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Studies on the mechanism of the antifungal action of benzoate

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1. A method is described for the determination of the pH of intracellular water based on the distribution of [^{14}C]benzoate (0.01 mM) between intra- and extra-cellular water. 2. Benzoate at higher concentrations (2–10 mM) enters the yeast cell in the undissociated form, and its neutralization within the cell can cause a shift of the pH of the intracellular water by more than 1 pH unit. 3. Benzoate causes an accumulation of the two hexose monophosphates of yeast glucose fermentation and a decrease in intermediates beyond phosphofructokinase, suggesting inhibition at this stage. Benzoate also causes a concomitant fall in [ATP]. 4. Phosphofructokinase is inhibited to a greater extent than hexokinase at acid pH. 5. There is a relationship between intracellular pH, phosphofructokinase inhibition and CO_2 production, suggesting that the antifungal action of benzoate is caused by an accumulation of benzoate at low external pH, which lowers the intracellular pH into the range where phosphofructokinase is sensitive. The subsequent inhibition of glycolysis causes a fall in [ATP] and thus restricts growth.

It has been known for over 100 years that benzoate inhibits fungal growth (Salkowski, 1875), and because of its antifungal action it has been widely used (at concentrations of 5–10 mM) for the preservation of acid foods such as fruit juices, pickles, wine and pharmaceutical preparations. Information on the mechanism of action of agents used as drugs or food preservatives is relevant to the assessment of the efficacy as well as of the risks involved. The extensive earlier literature on the use of benzoate at 0.1% (w/v) as an antifungal agent has often been reviewed (Wiley, 1908; Bosund, 1962; Sinskey, 1980).

The inhibitory effect of benzoate increases with a lowering of the pH in the suspending medium (see Bosund, 1962). In an acidic medium the undissociated form of the benzoic acid (to which the yeast cell membrane is permeable) enters the cell until the concentrations inside and outside the cell become equal. The neutralization of the undissociated form by the cell buffers cause an acidification of the cell interior which ultimately inhibits cell growth. However, the precise mechanism of antifungal action has not been elucidated, and the experiments described in this paper are designed to examine the effect of benzoate on yeast cells in an attempt to define its site of action.

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Experimental

Yeast

A strain of baker's yeast (*Saccharomyces cerevisiae*) was isolated from a commercial sample grown for 24 h at room temperature in medium containing 2% (w/v) Bacto-yeast extract, 0.5% (w/v) Bacto-peptone (both from Oxoid, Basingstoke, Hants., U.K.), 0.1 M-KH_2PO_4 and 2% (w/v) glucose. Of this medium 100 ml was placed in 1-litre Roux bottles, laid flat. The collected cells were washed twice with distilled water, and the sediment after centrifugation (2 min at 1500 *g*) was weighed and suspended in 3 vol. of water. The dry weight of the suspension was measured by drying 1 ml at 105°C. The wet weight was obtained by centrifuging (1 min at 3000 *g*) in a haematocrit-type tube and multiplying the volume by 1.07 (the assumed specific gravity of yeast cells). Incubations were done at 25°C either in conical Warburg vessels or in 25 ml Erlenmeyer flasks, sealed with a rubber cap. These were provided with a centre well to hold a

stick of freshly scraped yellow phosphorus for strictly anaerobic experiments. The incubation was terminated by the addition of 0.2 vol of 20% (v/v) $HClO_4$. The results presented in the Tables and Figures are from representative experiments.

An acid standard medium for yeast suspension (pH 2.5) contained 50 mM-tartaric acid and 50 mM-NaH_2PO_4. Buffers with other pH values were as described in legends to Figures and Tables. It should be pointed out that the buffers used contained reagents which do not enter yeast cells.

For enzyme studies a commercial brand of baker's yeast (Danubio, Madrid, Spain) was used. It was washed three times with water. Extracts were prepared by grinding with alumina (1:2, w/w), extracting with 10 vol. of 0.1 M-KCl/5 mM-dithioerythritol, centrifuging at 20000 *g* for 15 min at 4°C, and dialysing the supernatant against the same medium for 1 h. The extract was kept frozen until use.

Metabolite assays

The following methods were used for the determination of metabolites: pyruvate (Hohorst *et al.*, 1959); glucose 6-phosphate and fructose 6-phosphate (Hohorst, 1963); fructose 1,6-bisphosphate and triose phosphate (Michal & Beutler, 1974); ATP (Lamprecht & Trautschold, 1963); ADP and AMP (Adam, 1963).

Special reagents

[*ring*-U-^{14}C]Benzoic acid and [*carboxy*-^{14}C]inulin were obtained from Amersham International, Amersham, Bucks., U.K.

Hepes [4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid] and Mes (4-morpholine-ethanesulphonic acid) were obtained from Calbiochem, Lucerne, Switzerland. Other biochemicals were obtained from Sigma.

Enzyme assays

Phosphofructokinase and hexokinase activities in yeast extracts were assayed at 340 nm in a Gilford 2400 at room temperature, in a basal medium of 40 mM-Hepes/Mes adjusted to the desired pH values, 0.1 M-KCl, 5 mM-$MgCl_2$ and 0.5 mM-dithioerythritol. For the assay of phosphofructokinase the mixture also contained substrates and allosteric effectors at their physiological concentrations in yeast, as follows: 0.3 mM-fructose 6-phosphate, 1 mM-MgATP, 0.8 mM-ADP, 0.1 mM-AMP, 3 mM-P_i (from a solution at pH 6.5), 3 mM-NH_4^+, 0.3 mM-citrate, 0.15 mM-NADH and 1 unit each of aldolase, glycerol 3-phosphate dehydrogenase and triose phosphate isomerase (the enzymes dialysed against 10 mM-Tris/HCl, pH 8, for 2 h). In addition 0.9 mM-glucose 6-phosphate was added at the concentration corresponding to the equilibrium of glucose phosphate isomerase to prevent the latter from lowering the concentration of fructose 6-phosphate. For the assay of hexokinase the additions were 10 mM-glucose, 1 mM-MgATP, 0.5 mM-$NADP^+$ and 1 unit of glucose 6-phosphate dehydrogenase.

Calculation of the intracellular pH of yeast cell water from the distribution of benzoic acid between external medium and cells

The calculation is based on the assumption that the benzoate anion ($R-CO_2^-$) does not pass through the yeast membrane but that the undissociated acid ($R-CO_2H$) passes through readily and reaches the same concentration in both intra- and extra-cellular water. It is further assumed that extracellular pH, the total benzoic acid concentration ($R-CO_2H + R-CO_2^-$) in intra- and extra-cellular water, and the dissociation constant, K, of benzoic acid (6.30×10^{-5} at 25°C) are known. [Total benzoic acid] was obtained from measurements of radioactivity.

It follows from the definition of the dissociation constant K that

$$[R-CO_2H] = \frac{[\text{Total benzoic acid}]}{1 + \frac{K}{[H^+]}}$$

Hence at an external pH of 2.50 ($[H^+] = 2.31$ mM)

$$[R-CO_2H]_{out} = 0.98 \times [\text{Total benzoic acid}]_{out}$$

If external and internal $[R-CO_2H]$ are equal

$$[H^+]_{in} = K \times \frac{[R-CO_2H]_{out}}{[\text{Total benzoic acid}]_{in} - [R-CO_2H]_{out}}$$

In principle this derivation is the same as that of Conway & Downey (1950).

Obviously only those acids that dissociate differently in the external and internal water are suitable for the measurement of intracellular $[H^+]$, and that accuracy increases with increasing the difference between the concentrations of external and internal total acid. An acid is unsuitable if it is too weak in relation to extra- and intra-cellular $[H^+]$, as is dimethyloxazolidinedione in the case of yeast, or too strong, so that it is almost fully dissociated in both internal and external water.

Measurement of the distribution of [^{14}C]benzoate between intracellular and extracellular water

Yeast cells were suspended in 4 ml of the medium and shaken for 20 min in 25 ml Erlenmeyer flasks with 0.01 mM-[*ring*-U-^{14}C]benzoate (about 10^6 d.p.m.). Control tests indicated that the equilibrium concentration of benzoate had been obtained within less than 10 min at benzoate concentrations of between 0.0025 mM and 10 mM at pH values up to 7.0. At higher pH equilibrium took longer to reach (about 30 min), and therefore such experiments were

done for longer times. At the end of the incubation the flask contents were centrifuged at 1500 *g* for 2min in a conical tube. The supernatant was decanted and any supernatant adhering to the wall was mopped up. The pellet was resuspended in 1ml of 10% $HClO_4$. This was quantitatively transferred to a counting vial with 4ml of water and 10ml of Insta-gel (Packard). Correction for extracellular water was made by determining the inulin space of the pellet in a parallel flask. Radioactivity in the supernatant was measured in a 0.2ml sample to which 10ml of scintillation cocktail [5.5g of Permablend III (Packard Instruments, Caversham, Berks., U.K.), 60g of naphthalene, 400ml of 2-methoxyethanol and 600ml of toluene] had been added.

The water content of the yeast cells was taken to be 70% (Conway & Downey, 1950) of the wet weight.

Results and discussion

Effect of benzoate on intracellular pH

It is known (Bosund, 1960; Macris, 1975) that yeast cells rapidly take up benzoic acid from an acid medium and that large concentration gradients in favour of an accumulation of benzoate within the cell can be achieved. Hence the distribution of [^{14}C]benzoate with a p*K* of 4.2 can be used with confidence as an intracellular pH indicator in the pH range 2.7–5.7. In some experiments reported in this paper we have used benzoic acid as a pH indicator above pH5.7, but only as part of a spectrum of measurements.

Yeast suspensions in the tartaric acid medium of pH2.5 were incubated for 10min in air with benzoate at different concentrations. No oxidizable substrate was added. The distribution of [^{14}C]-benzoate was measured and the intracellular pH was calculated as described in the Experimental section. The pH value obtained with 0.01mM-benzoate represents the 'normal' pH value, because at that low concentration the uptake of benzoate is too small to affect intracellular pH significantly. With 2mM-benzoate the intracellular pH fell by more than 0.5pH unit, and at 10mM-benzoate the fall was 1pH unit (Table 1).

The intracellular pH value of 5.8 found in the presence of minimal benzoate concentrations is of the same order as those recorded by Conway & Downey (1950) (who used entirely different procedures) for yeast cells exposed to an acid medium.

Effect of variation of external pH on internal pH

It is remarkable that yeast cells maintain a 'normal' internal pH when suspended in a medium of pH2.5 (Table 1), and even when the pH of the suspending medium is varied between pH2.5 and 7.47 there is relatively little change in the intracellular pH (Table 2).

Effect of pH and [benzoate] on anaerobic CO_2 production by yeast cells

The anaerobic CO_2 production (Table 3) shows a broad optimum between pH3.5 and pH6.0, with a

Table 2. *Effect of external pH on intracellular pH of yeast*
The concentration of benzoate added to the external medium was 0.01mM when pH was below 6.96. It was 2mM in the more alkaline media. The incubation period was 20min when pH was below 6.96, but 120 min in the more alkaline media when equilibrium between extra- and intra-cellular benzoate was slow because of the low concentration of undissociated benzoic acid in the medium. No substrate was added. The pH of the medium was measured at the end of the incubation.

pH of external medium	Ratio $\frac{[\text{benzoate}]_{in}}{[\text{benzoate}]_{out}}$	pH in intracellular water
2.50	39.5	5.80
3.54	63.2	6.08
4.43	37.1	6.20
5.91	2.98	6.39
6.78	0.71	6.62
6.96	0.34	6.50
7.16	0.22	6.51
7.47	0.12	6.53

Table 1. *Effect of external benzoate concentration on pH of intracellular water (external pH2.5)*
Yeast cells (53mg) grown in the presence of glucose were washed and suspended in 4ml of tartaric acid medium and incubated for 10min in air at 25°C. [Benzoate] refers to the total benzoate concentration. No substrate was added.

External [benzoate] added (mM)	[Benzoate] after incubation (mM)		Ratio $\frac{[\text{benzoate}]_{in}}{[\text{benzoate}]_{out}}$	Internal pH
	External	Internal		
0.0025	0.0017	0.071	41.8	5.81
0.01	0.007	0.278	39.5	5.80
2.0	1.77	21.9	12.4	5.27
5.0	4.63	34.7	7.5	5.02
10.0	9.43	53.5	5.66	4.88

Table 3. *Effect of pH and benzoate on anaerobic CO_2 production by yeast cells*

For intracellular pH measurements about 120 mg (wet wt.) of yeast was suspended in 4 ml of medium. The gas space contained N_2 and the centre well yellow phosphorus. The incubation period was 30 min and 100 mM-glucose was added; 50 mM-3,3-dimethylglutaric acid/NaOH was used to vary the pH. The CO_2 measurements were made under similar conditions on the same batch of yeast, but in manometer flasks.

[Benzoate]	pH of medium	Ratio $\frac{[\text{benzoate}]_{in}}{[\text{benzoate}]_{out}}$	pH of intracellular water	CO_2 production (μmol/min per g wet wt.)
None	3.5	–	–	20.3
	4.0	–	–	22.1
	4.5	–	–	22.3
	5.0	–	–	21.6
	5.5	–	–	24.1
	6.0	–	–	23.5
	6.5	–	–	19.0
	7.0	–	–	17.9
2 mM	3.5	10.83	5.27	7.91
	4.0	11.51	5.44	11.0
	4.5	8.74	5.59	14.3
	5.0	5.56	5.79	20.7
	5.5	2.12	5.82	22.6
	6.0	1.20	6.07	25.0
	6.5	0.79	6.38	20.2
	7.0	0.54	6.72	17.9
5 mM	3.5	5.55	4.94	3.77
	4.0	4.51	4.99	7.19
	4.5	3.80	5.20	10.9
	5.0	3.60	5.59	15.1
	5.5	2.33	5.87	24.2
	6.0	1.23	6.08	23.2
	6.5	0.68	6.32	19.7
	7.0	0.56	6.74	17.9

slight decline at the more alkaline pH values, owing to the retention of CO_2 in the medium as bicarbonate. A high resistance of yeast fermentation to very low external pH was first observed by Rothstein & Demis (1953), and maximal values are already observed at pH 2.5 (results not shown).

In the presence of 2 mM-benzoate, 68–43% inhibition of CO_2 production is seen at the lower pH values (3.5–4.5), whereas in the presence of 5 mM-benzoate the inhibition is complete at pH 2.5 (results not shown) and is only overcome when the external pH approaches pH 5.5. This result demonstrates clearly the effectiveness of benzoate at the lower external pH values.

Effect of benzoate on the intermediary metabolites of yeast fermentation

In an attempt to pinpoint the site of action of benzoate on anaerobic fermentation, glycolytic metabolites in the presence and absence of benzoate were measured (Fig. 1). Over a 40 min time course benzoate caused an increase over the control values for both glucose 6-phosphate and fructose 6-phosphate. However, fructose 1,6-bisphosphate and triose phosphate were markedly decreased compared with their controls. Pyruvate was also decreased, and separation of cells from medium showed that much of the pyruvate was in the medium, whereas the phosphorylated intermediates were all found in the cell pellet. The accumulation of the two hexose phosphates and the fall in the concentrations of all intermediates measured beyond the stage of fructose 6-phosphate indicate that benzoate causes a block at the stage of phosphofructokinase.

Another striking effect of benzoate was the fall in the concentration of ATP (Fig. 2) and an approximately equivalent increase in the concentration of AMP. There was a relatively small increase in the concentration of ADP, but very little change in the concentration of total adenine nucleotides.

The block at the stage of phosphofructokinase raises the question whether this enzyme is specifically inhibited by benzoate. Tests on a highly purified enzyme with up to 50 mM-benzoate gave negative results (C. Domenech & A. Sols, unpublished work). The increase in the two hexose monophosphates in the presence of benzoate (2.36 μmol after 5 min) was

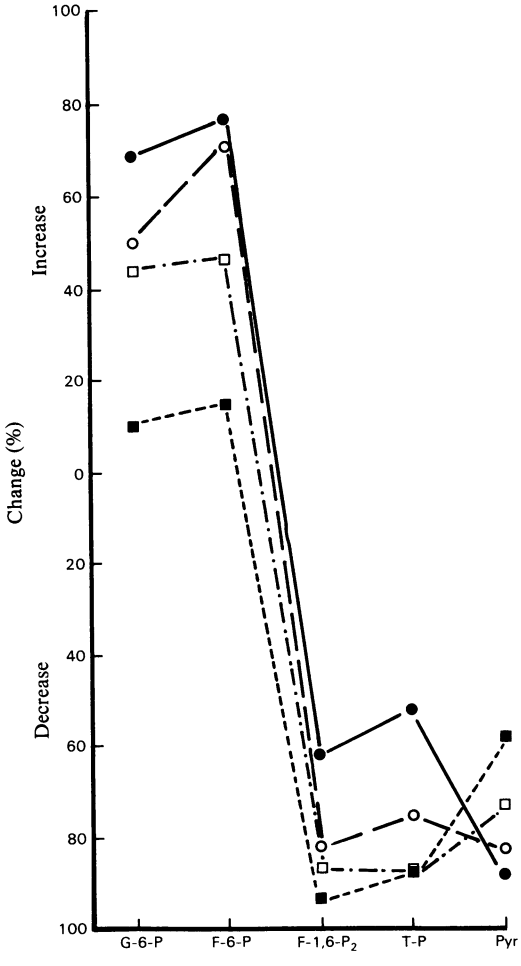

Fig. 1. *Effect of benzoate on intracellular metabolite concentrations of anaerobic fermentation of yeast*

Yeast cells were incubated anaerobically at 25°C in tartaric acid buffer (pH 2.5) in the presence of 10 mM-glucose and with or without 10 mM-benzoate for 5 min (■), 10 min (□), 20 min (○) and 40 min (●). Control values (μmol/g wet wt.) were as follows: glucose 6-phosphate (G-6-P), 5 min 1.9, 10 min 1.4, 20 min 1.0, 40 min 0.85; fructose 6-phosphate (F-6-P), 5 min 0.47, 10 min 0.33, 20 min 0.23, 40 min 0.18; fructose 1,6-bisphosphate (F-1,6-P_2), 5 min 1.1, 10 min 1.5, 20 min 1.7, 40 min 1.8; triose phosphate (T-P), 5 min 0.32, 10 min 0.38, 20 min 0.43, 40 min 0.46; pyruvate (Pyr), 5 min 0.59, 10 min 0.85, 20 min 1.8, 40 min 3.1.

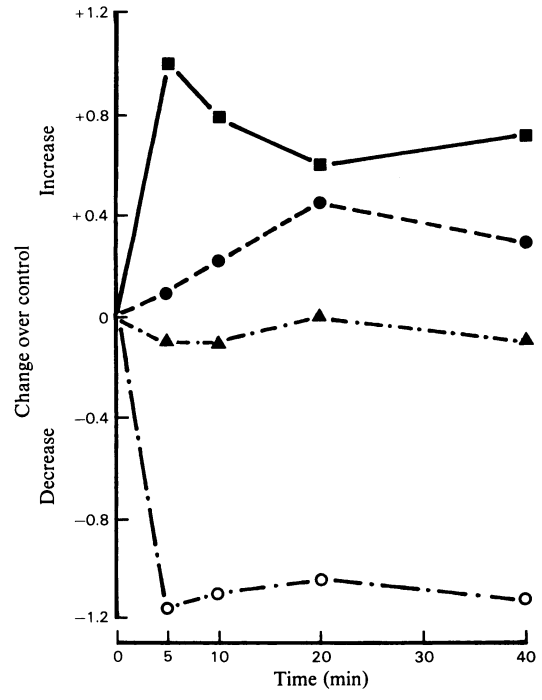

Fig. 2. *Effect of benzoate on adenine nucleotides during anaerobic fermentation*

Details were as for Fig. 1. ○, [ATP]; ●, [ADP]; ■, [AMP]; ▲, Total adenine nucleotides. Control values (μmol/g wet wt.) were as follows: ATP, 5 min 1.2, 10 min 1.2, 20 min 1.2, 40 min 1.2; ADP, 5 min 0.78, 10 min 0.71, 20 min 0.65, 40 min 0.54; AMP, 5 min 0.10, 10 min 0.07, 20 min 0.07, 40 min 0.07.

roughly equivalent to the loss of pyrophosphate bonds incurred by the conversion of ATP into AMP and ADP (2.13 μmol). Thus the available ATP was probably used for the hexokinase reaction and not by the phosphofructokinase reaction.

If benzoate does not directly interact with phosphofructokinase, then it has to be postulated that it brings about a change in the intracellular environment which inactivates phosphofructokinase to a much greater extent than hexokinase. It is relevant in this context that benzoate has antifungal properties only in an acid environment, at pH 5.0 or less. The question then arises whether a change in intracellular pH can account for the postulated preferential inhibition of phosphofructokinase.

Effect of pH on the activities of phosphofructokinase and hexokinase

Yeast phosphofructokinase is a multimodulated enzyme (Banuelos *et al.*, 1977; Sols *et al.*, 1981). Accordingly, the assay of the effect of pH on its activity was studied at concentrations of substrates and effectors within their physiological range as described in the Experimental section. Under these conditions phosphofructokinase was found to be markedly more sensitive to a decrease in pH in the range observed in yeast after treatment with benzo-

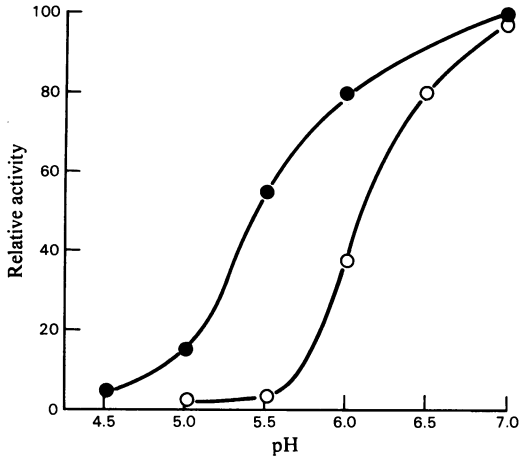

Fig. 3. *Effects of pH on the activities of phosphofructokinase and hexokinase of yeast*
Assays were performed under conditions resembling those *in vivo* (see the Experimental section): ○, phosphofructokinase; ●, hexokinase. Enzyme activity is expressed relative to the maximal activity at pH 7.0.

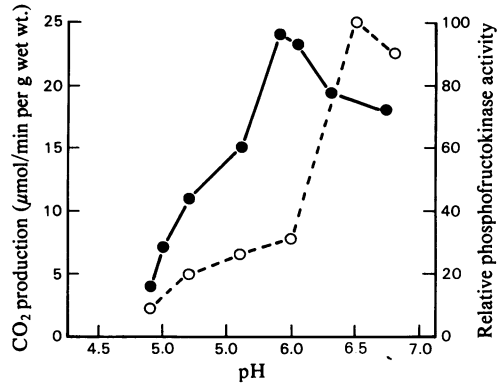

Fig. 4. *Relationship between CO_2 production, phosphofructokinase activity and intracellular pH*
The measurements were made as described in the Experimental section. CO_2 production and intracellular pH were measured in the presence of 5 mM-benzoate and 100 mM-glucose. ○, Phosphofructokinase activity in the presence of 3 μM-fructose 2,6-bisphosphate; ●, CO_2 production. Enzyme activity is expressed relative to the maximal activity at pH 6.5.

ate (Fig. 3). The pH–activity curve for hexokinase is similar to that reported by Sols *et al.* (1958). The greater sensitivity of the phosphofructokinase to a lowering of the pH may account for the observations in the intact cell.

Correlations between CO_2 production, phosphofructokinase activity and intracellular pH

When CO_2 production and phosphofructokinase activity are plotted against intracellular pH and pH in the medium respectively, the profiles are reasonably similar if phosphofructokinase activity is assayed in the presence of the recently discovered activator fructose 2,6-bisphosphate (Hers & Van Schaftingen, 1982) at 3 μM concentration, physiological for glycolysing yeast (C. Gancedo, personal communication) (Fig. 4). The differences in the yeasts used for the two kinds of observations can probably account for the relatively minor differences. It is therefore likely that the fall in intracellular pH caused by the accumulation of benzoic acid at low external pH inhibits glycolysis at the stage of phosphofructokinase, thus depleting the cell of ATP and in consequence restricting its growth.

Effects of acids other than benzoic acid on yeast

Many other acids act in the same way as benzoic acid provided that (1) their dissociation constants are of the same order of magnitude, (2) the undissociated acid is sufficiently lipophilic to cross the cell membrane, (3) the membrane is impermeable to the anion. Such acids as salicylate [whose antifungal action has been known since 1875 (von Meyer & Kolbe, 1875; Salkowski, 1875)], sorbate and acetate caused a fall of intracellular pH and inhibited the anaerobic fermentation of glucose by more than 95% when added to yeast cells suspended in the tartaric buffer at pH 2.5. The mechanism of action is therefore likely to be similar to the mechanism described for benzoate.

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