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KINETICS OF Ca^{2+} AND Sr^{2+} UPTAKE BY YEAST

EFFECTS OF pH, CATIONS AND PHOSPHATE

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Summary

The uptake of Ca^{2+} and Sr^{2+} by the yeast *Saccharomyces cerevisiae* is energy dependent, and shows a deviation from simple Michaelis-Menten kinetics. A model is discussed that takes into account the effect of the surface potential and the membrane potential on uptake kinetics.

The rate of Ca^{2+} and Sr^{2+} uptake is influenced by the cell pH and by the medium pH. The inhibition of uptake at low concentrations of Ca^{2+} and Sr^{2+} at low pH may be explained by a decrease of the surface potential.

The inhibition of Ca^{2+} and Sr^{2+} uptake by monovalent cations is independent of the divalent cation concentration. The inhibition shows saturation kinetics, and the concentration of monovalent cation at which half-maximal inhibition is observed, is equal to the affinity constant of this ion for the monovalent cation transport system. The inhibition of divalent cation uptake by monovalent cations appears to be related to depolarization of the cell membrane.

Phosphate exerts a dual effect on uptake of divalent cations: and initial inhibition and a secondary stimulation. The inhibition shows saturation kinetics, and the inhibition constant is equal to the affinity constant of phosphate for its transport mechanism. The secondary stimulation can only partly be explained by a decrease of the cell pH, suggesting interaction of intracellular phosphate, or a phosphorylated compound, with the translocation mechanism.

Abbreviation: DDA^+, dibenzyldimethylammonium.

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Introduction

In comparison to the extensive investigations on the mechanism of monovalent cation transport in yeast, relatively little is known about the uptake of divalent cations. Rothstein et al. [1] demonstrated the presence of a transport system for divalent cations in the yeast cell membrane, with a high affinity for Mg^{2+} and Mn^{2+} as compared to Ca^{2+} and Sr^{2+}. Also other divalent cations can be taken up, like Zn^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+} [2—4]. The transport system appeared to be dependent on phosphate [1,5].

Theuvenet and Borst-Pauwels [6] studied the kinetics of Sr^{2+} uptake by yeast. The uptake kinetics of divalent cations may be affcted by the presence of a negative surface charge. A model was developed for uptake via a single-site mechanism, where the apparent affinity constant K_m was no real constant, but increased with increasing concentrations of Sr^{2+} and progressive reduction of the surface potential. This model also accounted for the inhibition of Sr^{2+} uptake by monovalent cations. In this paper, the kinetics of Ca^{2+} and Sr^{2+} uptake, and the interaction of protons, alkali cations and phosphate with uptake of divalent cations have been studied in more detail.

Materials and Methods

Yeast cells, *Saccharomyces cerevisiae* strain Delft II, with a low phosphate content, were starved under aeration for 20 h. After starvation, the cells (2%, w/v) were incubated (unless otherwise stated) for 1 h in 45 mM Tris/succinate buffer of the desired pH, in the presence of 3% (w/v) glucose at 25°C. Nitrogen was bubbled through the suspension continuously.

The uptake of Sr^{2+} and Ca^{2+} (added to the medium as chloride salts) was studied using ^{89}Sr or ^{45}Ca as a tracer, with the technique described by Borst-Pauwels et al. [7]. Nine successive samples of the yeast suspension were taken within 2 min, washed with ice-cold 50 mM EDTA (adjusted to pH 8.5 with NaOH), filtered and in the case of ^{89}Sr dried with aceton; the radioactivity was determined by means of an end-window Geiger-Müller tube. In the experiments with ^{45}Ca, the filters were not dried and the radioactivity was determined by liquid scintillation analysis. Initial uptake rates were determined from the slopes of the tangents to the uptake curve at zero time.

Cells with a different cell pH were prepared by the following methods [8]: (1) variation of the length of the anaerobic preincubation with glucose from 5 min to 90 min; (2) preincubation of the cells with 1% (w/v) propanol under aerobic conditions, and (3) preincubation of the cells with glucose, and addition of various concentrations of butyric acid (adjusted to the desired pH with Tris) after 54 min of preincubation. In parallel experiments, the cell pH was determined after freezing and boiling the cells [9].

Uptake of [^{14}C]dibenzyldimethylammonium (DDA^+) was determined as described earlier [10].

For preparation of phosphate-rich cells, the following procedure was used. The starved cells (2%, w/v) were resuspended in 45 mM Tris/succinate buffer of pH 5.0 provided with 3% (w/v) glucose, 10 mM Tris-phosphate and 0.1 mM $MgCl_2$. The cells were kept anaerobically by bubbling nitrogen through the sus-

pension. After an incubation period of 1 h, the cells were washed twice and incubated for 20 min in buffer of pH 7.0 provided with 3% glucose under anaerobic conditions. If Mg^{2+} was omitted during the phosphate loading step, the cells lost appreciable amounts of K^+. This is possibly due to depletion of intracellular free Mg^{2+} by phosphate and a consequent inhibition of metabolism [11]. In the control experiment, the cells were incubated in a similar way, but without phosphate and Mg^{2+}.

Results

Uptake of Ca^{2+} and Sr^{2+} by yeast requires the presence of a metabolic substrate. Uptake does not occur immediately after addition of glucose, but only after a lag time of about 1—2 min. A similar phenomenon has been observed with Rb^+ uptake [8]. Addition of Ca^{2+} or Sr^{2+} to a suspension of metabolizing yeast cells did not cause efflux of K^+; in unbuffered suspensions, H^+ efflux could be observed, in accordance with findings of Conway and Gaffney [12]. During the lag period in which no Ca^{2+} and Sr^{2+} is taken up, also no net proton efflux occurs [8,13]. These findings point to a coupling of divalent cation uptake to proton efflux. just as was found for monovalent cation uptake [8]. The uptake kinetics of Ca^{2+} and Sr^{2+} show a deviation from simple Michaelis-Menten kinetics:

$$v = \frac{V \cdot s}{K_m + s} = V - K_m(v/s) \qquad (1)$$

If the data are plotted according to Hofstee [14], a concave plot is obtained for Ca^{2+} uptake (Fig. 1). Sr^{2+} inhibit Ca^{2+} uptake competitively.

The effect of 20 mM butyric acid (adjusted with Tris to pH 5.9) on the

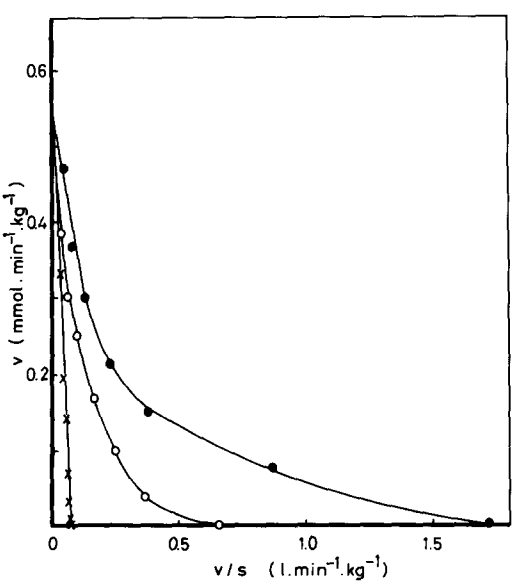
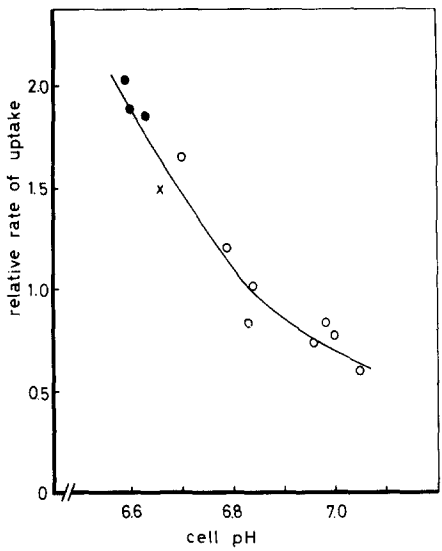


Fig. 1. Hofstee plot of Ca^{2+} uptake; ○, pH 6.0; X, pH 6.0, with 4 mM Sr^{2+} added; ●, pH 7.2.



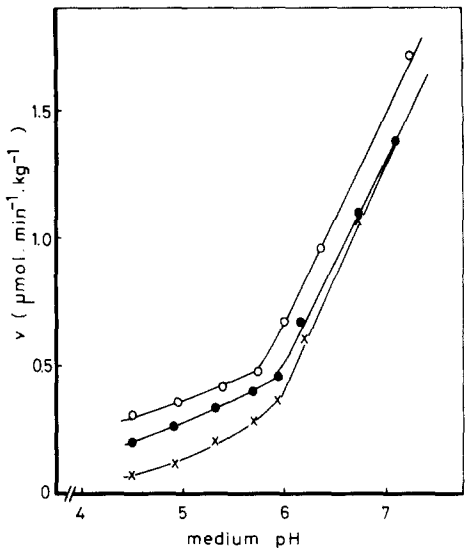
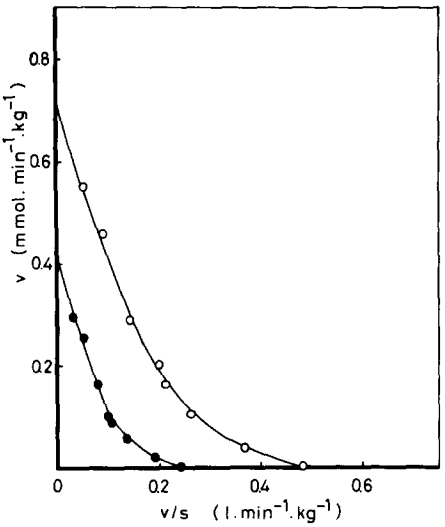


Fig. 2. Effect of the cell pH on the rate of Sr^{2+} uptake at medium pH 5.9; ○, data obtained by preincubating the cells anaerobically with glucose for 5—90 min; X, data obtained by preincubating the cells with propanol under aerobic conditions; ●, data obtained by preincubating the cells with glucose, and adding Tris/butyrate 6 min prior to uptake; data corrected for inhibition by $Tris^{+}$.

Fig. 3. Effect of the medium pH on the uptake of Ca^{2+} (○) and Sr^{2+} (●) at a concentration of 1 μM; X, data for Sr^{2+}, corrected for the effect of the cell pH.

kinetics of Sr^{2+} uptake was studied. Sr^{2+} uptake by these cells (cell pH 6.59) was compared to the control (cell pH 6.96) to which 20 mM Tris-HCl had been added, at a constant medium pH of 5.9. Uptake of Sr^{2+} is stimulated at low cell pH, but the stimulation is independent of the Sr^{2+} concentration. Similar results were obtained with other methods of lowering the cell pH, e.g. using propanol as a substrate under aerobic conditions, or by varying the period of anaerobic preincubation with glucose. A single relationship exists between the cell pH and the rate of Sr^{2+} uptake, independent of the way in which the cell pH is varied (Fig. 2); this resembles the results obtained with Rb^{+} uptake [8].

The effect of the medium pH on Ca^{2+} and Sr^{2+} uptake is shown in Figs. 1 and 3. The pH dependence is similar for Ca^{2+} and Sr^{2+}; uptake is inhibited at low pH. There seems to be an apparent competitive effect of protons, which may, however, not be due to real competition of H^{+} for the transport site, but may be caused by a reduction of the negative surface potential at low pH. Because of the reduction of the surface potential by Ca^{2+}, the effect of a decrease of the medium pH on the surface potential is less at high Ca^{2+} concentrations than at low Ca^{2+} concentrations. As a result, Ca^{2+} uptake will be inhibited by protons predominantly at low Ca^{2+} concentrations, and the concave deformation of the Hofstee plot is reduced at low pH. The effect of the decrease of the surface potential by protons on the rate of divalent cation uptake appears to be strongest at high pH, as is illustrated in Fig. 3; this was also found for Rb^{+} uptake [15]. The apparent biphasic character of the curves in Fig. 3 does not persist after correction for the effect of the cell pH, from the data in Fig. 2 (cf. Fig. 3, curve (x)).



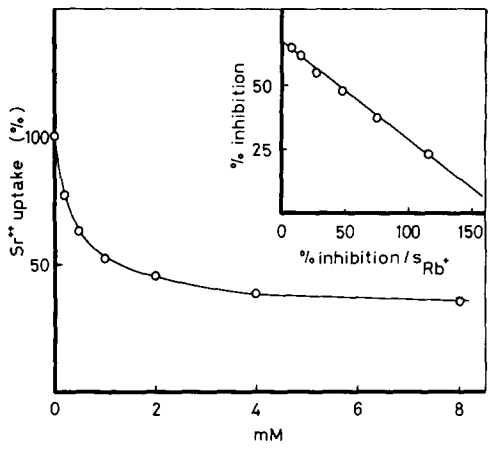
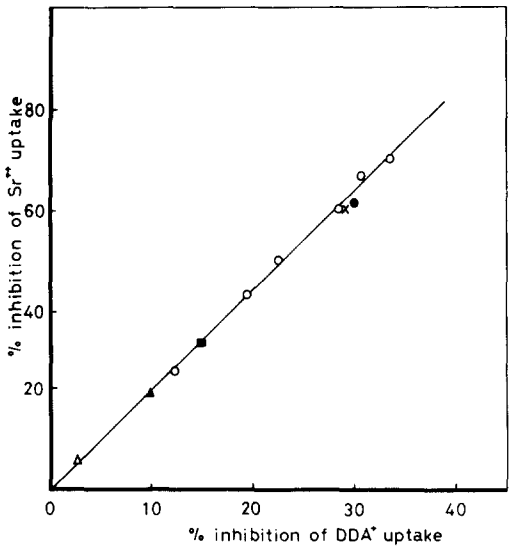


Fig. 4. Effect of Rb^+ on the kinetics of Sr^{2+} uptake at pH 5.9; the range of Sr^{2+} concentrations was 1 μM—10 mM; ○, control; ●, 1 mM Rb^+ added.

Fig. 5. Effect of Rb^+ on Sr^{2+} uptake at pH 5.9. The relative of Sr^{2+} uptake is plotted against the Rb^+ concentration. The concentration of Sr^{2+} was 1 μM. Inset: plot of the relative inhibition of Sr^{2+} uptake versus the quotient of this inhibition and the concentration of Rb^+ (in mM).

The uptake of Ca^{2+} and Sr^{2+} is inhibited by monovalent cations. At pH 5.9, the order of efficiency of inhibition by alkali cations is K > Rb > Cs > Na > Li at an equimolar concentration (0.5 mM) of these ions (see also Fig. 6). At this low concentration, Li^+ is hardly effective. The inhibition of Sr^{2+} uptake by Rb^+ is almost independent of the Sr^{2+} concentration (Fig. 4) and apparently of the non-competitive type. This is also true for the effect of Tris (not shown). However, a study of the dependence of the inhibition of Sr^{2+} uptake by Rb^+ on the Rb^+ concentration showed, that no linear plot of $1/v$ versus the Rb^+ concentration (Dixon plot) could be obtained. The inhibition of Sr^{2+} uptake by Rb^+ shows a saturation curve (Fig. 5). We have investigated, whether this inhibition was correlated with Rb^+ uptake via the transport system for monovalent cations [7,16] by comparing the Rb^+ concentration at which half-maximal inhibition of Sr^{2+} uptake is observed with the affinity constants of Rb^+ for the monovalent cation transport mechanism. In Fig. 5, a plot of the relative inhibition of Sr^{2+} uptake versus the quotient of this inhibition and the Rb^+ concentration is shown. From the slope of this plot the inhibition constant can be determined; the obtained value of 0.38 mM corresponds well with the value of about 0.3 mM for the affinity constant of the Rb^+ transport site. This suggests that the inhibition of Ca^{2+} and Sr^{2+} uptake by monovalent cations is indeed correlated with the uptake of monovalent cations, possibly due to depolarization of the yeast cell membrane. Rb^+ also inhibits the uptake of the lipophilic cation dibenzyldimethylammonium (DDA^+) that has been used as a probe for the determination of the membrane potential in small cells [17,18]. The inhibition of DDA^+ uptake depends in a similar way on the Rb^+ concentration as the inhibition of Sr^{2+} uptake. If the inhibition of Sr^{2+} uptake is plotted against the inhibition of DDA^+ uptake by the same concentration of Rb^+, a



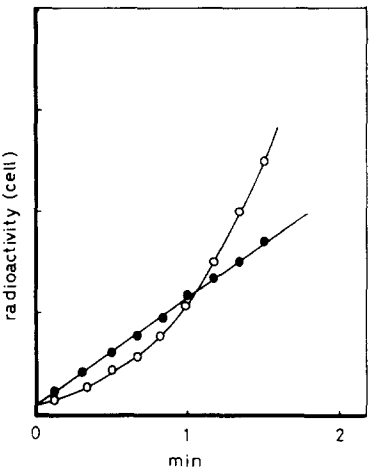


Fig. 6. Correlation between the inhibition of Sr^{2+} uptake and DDA^+ uptake by equal concentrations of: ○, Rb^+; ●, K^+; △, Li^+; ▲, Na^+; ■, Cs^+; X, phosphate. In the case of Rb^+, several concentrations in the range of 0.2—4 mM were tested, of the other alkali ions only 0.5 mM was tested (the inhibition of Sr^{2+} uptake by 0.5 mM Rb^+ was 41%); phosphate was used in a concentration of 0.2 mM. The concentration of Sr^{2+} and DDA^+ was 1 μM.

Fig. 7. Effect of phosphate on Sr^{2+} uptake at pH 5.9; ●, control; ○, 50 μM phosphate added.

straight line through the origin is obtained, on which also the inhibition of Sr^{2+} and DDA^+ uptake by other alkali cations is situated (Fig. 6).

If phosphate is added together with the divalent cation, a dual effect on the uptake of the divalent cation is observed: after an initial inhibition, uptake is markedly stimulated (Fig. 7). In a previous paper we showed [10] that uptake of monovalent cations was inhibited by phosphate, and that this inhibition could be attributed to phosphate uptake by the Na^+-independent phosphate transport system. Also the inhibition of Sr^{2+} uptake by phosphate shows a saturation curve and the phosphate concentration at which half-maximal inhibition is observed (14 μM) compares well with a K_m of 12 μM for phosphate uptake via the Na^+-independent mechanism at this pH [19]. The maximal inhibition of Sr^{2+} uptake by phosphate at pH 5.9 was about 65%. The curve has a similar appearance as the curve shown in Fig. 5. Complex formation between Ca^{2+} or Sr^{2+} and phosphate at pH 5.9 was determined with a Ca^{2+}-selective electrode. It could be calculated that under the experimental conditions applied, at 50 μM phosphate, the free Ca^{2+} or Sr^{2+} concentration was reduced with maximally 1%. Possibly, as was assumed in the case of monovalent cations, the initial inhibition of Sr^{2+} uptake by phosphate is due to a transient depolarization of the cell membrane by phosphate. The data for inhibition by phosphate of Sr^{2+} and DDA^+ uptake are included in Fig. 6.

The secondary stimulation of Sr^{2+} uptake by phosphate was investigated using phosphate-rich yeast cells. These cells accumulate Sr^{2+} much faster than phosphate-deficient cells. A comparison between the kinetics of Sr^{2+} uptake in phosphate-rich and phosphate-deficient cells (Fig. 8) shows that the stimulation

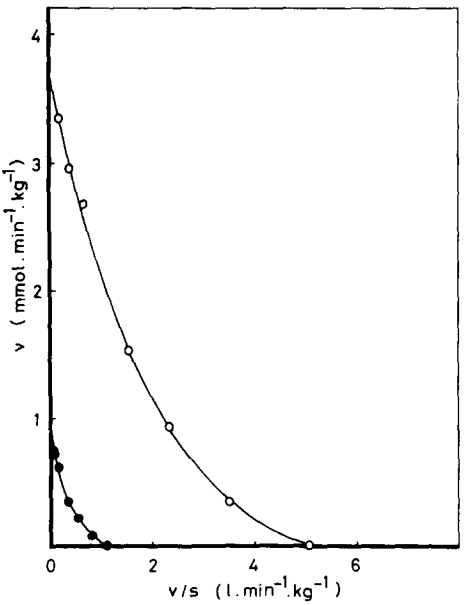


Fig. 8. Kinetics of Sr^{2+} uptake at pH 5.9; ○, phosphate-rich cells; ●, phosphate-deficient cells.

of Sr^{2+} uptake is virtually independent of the Sr^{2+} concentration. The difference between phosphate-rich cells and phosphate-deficient cells may be explained by assuming that either the rate of the translocation step, or the number of primary binding sites is increased.

Phosphate-rich cells differ from phosphate-deficient cells not only in phosphate content, but also in cell pH (6.85 as compared to 7.05). This difference in cell pH may be partly responsible for the increase in the rate of Sr^{2+} uptake, but from Fig. 2 it can be concluded that such a decrease in cell pH could not be responsible for a stimulation of the rate of Sr^{2+} uptake with more than about 50%. This would be insufficient to explain the more than 4-fold stimulation observed in Fig. 8. In addition it was found that the maximal rate of Rb^{+} uptake was only about 20% higher in phosphate-rich cells, which can very well be explained by the difference in cell pH. Experiments, in which DDA^{+} uptake in phosphate-rich cells and phosphate-deficient cells was determined, did not give any indication that an increase of the membrane potential might be responsible for the stimulation of Sr^{2+} uptake.

Discussion

The data presented in this paper confirm earlier observations [6] that the uptake of divalent cations shows a deviation from simple Michaelis-Menten kinetics. Theuvenet and Borst-Pauwels suggested that the concave Hofstee plot might be explained by a single-site uptake mechanism influenced by the negative surface potential [6,20]. In the case of divalent cation uptake, the rate of uptake is then given by

$$v = \frac{V \cdot s}{\frac{K}{y^2} + s} = \frac{V \cdot s}{K_m + s} \qquad (2)$$

where K is the (real) dissociation constant and y is related to the surface potential ψ_0 by $y = \exp(-q\ \psi_0/kT)$, where q is the absolute value of the charge of the electron, k is the Boltzmann constant and T the absolute temperature; for negatively charged membranes $y > 1$ [20,21]. The term 'surface potential' is used in this paper according to the definition given by McLaughlin [21]. V may be a function of the cell pH and the membrane potential. A reduction of the surface potential by addition of divalent cations will result in a decrease of y and an increase in the apparent affinity constant K_m (which is, consequently, no real constant, but depends on the divalent cation concentration). This model is supported by the finding that the concave deformation of the Hofstee plot is decreased by addition of not competing ions at a concentration where the surface potential is affected [6].

The uptake of Ca^{2+} and Sr^{2+} may be described by a model which both K_m and V increase with increasing ion concentrations. The model presented earlier [6] has been refined in one respect: the inhibition by monovalent cations may be described via an effect on the membrane potential rather than by a truly non-competitive inhibition. In addition, hyperpolarization by Ca^{2+} and Sr^{2+} may have to be taken into account. It is as yet difficult to make an estimate of the relative importance of the various effects via surface potential and membrane potential, since the dependence of the surface potential on the divalent cation concentration is not accurately known.

From Fig. 3 it can be calculated that a decrease of the medium pH from 7.2 to 4.5 causes an approximately 16-fold reduction in the rate of Ca^{2+} or Sr^{2+} uptake if a correction for the effect of the cell pH is taken into account. Since v/s at very low values of s is linearly related to y^2 (see Eqn. 2), from these data a 4-fold reduction of y between pH 7.2 and 4.5 can be estimated, if the inhibition of divalent cation uptake at low pH would be exclusively due to a reduction in surface potential. Considering the experimental inaccuracy, this value agrees rather well with the 3-fold reduction in y between pH 7.2 and 4,5, estimated by Theuvenet [15] from data on the effect of the pH on Rb^+ uptake. Similarly the reduction in the rate of Sr^{2+} or Ca^{2+} uptake at other pH values below 7.2 is compatible with the expected reduction in y estimated by Theuvenet [15]. This shows that after correction of the uptake rates for the effect of the cell pH, the dependence of the rate of divalent cation uptake upon the medium pH is determined by only one factor, and it seems possible to explain the inhibition of divalent cation uptake at low pH as a result of the reduction of the surface potential.

In some respects, the uptake of divalent cations resembles closely that of monovalent cations. Both uptake systems appear to require the presence of a metabolic substrate, but uptake occurs only after a lag time, parallel with the lag time observed for proton extrusion [8]. Also the dependence on the intracellular pH is the same for both systems. The similarity may indicate that also the uptake of divalent cations is mediated by a non-carrier mechanism. Both systems of uptake may be coupled to proton efflux, or may depend on a membrane potential generated by a proton pump [8]. The inhibitory effect of phosphate is similar for monovalent and divalent cation uptake [10]. The secondary stimulating effect of phosphate appears, however, to be specific for the divalent cation transport system. The enhancement of Rb^+ uptake in phosphate-rich

cells can be completely explained by the decrease in cell pH, but this is not the case for the stimulation of Sr^{2+} uptake. The enhancement of Sr^{2+} uptake by phosphate differs also from the stimulation of Na^+ uptake by phosphate via the Na^+-phosphate cotransport mechanism [22], in that stimulation is not direct, but only occurs after some time, indicating that phosphate has to be accumulated first before stimulation can occur. In fact, extracellular phosphate ions are not necessary for the stimulation of Sr^{2+} uptake. Also, arsenate ions, which stimulate Na^+ uptake, since they have afinity to the cotransport mechanism, do not enhance Sr^{2+} uptake. It is therefore not likely that the results might be explained by cotransport of phosphate and divalent cations. One may speculate that intracellular phosphate, or a phosphorylated compound, has to combine with the translocation mechanism to make it effective [1,5].

Some earlier observation on divalent cation uptake by yeasts [1—5,23,24] are confirmed by our results. A concave deformation of the Hofstee plot for divalent cations has also been found by Norris and Kelly [4]. The reported non-competitive inhibition of divalent cation uptake by monovalent cations [2] may, however, be only apparently non-competitive, and appears to be caused by depolarization of the yeast cell membrane by the uptake of the monovalent cation. Although the effect of phosphate on divalent cation uptake has been investigated [5], this is the first report of an initial inhibition of divalent cation uptake by phosphate. Since the transient inhibition, possibly due to a transient depolarization, occurs only within the first minute of uptake, the effect may well escape attention if uptake experiments are carried out over longer periods of time.

Acknowledgements

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