Transport of Benzoic and Propanoic Acids by *Zygosaccharomyces bailii*

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Uptake rates of benzoic and propanoic acid into *Zygosaccharomyces bailii* were proportional to the concentration of undissociated acid and showed no indication of limitation at high concentrations. Benzoic acid permeated 27 times faster than propanoic acid. Other low-M_r fatty acids were taken up at rates approximately related to their lipophilicity. Glucose stimulated uptake rate and inhibitors of glucose transport or metabolism removed the stimulation. It was concluded that the principal mechanism of uptake was diffusion of undissociated acid. The *Z. bailii* membrane had lower permeabilities than reported for other cell types and lipid bilayers. Growth in the presence of benzoic acid reduced permeability to benzoic acid further. Preservative-resistant yeast species had lower uptake rates of propanoic acid than sensitive ones. Degradation of the cell wall did not change the permeability to propanoic acid.

INTRODUCTION

The yeast *Zygosaccharomyces bailii* is very tolerant of the common weak-acid-type preservatives – benzoic, sorbic, propanoic and acetic acids and SO_2 – and often causes spoilage of acid foods and beverages containing these preservatives (Thomas & Davenport, 1985; Warth, 1986*b*). It is commonly accepted that lipophilic acids can permeate cell membranes passively in their undissociated form (Stein, 1981). Active transport systems for benzoic acid and for the aliphatic acids are also well known. At preservative concentrations used for growth inhibition, active transport is not likely to be important. Previous studies on the mechanism of uptake of benzoic acid (Macris, 1975) and of SO_2 (Macris & Markarkis, 1974) in *Saccharomyces cerevisiae* were interpreted as indicating active transport systems, although Stratford & Rose (1986) concluded that transport of SO_2 in *S. cerevisiae* was by simple diffusion.

Cells of *Z. bailii* grown in the presence of several weak-acid-type preservatives maintained, in the presence of an energy supply, a low intracellular concentration of preservative anion (Warth, 1977), and were more resistant to preservatives (Warth, 1986*a*, *b*). It was proposed that adapted cells formed a transport system for preservative anion, and that a major part of the effect of preservatives was the energy requirement to maintain a lowered intracellular preservative concentration. According to this model, the rate at which preservative enters the cell determines the energy demand and may influence the steady-state level of preservative anion in the cytoplasm. Knowledge of the rate, energetics and specificity of uptake of preservatives is therefore most important in elucidating mechanisms of preservative action, and in predicting the effects of conditions and different preservatives. Differences in the rate of uptake of preservative may explain differences in resistance between yeast species and the increased resistance of *Z. bailii* when grown in the presence of preservatives. Benzoic acid is the preservative most commonly used in low-pH products; however, it is taken up very rapidly by yeasts (Macris, 1975; Warth, 1977). Propanoic acid typifies the straight-chain aliphatic series of preservatives and has been used for much of this study as it permeates more slowly, allowing more accurate determination of kinetics.

METHODS

Growth. Zygosaccharomyces bailii FRR 1292 and other yeasts (see Table 4) were grown on 5% (w/v) glucose-yeast extract medium, pH 3·5, at 25 °C (Warth, 1985). Minimum inhibitory concentrations (MICs) of benzoic acid

were determined using microwell plates inoculated with exponential-phase cultures grown in the presence of benzoic acid (Warth, 1986a). Mid-exponential-phase cells, 0·2–1·0 mg dry wt (1 culture)$^{-1}$, were used for all experiments apart from the preparation of spheroplasts. Cells were centrifuged, washed twice with 0·05 M-potassium citrate buffer pH 3·5 and stored for up to 3 h at 5–10 °C.

Preservative uptake. Suspensions (2·0 ml) containing 12–24 mg dry wt of cells in 0·1 M-potassium citrate pH 3·50 were incubated together with any additions at 25 ± 0·2 °C for 15 min. The reaction was started by the addition of 20 µl 10 mM-preservative. Samples (200 µl) were filtered on 0·4 µm polycarbonate filters (Nuclepore) at intervals of approximately 15 s for benzoic and 30 s for propanoic acid, and the times at which each filtration was complete were noted. Filtration took from 1 to 1·5 s. More accurate and reproducible results were obtained by not washing the filter but applying corrections for the volume retained on the filter (0·14 µl), the interstitial volume of the cells [1·0 µl (mg dry wt)$^{-1}$] and the volume of cell ater [1·8 µl (mg dry wt)$^{-1}$]. This was possible because of the low volume of filtrate retained on this type of filter and the high uptake of preservative. Data were generally consistent with first-order kinetics. Uptake rate constants were calculated for each time interval, using the maximum uptake determined from samples taken at 10 and 15 min. In some circumstances where maximum uptake was not measured or was not constant, a value for maximum uptake was assumed so as to give a qualitative best fit. Values from data obtained at up to 50% of maximum uptake were averaged. Concentrations of preservative refer to the total concentration of dissociated plus undissociated forms.

Cell water, the interstitial volume and filter hold-up volumes were determined using [1-^{14}C]sorbitol (Amersham).

Permeability. The permeability of the cell surface was calculated from the uptake rate per mg dry wt, the area of the cell membrane, and the cell dry weight. Cell dimensions were measured using phase-contrast optics at × 2000 magnification. Nearly all cells and buds of *Z. bailii* closely approximated prolate ellipsoids. A cell wall thickness of 125 nm was assumed and volume and membrane area were calculated for each cell as prolate ellipsoids. Cell weight was calculated from the volume using a relative density of 1·096, determined by density-gradient centrifugation in Urographin.

Analyses. Propanoic and benzoic acids were determined by liquid scintillation counting using [1-^{14}C]propanoate and [7-^{14}C]benzoate respectively (1 GBq mol^{-1}, New England Nuclear). Sorbic acid was determined by HPLC at 250 nm using an RP8 reverse-phase column (250 × 3·7 mm) eluted with 50% (v/v) methanol in 1% acetic acid. The other low-M_r fatty acids were determined by gas chromatography using Chromosorb 101 at 170 °C and 185 °C.

Effect of concentration. Solutions of potassium benzoate (0·20 ml) containing 0·1 MBq [7-^{14}C]benzoate, and HCl (20 µl) equivalent to 80% of the potassium benzoate, were added simultaneously to 1·8 ml of the cell suspension. To avoid the effects of rapid osmotic changes at the higher concentrations tested with propanoic acid, the cells were incubated with potassium propanoate pH 3·5 for 15 min prior to the addition of [^{14}C]propanoate (0·4 MBq).

Uptake of fatty acids. A mixture of propanoic, 1-methylpropanoic and butanoic acids (0·5 M) was adjusted to pH 3·5, and added to the cell suspension to give an initial concentration of 5 mM each. In a separate incubation, a 100 mM solution of the potassium salts of pentanoic, hexanoic and sorbic acids was added to give 1 mM initial concentration of each in the cell suspension. Final concentrations were 3·4 mM for the first experiment and 0·55 mM for the second. Uptake was expressed relative to the concentration in the supernatant. Cells plus filters were extracted at 65 °C for 3 min with 100 µl 0·2 M-K$_2$CO$_3$ containing 1·0 mM-2-methylbutanoate as internal standard. The suspension was centrifuged after removal of the filters. A sample of the supernatant was acidified with 0·1 vol. 50% H$_2$SO$_4$ plus 1% H$_3$PO$_4$ and centrifuged; fatty acids were determined by gas chromatography and sorbic acid by HPLC.

Spheroplasts. Spheroplasts were prepared from a late-exponential-phase culture (2·8 g dry wt l^{-1}) using modifications of the method of Dickinson & Isenberg (1982). Cells were centrifuged and washed twice in 0·1 M-MES/0·1 M-EDTA, pH 6·0, and incubated (75 mg wet wt ml^{-1}) with 1 mg dithiothreitol ml^{-1} in this buffer at 30 °C with stirring for 30 min. A sample was taken and washed in 0·6 M-KCl/0·1 M-potassium citrate pH 3·5 before determination of propanoic acid uptake rate. These cells appeared normal by phase-contrast microscopy in water. The remainder were washed with 20 mM-citrate/phosphate pH 5·85 in 0·6 M-KCl and incubated in this buffer with 2 mg Novozym 234 ml^{-1} (Novo Enzyme Products, UK) at 25 °C with stirring. After 2 h, 74% of the cells were spherical and lysed on dilution with water, 24% were lysed and 2% retained their ovoid or budding shape. The suspension was then diluted slowly into 0·6 M-KCl/0·1 M-potassium citrate pH 3·5, centrifuged and washed three times to remove lysed cells. Propanoic acid uptake was determined for spheroplasts, dithiothreitol-treated cells and untreated cells in 0·6 M-KCl/0·1 M-potassium citrate pH 3·5.

RESULTS

Uptake kinetics and cell permeability. Uptake of benzoic and propanoic acids is shown in Fig. 1. Each followed first-order kinetics for at least the first 50% of the reaction. The uptake
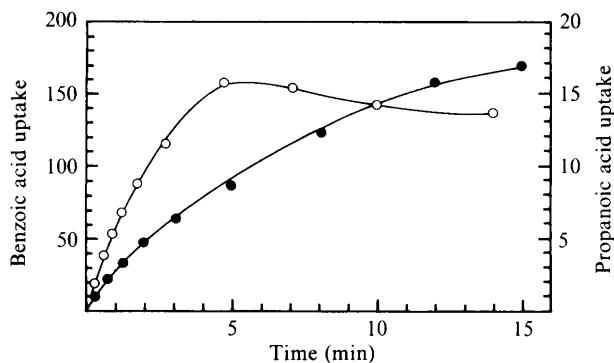
Fig. 1. Kinetics of uptake of benzoic acid (O) and propanoic acid (●) into *Z. bailii* at 25 °C. Uptake, expressed as nmol (mg dry wt)$^{-1}$ min^{-1}, was normalized by dividing by the actual concentration (mM) of preservative in the supernatant. The initial concentration was 0·1 mM.

Table 1. *Effect of various compounds on rates of uptake of benzoic and propanoic acids by Z. bailii*

Results are expressed as percentages of the respective value obtained in 0·1 M-citrate pH 3·50, and are shown ± SEM when at least three determinations on separate cell crops were averaged. Control values of uptake rates of benzoic and propanoic acids for cells grown in the absence of preservative were 86 ± 11 and 3·2 ± 0·37 nmol (mg dry wt)$^{-1}$ (mM undissociated acid in supernatant)$^{-1}$ min^{-1} respectively. For cells grown in the presence of benzoic acid, the uptake rates were 49 ± 6 and 2·9 ± 0·3 nmol mg^{-1} mM^{-1} min^{-1} respectively.

Addition	Cells grown without added preservative		Cells grown with 2 mM-benzoic acid	
	Benzoic acid	Propanoic acid	Benzoic acid	Propanoic acid
None	100	100	100	100
2·5% n-butanol	164 ± 25	202 ± 30		167
5% glucose	215 ± 24	158 ± 17	128	144
5% glucose + 2 mM-iodoacetate	130			
5% glucose + 4 mM-uranyl nitrate	129			
5% 2-deoxyglucose	110	133 ± 16		86
2 mM-iodoacetate	91	98		81
4 mM-uranyl nitrate		102		75
0·1 mM-p-chloromercuribenzoate		94		94

rate constant for benzoic acid [86 ± 11 nmol (mg dry wt)$^{-1}$ (mM undissociated acid in the supernatant)$^{-1}$ min^{-1}], was considerably greater than that of propanoic acid (3·2 ± 0·37). The average total cell volume and the average area of the membrane, determined from light microscope measurements and the cell density, were 65·8 ± 4 μm^3 and 82·7 ± 6 μm^2 respectively. From these data, permeabilities of the membrane to benzoic acid and propanoic acid of 26·9 ± 5 × 10^{-8} m s^{-1} and 1·55 ± 0·2 × 10^{-8} m s^{-1} respectively were calculated.

Cells grown in the presence of 2 mM-benzoic acid (adapted cells) had a slightly (10%) smaller membrane area and no significant difference in their permeability to propanoic acid. Permeability to benzoic acid, however, was 40% less.

Effect of various compounds on uptake rate. Butanol substantially increased the rates of uptake of both benzoic and propanoic acids (Table 1). Uptake rate was also stimulated by glucose, the effect being largely removed by the simultaneous addition of iodoacetate or uranyl nitrate. In the absence of glucose, deoxyglucose and other inhibitors of glycolysis or transport did not greatly affect uptake rates.
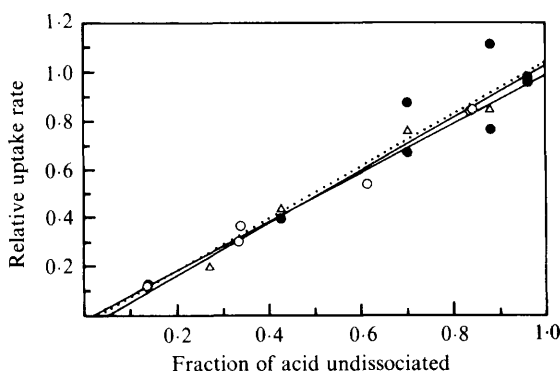
Fig. 2. Rates of uptake at various pH values, plotted against the fraction of acid undissociated at that pH. Control values, from the regression for each experiment, are expressed as nmol (mg dry wt)$^{-1}$ min^{-1}, normalized by dividing by the concentration (mM) of undissociated preservative in the supernatant: benzoic acid, 91·5 (○); propanoic acid, 4·11 (●); benzoic acid with cells grown in 2 mM-benzoic acid, 33·3 (△).

Table 2. *Effect of various compounds on the maximum amount of benzoic and propanoic acids accumulated by Z. bailii*

Results are the maximum uptake observed during the kinetic experiments in Table 1, and are expressed as percentages of the respective value obtained in 0·1 M-citrate pH 3·50. Under these conditions, cells grown in the absence of benzoic acid accumulated benzoic and propanoic acids to 155 ± 36 and 18·5 ± 0·7 nmol (mg dry wt)$^{-1}$ respectively; cells grown in the presence of 2 mM-benzoic acid accumulated the acids to 75 ± 19 and 19 ± 1 nmol (mg dry wt)$^{-1}$ respectively. These control values are normalized by dividing by the concentration (mM) of preservative in the supernatant, and are given ± SEM for three determinations on the same preparation of cells.

	Cells grown without added preservative		Cells grown with 2 mM-benzoic acid	
Addition	Benzoic acid	Propanoic acid	Benzoic acid	Propanoic acid
None	100	100	100	100
2·5% n-butanol	28	56		108
5% glucose	97	225	13	48
5% glucose + 2 mM-iodoacetate	134			
5% glucose + 4 mM-uranyl nitrate	84			
5% 2-deoxyglucose	68	60		71
2 mM-iodoacetate	47	61		71
4 mM-uranyl nitrate		59		51
0·1 mM-p-chloromercuribenzoate		48		89

Effect of various compounds on amount of uptake. Unadapted cells accumulated benzoate and propanoate (Table 2). Calculation of the apparent internal pHs, assuming impermeability of the anions, gave values of 6·20 and 6·02 respectively. Measurement by freeze–thawing gave a value of 5·95 ± 0·15. Adapted cells in the absence of glucose also accumulated high levels of both benzoate and propanoate, but these were considerably reduced in the presence of glucose. Internal pHs, in the absence and presence of glucose, were 6·13 ± 0·2 and 6·51 ± 0·27 respectively.

Effect of pH, concentration and temperature. Uptake rates were proportional to the fraction of the acid which was undissociated (Fig. 2). There was no positive intercept on the *y* axis, showing that the anion was not taken up at a rate comparable with that of the undissociated form. Uptake rate constants for both benzoic acid and propanoic acid were essentially independent of the concentration in the medium over a wide range (Fig. 3). In particular, there was no evidence
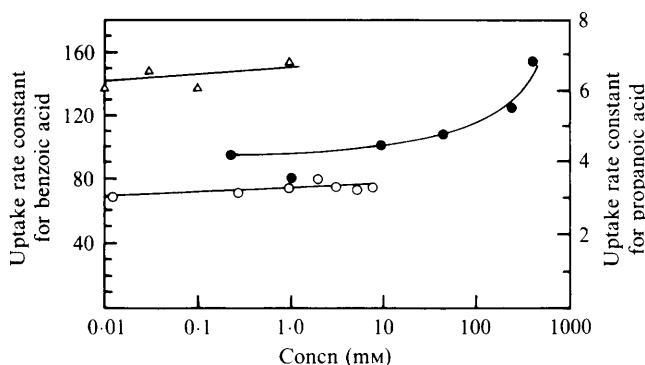
Fig. 3. Effect of concentration on the uptake rate constant. Uptake, expressed as nmol (mg dry wt)$^{-1}$ min^{-1}, was normalized by dividing by the actual concentration (mM) of preservative in the supernatant. ○, Benzoic acid; ●, propanoic acid; △, benzoic acid with cells grown in 2 mM-benzoic acid.

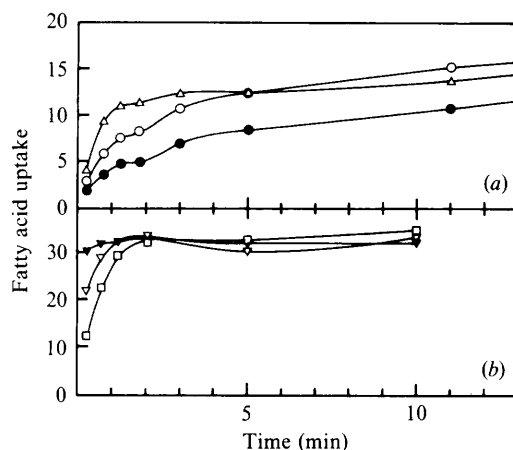Fig. 4 Uptake of low-M_r fatty acids by *Z. bailii*. (*a*) Propanoic (○), 2-methylpropanoic (●), and butanoic (△) acids. Intitial concentrations, 5 mM. (*b*) Pentanoic (□), hexanoic (▽) and sorbic (▼) acids. Initial concentrations, 1 mM. Uptake is expressed as nmol (mg dry wt)$^{-1}$, normalized by dividing by the actual concentration (mM) of preservative in the supernatant.

for a rate limitation at high preservative concentrations. The data in fact showed an increase. For adapted cells in the presence of glucose, the rate of benzoic acid uptake was proportional to the concentration of undissociated acid (Fig. 2), and the rate constant was independent of concentration over the range tested (Fig. 3).

The Arrhenius plot for propanoic acid uptake rate in *Z. bailii* was linear between 0·5 °C and 25 °C ($r^2 = 0.99$) and showed an activation energy of 105 kJ mol^{-1}. Two other yeasts, *Hansenula anomala* and *Kluyveromyces fragilis*, were also studied and gave values of 89 and 118 kJ mol^{-1} respectively.

Uptake of other low-M_r fatty acids. For the saturated straight-chain acids, uptake rate increased with chain length (Fig. 4). 2-Methylpropanoic acid was taken up more slowly than propanoic or butanoic acid. Sorbic acid (2,4-hexadienoic acid) was taken up more rapidly than hexanoic acid.

Effect of treatment with cell wall reagents on uptake. In order to determine if the permeation of weak-acid preservatives was limited by the cell wall or by the membrane, spheroplasts were prepared. Treatment of *Z. bailii* with dithiothreitol had no effect on the permeability (Table 3) or

Table 3. *Effect of treatment with cell-wall-degrading agents on the rate of uptake of propanoic acid by Z. bailii*

Treatment	$10^{-7} \times$ No. of cells ml^{-1}*	Uptake rate†	Corrected rate‡
None	5·0	3·8	3·8
Dithiothreitol	5·1	3·8	3·7
Dithiothreitol + Novozym	1·95	1·13	2·9

* Number of cells or spheroplasts per mg dry cells prior to treatment.

† Units: nmol (mg dry wt untreated cells)$^{-1}$ min^{-1}, normalized by dividing by the concentration (mM) of preservative in the supernatant.

‡ Uptake rate corrected for the number of intact cells or spheroplasts.

Table 4. *Rate of uptake of propanoic acid and sensitivity to benzoate in yeasts*

Organism	FRR no.	MIC of benzoic acid (mg l^{-1})	Uptake rate constant for propanoic acid*
Saccharomyces cerevisiae	1297	170	60
Kluyveromyces fragilis	1338	170	41
Kloeckera apiculata	2168	190	86
Hansenula anomala	2169	220	59
Saccharomyces cerevisiae	1298	290	20
Candida krusei	1302	440	15
Saccharomycodes ludwigii	2294	650	6·4
Schizosaccharomyces pombe	2535	570	2·7
Zygosaccharomyces bailii	1292	1250	3·7

* Units: nmol (mg dry wt)$^{-1}$ min^{-1}, normalized by dividing by the concentration (mM) of preservative in the supernatant.

appearance of the cells. Subsequent treatment with Novozym converted nearly all the cells into osmotically sensitive spheroplasts. This suspension had a substantially lower uptake rate. After correction for the number of intact spheroplasts the rate was still less than in untreated cells, indicating that an intact cell wall structure is not important in determining the permeability of the cell to preservatives.

Uptake rate in other yeast species. Generally, propanoic acid was taken up more rapidly in yeasts that were relatively sensitive to preservative, while species that were tolerant of preservative had low uptake rates (Table 4).

DISCUSSION

The proportionality over a range of pH values between the rate of uptake and the concentration of undissociated acid suggests that this is the dominant form in which benzoic and propanoic acids enter the cell. This result does not exclude transport by a proton–anion symport mechanism. It was not expected that the anion transport system postulated to account for low uptake of preservative (Warth, 1977) would be demonstrable under the conditions of these experiments. Furthermore, the cells used were grown in the absence of added preservative and therefore would not be expected to have the carrier. Uptake rate was proportional to concentration over a wide range, showing no evidence for carrier limitation at high concentrations. The tendency to an increased rate suggests that high concentrations of propanoic acid disturbed the membrane structure. Butanol also increased the uptake rate, an effect that is characteristic of passive uptake through the lipid pathway, while many mediated transfer processes are inhibited (Deuticke, 1977).

The relative rates of uptake for various acids were generally similar to the values predicted from their partition coefficients and to those found in erythrocytes (Klocke & Flasterstein, 1982) and lipid bilayers (Wolosin & Ginsburg, 1975; Walter & Gutknecht, 1986), and showed no examples suggestive of a specific transport system. Minor deviations were that 2-methylpropanoic acid permeated more slowly and sorbic acid more rapidly than expected from their octanol–water partition coefficients. Biological membranes generally show a greater degree of specificity (Deuticke, 1977) as diffusional and interfacial factors are also involved.

In absolute terms, the *Z. bailii* membrane was 40-fold less permeable to propanoic acid than toad bladder (Wolosin & Ginsburg, 1975), 1500-fold less than human erythrocyte membranes (Klocke & Flasterstein, 1982) and 20000-fold less than phosphatidylcholine bilayers (Walter & Gutknecht, 1986). The other yeast species studied had uptake rates up to 20 times that of *Z. bailii*. Destruction of cell wall integrity did not appear to increase the permeability of *Z. bailii*, confirming that the membrane was the rate-limiting barrier. The activation energy for propanoic acid uptake of 105 kJ mol^{-1} compares with values of 44 (Klocke & Flasterstein, 1982) and 92 kJ mol^{-1} (Deuticke, 1977) for acetic acid permeability in erythrocytes and is similar to that of a number of other passive permeants (Deuticke, 1977). It is also consistent with, but lower than, that often found for facilitated diffusion. This high value eliminates unstirred layer effects as being rate limiting, as these have activation energies of 17–20 kJ mol^{-1}.

The extent of uptake in uninduced cells was generally consistent with that calculated, assuming passive transport of free acid and impermeability of the anion, and values reported for the intracellular pH under the conditions of the experiment. Induced cells in the presence of glucose had much lower benzoate contents, as is consistent with efflux of preservative anions as previously proposed (Warth, 1977).

Results generally were obtained at 0·1 mM preservative concentration. This is advantageous in reducing possible effects of counter-ion flow, pH adjustment and osmotic balance. Benzoic and propanoic acids exert their preservative action on *Z. bailii* in the concentration range of 1–10 mM and 20–200 mM respectively (Warth, 1986b). Since rates were proportional to concentration, the data obtained should remain applicable at practical preservative concentrations.

The increased uptake rates observed in the presence of glucose, together with the effects of inhibitors on this increase, suggest the presence of an additional transport pathway requiring glucose transport. In several ways the system does not appear to be typical of active transport. It appears to lack specificity, as both benzoic and propanoic acid uptake rates were stimulated, there was no indication of carrier limitation at benzoic acid concentrations up to 1 mM, and it was not inducible, as the effect was seen in both unadapted and adapted cells. The response to pH showed that undissociated benzoic acid was the form transported. A possible alternative explanation is that the acids bound gratuitously to the glucose carrier. Further studies are required to characterize the system adequately.

The previous report of carrier-mediated transport of benzoic acid in *S. cerevisiae* (Macris, 1975) is not valid, as the conclusions were based upon uptake data taken over fixed time intervals, rather than on kinetic constants. Since benzoic acid uptake was rapid compared with the incubation time, such treatment does not give initial rates and leads to artifactual Michaelis–Menten constants.

A major stress on yeasts caused by weak-acid preservatives under acid conditions is the energy demand for maintenance of intracellular pH, while keeping the intracellular concentration of preservative anion low by allowing its exit from the cell (Warth, 1977). The maximum level of preservative that a species can tolerate may be determined by the rate at which preservative enters the cell and by the capacity of the cell to remove it. The present data for nine species of yeast showed that preservative-resistant species generally did have lower rates of uptake. Among a smaller set of species, there was no relationship between the maximum rate of energy generation and preservative resistance (Warth, 1988), suggesting that membrane permeability is of major importance in determining preservative tolerance in yeasts. The decreased permeability of cells of *Z. bailii* adapted to benzoic acid suggests that both formation of the anion transport system and a change in permeability of the membrane are responsible for the large

increase in preservative resistance observed. It is not known what changes in membrane structure or composition are responsible for the permeability differences.

Recently it was suggested that the very rapid rate of influx of benzoic acid would make efflux of anions from the cell a futile effort (Cole & Keenan, 1987). Efflux of anions requires energy to export an equivalent amount of protons to maintain cell pH. An indication of the feasibility of using energy to maintain lower than equilibrium values of benzoate in the cell can be obtained by comparing the uptake rate of benzoic acid from the present results with the increase in fermentation rate caused by benzoic acid in anaerobic chemostat cultures (Warth, 1988). Assuming the production of 1 mol ATP per mol CO_2 or ethanol formed, $0.7–1.8$ mol ATP was utilized per mol benzoic acid taken up. This value is comparable to, but slightly lower than, that required to maintain a normal proton-motive force, and confirms the potential practicability of the mechanism. Direct measurements under identical conditions are required to show the actual stoichiometry.

Transport of SO_2 in *S. cerevisiae* was studied by Stratford & Rose (1986), who concluded that uptake was by simple diffusion of the uncharged molecular species. There is a correlation between species in resistance to SO_2, benzoic acid and sorbic acid (Warth, 1985). The permeation of the undissociated form and the accumulation of anion, driven by the pH difference between the cell and the medium, are shared features of the antimicrobial action of the common weak-acid preservatives.

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