Demonstration of high-affinity Mn^{2+} uptake in *Saccharomyces cerevisiae*: specificity and kinetics

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The existence of multiple transport systems for Mn^{2+} in *Saccharomyces cerevisiae* has been demonstrated in this study. Mn^{2+} (supplied as MnCl_2) was accumulated by *S. cerevisiae* at all Mn^{2+} concentrations examined (25 nM–1 mM) but a log-log plot of uptake rates and total amounts accumulated revealed the existence of at least two Mn^{2+} -concentration-dependent transport systems. Over a low Mn^{2+} concentration range (25–1000 nM), high-affinity Mn^{2+} uptake occurred with a K_m value of 0.3 μM , while transformation of kinetic data obtained over the concentration range 5–200 μM revealed another system with a K_m of 62 μM . Meaningful kinetic analyses were not possible at higher Mn^{2+} concentrations because of toxicity: only about 30% of cells remained viable after 30 min incubation with 1000 μM MnCl_2 . Release of K^+ accompanied Mn^{2+} accumulation and this increased with increasing Mn^{2+} concentration. However, even in non-toxic Mn^{2+} concentrations, the ratio of Mn^{2+} uptake to K^+ release greatly exceeded electroneutral stoichiometric exchange. In 50 μM MnCl_2 , the ratio was 1:123 and this increased to 1:2670 in 1000 μM MnCl_2 , a toxic concentration. External Mg^{2+} was found to decrease Mn^{2+} accumulation at all concentrations examined, but to differing extents. Over the low Mn^{2+} concentration range (5–200 μM), Mg^{2+} competitively inhibited Mn^{2+} uptake with a half-maximal inhibitory concentration, K_i , of 5.5 μM Mg^{2+} . However, even in the presence of a 50-fold excess of Mg^{2+} , inhibition of Mn^{2+} uptake was of the order of 72% and it appears that the cellular requirement for Mn^{2+} could be maintained even in the presence of such a large excess of Mg^{2+} . Over the high Mn^{2+} concentration range (5–200 μM), the K_i for Mg^{2+} was 25.2 μM . At low Mn^{2+} concentrations, Zn^{2+} and Co^{2+} , but not Cd^{2+} , inhibited Mn^{2+} uptake, which indicated that the high-affinity Mn^{2+} uptake system was of low specificity, while at higher Mn^{2+} concentrations, where the lower-affinity Mn^{2+} transport system operated, inhibition was less marked. However, competition studies with potentially toxic metal cations were complicated due to toxic effects, particularly noticeable at 50 μM Co^{2+} and Cd^{2+} .

Keywords: Mn^{2+} , Mg^{2+} , *Saccharomyces cerevisiae*, transport kinetics, divalent cations

INTRODUCTION

Manganese is probably an essential metal for all micro-organisms and has been implicated in the regulation of growth and metabolism in fungi (Garraway & Evans, 1984; Kubicek & Rohr, 1986; Hockertz *et al.*, 1987a; Pilz *et al.*, 1991; Auling, 1994) as well as in bacteria (Auling, 1983; Archibald, 1986; Plonzig & Auling, 1987), mainly due to its influence on several key enzymes (Hughes, 1990;

Kendrick *et al.*, 1992; Frausto da Silva & Williams, 1993). Mn^{2+} has also been proposed as an important eukaryotic cell regulator (Williams, 1982) and, in fungi, it appears to be preferentially located in the vacuole, where it may be bound to polyphosphates (Okorokov *et al.*, 1980, 1985; Lichko *et al.*, 1980; Okorokov, 1985).

Although some divalent cations, e.g. Mg^{2+} , Ca^{2+} , may enter fungal cells via a monovalent cation transport

system (Borst-Pauwels, 1981; Jones & Gadd, 1990), this is probably of little or no importance in terms of cellular requirements, particularly since external K^+ will out-compete under most conditions of growth. More generally, divalent cations appear to enter into cells as a result of the electrochemical gradient ($\Delta\mu_{H^+}$) generated by the activity of the plasma membrane H^+ -ATPase (Borst-Pauwels, 1981; Sanders, 1990; Jones & Gadd, 1990; Gadd, 1993), although there is some evidence that an electrochemical K^+ gradient ($\Delta\mu_{K^+}$) may also be important (Okorokov, 1985; Ramos *et al.*, 1985). While the existence of a Ca^{2+} -ATPase has been proposed in fungi (Miller *et al.*, 1990), there is no evidence of a comparable mechanism for other essential divalent cations, although divalent cation efflux mechanisms have frequently been proposed (Nieuwenhuis *et al.*, 1981; Okorokov, 1985; Jones & Gadd, 1990).

The extent to which Mg^{2+} and Mn^{2+} may substitute for each other in fungal metabolism has long been a topic of speculation (Garraway & Evans, 1984) with considerable technical difficulties in attempting to elucidate such roles. Mn^{2+}/Mg^{2+} antagonism has been noted in reciprocal substitution in Mn^{2+}/Mg^{2+} -activated proteins as well as in Mn-induced mutagenicity in yeasts due to Mn^{2+}/Mg^{2+} antagonism in DNA metabolism (Auling, 1994). However, if Mn^{2+} is essential and specific for certain functions, then specific transport systems should exist for its accumulation regardless of the external ionic composition. Under most environmental and cultural conditions, external Mg^{2+} concentrations will be higher than Mn^{2+} concentrations and if Mn^{2+} transport was blocked by Mg^{2+} , then deleterious limitation would result. Specific transport systems would enable the accumulation of essential metal cations regardless of the external cationic composition, which may include potentially competing cations.

Many studies on divalent metal cation transport in fungi have been carried out with potentially toxic metals, often essential in low concentrations, like Cu, Zn and Mn, in order to understand mechanisms of accumulation, intracellular storage and relationship with tolerance (see Gadd, 1993). However, kinetic analyses are frequently complicated by the symptoms induced by toxic metals, which can include membrane disruption, and storage in intracellular organelles like the vacuole (Gadd & White, 1989). This may be particularly evident for metals such as Zn^{2+} and Mn^{2+} which are of relatively low toxicity to fungi (compared to, e.g. Cu^{2+} , Cd^{2+}), meaning that toxicity-related work necessitates the use of high non-physiological concentrations. Gadd & White (1989) have concluded that for many potentially toxic metals, the magnitude of affinity constants for transport is largely dependent on the metal concentration range used, with apparent low-affinity systems being detected at high concentrations. Although not enough work has been carried out at low micronutrient metal concentrations, high-affinity metal transport systems have been demonstrated in fungi, predominantly yeasts. A specific transport system for Mn^{2+} has been demonstrated in *Candida utilis* with a K_m of $16.4 \mu M$ (Parkin & Ross, 1986), with

Saccharomyces cerevisiae able to transport Zn^{2+} with a K_m of $3.7 \mu M$ (White & Gadd, 1987a). It is surprising that there is little detailed information on Mn^{2+} transport in *S. cerevisiae* since described high-affinity uptake systems for other cations appear less discriminatory against potential competing divalent cations in comparison to other yeasts, e.g. *C. utilis* (Ross, 1993). This property could affect the ability of *S. cerevisiae* to obtain adequate trace amounts of Mn^{2+} in the presence of competing cations. In addition, a depth of knowledge exists on intracellular Mn^{2+} storage in *Saccharomyces* spp. (Okorokov *et al.*, 1980; Nieuwenhuis *et al.*, 1981; Okorokov, 1985; Kihn *et al.*, 1988).

The objective of this work was therefore to characterize Mn^{2+} transport in *S. cerevisiae* over a range of external concentrations to find out whether this organism possesses multiple transport systems of differing affinities, and to determine their sensitivity to potentially competing divalent cations, particularly Mg^{2+} .

METHODS

Organism and culture conditions. *Saccharomyces cerevisiae* X2180-1B was routinely maintained on MYGP agar of composition ($g l^{-1}$): malt extract (Lab M), 3.0; yeast extract (Difco), 3.0; bacteriological peptone (Oxoid), 5.0; D-glucose, 10.0; agar (Lab M no. 2), 15; and incubated at $25^\circ C$. For experimental purposes, cultures were grown at $25^\circ C$ on an orbital shaker (100 r.p.m.) in a medium comprising ($g l^{-1}$): KH_2PO_4 , 2.72; $K_2HPO_4 \cdot 3H_2O$, 5.22; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.12; $FeSO_4 \cdot 7H_2O$, 0.0022; $ZnSO_4 \cdot 7H_2O$, 0.004; $MnSO_4 \cdot 4H_2O$, 0.004; $CuSO_4 \cdot 5H_2O$, 0.0004; D-glucose, 20.0; yeast extract (Difco), 1.0. Starter cultures were prepared by loop inoculation of the liquid medium from MYGP agar and grown as above for 24–48 h. Experimental cultures were inoculated to an initial OD_{550} of approximately 0.1. Cells were counted using a modified Fuchs–Rosenthal haemocytometer after appropriate dilution with distilled water.

Mn^{2+} uptake by *S. cerevisiae*. Late-exponential phase (18 h) cells were harvested by centrifugation (1200 g, 10 min) and washed three times with and finally resuspended in 5 mM PIPES buffer, adjusted to pH 6.5 using solid tetramethylammonium hydroxide, to a density of approximately 2×10^8 cells ml^{-1} . For experiments, cell suspensions were derived from this by dilution in PIPES buffer to a density of 5×10^6 cells ml^{-1} and contained D-glucose at a final concentration of 50 mM. The suspension was then equilibrated with stirring at $25^\circ C$ for 15 min. Mn^{2+} uptake was initiated by the addition of $MnCl_2 \cdot 4H_2O$ to the required concentration, with ^{54}Mn (Amersham International) added as a tracer to a final activity of 3.7–9.25 kBq ml^{-1} . Where required, $MgCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $CdCl_2$ or $ZnCl_2$ at the desired concentration was added to the cell suspension with the $MnCl_2 \cdot 4H_2O$ and ^{54}Mn . At intervals, 1 ml samples were removed and harvested by filtration through Whatman cellulose nitrate filters (0.45 μm , 25 mm diameter). Preliminary experiments were carried out with samples taken every 2 min during the first 10 min. This established the linear nature of initial uptake. The filters and cells were washed five times with 2.5 ml ice-cold 10 mM $MnCl_2 \cdot 4H_2O$ to remove bound ^{54}Mn . Filters were then placed in scintillation fluid (Ecoscint A, National Diagnostics) and radioactivity was measured using a Packard Tri-carb 4000 series scintillation counter.

Measurement of extracellular K^+ . K^+ concentrations in solutions were measured using a Corning K^+ -specific electrode

Fig. 1. Mn²⁺ uptake by *S. cerevisiae* X2180-1B measured over the Mn²⁺ concentration range of 0.025–1000 μM. A log-log plot is employed because of the wide range of uptake values and Mn²⁺ concentrations as well as revealing the existence of multiple uptake systems (Reed *et al.*, 1981). Rates of Mn²⁺ uptake (○) were calculated from determinations made over the initial 0.5–10 min of incubation with ⁵⁴Mn (at least three replicates); ●, amount of Mn²⁺ accumulated after 30 min incubation. Bars indicate SEM of individual points (three replicates) and when not shown were smaller than the dimensions of the symbols.

attached to a Kent/EIL 7055 pH meter (Eil Analytical Instruments) connected to a Servoscribe potentiometric chart recorder. Continuous measurements were standardized by known additions (White & Gadd, 1987b). To measure K⁺ efflux, cells at a density of 5×10^6 ml⁻¹ were equilibrated with 50 mM glucose as above. MnCl₂ was then added to the required concentration and the buffer K⁺ concentration was recorded as above.

Measurement of intracellular K⁺ and Mg²⁺ levels. Samples of cells (10 ml) were taken and washed three times with 5 mM PIPES buffer, pH 6.5. One millilitre of 6 M HNO₃ was added to the washed pellet and incubated at 90 °C. After 1 h incubation, 4 ml distilled-deionized water was added and if necessary the acid extracts were recentrifuged (1200 g, 10 min) to remove debris. K⁺ and Mg²⁺ concentrations were measured using a Pye Unicam SP9 atomic absorption spectrophotometer with reference to appropriate standards.

Effect of Mn²⁺ on yeast viability. Late-exponential phase cells (18 h) were aseptically harvested, washed and preincubated with stirring at a density of 5×10^6 cells ml⁻¹ in sterile PIPES buffer, pH 6.5, with 50 mM glucose at 25 °C for 15 min. Sterile MnCl₂·4H₂O was added to the required concentration and the suspension was incubated for a further 30 min. The number of viable cells was estimated by the conventional dilution series and spread-plate method using sterile distilled water as diluent.

RESULTS

Mn²⁺ accumulation in the absence of competing ions

Mn²⁺ was accumulated by *S. cerevisiae* at all the concentrations examined. At low concentrations (25–1000 nM MnCl₂), both the initial rate and the final amounts of Mn²⁺ accumulated by the cells increased with increasing concentrations of MnCl₂ up to 1000 nM (Fig. 1). When these data were transformed by Lineweaver–Burk analysis and the resultant straight line was fitted according to the least squares method, this transformation gave a $V_{\max}$ value of 3.4 pmol Mn²⁺ min⁻¹ per 5×10^6 cells and a K_m value of 0.3 μM MnCl₂ (correlation coefficient of fitted line = 0.9543).

Uptake of Mn²⁺ from higher concentrations of MnCl₂ (5–200 μM) (Fig. 1) differed from that at lower concentrations (25–1000 nM) in that the 'uptake profile' was different. The accumulation of Mn²⁺ at the higher

concentrations followed a linear pattern over the time-course of the experiment (30 min), whereas at lower concentrations the maximal rate of accumulation occurred within the first 10 min with a gradual decrease in rate after this time. Transformation of data obtained at MnCl₂ concentrations of 5–200 μM (Fig. 1) gave a $V_{\max}$ value of 57.6 pmol Mn²⁺ min⁻¹ per 5×10^6 cells and a K_m value of 62 μM MnCl₂ (correlation coefficient of line = 0.9949). At higher concentrations of MnCl₂ (600–1000 μM), Lineweaver–Burk transformation gave negative values of K_m and $V_{\max}$. This was despite the line of best fit having a correlation coefficient of 0.9830. On testing the viability of cells exposed to these higher concentrations, it was found that only $29.6 \pm 2.8\%$ of the cells exposed to 1000 μM MnCl₂ remained viable after 30 min (Table 1).

Induction of K⁺ release by MnCl₂

The amount of K⁺ released from cells, as measured using a K⁺-specific electrode, decreased with decreasing MnCl₂ concentration. The rate of K⁺ release increased as the concentration of MnCl₂ increased (Fig. 2); however, the ratio of Mn²⁺ uptake to K⁺ release was far in excess of a 1:2 stoichiometric electroneutral exchange. Even in the presence of 50 μM MnCl₂ (a non-toxic level), the ratio of Mn²⁺ uptake to K⁺ release was 1:123 while in the presence of 1000 μM MnCl₂ the ratio increased to 1:2670 (Table 2).

Mn²⁺ accumulation in the presence of Mg²⁺

The effect of MgCl₂ on the accumulation of Mn²⁺ by *S. cerevisiae* was investigated at low (25–1000 nM), high (5–200 μM) and potentially toxic (1 mM) concentrations of MnCl₂. Mg²⁺ was found to reduce Mn²⁺ accumulation at all concentrations examined, but to differing extents. Uptake of Mn²⁺ from 100 nM MnCl₂ in the presence of a 10-fold molar excess of Mg²⁺ led to a 54.3 % reduction in the amount of Mn²⁺ accumulated after 30 min, while a 50-fold molar excess led to a 71.5 % reduction. Similar results were obtained for uptake of Mn²⁺ from 1 μM MnCl₂, where the presence of equimolar MgCl₂ resulted in a

Table 1. Viability of *S. cerevisiae* X2180-1B after exposure to Mn^{2+}

Cells were suspended in 5 mM PIPES buffer, pH 6.5, to a density of 5×10^6 cells ml^{-1} and incubated with different concentrations of $MnCl_2 \cdot 4H_2O$ in the presence of 50 mM glucose for 30 min. Values shown are $\pm$ SEM.

$MnCl_2$ concn (μM)	$10^{-6} \times$ c.f.u. ml^{-1}	Viability (% of control)
0	5.26 ± 0.47	100.0 ± 6.35
400	5.33 ± 0.35	101.3 ± 4.72
600	4.63 ± 0.23	88.1 ± 2.79
800	4.60 ± 0.10	87.4 ± 1.34
1000	1.56 ± 0.20	29.6 ± 2.80

Fig. 2. Rates of Mn^{2+} uptake (○) and K^+ release (●) from *S. cerevisiae* X2180-1B at varying Mn^{2+} concentrations. Mn^{2+} uptake rates were derived using ^{54}Mn as described previously; rates of K^+ release were calculated from traces produced using a K^+ -specific electrode as described by White & Gadd (1987a, b).

58.7% reduction while a 5-fold molar excess of $MgCl_2$ resulted in an 85.1% reduction in the amount of Mn^{2+} accumulated after 30 min.

Transformation of the initial rates of Mn^{2+} uptake (according to Lineweaver-Burk) suggested that the inhibition caused by 1 μM $MgCl_2$ was competitive, the K_m value being 18.3 μM $MnCl_2$ as compared to 0.3 μM for uninhibited Mn^{2+} accumulation, while the V_{max} values were 6.0 and 3.4 μmol Mn^{2+} min^{-1} per 5×10^{-6} cells, respectively (Table 3). In competitive inhibition, while the K_m is increased the V_{max} should remain unchanged. The effects of Mg^{2+} over the low Mn^{2+} concentration range therefore do not readily fit usual models for competitive or non-competitive inhibition (where the K_m remains approximately constant while the V_{max} decreases). In the presence of 5 μM $MgCl_2$, the K_m was

Table 2. Ratios of the initial rate of Mn^{2+} uptake to the initial rate of K^+ released by *S. cerevisiae* X2180-1B in the presence of different concentrations of $MnCl_2$

Experimental conditions were as for Fig. 1. Rates of K^+ release were calculated from traces obtained using a K^+ -specific electrode (White & Gadd, 1987b). Initial rates of Mn^{2+} uptake were calculated as described in Methods. Typical results are shown from one of three experiments.

Concn of $MnCl_2$ (μM)	Ratio of Mn^{2+} uptake: K^+ release
50	1:123
100	1:99
200	1:317
400	1:1289
600	1:1175
1000	1:2670

also increased over the control, being 5.1 μM , and the V_{max} value increased to 9.6 μmol Mn^{2+} min^{-1} per 5×10^6 cells (Table 3). A secondary plot of the slope of the lines obtained in the primary double reciprocal plots against concentration of $MgCl_2$ indicated linear inhibition with a half-maximal concentration, K_i , of 5.5 μM $MgCl_2$ (correlation coefficient of line = 0.9491).

Inhibition of Mn^{2+} uptake by $MgCl_2$ also occurred at the higher $MnCl_2$ concentrations examined. Uptake of Mn^{2+} from 5 μM $MnCl_2$ in the presence of equimolar $MgCl_2$ resulted in a 55.5% inhibition of uptake over 30 min as compared to control values, while at 100 μM $MnCl_2$ equimolar $MgCl_2$ inhibited Mn^{2+} uptake by 78.4%. The presence of a 10- and 20-fold molar excess of $MgCl_2$ on Mn^{2+} accumulation from 5 μM $MnCl_2$ resulted in an inhibition of uptake by 90.4 and 91.1%, respectively. Thus, it appeared that inhibition of Mn^{2+} uptake from 5 μM $MnCl_2$ by Mg^{2+} was not increased significantly by increasing the concentration of $MgCl_2$ above 50 μM . Transformation of the data obtained revealed that in the presence of 5 μM $MgCl_2$, Mg^{2+} inhibition was of a competitive nature since the K_m value was increased while the V_{max} remained approximately the same in the presence of 5 μM $MgCl_2$. The new K_m and V_{max} values were

Table 3. Summary of the kinetic constants for Mn²⁺ uptake by *S. cerevisiae*

Concentration range of MnCl ₂ used	Concentration of MgCl ₂ (μM)	K _m (μM)	V _{max} (pmol Mn ²⁺ min ⁻¹ per 5 × 10 ⁶ cells)
25–1000 nM	0	0.3	3.4
5–200 μM	0	62.0	57.6
25–1000 nM	1	18.3	6.0
25–1000 nM	5	5.1	9.6
5–200 μM	5	106.5	47.9
5–200 μM	50	283.4	46.7
5–200 μM	100	108.4	13.5

Table 4. Cellular Mg content of *S. cerevisiae* X2180-1B after exposure to differing MnCl₂:MgCl₂ ratios

Cells were incubated in 5 mM PIPES buffer, pH 6.5, at 25 °C in the presence of 50 mM glucose for 30 min prior to harvesting and analysis. Intracellular Mg is expressed as nmol Mg per 5 × 10⁶ cells ± SEM (three replicates).

	MgCl ₂ concn (μM)	Intracellular Mg
MnCl ₂ (50 μM)	0	17.2 ± 0.4
	5	16.8 ± 0.3
	50	18.0 ± 0.4
	100	21.1 ± 0.5
	MnCl ₂ concn (μM)	Intracellular Mg
MgCl ₂ (50 μM)	0	17.3 ± 0.5
	5	23.2 ± 0.3
	50	18.0 ± 0.5
	100	17.0 ± 0.2

106.5 μM MnCl₂ and 47.9 pmol Mn²⁺ min⁻¹ per 5 × 10⁶ cells, respectively (correlation coefficient of line = 0.9631), as compared to 62.0 μM MnCl₂ and 57.6 pmol Mn²⁺ min⁻¹ per 5 × 10⁶ cells for control cells (Table 3). In the presence of 50 and 100 μM MgCl₂, the K_m values for Mn²⁺ transport were 283.4 μM and 108.4 μM MnCl₂, respectively, with corresponding V_{max} values of 46.7 and 13.5 pmol Mn²⁺ min⁻¹ per 5 × 10⁶ cells (correlation coefficients for these lines were 0.9948 and 0.9950, respectively; Table 3). This suggests that in the presence of 50 μM MgCl₂ the competition observed was also competitive, while in the presence of 100 μM MgCl₂ competition was of a non-competitive nature since the K_m value was increased only slightly while the V_{max} was reduced fivefold. A secondary plot of the slopes obtained in the primary double reciprocal plot indicated linear inhibition with a K_i value of 25.2 μM MgCl₂ (correlation coefficient of line = 0.9747).

Table 4 shows the intracellular levels of Mg after 30 min exposure to various MgCl₂:MnCl₂ ratios. In the presence of 50 μM MnCl₂, as the extracellular MgCl₂ level was increased so the internal level of Mg slightly increased. However in the presence of 50 μM MgCl₂, the addition

of 5 μM Mn²⁺ led to an increase in the Mg content of the cell of 25.4 % of the control value. The intracellular level of Mg decreased towards the control value as the concentration of MnCl₂ was increased. The effect of 0, 5, 50 and 100 μM MgCl₂ on Mn²⁺ accumulation from 1 mM MnCl₂ was also examined (results not shown). It was found that neither the initial rates nor the total amount of Mn²⁺ accumulated were dependent on MgCl₂ concentration, probably because of the toxic effects of MnCl₂ at this concentration.

Mn²⁺ accumulation in the presence of Cd²⁺, Co²⁺ and Zn²⁺

The accumulation of Mn²⁺ from various concentrations of MnCl₂ in the presence of 5 and 50 μM CdCl₂, CoCl₂ and ZnCl₂ was examined. At the lowest Mn²⁺ concentration (100 nM), 5 μM Cd²⁺ did not affect the initial rate of uptake but caused an increase in the total amount of Mn²⁺ accumulated (Fig. 3a). The presence of 50 μM Cd²⁺ caused a slight decrease in the total amount of Mn²⁺ accumulated as compared to control levels. Co²⁺ and Zn²⁺ both markedly reduced the initial rate and final amount of Mn²⁺ accumulated, Zn²⁺ to a greater extent than Co²⁺ (Fig. 3a). The effects of Cd²⁺, Co²⁺ and Zn²⁺ were most marked in the presence of 100 μM MnCl₂, where all the ions either increased or had no inhibitory effect on rate of Mn²⁺ uptake (Fig. 3b). The increase in Mn²⁺ accumulation caused by Co²⁺ was concentration-dependent, 5 μM CoCl₂ having no effect while 50 μM CoCl₂ caused an increase both in the rate and total amount of Mn²⁺ accumulated. Interestingly, the presence of 5 μM ZnCl₂ stimulated Mn²⁺ uptake while 50 μM ZnCl₂ decreased the total amount of Mn²⁺ accumulated (Fig. 3b).

DISCUSSION

In *Saccharomyces* spp., it is known that Mn is preferentially located in the vacuole, which is believed to function in the regulation of cytosolic Mn²⁺ homeostasis (Lichko *et al.*, 1980). The vacuolar membrane of *Saccharomyces* spp. possesses a Mn²⁺/H⁺ antiport system for such accumulation into the vacuole, which has an additional role in the storage and detoxification of other metal ions, including Ca²⁺, Cs⁺, Li⁺, Co²⁺ and Zn²⁺ (Eilam *et al.*, 1985; White & Gadd, 1986, 1987a; Cornelius & Nakashima, 1987;

Fig. 3. Uptake of Mn^{2+} by *S. cerevisiae* X2180-1B from (a) 100 nM and (b) 100 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ in the presence of: (i) 0 ($\circ$), 5 ($\square$), 50 ($\blacksquare$) μM CdCl_2 ; (ii) 0 ($\circ$), 5 ($\triangle$), 50 ($\blacktriangle$) μM CoCl_2 ; and (iii) 0 ($\circ$), 5 (∇), 50 ($\blacktriangledown$) μM ZnCl_2 . Cells were suspended in 5 mM PIPES buffer, pH 6.5, containing 50 mM D-glucose to a density of $5 \times 10^6 \text{ ml}^{-1}$. Values shown are means of three replicates; SEM values were smaller than the symbol dimensions in most cases.

Perkins & Gadd, 1993; Gadd, 1993; Ross, 1993). In this study, 1 ml cell suspension (density of $5 \times 10^6 \text{ cells ml}^{-1}$) took up $9.35 \times 10^{-3} \text{ nmol Mn}^{2+}$ during 30 min incubation in 100 nM MnCl_2 . If the cell volume, as measured by a Coulter analysis (Meikle *et al.*, 1988), is assumed to be 47 fl, this gives an intracellular concentration of Mn^{2+} of approximately 39.8 μM . If it is assumed that all the cellular Mn^{2+} is in an unbound ionic form, this represents an uphill transport concentration gradient of approximately 397:1 (inside:outside). However, it is unlikely that all the Mn^{2+} is present in an osmotically free form and Okorokov *et al.* (1975, 1980) have estimated that 78% of the total Mn^{2+} taken up by yeast was bound to cellular com-

ponents, with several reports demonstrating sequestration by vacuolar polyphosphate contributing significantly to the bound pool (Okorokov *et al.*, 1980; Borst-Pauwels, 1981; Kihn *et al.*, 1988; Gadd, 1986).

Mn^{2+} accumulation from low (5–1000 nM) MnCl_2 concentrations was shown to operate via a high-affinity system having a K_m value of 0.3 μM and a $V_{\max}$ of 3.4 pmol $\text{Mn}^{2+} \text{ min}^{-1}$ per 5×10^6 cells. The specific uptake of Mn^{2+} by the yeast *C. utilis* has a K_m value of 16.4 nM Mn^{2+} (Parkin & Ross, 1986). Comparable affinity constants for other metal transport systems in yeasts are 0.36 μM (Lawford *et al.*, 1980) and 1.3 μM (Failla *et al.*, 1976) for Zn^{2+} in *C. utilis*,

3.7 μM for Zn²⁺ uptake by *S. cerevisiae* (White & Gadd, 1987a), 3.1 μM for Cu²⁺ in *C. utilis* (Parkin & Ross, 1985) and between 1.1 and 4.4 μM for Cu²⁺ in *S. cerevisiae* (De Rome & Gadd, 1987; Lin & Kosman, 1990). The Mn²⁺ system operating in *C. utilis* appeared highly specific, being unaffected by a 100-fold molar excess of Mg²⁺, Zn²⁺, Ca²⁺, Co²⁺, Ni²⁺ and Cu²⁺. Uptake was, however, inhibited 30–40% by a 1000-fold molar excess of Mg²⁺, Zn²⁺, Ca²⁺, Co²⁺ and Ni²⁺ (Parkin & Ross, 1986). In the high-affinity Mn²⁺ system shown here for *S. cerevisiae*, a similar specificity was not seen. The large increase in the K_m value for Mn²⁺ transport at the lower Mn²⁺ concentrations in the presence of 1 μM MgCl₂ with only a slight increase in $V_{\max}$ tends to indicate competitive inhibition of this high-affinity system. However, even in the presence of a 50-fold molar excess of Mg²⁺, inhibition was only of the order of 71.5%. It appears in *S. cerevisiae*, therefore, that the cellular requirement for Mn²⁺ could be maintained, even in the presence of a large excess of Mg²⁺. The reduction in K_m at higher Mg²⁺ concentrations (although the K_m is still higher than the K_m for Mn²⁺ transport only), together with a slight increase in $V_{\max}$, could be due either to the Mg²⁺ accumulated affecting the electrochemical proton gradient and/or the surface potential, both of which would decrease the driving force available for Mn²⁺ accumulation (Borst-Pauwels & Severens, 1984), or, alternatively, the Mg²⁺ could be competing for the transport system in a non-concentration-dependent manner. In the filamentous fungus *Aspergillus niger*, a specific high-affinity Mn²⁺ transport system ($K_m = 3 \mu\text{M}$) has been detected at submicromolar concentrations of Mn²⁺ which functioned independently of the transport of Mg²⁺ and Ca²⁺ but was preferentially inhibited by Zn²⁺, Cu²⁺ and Cd²⁺ (Hockertz *et al.*, 1987b; Auling, 1994). In *Penicillium notatum*, Mn²⁺ uptake from nM concentrations occurred by a system with an apparent K_m of 4.4 nM which was insensitive to Mg²⁺, Zn²⁺, Ni²⁺, Cu²⁺ or Co²⁺ at 1000-fold excess but competitively inhibited by Cd²⁺ (Starling & Ross, 1990; Ross, 1993). Another uptake system in this organism, with a K_m of approximately 22 μM , operated at 10–100 μM Mn²⁺ and was competitively inhibited by a fivefold excess of the competing cations (Ross, 1993). In this present study, Co²⁺ and Zn²⁺ were found to inhibit Mn²⁺ uptake from low Mn²⁺ concentrations and to stimulate Mn²⁺ uptake from high Mn²⁺ concentrations.

Uptake of Mn²⁺ from the higher (5–200 μM) concentrations of MnCl₂ examined had a K_m value of 62 μM and a $V_{\max}$ of 57.6 pmol Mn²⁺ min⁻¹ per 5×10^6 cells. This compares with a K_m value of approximately 65 μM for the non-specific Mn²⁺ uptake system of *C. utilis* (Parkin & Ross, 1985) and a K_m and $V_{\max}$ of 860 μM and 0.51 μmol Mn²⁺ min⁻¹ per 10^9 cells, respectively, for *S. cerevisiae* 6175/11a (Bianchi *et al.*, 1981a,b) and a K_m value of approximately 100 μM for *S. cerevisiae* 431 (Norris & Kelly, 1977). The presence of 5, 50 and 100 μM MgCl₂ increased the K_m values to 106 μM , 284 μM and 108 μM , respectively. The K_i value for inhibition by MgCl₂ was 25.2 μM . This contrasts with the higher K_i values recorded for inhibition of Co²⁺ uptake below approximately

0.5 mM CoSO₄ of 125 μM MgSO₄ and a K_i value for Cd²⁺ uptake below 0.5 mM CdSO₄ of 1.4 mM MgSO₄ (Norris & Kelly, 1977). The slight increase in K_m and fivefold decrease in $V_{\max}$ in the presence of 100 μM Mg²⁺ is possibly indicative of non-competitive inhibition of Mn²⁺ transport. In the presence of 5 and 50 μM MgCl₂, a concentration-dependent increase in the K_m was observed with only a slight change in $V_{\max}$, indicating competitive inhibition.

Uptake of Mn²⁺ from the highest concentrations of MnCl₂ examined (400–1000 μM) gave negative K_m and $V_{\max}$ values. Viability studies indicated that toxic effects of Mn²⁺ were likely to have been responsible for this apparent deviation from Michaelis–Menten kinetics (White & Gadd, 1987a, b). These toxic effects are probably responsible for the anomalous uptake rates obtained from 1000 μM MnCl₂ in the presence of 5, 50 and 100 μM MgCl₂. The connection between K⁺ release and toxicity was clear and after 30 min exposure to 1 mM MnCl₂, plate counts on MYGP medium showed a 71.4% kill. The ratio of Mn²⁺ taken up to K⁺ released was far in excess of 1:2 (see Gadd, 1986), even at the non-toxic level of 100 μM MnCl₂. The mechanism by which the rate of K⁺ efflux from metabolizing yeast is controlled is poorly understood (Borst-Pauwels, 1981, 1988) and the effect of divalent cation uptake on this is unlikely to involve a simple 1:2 ratio (Ross, 1993). Okorokov *et al.* (1983a, b) also found intense K⁺ release on initiating Mn²⁺ uptake in *Saccharomyces carlsbergensis* using high concentrations (3 mM) of MnSO₄ and measuring the rate of uptake in a non-defined medium (meaning that the amount of free Mn²⁺ available for uptake was unknown). Okorokov *et al.* (1983a, b) proposed that the K⁺ efflux observed was used to drive the synthesis of high molecular mass polyphosphates which could be involved in sequestration of Mn²⁺ in the vacuole.

It has been shown that in the μM range the presence of Mn²⁺ can have an effect on internal Mg²⁺ concentrations. This would imply that the two ions are interacting in some way and that at these concentrations a more general divalent cation transport system may be operating (see Borst-Pauwels, 1981; Jones & Gadd, 1990; Gadd, 1993; Ross, 1993). The differing effects of Cd²⁺, Co²⁺ and Zn²⁺ on Mn²⁺ uptake from various concentrations of MnCl₂ may imply that different transport processes operate at different concentrations. At the lowest concentrations examined, all the competing metals except Cd²⁺ inhibited Mn²⁺ uptake, which indicates that the high-affinity Mn²⁺ system of *S. cerevisiae* is of low specificity regarding other divalent metal cations, while at the higher concentrations of MnCl₂, where a lower affinity Mn²⁺ transport system is more likely to operate, inhibition was less marked. Cd²⁺ and Co²⁺ at 50 μM even stimulated uptake at higher MnCl₂ concentrations as compared to control levels. This could be due to these ions affecting the electrochemical gradient positively, providing a greater driving force for the accumulation of Mn²⁺, although it is more likely that the increased level of Mn²⁺ accumulation observed may be due to toxic interactions of these metal ions with the cells. Part of the population of yeast cells may lose its

viability during incubation of the cells with the metal ions while the remainder remain viable (Gadd & Mowll, 1983; Belde *et al.*, 1988). This loss of viability may be due to a permeabilization of the cell membrane by the metal ions, thus exposing more binding surfaces. This would also explain the increase in both the rate and amount of Mn^{2+} accumulated from low Mn^{2+} concentrations in the presence of $5 \mu M$ Cd^{2+} , which is toxic (Kessels *et al.*, 1985; Belde *et al.*, 1988).

In summary, it appears that *S. cerevisiae* possesses a high-affinity Mn^{2+} transport system, functional at low Mn^{2+} concentrations, which is of low specificity, being inhibited by Mg^{2+} , Co^{2+} , Zn^{2+} and Cd^{2+} to varying extents. At higher concentrations of $MnCl_2$, a transport system of lower affinity is available to the cell. Despite the Mn^{2+} transport system of *S. cerevisiae* being relatively non-specific, transport of Mn^{2+} could still occur, even when there was an excess of competing divalent cations such as Mg^{2+} . This is important since micro-organisms must be able to acquire essential 'trace' divalent cations, even when there is an excess of other divalent cations present in their external environment.

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