Mechanism of Benzoic Acid Uptake by *Saccharomyces cerevisiae*

BASIL J. MACRIS

Department of Biology, Nuclear Research Center "Demokritos," Aghia Paraskevi, Athens, Greece

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A fast uptake of the preservative benzoic acid was observed in *Saccharomyces cerevisiae*, reaching saturation in about two min and then remaining constant at this level. The strong dependence of benzoic acid uptake on pH was due to the relative distribution of molecular and ionic forms in solution and not to the pH itself. The molecular form was the only one taken up by the cells. The specificity of the uptake mechanism was evidenced by the pattern of irreversible heat inactivation of the uptake system resembling protein denaturation by heat. Furthermore, the effect of temperature on the uptake was similar to that observed in enzymic reactions, whereas the kinetic data of uptake conformed to the Michaelis-Menten curve of saturation with a $K_m$ of $1.54 \times 10^{-2}$ M and $V_{max}$ of $3 \times 10^{-3}$ M/10 s. The evidence presented in this paper indicates that compounds of protein nature are involved in the uptake of this preservative.

Benzoic acid, usually in the form of sodium salt, has long been used as an additive to inhibit microbial growth in a variety of foods (4). The antimicrobial effect of benzoic acid is greatly pH dependent; it is increased by lowering the pH in the suspending medium (7, 9, 12).

In aqueous solutions, benzoic acid is present as molecular benzoic acid and as benzoate ion. The percent distribution of these forms at any pH can be calculated by means of the Henderson-Hasselbalch equation:

\[
\text{pH} = \text{pK} + \log \frac{[\text{base}]}{[\text{acid}]}
\]

and using $\text{pK} = 4.19$ at 25°C. Table 1 gives the percent distribution of molecular and ionic benzoic acid at different pH levels.

Bosund (2) showed that the amount of benzoic acid absorbed by the cells of bakers’ yeast (*Saccharomyces cerevisiae*) was roughly proportional to the concentration of the molecular benzoic acid in the external medium. Furthermore, Oka (11) concluded that, in the same yeast, benzoic acid and other similar inhibitors exist in the cell, both dissolved in the cell fluid and absorbed on solid phase, and that it is the absorbed quantity that determines the growth inhibitory effect.

The purpose of this paper was to study and clarify the mechanism of uptake of this common preservative, benzoic acid, in a common yeast, *S. cerevisiae*.

**MATERIALS AND METHODS**

Organism. The yeast used in this work was *S. cerevisiae* NRRL Y-635, previously designated as *S. ellipsosideus*.

Growth medium. The liquid growth medium was similar to that of Schultz and McManus (13) and contained (g/liter): sucrose, 12.5; KH$_2$PO$_4$, 0.6; KCl, 0.4; MgCl$_2$, 0.1; CaCl$_2$$\cdot$2H$_2$O, 0.1; NH$_4$Cl, 1.6; FeCl$_3$, 0.1; Na$_2$SO$_4$, 0.04. Also the following growth factors were added (mg/liter): inositol, 16; calcium pantothenate, 10.5; biotin, 0.05; thiamine, 0.05; pyridoxin, 0.05; nicotinic acid, 0.05. The pH was adjusted to 4.2 with 0.1 M citrate-phosphate buffer, pH 4.2, and the medium was filter sterilized.

Inoculation and incubation. Cells from a stock culture containing 0.5% yeast extract, 2.0% glucose, and 1.5% agar were used to inoculate 200 ml of growth medium in 500-ml Erlenmeyer flasks. The cells were grown aerobically with shaking (180 rpm) for 24 h at 30°C.

Harvesting. The cells were harvested by centrifugation at $3,000 \times g$ for 10 min, washed three times with deionized water, and concentrated to a dense suspension. The volume occupied by the cells in this suspension was measured by a modification of the method of Black and Gerhardt (1).

Measurement of uptake. The sodium salt of benzoic acid was used because of the low aqueous solubility of the free acid (4). The uptake was measured as follows: 0.2 ml of the dense cell suspension, containing about 10$^6$ cells, was incubated at 25°C with 0.4 ml of a solution containing the desired concentration of sodium benzoate and 0.4 ml of 0.25 M citrate-phosphate buffer. The incubation was terminated by filtering the cell suspension through a 0.6-μm membrane filter (Millipore Corp.). With this technique...
Benzoic acid was determined spectrophotometrically at 225 nm (8). To avoid interference of the ultraviolet-absorbing buffer compounds, the blank contained all the materials present in the incubation mixture except benzoic acid. The amount of benzoic acid taken up by the cells was calculated from the equation:

\[
\% \text{ uptake} = \left( \frac{C - B}{C} \right) \times 100,
\]

where \( C \) and \( B \) are the amounts of benzoic acid found in the control (incubation mixture without cells) and in the filtrate, respectively.

**RESULTS AND DISCUSSION**

The uptake of benzoic acid is strongly pH dependent and follows a saturation pattern which remains constant in the course of time (Fig. 1). The initial rate of uptake is linear and rapid, reaching a maximum in about two min. The size of the pool at saturation is about \( 1.20 \times 10^8 \), \( 2.50 \times 10^8 \) and \( 3.50 \times 10^8 \) benzoic acid molecules per yeast cell at pH levels 4.79, 4.36, and 4.01, respectively. The observed pattern of uptake could be explained on the basis that benzoic acid, being a nonspecific inhibitor, is not metabolized by the yeast cell and, therefore, the pool is not depleted with time.

The effect of pH on the uptake is exerted either directly by influencing the uptake system itself or indirectly by determining the dominant form of benzoic acid in solution. Figure 2A shows clearly that the molecular form is the only one taken up by the cells since the rate of uptake of this preservative is linearly proportional to the concentration of the molecular form present in the incubation mixture. The uptake system is not directly affected by pH because, when the same molecular benzoic acid concentration is used, the tested different pH levels result in the same rate of total benzoic acid uptake (Fig. 2B).

The data presented above show that the benzoic acid uptake system displays a high degree approximately 0.2 ml of a clear filtrate could be obtained in 3 s.

| pH  | Molecular form (%) | Ionic form (%) |
|-----|--------------------|----------------|
| 3.24| 90                 | 10             |
| 3.59| 80                 | 20             |
| 3.82| 70                 | 30             |
| 4.01| 60                 | 40             |
| 4.19| 50                 | 50             |
| 4.36| 40                 | 60             |
| 4.55| 30                 | 70             |
| 4.79| 20                 | 80             |
| 5.14| 10                 | 90             |

**TABLE 1. Percentage of distribution of the two benzoic acid species in aqueous solution at different pH levels**

**FIG. 1. Time course of benzoic acid uptake by the yeast S. cerevisiae as a function of pH. Total benzoic acid concentration, \( 10^{-3} \) M. The pH was adjusted to different levels: \( \bullet \), pH 4.01; \( \circ \), pH 4.36; \( \square \), pH 4.79.**

**FIG. 2. Effect of pH on uptake. (A) The total benzoic acid concentration was constant (\( 10^{-2} \) M). The pH was adjusted to 5.14, 4.79, 4.36, and 4.01, corresponding to molecular benzoic acid concentrations of 10, 20, 40 and 60% of the total benzoic acid. (B) The molecular benzoic acid concentration was constant (\( 4 \times 10^{-4} \) M). The pH levels necessary for adjusting the molecular benzoic acid concentration to the same level are shown.**
of structural specificity for the molecular form. This property can be explained on the basis that the molecular form, having no electric charge, was more soluble in the lipid portion of the cell membrane than in water, compared to the ionic form (3). However, our data show that when the cells were subjected to the effect of different temperatures, and their ability to take up molecular benzoic acid after cooling to 25°C was measured, a sharp decrease in the rate of uptake after 60°C, was found (Fig. 3A). The pattern of this irreversible heat inactivation resembles that of protein inactivation by heat. Furthermore, the effect of temperature on the uptake (Fig. 3B) shows a curve similar to that observed in enzymic reaction. These results, along with the conformity of kinetic data to Michaelis-Menten curve of saturation (Fig. 4), allowing the calculation of an apparent $K_m$ of $1.54 \times 10^{-3}$ M and a $V_{max}$ of $3 \times 10^{-3}$ M/10 s, indicate that uptake of the molecular form is specifically mediated by compounds of protein nature. In bacteria, there are indications that benzoic acid enters the cell by a facilitated diffusion process which requires the existence of protein carriers residing in the cell membrane (5, 6).

The above mechanism of uptake answers some of the questions associated with the mode of action of this common preservative (3). The mechanism by which pH affects the uptake and, finally, the toxicity of benzoic acid, beyond its theoretical significance, is also of practical importance; it allows the precise calculation of the concentration of the molecular form which is the active form of this preservative (3, 12).

Sulfur dioxide, another common acid food preservative, is transported by a similar mechanism in the same yeast (10). Both this and the evidence presented above indicate that a common mechanism operates during uptake of acid food preservatives.

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**Fig. 3.** Effect of temperature on uptake. (A) Thermal inactivation of the yeast (*S. cerevisiae*) uptake system. The cells were heated to the indicated temperatures for 2 min, cooled immediately down to 25°C, and incubated with $10^{-3}$ M total benzoic acid at pH 4.79 (corresponding to $2 \times 10^{-4}$ M molecular benzoic acid) for 2 min. (B) The cells were incubated at the indicated temperatures with $10^{-3}$ M total benzoic acid, pH 4.19 (corresponding to $5 \times 10^{-4}$ M molecular benzoic acid), for 30 s.
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