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L-Malic-acid permeation in resting cells of anaerobically grown *Saccharomyces cerevisiae*

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The study of permeation of L-malic acid in cells of *Saccharomyces cerevisiae* at pH 3.0 was carried out with (U-^{14}C)-labelled L-malic acid. Resting cells were used in these experiments. They were previously anaerobically grown on glucose. This study showed that this transport is the result of two competitive mechanisms, one for the uptake and one for the efflux. The uptake mechanism seems to be a simple diffusion of the L-malic acid in a non-dissociated form. The efflux mechanism seems to be an active transport of L-malic acid that is very dependent on the temperature. At the steady state, the result of uptake and efflux mechanisms leads to an intracellular concentration which is twice or three times the extracellular concentration.

Introduction

Trying to elucidate the mechanisms which could be responsible of the low utilization of L-malic acid during alcoholic fermentation of grape musts, we were interested in the transport of this acid in *Saccharomyces cerevisiae.*

Unfortunately, they are very few informations in the literature on the uptake of organic acids in *S. cerevisiae.* However, as a general rule, it is admitted that non-dissociated organic acids enter yeast cell by simple diffusion [1]. Polar fermentation end products have to utilize a transport system in order to leave yeast cells [2]. Active transport of this type in yeast could be driven by the electrochemical proton gradient across the plasma membrane [2].

Baranowski [3] has discovered a specific proteic uptake system of L-malic acid in *Saccharomyces bailii,* but not in *S. cerevisiae.* This uptake mechanism seems to be partly responsible of the higher L-malic acid degradation by this species [4].

In this paper, we present a preliminary study on the characteristics of the permeation system of L-malic acid in anaerobically grown cells of *S. cerevisiae* at pH 3.0, which is the pH range of enological conditions.

Material and Methods

Yeast strain. The enological strain of *S. cerevisiae* (K1) used for this experimentation was isolated during a continuous vinification of white wine in the south of France.

Culture conditions. Continuous cultures were carried out under anaerobiosis at 28°C, in 300 ml flasks with bubbling CO_2 outlet. The volume of medium in the fermenter was 250 ml. Samples of the outflow were kept at +4°C. Cultures were run at 28°C with permanent magnetic stirring, at a dilution rate of 0.048 h^{-1}.

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

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Growth media. This medium was a synthetic medium containing (in grams per liter of tartaric acid/tartrate buffer $5 \cdot 10^{-4}$M (pH 3.5)): nitrogen source: $(NH_4)_2SO_4$ (1.88); carbon source: L-malic acid (5), glucose (55); vitamins: calcium panthotenate ($15 \cdot 10^{-4}$), inositol ($2 \cdot 10^{-2}$), nicotinic acid ($2 \cdot 10^{-3}$), pyridoxine-HCl ($25 \cdot 10^{-5}$), thiamin-HCl ($25 \cdot 10^{-5}$), biotin ($1 \cdot 10^{-6}$); trace elements: H_3BO_3 ($5 \cdot 10^{-4}$), $CuSO_4 \cdot 5H_2O$ ($2.7 \cdot 10^{-5}$), KI (10^{-4}), $MnSO_4 \cdot H_2O$ ($3 \cdot 10^{-3}$), $NaMoO_4 \cdot 2H_2O$ ($2 \cdot 10^{-4}$), $Fe_2(SO_4)$ ($26 \cdot 10^{-4}$), $ZnSO_4 \cdot 7H_2O$ ($4 \cdot 10^{-4}$), $CoCl_2 \cdot 6H_2O$ ($12 \cdot 10^{-5}$), $NiSO_4 \cdot 6H_2O$ (10^{-4}); mineral salts: KH_2PO_4 (1.5), $MgSO_47H_2O$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.5); anaerobic growth factors; Tween 80 (2.16), sodium oleate ($2 \cdot 10^{-2}$), ergosterol ($6 \cdot 10^{-2}$).

Obtention of resting cells. Yeast cells were harvested at the steady state of the continuous fermenter by centrifugation, and rinsed twice with a Ringer solution without carbon source (NaCl: 8.5 g/l, KCl: 0.25 g/l, $NaHCO_3$: 0.2 g/l, $CaCl_2$: 0.3 g/l). Then, cells were incubated at 28° C in the same solution for 4 h in the presence of 10 μg/ml of cycloheximide. Cells were harvested by centrifugation and resuspended in a new Ringer solution in order to obtain a population of $4 \cdot 10^9$ cells/ml. The Ringer solution has been used as for optimization of cellular volume determination.

Mean cellular volume. Mean cellular volume used for the intracellular concentration estimation was given in μm^3 by a cell counter (Coulter-Counter Coultronics, ZBI model). A verification of mean cell volume values was made using the repartition of naphtol green B in the intercellular volume according to Reuß [5,6].

L-Malic acid uptake study. Incubation medium contained in 8.5 ml final volume: 8 ml of tartaric acid/H_3PO_4 0.1 M (pH 3.0), 80 μl of cycloheximide 1 mg/ml, 200 μl of (U-^{14}C)-labelled L-malic acid (spec. act. 46.4 μCi/μmol) and variable amounts of L-malic acid. After a 10 min temperature equilibration, 200 μl of the suspension containing $4 \cdot 10^9$ resting cells per ml were added. Kinetics of uptake were followed at 0° C by filtering aliquots of the suspension (1 ml) through RAWP millipore filters (pore size 1.2 μm). The filters were dried and solubilized in 1 ml of BTS 450 (Beckman) at 50° C for 12 h, and their radioactivity counted in 15 ml of the scintillation cocktail (ready-solv NA, Beckman) using an Intertechnique SL 3000 counter. Radioactivity of 0.5 ml of the filtrate was directly counted in 15 ml of the scintillation cocktail (ready-solv CP, Beckman).

L-Malic acid efflux study. $4 \cdot 10^9$ resting cells were preincubated for 5 min at 0° C in the same conditions as for L-malic acid uptake study, and rapidly filtered, rinsed with 5 ml of the Ringer solution at 0° C. Then, the filter and the cells retained on it were incubated at 28° C in the incubation medium with or without non labelled L-malic acid. Kinetics of L-malic acid efflux were followed as described in the above paragraph.

Estimation of the non-specific fixation. According to our experimental procedure, in order to estimate the non-specific fixation of a labelled compound on the filter and in the intercellular volume, we used [D-*glucose*^{14}-C]lactose instead of labelled L-malic acid. Lactose is well-known not to enter *S. cerevisiae* cells [7,8].

Results

Kinetics of L-malic acid permeation in resting cells of S. cerevisiae

We had checked previously that L-malic permeation was not an inducible phenomenon in *S. cerevisiae.* However, we utilized for all the experiments cells previously grown in the presence of exogenous L-malic acid in the culture medium.

Kinetics of L-malic acid permeation seem to be very fast, and complex: at 28° C, kinetics seem to

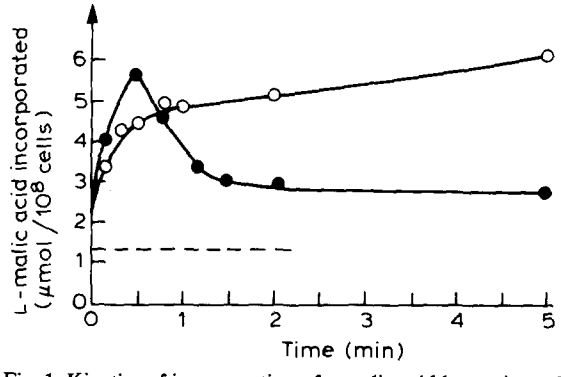

Fig. 1. Kinetics of incorporation of L-malic acid by resting cells of *S. cerevisiae* at two different temperatures (external malic acid concentration = 40 mM; pH 3.0). ●———●, $T = 28$° C; ○———○, $T = 0$° C; (— — —), non-specific fixation.

be the result of two phenomenons, a very rapid uptake (in 30 s–1 min), and an efflux leading to an equilibrium in about 3 min (Fig. 1, closed symbols).

In order to study separately these two phenomenons, we tried to reduce or inhibit the second one: kinetics made at 0 °C allowed us to reduce strongly the efflux phenomenon with no modifications on the initial uptake velocity (Fig. 1, open symbols).

Mechanism of L-malic acid uptake in resting cells of S. cerevisiae

On uptake kinetics made at 0 °C, we were able to define an initial uptake velocity V_i by the amount of L-malic acid entering yeast cells in 30 seconds corrected with the non-specific fixation (Fig. 1, open symbols). This initial velocity V_i appeared to be correlated with external L-malic acid concentration and did not show any saturation phenomenon for high L-malic acid concentrations (Fig. 2). These uptake kinetics seem to be of the first order (Fig. 2), and lead to the establishment of an L-malic acid concentration gradient with the higher concentration inside the cell. At the steady state (3 min), the intracellular L-malic acid concentration is (16–20)-times the extracellular L-malic acid concentration (Fig. 3). Addition of inhibitors of the proton gradient across the cytoplasmic membrane such as dicyclohexylcarbodiimide (DCCD) or 2,4-dinitrophenol in the presence of sodium azide, reduced the increase in intracellular concentration (Fig. 3).

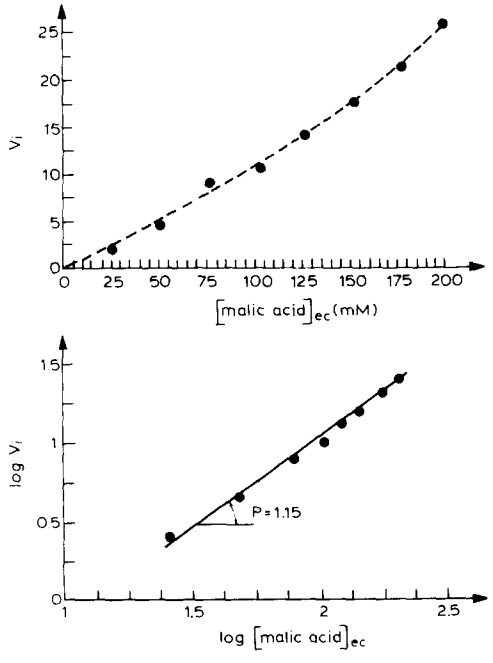

Fig. 2. Characterization of the uptake mechanism of L-malic acid in resting cells of *S. cerevisiae* (pH 3.0; $T = 0$ °C). V_i, initial velocity, i.e. μmol malic acid incorporated in 10^8 cells in 30 s. ec, extracellular.

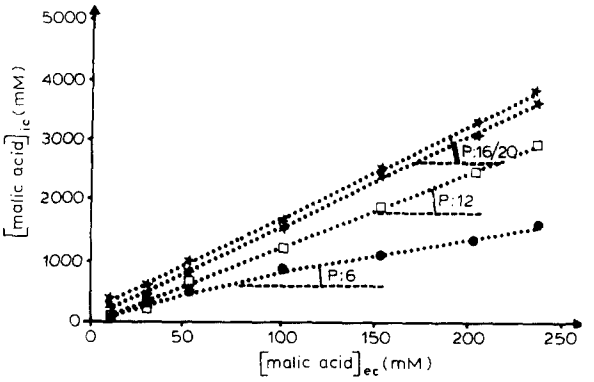

Fig. 3. Effect of inhibitors of the cytoplasmic proton gradient on the uptake mechanism (pH 3.0; $T = 0$ °C) at the steady-state of incorporation (5 min). ★———★, without inhibitors; □·····□, 10^{-4} M; DCCD; ●·····●, (10^{-4} M $NaN_3 + 10^{-4}$ M 2,4-DNP); *P*, average ratio intracellular/extracellular L-malic acid concentration. ic, intracellular; ec, extracellular.

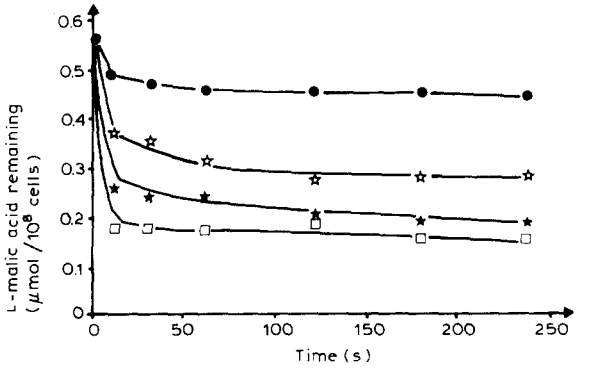

Fig. 4. Effect of temperature on the efflux mechanism of L-malic acid in resting cells of *S. cerevisiae* (pH 3.0; internal malic acid concentration: 130 mM), preloaded at $T = 0$ °C. ●———●, $T = 0$ °C; ☆———☆, $T = 10$ °C; ★———★, $T = 20$ °C; □———□, $T = 28$ °C.

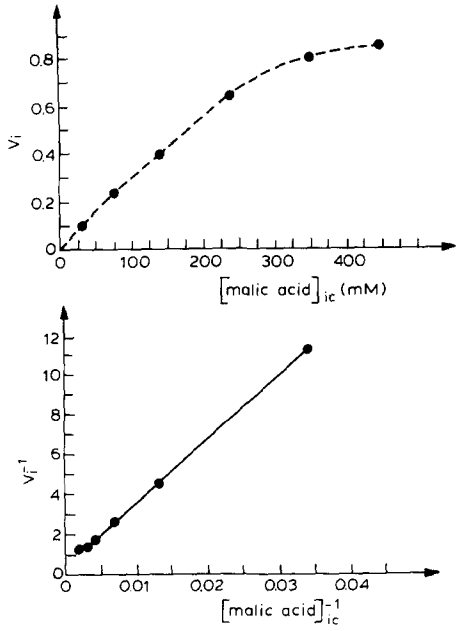

Fig. 5. Characterization of the efflux mechanism of malic acid in resting cells of *S. cerevisiae*. (pH 3.0; $T = 28°C$). V_i, initial velocity, i.e., µmol L-malic acid released from 10^8 cells in 10s.

Mechanism of L-malic acid efflux in resting cells of S. cerevisiae

It appears that L-malic acid efflux at 28° C is very fast, very dependent on temperature, and drastically reduced at 0° C (Fig. 4). It was possible to define an initial efflux velocity by the amount of L-malic acid leaving yeast cells in 10 s (Fig. 4).

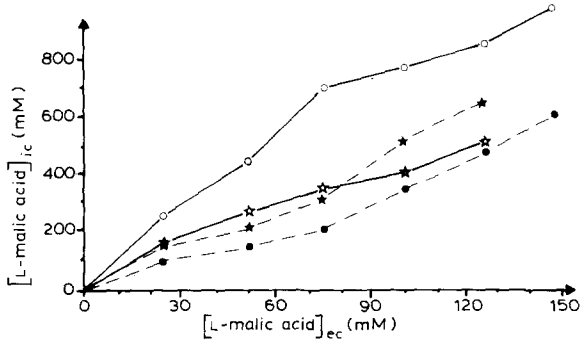

Fig. 6. Effect of glucose concentration on the incorporation of L-malic acid by resting cells *S. cerevisiae*, at the steady-state of incorporation (5 min). (pH 3.0; $T = 28°C$). ●———●, glucose 0 mM; ☆———☆, glucose 34 mM (6 g/l); ★———★, glucose 68 mM (12 g/l), ○———○, glucose 278 mM (50 g/l). ic, intracellular; ec, extracellular).

At high intracellular L-malic acid concentrations, this efflux shows Michaelis-type kinetics with a saturation phenomenon (Fig. 5).

Result of the two phenomenons

At 28° C and at pH 3.0, at the steady state (3–5 min), the result of uptake and efflux mechanisms leads to an intracellular concentration which is twice or three times the extracellular concentration (Fig. 6). Moreover, it seems that addition of glucose during these experiments increases the value of this ratio.

Discussion

The rapidity and the slight sensitivity to temperature of the L-malic acid uptake mechanism leads us to think that this uptake is only the result of a simple diffusion. The L-malic acid gradient concentration created, and the inhibition of this gradient by addition of inhibitors of the proton gradient across the cytoplasmic membrane, suggest that only the non-dissociated L-malic acid enters the cell. Indeed, a difference in pH between the inside and the outside of the cell (external pH: 3.0; internal pH: 5–6) in relation with the first dissociation constant of L-malic acid (pK_{a_1}: 3.45) could be responsible of the mechanism of concentration. The efflux of L-malic acid is very dependent on temperature. Moreover, it presents Michaelis-type kinetics with saturation phenomenon at high substrate concentrations. These results suggest that this L-malic acid efflux is mediated by an active transport system. On the other hand, glucose seems to inhibit this active transport of L-malic acid outside the cell. As a consequence, the intracellular concentration of L-malic acid increases in presence of glucose.

Characteristics and regulation of this possible active transport from the inside to the outside of the cell remain non completely elucidated and require more investigations.

These results are in accordance with the general rules on the uptake of organic acids in the acid pH range, and explain, by the discovery of an active transport for the L-malic acid efflux, results previously obtained on the excretion of this acid during fermentation [9–11].

As a general conclusion, it does not seem that

transport of L-malic acid across the membrane can be a limit for the metabolism of this acid by *S. cerevisiae.*

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