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Effect of Benzoic Acid on Glycolytic Metabolite Levels and Intracellular pH in *Saccharomyces cerevisiae*

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Low concentrations of benzoic acid stimulated fermentation rates in *Saccharomyces cerevisiae*. At concentrations near the maximum permitting growth, there was inhibition of fermentation, lowered ATP and intracellular pH, and relatively greater accumulation of benzoate. Changes in the levels of glycolytic intermediates suggested that fermentation was inhibited as a result of high ATP usage rather than of lowered intracellular pH. Specific inhibition of phosphofructokinase or of several other glycolytic enzymes was not observed.

Krebs et al. (3) studied the effects of benzoic acid on the intracellular pH (pH_i) and glycolytic metabolite levels of *Saccharomyces cerevisiae* and concluded that restriction of growth was caused by a fall in ATP levels as a result of inhibition of glycolysis. At low external pH, benzoic acid entering the cell caused a large reduction in pH_i which specifically inhibited phosphofructokinase.

With the preservative-resistant yeast *Zygosaccharomyces bailii*, however, the pattern of metabolite levels was distinctly different from that found in *S. cerevisiae*. This pattern indicated that phosphofructokinase was not limiting glycolysis and that the reduction in pH_i was comparatively small (11). A number of conditions were different in the two studies. This work was undertaken to compare the response of *S. cerevisiae* under conditions similar to those used for *Z. bailii* (11).

When grown in the presence of benzoic acid, several yeasts, including *S. cerevisiae*, exhibited an adaptive higher tolerance to this acid (10). Cells are very permeable to the undissociated form of benzoic acid and other weak-acid-type preservatives, but are commonly impermeable to the anion. For cells in an acidic medium, this leads to the accumulation of high levels of preservative anion and a transient reduction in pH_i. However, cells already adapted to benzoate appeared to have an induced transport system for benzoate anion (10). In the presence of glucose, lower levels of preservative anion were accumulated, but additional energy was required to maintain the pH_i (5, 8). The difference in tolerance and transport properties between the adapted and unadapted cells indicates that the stresses are different. This study has used yeast cells grown in the presence of benzoic acid.

MATERIALS AND METHODS

Growth. *S. cerevisiae* FRR 1297 was grown semiaerobically in 5% glucose–yeast extract medium (6), containing 0.25 mM benzoic acid to induce the preservative anion transport system (5). Cells were centrifuged, washed twice in 0.1 M potassium citrate buffer (pH 3.5), and kept at 10°C for 1 to 3 h prior to incubation. Inhibition of growth rate was determined by using 5-ml aerobic cultures in screw-cap tubes (6). MICs were determined by using microwell plates with inocula grown in the presence of benzoic acid (7).

Incubation. Cells were incubated at 25°C in screw-cap tubes fitted with a septum and flushed with N_2. Glucose (20%) was added after 10 min, and 200 mM potassium benzoate was added 2 min later. Final concentrations were as follows: cells, 50 g (wet weight) per liter; glucose, 50 g/liter; potassium citrate (pH 3.5), 0.1 M; and benzoic acid, 0 to 2.5 mM. All analyses were as described in the accompanying paper (11).

RESULTS AND DISCUSSION

Growth inhibition. Growth of *S. cerevisiae* in the presence of benzoic acid increased the MIC of benzoic acid, determined by using microwell plates, from 0.82 to 1.40 mM (10). In stirred cultures, increasing the concentration of benzoic acid steadily reduced the growth rate of the yeast, giving an MIC of 1.4 mM (Fig. 1). Under anaerobic conditions, both the growth rates and the MIC were lower (Fig. 1). The cells used for the remainder of this study were grown in the presence of benzoic acid and incubated anaerobically.

Effect of benzoic acid concentration on fermentation rate, ATP level, pH_i, and benzoate accumulation. Benzoic acid at low concentrations (up to 0.4 mM) stimulated ethanol production (Fig. 2). ATP levels and pH_i dropped slightly (Fig. 2 and 3), and only small amounts of benzoate were accumulated (Fig. 3). However, between 0.3 and 0.5 mM benzoic acid, the fermentation rate reached a maximum, the ATP level and pH_i remained constant, and the intracellular benzoate concentration started to increase. Finally, over the range from 0.5 to 1 mM benzoic acid, the fermentation rate, ATP level, and pH_i declined in parallel, while the accumulation of benzoate increased greatly. At higher benzoate levels there was little further change. Fermentation rates and ATP concentrations were low but not zero, pH_i was near 5.9, and cells contained high concentrations of benzoate. Cells incubated in the absence of both glucose and benzoic acid had only slightly higher levels of ATP than did cells in glucose plus high benzoic acid concentrations (Fig. 2), but their pH_i (5.4) was lower (Fig. 3). Also, benzoate accumu-

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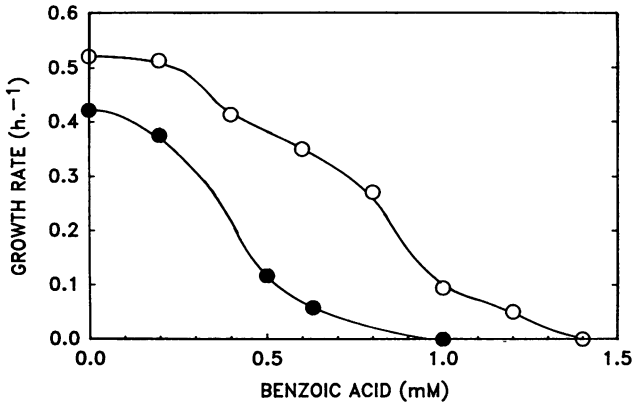

FIG. 1. Effect of benzoic acid on the growth rate of *S. cerevisiae* at pH 3.50 and 25°C. Symbols: ○, aerobic; ●, anaerobic.

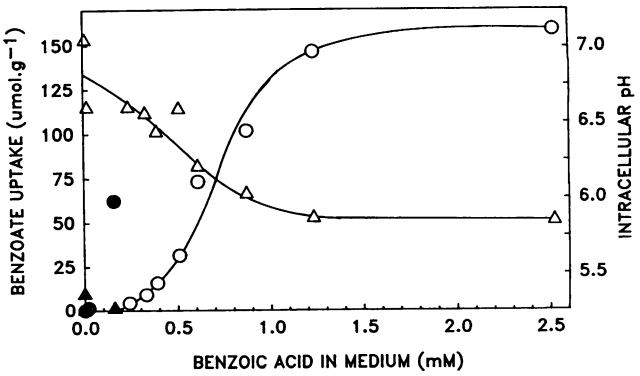

FIG. 3. Effect of benzoic acid on pH_i (△, ▲) and benzoate accumulation (○, ●) in *S. cerevisiae* incubated in 5% glucose at pH 3.50. Solid symbols indicate incubation with no glucose added.

lation was much higher in the absence of glucose than in its presence (Fig. 3).

Thus, the response of *S. cerevisiae* to benzoic acid was very similar to that of *Z. bailii* (7, 9, 11), except that the MIC of benzoic acid for *S. cerevisiae* was lower and the biochemical changes were seen at correspondingly lower benzoic acid concentrations. These results are consistent with a model in which the cell can export the benzoate anion. Undissociated benzoic acid rapidly enters the cell and dissociates, releasing a hydrogen ion. If the benzoate anion leaves the cell, a cycling ensues and further energy is required to export the hydrogen ions.

At lower external benzoic acid concentrations (≤0.5 mM), benzoate accumulation was much lower than would be expected from equilibrium conditions wherein the membrane was permeable to undissociated benzoic acid but not to the anion. The uptake at 0.5 mM benzoic acid was only one-sixth of that calculated from the measured pH_i of 6.5.

S. cerevisiae responded to the above energy demand with an increased fermentation rate, and cells were able to keep both ATP levels (Fig. 2) and pH_i (Fig. 3) relatively high. The growth rate was reduced (Fig. 1), probably because the energy needed to keep the benzoate content low was therefore unavailable for growth. At higher benzoic acid concentrations, however, fermentation became inhibited (Fig. 2), leading to a marked decline in both ATP levels (Fig. 2) and pH_i (Fig. 3) and a relatively sharp increase in benzoate accumulation (Fig. 3). At these high benzoic acid concentrations, growth was fully inhibited but cells did not die rapidly. Minimal levels of fermentation and ATP were still evident (Fig. 2). The pH_i was reduced but remained considerably higher than that of the external medium (Fig. 3). This state is similar to that of cells incubated without glucose and indicates that similar homeostatic mechanisms for survival may operate.

This inhibition of fermentation in the presence of high benzoic acid concentrations did not appear to be due to lowered pH, as the lowest pH_i reached was still higher than that found in cells in the absence of glucose and preservative.

Glycolytic metabolite levels. Glucose 6-phosphate and fructose 6-phosphate levels decreased by 75% between 0 and 1.25 mM benzoic acid (Fig. 4), suggesting that transport or phosphorylation of glucose may be limiting. The 2.5-fold increase in the fructose 1,6-bisphosphate level showed that inhibition of phosphofructokinase does not limit glycolysis and suggested instead that aldolase might be rate limiting. Triosephosphate levels, however, did not decline significantly. The value of [fructose 1,6-bisphosphate]/[triosephosphate] was near the predicted equilibrium value, and thus the

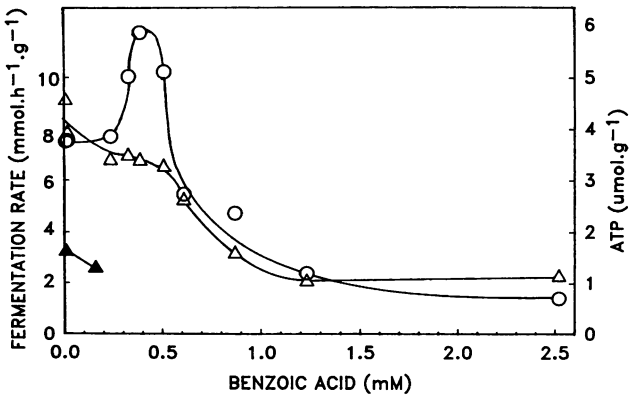

FIG. 2. Effect of benzoic acid on the rate of ethanol production (○) and the ATP level (▲, △) in *S. cerevisiae*. Solid symbols indicate incubation with no glucose added.

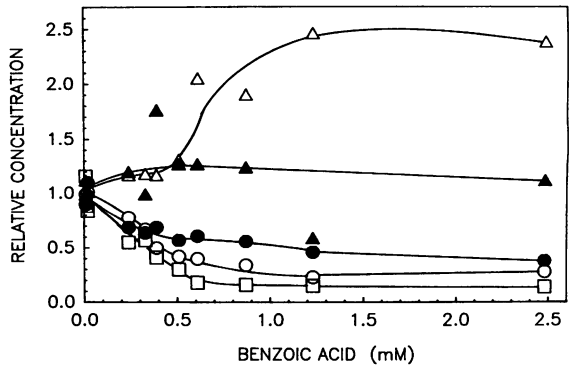

FIG. 4. Effect of benzoic acid on glycolytic metabolite levels in *S. cerevisiae*. All values are compared with normalized concentrations of 1.0. Actual concentrations (micromoles per gram) in the absence of benzoic acid: glucose 6-phosphate (○), 3.65; fructose 6-phosphate (●), 0.74; fructose 1,6-bisphosphate (△), 1.60; triosephosphates (▲), 0.31; pyruvate (□), 4.89.

observed increase in fructose 1,6-bisphosphate levels was not sufficient to indicate significant inhibition of aldolase. Pyruvate was decreased to a low level by 0.6 mM benzoic acid, indicating that glycolysis was inhibited at some stage between glyceraldehyde 3-phosphate and pyruvate.

The patterns of metabolite changes do not show a clear indication of a blockage at any specific step in glycolysis. In particular, they do not correspond to those shown by a variety of glycolytic mutants of *S. cerevisiae* (1). The changes, however, were generally similar to those found in *Z. bailii*, for which, on the basis of more extensive data, it appeared that glycolysis was inhibited principally at pyruvate kinase and phosphoglycerate kinase (11). It was suggested (11) that this inhibition was directly or indirectly due to the high ATP demand. The response of *S. cerevisiae* to benzoic acid differed from that of *Z. bailii* in that glucose 6-phosphate and fructose 6-phosphate levels declined. This drop appears unlikely to be caused directly by the reduction in ATP, since cellular ATP levels remained above the reported K_m values and the equilibria are favorable (4). Levels of the hexosephosphates were lower than those found in *Z. bailii* (11) but are typical of those reported previously for *S. cerevisiae* (2, 3).

The changes in the levels of glycolytic metabolites, particularly fructose 1,6-bisphosphate, observed by Krebs et al. (3) were very different from those found in the present study. Several factors may be responsible for this. There may be differences in the strains of *S. cerevisiae* used, particularly since Krebs et al. (3) found a much lower pH_i at 2 mM benzoic acid and pH 3.5. Also, in the experiments by Krebs et al. (3), the cells were not grown in the presence of benzoic acid and did not show a stimulation of fermentation at low benzoic acid levels. Finally, their metabolite levels were determined after exposure of cells to 10 mM benzoic acid at pH 2.5. This concentration of benzoic acid is far beyond the MIC for *S. cerevisiae*. The biochemical changes they observed may therefore be secondary to those that inhibit growth at lower concentrations.

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