The Uptake and Cellular Distribution of Zinc in *Saccharomyces cerevisiae*

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Zn$^{2+}$ uptake by *Saccharomyces cerevisiae* was biphasic. The first phase was independent of metabolic energy, consisting of adsorption to the cell surface, and followed a Freundlich isotherm. The second phase was dependent on metabolic energy, ATPase activity and the transmembrane proton gradient, and consisted of uptake into the cell. Energy-dependent uptake showed Michaelis-Menten kinetics with a $K_m$ of 3.7 µM-Zn$^{2+}$ and a $V_{max}$ of 1.6 nmol min$^{-1}$ per 10$^7$ cells at Zn$^{2+}$ concentrations below 80 µM but deviated at higher concentrations. K$^+$ and Mg$^{2+}$ inhibited energy-dependent Zn$^{2+}$ uptake while Na$^+$ and Ca$^{2+}$ did not. The effect of heavy metals was complex and included both inhibition and stimulation of Zn$^{2+}$ uptake. K$^+$ efflux accompanied Zn$^{2+}$ uptake at all Zn$^{2+}$ concentrations but there was no simple stoichiometric relationship between the two. Toxic effects of Zn$^{2+}$ such as inhibition of H$^+$ efflux and K$^+$ uptake and reduction of viability were observed at all Zn$^{2+}$ concentrations and toxicity appeared to be a major factor in K$^+$ efflux. Toxicity also affected the kinetics of Zn$^{2+}$ uptake, being a major cause of deviation from Michaelis-Menten kinetics. Zn$^{2+}$ was compartmented within the cell: 56% of the total intracellular pool was in the soluble vacuolar fraction, 39% was bound to insoluble components and only 5% was found in the cytosol. Isolated yeast vacuoles possessed an ATP-dependent Zn$^{2+}$ uptake system whose properties were consistent with a Zn$^{2+}$/H$^+$ antiport.

INTRODUCTION

Many yeasts and fungi exhibit energy-dependent uptake of zinc (Fuhrmann & Rothstein, 1968; Paton & Budd, 1972; Failla et al., 1976; Mowll & Gadd, 1983), an element that possesses a number of important biological functions such as stabilization of proteins and membrane systems and as a component of several metalloenzymes (Chlebowski & Coleman, 1977; Failla, 1977). There is little detailed work pertaining to *Saccharomyces cerevisiae* particularly with regard to the mechanism of uptake and the intracellular fate of accumulated zinc and, indeed, for divalent cation uptake in general, a coherent picture is still not available (see Borst-Pauwels, 1981). This is in part due to the many factors that can affect the kinetics of divalent cation uptake in yeast such as surface potential effects (Roomans et al., 1979; Borst-Pauwels & Theuvenet, 1984), the possible existence of multiple transport systems (Norris & Kelly, 1977; Borst-Pauwels, 1981), toxicity and other factors pertaining to the physiological state of the cells and varying external conditions (see Gadd, 1986).

ATPase-dependent H$^+$ efflux is considered to be involved in cation uptake into cells by its role in establishing the plasma-membrane potential (Okorokov, 1985). A direct role of the plasma-membrane ATPase in divalent cation uptake by yeast has been discounted (see Borst-Pauwels, 1981) but divalent cation uptake can be decreased or prevented by factors that cause membrane depolarization, e.g. high external K$^+$ or uncouplers, or can be increased if the membrane is hyperpolarized by certain ionophores (Borst-Pauwels, 1981; Gadd & Mowll, 1985).

**Abbreviations:** DDG, 2-deoxy-D-glucose; DCCD, N,N'-dicyclohexylcarbodiimide; DES, diethylstilboestrol; DEAE dextran, diethylaminoethyl dextran.

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K⁺ efflux often accompanies heavy metal uptake in fungi and can show an approximate ratio of 1 M⁰⁺₂: 2 K⁺⁺ (Fuhrmann & Rothstein, 1968; Norris & Kelly, 1977; Lichko et al., 1980; Mowll & Gadd, 1984; Gadd & Mowll, 1985) indicating a compensatory mechanism for the maintenance of ionic balance, and it has been proposed that K⁺ efflux from yeast can act as a driving force for heavy metal uptake (Okorokov et al., 1983a, b). However, K⁺ release may not occur in all cases (Norris & Kelly, 1977; Mowll & Gadd, 1983) and, where it does occur, there is often no stoichiometric relationship between the amounts of metal taken up and the K⁺ released (Passow & Rothstein, 1960; Norris & Kelly, 1977; Gadd & Mowll, 1983; Kessels et al., 1985), which can be due to toxic disruptive effects of the metal on the cell membrane (Kuyers & Roomans, 1979; Borst-Pauwels, 1981; Gadd & Mowll, 1985).

As regards the intracellular fate of zinc in fungi, electron-dense bodies have been observed in Neocosmospora vasinfecta (Paton & Budd, 1972) and there is one report of a metallothionein induced by Zn²⁺ in Candida utilis (Faiella et al., 1976). For Mn²⁺ and Ca²⁺, it has been found that the vacuole is the major cellular compartment (Okorokov et al., 1977; Eilam et al., 1985) which is apparently advantageous to the cell in that the metal is localized and potentially damaging effects are limited (Matile & Wiemken, 1976). The vacuolar membrane possesses an ATP-dependent transport system for basic amino acids (Boller et al., 1975; Ohsumi & Anraku, 1981) and Ca²⁺ (Ohsumi & Anraku, 1983; Eilam et al., 1985) and a range of heavy metals can affect vacuolar ATPase activity in vitro and alter the H⁺ gradient across the vacuolar membrane (Okorokov, 1985).

In this study, we have examined and characterized Zn²⁺ uptake by S. cerevisiae, particularly at low external concentrations, which are probably of the greatest physiological importance, and have also examined the influence of toxicity on uptake at higher Zn²⁺ concentrations. K⁺ efflux was also investigated and attention given to the intracellular location of zinc after uptake and the mechanisms involved in this partitioning within the cell.

**METHODS**

Organism and culture. Saccharomyces cerevisiae X2180-1B was routinely maintained at 25 °C on a solid medium comprising (g l⁻¹): malt extract (Lab M), 3.0; yeast extract (Difco), 3.0; bacteriological peptone (Oxoid), 5.0; glucose 10.0; agar (Lab M no. 2), 15.0. For experimental purposes, cultures were grown at 25 °C on an orbital shaker (100 r.p.m.) in a medium comprising (g l⁻¹): KH₂PO₄, 2.72; K₂HPO₄, 3H₂O, 5.22; (NH₄)₂SO₄, 2.0; MgSO₄. 7H₂O, 0.12; FeSO₄. 7H₂O, 0.0022; ZnSO₄. 7H₂O, 0.004; MnSO₄. 4H₂O, 0.004; CuSO₄. 5H₂O, 0.0004; D-glucose, 20.0; yeast extract (Difco), 1.0.

 Cultures were harvested by centrifugation (5 min, 1200 g) and washed three times with distilled water. Where appropriate, cells were starved by resuspending to approximately 10⁷ ml⁻¹ in growth medium without glucose or yeast extract and incubating at 25 °C with stirring for 30 min.

Zn²⁺ uptake by S. cerevisiae. Exponential-phase cells (5 x 10⁶ ml⁻¹) were harvested by centrifugation (5 min, 1200 g), washed twice in distilled water and resuspended to 5 x 10⁶ ml⁻¹ in 5 mM-PIPES/tetramethylammonium hydroxide ('PIPES buffer'), pH 6.5. Glucose was added to 50 mM and the suspension equilibrated with stirring at 25 °C for 15 min. Zn²⁺ uptake was initiated by addition of a solution of ZnCl₂, with ⁶⁵Zn (Amersham) (final concentration of 20 pM and incubated for 60 min. Cells were removed from the loading suspension by centrifugation (5 min, 1200 g) washed three times with distilled water and resuspended to 5 x 10⁶ ml⁻¹ in 5 mM-PIPES buffer, pH 6.5, either without additions, with 50 mM-glucose, with 20 mM-non-radioactive ZnCl₂ or with both 50 mM-glucose and 20 mM-non-radioactive ZnCl₂. Cells were incubated at 25 °C with stirring. Samples were removed at intervals and cells separated by centrifugation through an oil-phthalate layer into 40% perchloric acid; the radioactivity present both in supernatant and pellets was measured as described above.

Cellular distribution of Zn²⁺. Metals were differentially extracted from cell compartments by a modification of the method of Huber-Walchli & Wiemken (1979). The quantity of diethylaminoethyl dextran (DEAE dextran)
required to give complete permeabilization of yeast cells was determined microscopically using the uptake of methyl green dye as an indication of cell permeability.

Cells were pre-loaded with 65Zn by incubating for 20 min under the same conditions as for uptake at a density of $5 \times 10^7$ ml$^{-1}$. Cells were harvested by centrifugation (1 min, 8000 g) of 1 ml of uptake suspension through 0-5 ml 3% (w/v) Nycolen (Nyeagaik UK) in a 1-5 ml microcentrifuge tube. After removal of the supernatant, all further operations were done in the microcentrifuge tube.

Exchangeable Zn$^{2+}$ at the cell surface was removed by washing with 3 x 1 ml tracer-free ZnCl$_2$ solution, of the concentration used for loading, at 0-4 °C followed by washing once with 10 mM-Tris/MES, pH 6-0, at 0-4 °C. Cells were incubated in the washing medium for 1 min and separated by microcentrifugation at each step. All the supernatants were combined and retained.

The cell membrane was permeabilized by resuspending the pellet in 1 ml 10 mM-Tris/MES buffer, pH 6-0, with 0-7 M-sorbitol at 25 °C. DEAE dextran in the same buffer (40 μl, 10 mg ml$^{-1}$) was added, mixed and incubated for 30 s at 25 °C. Cells were separated by centrifugation (30 s, 8000 g) and the supernatant removed and retained. The permeabilized cells were washed with 3 x 0-5 ml 0-7 M-sorbitol in 10 mM-Tris/MES buffer, pH 6-0, at 0-4 °C, incubating for 1 min at each wash and separating by centrifugation as described. Supernatants were retained and combined with that from the permeabilization step.

The vacuole contents were extracted by suspending in 60% (v/v) methanol at 0-4 °C for 30 s, centrifuging (30 s, 8000 g) and removing the supernatant. This was repeated three times and followed by three further washes in 10 mM-Tris/MES, pH 6-0, at 0-4 °C, incubating for 1 min and centrifuging as above. The supernatants were combined with those from the methanol washes. The remaining pellet was resuspended in 0-5 ml 10 mM-Tris/MES, pH 6-0, the suspension removed and the tube washed with a further 0-5 ml of the same buffer. The suspension and washings were combined. Radioactivity in each of the four fractions was measured as described above.

To assay the soluble and bound fractions of protoplasts, 1-2 M-sorbitol was added to the initial tracer-free ZnCl$_2$ surface wash and the separate DEAE dextran cytosol extraction and subsequent washings were also omitted. 

Measurement of K$^+$ concentration and pH. This was done using a Corning potassium-specific electrode and a Russell combination pH electrode with gel electrolyte (Russell pH Ltd), respectively, attached to a Kent/EIL 7055 pH meter (EIL Analytical Instruments) connected where necessary to a Servoscribe potentiometric chart recorder for continuous measurements. Continuous measurements were standardized by known additions. For measurement of K$^+$ uptake by S. cerevisiae, washed, harvested, starved cells were washed three times with distilled water and resuspended in 20 ml 5 mM-PIPES buffer, pH 6-5, at 5 x 10$^{-7}$ ml$^{-1}$ with 50 μM-KCl, incubated for 15 min with stirring at 25 °C and with glucose added to a final concentration of 50 mM. Where appropriate, ZnCl$_2$ was added 10 min before the glucose. A continuous recording was made of the K$^+$ concentration in the buffer. To record H$^+$ extrusion, a similar procedure was adopted using the pH electrode and omitting KCl from the buffer.

To measure K$^+$ efflux, cells were equilibrated in the presence of 50 μM-KCl and 50 μM-glucose as above, ZnCl$_2$ was added to the required concentration and the buffer K$^+$ concentration recorded as above.

Isolation of yeast protoplasts. Harvested cells were suspended at 10$^8$ ml$^{-1}$ in a buffer comprising 100 mM-MES/tetramethylammonium hydroxide with 1-2 M-sorbitol and 10 mg Novozym 234 ml$^{-1}$ (Novo Enzymes Ltd) and incubated at 25 °C with stirring for 2 h. Protoplasts were separated by centrifugation (2 min, 400 g) and resuspended in 1-2 M-sorbitol. Cenfriugation was repeated as necessary to remove debris.

Isolation of vacuoles. The method was a modification of that of Durr et al. (1975). A purified suspension of yeast protoplasts (10$^9$ ml$^{-1}$) in 1-2 M-sorbitol was cooled to 0-4 °C on ice and diluted dropwise 10 times with a buffer comprising 10 mM-Tris/MES, pH 6-0, and 0-7 M-sorbitol at 0-4 °C in a 1 ml plastic centrifuge tube to give a total volume of 10 ml. DEAE dextran in the same buffer (40 μl, 10 mg ml$^{-1}$) was added, mixed and incubated for 60 s at 0-4 °C. The preparation was then incubated at room temperature for 4-6 min. At the end point at least 95% of the protoplasts present were lysed as determined microscopically. Nycolen in the same buffer [1-25 ml, 40% (w/v)] was then mixed with the suspension and a further 1-0 ml of buffer with 40% (w/v) Nycolen layered underneath. The preparation was centrifuged for 5 min at 400 g. Vacuoles were removed from the interface of the two layers with a minimal volume of buffer and diluted to the required concentration with 5 mM-PIPES buffer, pH 6-5, with 0-7 M-sorbitol at 0-4 °C. The integrity of vacuoles was confirmed by phase-contrast microscopy and by the uptake of neutral red stain.

Uptake of Zn$^{2+}$ by isolated vacuoles. Vacuoles were suspended at 5 x 10$^7$ ml$^{-1}$ in 10 ml of a buffer comprising 5 mM-PIPES buffer, pH 7-0, and 0-7 M-sorbitol and incubated for 15 min at 25 °C with stirring. ATP was added as 0-1 ml of a solution of 100 mM-ATP with 100 mM-MgCl$_2$ in the above buffer, with the pH adjusted to 7-0 after ATP addition (a final concentration of 1 mM each of ATP and MgCl$_2$), and the suspension was incubated for a further 15 min (where ATP was omitted, 0-1 ml of 100 mM-MgCl$_2$ was added). Uptake of Zn was initiated by addition of ZnCl$_2$, labelled with $^{65}$Zn at 250 Bq nmol$^{-1}$, to the required concentration. Where necessary, DCCD was added 5 min before addition of ATP; other inhibitors were added 5 min before addition of labelled ZnCl$_2$. Vacuoles incubated under these conditions retained their integrity as determined by phase-contrast microscopy and uptake of neutral red stain.
Samples were taken by centrifugation through 1.0 ml of 5 mM-PIPES buffer, pH 6.5, with 0.7 M-sorbitol and 3% (w/v) Nycodenz; radioactivity in the pellet was counted as described above.

**Effect of Zn**

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Results and Discussion

Energy-dependence of Zn**

Uptake of Zn** was biphasic consisting of an initial rapid, non-linear phase of approximately 2 min followed by a second linear phase which continued for the duration of the experiment (30 min) (Fig. 1). This biphasic pattern is in agreement with other studies on the uptake of divalent metal cations (Ponta & Broda, 1970; Paton & Budd, 1972; Failla et al., 1976; Failla & Weinberg, 1977; Gadd, 1981, 1986; Mowll & Gadd, 1983; Trevors et al., 1986).

Zn** uptake in the first phase was only slightly reduced by incubation in the absence of glucose, in the presence of 0.2 mM-KCN or by incubating at 0-4 °C (Fig. 2a) and was thus independent of metabolic energy. Similarly, uptake in this phase was only slightly reduced by DCCD and DES, showing independence of ATPase activity (Serrano, 1980) (Fig. 2b). The uncoupler DNP effected a greater reduction in this phase of uptake (Fig. 2b). However, it was used at a very high concentration and consequently secondary effects e.g. reduction of pH, were probably important.

Zn** uptake in the second phase was stimulated by glucose, but not by the transportable glucose analogue DDG, and was completely inhibited in the presence of 0.2 mM-KCN or at 0-4 °C, confirming dependence on metabolic energy (Fig. 2a). As can be seen from Fig. 2b, it was also inhibited by DCCD and DES indicating respectively dependence on ATPase activity and particularly plasma-membrane-bound ATPase activity (Serrano, 1980). Complete inhibition by DNP of Zn** uptake in this phase indicated that it required a trans-membrane proton gradient.

The energy-independent component of uptake could be estimated by extrapolating the energy-dependent phase to the vertical axis; this estimate, and also surface-bound Zn** removed by washing cells loaded with labelled Zn**, were plotted against external Zn** concentration (Fig. 3). These two sets of data fit the same Freundlich isotherm of the form [bound Zn**] = 0.178[free Zn**]*0.52 where [bound Zn**] has units of nmol per 107 cells and [free Zn**] has units of nmol ml⁻¹. This phase of Zn** uptake, therefore, comprised largely adsorption to the cell surface while the second comprised energy-dependent transport of Zn** into the cell.

**Kinetics of Zn**

Zn** uptake conformed to Michaelis-Menten kinetics at Zn** concentrations below approximately 80 μM but deviated strongly above this concentration (Fig. 4). The Kₘ was 3.7 μM and the Vₐₘₐₜ was 1.6 nmol min⁻¹ per 10⁷ cells. Previous studies of energy-dependent heavy metal uptake have also generally found saturation kinetics; however, the parameters vary widely (Borst-Pauwels, 1981). The Kₘ values for Zn** uptake were generally higher in studies where the external Zn** concentration was high (Fuhrmann & Rothstein, 1968; Ponta & Broda, 1970; Paton & Budd, 1972), which might be expected in view of our observed deviation from Michaelis-Menten kinetics at higher Zn** concentrations. For this reason, and also because Zn** uptake systems with low affinities would be of little value to organisms except in highly polluted habitats, the lower Kₘ values observed here and in other studies (Failla et al., 1976; Failla & Weinberg, 1977; Lawford et al., 1980) probably reflect physiologically important Zn** uptake systems.

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K** and Mg** had an inhibitory effect on Zn** uptake (Fig. 5a, b) whereas Na** and Ca** did not (results not shown). Both K** and Mg** are strongly taken up by cells whereas there is little

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Zinc uptake and distribution in yeast

Fig. 1. Uptake of Zn$^{2+}$ by S. cerevisiae from 20 μM-ZnCl$_2$ in PIPES buffer, pH 6.5, at 25 °C in the presence of 50 μM-glucose. Bars indicate SEM of individual points (four replicates).

Fig. 2. Uptake of Zn$^{2+}$ by S. cerevisiae from 20 μM-ZnCl$_2$ in PIPES buffer, pH 6.5. (a) Metabolic dependence: ○, 50 mM-glucose (control); ●, no additions; ■, 50 μM-DDG; ▲, 50 mM-glucose and 0.2 mM-KCN; △, 50 mM-glucose incubated at 0-4 °C (all others were at 25 °C). (b) Dependence on plasma-membrane ATPase activity and H$^+$ gradient: ○, 50 mM-glucose alone (control); ●, 50 mM-glucose and 100 μM-DES; □, 50 mM-glucose and 100 μM-DCCD; ■, 50 mM-glucose and 2 mM-DNP. Bars indicate SEM (four replicates).

Fig. 3. Surface adsorption of Zn$^{2+}$ by S. cerevisiae estimated by the intercept of energy-dependent Zn$^{2+}$ uptake with the vertical axis (○) and washing with a non-radioactive ZnCl$_2$ solution in differential extraction (●). Cells were loaded at various ZnCl$_2$ concentrations under conditions as described for Fig. 1. The line indicated is a least-square regression line for the transformed data.

Fig. 4. Kinetics of energy-dependent Zn$^{2+}$ uptake by S. cerevisiae. (a) Zn$^{2+}$ uptake rates at various Zn$^{2+}$ concentrations; other conditions as in Fig. 1 (the rates were derived by linear regression on the energy dependent phase of individual uptake curves). (b) The same data transformed according to Woolf.
uptake of either Na\(^+\) or Ca\(^{2+}\), so that it would appear that inhibition of Zn\(^{2+}\) uptake by these cations is related to their uptake by the cell. Similar inhibition of heavy metal uptake by K\(^+\) has been observed previously (Fuhrmann & Rothstein, 1968; Gadd & Mowll, 1985), and has been attributed to partial depolarization of the plasma-membrane resulting from K\(^+\) uptake. This was presumably also a cause of inhibition of Zn\(^{2+}\) uptake by Mg\(^{2+}\) but competition may be a further factor (Fuhrmann & Rothstein, 1968) and also reduction of nett surface charge by bound cations (Borst-Pauwels, 1981; Borst-Pauwels & Theuvenet, 1984).

The effect of heavy metal cations on Zn\(^{2+}\) uptake was complex and varied with the different heavy metals and their concentrations and with the Zn\(^{2+}\) concentration. Several metals both inhibited and stimulated Zn\(^{2+}\) uptake at different concentrations (Table 1). Fig. 6 shows the effect of Mn\(^{2+}\) and Ni\(^{2+}\) in greater detail as examples. It can be seen that Mn\(^{2+}\) was inhibitory over the range studied (Fig. 6a). Ni\(^{2+}\) was also inhibitory at low concentrations but stimulated Zn\(^{2+}\) uptake at higher concentrations (Fig. 6b).

**K\(^+\) efflux during Zn\(^{2+}\) uptake**

K\(^+\) efflux from cells occurred at all concentrations of added Zn\(^{2+}\) (Figs 7 and 8). K\(^+\) efflux at Zn\(^{2+}\) concentrations below 50 \(\mu\)M was followed by a phase of uptake which restored the K\(^+\) level to that observed initially (Fig. 7). At concentrations above 50 \(\mu\)M, K\(^+\) efflux was not reversed and...
Fig. 7. K⁺ efflux from cells of *S. cerevisiae* in response to Zn²⁺. The tracings shown are examples of K⁺ efflux from cells preloaded with K⁺ in 5 mM-PIPES buffer, pH 6.5 with 50 mM-glucose and 50 μM-KCl. ZnCl₂ solution was added at the point indicated to yield the final concentration (μM) specified for each trace. The cell density was 2–46 × 10⁷ cells ml⁻¹.

Fig. 8. Zn²⁺ uptake, K⁺ efflux and toxic effects of Zn²⁺ over the concentration range used in uptake experiments. ○, Zn²⁺ uptake rate (data from Fig. 4) plotted according to Woolf; ●, initial K⁺ efflux rate, □, maximum nett K⁺ efflux, in response to Zn²⁺ uptake (conditions as in Fig. 7); △, inhibition of cellular H⁺ efflux, ▴, inhibition of K⁺ uptake, by Zn²⁺ as a percentage of the rate in the absence of Zn²⁺; ▲, viability of cells incubated in the presence of various concentrations of ZnCl₂. The number of viable units was estimated by conventional plate-count methods and expressed relative to a control incubated in the absence of added ZnCl₂. All experiments were done in 5 mM-PIPES buffer, pH 6.5, with 50 mM-glucose at 25 °C. All points are the mean of at least two values; bars indicate SEM.

Table 1. Effect of heavy metals on the Zn²⁺ uptake rate

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Metal concn (μM)</th>
<th>Zn²⁺ uptake rate</th>
<th>Percentage of control</th>
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<tr>
<td></td>
<td></td>
<td>nmol min⁻¹ per 10⁷ cells</td>
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<tr>
<td>Ni²⁺</td>
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<td>60.8</td>
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The Zn²⁺ concentration was 20 μM throughout. The mean control Zn²⁺ uptake rate was 0.153 nmol min⁻¹ per 10⁷ cells.
was followed by a phase of equilibrium or slow efflux. The initial rate of K+ efflux was constant at external Zn2+ concentrations below approximately 25 μM but increased above this value (Figs 7 and 8). The total efflux, however, increased with Zn2+ concentration over the whole concentration range but in two phases, and the Zn2+ concentration ranges of these corresponded to the two phases of the K+ efflux rate.

Both H+ efflux and K+ uptake were progressively inhibited by Zn2+ over the concentration range 0–100 μM, 50% inhibition of H+ efflux occurring at approximately 37 μM-Zn2+ and 50% inhibition of K+ uptake at approximately 14.5 μM-Zn2+ (Fig. 8). Complete inhibition of H+ efflux did not occur in this range but complete inhibition of K+ uptake occurred at an external Zn2+ concentration of approximately 45 μM. There was also a progressive reduction in viability of cells incubated for 120 min with Zn2+ at concentrations over this range. These inhibitory effects of Zn2+ clearly contributed to K+ efflux over the whole range of Zn2+ concentration. However, this was most clearly apparent at Zn2+ concentrations above approximately 25 μM, where the Zn2+ concentration dependence of total K+ efflux paralleled that of inhibition of K+ uptake. The Zn2+ concentration that produced inhibition of K+ uptake following efflux also corresponded to that which inhibited K+ uptake by K+ starved cells (Fig. 8). The initial rate of K+ efflux continued to increased above this Zn2+ concentration, presumably indicating that membrane damage continued to increase.

Below 25 μM-Zn2+ the effect of toxicity on K+ efflux was not apparent. As mentioned previously, the rate of K+ efflux in this range was constant while the total efflux showed an increase, but neither total efflux nor efflux rate showed a constant stoichiometric relationship to Zn2+ uptake (Fig. 8). Also, the time courses of Zn2+ uptake (Fig. 1) and K+ efflux (Fig. 7) differed, so that it is apparent that Zn2+ uptake and K+ efflux were not tightly coupled under these conditions. However, a stoichiometric relationship between K+ efflux and M2+ uptake has been observed with some metals and fungi (see Gadd, 1986), but not in all cases, particularly where toxic symptoms may be manifest (Norris & Kelly, 1977; Gadd & Mowll, 1983; Kessels et al., 1985). Variation between different heavy metals was also shown in a recent study where Mn2+ and Zn2+ stimulated K+ efflux from Saccharomyces carlsbergensis while Co2+ and Ni2+ did not (Okorokov et al., 1983a, b). The conditions of these experiments, however, differed from those here, but they underline the complex nature of K+ efflux during heavy metal uptake by yeast, and consequently coupling between Zn2+ uptake and K+ efflux cannot be ruled out in the present study.

Similar biphasic K+ loss to that seen in the present study has been observed in cells incubated with both Hg2+ (Kuypers & Roomans, 1979) and ethidium bromide (Theuvenet et al., 1983) where both graded and all-or-none K+ efflux occurred. K+ efflux showed a similar relationship to ethidium bromide concentration to that seen to Zn2+ concentration here, which supports the view that a major component of K+ efflux resulted from a non-specific mechanism.

Both partial depolarization of the plasma-membrane due to inhibition of H+ efflux and also increased membrane permeability would affect the kinetics of Zn2+ uptake to produce, in the first case, progressive inhibition, and in the second a progressive stimulation, of Zn2+ uptake. Progressive inhibition would probably not be distinguishable in kinetic studies but represents a possible effect of toxic cations, in addition to the masking of surface charge which occurs with non-toxic cations (Roomans et al., 1979; Borst-Pauwels & Theuvenet, 1984). Increased membrane permeability would cause deviation from Michaelis–Menten kinetics and such deviation occurred at Zn2+ concentrations where high K+ efflux was clearly apparent.

Such influence on membrane permeability and H+ efflux by other heavy metals are the likely cause of the complex effects produced by these ions on Zn2+ uptake (Table 1, Fig. 6), especially as the magnitude of the effects on membrane permeability and H+ efflux will probably vary between different metals.

**Intracellular distribution of Zn2+ and Zn2+ efflux from cells**

The Zn2+ content of each of the three intracellular compartments of whole yeast cells – cytosol, vacuole and the membrane and organelle-bound fraction – was proportional to the total cell content over the range investigated, and the fractional distribution of Zn2+ was constant and
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Fig. 9. Zn$^{2+}$ content of intracellular compartments of *S. cerevisiae* cells loaded at various concentrations of ZnCl$_2$ for 10 min (□, ○, △) and 120 min (■, ●, ▲) in 5 mM-PIPES buffer, pH 6.5, with 50 mM-glucose at 25 °C at a cell density of $5 \times 10^7$ ml$^{-1}$. ○, ●, Cytosol fraction; □, ■, vacuole fraction; △, ▲, bound fraction (insoluble). Least square regression lines on the contents of each compartment are shown. All points shown are the mean of four determinations.

Fig. 10. Fractional distribution of Zn$^{2+}$ between intracellular compartments in (a) whole cells and (b) protoplasts of *S. cerevisiae*: C, cytosol; V, vacuole; B, bound (insoluble) fraction. The data used in (a) are from Fig. 9. Bars indicate SEM.

independent both of total loading and of the duration of incubation (Fig. 9). The main compartment of Zn$^{2+}$ in the cell was the vacuole, and most of the remainder was in the firmly bound fraction with only a very small fraction in the cytosolic compartments in whole cells (Fig. 10a). It was impossible, due to the fragility of unprotected vacuoles, to separate the cytosolic and vacuolar fractions of protoplasts, but as the firmly bound fraction was only slightly smaller than that of whole cells (Fig. 10b) it is probable that this mainly comprised intracellular or plasmamembrane-bound Zn$^{2+}$ rather than wall-bound Zn$^{2+}$.

Compartmentation into the vacuole has also been found to occur for other physiologically important metals – Mn$^{2+}$ (Okorokov *et al.*, 1977), Ca$^{2+}$ (Eilam *et al.*, 1985), K$^+$ and Mg$^{2+}$ (Lichko *et al.*, 1982) – and this is consistent with the role of the vacuole as a reserve of physiologically important but potentially damaging materials (Matile & Wiemken, 1976).

As in past studies of Zn$^{2+}$ uptake by yeasts (Failla *et al.*, 1976; Lawford *et al.*, 1980) there was no efflux of Zn$^{2+}$ from loaded cells, either in the presence of 50 mM-glucose or 20 μM-Zn$^{2+}$ or both, or in the absence of both (results not shown). This was probably due to the compartmentation of Zn$^{2+}$ into the vacuole and bound fraction.

**Uptake of Zn$^{2+}$ by isolated vacuoles**

Compartmentation of Zn$^{2+}$ such as described above requires an energy-dependent Zn$^{2+}$ transport process to establish and maintain it, and isolated vacuoles were found to possess such a transport system for Zn$^{2+}$ (Fig. 11). Uptake was stimulated by 1.0 mM-ATP and inhibited by 30 μM-nigericin and 40 μM-DCCD. Since Zn$^{2+}$ uptake by vacuoles was stimulated by ATP and inhibited by DCCD, it was apparent that an ATPase activity was required. Inhibition by nigericin indicated that a proton gradient, presumably across the vacuolar membrane, was also necessary for Zn$^{2+}$ uptake by vacuoles.

Basic amino acids and Ca$^{2+}$ are taken up by yeast cells via a proton antiport system (Ohsumi & Anraku, 1981, 1983; Eilam *et al.*, 1985) and several heavy metals induce dissipation of the H$^+$ gradient in vacuolar preparations (Okorokov *et al.*, 1985). Consequently, it has been proposed
that heavy metals are taken up by vacuoles by means of a similar system (Okorokov, 1985; Okorokov et al., 1985) and this is fully consistent with our results, ATPase activity maintaining the necessary H⁺ gradient with the result that absence of ATP or presence of DCCD or nigericin inhibited uptake. Here nigericin acted as an uncoupling agent, inhibiting Zn²⁺ uptake by dissipation of the H⁺ gradient.

To conclude, S. cerevisiae is able to take up Zn²⁺ from the external medium into specific compartments of the cell and these processes of uptake and distribution of Zn²⁺ are mediated by energy-dependent transport systems at the plasma-membrane and at the vacuolar membrane. However, significant toxic effects accompany the uptake of Zn²⁺ and appear both to affect the kinetics of Zn²⁺ uptake and to be largely responsible for the K⁺ efflux which accompanies it. Moreover, it should be remembered that Zn²⁺ has low toxicity when compared with other heavy metals and that it is likely that toxic effects of this type will be of much greater importance in the uptake of these other heavy metals.

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