excreted pyruvate after a glucose pulse (Figure 2 and Table 4). Pyruvate excretion is indicative of a high flux through the glycolytic pathway upon transition to glucose excess. Apparently, catabolism and anabolism of pyruvate cannot keep pace with its supply via glycolysis. *S. cerevisiae* also excreted malate, citrate, 2-oxoglutarate and succinate. Whereas the concentrations of malate and citrate were the same before and after glucose addition, the amounts of 2-oxoglutarate and succinate in the culture fluid increased with time upon a glucose pulse. Excretion of TCA-cycle intermediates was not observed with *C. utilis*. The presence of these compounds in cultures of *S. cerevisiae* is probably not a result of cell lysis. This can be concluded from the fact that in cultures of both *S. cerevisiae* and *C. utilis* only 15–20 mg/l of protein was found in the culture fluid under steady-state conditions (at a cell concentration of 6·25 g/l). Assuming that this protein is due to lysis of 40 mg of cells and that the

specific cell volume is 2 ml/g cells, the intracellular concentration of, for example, 2-oxoglutarate should have been as high as 750 mM.

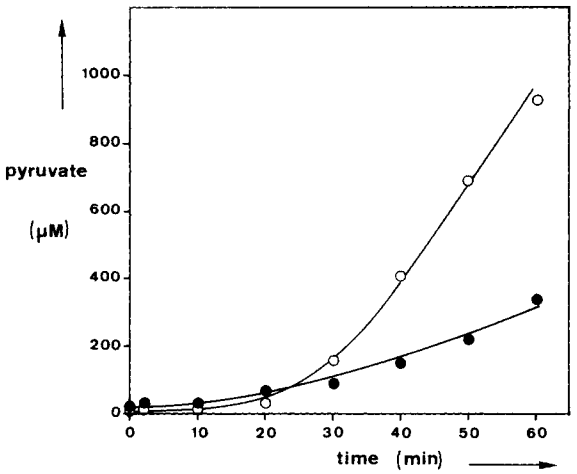

Figure 2. Typical plots of the pyruvate concentrations in the medium during glucose pulse experiments. Cells were pre-grown under glucose limitation at $D = 0{\cdot}2\ h^{-1}$. *S. cerevisiae* (●), *C. utilis* (○).

DISCUSSION

The tendency of *S. cerevisiae* towards alcoholic fermentation in the presence of excess glucose is an important phenomenon in the industrial production of bakers' yeast. This process relies on cultivation under glucose limitation in order to circumvent ethanol production. In large reactors this unwanted ethanol production is difficult to avoid due to imperfect mixing, resulting in sugar gradients. The cause of this ethanol production has been ascribed to a limited respiration capacity of *S. cerevisiae* (Fiechter *et al.*, 1981; Rieger *et al.*, 1983). However, the enzymic steps in glucose metabolism, associated with this limitation, have so far not been resolved. It is not known, for example, whether this would result from a bottleneck in the production of reducing equivalents (in the TCA cycle) or in their reoxidation (in the electron transport chain).

So far no comparative studies have been published on the behaviour of Crabtree-positive and Crabtree-negative yeasts upon transfer from sugar limitation to sugar excess. In our study *S. cerevisiae* and *C. utilis* were pre-grown under identical, controlled conditions. Therefore, possible effects of environmental factors such as medium composition, pH, oxygen tension and growth rate can be ruled out. The results clearly quantify the important differences between the activities of the two yeasts after the transition from glucose limitation to glucose excess (Figure 1, Tables 1 and 4). Whereas in *C. utilis* glucose is mainly catabolized via respiration, in *S. cerevisiae* a major portion of the glucose is fermented to ethanol (Figure 1). This different behaviour is apparently not caused by differences in glucose uptake rate or respiratory potential. Immediately after transfer to glucose excess these parameters reached approximately the same values in both yeasts (Table 1). Only after prolonged exposure to glucose excess (phase II) differences in glucose uptake and oxygen consumption became apparent (Table 1). The occurrence of alcoholic fermentation in *S. cerevisiae* after a glucose pulse does not seem to serve a physiological function. The rate of ATP synthesis is probably in excess of that required for growth (Table 3), at least initially (phase I).

From Table 2 it can be concluded that in phase I *C. utilis* accumulates reserve material in the form of glycogen. Hence, the glycolytic flux will be lower in this yeast than in *S. cerevisiae*, since in this phase glucose consumption was approximately equal in both organisms (Table 1). During phase II almost no decrease in the protein content was detected in *C. utilis*. Production and consumption of reserve material are probably in equilibrium in this phase. It is also during this phase that ethanol production (Figure 1) and pyruvate excretion (Figure 2) set in. This indicates that also in *C. utilis* the glycolytic flux becomes higher than the conversion of pyruvate in assimilatory and dissimilatory pathways. However, because the ethanol production rate was lower in *C. utilis* than in *S. cerevisiae* (Figure 1) it is probable that the activity of pyruvate decarboxylase, the key enzyme of alcoholic fermentation, is lower in *C. utilis* (Holzer, 1961).

The pronounced difference in the excretion of TCA-cycle intermediates between the two yeasts (Table 4) suggests that bottlenecks in glucose metabolism in *S. cerevisiae* may occur at the level of TCA-cycle activity or beyond. For instance, consumption of TCA-cycle intermediates for amino acid production could be a rate-limiting step.

Although our results do not exclude the possibility of a respiratory limitation in *S. cerevisiae*, the hypothesis of a limiting assimilatory capacity may equally well explain the observed behaviour of this yeast. For example, in the case of a bottleneck in the assimilation, inhibition of respiration could occur via respiratory control in the mitochondria, by a limited availability of ADP.

Table 4. Concentrations (mM) of metabolites in the culture fluid during steady state (SS) and glucose pulse experiments after pre-cultivation at $D = 0{\cdot}1\ h^{-1}$ (Table 4A) or $D = 0{\cdot}2\ h^{-1}$ (Table 4B).
Table 4A: Cells pre-cultured at $D = 0{\cdot}1\ h^{-1}$.

	S. cerevisiae				*C. utilis*			
	SS	10 min	30 min	50 min	SS	10 min	30 min	50 min
Glycerol	0·00	0·30	1·80	3·00	0·00	0·00	0·00	0·00
Ethanol	0·30	3·90	15·20	33·60	0·00	0·00	0·30	0·90
Acetate	0·30	1·10	4·70	8·40	0·00	0·60	1·50	2·40
Pyruvate	0·02	0·05	0·13	0·31	0·00	0·03	0·29	0·86
Malate	0·21	0·21	0·22	0·26	0·02	0·02	0·02	0·03
Citrate	0·03	0·04	0·03	0·04	0·00	0·00	0·00	0·00
2-Oxoglutarate	0·07	0·08	0·14	0·21	0·00	0·00	0·00	0·00
Succinate	0·09	0·12	0·14	0·17	0·00	0·00	0·00	0·00

Table 4B. Cells pre-cultured at $D = 0{\cdot}2\ h^{-1}$.

	S. cerevisiae				*C. utilis*			
	SS	10 min	30 min	50 min	SS	10 min	30 min	50 min
Glycerol	0·00	0·30	1·50	1·80	0·00	0·00	0·00	0·00
Ethanol	0·20	5·50	20·00	39·00	0·00	0·10	0·40	2·00
Acetate	0·10	0·90	3·40	6·00	0·00	0·20	0·30	0·20
Pyruvate	0·03	0·07	0·15	0·25	0·00	0·02	0·20	0·93
Malate	0·15	0·16	0·17	0·17	0·00	0·00	0·00	0·00
Citrate	0·03	0·03	0·03	0·05	0·00	0·00	0·00	0·00
2-Oxoglutarate	0·06	0·10	0·15	0·20	0·00	0·00	0·00	0·00
Succinate	0·08	0·09	0·11	0·17	0·00	0·00	0·00	0·00

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