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Cadmium accumulation and its effects on intracellular ion pools in a brewing strain of *Saccharomyces cerevisiae*

KJ Blackwell and JM Tobin

School of Biotechnology, Dublin City University, Dublin 9, Ireland

The presence of glucose resulted in a two- to three-fold increase in levels of Cd^{2+} accumulated by *Saccharomyces cerevisiae* after 5 h compared with those observed in the absence of glucose. However, time-dependent Cd^{2+} uptake continued in the absence of glucose over 5 h, resulting in an appreciable increase in cellular Cd^{2+} levels. Substantial K^+ efflux but little Mg^{2+} and negligible Ca^{2+} release was observed. Cell fractionation revealed that the bulk of intracellular Cd^{2+} was located in the vacuolar (25%) and bound (60%) fractions. Accumulation of Cd^{2+} ions impacted most noticeably on K^+ rather than Mg^{2+} levels in intracellular compartments. Cytoplasmic and particularly vacuolar K^+ levels decreased as Cd^{2+} sequestration continued resulting in increased extracellular levels. In contrast, corresponding intracellular Mg^{2+} pools were only modestly affected with a slight increase and decrease observed in the cytoplasmic and vacuolar fractions respectively. However, levels of bound Mg^{2+} decreased in response to continued Cd^{2+} accumulation.

Keywords: cadmium; magnesium; potassium; intracellular localisation; *Saccharomyces cerevisiae*

Introduction

Interest in metal accumulation by microorganisms has increased in recent years because of concern over possible toxic metal transfer from microorganisms through the food chain to higher organisms (including humans), and in relation to the bioremediation potential of microorganisms for removal and/or recovery of metal waste [1,3]. *Saccharomyces cerevisiae* possesses good metal-binding potential [16,19,20]. However the cost of producing biomass exclusively for an industrial scale metal removal process could be a serious economic disadvantage [8], and it would be therefore desirable to utilise existing waste microbial biomass, for example, from the brewing industry.

Metal uptake by microorganisms occurs in two stages: the first stage consisting of passive adsorption of metal ions to the external cell surface, and a second stage in which metal ions are subsequently transported through the cell membrane into the cell itself [1,3,7]. The plasma membrane bound H^+-ATPase creates a transmembrane electrochemical proton gradient, and the electrical component of this transmembrane gradient, the membrane potential, is the primary driving force for cation transport into the cell [12,21]. As ion transport is not driven directly by the hydrolysis of ATP but is dependent on the electrochemical gradient that is the result of H^+-ATPase activity, it is possible for ion accumulation to continue when energy metabolism has ceased provided such a transmembrane gradient can be maintained [12]. However, regeneration of the gradient would be essential for sustained transport, ie, a metabolisable substrate would be required.

Cd^{2+} has no biological function [12] and can exert toxic effects at low concentrations, including inhibition of growth [13] and cell death [9]. Despite these effects, Cd^{2+} ions can be accumulated to an appreciable extent by viable and nonviable biomass. Little is known about the consequent effects of Cd^{2+} uptake on levels of biological ions in subcellular compartments, ie, cytoplasmic, vacuolar and bound compartments. Accordingly, the purpose of this study was firstly to investigate the uptake and intracellular compartmentation of cadmium by a brewing strain of *S. cerevisiae* in both the presence and absence of glucose as an energy source. Secondly, consequent changes to Mg^{2+} and K^+ in intracellular compartments and their extracellular release were investigated in the presence and absence of Cd^{2+}, and mass balances were successfully applied to each ion pool in order to correlate total accumulation/release levels with changes to intracellular and extracellular concentrations.

Materials and methods

Organism, media, and growth conditions

A brewing strain of *S. cerevisiae* was routinely maintained on malt extract agar (Oxoid, Hampshire, UK). For experimental purposes, cultures were grown in a liquid medium comprised of (in g L^{-1}): KH_2PO_4, 2.72; K_2HPO_4, 3.98; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.0022; $ZnSO_4 \cdot 7H_2O$, 0.004, $MnSO_4 \cdot 4H_2O$, 0.004; $CuSO_4 \cdot 5H_2O$, 0.004; D-glucose, 20.0; and yeast extract, 1.0. Cultures were grown at 25°C on an orbital shaker (150 rpm).

Cd^{2+} uptake experiments

The medium of the above composition was inoculated with a 24-h starter culture, and cells from early stationary phase (18 h) were harvested by vacuum filtration through Whatman No. 1 filter paper, washed twice with distilled deionised water, and pressed dry with filter paper. Biomass was added to 200 ml of 10 mM 2-[-N-morpholine] ethanosulphonic acid (MES buffer), previously adjusted to pH 5.5 with 1 M NaOH to a final concentration of 0.2 g (dry

Correspondence: Dr JM Tobin, School of Biotechnology, Dublin City University, Dublin 9, Ireland
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weight) 200 ml^{-1}, and allowed to equilibrate for 90 min with rotary shaking at room temperature. Where required, glucose (2% w/v) was added 5 min prior to addition of Cd^{2+} (as nitrate salt) to the desired final concentration. At specified intervals, 5-ml samples were removed, the biomass separated by centrifugation (1200 × *g*, 5 min) and the supernatants were retained for metal analysis.

Subcellular location of metal ions

Subcellular fractionation of cells followed a modification of the protocol used by Huber-Walchi and Wiemken [11] and White and Gadd [20]. The wash fraction was obtained by washing pellets twice, with 1.5 ml of 10 mM Tris-MES buffer pH 6.0, at 0–4°C. The cytoplasmic membrane was permeabilised by resuspending the pellet in 1 ml of 10 mM Tris-MES buffer, pH 6.0, 0.7 M sorbitol at 25°C. DEAE-dextran (40 μl, 10 mg ml^{-1}) was added to the same buffer, mixed and incubated for 30 s at 25°C. Cells were separated by centrifugation (8000 × *g*, 40 s) and the supernatant was removed and retained. The permeabilised cells were then washed three times with 0.7 M sorbitol in 10 mM Tris-MES buffer, pH 6.0, at 0–4°C, with incubation for 1 min at each wash. Supernatants were removed and retained after centrifugation at each wash and were combined with that from the initial permeabilisation step.

The vacuolar membrane was permeabilised by suspending the remaining pellet in 60% (v/v) methanol at 1–4°C. After incubation for 30 s, cell fragments were centrifuged (8000 × *g*, 40 s) and the supernatant was retained and finally combined with those from further washes with 60% (v/v) methanol (three times, 1 ml each) followed by 10 mM Tris-MES, pH 6.0 (three times, 1 ml each). Suspensions were incubated for 1 min at 0–4°C for each wash.

The remaining pellet (termed the bound fraction) was digested in 0.5 ml of 6 M HNO_3 for 1 h at 100°C, and subsequently diluted by addition of 3 ml distilled deionised water. All fractions were analysed for Cd^{2+}, Mg^{2+}, Ca^{2+} and K^+ as described below.

Metal analysis

Cd^{2+}, Mg^{2+}, Ca^{2+} and K^+ were analysed using a Perkin-Elmer 3100 atomic absorption spectrophotometer, fitted with a 10-cm single slot burner head, with an air-acetylene flame. Metal concentrations were determined by reference to appropriate standard metal solutions.

Dry weights

Cell dry weights were determined after drying in foil cups overnight at 55°C.

Results

Cd^{2+} accumulation by S. cerevisiae

Live cells of *S. cerevisiae* adsorbed cadmium from solution in both the presence and absence of 2% (w/v) glucose at all three Cd^{2+} concentrations studied (Figure 1). In the absence of glucose, there was a phase of rapid Cd^{2+} uptake in the first minutes of contact, followed by a period of slow, sustained Cd^{2+} uptake. After 5 h cellular Cd^{2+} levels were 25, 38 and 56 μmol g^{-1} (dry weight) at initial Cd^{2+} concentrations of 100, 200 and 250 μM respectively

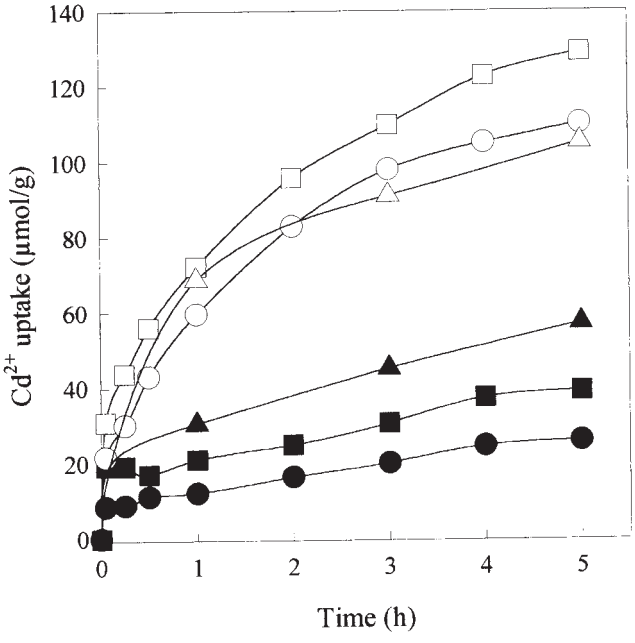

Figure 1 Influence of glucose on Cd^{2+} uptake by live *S. cerevisiae* cells. Cells were incubated in 10 mM MES buffer with 100 μM (● and ○), 200 μM (■ and □), and 250 μM (▲ and △) Cd^{2+} in the presence (open symbols) and absence (closed symbols) of 2% glucose. Each point is an average of two determinations and typical results from one of three experiments are shown.

(corresponding to *ca* 25%, 19% and 22.4% Cd^{2+} removal, respectively). In the presence of glucose, Cd^{2+} uptake was most rapid during the first hour of contact, with continuous Cd^{2+} accumulation over the next 4 h, although the rate of active accumulation decreased with time. After 5 h of incubation in the presence of metal and glucose, cellular levels of Cd^{2+} were approximately 110, 128 and 102 μmol g^{-1} (dry weight) for initial Cd^{2+} concentrations of 100, 200 and 250 μM (corresponding to *ca* 100%, 64% and 41% Cd^{2+} removal respectively).

Displacement of Mg^{2+}, Ca^{2+} and K^+ ions by Cd^{2+}

In native freshly-harvested biomass total cellular levels of Mg^{2+}, K^+ and Ca^{2+} were determined to be approximately 225, 620 and less than 4 μmol g^{-1} (dry weight) respectively. It was found that Ca^{2+} release in response to Cd^{2+} uptake was negligible, presumably due to the very low levels present initially and its analysis was discontinued. K^+ and Mg^{2+} ions (approximately 70 and 6 μmol g^{-1}, respectively) were released from the biomass during the 1.5-h incubation period. No net release of Mg^{2+} was observed at any of the Cd^{2+} concentrations examined in the presence or absence of glucose. In contrast, Cd^{2+} ions induced a net release of K^+ both in the presence and absence of glucose (Figure 2). In the absence of glucose, net release of K^+ (when compared with controls) did not become apparent until 1 h had elapsed, whereas it was immediate in the presence of glucose. Substantially greater amounts of K^+ were released in the presence of glucose, which correlated with increased active Cd^{2+} accumulation.

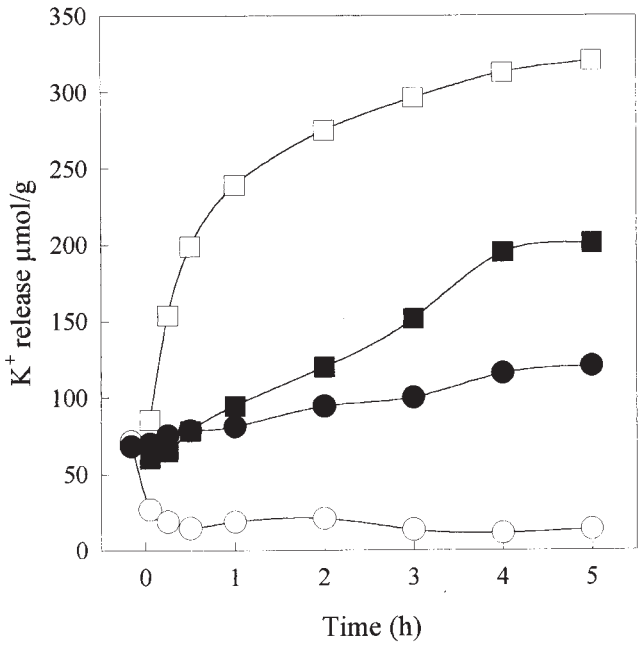

Figure 2 Cadmium-induced K^+ release. Extracellular K^+ levels in the presence (□ and ■) and absence (○ and ●) of 200 μM Cd^{2+} are shown. Cells were incubated in 10 mM MES buffer in the presence (open symbols) and absence (closed symbols) of 2% glucose. Glucose was added 7 min prior to Cd^{2+} addition at 0 h. Each point is an average of two replicates and typical results from one of three experiments shown.

Subcellular location of Cd^{2+} and its effect on intracellular levels of Mg^{2+} and K^+

Cd^{2+} ions were present in all subcellular fractions examined irrespective of glucose addition, and specific levels of Cd^{2+} present in each fraction increased with time (Table 1). While the presence of glucose resulted in higher levels of Cd^{2+} accumulation, the percentage of Cd^{2+} in each fraction expressed as a percentage of total cellular Cd^{2+} remained effectively constant at each time point over the 5-h period in both the presence and absence of glucose. The level of Cd^{2+} in each fraction as a percentage of total cellular Cd^{2+} was approximately 5–10, 2–3, 22–28 and 60–65 for the wash, cytoplasmic, vacuolar and bound fractions respectively. Mass balances between overall Cd^{2+} uptake, as determined from the decrease in extracellular Cd^{2+} levels and the sum of Cd^{2+} levels present in subcellular fractions, agreed to within 10%. Small quantities of Cd^{2+} ions were detected in the wash fraction which increased with time. Cytoplasmic Cd^{2+} levels were the lowest of the four fractions and were some 30–65% higher in the cytoplasm of cells incubated with glucose. The influence of glucose on Cd^{2+} accumulation was most apparent in the vacuolar and bound fractions. Cd^{2+} in vacuoles in cells incubated in the absence of glucose was apparent within 1 h and continued to increase over the next 4 h. By comparison, vacuolar Cd^{2+} levels of cells incubated with glucose were three-fold greater at 1 h and approximately double at successive time points. The bound fraction at each time point consistently contained the greatest amount of Cd^{2+} (almost two thirds of total cellular Cd^{2+}). Overall, total cellular Cd^{2+} uptake in the presence of glucose was approximately twice that without glucose at each of the time points examined.

Cd^{2+} sequestration had a greater impact on subcellular levels of K^+ than Mg^{2+}. Although no net extracellular Mg^{2+} release was observed, there were changes to Mg^{2+} levels in subcellular compartments. Mg^{2+} ions in wash fractions increased over 5 h (in the presence of Cd^{2+}), by 21 and 27.6 μmol g^{-1} in the absence and presence of glucose respectively (Table 2). This was complemented by a decrease in bound Mg^{2+} of approximately similar magnitude in both the presence and absence of glucose. Small increases in cytoplasmic and appreciable loss of vacuolar Mg^{2+} occurred in the presence of Cd^{2+} ions in both the presence and absence of glucose. Depletion of vacuolar Mg^{2+} was most noticeable in the absence of glucose, where it was approximately twice that observed in the absence of glucose. Bound Mg^{2+} decreased in the presence and absence of glucose, possibly as a result of a transfer of Mg^{2+} from the pellet to the wash fraction in the presence of Cd^{2+} ions.

As shown in Figure 2, the presence of Cd^{2+} ions induced substantial K^+ efflux. Net extracellular K^+ release was greater from cells incubated at 250 μM Cd^{2+} than at 200 μM (results not shown). Mass balances on total K^+ in the system (sum of total cellular K^+ and overall K^+ release) at each time point agreed to within 10%. Large amounts of K^+ were detected in the wash fraction (55–76 μmol g^{-1}) in the presence and absence of glucose and Cd^{2+} ions, which remained effectively unchanged over the course of the experiment (Table 3). Cytoplasmic K^+ levels were the highest of the three ions examined, and decreased over time in the presence of Cd^{2+}, with a greater decrease (two-fold)

Table 1 Subcellular distribution of Cd^{2+} ions in *S. cerevisiae* cells incubated at an initial concentration of 250 μM Cd^{2+}. Mean values ± standard error of the means from four replicate determinations are shown

	Intracellular Cd^{2+} levels (μmol g^{-1})							
Time	0 h		1 h		3 h		5 h	
2% glucose	–	+	–	+	–	+	–	+
Wash	0.0	0.0	2.5 ± 0.2	3.8 ± 0.1	3.9 ± 0.1	7.2 ± 0.2	6.3 ± 0.4	9.7 ± 0.1
Cytosolic	0.0	0.0	0.9 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	2.5 ± 0.1
Vacuolar	0.0	0.0	6.9 ± 0.2	20.0 ± 0.6	12.2 ± 0.2	26.0 ± 0.7	15.9 ± 0.4	31.2 ± 0.8
Bound	0.0	0.0	20.3 ± 0.3	48.2 ± 0.8	33.4 ± 0.6	62.2 ± 1.5	37.9 ± 0.5	67.1 ± 0.6
Sum	0.0	0.0	30.6 ± 0.8	73.5 ± 1.6	51.1 ± 1.0	97.6 ± 2.5	62.0 ± 1.4	110.5 ± 1.6
Total cells	0.0	0.0	30.5 ± 0.4	68.8 ± 2.1	45.1 ± 0.9	90.9 ± 1.7	56.9 ± 2.1	105 ± 1.9

Table 2 Subcellular distribution of Mg^{2+} ions in *S. cerevisiae* cells incubated at an initial concentration of 250 μM Cd^{2+}. Mean values ± standard error of the means from four replicate determinations are shown

	Intracellular Mg^{2+} levels during Cd^{2+} accumulation (μmol g^{-1})							
Time	0 h		1 h		3 h		5 h	
2% glucose	–	+	–	+	–	+	–	+
Wash	8.9 ± 0.6	6.2 ± 0.4	16.7 ± 0.5	18.4 ± 0.6	18.9 ± 0.4	23.5 ± 0.5	29.9 ± 1.3	33.8 ± 1.3
Cytosolic	4.3 ± 0.1	4.2 ± 0.2	6.5 ± 0.4	5.9 ± 0.2	7.1 ± 0.7	6.1 ± 0.4	6.2 ± 0.2	6.4 ± 0.1
Vacuolar	136.7 ± 1.6	141.7 ± 8.5	118.2 ± 2.8	132.7 ± 3.8	118.7 ± 2.8	133.5 ± 3.7	111.8 ± 1.7	125.6 ± 2.1
Bound	73.7 ± 0.8	72.5 ± 0.8	61.4 ± 1.6	64.3 ± 1.3	62.8 ± 0.9	53.9 ± 1.5	53.0 ± 1.3	50.2 ± 1.1
Sum	223.6 ± 3.1	224.6 ± 9.9	201.8 ± 5.3	221.3 ± 5.9	207.5 ± 4.8	217.0 ± 6.1	200.9 ± 4.5	216.0 ± 4.6
Total cells	215.3 ± 4.5	221.3 ± 4.2	209.7 ± 7.3	216.4 ± 4.4	210.6 ± 2.7	212.9 ± 4.9	206.4 ± 3.7	208.6 ± 5.7

Table 3 Subcellular distribution of K^+ ions in *S. cerevisiae* cells incubated at an initial concentration of 250 μM Cd^{2+}. Mean values ± standard error of the means from four replicate determinations are shown

	Intracellular K^+ levels during Cd^{2+} accumulation (μmol g^{-1})							
Time	0 h		1 h		3 h		5 h	
2% glucose	–	+	–	+	–	+	–	+
Wash	54.9 ± 13.0	69.3 ± 2.2	75.7 ± 5.5	75.8 ± 5.0	59.8 ± 2.6	66.9 ± 2.9	61.1 ± 1.4	72.7 ± 3.2
Cytosolic	36.0 ± 1.0	48.9 ± 1.7	27.1 ± 1.0	27.9 ± 0.8	27.3 ± 1.3	30.8 ± 0.3	19.4 ± 0.9	21.6 ± 1.0
Vacuolar	430.1 ± 14.7	450.6 ± 15.4	326.8 ± 7.1	271.2 ± 8.5	262.2 ± 4.1	218.1 ± 3.7	212.1 ± 7.0	200.1 ± 4.4
Bound	6.6 ± 1.5	8.7 ± 1.8	8.1 ± 1.6	14.6 ± 2.8	8.9 ± 2.1	12.7 ± 1.9	5.7 ± 2.0	12.7 ± 1.9
Sum	527.6 ± 30.2	577.5 ± 21.1	437.7 ± 15.2	389.5 ± 17.1	358.2 ± 10.1	328.5 ± 8.8	298.3 ± 11.3	307.1 ± 10.5
Total cells	548.3 ± 21.5	585.7 ± 8.5	453.6 ± 8.9	362.4 ± 7.3	372.7 ± 12.6	336.9 ± 4.3	278.8 ± 14.6	303.4 ± 5.9

noted in the presence of glucose due to the higher levels initially present at 0 h. Bound K^+ was minimal (less than 2% of total cellular K^+), suggesting that K^+ is predominantly a 'soluble' ion rather than associated with solid cellular material. The bulk of cellular K^+ (70%) was localised in the vacuole, approximately 440 μmol g^{-1} at 0 h and it was here that the greatest changes were observed. The presence of Cd^{2+} ions induced substantial K^+ loss from cell vacuoles and was most apparent in the presence of glucose. This vacuolar loss of K^+ was in most cases approximately equivalent to or greater than the observed net extracellular increase at each time point.

Discussion

In the present work, a period of starvation was included prior to addition of metal ions for the purpose of depleting cellular energy reserves. This, coupled with the absence of a metabolisable substrate would lead to the expectation that metal uptake could not have been metabolically mediated. However the observed time-dependent nature of uptake suggests the presence of residual enzyme activity. That a significant amount of cadmium was ultimately sequestered in the vacuole further suggests some form of energy-driven ion transport process [3,19]. Diffusion processes are also likely involved [10] as Cd^{2+} accumulation increased with increasing external Cd^{2+} concentration, most markedly in the glucose-free studies.

Stimulation of Cd^{2+} uptake by glucose may result from the biosynthesis of transport proteins and/or plasma membrane H^+-ATPase activity [12]. Cells incubated in the presence of glucose with 100 μM Cd^{2+} were able to remove virtually all metal present within 5 h. Further increasing external metal concentration resulted in a slight increase in Cd^{2+} accumulation at 200 but not at 250 μM. While increased toxicity effects at 250 μM Cd^{2+} might limit uptake as observed after 1 h, absolute accumulation levels are lower than at 200 μM Cd^{2+}. It is likely that the rate-limiting step is the actual transport of Cd^{2+} ions into the cell [18], and is independent of external Cd^{2+} above certain threshold concentrations.

Cd^{2+} ions induced negligible release of Ca^{2+} and Mg^{2+} ions in the presence or absence of glucose suggesting that Cd^{2+} ions bind to sites not previously occupied by these ions. Loss of K^+ as observed here is a feature of heavy metal accumulation [5,9,16] and has been used as an indicator of metal toxicity towards cells. K^+ release here appears to be an integral part of intracellular Cd^{2+} accumulation and is more marked in the presence of glucose correlating with increased Cd^{2+} accumulation. However, there is no evidence of a stoichiometric balance between K^+ release and Cd^{2+} uptake. Increased K^+ efflux further enhances divalent cation accumulation by increasing the membrane potential [4,12].

Cd^{2+} ions were localised intracellularly in both the presence and absence of glucose. It is likely that the Cd^{2+} ions released by washing cells represent loosely bound Cd^{2+} or ions that were taken up into waterfilled spaces in the yeast cell wall [1]. All cytoplasmic levels of Cd^{2+} in this study were maintained at low levels, presumably due to increased transport of Cd^{2+} ions to the vacuole where a two-fold increase in Cd^{2+} levels was observed over cells incubated

without glucose. However, the majority of Cd^{2+} was localised in the bound fraction. This distribution indicates the preferred tendency of cadmium to bind with cellular material than to be located in 'soluble pools' within the cell [13,20]. In contrast, Mn^{2+} and to lesser extent Sr^{2+} were previously observed to be localised in vacuoles (90 and 70% respectively) with the remainder of soluble metal present in the cytosolic compartment of *S. cerevisiae* Delft II [15]. Levels of bound metal were not reported.

Concomitant with intracellular Cd^{2+} accumulation were changes in the subcellular distribution of Mg^{2+} and K^+ ions. The increase in Mg^{2+} levels in the wash fraction along with the drop observed in the bound fraction may be indicative of Cd^{2+} destabilising bound Mg^{2+}. Overall, Mg^{2+} was not generally displaced by Cd^{2+} with *ca* 90% Mg^{2+} being retained by the cell. Small cytoplasmic variations possibly result from larger changes observed in vacuolar Mg^{2+} levels and it appears that the low extracellular Mg^{2+} concentrations observed may be due to loss of Mg^{2+} ions from the vacuolar compartment. This contrasts with literature reports of simple biosorption type binding [6] but in those studies a non-metabolising mould biomass was used.

Changes to subcellular compartmentation of K^+ were confined to the cytoplasmic and vacuolar fractions as bound K^+ and that present in the wash fraction did not change appreciably with time in the presence of Cd^{2+}. K^+ loss from cytoplasmic and vacuolar fractions is consistent with previous reports [14,17] and it appears that K^+ is primarily a soluble ion and the observed decreases may be attributed to K^+ extrusion in response to the toxic effects of Cd^{2+} and resulting lesions in the plasma membrane [2,21].

These results underscore the importance of energy-driven accumulation processes which here resulted in two- to four-fold increases in cellular cadmium levels. However, potential benefits for detoxification applications are limited by corresponding increases in cell toxicity.

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