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Effect of ethidium bromide and DEAE-dextran on divalent cation accumulation in yeast. Evidence for an ion-selective extrusion pump for divalent cations

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The larger accumulation of Mn^{2+} than of Sr^{2+} in *Saccharomyces cerevisiae* is ascribed to the operation of a specific extrusion pump, presumably a Ca^{2+} pump, which has a higher affinity for Sr^{2+} than for Mn^{2+}. The differences in accumulation levels of Mn^{2+} and Sr^{2+} attained after prolonged incubation are completely abolished in cells of which the plasmamembrane has been permeabilized with the polybase DEAE-dextran under isotonic conditions. In the permeabilized cells Sr^{2+} and Mn^{2+} accumulation levels are attained as for Mn^{2+} in intact cells. It is suggested that the accumulation of divalent cations into the permeabilized cells mainly represents their accumulation into the vacuoles. Also the cationic dye ethidium abolishes the differences in Mn^{2+} and Sr^{2+} accumulation. The dye increases the accumulation of Sr^{2+} but decreases that of Mn^{2+} somewhat. It cannot be distinguished yet whether its action is due to an impairment of the efflux pump or to an increase in the permeability of the plasmamembrane facilitating the divalent cations to be accumulated into the vacuoles. Ethidium does not affect the initial rates of divalent cation uptake into the vacuoles, but it effectively reduces the ultimate accumulation of the divalent cations in the DEAE-dextran permeabilized cells, possibly by competing with the divalent cations for intravacuolar binding sites. Similar results are obtained for the accumulation of Ca^{2+}. It is concluded that the efflux pump enables the yeast cell to regulate accumulation levels of the various divalent cations to different extents.

Introduction

The accumulation of divalent cations in yeast is a complicated and ion-selective process. Ultimate accumulation levels attained for Mg^{2+} or Mn^{2+} are far more greater than for Ca^{2+} or Sr^{2+} [1]. Obviously, the cells are able to adjust intracellular concentrations of divalent cations that meet specific demands of regulatory processes in the cell. In their cytoplasm, for example, relatively high concentrations of Mg^{2+} are needed for the optimal functioning of the plasmamembrane H^+-translocating ATPase [2]. Ca^{2+}, on the other hand, inhibits this to the cell vitally important system and its concentration should therefore be kept much lower than that of Mg^{2+} [2].

In a previous study we showed that the influx system for the divalent cations is rather unspecific [3]. Ion-selectivity in divalent cation accumulation in yeast is therefore caused by other transport systems also involved in this process.

In numerous eukaryotes and prokaryotes active

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Abbreviations: DEAE-dextran, diethylaminoethyl-dextran; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

transport systems that function to lower the cytoplasmic Ca^{2+} concentration have been found [4–6]. In the related fungus, *Neurospora crassa*, the existence of a Ca^{2+} extrusion pump has already been demonstrated [7,8]. In yeast, the lower accumulation of Sr^{2+} or Ca^{2+} as compared to Mg^{2+} or Mn^{2+} is also in accordance with the presence of a specific Ca^{2+} (Sr^{2+}) pump in their plasmamembranes. Indications for the existence of a Ca^{2+}-pumping system located in the yeast plasmamembrane has been obtained by Okorokov and Fuhrmann (personal communication) in studies carried out with plasmamembrane vesicles. Apart from this extrusion system also intracellular transport systems are involved in maintaining a low cytoplasmic Ca^{2+} (Sr^{2+}) concentration. In this connection no important role of the mitochondria is expected, as in vitro experiments revealed that the mitochondria of *S. cerevisiae* are incapable of Ca^{2+} uptake [9]. The vacuoles, on the other hand, contain a transport system that mediates the translocation of Ca^{2+} from the cytoplasm into the vacuoles [10], and as a matter of fact recently the involvement of the vacuoles in maintaining cytoplasmic Ca^{2+} homeostasis has been demonstrated [11].

Thus, apart from an influx system that shows no or hardly any ion-selectively, at least two additional transport systems seem to be involved in divalent cation accumulation into yeast. The present study is aimed at elucidating their role in the accumulation of Mn^{2+}, Ca^{2+} and Sr^{2+} in yeast. In this study we have made use of two cationic agents: the dye ethidium and the polybase DEAE-dextran. The polybase is used to permeabilize the plasmamembrane of the cells. If the treatment is applied under isotonic conditions, the vacuoles of the cells remain intact [12]. Thus in these permeabilized cells accumulation of divalent cations into the vacuoles may be studied without interference of transport systems in the plasmalemma. The cationic dye ethidium is used, as this dye stimulates the uptake of divalent cations in yeast [13]. This dye is greatly accumulated into the cells [13] and we have now examined whether an intracellular interaction of ethidium with the vacuolar and/or plasmamembrane of the yeast cells leads to the stimulation of divalent cation uptake.

Materials and Methods

Preparation of yeast cells. Yeast cells, *Saccharomyces cerevisiae*, strain Delft II with a low phosphate content were starved in distilled water overnight by aeration. After starvation, the cell (2%, w/v) were washed and incubated for 1 h in 45 mM Tris succinate (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 suspension. In this way phosphate-rich cells were obtained with an appreciably higher capacity to accumulate divalent cations than the originally phosphate-poor cells [14,15]. The phosphate-loaded cells were subsequently washed twice with distilled water and resuspended in 45 mM Tris succinate (pH 7.0), provided with 3% (w/v) glucose. After a preincubation of 20 min at 25°C under anaerobic conditions the pH dropped to pH 6.5 and the uptake of divalent cations was examined as described below.

Preparation of acetone-permeabilized cells. After the fermentation period of 20 min (see above) the cells were collected by centrifugation and resuspended in ice-cold acetone. This acetone treatment was repeated for a second time and the permeabilized cells were collected by centrifugation, air dried and stored at 4°C until further use. Acetone-permeabilized ethidium pretreated cells were prepared by the same procedure. After the 20 min fermentation period, the cells were incubated for an additional period of 5 min in the presence of 3 mM ethidium. Subsequently the cells were washed thrice with buffer without the dye prior to the acetone treatment.

DEAE-dextran treatment. The phosphate-loaded cells were resuspended to a density of 2% (w/v) in 45 mM Tris succinate (pH 6.0), provided with 0.7 M sorbitol and 3 mg/ml DEAE-dextran. After a 5 min incubation at 25°C the cells were collected by centrifugation and washed twice with 45 mM Tris Succinate (pH 7.0), provided with 0.7 M sorbitol. subsequently the cells were preincubated 20 min in the same buffer (+sorbitol) but without DEAE-dextran, provided with 3% (w/v) glucose anaerobically and at 25°C. Thereafter, the uptake of divalent cations was measured, also under isotonic conditions, that means under conditions that the

vacuoles remain intact [12].

It was assured that the DEAE-dextran treatment resulted in the complete permeabilization of the plasmamembranes of the yeast cells. An almost complete loss of cellular K^+ occurred if the treatment was applied in the absence of sorbitol, whereas in the presence of sorbitol only 30–40% of the cellular K^+ was lost. K^+ loss was measured with a K^+-sensitive electrode (Philips, G15K) connected to a Philips PW 9421 pH meter.

Divalent cation uptake. Uptake of Mn^{2+} and Sr^{2+} (added to the suspension as chloride salts) was studied using ^{54}Mn and ^{89}Sr, respectively, as a tracer with the technique described by Borst-Pauwels et al. [16]. After the 20 min preincubation in the presence of glucose, radioactively labeled Mn^{2+} or Sr^{2+} was added to the yeast suspension to a final concentration of 1 mM of the divalent cation, unless stated otherwise. Samples of the yeast suspension were washed with ice-cold 50 mM EDTA (adjusted to pH 8.5 with NaOH), filtered and dried with acetone; the radioactivity of ^{89}Sr was determined by means of an end-window Geiger-Mueller tube; in the case of ^{54}Mn a scintillation probe (containing a Na-Tl crystal) was used.

Uptake of Ca^{2+} was measured with a Ca^{2+} electrode (Orion, model 93-20-01) connected to a Pye Model 292 pH meter at room temperature. Further conditions were the same as applied in Mn^{2+} and Sr^{2+} uptake, except that the initial Ca^{2+} concentration was 8 μM and the cell density was 1% (w/v). Because the Ca^{2+}-electrode was sensitive to ethidium, effects of this dye on Ca^{2+} uptake and binding were studied with cells which have been preloaded with the dye and after removal of external dye by repeatedly washing of the cells. The addition of DEAE-dextran to the cell suspension caused a shift in the electrode response. The results were corrected for this shift, which was determined under the Ca^{2+} uptake conditions, except that no yeast cells were present.

Chemicals. Ethidium bromide was purchased from Boehringer, Mannheim, F.R.G. and DEAE-dextran from Pharmacia, Uppsala, Sweden. The chloride salts of ^{89}Sr and ^{54}Mn were obtained from Amersham International, Amersham, U.K. All other chemicals were reagent or analytical grade and obtained from commercial sources.

Results

Effect of nonradioactive Mn^{2+} and Sr^{2+} on the time-course of the accumulation of their radioisotopes

Fig. 1 shows the time-course of the accumulation of a trace amount of ^{54}Mn in the presence of either 1 mM nonradioactive Mn^{2+} or Sr^{2+}. Initially ^{54}Mn was accumulated into the cells at almost the same rate. In the presence of nonradioactive Mn^{2+} the net uptake rate decreased strongly during the first minutes of accumulation (Fig. 1B) but increased again after further incubation to reach values that were almost the same as the initial rate of uptake. In the presence of nonradioactive Sr^{2+} the net uptake rate decreased less pronounced and as a consequence, more ^{54}Mn was retained by the cells than in the presence of 1 mM

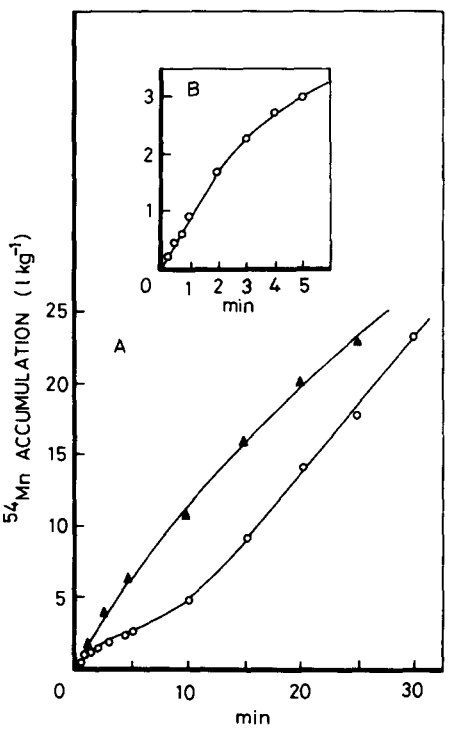

Fig. 1. (A) Time-course of ^{54}Mn accumulation in *S. cerevisiae* at pH 6.5. In the presence of 1 mM $MnCl_2$ (○) or 1 mM $SrCl_2$ (▲). The accumulation is expressed as the quotient of radioactivity accumulated per kg dry weight of cells and the radioactivity of the medium per liter medium. (B) Time-course of ^{54}Mn accumulation in the presence of 1 mM $MnCl_2$ during the first 5 min at extended scale. Each point represents the mean of triplicates.

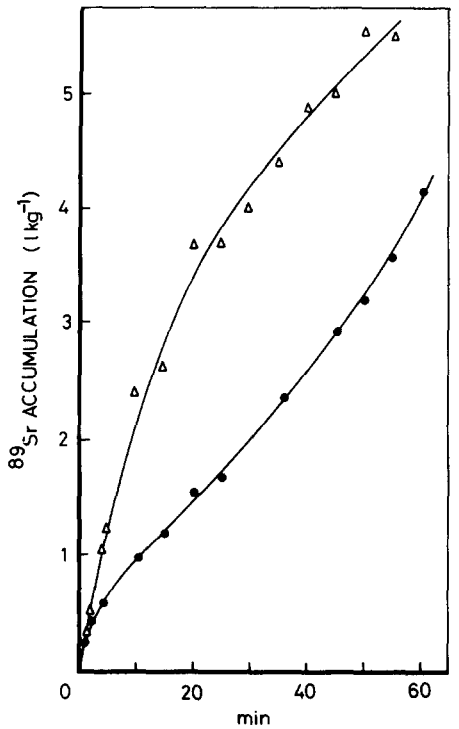

Fig. 2. Time-course of ^{89}Sr accumulation of *S. cerevisiae* at pH 6.5. In the presence of 1 mM $MnCl_2$ (●) or 1 mM $SrCl_2$ (△), see also legend to Fig. 1.

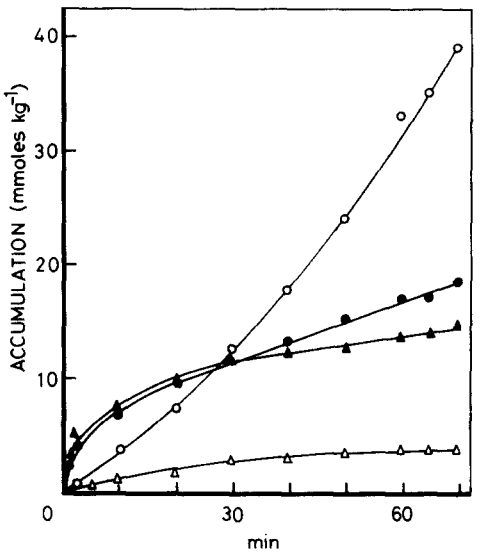

Fig. 3. Effect of ethidium upon the accumulation of Mn^{2+} and Sr^{2+} in *S. cerevisiae* at pH 6.5. ○, Mn^{2+}; △, Sr^{2+}. Open symbols, controls; closed symbols, in the presence of 3 mM ethidium. Uptake was studied at 1 mM concentration of the divalent cations. Each point represents the mean of duplicates.

Mn^{2+}. Furthermore there was no recovery of the ^{54}Mn uptake as occurred in the presence of 1 mM Mn^{2+}. Fig. 2 shows that also the accumulation of a trace amount of ^{89}Sr was higher in the presence of 1 mM nonradioactive Sr^{2+} than in the presence of 1 mM Mn^{2+} and that in either case the ^{89}Sr accumulation levels attained were much lower than those of ^{54}Mn under similar conditions (compare Figs. 1 and 2). Just as found for ^{54}Mn uptake, also the net uptake of ^{89}Sr in the presence of 1 mM Mn^{2+} decreased initially much stronger than in the presence of 1 mM Sr^{2+}. The subsequent increase in the net uptake rate as found in ^{54}Mn accumulation was small and therefore less clear in this experiment but appeared to be reproducible.

Effect of ethidium on Mn^{2+} and Sr^{2+} accumulation

Fig. 3 shows the effect of ethidium on the time-course of the accumulation of Mn^{2+} and Sr^{2+}. Just as was found earlier with a brewing yeast [3] the initial rates of Mn^{2+} and Sr^{2+} uptake in the absence of the dye were approximately the same, 0.48 ± 0.12 and 0.43 ± 0.06 mmol · kg^{-1} · min^{-1}, respectively, but the Mn^{2+} accumulation exceeded that of Sr^{2+} many-fold after an 1 h incubation period.

Ethidium increased the initial rates of both Mn^{2+} and Sr^{2+} uptake and abolished the differences in accumulation after prolonged incubation. The accumulation of Mn^{2+} after 1 h incubation was somewhat reduced as compared to the control. The accumulation of Sr^{2+}, on the other hand, was greatly increased. Based upon these findings we have considered the possibility that the dye either inhibited the extrusion pump or increased the permeability of the plasma-membrane for divalent cations. In either case the dye would facilitate the uptake of the divalent cation into the vacuoles. If this is true, divalent cation accumulation into these organelles should show no, or only little, ionspecificity. This was examined further in DEAE-dextran permeabilized cells.

Uptake of Mn^{2+} and Sr^{2+} in DEAE-dextran-permeabilized cells

Fig. 4 shows that in DEAE-dextran-permeabilized cells under isotonic conditions indeed uptake

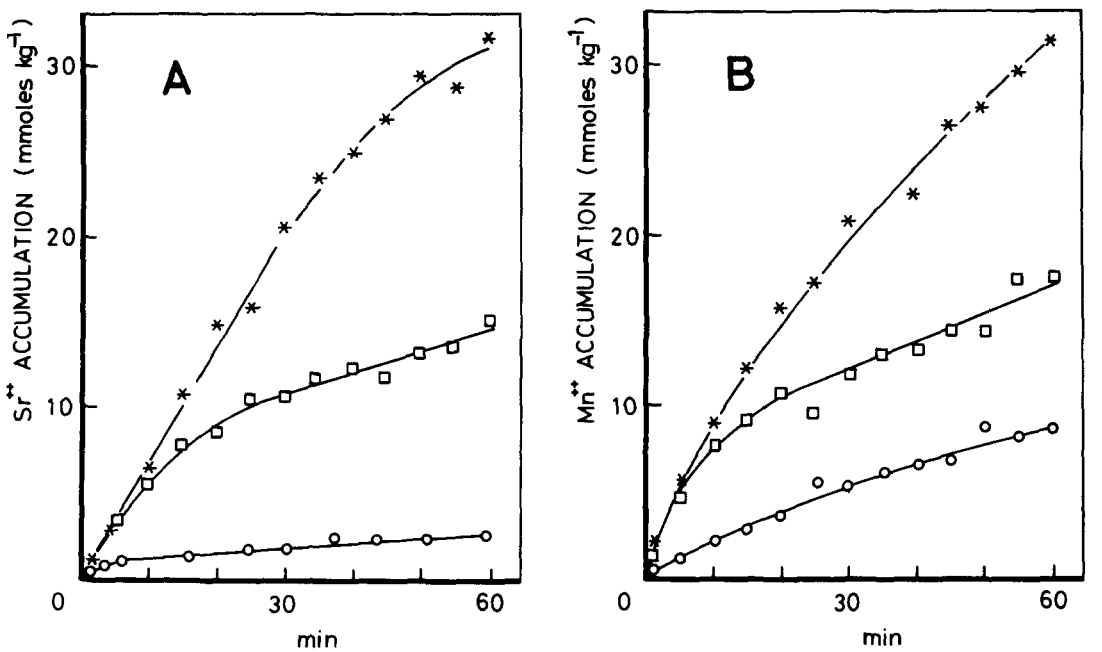

Fig. 4. Accumulation of Mn^{2+} and Sr^{2+} into DEAE-dextran-permeabilized cells in the presence of 0.7 M sorbitol at pH 6.5. Effect of ethidium. (A) Sr^{2+} uptake; (B) Mn^{2+} uptake. ○, in control intact cells; *, □, in DEAE-dextran-permeabilized cells; *, in the absence and, □, in the presence of 3 mM ethidium. See legend to Fig. 1.

of divalent cations occurred. For a proper comparison of results also the uptake of Mn^{2+} and Sr^{2+} by intact cells was studied in the presence of 0.7 M sorbitol. Sorbitol appeared to reduce the uptake of both cations appreciably, as follows from a comparison of the data in Figs. 3 and 4. Not only the initial rates of uptake, but also the accumulation levels found after 1 h incubation were lowered. After destruction of the barrier properties of the plasmamembrane with DEAE-dextran, accumulation of the divalent cations was greatly increased, showing the ability of the vacuoles to accumulate the divalent cations to a great extent. Contrary to the finding in intact cells, no selectivity in divalent cation uptake was observed in the DEAE-dextran-permeabilized cells.

Ethidium had no effect upon the initial rates of divalent cation uptake by the DEAE-dextran treated cell. The ultimate accumulation of Mn^{2+} and Sr^{2+} was, however, strongly reduced. Apparently ethidium did not impair the vacuolar divalent cation transporter but reduced the capacity of the vacuoles to accommodate the divalent cations.

Effect of DEAE-dextran on Ca^{2+} uptake into ethidium-pretreated yeast cells

Fig. 5A shows that addition of metabolizing control cells to the Ca^{2+}-containing solution, resulted in an immediate reduction in the Ca^{2+} concentration, which should be ascribed to binding of Ca^{2+} to the outerside of the cells. No net Ca^{2+} uptake was detectable in these cells, whereas in using $^{45}Ca^{2+}$ as a tracer under the uptake conditions applied an appreciable accumulation of radioactive Ca^{2+} into the cells was measured (data not shown). Apparently influx and efflux of Ca^{2+} were in balance under these conditions. A great net Ca^{2+} uptake was, however, observed in the ethidium-pretreated cells. Permeabilization of the plasmalemma with DEAE-dextran further increased the rate of net Ca^{2+} influx. The permeabilization by the polybase caused Ca^{2+} to be accumulated into the control cells at almost the same rate as in ethidium-pretreated cells. The subsequent accumulation of Ca^{2+} into the vacuoles, however, was greatly reduced in the ethidium-loaded cells.

Obviously, ethidium reduced the accumulation

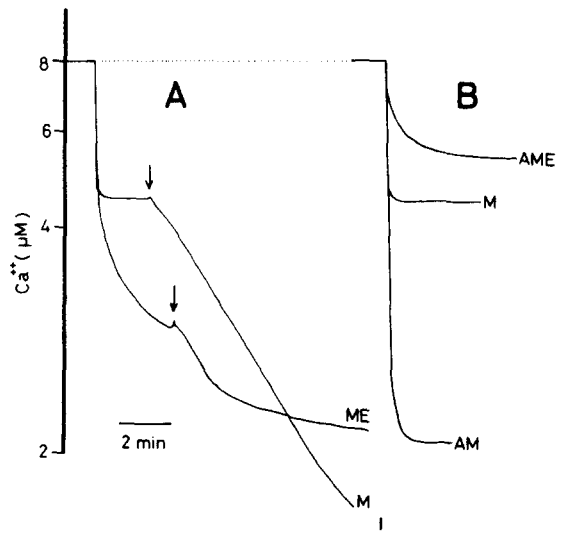

Fig. 5. Effect of DEAE-dextran upon Ca^{2+} uptake in control cells and ethidium-pretreated yeast cells and the binding of Ca^{2+} to the cells after their complete permeabilization with acetone. (A) Ca^{2+} uptake. 1 ml of a 10% (w/v) yeast suspension was added to 10 ml 45 mM Tris succinate (pH 6.5) containing 8 μM $CaCl_2$, 0.77 M sorbitol and 3% (w/v) glucose at zero time. The arrow indicates the addition of DEAE-dextran in a final concentration of 3 mg/ml. M, metabolizing control cells; ME, metabolizing ethidium-pretreated cells. (B) Ca^{2+} binding, at the same cell density as in (A). AM, acetone-permeabilized cells; AME, acetone-permeabilized ethidium-pretreated cells. Ca^{2+} was measured with a Ca^{2+}-electrode.

of Ca^{2+} (Fig. 5) and also of Mn^{2+} and Sr^{2+} (see Fig. 4) into the vacuoles. The possibility that this was due to a competition of ethidium and the divalent cations for binding to intravacuolar binding sites of divalent cations was further examined. Fig. 5B shows that the binding of Ca^{2+} in acetone-permeabilized cells was greatly increased as compared to in intact metabolizing yeast cells. In the ethidium-pretreated cells the binding was even lower than that to the intact cells. The difference in Ca^{2+} levels measured with intact cells and acetone-permeabilized cells corresponded to the amount of Ca^{2+} bound inside the cells. The higher Ca^{2+} level measured with acetone-permeabilized ethidium-pretreated cells than with intact cells shows that ethidium completely prevented this intracellular binding of Ca^{2+} and, in addition, reduced the binding of the divalent cation to the outer surface of the cells.

Discussion

In agreement with recent results of Eilam et al. [11] our present results demonstrate the ability of the vacuoles to accommodate large amounts of divalent cations. In cells permeabilized with DEAE-dextran accumulation levels of divalent cations are attained comparable with that of Mn^{2+} in intact cells. Permeabilization of the cells with this polybase under isotonic conditions keeps the vacuoles intact [12] and directly accessible to the divalent cations. The great increase in initial rates of uptake on destructing the plasmalemma indicates that this membrane is the rate-limiting barrier for divalent cation uptake. Little or no differences are found in the uptake rates and the ultimate accumulation of the two divalent cations in the permeabilized cells. The large difference in Mn^{2+} and Sr^{2+} accumulation by intact cell might be attributed to the operation of a Sr^{2+} (Ca^{2+}) extrusion system in the plasmalemma of the cells. Nieuwenhuis et al. [3] already earlier showed that the decrease in the net rate of Sr^{2+} uptake during Sr^{2+} accumulation is due to an extrusion of Sr^{2+} and not to a decrease in influx. This extrusion system however, appears also able of extruding Mn^{2+} as may be deduced from the data in Fig. 1. The net uptake rate of ^{54}Mn in the presence of 1 mM nonradioactive Mn^{2+} is reduced appreciably during the first minutes of accumulation into the cells. At pH 7.0 the net uptake rate even decreases to almost zero and during several minutes the further accumulation of ^{54}Mn remains arrested (data not shown). Apparently in that period ^{54}Mn influx becomes progressively counterbalanced by an efflux of this cation. If this uptake is studied in the presence of 1 mM Sr^{2+} instead of 1 mM nonradioactive Mn^{2+}, ^{54}Mn uptake decreases less pronounced, showing that cellular Sr^{2+} inhibits the ^{54}Mn extrusion more effectively than Mn^{2+} accumulated into the cell despite the fact that Mn^{2+} is accumulated more rapidly and to much higher extents than Sr^{2+}. This indicates that Sr^{2+} has a higher affinity for the extrusion system than Mn^{2+}. Also data on the ^{89}Sr uptake (Fig. 2) are compatible with this notion. ^{89}Sr uptake is higher in cells that accumulate 1 mM nonradioactive Sr^{2+} than in cells accumulating 1 mM Mn^{2+}. Furthermore, under both conditions accumulation

of Sr^{2+} is appreciably lower than of ^{54}Mn.

After prolonged accumulation of nonradioactive Mn^{2+} the net rates of both ^{54}Mn and ^{89}Sr uptake increase again. As shown previously an increase in influx rate is not involved [3]. Apparently the extrusion of the cations becomes gradually impaired at the higher intracellular Mn^{2+} concentrations. It may be hypothesized that an allosteric interaction of Mn^{2+} with the extrusion system is involved or that the energization of this transport system becomes impaired at high cytoplasmic Mn^{2+} concentrations. Another possibility could be that Mn^{2+} releases vacuolar Ca^{2+} into the cytoplasm that causes a competitive inhibition of Mn^{2+} and Sr^{2+} extrusion. It was, however, out of the scope of the present study to solve this particular problem.

There is little doubt that also yeast cells have a transport system in their plasmalemma that extrudes Ca^{2+}. Functionally inverted plasmamembrane vesicles isolated from our yeast catalyze Mg^{2+}/ATP-dependent Ca^{2+} uptake (Peters, unpublished results). To exert a physiological role this transporter should have a high affinity for Ca^{2+} (Sr^{2+}) and no or a much lower affinity for Mg^{2+} (Mn^{2+}). The findings described above are compatible with this notion.

Compounds that inhibit the extrusion of the divalent cations or permeabilize the plasmalemma and leave the vacuoles intact are expected to increase the initial rates of divalent cation uptake and to abolish the difference in Mn^{2+} and Sr^{2+} accumulation observed in intact yeast cells. An example of a permeabilizing compound is the non-penetrating [11,12] polybase DEAE-dextran. The penetrating cationic dye ethidium also abolishes the differences in divalent cation accumulation in yeast (Fig. 3). This dye is known to stimulate Ca^{2+} accumulation in yeast and to provoke a concomitant loss of cellular K^+ [17]. Its efficacy to cause K^+ loss appears to be related to the amount of dye taken up by the cells [17,18]. Both the accumulation of the dye and the dye provoked K^+ loss are greater in metabolizing cells than in non-metabolizing cells. It is therefore likely that its effect on cation fluxes in yeast is exerted by an intracellular action of the dye. The finding that Ca^{2+} accumulation is still stimulated, despite the fact that the external dye has been washed away (Fig. 5A) agrees with this notion. The mechanism of this stimulation is, however, still unclear. There are two possibilities. Intracellular ethidium may act as a permeabilizing agent like DEAE-dextran does from the outerside of the cells. The other possibility would be that cytoplasmic ethidium impairs the extrusion of the divalent cations. This may be expected if their extrusion is mediated by a carrier that exchanges the divalent cations for H^+ as is found for Ca^{2+} extrusion in *Neurospora crassa* [7,8]. Ethidium namely causes an acidification of the cells [17] that would decrease this divalent cation-H^+ exchange and result in an enlarged accumulation of Ca^{2+} and Sr^{2+}.

The present study clearly demonstrates that the differential accumulation of divalent cations reflects their differential compartmentation in the cells which is made possible by the specific extrusion of Sr^{2+} and Ca^{2+} by the pump in the plasmalemma of the yeast cells, preventing an appreciable accumulation of Sr^{2+} and Ca^{2+} into the vacuoles.

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