Effect of Benzoic Acid on Metabolic Fluxes in Yeasts: A Continuous-Culture Study on the Regulation of Respiration and Alcoholic Fermentation

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Addition of benzoate to the medium reservoir of glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066 growing at a dilution rate (D) of $0.10 h^{-1}$ resulted in a decrease in the biomass yield, and an increase in the specific oxygen uptake rate (qO_2) from 2·5 to as high as $19.5 mmol g^{-1} h^{-1}$. Above a critical concentration, the presence of benzoate led to alcoholic fermentation and a reduction in qO_2 to $13 mmol g^{-1} h^{-1}$. The stimulatory effect of benzoate on respiration was dependent on the dilution rate: at high dilution rates respiration was not enhanced by benzoate. Cells could only gradually adapt to growth in the presence of benzoate: a pulse of benzoate given directly to the culture resulted in wash-out.

As the presence of benzoate in cultures growing at low dilution rates resulted in large changes in the catabolic glucose flux, it was of interest to study the effect of benzoate on the residual glucose concentration in the fermenter as well as on the level of some selected enzymes. At $D = 0.10 h^{-1}$, the residual glucose concentration increased proportionally with increasing benzoate concentration. This suggests that modulation of the glucose flux mainly occurs via a change in the extracellular glucose concentration rather than by synthesis of an additional amount of carriers. Also various intracellular enzyme levels were not positively correlated with the rate of respiration. A notable exception was citrate synthase: its level increased with increasing respiration rate.

Growth of *S. cerevisiae* in ethanol-limited cultures in the presence of benzoate also led to very high qO_2 levels of 19–$21 mmol g^{-1} h^{-1}$. During growth on glucose as well as on ethanol, the presence of benzoate coincided with an increase in the mitochondrial volume up to one quarter of the total cellular volume.

Also with the Crabtree-negative yeasts *Candida utilis*, *Kluyveromyces marxianus* and *Hansenula polymorpha*, growth in the presence of benzoate resulted in an increase in qO_2 and, at high concentrations of benzoate, in aerobic fermentation. In contrast to *S. cerevisiae*, the highest qO_2 of these yeasts when growing at $D = 0.10 h^{-1}$ in the presence of benzoate was equal to, or lower than the qO_2 attainable at μ_{max} without benzoate. Enzyme activities that were repressed by glucose in *S. cerevisiae* also declined in *K. marxianus* when the glucose flux was increased by the presence of benzoate.

The maximal aerobic fermentation rate at $D = 0.10 h^{-1}$ of the Crabtree-negative yeasts at high benzoate concentrations was considerably lower than for *S. cerevisiae*. This is probably due to the fact that under aerobic conditions these yeasts are unable to raise the low basal pyruvate decarboxylase level: cultivation without benzoate under oxygen-limited conditions resulted in rates of alcoholic fermentation and levels of pyruvate decarboxylase comparable to those of *S. cerevisiae*.

KEY WORDS — Benzoic acid: yeasts; Crabtree effect; respiration; fermentation; mitochondria; metabolic flux.

INTRODUCTION

In yeasts, glucose catabolism can occur via respiration, via alcoholic fermentation, or by a combination of these two pathways. Which pathway prevails depends on a number of factors, including strain and culture conditions. For instance,

Saccharomyces cerevisiae cultivated in aerobic glucose-limited chemostat cultures shows respirative growth at low dilution rates. Fermentation, together with respiration at an approximately constant specific rate occurs at high growth rates (Barford and Hall, 1979; Rieger *et al.*, 1983). The occurrence of alcoholic fermentation under aerobic conditions is known as the Crabtree effect. The

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Crabtree effect may occur under steady-state conditions (long-term effect, Fiechter *et al.*, 1981) or may be triggered during transient state (short-term effect, Petrik *et al.*, 1983; van Urk *et al.*, 1988): when glucose-limited cultures of *S. cerevisiae* are suddenly exposed to excess glucose, alcoholic fermentation is observed (Woehrer and Roehr, 1981; Verduyn *et al.*, 1984). However, the majority of yeasts, for example representatives of the genera *Candida, Hansenula, Kluyveromyces* and *Rhodotorula*, are Crabtree-negative; they do not perform aerobic alcoholic fermentation in the presence of high sugar concentrations, irrespective whether chemostat or batch cultivation is employed.

It is generally assumed that the major difference between *S. cerevisiae* and Crabtree-negative yeasts such as *Candida utilis* is the respiratory capacity (mmol O_2 consumed $g^{-1} h^{-1}$). Crabtree-negative species can attain considerably higher respiration rates than *S. cerevisiae* (Alexander and Jeffries, 1990; Barford, 1990). However, it has been shown recently that addition of butyrate to glucose-limited cultures of *S. cerevisiae* growing at a low dilution rate increased the *in situ* qO_2 from 2·5 to approximately 16 mmol $g^{-1} h^{-1}$ (Verduyn *et al.*, 1992). This was considerably higher than the qO_2 observed with glucose-limited cultures growing without butyrate at a rate approaching μ_{max}. This increase in qO_2 in the presence of butyrate was accompanied by a decrease in the biomass yield, as a larger part of the glucose was dissimilated. This observation prompted us to study the effects of weak acids on respiration in more detail. Benzoate, rather than butyrate, was chosen as a model acid, since benzoic acid was not metabolized by the yeasts used in this study. This is in contrast to butyrate, for example, which could serve as a carbon source for *C. utilis* (Verduyn *et al.*, unpublished).

The experiments described in this paper were aimed at answering the following questions:

(1) Is the maximal respiratory capacity of Crabtree-positive yeasts like *S. cerevisiae* intrinsically lower than that of Crabtree-negative yeasts?

(2) Can aerobic alcoholic fermentation (i.e. a Crabtree effect) also be provoked in Crabtree-negative yeasts?

(3) Can changes in metabolic fluxes that are observed during growth of yeasts in the presence of weak acids be explained in terms of enzyme levels, particularly with respect to key enzymes of respiratory pathways?

METHODS

Micr016ganisms and growth conditions

Saccharomyces cerevisiae CBS 8066, *Candida utilis* CBS 621, *Hansenula polymorpha* ATCC 46059 and *Kluyveromyces marxianus* CBS 6556 were grown in carbon-limited chemostat cultures at pH 5·0 and 30°C (except *K. marxianus*, which was grown at 37°C) in a fermenter as described by Harder *et al.* (1974), on a mineral medium containing per litre: $(NH_4)_2SO_4$, 5 g KH_2PO_4, 3 g; $MgSO_4.7H_2O$, 0·5 g; EDTA, 15 mg; $ZnSO_4.7H_2O$, 4·5 mg; $CoCl_2.6H_2O$, 0·3 mg; $MnCl_2.4H_2O$, 1 mg; $CuSO_4.5H_2O$, 0·3 mg; $CaCl_2.2H_2O$, 4·5 mg; $FeSO_4.7H_2O$, 3 mg; $NaMoO_4.2H_2O$, 0·4 mg; H_3BO_3, 1 mg- KI, 0·1 mg; and 0·025 ml silicone antifoam (BDH). Filter-sterilized vitamins were added after heat sterilization (120°C) of this medium. Final vitamin concentrations per litre were: biotin, 0·05 mg; calcium pantothenate, 1 mg; nicotinic acid, 1 mg; inositol, 25 mg; thiamine HCl, 1 mg; pyridoxine HCl, 1 mg; and para-aminobenzoic acid, 0·2 mg.

Stirrer rate was 800 rpm, with an air flow rate of 2 l min^{-1}, to give a dissolved-oxygen tension of at least 50% of air saturation under all experimental conditions. The condensor of the fermenter was connected to a cryostat and cooled at 2°C. The carbon source consisted of 10 g l^{-1} glucose or 7·5 g l^{-1} ethanol. When ethanol served as the carbon source, the medium reservoir was placed in a refrigerator. Sodium benzoate, sterilized as a 20% solution at 110°C for 15 min, was added to the medium reservoir.

For oxygen-limited conditions, the air supply was replaced by nitrogen gas (Air Products, The Netherlands) with a flow rate of 1 l min^{-1} via a flow controller. No special precautions were taken to prevent diffusion of oxygen into the system. The tubing consisted of silicone and the medium reservoir was not flushed with nitrogen.

Enzyme assays

Spectrophotometric assays were carried out at 30°C with freshly-prepared extracts as described by Postma *et al.* (1989a). Reaction rates were linearly proportional to the amount of enzyme added. Preparation of cell-free extracts, and assays of pyruvate decarboxylase (PDC; EC 4.1.1.1), alcohol dehydrogenases (ADH; EC 1.1.1.1, with either 100 mM-ethanol or 25 mM-pentanol as substrate), acetaldehyde dehydrogenases (EC 1.2.1.4

and 1.2.1.5) and acetyl-CoA synthetase (EC 6.2.1.1) were performed as described by Postma *et al.* (1989a). NADH dehydrogenase (EC 1.6.99.3) and cytochrome *c* oxidase (EC 1.9.3.1) were assayed according to Bruinenberg *et al.* (1985), citrate synthase (EC 4.1.3.7) according to Srere (1969) and hexokinase (EC 2.7.1.1) according to Postma *et al.* (1988). One unit is defined as the amount of enzyme catalyzing the conversion of 1 µmol substrate min^{-1}. Specific activity is expressed as units mg protein^{-1}. Catalase units are expressed as ΔA_{240} min^{-1}.

Protein concentrations of cell-free extracts were determined according to the Lowry method with bovine serum albumin (Sigma, fatty-acid free) as a standard.

Analytical methods

Dry weight of cultures was determined via filtration as described by Postma *et al.* (1989a). This parameter was regularly checked, both in the culture samples and in the effluent, to establish whether representative sampling of biomass from the fermenter occurred. The biomass concentration in the outlet was always within 3% of that in the fermenter.

Pyruvate and acetate in culture supernatants were analyzed by HPLC as described previously (Verduyn *et al.*, 1990a). Glycerol was analyzed by the same method. Glucose in medium reservoirs was assayed with the Boehringer GOD-PAP glucose kit (no. 676543) or with HPLC on the system described above. Residual glucose concentrations in the fermenter were assayed with the Boehringer glucose/hexokinase kit (no. 716251) after fast sampling of culture fluid in liquid nitrogen as described by Postma *et al.* (1988). Ethanol was assayed colorimetrically as described by Verduyn *et al.* (1990a) or with HPLC. Benzoate can also be assayed with this HPLC set-up; however, it eluted late (retention time approximately 40 min), which resulted in broad peaks. Concentrations below 1·5 mM could not be determined accurately. Between 1·5 and 2·5 mM, the accuracy was 10–20% (depending on the concentration) and it increased to better than 10% at benzoate concentrations higher than 2·5 mM.

Gas analysis

Analysis of oxygen consumption and carbon dioxide production in chemostat cultures was performed according to Postma *et al.* (1989a). Cultures were checked for the occurrence of oscillations (Parelukar *et al.*, 1986; Sonnleitner, 1991). When oscillations occurred, the experiments were postponed till the oscillations had dampened out. If this did not occur within 10 volume changes, cultivation was terminated and restarted with a clean fermenter.

Polarographic measurements

In order to establish the instantaneous maximum respiratory capacity (qO_2^{max}) of a culture in the presence of excess substrate, 5-ml samples were taken from the fermenter, washed once at room temperature with 10 ml complete medium without the carbon source, and resuspended to 5 ml with medium not containing the carbon source. This suspension was continuously stirred and aerated to prevent oxygen-limited conditions. A 100 µl sample was then added to a stirred vessel (Biological Oxygen Monitor, Yellow Springs, USA) containing 4·8 ml air-saturated medium (pH 5·0, 30°C). Endogenous respiration was followed for 5 min with a Clark-type oxygen electrode. The instantaneous respiratory capacity was then determined following the addition of 100 µl glucose or ethanol to a final concentration of 20 mM, assuming a dissolved-oxygen concentration of 0·235 µmol ml^{-1} at 30°C.

ATP assays

For the determination of ATP in biomass, a fresh cell sample was immediately diluted 100-fold in 0·9% NaCl and processed with a Lumac 3M Biocounter according to the instructions of the manufacturer. Benzoate did not interfere with this assay.

Electron microscopy

For electron micrographs, cells were fixed and stained as described in Visser *et al.* (1990). The mitochondrial volume was calculated from the micrographs according to the method of Weibel and Bolender (1973).

Chemicals

Sodium benzoate was obtained from Baker.

RESULTS

Effect of benzoate on glucose-limited cultures of S. cerevisiae at D = 0·10 h^{-1}

Weak acids such as benzoate may 'uncouple' energy generation from biomass formation by dissipating the proton-motive force across the plasma
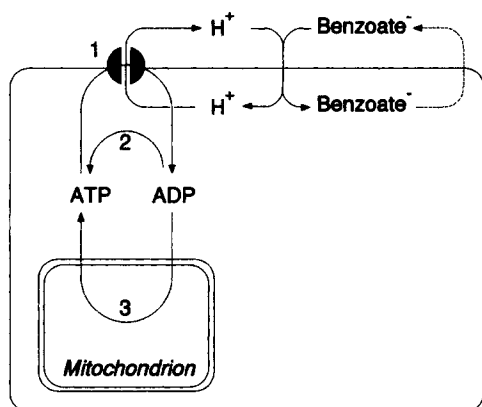
Figure 1. Schematic representation of uncoupling by benzoic acid at the level of the plasma membrane. 1: Plasma membrane ATPase; 2: ATP generation via substrate-level phosphorylation, and 3: ATP generation via respiration.

Figure 2. Biomass yield (■), specific oxygen consumption rate (○) and glucose flux (△) as a function of the residual benzoate concentration during glucose-limited growth of *S. cerevisiae* CBS 8066. The dilution rate was 0.10 h^{-1}. The arrow indicates the benzoate concentration at which the culture washed out.

membrane, due to influx of the undissociated acid. The resultant drop in cytosolic pH can be neutralized by the action of plasma membrane ATPase, which hydrolyzes ATP and pumps protons out of the cell. Thus, additional ATP has to be generated to compensate the influx of acid. This may be achieved via an increased respiration rate, as schematically shown in Figure 1.

Addition of benzoate to the medium reservoir of glucose-limited cultures of *S. cerevisiae* CBS 8066 growing at a dilution rate (D) of 0.10 h^{-1} resulted in a concentration-dependent decrease of the biomass yield from 0.51 g biomass g glucose^{-1} without benzoate, to 0.15 g g^{-1} at a residual concentration of 10 mM-benzoate (Figure 2). The specific oxygen consumption rate (qO$_2$) increased from 2.5 ± 0.2 in the absence of benzoate, to a value as high as $19.5 \pm 1 \text{ mmol g}^{-1} \text{h}^{-1}$ at a residual benzoate concentration of 10 mM (Figure 2). The highest measured value of the specific oxygen uptake rate in the presence of benzoate will be indicated as the 'critical respiratory capacity' (Table 1). Up to the corresponding benzoate concentration (10 mM), biomass and CO$_2$ were the only main products. The respiratory quotient (RQ) was between 0.95 and 1.10 for all steady states. When the benzoate concentration was increased further to 10.5 mM, a large drop in cell yield occurred and ethanol formation became apparent. The yield declined to 0.09 g biomass g glucose^{-1}, with an ethanol production rate of $6.4 \text{ mmol g}^{-1} \text{h}^{-1}$. However, a normal steady state could be established. The qO$_2$ decreased to a constant value of $13 \pm 1 \text{ mmol g}^{-1} \text{h}^{-1}$ (Figure 2).
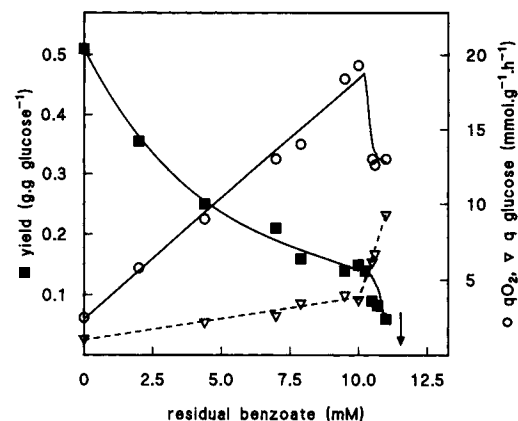
Table 1. Definition of various specific oxygen uptake rates

Term	Definition
qO$_2$	Specific oxygen uptake rate, measured *in situ* via analysis of the exhaust gas of cultures
qO$_2^{max}$	qO$_2$ of washed cell samples (i.e. not containing benzoate) upon addition of excess substrate. qO$_2^{max}$ values were assessed by oxygen probe measurements
qO$_2^{crit}$	Highest qO$_2$ measured *in situ* in the presence of benzoate. The value of qO$_2^{crit}$ was assessed by analysis of the culture exhaust gas via gradual increase of the benzoate concentration in the medium reservoir

A further increase in the benzoate concentration resulted in increased fermentation, and a further decrease in the biomass yield to 0.06 g biomass g glucose^{-1}, but the cells maintained the same qO$_2$. Cultures were run for another 150 h but no changes in dry weight, qO$_2$ or q ethanol were observed.

The above phenomena were independent of the reservoir glucose concentration: the same fluxes were obtained when the glucose concentration in the medium-reservoir was doubled.

The presence of benzoate resulted in an increase in the pyruvate concentration in the culture from 0.07 mM, without benzoate, to 0.17 mM at 10 mM

residual benzoate. Acetate was also encountered, its concentration increased from $1 \cdot 1$ mM to $1 \cdot 7$ mM between benzoate concentrations of 0 and 10 mM. Although these are comparatively small increases, it can be calculated that the specific production rates of pyruvate and acetate increased seven-fold and five-fold, respectively, in the presence of 10 mM residual benzoate, as compared to cultures without benzoate.

In all steady-state situations $90 \pm 5\%$ of the benzoate added to the medium reservoir was recovered in the medium. If it is assumed, for simplicity, that all intracellular benzoate is present in the cytosol, it can be calculated, with a cytosolic volume of $2 \cdot 0$ ml g cells^{-1} (Valle et al., 1986; Warth, 1988), that the total intracellular concentration (anion and acid) would be $0 \cdot 37$ M at a residual benzoate concentration in the culture fluid of 10 mM; in other words, an accumulation factor of 37. Although this concentration factor is considerable, it is still significantly lower than expected if the weak acid is distributed according to the ΔpH. In that case, it can be calculated that benzoate $(pK = 4 \cdot 19)$ should accumulate $357 \times$, assuming an intracellular pH of approximately $7 \cdot 0$ (cf Verduyn et al., 1990a for calculations and references). Also from the experimental data of Warth (1988) for a number of yeasts, an accumulation factor for benzoate of only 15–$20 \times$ can be calculated.

Effect of a benzoate pulse on S. cerevisiae

In order to establish if, and to what extent, an instantaneous increase in qO_2 occurs upon addition of a non-metabolizable weak acid, a pulse of benzoate was given directly to a glucose-limited culture, pregrown without benzoate.

Injection of 10 mM-benzoate to a glucose-limited chemostat culture $(D = 0 \cdot 11 \text{ h}^{-1})$ of S. cerevisiae CBS 8066, with the medium supply left running to provide a carbon and energy source, resulted in an immediate decrease in dry weight, and accumulation of glucose (Figure 3A). The intracellular ATP concentration increased from the steady-state value of $2 \cdot 2$ mM to approximately 4 mM and subsequently declined to 1 mM (Figure 3A). During the initial period after the pulse, acetate accumulated up to 25 mM. The pyruvate concentration in the culture increased six-fold. Remarkably, however, alcoholic fermentation remained very low during the first 10 h after the pulse. The in-situ specific oxygen uptake rate declined from $2 \cdot 5$ mmol O_2 g^{-1} h^{-1} to approximately 1 mmol g^{-1} h^{-1}. Nevertheless, measurement
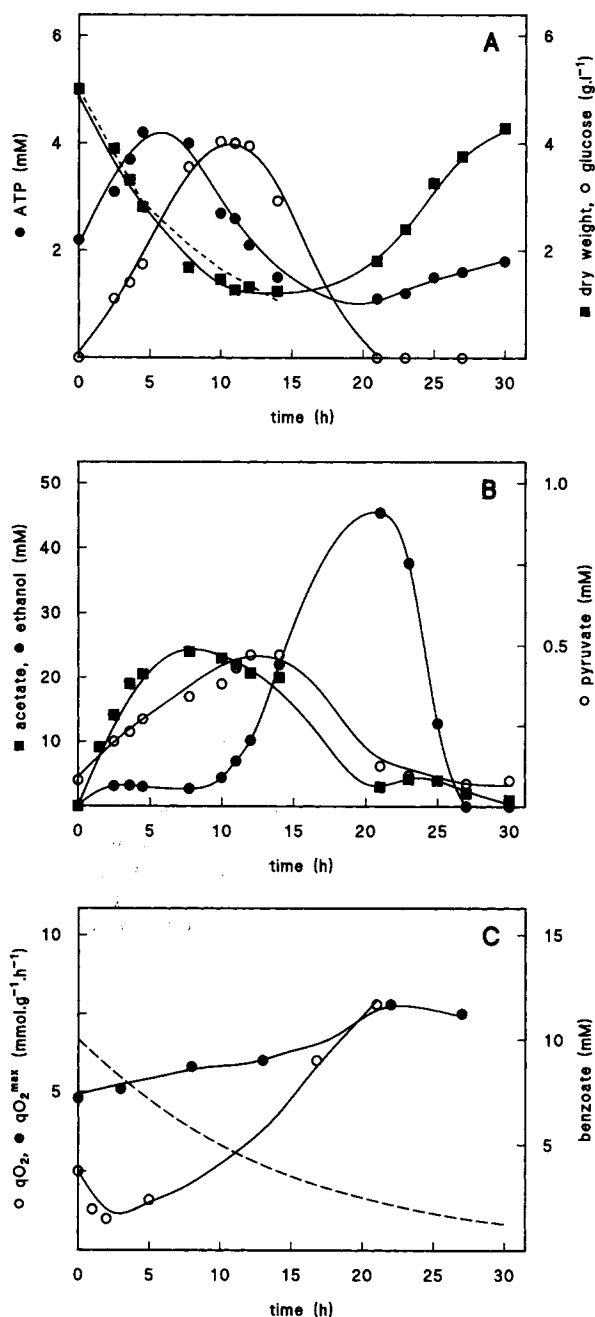
Figure 3. (A) Dry weight ($\blacksquare$), glucose concentration ($\bigcirc$) and intracellular ATP concentration ($\bullet$) during a benzoate pulse (10 mM) to a glucose-limited chemostat culture $(S_r = 10 \text{ g l}^{-1})$ of S. cerevisiae CBS 8066 at $D = 0 \cdot 11$ h^{-1}. The broken line shows the theoretical wash-out curve of biomass, assuming no growth. (B) Concentrations of acetate ($\blacksquare$), ethanol ($\bullet$) and pyruvate ($\bigcirc$). Conditions as in (A). (C) In-situ specific oxygen consumption rate ($\bigcirc$) and instantaneous maximum qO_2 of washed cells ($\bullet$). Conditions as in (A).

of the oxygen consumption rate of cell samples after removal from the fermenter and washing indicated that the cells had retained a normal instantaneous qO_2^{max} (for definitions see Table 1) of approximately 5 mmol g^{-1} h^{-1} (Figure 3B). After several hours, a rapid increase in ethanol production was observed, with a concomitant decline in residual glucose concentration. In the second phase, rapid uptake of ethanol occurred, accompanied by an increase in biomass concentration and a gradual return of the intracellular ATP concentration to the normal steady-state value (Figure 3A). This phase was associated with increased *in-situ* qO_2 as well as increased qO_2^{max} values (Figure 3C). It can be concluded from these experiments that cells have to adapt gradually to benzoate: the phenomena observed upon a pulse of benzoate are different as compared to a gradual increase in the benzoate concentration in the medium reservoir (Figure 2). Sudden exposure to a high concentration of benzoate results in inhibition, rather than stimulation of respiration.

Effect of growth rate on benzoate-mediated modulation of metabolic fluxes in glucose-limited S. cerevisiae cultures

Analogous to the experiments described above, the effect of benzoate additions to the medium reservoir was studied at other dilution rates in order to determine the critical respiratory capacity at a given dilution rate. This critical respiratory capacity is defined as the highest oxygen uptake rate measured *in-situ* in the presence of benzoate (Table 1). The critical respiratory capacity appeared to be maximal between $D = 0.10$ and 0.20 h^{-1} (Figure 4). Between dilution rates of 0.20 h^{-1} and 0.38 h^{-1}, the highest attainable dilution rate without alcoholic fermentation in cultures without benzoate (Postma *et al.*, 1989a), the critical respiratory capacity qO_2 decreased from 20 ± 1.5 to 12 ± 1 mmol g^{-1} h^{-1} (Figure 4). Addition of benzoate to cultures growing at $D > 0.38$ h^{-1} did not result in an increase in qO_2 but only in a decrease in yield and an increase in ethanol production (results not shown).

The amount of benzoate which had to be added with increasing dilution rate to achieve a given amount of reduction of the biomass yield, or a given increase in qO_2 as compared to growth without benzoate, increased with growth rate. For instance, the residual benzoate concentrations required for a 50% reduction of the biomass yield at dilution rates of 0.1 and 0.2 h^{-1} (yield 0.51 g g glucose^{-1} in the
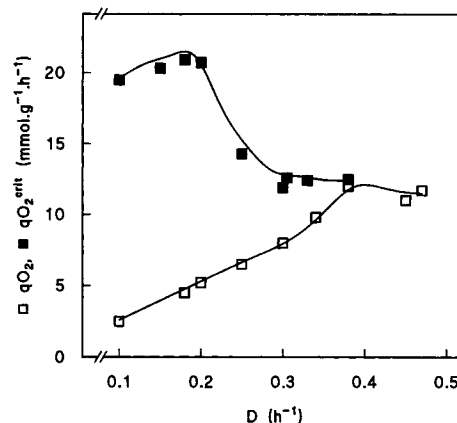

Figure 4. Critical specific oxygen consumption rate (■) in the presence of benzoate and qO_2 in the absence of benzoate (□) as a function of the dilution rate during glucose-limited growth of *S. cerevisiae* CBS 8066.

absence of benzoate) were approximately 4.3 mM (Figure 2) and 9 mM, respectively (not shown). However, the total amount of benzoate which can be tolerated by the culture quickly declines at $D > 0.2$ h^{-1}. This is due to the fact that the difference between the critical respiratory capacity and the *in-situ* qO_2 becomes smaller with increasing growth rate (Figure 4). These results suggest that the effect of benzoate probably is a typical growth rate-independent maintenance energy effect (Pirt, 1965), as shown previously for propionate with anaerobic glucose-limited cultures of *S. cerevisiae* CBS 8066 (Verduyn *et al.*, 1990b).

Regulation of glucose transport in S. cerevisiae

Since addition of benzoate to the medium feed of glucose-limited chemostat cultures led to a large increase in glucose flux (Figure 2), it was of interest to study the residual glucose concentration(s) in the fermenter. During aerobic glucose-limited growth at $D = 0.10$ h^{-1}, two glucose transport systems are present, with K_m values of 1 and 20 mM, and V_{max} values of approximately 160 and 120 μmol g biomass^{-1} min^{-1}, respectively (Postma *et al.*, 1989b). The *in-situ* glucose flux ($q_{glucose}$) depends on the residual substrate concentration in the fermenter and the kinetic properties of the glucose transporters, i.e K_m and V_{max}, according to: $q = \sum (V_{max}{}^{*}s) / (K_m + s)$. In order to estimate the *in-situ* transport activity at a fixed dilution rate of 0.10 h^{-1}, but at different glucose fluxes, the residual glucose concentration in the fermenter was determined in steady
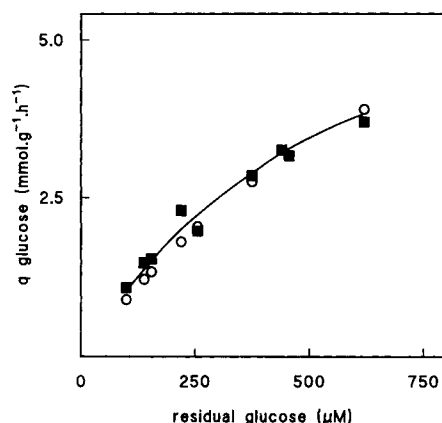
Figure 5. *In-situ* glucose flux (q glucose) as a function of the residual glucose concentration in the fermenter (after fast sampling) during glucose-limited growth of *S. cerevisiae* CBS 8066 in the presence of increasing amounts of benzoate (■) and q glucose calculated from the kinetic parameters of glucose carriers in *S. cerevisiae* (see text) (○). The dilution rate was $0.10\,h^{-1}$.

states at different residual benzoate concentrations. The residual glucose concentration in the fermenter increased with increasing amounts of benzoate from 100 μM without benzoate to 600 μM at a residual benzoate concentration of 10 mM. This was associated with an increase in the glucose flux from 1·1 to 3·9 mmol $g^{-1}\,h^{-1}$ (Figure 5), the highest flux observed without alcoholic fermentation (Figure 2). With the equation shown above, the transport activity, which is the sum of the activities of the two carriers involved, can be calculated. The data thus obtained show a good fit with the glucose flux as calculated from the equation shown above (Figure 5). For example, it can be calculated that with a residual glucose concentration of 600 μM, the activity of the glucose carriers results in a $q_{glucose}$ of 3·8 mmol $g^{-1}\,h^{-1}$ (of which only 5% is due to the low-affinity carrier).

Enzyme levels in glucose-limited cultures of
S. cerevisiae *at* D = 0·10 h⁻¹

To investigate whether the increase in glucose flux correlated with an adjustment of enzyme levels, several key enzymes in different parts of the central metabolism, i.e. glycolysis, tricarboxy acid (TCA) cycle and respiratory chain, were assayed. The specific activity of the various enzymes as a function of the glucose flux is shown in Figure 6A–C.
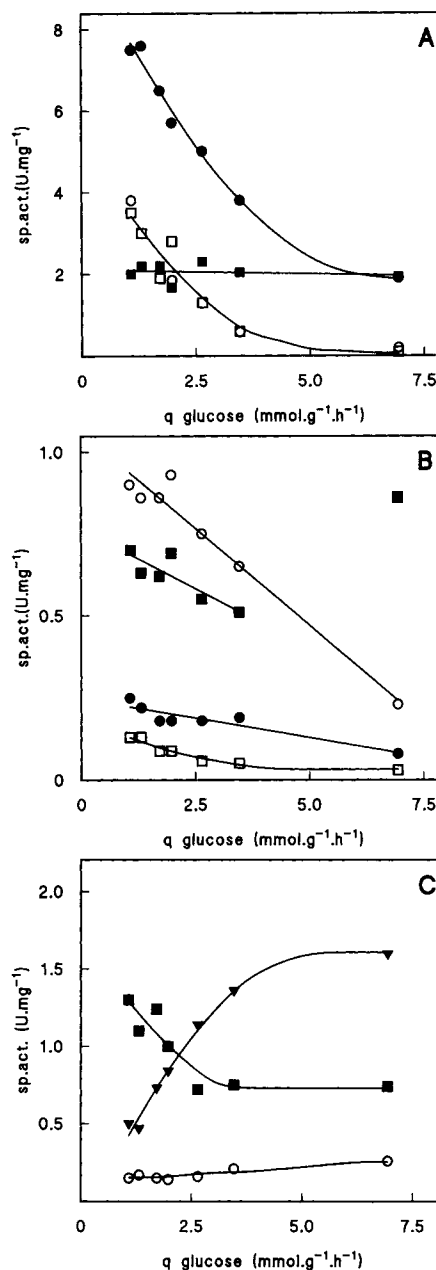
Figure 6. Specific activities (U mg protein⁻¹) of selected enzymes as a function of the glucose flux following addition of benzoate to glucose-limited *S. cerevisiae* CBS 8066. The first point represents the value without benzoate. The dilution rate was $0.10\,h^{-1}$. (A) Hexokinase (■), catalase (□), ethanol-dependent alcohol dehydrogenase (●) and pentanol-dependent alcohol dehydrogenase (○). Only a few assays of the latter have been made. (B) Pyruvate decarboxylase (■), NAD^+ (○) and $NADP^+$ (●)-dependent acetaldehyde dehydrogenase, and acetyl-CoA synthetase (□). (C) NADH dehydrogenases (■), citrate synthase (▼) and cytochrome *c* oxidase (○).

Hexokinase activity was not significantly affected with increasing benzoate concentrations at $D = 0.10 \, h^{-1}$. Its activity was approximately 2 U (mg protein)$^{-1}$, similar to that reported in glucose-limited chemostat cultures between $D = 0.10$ and $0.39 \, h^{-1}$ for this strain (Postma *et al.*, 1989a). Catalase activity declined drastically, almost 40-fold, from 3.5 to 0.1 U mg protein^{-1} (Figure 6A).

Several isoenzymes of ADH occur in *S. cerevisiae*. Two of these enzymes, which are localized in the cytosol and in the mitochondria, respectively, are glucose-repressible. The cytosolic, repressible ADH which functions in the oxidation of ethanol, has a high activity towards pentanol (Bränden *et al.*, 1975). This also holds for the mitochondrial ADH. The function of the latter enzyme is not known (Fraenkel, 1982). In addition to these repressible ADHs, a constitutive ADH is found in the cytosol. This enzyme, which functions in the formation of ethanol from acetaldehyde, has virtually no reactivity toward pentanol (Lutstorf and Magnet, 1968; Verduyn *et al.*, 1988). The levels of both ethanol- and pentanol-dependent ADH activity decreased strongly with the glucose flux (Figure 6B).

Of the enzymes of the pyruvate 'bypass' (PDC, acetaldehyde dehydrogenases and acetyl-CoA synthetase) (Holzer and Goedde, 1957), PDC was not significantly affected (Figure 6B). However, the other enzymes of the bypass, the NAD^+- and $NADP^+$-dependent acetaldehyde dehydrogenases and acetyl-CoA synthetase, decreased by a factor of three (Figure 6B) with increasing glucose fluxes. Citrate synthase activity increased in specific activity from 0.5 to 1.6 U mg protein^{-1}. A small decrease in NADH-dehydrogenase activity was observed, whereas cytochrome *c* oxidase activity was almost constant (Figure 6C).

Effect of benzoate on ethanol-limited cultures of S. cerevisiae

It is often assumed that the inability of *S. cerevisiae* strains to respire sugars at a high rate resides at the level of pyruvate, i.e. is caused by a limitation in the rate of supply of pyruvate to the TCA cycle, or by a low oxidation rate of pyruvate (reviewed by Käppeli, 1986; Ratledge, 1991). It was therefore investigated to what extent benzoate affects respiration on a growth substrate that is not metabolized via pyruvate as a key intermediate.

Addition of benzoate to the medium reservoir of an ethanol-limited culture of *S. cerevisiae* growing
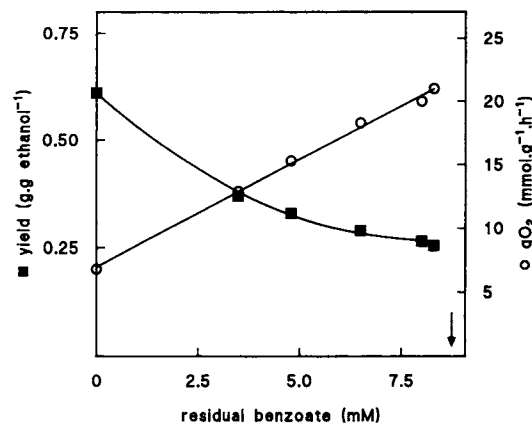

Figure 7. Biomass yield (■) and specific oxygen consumption rate (○) as a function of the residual benzoate concentration during ethanol-limited growth of *S. cerevisiae* CBS 8066. The dilution rate was $0.10 \, h^{-1}$. The arrow indicates the benzoate concentration at which the culture washed out.

at $D = 0.10 \, h^{-1}$ also resulted in a decrease in the biomass yield and a linear increase in qO_2 to a value of $21 \pm 1.5 \, \text{mmol g}^{-1} h^{-1}$ (Figure 7) at a residual benzoate concentration of 8 mM. A further addition of 1 mM-benzoate to the reservoir resulted in accumulation of ethanol and acetate and wash-out. Ethanol-limited chemostat cultures without benzoate exhibited a yield of 0.61 ± 0.02 g biomass (g ethanol)$^{-1}$ between dilution rates of 0.10 and $0.19 \, h^{-1}$. The μ_{max} was $0.21 \, h^{-1}$, as also reported by Postma *et al.* (1989b). The qO_2 in cultures without benzoate increased linearly, from 6.8 ± 0.5 at $D = 0.10 \, h^{-1}$, to a maximal value of $14 \pm 1 \, \text{mmol g}^{-1} h^{-1}$ at $D = 0.19 \, h^{-1}$ (Figure 7). In the presence of benzoate, the highest qO_2 that could be obtained was 21.5 at $D = 0.10 \, h^{-1}$ and $19 \pm 1 \, \text{mmol g}^{-1} h^{-1}$ at $D = 0.19 \, h^{-1}$. When the results are plotted in terms of ethanol fluxes it is remarkable that the observed ethanol flux in the presence of benzoate is similar for all dilution rates and that it is higher than the maximal ethanol flux achieved without benzoate (Figure 8).

The enzymes measured previously in glucose-limited cultures were also assayed in ethanol-limited cultures grown in the presence or absence of benzoate. The results (Table 2) differ considerably from those observed for glucose-limited cultures. In the latter, a significant decrease in, for example, catalase and acetaldehyde dehydrogenase levels was observed. In ethanol-limited cultures, catalase, for instance, was not affected by the presence of benzoate, and an increase was observed in the level of acetaldehyde
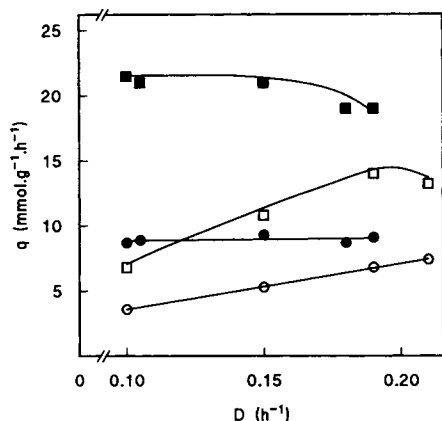
Figure 8. Critical respiratory capacity (■) and ethanol flux (●) in the presence of benzoate, and qO_2 (□) and ethanol flux (○) in the absence of benzoate during ethanol-limited growth of *S. cerevisiae* CBS 8066.

dehydrogenase(s). Hexokinase activity was only a third of that in glucose-limited chemostat cultures. From these results it can be concluded that, in ethanol-limited cultures, repression of the assayed enzymes does not occur. This is in sharp contrast to the results with glucose-limited cultures (Figure 6A–C).

Mitochondrial volume in S. cerevisiae

The *in-situ* respiration rate (qO_2) and the maximum instantaneous respiration capacity (qO_2^{max}), measured in cultures grown in the absence of benzoate, depends on the carbon source. Both these values are higher in ethanol-limited cultures than in glucose-limited cultures grown at the same dilution rates (Table 3). Measurement of the mitochondrial volume indicated that ethanol-limited cells had a higher mitochondrial volume than glucose-limited cells. Growth of glucose- and ethanol-limited cultures in the presence of benzoate resulted in an increase of the mitochondrial volume up to a maximum of 26% of the cell volume (Table 3). The large mitochondrial volume of cells grown in the presence of benzoate is readily apparent from electron micrographs (not shown).

Effect of benzoate on glucose-limited cultures of Crabtree-negative yeasts

The *in-situ* qO_2 for Crabtree-negative yeasts, grown in carbon-limited chemostat cultures without benzoate, usually increases linearly with the dilution

rate (for examples see Alexander and Jeffries, 1990; Barford, 1990). For *C. utilis*, for example, the *in-situ* specific oxygen uptake rate in glucose-limited cultures in the absence of benzoate increased from $2\cdot5\pm 0\cdot2$ to 14 ± 1 mmol O_2 g^{-1} h^{-1} when the dilution rate was increased from $0\cdot10$ to $0\cdot55$ h^{-1} (Figure 9). At high dilution rates considerable accumulation of pyruvate occurred, with a maximal concentration of $2\cdot5$ mM at $D=0\cdot55$ h^{-1} (Figure 9).

Addition of benzoate to the medium reservoir of glucose-limited chemostat cultures of *C. utilis*, and of other Crabtree-negative yeasts such as *K. marxianus* and *H. polymorpha*, resulted in a similar behaviour as observed with *S. cerevisiae*: a decrease in yield and an increase in qO_2. Surprisingly, alcoholic fermentation also occurred for all three species when the critical respiratory capacity was reached. The critical respiratory capacity of *C. utilis* increased from $9\cdot0\pm1\cdot0$ mmol g^{-1} h^{-1} at $D=0\cdot10$ h^{-1} to $14\cdot1\pm 1\cdot0$ mmol g^{-1} h^{-1} at $D=0\cdot55$ h^{-1} (Figure 9). Growth in the presence of benzoate at this dilution rate did not result in an increase in qO_2 but in a gradual wash-out.

The pattern of qO_2^{crit} as a function of the dilution rate as observed with *C. utilis* (Figure 9) was rather different from that of *S. cerevisiae* (Figure 4). In *C. utilis* the qO_2^{crit} increased with increasing dilution rate and became equal to the *in-situ* qO_2, without benzoate, at μ_{max} (Figure 9). In *S. cerevisiae*, however, the qO_2^{crit} declined with the dilution rate and became equal to the *in-situ* qO_2 at a dilution rate below μ_{max} (Figure 4).

The qO_2^{crit} may be regarded as a reflection of the highest attainable level of a rate-limiting key respiratory enzyme. The maximum level of this hypothetical enzyme, in turn, will depend on its maximum rate of synthesis (assuming that turnover is low). In order to compare the regulation of respiratory activity of *S. cerevisiae* and *C. utilis*, it therefore is relevant to compare the product of the critical oxygen consumption rate and the dilution rate. In chemostat mathematics this product is known as productivity, in this case of (a) critical component(s) of the respiratory machinery. The results shown in Figure 10 accentuate the differences in the regulation of the synthesis of the respiratory machinery in *S. cerevisiae* and *C. utilis* as reflected in Figures 4 and 9.

For *K. marxianus* and *H. polymorpha*, the critical respiratory capacity was only determined at $D=0\cdot10$ h^{-1}. Values of $12\cdot0$ for *K. marxianus*, and $11\cdot5$ mmol g^{-1} h^{-1} for *H. polymorpha* were observed (Table 4), much lower than the value of $19\cdot5$ mmol g^{-1} h^{-1} for *S. cerevisiae* (Table 4).

Table 2. Specific enzyme activities (U mg protein^{-1}) in ethanol-limited chemostat cultures of *S. cerevisiae* CBS 8066, in the absence of benzoate and in the presence of 7·8 mM residual benzoate, respectively, in relation to the specific ethanol and oxygen consumption rates (q, mmol g^{-1} h^{-1})

	No benzoate		With benzoate
Dilution rate (h^{-1})	0·10	0·19	0·10
Specific consumption rates			
q Ethanol	3·6	6·8	8·3
qO$_2$	6·8	14·0	20·0
Enzyme activities			
Acetaldehyde dehydrogenase			
NAD$^+$	0·48	0·92	1·07
NADP$^+$	0·17	0·23	0·38
Acetyl-CoA synthetase	0·46	0·37	0·28
Alcohol dehydrogenase			
Ethanol	9·0	nd	6·8
Pentanol	2·9	nd	3·1
Catalase	3·0	3·3	3·6
Citrate synthase	1·10	1·22	1·27
Cytochrome *c* oxidase	0·24	0·21	0·26
Hexokinase	0·56	nd	0·66
NADH dehydrogenase	1·00	1·22	1·21

nd: Not determined.

Table 3. *In-situ* respiration rate (qO$_2$), instantaneous qO$_2$ of washed cells in the presence of excess glucose (qO$_2^{max}$), and mitochondrial volume (as percentage of total cell volume) in *S. cerevisiae* CBS 8066 grown in glucose- or ethanol-limited cultures in the absence or presence of benzoate. The dilution rate was 0·10 h^{-1}

Carbon source	Glucose		Ethanol	
Benzoate (mM)	0	9	0	7·8
qO$_2$ (mmol g^{-1} h^{-1})	2·5	17	6·6	20
qO$_2^{max}$ (mmol g^{-1} h^{-1})	4·5	nd	9·0	nd
Mitochondrial volume (%)	13	22	19	26

nd: Not determined.

The highest fermentation rate observed with Crabtree-negative yeasts in the presence of benzoate at low dilution rates was considerably lower than that for *S. cerevisiae*. In order to establish whether the enzyme patterns as observed for *S. cerevisiae* upon addition of benzoate (Figure 6A–C) also occur in a Crabtree-negative species, the same enzymes were measured for *K. marxianus* at a dilution rate of 0·10 h^{-1}. This was done for two situations: firstly without benzoate and secondly at the critical respiration rate induced by benzoate. The enzyme patterns were similar to those observed with *S. cerevisiae*, with a large decline in all the glucose-repressible enzymes. In contrast to *S. cerevisiae, K. marxianus* also showed a decrease in cytochrome *c* oxidase (Table 5).

Regulation of alcoholic fermentation in yeasts

The Crabtree-negative yeasts investigated in this study are facultatively fermentative (Barnett *et al.*,
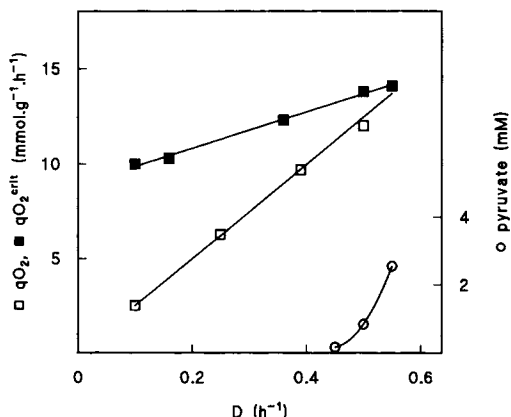
Figure 9. Critical respiratory capacity in the presence of benzoate (■), and qO₂ (□) and pyruvate concentration (○) in the absence of benzoate during glucose-limited chemostat growth of *C. utilis* CBS 621.
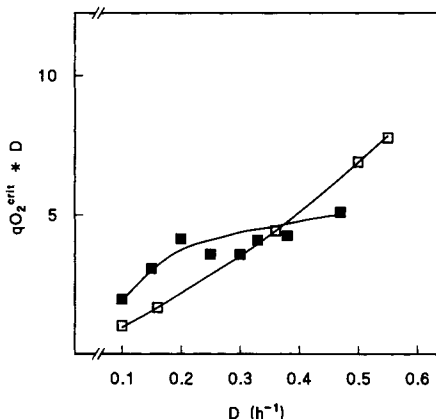

Figure 10. Calculated maximal synthesis rate of the respiratory machinery (critical qO₂*D) as a function of the dilution rate in glucose-limited cultures of *S. cerevisiae* CBS 8066 (■) and *C. utilis* CBS 621 (□).

1990). When grown under oxygen limitation, for example in shake-flask cultures, these yeasts exhibit alcoholic fermentation at a rate comparable to that obtained with *S. cerevisiae* (Van Dijken and Scheffers, 1986). However, the rates of aerobic alcoholic fermentation that could be provoked in Crabtree-negative yeasts by high benzoate concentrations were far lower than those observed with *S. cerevisiae*. It was therefore decided to compare the benzoate-triggered aerobic alcohol fermentation with an oxygen-limited situation, particularly with respect to enzyme levels. To this end, *S. cerevisiae* and the Crabtree-negative yeasts *C. utilis, H.*

polymorpha and *K. marxianus* were grown under oxygen limitation at $D = 0\cdot1$ h^{-1}, as described in Materials and Methods. For all yeasts, steady states were obtained that were characterized by a low biomass yield and the presence of considerable quantities of ethanol and glycerol (Table 6) and, to a much lesser extent, pyruvate and acetate. The actual oxygen uptake rate could not be accurately measured under these conditions, but assay of washed cell samples showed that the cells had retained a low respiration capacity (Table 6). From Table 7 it can be seen that oxygen-limited conditions led to ethanol production rates considerably higher than those observed in the aerobic cultures with benzoate. Furthermore, the PDC level was higher under oxygen-limited conditions. In contrast, ADH levels were not positively correlated with the fermentation rate. Interpretation of ADH assays is complicated, however, by the fact that several isoenzymes of ADH may occur at the same time, as mentioned above. In conditions of a low glucose flux, i.e. in aerobic cultures grown in the absence of benzoate, a high ADH activity was found. In cultures with an increased glucose flux, repression of assimilatory and mitochondrial ADHs occurred (Table 7).

DISCUSSION

Metabolic effects of weak acids

The effect of addition of various acids to the medium reservoir of anaerobic glucose-limited cultures of *S. cerevisiae* CBS 8066 and H1022 has been studied by Verduyn *et al.* (1990a,b). Addition of weak acids, including acetate, propionate and butyrate, resulted in a decreased cell yield and an increased ethanol formation. The uncoupling effect of the weak acids appears to be directly related to their liposolubility. A higher solubility will lead to a higher diffusion rate and hence to an increase in the amount of ATP required per time unit to compensate for the influx of the acid (Warth, 1988, 1989; Verduyn *et al.*, 1990a,b). Furthermore, the extent of uncoupling was directly proportional to the residual concentration of acid in the fermenter (Verduyn *et al.*, 1990b). The extent to which the acids affected the bioenergetics of growth could be quantified in terms of loss of ATP per amount of residual acid (Verduyn *et al.*, 1990a,b). Assuming that the P/O-ratio did not change as a result of the presence of benzoic acid, it could be calculated that the amount of ATP lost per amount of acid added was similar in anaerobic and aerobic cultures (Verduyn, 1992). This suggests that the uncoupling effect of acids

Table 4. *In-situ* qO_2 in glucose-limited chemostat cultures of various yeasts in the absence of benzoate, and instantaneous maximum qO_2 (qO_2^{max}) in washed-cell samples from these cultures, and critical qO_2 (qO_2^{crit}) in the presence of benzoate. The dilution rate was $0.10\ h^{-1}$

| | No benzoate | | With benzoate |
| | qO_2 | qO_2^{max} | qO_2^{crit} |
Organism	$(mmol\ g^{-1}\ h^{-1})$		$(mmol\ g^{-1}\ h^{-1})$
C. utilis	2·5	4·9	9·0
K. marxianus	3·1	7·6	12·5
H. polymorpha	3·3	nd	11·5
S. cerevisiae	2·5	4·5	19·5

nd: Not determined.

Table 5. Specific enzyme activities ($U\ mg\ protein^{-1}$) in glucose-limited chemostat cultures of *K. marxianus* CBS 6556 grown in the absence of benzoate, or in the presence of an amount of benzoate that gave rise to the critical oxygen uptake rate. The dilution rate was $0.10\ h^{-1}$

	No benzoate	With benzoate
Acetaldehyde dehydrogenase		
NAD$^+$	0·63	0·06
NADP$^+$	0·13	0·03
Acetyl-CoA synthetase	0·29	0·03
Alcohol dehydrogenase		
Ethanol	2·10	0·21
Pentanol	3·30	0·18
Catalase	13·70	4·60
Citrate synthase	0·35	2·30
Cytochrome *c* oxidase	0·57	0·14
Hexokinase	nd	2·30
NADH dehydrogenase	0·94	0·62
Pyruvate decarboxylase	0·05	0·04

nd: Not determined.

Table 6. Substrate consumption and product formation during oxygen-limited growth of *C. utilis* CBS 621, *K. marxianus* CBS 6556 and *S. cerevisiae* CBS 8066 in glucose-limited chemostat cultures. The dilution rate was $0.10\ h^{-1}$

Organism	*C. utilis*	*K. marxianus*	*S. cerevisiae*
Yield ($g\ g^{-1}$)	0·11	0·10	0·13
q Glucose ($mmol\ g^{-1}\ h^{-1}$)	5·1	5·3	4·3
Ethanol (mM)	90	79	86
Glycerol (mM)	6·9	6·4	6·4
Pyruvate (mM)	0·56	0·15	0·08
Acetate (mM)	0·58	1·1	0·70

Table 7. Specific rates of glucose consumption and ethanol production (mmol $g^{-1} h^{-1}$), and specific activities (U mg protein^{-1}) of pyruvate decarboxylase (PDC) and alcohol dehydrogenases (ADH) during aerobic growth in the absence and presence of benzoate, and during oxygen-limited growth (without benzoate) in glucose-limited cultures of *C. utilis* CBS 621, *K. marxianus* CBS 6556 and *S. cerevisiae* CBS 8066. The dilution rate was $0.10 h^{-1}$

Cultivation condition	Aerobic		Oxygen-limited (no benzoate)
	No benzoate	With benzoate	
C. utilis			
q Glucose	1·1	3·2	5·1
q Ethanol	0	1·9	8·0
PDC	0·08	0·14	0·56
ADH	4·80	3·40	nd
K. marxianus			
q Glucose	1·2	4·3	5·3
q Ethanol	0	3·3	7·5
PDC	0·05	0·15	0·35
ADH	2·10	1·3	1·5
S. cerevisiae			
q Glucose	1·1	9·3	4·3
q Ethanol	0	11·3	6·8
PDC	0·61	0·86	0·92
ADH	8·00	1·90	nd

nd: Not determined.

mainly occurs at the level of the plasma membrane (Figure 1; Verduyn, 1992).

We chose benzoic acid as a model acid for studies under aerobic conditions, as it cannot be metabolized by the yeasts used in this study. Benzoate also has some practical interest as it is commonly used as a food preservative (Jay, 1978; Lueck 1980). Literature data indicate that the action of benzoate is probably more complex than a simple combination of influx of the undissociated acid by diffusion and subsequent proton extrusion via the plasma membrane ATPase. It has been suggested (Warth, 1977, 1988) that yeasts can induce an active (energy-dependent) transport system for the removal of the benzoate anion (and some other medium-length weak acid anions, including sorbate) by an as yet unresolved mechanism. This would then impose an additional energy drain on the organism.

Regulation of respiration in S. cerevisiae during glucose-limited growth

In cultures without benzoate, the qO_2 of *Saccharomyces* strains increases to a maximum with increasing dilution rates and then becomes constant

(Rieger *et al.*, 1983; Barford, 1990). In order to describe the phenomenon of a constant respiration rate at higher dilution rates, terms such as 'saturated' respiration (Barford, 1981); 'bottlenecks in respiration' (Sonnleitner and Käppeli, 1986) and 'respiration-limited' (e.g. Alexander and Jeffries, 1990) have been used. However, the bottleneck(s) in respiration has (have) not been identified. Barford (1990) has suggested that the drain of mitochondrial intermediates for anabolism limits the amount of intermediates available for respiration. This would be caused by a limiting supply rate of precursors for these intermediates from the cytosol to the mitochondria. However, if this was the case, this still implies that the total transport rate of these intermediates must increase with increasing D to maintain a constant qO_2.

We expected that when qO_2 reaches its maximum in the presence of benzoate, i.e. reaches the critical respiration capacity, it would become constant and additional energy generation might still occur via alcoholic fermentation. However, once fermentation set in, a significant decrease in qO_2 was observed (Figure 2). Apparently a strong repression

of respiration occurred. The glucose flux in steady state at the maximal benzoate concentration which did not provoke alcoholic fermentation was $3.9 \, \text{mmol g}^{-1} \text{h}^{-1}$ (Figure 2). In cultures without benzoate, qO_2 became constant and fermentation started above $D = 0.38 \, \text{h}^{-1}$ (Postma *et al.*, 1989a), at a $q_{glucose}$ of 4.5. These data suggest that glucose flux determines the critical respiration capacity that can be reached. This infers that the concentration of the intracellular metabolite(s) responsible for glucose repression, which have not been identified so far (Gancedo, 1987), is similar at a given glucose flux, independent of the dilution rate.

The critical respiratory capacity of *S. cerevisiae* CBS 8066 was not constant over the entire range of growth rates, but exhibited a maximum between $D = 0.10$ and $0.20 \, \text{h}^{-1}$ (Figure 4). The maximum value of $20–21.5 \, \text{mmol O}_2 \, \text{g}^{-1} \text{h}^{-1}$ observed here is much higher than values previously reported in the literature for the maximal respiration rate in *S. cerevisiae*. For instance, values of 8 and 12 mmol $O_2 \, \text{g}^{-1} \text{h}^{-1}$ were found for *S. cerevisiae* H1022 (Rieger *et al.*, 1983) and strain UNSW 703100 (Barford, 1981), respectively. The net synthesis rate of critical respiratory components can be calculated by multiplying the critical respiratory capacity by the dilution rate. For *S. cerevisiae*, the relation between dilution rate and synthesis rate follows approximately a saturation curve (Figure 10). At dilution rates above $0.2 \, \text{h}^{-1}$ the synthesis rate was already sub-maximal, and could not be increased further. Therefore, a high critical respiration capacity cannot be maintained at higher dilution rates, as this would require a continuous increase in the synthesis rate of critical respiratory components with the growth rate. In this respect, in Crabtree-negative yeasts such as *C. utilis*, the regulation of respiration seems to be different. As mentioned in the introduction, it is generally assumed that the respiration capacity of Crabtree-negative species is considerably higher than that of, for instance, *S. cerevisiae* (Alexander and Jeffries, 1990; Barford, 1990). This observation is based on the analysis of qO_2 at dilution rates close to μ_{max}. The higher qO_2 is taken to explain the absence of the Crabtree effect as these yeasts can generate sufficient energy for growth from respiration alone. Therefore terms like respiration-sufficient and respiration-limited are sometimes used with respect to Crabtree-negative and -positive species, respectively (e.g. Alexander and Jeffries, 1990).

Quite unexpectedly, a comparison of the critical respiratory capacities provoked by benzoic acid shows that—at low dilution rates—the respiratory capacity of *S. cerevisiae* is significantly higher than that of *C. utilis* and other Crabtree-negative yeasts (Table 4). However, at higher dilution rates the apparent synthesis rate of the respiratory system of *C. utilis* did not level off as in *S. cerevisiae*, but rather increased almost linearly with the dilution rate (Figure 9). Hence it can be concluded that the respiratory capacity of *S. cerevisiae* is not intrinsically lower than that of Crabtree-negative yeasts: this is only the case at higher dilution rates.

It is commonly assumed that repression of respiration is associated with growth on sugars, which are metabolized via pyruvate (Gancedo and Serrano, 1989). The critical oxygen uptake rates observed in ethanol-limited cultures grown at $D = 0.10 \, \text{h}^{-1}$ in the presence of benzoate were slightly higher (10%) than those in glucose-limited cultures, both in the absence and in the presence of benzoate (compare Figures 2 and 7). However, this difference is too small to decide whether during ethanol and glucose catabolism, the respiratory capacity is limited by the same step, for instance in the TCA cycle, respiratory chain or the mitochondrial ATP/ADP-translocation, or whether glucose catabolism is limited by a different step, for instance in transport or oxidation of pyruvate.

Effect of benzoate pulses on the physiology of *S. cerevisiae*

In the course of our studies on the effects of benzoate in yeast physiology, it was observed that gradual adaptation of the yeasts to the presence of benzoate took place: the qO_2^{crit} could only be reached via gradual adaptation of cultures to increasing reservoir benzoate concentrations. It was therefore of interest to investigate the effects of a sudden exposure to high benzoate concentrations via a direct addition to the culture. Such a direct pulse resulted in phenomena completely different from those observed upon stepwise increases of the reservoir concentration. Under the latter condition, growth in the presence of 10 mM-benzoate resulted in a completely respiratory metabolism with a very high qO_2 (Figure 2). However, a direct pulse of benzoate resulted in a severe reduction of *in-situ* qO_2. This qO_2 is probably due to oxidation of reducing equivalents formed in the production of acetate, which accumulated up to 25 mM (Figure 3B). It appears that functioning of the TCA cycle (or supply of its intermediates) is inhibited under these conditions, but that oxidation of reducing equivalents can still take place, at least to some extent. The effect of a pulse-wise addition of benzoate to the

fermenter on the respiration rate was completely reversible (Figure 3C): washed cell suspensions had the same oxygen uptake rate (4–5 mmol g^{-1} h^{-1}, Table 3) in the presence of excess glucose as normally observed in cells grown without benzoate at this D. A very unusual feature is the fact that virtually no ethanolic fermentation occurred, despite considerable glucose accumulation (Figure 3A). In all other cases we have encountered so far, glucose accumulation resulted in a high specific ethanol production rate. For instance, glucose pulses to S. *cerevisiae* CBS 8066 led to both increased specific oxygen uptake rates and considerable alcoholic fermentation (van Urk *et al.*, 1988). Similar results have been obtained with other *Saccharomyces* strains (Rieger *et al.*, 1983). The reason for the low ethanol production rate of S. *cerevisiae*, despite significant glucose accumulation, after a pulse of benzoate, remains to be established.

Effect of benzoate on enzyme levels in S. cerevisiae

It has previously been shown that enzyme levels in glucose-limited S. *cerevisiae* were affected by the glucose flux. With increasing dilution rates, i.e. increasing glucose flux, repression of a number of enzymes was observed (Postma *et al.*, 1989a). Also at a fixed dilution rate, but at different glucose fluxes as a result of growth in the presence of benzoate, a decline in the activity of a number of enzymes was observed (Figure 6A–C). It can be argued that the synthesis of these enzymes is affected by benzoate. In order to establish whether for instance catalase synthesis is influenced by benzoate, levels of this enzyme were assayed in ethanol-limited cultures of S. *cerevisiae* grown without or with 8 mM residual benzoate. The catalase activities were similar (Table 2). This indicates that catalase is indeed not subject to repression in ethanol-limited cultures and furthermore that its total activity in biomass is not affected by benzoate. The same is true for acetaldehyde dehydrogenases, the activity of which actually increased when the ethanol flux was increased, either by increasing the dilution rate, or by adding benzoate at a fixed D (Table 2). However, it should be kept in mind that in the enzyme assays no benzoate is present and thus the possibility cannot be excluded that benzoate inhibits an enzyme activity *in vivo*.

The activity of hexokinases, which have no apparent major role in ethanol metabolism, were considerably lower than in glucose-limited cultures. Less clear is the behaviour of ADH(s) and acetyl-CoA synthetase, which showed some decrease at

$D = 0.10$ h^{-1} in the presence of benzoate. A decrease in enzyme activities was also observed at a higher dilution rate (0.19 h^{-1}) in the absence of benzoate. It is clear that the regulation of enzyme activity during growth on ethanol is quite different from that during growth on glucose, both with and without benzoate.

Some enzymes of the TCA cycle and of the respiratory chain were also assayed. Interestingly, citrate synthase increased three-fold in glucose-limited cultures grown in the presence of benzoate, although glucose repression of this enzyme has been reported in batch cultures (Wales *et al.*, 1980). However, it is still possible that benzoate might bind to this enzyme and that the *in-situ* activity is reduced, hence a larger amount of enzyme would be required. However, in ethanol-limited cultures, its activity was uniformly high (with and without benzoate addition; Table 2). A similar large increase was also observed for citrate synthase in K. *marxianus* upon addition of benzoate (Table 5). NADH-dehydrogenase activity showed some decline with increasing glucose flux, whereas cytochrome c oxidase activity increased slightly. Hence it can be concluded that, although only a small selection of enzymes have been assayed in this work, the results (Figure 6A–C) suggest that the increased glycolytic and respiratory fluxes in the presence of benzoate must be mainly due to a rise in the concentration of intracellular intermediates. Furthermore, there appears to be no major difference in the enzyme patterns of S. *cerevisiae* and K. *marxianus* (compare Figure 6A–C and Table 5). Not only in glucose-limited cultures, but also in ethanol-limited cultures, levels of intermediates probably are decisive for metabolic fluxes. However, in contrast to growth on glucose, repression of enzymes did not occur during growth on ethanol in the presence of benzoate: the levels of various enzymes were almost independent of the ethanol flux.

Mitochondrial volume in relation to respiration rate

The large increase of the mitochondrial volume observed upon addition of benzoate to glucose-limited cultures of S. *cerevisiae* (Table 3) is unlikely to be solely due to an increase in the amount of mitochondrial protein. Of the mitochondrial enzymes assayed, only citrate synthase increased significantly (Figure 7C). Cells grown on ethanol showed a larger mitochondrial volume than cells grown on glucose in the absence of benzoate, but had a considerably higher *in-situ* qO_2 and instantaneous maximum qO_2 (Table 3). The increase in mitochondrial volume possibly serves to increase the area

available for respiration-linked processes, which might serve to accommodate increased amounts of the electron transport chain or various carriers (e.g. for pyruvate and/or ADP/ATP exchange).

Regulation of alcoholic fermentation

Our results show that in Crabtree-negative yeasts alcoholic fermentation can be provoked under aerobic conditions which impose a large demand for ATP, as is the case during growth in the presence of benzoate (Tables 6 and 7). However, the observed maximal fermentation rate at low dilution rates, as induced by benzoate, was lower in Crabtree-negative yeasts than in *S. cerevisiae* (Table 7). This appears to be due to the inability of the Crabtree-negative species to induce higher PDC levels upon transition to partly fermentative conditions (Table 7). It has already been proposed that the level of PDC is rate-limiting for the fermentation (Schmitt and Zimmerman, 1982; Sharma and Tauro, 1986). A good correlation between fermentation rate and PDC level was also observed in glucose-limited chemostat cultures of *S. cerevisiae* 8066 (Postma *et al.*, 1989a). A further important enzyme in the context of fermentative capability is ADH. The activity of ADH was considerably higher than that of PDC. Although ADH activity may be due to several isoenzymes (Lutstorf and Magnet, 1968; Fraenkel, 1982), the reaction from acetaldehyde to ethanol proceeds approximately ten times faster than the oxidation of ethanol (Verduyn *et al.*, 1988). Thus, even if only 25% of the total ADH activity is due to the fermentative enzyme, it is unlikely that ADH will become rate-limiting with respect to alcoholic fermentation.

In summary, our results show that the increase in the catabolic flux due to the presence of benzoate is regulated at the level of metabolite concentrations rather than via enhanced enzyme synthesis. Under appropriate cultivation conditions the respiration of *S. cerevisiae* can attain values higher than those observed in Crabtree-negative yeasts. The question why benzoate can trigger such high respiration rates in *S. cerevisiae* only at low growth rates (Figure 4) cannot be answered at present. In our opinion answering this question is a necessity for an understanding of the Crabtree effect.

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