The Specific Uptake of Manganese in the Yeast *Candida utilis*

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(Received 9 December 1985; revised 21 February 1986)

Uptake of ^{54}Mn from 10 nM- Mn^{2+} was shown to be both energy- and pH-dependent. Analysis of the uptake kinetics revealed an apparent half-saturation constant, K_i , of 16.4 nM- Mn^{2+} and a maximal rate of transport, $V_{\max}$, of 1.01 nmol Mn^{2+} (g dry wt) $^{-1}$ min $^{-1}$. Mn^{2+} uptake was highly specific, being unaffected by 100-fold molar excess of Mg^{2+} , Zn^{2+} , Ca^{2+} , Co^{2+} , Ni^{2+} and Cu^{2+} ; however, uptake was inhibited 30 to 40% by 1000-fold molar excess of Mg^{2+} , Zn^{2+} , Ca^{2+} , Co^{2+} and Ni^{2+} . Zn^{2+} competitively inhibited Mn^{2+} uptake, the K_i value being approximately 500-fold greater than the K_i for Mn^{2+} . Efflux studies indicated that metabolic exchange of ^{54}Mn occurred to a small extent. Cellular Mn^{2+} contents remained relatively constant during growth in batch culture. The Mn^{2+} transport system observed appears to be analogous to the specific metal transport systems reported in bacteria.

INTRODUCTION

Manganese is required by yeasts at trace concentrations for optimal growth and fermentation (Jones & Greenfield, 1984). A number of reports detailing the accumulation of manganese in yeasts have appeared [Rothstein *et al.*, 1958; Furhmann & Rothstein, 1968; Okorokov *et al.*, 1977; Parkin & Ross, 1985*a*; see also Borst-Pauwels (1981) for a review]. Mn^{2+} is transported by an energy-dependent system primarily responsible for Mg^{2+} transport but which exhibits a broad specificity to other bivalent metal ions including Co^{2+} , Zn^{2+} and Ni^{2+} .

If other bivalent trace elements are exclusively taken into cells simply as alternative low-affinity substrates of the Mg^{2+} -transporter, the problem arises as to how a cell might obtain essential elements when, as in normal growth medium, there is a great excess of Mg^{2+} (often up to 10000-fold). Silver & Jasper (1977) suggested that in bacterial systems, for each essential metal ion, there might exist a specific transporter. Indeed, a number of studies in *Escherichia coli* have demonstrated, in addition to a non-specific Mg^{2+} -transporter (Webb, 1970; Park *et al.*, 1976), the presence of two separate transport systems for Mg^{2+} (Park *et al.*, 1976) and Mn^{2+} (Silver & Kralovic, 1969). These energy-linked transporters exhibited both high affinity and high specificity and were generally detected only in cells grown with very low metal concentrations, which presumably induce such pathways.

Similarly, highly specific active transport systems for Mn^{2+} have been observed in *Bacillus* spp. (Silver & Jasper, 1977), *Rhodopseudomonas capsulata* (Jasper & Silver, 1978), *Staphylococcus aureus* (Perry & Silver, 1982) and *Lactobacillus plantarum* (Archibald & Duong, 1984). In eukaryotic cells, Silver & Jasper (1977) reported specific manganese transporters in *Euglena gracilis* and in human KB cells.

Failla *et al.* (1976) demonstrated the presence of a highly specific Zn^{2+} transporter in the yeast *Candida utilis*. This contrasts with the relatively non-specific bivalent cation transport system observed in *Saccharomyces cerevisiae* (Borst-Pauwels, 1981). Parkin & Ross (1985*b*) suggested that copper influx in *C. utilis* does not occur via a general Mg^{2+} transporter but is facilitated by a separate and much more specific mechanism. These two studies in *C. utilis* used metal concentrations around 1 $\mu\text{mol l}^{-1}$.

Abbreviations: DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

Previous studies of Mn^{2+} uptake in *Saccharomyces* spp. (Norris & Kelly, 1977; Okorokov *et al.*, 1979; Niewenhuis *et al.*, 1981) have generally used media containing relatively high Mn^{2+} concentrations (in the range 0.2 mM to 3 mM). Under these conditions saturation of specific high-affinity transport systems may occur thus masking their detection. Hence previous authors, in studying the uptake of Mn^{2+} in yeasts, may well have observed Mn^{2+} transport via the low-affinity Mg^{2+} transport system. This view is supported by studies of Mn^{2+} uptake in *C. utilis* (Parkin & Ross, 1985a): kinetic analysis of Mn^{2+} transport from Mn^{2+} concentrations up to 50 μ M revealed competitive inhibition by Mg^{2+} , Co^{2+} and Zn^{2+} with similar affinity constants for both Mn^{2+} and Mg^{2+} .

In the present investigation, using the radionuclide ^{54}Mn , the possibility that a specific mechanism exists for the transport of manganese in *C. utilis* was investigated.

METHODS

Organism and growth. *Candida utilis* 708 was obtained from the National Collection of Yeast Cultures, Norwich, UK. Cultures were maintained at 4 °C on MYGP slopes of the following composition (g l⁻¹): malt extract (Oxoid), 3; yeast extract (Oxoid), 3; glucose, 10; mycological peptone (Oxoid), 5; agar (Oxoid, no. 3), 12. Cells were grown in the medium described by Ross (1977) except that the vitamins were replaced with 0.1% yeast extract. Medium (400 ml) in a 1 litre shake-flask was inoculated with 0.1 ml of a 24 h starter culture; subsequent growth was at 30 °C on an orbital shaker (200 cycles min⁻¹).

Uptake of Mn^{2+} . Cultures were harvested during mid-exponential phase after approximately 18 h growth when the culture had reached a dry weight of 0.3 mg ml⁻¹ as determined from a curve relating OD₆₆₀ to dry weight. Dry weights were determined by filtering known volumes of cell suspension through preweighed membrane filters and drying for 16 h at 105 °C. Cells were collected by centrifugation (4000 g for 5 min), washed twice with distilled water and resuspended in buffer containing 50 mM-MES and 50 mM-glucose, pH 5.5, at 30 °C. The cell density was adjusted to approximately 0.1 mg ml⁻¹ so that only a small fraction of the total added Mn^{2+} was taken up during the assay. Cells were allowed to equilibrate in this medium for 30 min before adding Mn^{2+} . For experiments measuring ^{54}Mn accumulation in batch culture, the medium of Failla *et al.* (1976) was used, supplemented with trace elements as follows (g l⁻¹): $MgSO_4 \cdot 7H_2O$, 0.74; $CaCl_2 \cdot 6H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.00175; $CuSO_4 \cdot 5H_2O$, 0.0001. The inhibitors carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP) were added 15 min before the Mn^{2+} . Solutions of the chloride salts of Mg^{2+} , Zn^{2+} , Co^{2+} , Cu^{2+} , Ni^{2+} and Ca^{2+} were added 1 min before the addition of Mn^{2+} to give a final volume of 20 ml in 100 ml flasks on a shaking water bath at 30 °C. $^{54}MnCl_2$ [Amersham; 0.275 μ Ci ml⁻¹ (10.175 kBq ml⁻¹)] was added to give a final ^{54}Mn concentration of 10 nM unless otherwise stated. At intervals, 1 ml samples were removed and filtered through 0.45 μ m filters (Whatman), and then washed rapidly with 10 ml of a chilled solution of 100 μ M non-radioactive $MnCl_2$ in order to remove $^{54}Mn^{2+}$ adsorbed on to the cell walls (Borst-Pauwels, 1981). Radioactivity on the filters was then counted in a Packard Auto-Gamma scintillation spectrometer. All glassware used in uptake experiments was acid-washed to minimize metal contamination. Chemicals used were of high-purity AnalaR grade; MES and CCCP were obtained from Sigma.

RESULTS

Energy dependence of uptake

Mn^{2+} uptake by cells harvested from exponentially growing cultures proceeded almost linearly at 30 °C in the presence of glucose (Fig. 1). However, at 4 °C or in the absence of glucose, uptake was not observed. Similarly, in the presence of the metabolic inhibitors CCCP and DNP, no uptake occurred. Thus it appears that Mn^{2+} transport is an energy-requiring process. Non-specific binding of the metal to anionic species on the cell surfaces occurred almost instantaneously, reaching about 7% of the total Mn^{2+} bound after 20 min. This may well reflect the efficacy of the cell washing procedure when compared to a value of nearly 20% Mn^{2+} bound in 20 min in the same species using 2 mM- $CaCl_2$ as a washing solution (Parkin & Ross, 1985b).

pH dependence of uptake

Mn^{2+} transport was highly dependent upon the pH of the suspending medium. At pH 5.5 cells accumulated 5.65 ± 0.36 nmol Mn^{2+} (g dry wt)⁻¹ over 20 min from 10 nM- Mn^{2+} (values given are the mean $\pm$ SE of three experiments). As the pH was decreased to 3.0 Mn^{2+} uptake decreased

Fig. 1

Fig. 2

Fig. 1. Energy dependence of Mn^{2+} uptake. Mn^{2+} accumulation from 10 nM- Mn^{2+} in the presence (●, ▲, ■, ○) and absence (△) of glucose, and in the presence of 100 μ M-CCCP (■) or 2 mM-DNP (○); uptake at 4 °C. Values shown are the mean $\pm$ SE of three experiments (for clarity, only the maximum SE bars have been included for uptake in the absence of glucose, at 4 °C and in the presence of CCCP or DNP).

Fig. 2. Mn^{2+} efflux from Mn^{2+} preloaded cells. Mn^{2+} -loaded cells were suspended at a density of 0.5 mg dry wt ml⁻¹ in Mn^{2+} -free buffer containing 50 mM-glucose and incubated at 30 °C in the absence (□, ○) and presence (●, ▲) of 100 μ M-CCCP, with the addition of 1 mM- $MnCl_2$ (arrow) at 22 min (▲, ○). Values shown are the mean $\pm$ SE of three experiments.

Table 1. *Effect of divalent cation competition on Mn^{2+} uptake*

Values shown (mean $\pm$ SE of three experiments) are for uptake after 20 min from 10 nM- Mn^{2+} in the presence of competing cations as indicated. The control value for uptake in the absence of any competing cation was 5.16 ± 0.38 nmol Mn^{2+} (g dry wt)⁻¹. ND, Not determined.

Competing cation	Mn^{2+} uptake (percentage of control) in the presence of:	
	1 μ M Competing cation (100-fold excess)	10 μ M Competing cation (1000-fold excess)
Mg ²⁺	90.6 $\pm$ 3.7	73.6 $\pm$ 13.8
Zn ²⁺	96.4 $\pm$ 13.0	57.4 $\pm$ 5.7
Co ²⁺	108.7 $\pm$ 14.6	70.3 $\pm$ 9.8
Ni ²⁺	107.5 $\pm$ 13.4	61.8 $\pm$ 3.5
Ca ²⁺	98.5 $\pm$ 9.0	91.9 $\pm$ 8.5
Cu ²⁺	102.6 $\pm$ 1.4	ND

sharply to 0.29 ± 0.02 nmol Mn^{2+} (g dry wt)⁻¹. At pH 7.0 and above a massive increase to over 15 nmol Mn^{2+} (g dry wt)⁻¹ cells was recorded. It is probable that at these high pH values insoluble complexes of manganese hydroxide are precipitated on the cell surfaces.

Specificity of uptake

Table 1 shows the effect of bivalent cation competition on ⁵⁴Mn uptake. Oxygen uptake studies revealed that, with the exception of Cu²⁺ at 10 μ M, none of the ions were toxic at the concentrations used here (data not shown). Uptake was unaffected by 100-fold molar excess of other bivalent cations; in the presence of 1000-fold excess of Mg²⁺, Zn²⁺, Co²⁺ and Ni²⁺ uptake was still 57 to 74% of the original value, while 1000-fold excess Ca²⁺ exerted a somewhat less inhibitory effect.

Fig. 3. ^{54}Mn accumulation during growth in batch culture. ▲, Cell density of culture; ■, total ^{54}Mn accumulated by culture; ●, cellular ^{54}Mn content. Values shown are the mean $\pm$ SE of three experiments.

Uptake kinetics

In studies of the uptake of metal ions from very low external concentrations, it is important to assess the extent to which metal complexation to glassware and media components may affect subsequent kinetic evaluations. The MES buffer chosen has negligible metal-complexing capacity (Good *et al.*, 1966) and has been previously used in metal uptake experiments (Parkin & Ross, 1985*b*). Prolonged uptake experiments, using a high cell density suspension, recovered almost 100% of added radioactivity, demonstrating that virtually all the ^{54}Mn was available to the cells (data not shown).

The uptake kinetics were determined from the linear rates of uptake over 20 min from Mn^{2+} concentrations in the range 2.5 to 50 nM. If the mean data of three separate experiments are plotted by the Lineweaver-Burk method, and the resultant straight line is fitted by the least-squares method, the intercepts on the y- and x-axes give estimates of the maximal rate of transport, $V_{\max}$, and apparent half-saturation constant, K_t , respectively. The degree of uncertainty in these estimates can be quantified by calculating the standard error of estimate (SEE) giving a mean value for K_t of 16.4 nM- Mn^{2+} (range 12.3 to 24.4 nM) and for $V_{\max}$ of 1.01 nmol (g dry wt) $^{-1}$ [range 0.76 to 1.50 nmol (g dry wt) $^{-1}$ min $^{-1}$]. Since Zn^{2+} , at 10 μM , appeared to inhibit uptake by over 40%, Mn^{2+} uptake was examined over the range 2.5 to 50 nM in the presence of 100 μM - Zn^{2+} . The initial uptake rates were plotted as before to determine the nature of this inhibition (data not shown). Inhibition was competitive giving an inhibition constant (K_i) value of 8.0 μM (range 7.3 to 8.9 μM) as calculated from the slope of the line showing inhibition.

^{54}Mn efflux

To investigate the possibility of a metabolic efflux system cells were pre-loaded with ^{54}Mn and resuspended in ^{54}Mn -free buffer containing glucose. When 1 mM- MnCl_2 was added at 22 min 25% of the cellular Mn^{2+} was released into the medium during the following 13 min. This release was markedly reduced in the presence of CCCP (Fig. 2).

^{54}Mn accumulation during growth in batch culture

This is shown in Fig. 3. The cell Mn^{2+} content remained fairly constant throughout the growth period. It should be noted that the medium was closely maintained at pH 6.0 during this time by the addition of KOH or HCl. Failure to do so resulted in a drop in pH to 3.8 over 3.5 h during late-exponential phase coupled with a 50% reduction of the cell Mn^{2+} . This dilution effect may well be due to reduced Mn^{2+} uptake under low pH conditions during culture growth.

DISCUSSION

Dependence upon a cellular energy source is a fairly well-documented feature of metal cation transport systems in micro-organisms (Norris & Kelly, 1977; Ross, 1977; Borst-Pauwels, 1981;

Gadd & White, 1985). The lack of ^{54}Mn influx in the presence of the uncoupling agents CCCP and DNP is indicative of a metabolically dependent process. In our system, at a cell density of 10^7 cells ml^{-1} , occupying a packed cell volume of approximately 0.1% (v/v) of the medium (assuming intercellular spaces to be minimal), 10^7 cells took up 1.53×10^{-3} nmol during 20 min incubation in 10 nM- ^{54}Mn . Assuming all cellular Mn^{2+} to be in an unbound, ionic form, this represents an uphill transport concentration gradient of approximately 180:1 (inside:outside). However, it is unlikely that all the Mn^{2+} is present in an osmotically free form. Okorokov *et al.* (1977) estimated that 78% of the total Mn^{2+} was bound to cellular components. Other reports suggest that bivalent cations may be compartmentalized within cell vacuoles and sequestered to polyphosphates (Borst-Pauwels, 1981). Mn^{2+} uptake, at the Mn^{2+} concentrations studied, appears to be via a highly specific mechanism, unlike the low affinity divalent cation transporter previously observed (Parkin & Ross, 1985a). The degree of cation specificity is not as great as that observed by Silver *et al.* (1970) for Mn^{2+} transport in *E. coli*, which was largely unaffected by 10^6 -fold molar excess of Mg^{2+} , Ca^{2+} or Sr^{2+} . Nevertheless the present yeast system exhibits considerable discrimination for Mn^{2+} in the presence of 1000-fold excess of Mg^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+} or Ca^{2+} .

As far as we are aware, the kinetic data available in the literature for Mn^{2+} uptake in yeasts pertains to uptake via the Mg^{2+} transporter and, as would be expected for such a system, gives affinity constants several orders of magnitude greater than the K_t value of 16.4 nM- Mn^{2+} observed in this study. The only comparable affinity constants calculated for micronutrient transport systems in yeasts are 0.36 μM - Zn^{2+} (Lawford *et al.*, 1980) and 1.3 μM - Zn^{2+} (Failla *et al.*, 1976) for Zn^{2+} -specific uptake in *C. utilis*. However, in bacteria, K_t values for Mn^{2+} -specific transport ranging from 0.05 to 2 μM - Mn^{2+} have been reported (Silver & Kralovic, 1969; Silver, 1978; Archibald & Duong, 1984). The maximal Mn^{2+} uptake rate in our strain of *C. utilis* [1.01 nmol (g dry wt) $^{-1}$ min $^{-1}$] is somewhat lower than in previously described systems (Silver, 1978), and this may reflect a difference in Mn^{2+} requirements between the various cell types or may simply be due to variations in experimental techniques. Zn^{2+} competitively inhibited Mn^{2+} uptake; however, the inhibitory constant, K_i , was some 500 times greater than the K_t for Mn^{2+} . It is probable that the other cations might also exert some reduced competitive effect as it is unlikely that the specificity will be complete in a mechanism of this type. Silver *et al.* (1970) demonstrated competitive inhibition of Mn^{2+} transport by both Co^{2+} and Fe^{2+} in *E. coli* whilst Cd^{2+} has also been shown to inhibit competitively Mn^{2+} uptake in *Lactobacillus plantarum* (Archibald & Duong, 1984) and *Bacillus subtilis* (Laddaga *et al.*, 1985). Cd^{2+} was not included in this study as yeasts have no essential requirement for this ion and a number of deleterious effects of Cd^{2+} on cells have been reported (Gadd & Mowll, 1983; Grafl & Schwantes, 1983; Jones & Greenfield, 1984).

Energy-linked systems for Mg^{2+} efflux exist in *E. coli* (Lusk & Kennedy, 1969) and Mn^{2+} and Sr^{2+} egress has been observed in *S. cerevisiae* (Nieuwenhuis *et al.*, 1981). Our results suggest that a metabolism-dependent exchange of ^{54}Mn occurs, albeit to a relatively small extent. It has been suggested that displacement of the metal ions from intracellular sites may occur (Jasper & Silver, 1977) and this possibility is not precluded.

Fluctuations in cellular metal levels during batch growth have been observed for Zn^{2+} in *C. utilis* (Failla & Weinberg, 1977) and Cu^{2+} in *E. coli* (Baldry & Dean, 1980). Both studies demonstrated an uptake peak during late-exponential phase, the former study also demonstrating rapid uptake during the lag phase of growth. However, Mn^{2+} uptake in *C. utilis* does not appear to be under such regulation, the cell Mn^{2+} level remaining constant around 0.35 nmol (g dry wt) $^{-1}$ in our experiments. Attempts to culture cells in the medium described by Failla & Weinberg (1977) resulted in a pH drop to 3.8 during late-exponential phase of growth. Under these conditions Mn^{2+} uptake would be severely limited.

Thus in conclusion, there exists in *C. utilis* a Mn^{2+} transport system which is energy-dependent, substrate-specific and of high affinity. This system is only detected at submicromolar concentrations of the metal and is independent of the non-specific Mg^{2+} transporter (Parkin & Ross, 1985b).

The authors gratefully acknowledge the Science and Engineering Research Council for the receipt of a studentship for M.J.P.

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