Mechanism of Action of Benzoic Acid on *Zygosaccharomyces bailii*: Effects on Glycolytic Metabolite Levels, Energy Production, and Intracellular pH

ALAN D. WARTH

Division of Food Processing, Commonwealth Scientific and Industrial Research Organization, P.O. Box 52, North Ryde, New South Wales 2113, Australia

Received 7 March 1989/Accepted 8 September 1991

The effects of benzoic acid in the preservative-resistant yeast *Zygosaccharomyces bailii* were studied. At concentrations of benzoic acid up to 4 mM, fermentation was stimulated and only low levels of benzoate were accumulated. Near the MIC (10 mM), fermentation was inhibited, ATP levels declined, and benzoate was accumulated to relatively higher levels. Intracellular pH was reduced but not greatly. Changes in the levels of metabolites at different external benzoic acid levels showed that glycolysis was limited at pyruvate kinase and glyceraldehyde dehydrogenase-phosphoglycerate kinase steps. Inhibition of phosphofructokinase and several other glycolytic enzymes was not responsible for the inhibition of fermentation. Instead, the results suggest that the primary action of benzoic acid in *Z. bailii* is to cause a general energy loss, i.e., ATP depletion.

Although benzoic acid and other weak-acid-type preservatives have been commonly used to preserve acidic foods, beverages, and pharmaceuticals for many years, there is uncertainty about which biochemical events are important in inhibiting cell growth under industrially relevant conditions. Inhibition of a variety of enzymes in vitro has been reported for sorbic acid (24), but there has not been clearly related to growth inhibition or cell death. There are few reports of significant inhibition of enzymes by benzoic acid or the aliphatic acids. In fact, glycolytic metabolism is stimulated by concentrations of preservative that inhibit cell growth (6, 26). Preservatives inhibit transport in a variety of cells (12). This may account for their effectiveness in preventing the growth of many sensitive bacteria and yeasts under some conditions, but it is not a likely cause of growth inhibition under conditions such as anaerobic fermentation of nutrient-rich foods (29). On the other hand, weak acids are well known to reduce cytoplasmic pH, particularly under the acidic conditions in which the weak-acid-type preservatives are used and are most effective. Krebs et al. (14) suggested that benzoic acid inhibited the growth of *Saccharomyces cerevisiae* by lowering the intracellular pH, which inhibited glycolysis, specifically by inhibition of phosphofructokinase. *Zygosaccharomyces bailii* is a yeast which tolerates high levels of preservative and causes spoilage of beverages and other acidic foods (25, 29). When grown in the presence of benzoic acid, *Z. bailii* in particular, as well as several other yeasts, tolerates higher levels of the acid. These cells accumulate less benzoate than expected from their intracellular pH, and benzoic acid at sublethal concentrations stimulates fermentation rates and reduces growth yields (26, 30). It was suggested that cells grown in the presence of benzoic acid became permeable to or transported benzoate anion, thus reducing the concentration of benzoate in the cell. Benzoic acid would then cycle in and out of the cell in undissociated and dissociated forms, respectively, and the intracellular pH would tend to drop. The energy demand for export of protons and maintenance of intracellular pH may stimulate fermentation rates and lower the growth yields.

In the present study, the effects of benzoic acid on fermentation rate, ATP level, intracellular pH, and benzoate accumulation level were determined over a range of concentrations up to that causing full inhibition of growth to elucidate the events that may be critical in producing growth inhibition in *Z. bailii*. Metabolite levels were measured to determine which reaction steps in glycolysis were affected.

**MATERIALS AND METHODS**

**Growth.** *Z. bailii* FRR 1292 was grown in yeast extract medium (pH 3.5) (27) containing 5% glucose in place of the fructose. Benzoic acid (2 mM) was added (26). Cultures were stirred at 25°C in plugged flasks containing 30% headspace and harvested at the late exponential phase (0.3 to 1 mg [dry weight] per ml). Cells were washed twice in 0.1 M potassium citrate buffer (pH 3.5) and kept at 10°C for 1 h prior to incubation. MICs were determined by using microwell plates with inocula grown in the presence of benzoic acid (28). Growth rates were measured at 25°C by using shaken screw-cap tubes.

**Incubation.** Cells were incubated at 25°C in screw-cap tubes fitted with a septum and flushed with N₂. Glucose (20%) was added after 10 min, and 200 mM potassium benzoate and 0.8 equivalent of 1 M HCl were added 2 min later. Final concentrations were as follows: cells, 13 g (dry weight) per liter; glucose, 50 g/liter; potassium citrate (pH 3.5), 0.1 M; benzoic acid, 0 to 18 mM. Three independent experiments were done, and the same trends were found in each; results are plotted for one experiment only.

**Analyses.** To determine the fermentation rate, samples (0.5 ml) were withdrawn 3 and 13 min after the addition of benzoate, diluted with cold 1% 2-propanol, frozen with dry ice, and stored at −180°C. For analysis, the samples were rapidly thawed and filtered. Ethanol was determined by gas chromatography (27), and glycero alcohol was determined enzymatically (Boehringer test combination; Boehringer Mannheim Australia Ltd.). Samples for metabolite determinations were

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† Deceased 21 June 1990. All revisions were completed by Dr. Kenneth W. Nickerson, University of Nebraska. Address reprint requests to Dr. Michael Eyles, Commonwealth Scientific and Industrial Research Organization.
withdrawn at 14 min. Adenine nucleotides were extracted by injecting the sample rapidly into an equal volume of ethanol at 78°C. After 5 min, the samples were cooled, diluted fourfold, and stored at −18°C. ATP was measured by bioluminescence by using a model M1070 luminometer and Lumit reagent (Lumac B.V., The Netherlands). AMP + ADP + ATP and ADP + ATP were determined as ATP by using pyruvate kinase and adenylyl kinase (1). For other metabolites, the sample was mixed with an equal volume of 2 M HClO₄ at 0°C for 30 min, pH was adjusted to 6.2 to 6.7 with 2 M K₂CO₃, and the mixture was frozen with dry ice and thawed twice and centrifuged. Phosphoenolpyruvate and glyceral 2-phosphate were determined within 4 h, and other metabolites were determined after storage at −180°C.

The following glycolytic metabolites were measured enzymatically: glucose 6-phosphate and fructose 6-phosphate (19), fructose 1,6-bisphosphate and triosephosphates (20), glyceral 3-phosphate (11), glyceral 2-phosphate and phosphoenolpyruvate (15), pyruvate (16), acetaldehyde (2), and NAD (13). Pi was estimated by the method of Lowry and Lopez (17). Intracellular concentrations of metabolites were calculated by using a value of 1.8 μl of cell water per mg (dry weight) (26). The amount of benzoic acid in cells and in the medium was determined by high-pressure liquid chromatography (27) after centrifugation for 2 min at 20,000 × g. A correction was applied for interstitial fluid.

**Intracellular pH determination.** Since determination of intracellular pH by distribution of weak acid requires the assumption of impermeability of the anion, intracellular pH was estimated by the freeze-thaw method (4, 26, 30). Cells (250 mg [wet weight]) were rapidly filtered on Nuclepore membranes and washed with 0.5 ml of cold water, and the pellet was scraped off into a polyethylene cap (9 mm [inner diameter]) and frozen in liquid N₂. The caps were then thawed in air or in hexane and immediately refrozen. After six cycles, the temperature was quickly raised to 25°C and the pH was determined by using a flat, combination electrode. No addition of fluid was necessary, as the pellet became a viscous fluid.

**Calculation of mass action substrate/product ratios.** Levels of several metabolites were below the limit of detection by the methods used. Glyceroldehyde 3-phosphate and glyceral 2,3-bisphosphate concentrations were calculated by assuming equilibrium of these metabolites at zero and phosphoglycerate mutase, respectively, as these steps are not commonly limiting (22). The lack of data for glyceral 1,3-bisphosphate means that glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions cannot be distinguished. Glyceroldehyde-3-phosphate dehydrogenase has been taken as being in equilibrium, but the results may apply to either step. The enolase reaction equilibrium requires that the phosphoenolpyruvate concentration in the sample with the lowest benzoic acid concentration be less than 0.039 mM, which is less than the limit of detection (0.1 mM). This value was used at each benzoate concentration. Modeling of higher values allowed at other benzoic acid concentrations did not significantly affect any conclusions.

NAD+/NADH ratios were calculated from ethanol and acetaldehyde concentrations, assuming equilibration by alcohol dehydrogenase.

**RESULTS**

**Growth inhibition.** The MIC of benzoic acid for *Z. bailii*, determined by using microwell plates, was 10 mM (28). Benzoic acid progressively reduced the growth rate, causing full inhibition at 10 mM. In the absence of benzoic acid, *Z. bailii* grew faster under aerobic conditions; however, in the presence of 1 mM benzoic acid, there was no difference between the aerobic and anaerobic growth rates. The following experiments were done under anaerobic conditions.

**Fermentation rate and ATP level.** The rate of ethanol production was stimulated at benzoic acid concentrations up to 4 mM and then was progressively inhibited (Fig. 1). Glycolysis was not diverted to glycerol production, because glycerol production was reduced by benzoate (Fig. 1). ATP levels declined with increasing benzoic acid concentrations, and both ethanol production and the ATP level were very low near the MIC (Fig. 1). The energy charge (½ADP + ATP)/(AMP + ADP + ATP) declined from 0.97 at low benzoic acid concentrations to 0.49 at 7 mM benzoic acid.

**Benzoate accumulation and intracellular pH.** Benzoate was accumulated in cells to approximately 10 times the concentration of benzoic acid in the medium (Fig. 2). A steeper rise between 6 and 8 mM benzoic acid corresponds to the range of inhibition of glycolysis. The maximum concentration of benzoate in the cytoplasm was 127 mM. The intracellular pH was reduced, but not greatly, and was 6.3 at fully inhibitory benzoic acid concentrations (Fig. 2). The cytoplasmic pH depended upon the length of time the washed cells were stored before the experiment. In two other experiments, pHs
were 6.6 and 6.7 in the absence of benzoic acid and 6.0 and 6.1 at high concentrations of benzoic acid.

The concentration of benzoate in the cells was much lower than that predicted from the pHs of the medium and the cytoplasm, on the assumption of an equilibrium in which the cell membrane is permeable to benzoic acid but impermeable to benzoate. At a cytoplasmic pH of 6.77 and a concentration of benzoic acid in the medium of 5.8 mM, a cytoplasmic concentration of benzoate of 1.5 M would have been expected, whereas 45 mM was found. In the absence of glucose, the pH dropped to 5.9 but benzoate was accumulated to a much higher ratio (Fig. 2).

**Glycolytic metabolite levels.** The levels of glycolytic metabolites are shown in Fig. 3 to 5. Glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate levels remained unchanged up to 5 mM benzoic acid and then increased (Fig. 3). Triosephosphate concentrations increased over the full range of benzoic acid concentrations, and glyceraldehyde 3-phosphate concentrations increased up to 5 mM benzoic acid (Fig. 4). Glyceraldehyde 2-phosphate and phosphoenolpyruvate levels were below the limit of detection (0.05 mM) at all benzoic acid concentrations, and glyceraldehyde 1,3-bisphosphate was not determined. The pyruvate concentration (Fig. 4) decreased, and the acetaldehyde concentration (Fig. 5) showed no clear trend. NAD levels remained constant at all benzoic acid concentrations, whereas P_i levels increased over the inhibitory range (Fig. 5).

**Limitation of glycolysis at specific steps.** Comparison of the mass action ratios with published equilibrium values (7) showed that the major deviations from equilibrium were at the transport/hexokinase, pyruvate decarboxylase, and phosphofructokinase steps, whereas aldolase and phosphoglycerate kinase and pyruvate kinase steps were limiting to a lesser degree (Table 1). Phosphotriose isomerase and phosphoglycerate mutase reactions are not commonly rate limiting (22) and were taken as being in equilibrium, whereas equilibration of the alcohol dehydrogenase step was assumed in order to calculate NAD^+/NADH ratios.

With increasing benzoate concentrations, the phosphoglycerate and pyruvate kinase steps became progressively more limiting (Table 1) and appear a priori to be the sites of inhibition of glycolysis. Transport/hexokinase and the phosphofructokinase steps became progressively less limiting, as may result from relief of metabolic inhibitory controls in response to the energy demand or in compensation for inhibition at other stages of glycolysis. However, these results were caused largely by variations in the intracellular ATP levels. Appropriate elevation and depletion of the levels of the other substrates and products were not clearly evident. Modeling the reactions with a constant ATP/ADP ratio (Table 1) almost eliminated the apparent inhibitions of pyruvate and phosphoglycerate kinases and removed the apparent stimulation of hexokinase and phosphofructokinase. The model did not suggest any significant inhibition of aldolase or pyruvate decarboxylase.

**DISCUSSION**

*Z. bailii* exhibits a remarkable resistance to benzoic acid. At low preservative concentrations, *Z. bailii* adopts a strategy of keeping the intracellular concentration of preservative anions low. Maintenance of the benzoate content below the chemical equilibrium value requires energy. It has been shown (18, 31) that *Z. bailii* is permeated rapidly by undissociated benzoic acid. Therefore, a low benzoate content can be achieved only by removal of anion at a rate comparable to the rate of inflow of benzoic acid. It is probable that the anion can leave without direct energy usage by flowing down the electrochemical gradient. However, energy is still required to maintain the intracellular pH, and the observed stimulation of fermentation is probably a response to this energy demand. The accompanying reduced growth yields (30) and lower growth rates (30) are due to diversion of
energy and not, under these low-benzoate conditions, to inhibition of glycolysis.

At benzoic acid concentrations between 5 and 10 mM, fermentation and growth rates were progressively inhibited. Examination of the glycolysis intermediates at each reaction step (Table 1) showed that as the benzoic acid concentration increased, glycolysis became more limiting at the phosphoglycerate kinase and pyruvate kinase reaction steps, whereas phosphofructokinase and the transport/hexokinase step became less restrictive. Both of these effects resulted from the change in ATP levels, since there were not significant changes in the concentrations of the other substrates and products.

Apart from the effects on the kinases, there were no indications of specific inhibitions of glycolytic steps. In particular, there were no elevated levels of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, and alcohol dehydrogenase steps were assumed to be in equilibrium. The phosphoenolpyruvate concentration was assumed to be constant at 0.039 mM. Large numbers indicate rate-limiting deviations from equilibrium.

![Table 1](https://example.com/table1.png)

**TABLE 1. Effect of benzoic acid on the mass action substrate/product ratios for glycolytic reaction steps in Z. bailii**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>S/PK (a)</th>
<th>Relative S/PK value (b) at benzoic acid concn of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.20</td>
<td>1.64</td>
</tr>
<tr>
<td>Transport/hexokinase</td>
<td>1.8 × 10^6</td>
<td>1.00</td>
</tr>
<tr>
<td>Phosphohexose isomerase</td>
<td>2.5</td>
<td>1.00</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>1.1 × 10^3</td>
<td>1.00</td>
</tr>
<tr>
<td>Aldolase</td>
<td>87</td>
<td>1.00</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>68</td>
<td>1.00</td>
</tr>
<tr>
<td>Enolase</td>
<td>1.0</td>
<td>1.00</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>20</td>
<td>1.00</td>
</tr>
<tr>
<td>Pyruvate decarboxylase</td>
<td>6.6 × 10^4</td>
<td>1.00</td>
</tr>
<tr>
<td>Constant ATP/ADP (c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transport/hexokinase</td>
<td>1.00</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>1.00</td>
<td>0.75</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>1.00</td>
<td>0.74</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>1.00</td>
<td>0.59</td>
</tr>
</tbody>
</table>

\(a\) The mass action substrate/product ratios \(S/P\), taken from the lowest benzoic acid concentration (0.2 mM) in Fig. 3 to 5, were compared with published \(K\) values. Phosphohexose isomerase, glycerol dehydrogenase, phosphoglycerate mutase, and alcohol dehydrogenase steps were assumed to be in equilibrium. The phosphoenolpyruvate concentration was assumed to be constant at 0.039 mM. Large numbers indicate rate-limiting deviations from equilibrium.

\(b\) S/PK values from Fig. 3 to 5 at increasing concentrations of benzoic acid relative to those at 0.2 mM benzoic acid. Large numbers (>1.0) indicate a more limiting reaction, i.e., apparent inhibition, while small numbers (<1.0) indicate a less limiting reaction, i.e., apparent stimulation.

\(c\) Modeling calculated by using \([ATP]\)/[ADP] = 5.0 mM at each benzoic acid concentration.

In contrast, Cole and Keenan (10) studied the effects of benzoic and sorbic acids on the intracellular pH of Z. bailii and did not find evidence that these weak acids were actively extruded. They measured intracellular pHs by monitoring the distribution of benzoic and propanoic acids and by a fluorescence technique. They concluded that resistance can be explained in part by active ejection of acids produced during metabolism, and that Z. bailii has the ability to tolerate chronic reductions in intracellular pH. However, active ejection of metabolic acids is probably an insufficient explanation because of the high rate of benzoic acid permeation and the stoichiometry of 1 to 2 mol of ATP per mol of benzoic acid entering the cell (31).

The cellular pH values found in the present study are much higher than those reported by Krebs et al. (14) and Cole and Keenan (10). Although the organisms and conditions were not identical, it appears likely that the difference is due to the methods used. The several methods available for the determination of intracellular pH in yeasts do not give consistent results (3). The most commonly used method, equilibration of a lipophilic weak acid, depends on the cell's being permeable to the undissociated acid and relatively impermeable to the anion. This condition has rarely been demonstrated. With preservative-adapted Z. bailii, it is not applicable (26), and another method is required. The intracellular pH was estimated in the present and previous (26) studies by freeze-thawing with liquid N\(_2\). Of the potential errors associated with this procedure, nearly all would be expected to give underestimates of the actual cytoplasmic
pH. Also, for yeast cells at external pHs near 3.5 with an adequate energy supply, several studies have found near-neutral internal pH values. For *S. cerevisiae*, nuclear magnetic resonance studies gave a value of 7.1 (21), compared with 6.7 from freeze-thawing (5), and 6.4 from the weak-acid method (8). Lower values have frequently been obtained, but these can be attributed to an inadequate energy supply.

Above the MIC for growth, cells contained high concentrations of benzoate. This is an appropriate response to conserve energy for long-term survival. Cells can retain viability under these conditions for many days while the high benzoate anion concentration could well cause direct inhibition of glycolysis and other cell functions. However, the intracellular pH values did not drop to levels which would require no energy to maintain, at least not within the time scale of this experiment. The residual fermentation rates, although low, may be necessary for survival.

In summary, the action of benzoic acid on *Z. bailii* depends upon its concentration. At low concentrations, growth rates are reduced and fermentation rates are stimulated because energy is used to lower the internal concentration of benzoate. At higher concentrations, fermentation itself becomes inhibited, leading to a collapse of the system. The reasons for this inhibition of fermentation are not fully clear. Internal benzoate concentrations increased in parallel with the inhibition of fermentation, but they may not be the major direct cause of inhibition. pH reduction also does not appear to be a prime cause of inhibition in *Z. bailii*, since the internal pH was not severely depressed. Instead, the pattern of glycolytic intermediate changes suggests that ATP limitation was important. This ATP limitation may be direct or may result from regulatory mechanisms designed to conserve the remaining energy.

REFERENCES