ACCUMULATION AND SUBCELLULAR LOCALISATION OF METAL CATIONS BY Saccharomyces cerevisiae

Ph D Research Thesis by

KEVIN J BLACKWELL B Sc

Supervisor

Dr JOHN M TOBIN

SCHOOL OF BIOLOGICAL SCIENCES

DUBLIN CITY UNIVERSITY

SEPTEMBER, 1998

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work

Signed Kari Blackwell ID No 93701055

Date 10th September, 1998

Dedicated to all my family, and friends you know who you are

"I am but wounded, I am not slain,
I'll layne down and bleed awhile,
And then I'll rise and fight again"

Old Scottish border ballad

Imagine the following scene

Its a beautiful, bright, warm summer's day In the corner of a meadow, next to its burrow, an academically minded rabbit is furiously tapping away on a laptop computer A fox is attracted by the commotion

"tap, tap, tap, tap, tap" Pause "tap, tap, tap "
The astonished fox observes the rabbit for some minutes before saying anything
"Mr Rabbit, what exactly are you doing?"
"Oh, Mr Fox, I am writing my graduate thesis entitled How Rabbits Eat Foxes "
The sceptical fox laughs and says "You cannot be serious?"
"But I am" replied the Rabbit "Come into my burrow and I will show you my data"
Five minutes later, the rabbit comes out alone, gnawing on a fox bone

A little later, a wolf passing through the meadow happens upon the rabbit
"tap, tap, tap, tap, tap" Pause "tap, tap, tap "
"Little rabbit, what are you doing?"
"I am writing a thesis on How Rabbits Eat Wolves"
"Such a thing is an impossibility" said the wolf imperiously
"Not so" said the rabbit "Come into my burrow and see the data for yourself "
Ten minutes later, comes back out, tossing a wolf's bone over his shoulder

Some hours later, a bear is attracted by the rabbit's tapping
"tap, tap, tap, tap, tap" Pause "tap, tap, tap "
"Mr Rabbit, what are you up to?"
"I am writing my thesis on How Rabbits Eat Bears"
"You crazy rabbit That just does not happen "
"I disagree" says the rabbit "Come into my burrow and I will show you my data"

The bear follows the rabbit into the burrow and is met by an incredible sight In one corner is a pile of fox bones In another corner is a pile of wolf bones And stretched out in the opposite corner was a huge lion, belching and cleaning his teeth with a toothpick

The moral of the story is
It does not matter what your thesis is about
It does not matter what you use for your data
It does not even have to make sense
But what is important is who you have as a thesis advisor

TABLE OF CONTENTS

ABSTRACT

Accumulation and subcellular localisation of metal cations by Saccharomyces cerevisiae

Ph.D. Research Thesis by

Kevin J. Blackwell, B.Sc.

ABSTRACT

Uptake of Cd^{2+} ions in the presence or absence of glucose by a brewing strain of *Saccharomyces cerevisiae* was examined in unbuffered, buffered and pH controlled systems Levels of Cd^{2+} taken up, and K^+ release were influenced by the degree of pH control in each system A time-dependent component of uptake was observed in the absence of glucose which was attributed to a degree of metabolic activity Subcellular localisation studies determined that Cd^{2+} was predominantly bound by insoluble cellular material (approximately 70%) whereas Mn^{2+} was localised to soluble pools, particularly the vacuole (less than 45% bound by insoluble cellular material) In the presence and absence of glucose, Sr^{2+} was sequestered mainly to bound (50-68%) or soluble (65-85%) subcellular pools, respectively, but the converse was observed with Cu^{2+} localisation Perturbation of subcellular Mg^{2+} pools was observed in the presence of glucose only, and consisted of Mg^{2+} displacement from vacuolar or bound pools to other subcellular compartments Vacuolar K^+ loss was recorded during accumulation of all cations in the absence of glucose but only during Cd^{2+} uptake in the presence of glucose Growth inhibitory effects of Mn^{2+} on *S cerevisiae* NCYC 1383 were eliminated by supplementing with excess Mg^{2+} ions but not with Ca^{2+} or K^+ Intracellular Mg levels were amenable to manipulation and it was observed that Mg-enriched cells accumulated less Mn^{2+} (by approximately half) than their unsupplemented counterparts In addition, cells possessing low intracellular Mg levels were more susceptible to Mn^{2+} toxicity Toxicity was dependent on the cellular Mg Mn ratio, a critical ratio of 2 0 was identified below which toxicity became acute An alternative technique for differential extraction of subcellular ion pools was developed utilising specific biochemical markers to characterise separation of cytoplasmic and vacuolar ion pools, and the results obtained compared with the original method Some differences arose between levels of metal cations recovered but similar trends in subcellular localisation patterns were evident It was concluded that either protocol could be used to characterise subcellular sequestration of accumulated metal cations

LIST OF FIGURES

LIST OF TABLES

Table 3 4 7 6 Distribution of G-3-PDH and CpY activity in subcellular pools of cells incubated with 5 μM Cu^{2+}

Table 3.4.7.7 Cu^{2+} levels in subcellular pools of control cells

Table 3.4.7.8 Cu^{2+} levels in subcellular pools of cells incubated with 5 μM Cu^{2+}

PUBLICATIONS

Blackwell, K J , Singleton, I , Tobin, J M (1995) Metal cation uptake by yeast a review *Applied Microbiology and Biotechnology*, **43**, 579-584

Blackwell, K.J. and Tobin J M (1996) The influence of buffering on Cd^{2+} uptake by *Saccharomyces cerevisiae* Abstracts from the proceedings of the Fifth Environmental Researchers Colloquium, University College Cork, January 1995 *Biology and Environment*, **95B**, 123-168

Blackwell, K.J., Tobin, J M , Avery, S V (1997) Manganese uptake and toxicity in magnesium-supplemented and unsupplemented *Saccharomyces cerevisiae Applied Microbiology and Biotechnology*, **47**, 180-184

Blackwell, K J., Avery, S V , Tobin, J M (1998) Manganese toxicity towards *Saccharomyces cerevisiae* Dependence on intracellular and extracellular magnesium concentrations *Applied Microbiology and Biotechnology*, **49**, 751-757

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr John Tobin for the opportunity to complete this period of study in Dublin City University, and for the advice, encouragement and professionalism shown towards me during my years of research

I extend my thanks to the head of the department, Professor Richard O Kennedy and all the staff of the School of Biological Sciences for their courtesy and assistance when required throughout my time here

To Dr Simon Avery, I am forever grateful for not one, but two opportunities to work under his guidance in Oxford Brookes University A big thank you to all the inhabitants of the Tonge Building who made my stay so enjoyable I had a ball

To all the souls who had the privilege to be involved with A112 From the outset, Joe, Marianne and Robert made each day memorable, and kept me on the straight and narrow (most of the time!!) Guys, it was a pleasure and I treasure the friendship we forged together In more recent times, the newer recruits made sure there was never a dull moment And to my longstanding buddies, Donal and Paula, we chose similar paths, and may they lead to success and prosperity It was an honour to know you guys

To all postgrads of the department, past and present, whose paths I was lucky enough to cross, I say thanks for the memories and great times we had together

Finally, a special thank you to my parents and family, without whose help, encouragement, support and love, I would not be where I am today You were always there for me when I needed you

CHAPTER 1

INTRODUCTION

Accumulation and subcellular localisation of metal cations by Saccharomyces cerevisiae

Ph D. Research Thesis

Kevin J Blackwell B.Sc.

CHAPTER 1. INTRODUCTION.

1 1 Metals and microorganisms

The study of the interactions between metals and microorganisms has long been of scientific interest and has been the subject of a number of reviews (Gadd, 1993, Volesky and Holan, 1995, 1995) Metals are directly or indirectly involved in all aspects of microbial growth, metabolism and differentiation Many metals are essential, for example Zn^{2+} and Mn^{2+}, which play important roles in growth, budding and nuclear development of *Saccharomyces cerevisiae* (Loukin and Kung, 1995), whereas others, such as Cd^{2+} or Ag^{+}, have no known biological function In an environmental context, accelerating pollution by toxic metals, metalloids, radionuclides and organometal(oid)s has influenced research towards the biotechnological potential of utilising biological material for metal removal and/or recovery from industrial waste streams However, the recent discovery of thriving biological communities in metal-rich ecosystems around mid-ocean ridges where a supply of heavy metals is actually essential for life has opened new areas of research and underlines the complex nature of metals in the biosphere (Nisbet and Fowler, 1995) All material of biological origin possesses an acknowledged potential for accumulating metal cations Plants (Kramer *et al*, 1996, Raskin *et al*, 1997, Chaney *et al*, 1997), bacteria (Scott and Palmer, 1990, Urrutia Mera *et al*, 1992, Golab *et al*, 1995), algae (Aksu and Kutsal, 1987, Crist *et al*, 1990, Sloof *et al*, 1995), fungi (Tobin *et al*, 1994, Brady and Tobin, 1995, Sag and Kutsal, 1996a,b) and yeasts (Avery and Tobin, 1992, 1993, White and Gadd, 1986) have all been shown to accumulate a wide variety of metal cations as a result of physico-chemical mechanisms, and transport systems of varying specificity

Conventional physico-chemical methods for the treatment of metal bearing waste streams involved precipitation/crystallisation, coagulation/flocculation, adsorption, ion exchange and electrochemical deposition, but these techniques become less effective as metal ion concentrations fall to the low parts per million range and where large volumes of liquor are to be treated (Brady and Tobin, 1995) Those processes involving precipitation/ crystallisation are relatively slow in requiring about seven days for the precipitation of metal contaminants (Azab *et al*, 1990) Precipitation is controlled by pH and applied dose of flocculants such as ferric chloride, sodium carbonate, aluminum hydroxide and EDTA, although addition of polyelectrolytes may improve precipitation efficiency (Enzminger,

1991) Treatments based on adsorption, electrochemical separation and ion exchange can be expensive, complex, difficult to control and also involve problems with the disposal of the extracted waste materials (Azab *et al*, 1990) In recent years, methods based on biological systems which include the use of immobilised biomass (Brady *et al*, 1994b, Wong and Fung, 1997, Stoll and Duncan, 1997) or its derivatives (Spears and Vincent, 1997) are being considered as alternatives to, or as an additional polishing step, alongside conventional chemical treatments (Gadd, 1990a, Volesky, 1990) Metal accumulation by some types of biomass has been reported to exceed that of activated charcoal filters or certain ion exchange resins (Tsezos and Volesky, 1981, Azab *et al*, 1990) and a number of biological processes for treatment of liquid, metal-bearing streams have been predicted to be at least as cost effective and in some cases, considerably less expensive than conventional chemical precipitation or ion exchange processes currently in use (Eccles, 1995)

1 2 Essentiality and toxicity of metal ions

In microbial cells, essential metal ions have many functions, some of which remain to be characterised Known essential cations include K, Mg, Ca, Na, Mn, Fe, Zn, Co, Ni, Cu and Mo Cations such as K and Mg are present at macro levels within the cell (Walker, 1994) whereas the majority are present in trace amounts The importance of the presence of trace metals in cellular metabolism is underlined by the fact that it has been estimated that one third of all known enzymes contain a metal ion as a functional participant (Dedyukhina and Eroshin, 1991) Their functions are thought to reflect their fundamental chemical properties of charge, size, redox potential, and rates of ligand exchange (Hughes and Poole, 1989) Important roles include the formation of charge and concentration gradients across membranes which may be used for solute transport e g the K^+ gradient (Okorokov *et al*, 1983), osmoregulation and stabilisation of cellular structures However, virtually all metals, whether essential or not can exhibit toxic effects above certain threshold concentrations which vary depending on speciation and chemical properties of the metal in question

When metals with no known biological function such as Pb, Tl, Hg, Au, Al, Cd, and Ag compete with, or replace a functional metal ion, toxicity results (Hughes and Poole 1989) Toxic metals exert harmful effects principally as a consequence of their strong

coordinating abilities (Gadd, 1993) Toxic effects include the blocking of functional groups of biologically important molecules, the displacement and/or substitution of essential metal ions from biomolecules, conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organelle integrity (Gadd, 1993, Volesky and Holan, 1995) On a macro scale, effects include reduction in growth rates, extension of lag phase and perturbations in morphology and physiology (Hughes and Poole, 1989)

Attempts to rapidly assess toxic effects of heavy metals have centered on measurement of ion leakage (Koch *et al*, 1995) from cells and plasma membrane H^+-ATPase activity (Karamushka *et al*, 1996) The plasma membrane ATPase is one of the targets for action of heavy metals, and its activity as indicated by glucose-dependent H^+ efflux can serve as a convenient, relative indicator of toxicity (Karamushka *et al*, 1996, Gruzina *et al*, 1997) Release of intracellular K^+ ions has been used as an indicator of toxicity but its interpretation requires careful consideration, because as K^+ is present intracellularly at high concentrations, its release and viability may be unconnected Uptake of metal cations at non-toxic concentrations may be accompanied by K^+ efflux to maintain ionic balance It is therefore necessary to establish whether K^+ loss is due to toxicity or normal physiological processes (Gadd *et al*, 1986) Analysis of respiration rates and tests relying on diffusion processes in agar also have applications in the study and assessment of metal toxicity towards yeast and fungi However, high concentrations of Cd^{2+} and Zn^{2+} ions (> 1 5 mM) were reported to stimulate respiration in the salt water algae *Ulva lactuca*, possibly via an alternative respiratory pathway (Webster and Gadd, 1996b) This finding, albeit at metal concentrations that are rarely environmentally significant has implications concerning the use of algae in bioassays for metal toxicity

1 3 Biosorption

Metal uptake is essentially a biphasic process consisting of a metabolism-independent and metabolism-dependent step The metabolism-independent step is known as biosorption, and can be defined as the passive sorption and complexation of metal ions by microbial biomass, or material derived from it (Eccles, 1995) This biosorption step is rapid (Avery and Tobin, 1992, Brady and Duncan, 1994a), typically only a few minutes in duration (White and Gadd, 1987a) and is independent of temperature (Mowll and Gadd, 1984),

metabolic energy (de Rome and Gadd, 1987), the presence of a metabolisable energy source and the presence of metabolic inhibitors (Mowll and Gadd, 1984, White and Gadd, 1987a) Almost always this initial binding is thought to involve the microbial cell wall, although in the case of bacteria extracellular polymers may also be responsible (Collins and Stotzky, 1989) Binding is attributed to ion-exchange, adsorption, complexation, precipitation and crystallisation within the multilaminate, microfibrillar cell wall structure (Tobin *et al*, 1984, Remacle, 1990) Biosorption is exclusively responsible for metal accumulation by non-viable biomass (Tobin *et al*, 1984, Avery and Tobin, 1992) due to the absence of metabolic activity necessary for intracellular metal accumulation The amount of biomass present influences specific metal uptake with increased metal uptake per gram of biomass recorded at lower biomass loadings (Veglio *et al*, 1997) Brady and Duncan (1994a) found that if the metal to biomass ratio was below 100 nmol g^{-1} then metal accumulation was due almost entirely to biosorption of metal ions by the cell wall

1.4 Bioaccumulation

Bioaccumulation can be defined as processes responsible for uptake of metal ions by living cells, and thus includes biosorptive mechanisms, together with intracellular accumulation and bioprecipitation mechanisms (Eccles, 1995) Bioaccumulation is influenced by factors such as temperature and the presence of metabolic inhibitors (Okorokov *et al*, 1977, Mowll and Gadd, 1984, de Rome and Gadd, 1987), and greater amounts of metal may be accumulated by this means in some organisms, especially yeasts (Norris and Kelly, 1977, Avery and Tobin, 1992) However, transport may not be as significant a component of total uptake as general biosorption for other organisms, most notably the filamentous fungi (Gadd, 1990b)

Suggested mechanisms for transport of metal ions into microbial cells include carrier mediation, ion channels/pumps and endocytosis (Ford and Mitchell, 1992) The rate of passive diffusion across the plasma membrane is governed in part by the physical properties of the membrane and its composition (Van der Rest *et al*, 1995) The interaction between proteins and lipids in biological membranes enhances the overall passive diffusivity but is thought to account for only a very small fraction of ion uptake (Jones and Gadd, 1990) However, uptake of thorium by metabolising and resting yeast was attributed to permeation across the cell membrane by a diffusion mechanism, as the

addition of glucose had no stimulatory effect on levels of metal accumulated (Gadd and White, 1989) Ion channels allow the downhill flow of solutes across the plasma membrane In *S cerevisiae* voltage-dependent K^+ channels and unspecific channels activated by stretching of the bilayer have been identified, and are thought to play a role in osmoregulation via ion conductance and signal transduction (Van der Rest *et al*, 1995) Their exact role in transport of heavy metal cations remains to be quantified However, the main mechanism of primary ion transport in living cells derives from active conversion of the chemical energy of ATP into electrochemical energy Hydrolysis of ATP by plasma membrane ATPase results in the generation of an electrochemical gradient of protons (Δp) across the cell membrane, negative and alkaline inside This Δp is composed of an electrical ($\Delta\psi$) and a chemical (ΔpH) potential which are interconvertible and can each drive ion transport across the plasmalemma The plasma membrane-bound H^+-ATPase that pumps protons from the cell has been widely implicated in metal cation transport (Jones and Gadd, 1990, Van der Rest *et al*, 1995) Secondary transport systems which utilise existing cellular (electro)chemical gradients of other solutes (or ions) to supply energy for translocation of ionised substances include the energy of the K^+ gradient, which is coupled to the H^+ gradient Dissipation of the K^+ gradient or increasing exogenous K^+ levels has been shown to result in decreased cation uptake (Borbolla *et al*, 1985, Okorokov, 1985) Sequestration of cations to the vacuole is facilitated by the vacuolar ATPase, a secondary transporter which functions as an electrogenic pump transporting H^+ ions into the vacuole creating a Δp across the tonoplast The mechanism of transport across the tonoplast is thought to involve a cation/proton antiport

Generally, transport systems encountered in microorganisms are of varying specificity through which both essential and non-essential ions may be taken up Various metabolic inhibitors that interfere with membrane potential have been shown to reduce metal accumulation (Norris and Kelly, 1977, White and Gadd, 1987a)

1.5 Factors affecting biomass metal accumulating capacity

The ability of biological material to remove metal cations from solution and maximisation of uptake is influenced by a number of factors ranging from biomass preparation to experimental conditions employed A number of these factors is discussed below

1.5 1 Viable as against inactive biomass

There is considerable disagreement in the literature concerning whether live or inactivated biomass is the better metal sorbent However, it is clear from published data that metal uptake capacity is dependent on the experimental conditions (Miekle *et al*, 1990) and strain of organism under investigation (Tobin *et al*, 1994) Different yeast strains were reported to accumulate varying amounts of metal (Junghans and Straube, 1991) with uptake by living cells only slightly higher than that of cells rendered non-viable by heat treatment In contrast, Volesky *et al* (1993) observed Cd^{2+} uptake in excess of 625 µmol g^{1} for aerobic bakers yeast compared to dry non-living yeasts which did not accumulate more than 180 µmol g^{1} Cd^{2+} Conversely, dried and ground cells of *S cerevisiae* removed 40% more uranium and zinc than corresponding live cultures (Volesky and May-Philips, 1995), with similar findings reported in other work (Omar *et al*, 1996) Uptake of Sr^{2+} by live brewing and laboratory strains of *S cerevisiae* did not exceed 40 µmol g^{1} whereas denatured biomass (dried and ground) accumulated between 100 and 200 µmol g^{1} at similar external Sr^{2+} concentration (Avery and Tobin, 1992) However, it was noted that live biomass proved to be a more efficient metal sorbent than denatured biomass at low (10 µM) Sr^{2+} concentrations

1.5.2 Chemical treatments

Biomass has been treated with a variety of chemicals in an attempt to improve metal accumulating capacity, with varying degrees of success Acid washing improved the Cu^{2+} biosorptive capacity of *Aspergillus oryzae* (Huang and Huang, 1996) The washed biomass was found to contain a higher percentage of surface nitrogen, possibly as a result of partial dissolution of polysaccharide compounds in the outer layer of fungal cell wall exposing additional binding sites Biomass could be regenerated by acid stripping and showed no loss of biosorptive capacity A similar improvement in Ag^{+} biosorption by *Aspergillus niger* was observed after alkali treatment (Akthar *et al*, 1995) The strength of acid or alkali wash solutions varied between 0 001 and 0 1 M in most studies Metal accumulation by *S cerevisiae* cells was found to be improved following exposure to acid, alkali, heat (boiling), formaldehyde or mercuric chloride (Strandberg *et al*, 1981, Ting and Teo, 1994) However, chemical treatment sometimes brings about no improvement in biosorptive capacity as observed with acid washing of *Rhizopus oryzae* (Huang and

Huang, 1996), and Singleton and Simmons (1996) reported a 50% decrease in Ag^+ biosorption by *S cerevisiae* cells following boiling in 0 1 M NaOH or 0 01 M sodium dodecyl sulphate (SDS) With respect to metal uptake studies using live, freshly harvested biomass, washing of the biomass with buffer or distilled water reduced metal uptake capacity Unwashed yeast biomass adsorbed up to two-fold more uranium than did washed biomass which was attributed to precipitation of uranium by low molecular weight components (remains of fermentation products) loosely associated with the biomass (Omar *et al* , 1996, Riordan *et al* , 1997)

1 5 3 Culture age

The influence of culture age on biosorptive capacity is varied and strain dependent (Azab *et al* , 1990) but in general, younger cells of the same biomass type are reported to possess greater metal binding capacity than older cultures Simmons and Singleton (1996) observed that Ag^+ biosorption by 1 day old cells of *S cerevisiae* was double that of 4 day old cells Similar results were reported for uranium accumulation by brewers and bakers yeasts, where a three-fold increase in metal levels was noted for log phase cultures (9-12 h) compared with 24 h cells (Volesky and May-Phillips, 1995) By contrast, Cd^{2+} uptake by bacterial cells was not found to be age dependent between one and seven day old cultures However, Junghans and Straube (1991) reported that stationary phase yeast cells possessed the highest biosorptive capacity and attributed this finding to increases in the phosphorus and protein levels in yeast cell walls as growth rate decreases (McMurrough and Rose, 1967)

1 5.4 Supplementation of growth medium

The composition of growth medium has been found to influence the cultures metal uptake characteristics The type of fermentable sugar used during growth of *S cerevisiae* was found to affect subsequent metal uptake levels Cultures grown on glucose and sucrose generally exhibited greater uptake than cultures that metabolised fructose (Engl and Kunz, 1995) Similarly, Cd^{2+} accumulation by *Candida utilis* from the cultivation broth was dependent on the carbon source used Cells grown with glucose adsorbed higher levels of Cd^{2+} compared with cells grown on xylose (Kujan *et al* , 1995) Biomass of *Neurospora crassa* grown in nitrate N-medium was more effective than that grown in ammonium N-

medium in removing Co^{2+} during growth Cell wall preparations from nitrate N-medium bound 4-5 fold more Co^{2+} compared with preparations from ammonium N-medium (Karna *et al*, 1996) Additionally, uptake of Cs^{+} by cyanobacteria was markedly influenced by the nitrogen source supplied during culture growth (Avery *et al*, 1992b) Supplementation of growth medium with cysteine (Engl and Kunz, 1995) or sulphate (Lin *et al*, 1993b) also resulted in higher metal binding capacity compared with unsupplemented cultures In contrast, sorption of Cu^{2+} and Pb^{2+} was reduced in cultures supplemented with ammonium or phosphate (Engl and Kunz, 1995)

1.5.5 Cell viability during metal contact

Maintenance of cell viability during the course of experiments is essential when considering metabolism-dependent metal uptake (Gadd and Mowll, 1983) Maintenance of viability is dependent on the test ion in question and its concentration, with variable tolerance levels exhibited by different strains White and Gadd (1987) observed a progressive reduction in viability (to 50%) of *S cerevisiae* cells incubated over a range of zinc concentrations up to 100 mM Viability loss was correlated with indicators of zinc toxicity such as inhibition of H^{+} efflux and K^{+} uptake but not with Zn^{2+} uptake No loss of viability was observed for cells of *Aureobasidium pullulans* up to Cd^{2+} concentrations of 0 5 mM (Mowll and Gadd, 1984) whereas *S cerevisiae* cells experienced rapid viability loss (90% decrease within 5 minutes) at a similar Cd^{2+} concentration (Gadd and Mowll, 1983) At 0 05 mM Cd^{2+} loss of viability was rapid in the presence of glucose and Cd^{2+} uptake was double that of cells incubated in the absence of glucose (where cells remained viable), suggesting that intracellular metal accumulation was necessary for toxicity to result

1.5.6 Glucose enhanced metal accumulation

Bioaccumulation and subcellular localisation of metal cations are generally reported to be enhanced by the presence of a metabolisable substrate such as glucose (Okorokov *et al*, 1977, Avery and Tobin, 1992, Volesky and Holan, 1995), becoming evident within minutes (Norris and Kelly, 1977, Avery and Tobin, 1992) and can continue for several hours (Avery and Tobin, 1992, Mowll and Gadd, 1983) Localisation of cations in yeasts, in the presence of a metabolisable substrate, is primarily determined by active transport in

response to electrochemical proton gradients (Δp), generated by membrane bound H^+-ATPases, across the cytoplasmic and vacuolar membranes (Jones and Gadd, 1990) Hydrolysis of glucose enables regeneration of Δp resulting in prolonged uptake until such time as either substrate (glucose or metal ion) is exhausted or accumulated cellular metal attains levels inhibitory to normal cellular metabolism (Ross, 1977, White and Gadd, 1987) Metal accumulation by cells in the presence of glucose can be up to three-fold greater than in the absence of glucose (Norris and Kelly, 1977, Avery and Tobin, 1992) Non-metabolisable analogs of glucose although absorbed by the cell, do not stimulate metal uptake (White and Gadd, 1987), indicating that metal uptake is not linked to substrate transport across cell membranes

1.5.7 Time-dependent metal uptake in the absence of glucose

There is a growing number of studies reporting intracellular heavy metal accumulation in the absence of a metabolisable substrate The time-dependent accumulation of thorium and uranium by *Aspergillus flavus* biomass (Hafez *et al*, 1997) along with Pb^{2+} (Suh *et al*, 1998), Ag^+ (Simmons and Singleton, 1996), Cd^{2+} (Volesky *et al*, 1993) and uranium (Volesky and May-Philips, 1995) by *S cerevisiae* cells have been observed in the absence of glucose during an incubation period between 2 h and 4 days Earlier data concerning Mn^{2+} (Rothstein and Hayes, 1956) and Cd^{2+} uptake (Norris and Kelly, 1977) show similar patterns Electron microscopy has confirmed the subcellular localisation of metal deposits to the cell cytoplasm and vacuoles in addition to deposits on the cell wall (Volesky and May-Philips, 1995, Simmons and Singleton, 1996, Hafez *et al*, 1997, Suh *et al*, 1998)

1 5.8 Influence of physiological activity on metal uptake

Some authors observed no enhancement of metal cation uptake in the presence of glucose as compared with cells incubated without glucose, which may be attributable to the cultures physiological state (Volesky *et al*, 1993) Thorium uptake by *S cerevisiae* was virtually identical in the presence and absence of glucose (Gadd and White, 1989) Similar finding are reported for Mn^{2+} accumulation by *Penicillium notatum* (Starling and Ross, 1990) and uptake of Cd^{2+}, Co^{2+} and Cu^{2+} by *S cerevisiae* (Brady and Duncan, 1994a) In each case, freshly grown cells were used for experiments after only a short (30 minute) equilibrium period It is likely that this period was not sufficient to exhaust cellular energy

reserves or allow metabolic activity to decrease to a basal/resting state Brady and Duncan (1994a) cited the absence of a starvation step to explain the absence of increased metal uptake in the presence of glucose Furthermore, Starling and Ross (1990) observed that glucose did not stimulate metal uptake unless the biomass was substantially starved for up to 18 h prior to initiation of experiments The importance of cells metabolic activity at time of addition of test metal ion was further highlighted by Stoll and Duncan (1996) who reported pretreatment of *S cerevisiae* cultures with glucose for 30 minutes (no glucose present during metal uptake) increased metal removal compared with cultures that did not receive a glucose supplement, or that received a glucose supplement simultaneously with test ions It has also been reported that pretreatment with phosphate, in addition to glucose was observed to improve metal uptake capacity (Fuhrmann and Rothstein, 1968)

1 6 Cellular cation release during metal accumulation

Concomitant with metal uptake, ion release from biomass (both living and denatured) is frequently observed Release of K^+, H^+, Ca^{2+} and Mg^{2+} (Belde *et al*, 1988, Avery and Tobin, 1992, 1993, Norris and Kelly, 1977, Okorokov *et al*, 1983, Brady and Duncan, 1994c) has been widely reported K^+ is the most abundant ion in the yeast cell followed by Mg^{2+} (Walker, 1994), and these are the main ions mobilised during metal accumulating processes Concentration gradients of these ions exist within cells with cytosolic concentrations maintained at a much lower level than vacuolar concentrations (Okorokov *et al*, 1980, Lichko *et al*, 1980) Cellular levels of Ca^{2+} vary depending on organism and growth medium but are generally at least an order of magnitude lower than Mg^{2+} and K^+ levels (Jones and Gadd, 1990, Avery and Tobin, 1992) Free cytosolic Ca^{2+} in yeast is maintained at submicromolar levels (as higher concentrations result in toxicity) with 90% of cellular Ca confined to a non-exchangeable pool in the vacuole (Cunningham and Fink, 1994) Small amounts of Na^+ are present in some organisms but appreciable release in response to heavy metal binding has rarely been observed (Avery and Tobin, 1992, Perkins and Gadd, 1993a)

1.6.1 K^+ release

The role of K^+ release during metal ion uptake is somewhat unclear Some studies suggest that it is a symptom of cellular, probably cytosolic membrane damage by heavy metals

while in other cases, there appears to be a regulated exchange for accumulated metal ions Stoichiometric exchanges of K^+ in *S cerevisiae* for accumulated metal cations ($2K^+$ released for each divalent cation taken up) have been reported during uptake of Co^{2+} (Norris and Kelly, 1977) and Cu^{2+} ions (de Rome and Gadd, 1987) at concentrations of 200 µM and 10 µM respectively Furthermore, accumulation of the monovalent ion Li^+ by *S cerevisiae* at concentrations between 5-10 mM induced release of one K^+ ion for each Li^+ ion taken up (Perkins and Gadd, 1993a) A similar relationship was observed with Cs^+ accumulation by *Chlorella emersonii* (Avery *et al*, 1992a) Yeast-like cells of *Aureobasidium pullulans* exhibited release of $2K^+$ for each ion of Cu^{2+} (Gadd and Mowll, 1985) and Cd^{2+} accumulated (Mowll and Gadd, 1984) at concentrations of between 10-80 µM Cu^{2+} and up to 0 5 mM Cd^{2+} It is suggested that this regulated K^+ release is an attempt by the cells to maintain an electroneutral ionic balance across the cell membrane (Mowll and Gadd, 1984, Ramos *et al*, 1985)

A number of other studies have observed K^+ release to be a general response to metal accumulation with no simple stoichiometric relationship evident Rapid loss of cellular K^+ from *S cerevisiae* occurred within a short contact time (10-20 minutes) with Cd^{2+} ions at concentrations ranging from 20-500 µM (Norris and Kelly, 1977, 1979, Gadd and Mowll, 1983, Belde *et al*, 1988) Other cations such as Zn^{2+} at concentrations between 0 1-1 0 mM (Mowll and Gadd, 1983, White and Gadd, 1987), Cu^{2+} at concentrations between 25-200 µM (Norris and Kelly, 1979, Brady and Duncan, 1994b) and Mn^{2+} ions at concentrations between 50-1000 µM (Gadd and Laurence, 1996) induced a similar uncontrolled K^+ efflux This uncontrolled K^+ release is attributed to membrane disruption as a result of metal cations binding to organic ligands (Gadd and Mowll, 1983, Kessels *et al*, 1987, Oshumi *et al*, 1988, Assmann *et al*, 1996) A similar conclusion was drawn from observations of intracellular K^+ loss from the microalgae *Ulva lactuca* in the presence of 30 µM Cu^{2+} and Cd^{2+} ions (Webster and Gadd, 1996a)

However, no K^+ release from *S cerevisiae* was detected in the presence of between 10 and 50 µM Mn^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Tl^+ (Avery and Tobin, 1993) and between 0 1 and 1 0 mM Sr^{2+} (Avery and Tobin, 1992) even though some of these metal ions provoked K^+ loss from *S cerevisiae* in other studies at similar concentrations (see previous paragraph) Zn^{2+} uptake by viable cells of *Sporobolomyces roseus* and *S cerevisiae* was not accompanied by K^+ release in either yeast, but Zn^{2+} levels which affected viability of *S*

cerevisiae (> 0 1 mM) induced release of K^+ (Mowll and Gadd, 1983) A further study by White and Gadd (1987a) reported that K^+ efflux induced by Zn^{2+} accumulation by viable *S cerevisiae* cells, was reversible at Zn^{2+} concentrations less than 50 µM, but above this Zn^{2+} concentration, K^+ efflux was irreversible and was followed by a phase of equilibrium or slow efflux Similarly, 30 µM Zn^{2+} and Co^{2+} did not induce release of intracellular K^+ from *U lactuca* which may be attributed to differing effects on membrane permeability by different ions (Webster and Gadd, 1996a) These conflicting and often contradictory observations underline the fact that K^+ efflux in response to metal cations is a complex phenomena, with no defined or apparently consistent mechanism It can furthermore be influenced by factors that vary between different metal cations, their applied concentrations, microbial strains, and include transport and damage to cell membrane

1.6.2 Mg^{2+} and Ca^{2+} release in response to heavy metal accumulation

Mg^{2+} and to a lesser degree Ca^{2+} may also be released or displaced from biomass during metal uptake Due to the low cellular levels of Ca^{2+} present in yeast (Jones and Gadd, 1990, Avery and Tobin, 1992), its exchange for accumulated heavy metal ions is not as significant when compared with the filamentous fungi *A niger* (Akthar *et al* , 1995) and *R arrhizus* (Brady and Tobin, 1994, 1995)

Where extensive Ca^{2+} and Mg^{2+} release was reported, it generally occurred with biosorption of heavy metal ions by dried, denatured biomass, and may display a close stoichiometric relationship to levels of accumulated heavy metals The mechanism of Ag^+ biosoption (up to 200 µM) by *A niger* was reported to be exclusively by stoichiometric exchange with Ca^{2+} and Mg^{2+} ions of the biosorbent, with Ca^{2+} ions accounting for approximately 55% of bound Ag^+ (Akthar *et al* , 1995) Accumulation of Cu^{2+} ions by the brown marine algae *Ecklonia radiata* was almost exclusively attributed to ion exchange for Ca^{2+} and Mg^{2+} ions although small amount of H^+ were also released (Matheickal *et al* , 1997) Similarly, uptake of Sr^{2+} and Cu^{2+} by dried algal biomass of *Vaucheria* was an ion exchange phenomenon Adsorption of Sr^{2+} released an equivalent amount of Ca^{2+} and Mg^{2+} ions indicating electrostatic interactions Cu^{2+} binding was not entirely accounted for by release of Ca^{2+} and Mg^{2+} ions as substantial levels of H^+ ions were also displaced contributing to the stoichiometric exchange (Crist *et al* , 1990) Sr^{2+} accumulation by *R arrhizus* could also be accounted for solely by release of Mg^{2+} and

Ca^{2+}, whereas release of these ions accounted for only a small portion of uptake of Mn^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+} (Brady and Tobin, 1995)

The effect of metal cation accumulation by viable *S cerevisiae* biomass on release of Ca^{2+} and Mg^{2+} ions is variable Li^{+} accumulation had no effect of cellular Ca^{2+} and Mg^{2+} levels (Perkins and Gadd, 1993a) and cell Mg^{2+} levels remained unchanged during accumulation of 200 μM Cd^{2+} or Co^{2+} (Norris and Kelly, 1977) The same authors later report a gradual loss of cell Mg^{2+} (a 67% reduction within 30 minutes) after initiation of Cu^{2+} or Cd^{2+} uptake by *S cerevisiae* (Norris and Kelly, 1979) Other yeast strains tested showed variations in cell Mg^{2+} levels of between 0 and 50% at 200 μM Cd^{2+} which are likely to be dependent on a particular strains tolerance to any given metal cation (Norris and Kelly, 1979) Substantial loss of cell Mg^{2+} in addition to K^{+} release was reported at a Cd^{2+} concentration of 0 5 mM (Gadd and Mowll, 1983) Viability loss accompanied release of intracellular ions and was attributed to membrane disruption by accumulated metal cations Brady and Duncan (1994b) observed slow release of approximately 60% of cellular Mg^{2+} during 1 h contact with 200 μM Cu^{2+} but little loss of Ca^{2+} (cellular K^{+} was rapidly lost also) Co^{2+} accumulation induced a lesser loss of physiological cations During adsorption from 100 μM Sr^{2+} to live *S cerevisiae* biomass, the total charge (sum of Mg^{2+}, Ca^{2+} and H^{+}) released from cell walls was approximately 45% of that taken up (Avery and Tobin, 1992) Levels of Mg^{2+} release were only marginally greater than Ca^{2+} release Increased Sr^{2+} concentrations (10-fold) only modestly increased physiological ion release By comparison, substantially greater amounts of Mg^{2+} were released by denatured yeast (5-40 fold greater) at similar Sr^{2+} levels, but Ca^{2+} and H^{+} displacement was much reduced, representing less than 5% of total ion release Biosorption of Mn^{2+}, Cu^{2+}, Zn^{2+}, and Tl^{+} by live *S cerevisiae* resulted in release of small amounts of Ca^{2+} and Mg^{2+}, representing between 15-25% of accumulated ions on a charge basis (Avery and Tobin, 1993) Ca^{2+} and Mg^{2+} release from viable biomass as a consequence of heavy metal uptake is a more complex process than for inactivated biomass No clear trends are apparent other than to suggest that metal uptake by live biomass is less of an ion-exchange process, with cellular transport systems playing an important role in heavy metal uptake, and conservation of cellular physiological ion levels

1 7 Intracellular fate of toxic metals

The diversity of intracellular organelles and biomolecules provides a wide range of potential binding sites (Borst-Pauwels, 1981, Strandberg *et al*, 1981) The cell wall is the initial point of contact between the biomass and metal species, and considerable amounts of metal may be bound here (Gadd and White, 1989, Yazgan and Ozcengiz, 1994) Entry to the cell interior is mediated by membrane transport systems or diffusion processes (Okorokov, 1985, Gadd and Laurence, 1996) Biomolecules in the cytosol, including some specific metal scavengers can trap metal cations (Butt and Ecker, 1987, Lin *et al*, 1993a,b), but a large proportion of accumulated cellular metal ions are sequestered to the vacuole by relatively non-specific transport systems (Okorokov *et al*, 1977, Nieuwenhuis *et al*, 1981) Electron microscopy is being recognised as a powerful aid to researchers in determining localisation of subcellular metal deposits (Volesky *et al*, 1993, Suh *et al*, 1998)

1.7.1 Metal binding by the cell wall

The cell wall is the first line of defense against heavy metal cations and is capable of trapping considerable amounts of metal ions The yeast cell wall is a living, dynamic structure, interacting with the underlying cytoplasm and external environment, which changes with culture conditions and growth stage Its functions include providing physical protection, osmotic stability and enzyme support, binding of compounds, cell/cell adhesion and acting as a selective permeability barrier (Stratford, 1994) It is comprised mainly of mannoproteins (40%) and glucans (58%) with some chitin (2%) (Gooday, 1993, Kilis, 1994), arranged in a bilayer morphology comprising of an inner, amorphous layer and an outer fibrillar layer The outer layer is comprised mainly of mannoprotein and determines the cell surface properties such as surface charge, while the inner layer, which is responsible for the cells mechanical strength is comprised of different glucans (Remacle, 1990, Stratford, 1994) There is no clear distinction between the two layers and the entire structure is highly elastic

The adsorptive capacity of the yeast cell wall for heavy metals is not determined by its protein components, but rather by the structural organisation of the entire protein-carbohydrate complex, and by the degree of dissociation of the negatively-charged functional groups and their accessibility to metal cations (Davidova and Kasparova, 1992)

Functional groups of the cell wall capable of binding metal cations include electronegative groups such as hydroxyl and sulphydryl moieties, anionic groups such as dissociated carboxyl and phosphate groups and nitrogen containing amino groups (Kihn *et al*, 1988, Tobin *et al*, 1990, 1994), although their relative significance is difficult to assess Protein and carbohydrate fractions of yeast cell walls are involved in binding Cu^{2+}, Cd^{2+} and Co^{2+} ions (Brady and Duncan, 1994c) Isolated components of yeast cell walls (mannans, glucans and chitin) were observed to accumulate greater quantities of metal than intact cell walls (Brady and Duncan, 1994c), although Simmons and Singleton (1996) reported a reduction in Ag^{+} biosorption by isolated cell walls as compared to whole cell biosorption Cell wall components exhibited a 30% reduction in metal accumulating capacity when the protein fraction of cell walls was removed by enzymatic digestion, which suggests that the outer mannan-protein layer of the cell wall is more important than the inner glucan-chitin layer in heavy metal cation accumulation (Brady *et al*, 1994c)

The major fraction of Hg^{2+} accumulated by *S cerevisiae* was tightly bound to the cell wall by proteins associated with cell wall glucan (Murray and Kidby, 1975) Some Hg^{2+} did penetrate to the cytoplasm but only a minor fraction was present as low molecular weight components suggesting that the majority was associated with high molecular weight moieties such as polypeptides or cellular organelles One quarter of Mn^{2+} ions accumulated by *S cerevisiae* cells in the presence of glucose were found to be wall associated (Okorokov *et al*, 1977), whereas in the absence of an energy source, only a small fraction (3-9%) of this cation was bound by the cell wall (Kihn *et al*, 1988) Cells of *S cerevisiae* exposed to 10 μM Cu^{2+}, localised Cu^{2+} to an exchangeable pool in the cell wall (Lin *et al*, 1993a) Uptake of Pb^{2+} by viable *S cerevisiae* cells involved rapid binding to cell wall (within 3 minutes) and membrane (within 50 minutes) followed within two hours by penetration into the cytoplasm (Suh *et al*, 1998) Similarly, accumulation of Pb^{2+} by an active culture of the cyanobacterium *Anabaena cylindrica* was observed to consist of a very fast adsorption mechanism in the cell envelope, a time-dependent deposition reaction on the cell surface and a further time-dependent adsorption mechanism on polyphosphate bodies inside the cell (Swift and Forciniti, 1997) The cell envelope did not saturate with adsorbed lead immediately, but lead levels gradually increased with time of incubation Lead did not diffuse through the cell envelope into the cytoplasm of resting (inactive cells) although some time-dependent deposition in the cell envelope continued to

occur (Swift and Forciniti, 1997) Uranium was reported to deposit as fine, needle-like crystals on the cell wall of *S cerevisiae* (Volesky and May-Phillips, 1995) and *A niger* (Hafez *et al*, 1997) though penetration into the cell was observed with prolonged incubation (Volesky and May-Phillips, 1995) Thorium also deposited in the outer cell wall of stationary phase *S cerevisiae* (Gadd and White, 1989) and on the surface of *A niger* (Hafez *et al*, 1997) However, exponential phase *S cerevisiae* cells tended to localise more thorium intracellularly than was precipitated in the cell wall (Gadd and White, 1989)

1 7.2 Cytosolic metal sequestration

Entry of heavy metal cations to the cytosol is gained by passage across the plasma membrane through diffusion (facilitated or passive) or transport systems of low specificity (Melhorn, 1986) Alteration of plasma membrane fatty acid composition can increase the cells susceptibility to metal cation permeabilisation (Avery *et al*, 1996) Damage can occur to the plasma membrane as a result of metal binding resulting in increased permeability of the cell to external material and loss of mobile cellular solutes such as K^+, Mg^{2+} and Ca^{2+} (Norris and Kelly, 1977, de Rome and Gadd, 1987, Avery and Tobin, 1992)

In viable cells, regulation of ion concentrations in the yeast cytosol is based on activity of transporters of the plasmalemma and tonoplast (Borst-Pauwels, 1981) Plasmalemma transporters accumulate ions against a concentration gradient and prevent diffusion down the same gradient Tonoplast transporters regulate and stabilise cytosolic concentrations in conjunction with processes based on complex formation whereby accumulated free ions are converted to osmotically free ions (low molecular weight complexes) or to insoluble forms (Okorokov, 1985) Cytosolic metal concentrations are maintained at concentrations lower than in vacuolar or membrane/organelle fractions (Avery and Tobin, 1992, White and Gadd, 1987, Nieuwenhuis *et al*, 1981) Mn^{2+} ions that entered the cell were rapidly precipitated resulting in low concentrations of free Mn^{2+} (Kihn *et al*, 1988, Fracasanu *et al*, 1996) In addition, only osmotically free ions could be exported via an efflux system, and bound Mn had to be converted to the soluble form before it could leave the cell (Fracasanu *et al*, 1996)

Many cytoplasmic biomolecules have the ability to bind metals (Butt and Ecker, 1987, Lin *et al*, 1993a,b) In addition to the polypeptides of the metallothionein family,

another group of molecules present in certain fungi and yeasts are the short, cysteine-containing γ-glutamyl peptides (Jamison, 1995). Synthesis of these molecules can be induced by the presence of heavy metals (Gadd, 1993). Intracellular Cu^{2+} ions were associated with copper metallothionein, Cu/Zn superoxide dismutase and a Cu-glutathione complex (Lin *et al.*, 1993a,b). Additionally, bacterial cells tolerant to Cu^{2+} that possessed a high molecular weight Cu-binding moiety rich in aspartate and glutamate residues, could also tolerate high levels of Cd^{2+}, indicating that specific metal binding proteins are not required for different metal cations (Capasso *et al.*, 1996). Cellular metabolites such as nucleotides, RNA (Cabral, 1992; Huang *et al.*, 1990), inorganic phosphate (Volesky *et al.*, 1993) and sulphydryl moieties (Scott and Palmer, 1990) all possess significant metal binding capacity.

1.7.3 Vacuolar metal sequestration

The vacuole has an important role in the regulation of cytosolic concentration of metal ions both for essential metabolic functions and detoxification of potentially toxic metal species (Niewenhuis *et al*, 1981; Borst-Pauwels, 1981; Okorokov *et al*, 1983, 1985). Many ions including inorganic phosphate and monovalent and divalent cations are preferentially located in vacuoles (Gadd, 1993). The main solute transport system across the tonoplast is by a H^+/ion antiport mechanism which can be stimulated by Mg^{2+} and Mn^{2+} ions (Okorokov *et al.*, 1985). As the vacuole is the predominant intracellular metal sequestering compartment in yeast (Okorokov *et al*, 1977, 1980; Niewenhuis *et al*, 1981), it is intrinsic for intracellular metal accumulation. Studies with mutant *S. cerevisiae* cells lacking a vacuole or deficient in vacuolar acidification systems, exhibited reduced Ca^{2+}, Zn^{2+}, Sr^{2+} and Mn^{2+} accumulation when compared with wild type strains. Iron transport was not affected in vacuolar acidification deficient mutants, indicating its transport into the vacuole was not mediated by antiport with protons (Bode *et al.*, 1995). Metal uptake experiments conducted on isolated vacuoles of *S. cerevisiae* established that ATPase activity, and the presence of a proton gradient were necessary for Zn^{2+} accumulation (White and Gadd, 1987a).

Localisation of metal ions in the vacuole enables low non-toxic cytosolic concentrations to be maintained. 70% Sr^{2+} and 90% Mn^{2+} accumulated intracellularly by *S. cerevisiae* were sequestered to the vacuole (Nieuwenhuis *et al.*, 1981). The fractional

distribution of cellular Zn^{2+} in *S cerevisiae* was observed to be constant, and independent of loading and duration of contact, with the majority of approximately 60% sequestered to the vacuole Of the remainder, only 5% was localised to the cytosol (White and Gadd, 1987a) Electron dense metal deposits of Cd^{2+} were predominantly accumulated within vacuoles of *S cerevisiae* (Volesky *et al*, 1993) Deposits were associated with phosphorous, possibly as cadmium phosphate, and enlarged with increased time of exposure, but no apparent alteration of the cell interior and organelles was observed This is in contrast with cells exposed to uranium, where uranium was accumulated throughout the cell, resulting in increased vacuolar size and number, and contraction of the cytoplasm from the cell wall (Volesky and May-Phillips, 1995) The vast majority of accumulated monovalent metal cations was localised to the vacuole 85% of cellular Li^{+} was sequestered to the vacuole, which resulted in vacuolar enlargement (Perkins and Gadd, 1993a), and 90% of cellular Cs^{+} was found in the vacuolar fraction (Perkins and Gadd, 1993b) Exponential phase cells of *S cerevisiae* localised thorium mainly in vacuoles (Gadd and White, 1989), while the green algae *Chlorella salina* possessed higher concentrations of Co^{2+}, Mn^{2+} and Zn^{2+} in the vacuole than in the cytosol (Garnham *et al*, 1992) Avery and Tobin (1992) found that the relative importance of the cytosolic and vacuolar compartments in intracellular localisation of Sr^{2+} was dependent on whether uptake was active or passive With live, non-metabolising yeast, intracellular Sr^{2+} was predominantly located in the cytoplasm, where levels were approximately 2-fold higher than in the vacuole Although Sr^{2+} uptake in the presence of glucose resulted in elevated levels of the cation in the cytoplasm, increases were more evident in the vacuole, which now possessed 2-4 fold higher Sr^{2+} levels than observed in the cytoplasm

1.8 Mechanisms of microbial metal resistance

It is likely that toxic metal resistance systems arose soon after life began in a world already polluted by volcanic activity and other geological sources Toxic heavy metal resistance determinants are pre-existent to recent human activities that create polluted environments (Silver and Phung, 1996) Survival of microorganisms in the presence of toxic metals depends on their intrinsic biochemical and structural properties, physiological and/or genetic adaptation, environmental modification of metal speciation, availability and toxicity Reduction in metal uptake due to changes in cell structure, coupled with induced

production of intracellular metal-binding molecules and other genetic mechanisms all serve to increase tolerance to heavy metals

1.8.1 Altered metal uptake

Cell wall construction is crucial for survival of microorganisms in metal polluted environments as the structural features of the cell wall provide a mechanism to immobilise metals and prevent their entry into the cell (Remacle, 1990) Significant variations are observed in fungal wall features depending on stages of the life cycle (McMurrough and Rose, 1967) and on culture conditions, which can affect metal accumulating capacity (Engl and Kunz, 1995)

Ono *et al* (1988) observed that Hg^{2+}-resistant mutants possessed greater cell wall binding capacity than parental strains Similarly, Bianchi *et al* (1981) reported that a strain of *S cerevisiae* resistant to Mn^{2+} accumulated greater levels than the sensitive wild type strain This difference was attributed to the fact that Mn^{2+} ions had a greater depolarising effect on the membrane potential of the sensitive strain compared with the resistant one, resulting in reduced Mn^{2+} accumulation The resistant strain was also thought to carry a modification of a Mn-sensitive macro-molecule involved in the electrogenic pumping process responsible for Mn^{2+} uptake, rendering it less sensitive to Mn^{2+} toxicity

However, reduced metal accumulation by metal tolerant strains is the main mechanism of resistance for many organisms (Joho *et al*, 1983, Trevors, 1987) Training by repeated culturing on elevated metal concentrations resulted in increased resistance to Co^{2+}, Cu^{2+} and Cd^{2+} as a consequence of reduced metal uptake (Belde *et al*, 1988, White and Gadd, 1986) and altered intracellular distribution (White and Gadd, 1986) Cd^{2+} accumulation by a resistant strain of *S cerevisiae* was four-fold lower than that observed with a sensitive strain, where increased binding was attributable to increased susceptibility of the cell to Cd^{2+} permeabilisation (Belde *et al*, 1988) Resistance to Co^{2+} and Ni^{2+} by a mutant yeast strain was due to a reduction in uptake of these metals via a Mg^{2+} transport system (Joho *et al*, 1991) A copper-tolerant strain of *S cerevisiae*, obtained after serial subculturing in copper-containing media, was found to accumulate less metal than the parent strain (Brady *et al*, 1994a) While initial Cu^{2+} accumulation within the first 5 minutes was similar in both copper-tolerant and parent strains, the tolerant strain (unlike the parent strain) did not accumulate further metal over the next 55 minutes A mechanism

preventing metal uptake was proposed, which implied that tolerant cells limit Cu^{2+} accumulation by modifications to the cell membrane Copper-tolerant cells exhibited reduced mechanical strength, and changes to cell surface ultrastructure Similarly, Gadd *et al* (1984) reported that decreased uptake by copper resistant mutants of *S cerevisiae* was dependent on changed membrane transport properties and was not attributable to alterations to the cell wall A Cd-resistant strain was reported to possess increased levels of Cd^{2+} in the cytosol bound to low molecular weight protein residues, whereas almost all Cd^{2+} bound by the sensitive wild type strain was associated with insoluble cellular material, indicating altered intracellular sequestration as mechanism of tolerance (Joho *et al*, 1985a,b) Cd-resistant bacterial strains isolated from soil exhibited decreased Cd^{2+} uptake as mechanism of resistance The sensitive strain accumulated up to 8-fold more Cd^{2+} The strains with the highest level of resistance accumulated the least amount of Cd^{2+}, which was attributed to the existence of a Cd^{2+} efflux system (Kanazawa and Mori, 1996)

Bacteria and algae possess additional mechanisms which result in lower cellular metal levels Production of exopolysaccharides enables binding of large quantities of metal extracellularly, due to the negatively charged groups present (Scott and Palmer, 1990, Geddie and Sutherland, 1993, Loaec *et al*, 1997) Sulphate reducing bacteria utilise the production of H_2S to precipitate metal extracellularly in an insoluble form (Hughes and Poole, 1989, Ford and Mitchell, 1992)

1.8 2 Genetic adaptation

Many resistance mechanisms result from genetic adaptation Plasmids can encode resistance to a range of metal ions that are highly specific, but more frequently, related resistance systems have been discovered on chromosomal genes The largest group of metal resistance systems function by energy-dependent efflux of toxic ions Some of these systems are ATPases while others are chemiosmotic cation/proton antiporters (plasmid and chromosomally mediated) Others function by enzymatic detoxification, converting metal species to a less toxic or available species (Silver and Phung, 1996)

Metal-binding proteins are important regulators of cytosolic concentrations of metal cations, the best known of which are the metallothioneins These are small, cysteine-rich polypeptides which can bind essential as well as inessential metals Three

classes of metallothionein (MT) have been defined Class I and II are products of genes, while class III (also known as phytochelatins or γ-glutamyl peptides) are enzyme synthesised polypeptides Transcription of class I and II MT can be induced by copper and to a lesser degree by silver ions Class III MT are the smallest and similar to glutathione with the addition of extra dipeptide residues In fungi, only certain species are capable of their synthesis which can be induced by a wide range of heavy metals (Tomsett, 1993) In *S cerevisiae*, MT encoded by CUP1 brought about metal detoxification by chelating Cu^{2+} and Cd^{2+} ions as strains resistant to both metal produce the same MT This gene is usually present in a higher copy number in resistant strains (3-14) whereas wild type (sensitive) strains contained only a single copy (Tohoyama *et al*, 1995)

A number of chromosomal genes involved in metal resistance in yeasts have been identified Those involved in copper resistance include CUP1, CUP2, cupA and cupB genes (Cervantes and Corona, 1994) Some of these gene products such as that of CUP1 code for metallothionein (Butt and Ecker, 1987) but other gene products are not yet known Copper tolerance by mutants of *A nidulans* (through reduction in ion uptake) containing these genes does not appear to result from increased synthesis of metal binding proteins, despite evidence that they are present in the cell Three genes responsible for cadmium resistance have been identified in *S cerevisiae* There has been little characterisation of CAD1 gene, but resistance conferred by the CAD2 gene again does not appear to result from increased synthesis of metal-binding proteins, rather most of the cadmium appears to be bound to structural components including the cell wall (Trevors *et al*, 1986, Tomsett, 1993) The ZRC1 gene in *S cerevisiae* conferring resistance to cadmium and zinc, codes for a protein that is not cysteine-rich like metallothionein, and is believed to be a membrane protein that increases ion efflux Cobalt resistance has also been studied but exact mechanism is unclear and may involve more efficient compartmentation (Tomsett, 1993) Lapinskas *et al* (1996) have reported that a gene which functions in Ca^{2+} metabolism also acts in sequestration of Mn^{2+} ions to golgi apparatus, limiting its intracellular availability Mn^{2+} toxicity is reduced not by reducing overall Mn^{2+} accumulation but by more efficient intracellular sequestration

The best understood plasmid-encoded systems are those in bacteria conferring resistance to mercuric compounds, arsenate and cadmium Organomercurial compounds

are enzymatically converted to volatile Hg^0 (Hughes and Poole, 1989), whilst others such as arsenic, selenium, thallium, and antimony can be methylated by a variety of microorganisms as a means to removal by volatilisation (Ford and Mitchell, 1992) Arsenate resistance is achieved via an inducible ATP-driven transport system that serves to eject toxic ions from the cell Similarly, cadmium resistance is also based on an efflux system, catalysing exchange of cellular Cd^{2+} ions for protons (Tynecka *et al*, 1981, Nies and Silver, 1995) The cadmium efflux system may be able to transport zinc, although this has not been directly demonstrated (Hughes and Poole, 1989) In yeast, efflux of Cd^{2+} from Cd^{2+}-loaded cells of *A pullulans* was significantly enhanced by the addition of glucose (Mowll and Gadd, 1984), resulting in loss of intracellular Cd^{2+} within a short period of time

1.8.3 Free radicals

Indirect mechanisms of metal toxicity may involve generation of free radicals which are deleterious to cells Studies have shown that metals such as Fe, Cu and Cr undergo redox cycling while Cd, Hg, Pb and Ni deplete glutathione and protein-bound sulphydryl groups, resulting in production of reactive oxygen species (ROS) as superoxide ion, hydrogen peroxide and hydroxyl radical (Stohs and Bagchi, 1995, Vulpe and Packman, 1995) Major targets of radicals are cell membranes where lipid peroxidation is initiated with consequent loss of cell integrity (Melhorn, 1986, Gadd, 1993) ROS may also damage cellular constituents such as DNA and proteins, in addition to lipids Many aerobic organisms possess defense mechanisms against ROS that involve the metalloenzymes Cu/Zn- and Mn-superoxide dismutase and free radical scavengers such as glutathione (GSH) (Jamieson, 1995) Other genes have also been identified that in addition to responding to oxidative stress, confer degrees of resistance to certain metals and are involved in regulating some aspects of metal homeostasis (Mordas-Ferreira *et al*, 1996)

Exposure of *S cerevisiae* to Cd^{2+} ions constitutes oxidative stress, and strains deficient in components of the defense system against oxidative stress were hypersensitive to cadmium-induced killing It was postulated that the threshold dose for Cd^{2+} toxicity may be due to the saturation of cellular defenses and/or repair mechanisms for oxidative stress (Brennan and Schiestl, 1996) Addition of GSH reduced growth inhibitory effects of Cd^{2+} to a *Chlorella* species Complexation of Cd^{2+} was not responsible indicating that an

intracellular mechanism involving GSH and its metabolites was involved It is possibly that production of phytochelatins from GSH resulted in reduction of toxicity (Kaplan *et al* , 1995)

1.9 Environmental factors affecting metal accumulation

The conditions present in a given environment determines metal speciation and therefore metal bioavailability and toxicity A number of contributing factors is discussed below

1.9.1 Influence of exogenous ions on metal uptake

Exogenous cations and anions in addition to the metal cation of interest generally reduce levels of metal uptake Inhibition of cation accumulation by competing ions depends on a range of factors such as (i) concentration of test and inhibitory ion, (ii) charge density on cell surface, (iii) ionic strength of medium, and (iv) affinity of test ion for negatively charged groups on the cell surface (Borst-Pauwels and Severens, 1984) Decreased metal uptake is thought to be in response to increased competition between like charged species for binding both to negatively charged groups on the cell surface and carrier sites on the cell membrane, with preference given to particular ions (Norris and Kelly, 1977) Elimination of energy-dependent transport as a result of metal toxicity, in addition to a reduction of net surface potential *i e* screening negatively charged binding sites may also be involved (Norris and Kelly, 1979, Borst-Pauwels and Theuvenet, 1984)

Studies have shown that reduction in test ion uptake occurs over a range of co-ion concentrations Zn^{2+} uptake by *S cerevisiae* was inhibited more than 50% by equimolar Mg^{2+} or 20-fold excess K^{+} which was attributed to preferential accumulation of theses ions (White and Gadd, 1987a) Similarly, equimolar or greater concentrations of the monovalent ions K^{+} and Rb^{+} reduced cellular levels of Sr^{2+} in *S cerevisiae* and was correlated with uptake of these cations (Roomans *et al* , 1979) Cd^{2+} and Co^{2+} uptake by *S cerevisiae* has been reduced by a wide range of ions (Norris and Kelly, 1977, Mowll and Gadd, 1984, Kanazawa and Mori, 1996) Ca^{2+} was found to strongly depress Cd^{2+} accumulation and inhibition was attributed to its size similarity in terms of ionic radius to Cd^{2+} (Norris and Kelly, 1977) and that Cd^{2+} ions were taken up by a system normally required for Ca^{2+} accumulation (Mowll and Gadd, 1984) Ni^{2+} and Mn^{2+} uptake was reduced in the presence of Mg^{2+} and Zn^{2+} ions at similar concentrations (Norris and Kelly,

1979, Parkin and Ross, 1985) with Mg^{2+} being a potent inhibitor of Mn^{2+} accumulation (Parkin and Ross, 1985) Bioaccumulation of Mn^{2+} by intact mycelium of *P notatum* was unaffected by presence of 1000-fold Mg^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} ions, but protoplasts showed reduced transport of Mn^{2+} in the presence of Mg^{2+} ions (Starling and Ross, 1990) Furthermore, inorganic anions can form coordination complexes with heavy metal cations resulting in inhibition of metal uptake (Tobin *et al* , 1987, Hughes and Poole, 1991, Avery and Tobin, 1993)

However, in some studies uptake inhibition was not observed White and Gadd (1987a) reported no reduction of Zn^{2+} accumulation in the presence of equimolar Na^{+} or Ca^{2+} which was attributed to the fact that only minor uptake of Na^{+} and Ca^{2+} ions present occurred Similarly, Norris and Kelly (1979) reported that neither equimolar Mg^{2+} or Ni^{2+} reduced Zn^{2+} uptake, and the presence of a range of light metal ions (Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}) did not significantly affect Cu^{2+} adsorption by marine algae *E radiata* (Matheickal *et al* , 1997) These findings suggest that specific transport systems exist for some cations but not others, or that certain cations possess higher affinities for a general transport system (Norris and Kelly, 1979)

1 9.2 pH effects

The influence of pH on metal uptake by yeasts (Junghans and Straube, 1991, Brady and Duncan, 1994a), algae (Wood and Wang, 1984) and bacteria (Nakajima and Sakajuchi, 1993) is very similar, with extremes of pH generally decreasing the rate and extent of metal uptake (Sag *et al* , 1995) At a pH < 2 0 metal uptake by yeasts was undetectable (Brady and Duncan, 1994a, Junghans and Straube, 1991), and Omar *et al* (1996) reported that increasing pH from 2 5 to 4 5 favoured biosorption of uranium by *S cerevisiae* At low pH, H^{+} ions compete with metal cations for cellular binding sites and reduce potential metal interaction with cells (Gadd, 1993) Variations in external pH can affect the degree of protonation of potential ligands that contribute to metal binding (Tobin *et al* , 1984), therefore the affinity of the cell surface for metal ions will also be affected by changes in ambient pH A pH of between 4 0 and 8 0 is widely accepted as being optimal for metal uptake (Brady and Duncan, 1994a, Fourest and Roux, 1992) for almost all types of biomass

Hydrolysis reactions occur with nearly all the metallic cations and because of the diversity of the hydroxide complexes which can be formed in solution, the resulting chemical behaviour of a given metal can be a complicated function of pH and concentration (Baes and Mesner, 1976) Hydroxide complexes are often polynuclear, reducing available free metal ions for sequestration Increased pH can result in precipitation of metal hydroxides or oxides, with similar results (Brady and Duncan, 1994a) Toxicity of some heavy metals varies with pH because the hydrolysed forms of these metals which occur at higher pH values, bind to the cell surface and alter the net charge of the cell Alteration in charge may affect various physiological functions of the cell as well as interactions with other cells and the immediate environment (Collins and Stotzky, 1992)

1.9.3 Temperature

Temperature effects are confined to metabolism-dependent metal accumulation and relate to the effect of temperature on the physiological state of cells (Norris and Kelly, 1977) rather than on chemical speciation or availability of metal cations (Baes and Mesner, 1976) Accumulation processes that depend on cellular metabolism would be those most likely inhibited at low temperature, whereas high temperatures may affect the integrity of cell membranes and hinder compartmentalisation (Brady and Duncan, 1994a) At low temperature (0-5°C), little or no metal is sequestered through metabolic processes by viable biomass (Norris and Kelly, 1977, 1979) Most laboratory experiments are carried out in the temperature range 25-35°C which has been reported optimal for metal accumulation (Failla *et al*, 1976, Brady and Duncan, 1994a) Metal binding through biosorptive action is unchanged across the temperature range 4-35°C (Norris and Kelly, 1977, Sag *et al*, 1995, Omar *et al*, 1996)

1 9.4 Particulate matter

Dissolved and particulate matter in the environment and in growth media, generally reduces metal availability by complexation and binding (Babich and Stotzky, 1977) Proteins (Huang *et al*, 1990), humic acids (Wood and Wang, 1983) and cellular metabolites released as a consequence of induced membrane damage (Cabral, 1992) can bind appreciable quantities of free ions, making them unavailable for uptake Release of

carbohydrate and protein moieties from cells during incubation with metal cations can lead to complexation of metal cations and a reduction in the concentration of free ions for uptake (Simmons *et al*, 1995)

1.9 5 Other factors

Metal uptake can be influenced by ionic strength of the medium (Borst-Pauwels and Severens, 1984), being modestly inhibited by elevated ionic strength (Brady and Duncan, 1994a,c) Metabolic inhibitors affect metabolism-dependent accumulation (Norris and Kelly, 1977, White and Gadd, 1987a) through interference with membrane transport systems Oxidation-reduction potential (E_h) of an environment affects metal speciation and availability Insoluble metal sulphides may form in reducing environments which exhibit little or no toxicity, sulphide precipitation of heavy metals is a resistance mechanism found in several bacteria and fungi (Hughes and Poole, 1989, Ford and Mitchell, 1992)

1 10 Hard and soft theory of metal classification

Metals can be classified according to the principle of "hard" and "soft" acids and bases (Pearson, 1963) or as class A, class B and borderline ions (Nieboer and Richardson, 1980) on the basis of ligand preference, a property which can underline biological activity Both classification systems are based on thermodynamic data of metal ion chemistry, namely on trends in the magnitude of equilibrium constants that describe the formation of metal ion/ligand complexes

Metal ions can act as Lewis acids by accepting electron pairs from ligands The majority of all inorganic ligands are from groups V, VI, and VII in the periodic table According to Pearson (1963), metal ions can be divided into two classes depending on whether they form their most stable complexes with the first ligand atom of each group (N, O, F), class A, or whether they form more stable complexes with the second or subsequent member of each group (P, S, I), class B Refinement of this principle by Nieboer and Richardson (1981) determined that in biological systems, class A metal ions have the following preference for metal-binding donor atoms in ligands O > N > S, and in contrast, class B metal ions exhibit the opposite preference sequence S > N > O Borderline ions form an intermediate group with varying affinity for class B metal-binding donor atoms and ligands Therefore in a biological context, class A metal ions

show an almost absolute preference for binding to ligands with oxygen donor atoms such as carboxylate, carbonyl, alcohol, phosphate and phosphodiester groups Class B metal ions seek out nitrogen and sulphur centres such as sulphydryl, disulphide, thioether and amino groups Borderline ions are able to form complexes with all of the above ligands, but preferences do exist which reflect the degree of class A or class B character of a particular ion (Nieboer and Richardson, 1981)

Class A metal ions are generally small, exhibit low polarisability (related to electronegativity, ionisation potential and ionic radius) and tend to participate in principally ionic (electrostatic) bonding to ligands Class B metal ions possess high polarisability and bind to ligands in a predominantly covalent manner (Gadd, 1992) There is a sharp separation between class A and borderline metal ions but the distinction between class B and borderline metal ions is less clear In a mixture of hard and soft metal ions, soft metal ions may outcompete hard metal ions for binding ligands Class B metal ions are generally regarded as more toxic than class A ions Indeed a number of macro-nutrient metals (such as Na, Mg, Ca and K) belong to class A and the micro-nutrient metals (Mn, Fe, Cu and Zn amongst others) belong to the borderline group (Hughes and Poole, 1989, 1991)

1 10.1 Effect of metal chemistry on uptake

A number of studies have attempted to relate differing mechanisms, relative levels of metal uptake or toxicity to the chemical characteristics of the metal ion in question Avery and Tobin (1993) investigated biosorption of a range of hard (class A) and soft (class B) metal ions by live *S cerevisiae* cells The harder ions Sr^{2+}, Zn^{2+} and Mn^{2+} were absorbed to a lesser degree than the softer ions, Cu^{2+} and Cd^{2+}, and tended to display saturation of uptake at lower external metal concentrations Relative covalent bonding (as indicated by H^{+} displacement) of metals was greater at low metal concentration, while weaker electrostatic interactions (Mg^{2+} and Ca^{2+} displacement) became increasingly important at higher concentrations However, the actual levels of Mg^{2+}, Ca^{2+} and H^{+} release were less than 4 µmol g^{-1} compared with metal uptake levels of approximately 15 µmol g^{-1} for the hard metal ions, but were approximately 2-fold greater for the softer metal ions which were accumulated to about 25 µmol g^{-1} Similarly, binding of the hard ion Sr^{2+} was accompanied by an equivalent release of

Ca^{2+} and Mg^{2+} ions from *Vaucheria* biomass, demonstrating electrostatic interactions, whereas uptake of the softer ion Cu^{2+} released additional protons indicating covalent interactions (Crist *et al*, 1990) Ag^{+} binding by *S cerevisiae* provoked considerable H^{+} release indicating the covalent nature of binding (Ag is soft metal) Factors which decreased biosorption of Ag^{+} resulted in reduced H^{+} release (Singleton and Simmons, 1996) The amount of Ca^{2+} and Mg^{2+} displaced by metal binding to *R arrhizus* decreased with increasing class B character of the test ions (Sr < Cd < Cu), and H^{+} displacement increased which was indicative of the increasing covalent nature of binding (Brady and Tobin, 1994)

The potential of test ions to displace another preloaded borderline metal ion from *R arrhizus* was observed to increase with increasing covalent index of test ion, which is an indicator of degree of class B character (Brady and Tobin, 1995) Hence, soft metals were able to displace metals of lesser class B character Distinctly hard (Sr^{2+}) and soft (Cu^{2+}) ions bound to different sites on the biomass and did not interfere with the uptake of one another, but the issue was less clear with borderline ions In addition, levels of metal uptake were observed to increase with increasing covalent index Tsezos *et al* (1996) reported during accumulation of metal cations from a two component solution, elements belonging to either the hard or soft classes exhibited competition effects amongst members of their class Uptake of borderline elements were affected by the presence of either hard or soft elements However, uptake of a hard cation in the presence of a soft ion (or vice versa) did not affect accumulation of either metal ion and was attributed to cation binding at different sites on the biomass Studies with immobilised *S cerevisiae* showed that in a mixed metal solution, the softer of the two metals present was preferentially bound by the biomass (Wilhelmi and Duncan, 1995) When uptake from a solution of Cu^{2+} and a second ion was examined, the molar ratios of bound Cu^{2+} to bound co-ion increased as the co-ion class B character decreased (Cu > Cd > Co > Zn, Nieboer and Richardson, 1981) as follows, Cu Cd = 3 1, Cu Co = 4 1, Cu Zn = 6 1

Culture supplementation studies by Engl and Kunz, (1995) have highlighted the link between metal chemistry and levels of metal uptake *S cerevisiae* cultures supplemented with cysteine, ammonium and phosphate led to the incorporation of sulphur and nitrogen groups, nitrogen, and oxygen-containing phosphate moieties,

respectively. These cultures were challenged with a metal solution containing equimolar levels of metal ions of increasingly class B (soft) nature (Zn < Cd < Cu < Pb) according to Nieboer and Richardson (1980) classification. Metal uptake patterns by control cultures were such that ions possessing the most class B (soft) character were taken up to the greatest extent from an equimolar solution. Cysteine supplementation resulted in increased accumulation of the softer metals, Cu and Pb, due to presence of increased -S and -N sites, the preferred ligands for class B ions. Ammonium supplementation had little effect on uptake when compared with controls as -N groups are of intermediate affinity to -S and -O ligands. Phosphate supplementation resulted in reduced accumulation of the three softer metals compared with unsupplemented controls but led to an increase in uptake of Zn, the ion possessing the most class A (hard) character. This was attributable to the preferential binding of class A (hard) metals to -O containing ligands.

1.11 Introduction to experimental work

The experimental work undertaken in this thesis serves to add to the knowledge available in the literature in a number of ways. Intracellular localisation of divalent metal cations in yeast has been investigated by a number of researchers, but effects of subcellular metal sequestration on cellular physiological ion pools has not been widely reported. One of the objectives of this work was to examine metal cation sequestration in the presence and absence of a metabolisable energy source to ascertain differences, if any, in the mechanism of sequestration. In addition, the cations used in this study were chosen to investigate the application of the Hard and Soft principle to active accumulation and subcellular sequestration of metal cations. Previous studies have primarily concentrated on the biosorptive (cation binding to the cell surface) nature of the Hard and Soft principle.

The presence of charged species in addition to the metal cation of interest generally brings about a reduction in cellular metal levels, with excess levels of the physiological cations Ca^{2+} and Mg^{2+} particularly noted for alleviating metal toxicity. In the present work, Mg^{2+} ions were observed to have a marked influence on Mn^{2+} uptake and toxicity. This specific protective effect was investigated to determine how Mg^{2+} ions act to lessen the toxic effects of Mn^{2+} though mechanisms other than purely

competitive interaction The finding linking metal toxicity with cellular Mg status in yeast had not been previously reported

Subcellular fractionation protocols employed in this work and elsewhere did not include verification of the separation of subcellular ion pools Therefore a study was undertaken to check the efficacy of existing protocols making use of specific biochemical markers However, obstacles were encountered which ultimately led to the development of an alternative method for the differential extraction of subcellular ion pools

The following sections serve to introduce in more detail the background to the work undertaken

1.11.1 Effect of environment on metal accumulation

In recent years, interest in processes involved in heavy metal accumulation by microorganisms has increased because of concern over possible transfer of toxic metals from microorganism through food chains to higher organisms, and in relation to the bioremediation potential of microorganisms for metal removal and/or recovery (Hughes and Poole, 1989, Tobin *et al* , 1984) For an industrial-scale metal treatment process, the cost of producing biomass can be a serious disadvantage (Gadd, 1990a) and consequently, the use of waste biomass would be desirable The brewing industry is one such industry that produces large quantities of waste biomass that could be potentially utilised in metal removal and/or recovery systems The present work examines metal uptake by a brewing strain of *Saccharomyces cerevisiae* and attempts to characterise its potential application for metal recovery processes

Differing experimental conditions are known to influence metal uptake by yeast (Meikle *et al* , 1990) The pH at which uptake occurs is one such physico-chemical parameter that affects metal accumulation Extremes of pH are undesirable for maximal metal removal (Sag *et al* , 1995), with a range between 4 0 and 8 0 widely accepted as optimal for metal removal by most types of biomass (Brady and Duncan, 1994a, Fourest and Roux, 1992) In the present work, metal uptake was investigated in three systems where extracellular pH was under varying degrees of control The objective of this set of experiments was to assess the effect of extracellular pH on levels of metal uptake and

identify a suitable experimental system for subsequent subcellular localisation experiments

1 11 2 Accumulation and subcellular localisation of metals

Cells possess the ability to accumulate a wide range of metal cations from their environment in order to fulfil essential functions in cellular metabolism (Hughes and Poole, 1989, Dedyukhina and Eroshin, 1991) However, toxic effects result when physiological levels are exceeded An understanding of the mechanisms of metal accumulation and subcellular localisation can facilitate assessment of the impact of metals on biological systems

Metal cation accumulation by live yeast 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 across the cell membrane into the cell itself This second stage is enhanced by the presence of a metabolisable energy source, required by cellular transport mechanisms to compartmentalise metal cations to intracellular organelles In the present study, subcellular sequestration of a range of metal cations in the presence and absence of glucose by a brewing strain of *Saccharomyces cerevisiae* over a 5 h incubation period was investigated Particular attention was paid to the effect of metal cation accumulation on levels of physiological ions, Mg^{2+} and K^{+} in subcellular compartments

Metal cations chosen for investigation were Sr^{2+}, Cd^{2+}, Mn^{2+} and Cu^{2+} Strontium is a trace element and is thought not to have a direct biological role However, its chemical similarity to Ca^{2+} allows it to substitute for this ion in cell cycle control (Loukin and Kung, 1995) In addition, the long half-life of the ^{90}Sr radioisotope and its presence in wastewaters from nuclear reactors has given rise for concern over the fate of this radionuclide in the environment Cadmium compounds are used in a wide variety of industrial processes including electronics, battery manufacturing and metal plating, and in the chemical industry for a number of purposes such as stabilisation of formulations for vinyl chloride and cosmetics (Volesky, 1990) Within cells, Cd^{2+} affects ordering and structure of the plasma membrane, resulting in a more rigid structure and reduced ATPase activity (Fodor *et al* , 1995) Despite the fact that Cd^{2+} has no biological function (Jones and Gadd, 1990) it can be accumulated to an appreciable

extent by viable and non-viable biomass Manganese is an essential trace element, whose uptake is mediated by a number of transport systems (Gadd and Laurence, 1996, Jasper and Silver, 1977) Copper is ubiquitous in nature and has widespread use in technological processes Cu^{2+} is a trace element and is the strongest Lewis acid of the divalent metal ions, making it one of the more toxic elements (Beveridge *et al* , 1997)

Attempts to relate chemical characteristics of test metal ions to mechanism and levels of uptake has received relatively little attention when compared to the extensive literature available on metal-microbe interactions The selection of these four cations was influenced by their respective metal chemistry which spans the hard and soft spectrum (Nieboer and Richardson, 1980), Sr^{2+} is the hardest, Class A ion, and Class B character increases from Mn^{2+} to Cd^{2+} and Cu^{2+}

The purpose of the present investigation was to compare accumulation and subcellular sequestration of a number of different metal cations by actively metabolising and resting cells of *Saccharomyces cerevisiae* Physiological cation displacement from the biomass was determined and perturbations (if any) within subcellular ion pools characterised The extent to which these interactions that could be accounted for by the hard and soft principle was assessed

1 11.3 Influence of intracellular and extracellular magnesium concentrations on manganese uptake and toxicty

Trace metals play important roles in cellular metabolism, due primarily to their requirement as cofactors for a large number of enzymatic processes (Hughes and Poole, 1989, Dedyukhina and Eroshin 1991, Gadd, 1992) The bioavailability of these metals in the environment is usually low and cells require active transport systems foi their accumulation However, essential metal ions can become toxic when intracellular concentrations exceed physiological levels Toxic effects are generally manifested as a result of the metals strong co-ordinating abilities, and can include blocking of functional groups and substitution/displacement of essential metals on important biomolecules, denaturation of enzymes and disruption of cellular and organellar integrity (Gadd, 1993, Hughes and Poole, 1989) On a macroscopic scale, metal-induced perturbations of cellular physiology, morphology and growth rates can be observed (Hughes and Poole, 1989)

Manganese is an essential trace element, required at a concentration of 2-10 μM for optimal yeast growth (Jones and Gadd, 1990) A number of transport systems of varying affinity and specificity are known to mediate the uptake of Mn^{2+} (Gadd and Laurence, 1996, Norris and Kelly, 1979, Jasper and Silver, 1977, Fuhrmann and Rothstein, 1968) Mn^{2+} transport into yeast cells can be passive and driven by the concentration gradient (Kihn *et al*, 1988) or energy dependent being stimulated by glucose (Okorokov *et al*, 1977, Okorokov, 1985) Accumulated Mn^{2+} is preferentially sequestered in vacuoles (Lichko *et al*, 1980, Nieuwenhuis *et al*, 1981)

Magnesium is the second most abundant cation in microorganisms, exceeded only by K^+ (Jones and Gadd, 1990) The high cellular requirement for Mg^{2+} is a consequence of its role in stabilising ribosomal and nucleic acid structures, in maintaining structural integrity of cellular and organellar membranes, and as a cofactor for over three hundred enzymes (Walker, 1994) Mn^{2+} is a potential intracellular competitor of Mg^{2+} for binding to biological molecules such as Mg^{2+}-requiring enzymes, ATP and nucleic acids, principally as a result of its similar chemical properties (including ionic radius) Conversely, a number of proteins are known to exist that have a specific requirement for Mn^{2+}, which in some cases cannot be met by Mg^{2+} (Hughes and Poole, 1989, Walker, 1994, Supek *et al*, 1996) The similarity between Mg^{2+} and Mn^{2+} is further illustrated by transport of Mn^{2+} into yeast via the Mg^{2+} transport system, although cells with a specific requirement for Mn^{2+} have transport systems that are unaffected by large excesses of Mg^{2+} (Jasper and Silver, 1977)

A general protective effect of extracellular Mg^{2+} against the toxicity of metal cations such as Cu^{2+} (Karamushka and Gadd, 1994) and Cd^{2+} (Kessels *et al*, 1985) has been described Mn^{2+} uptake by yeast can be reduced by addition of extracellular Mg^{2+} (Bianchi *et al*, 1981) In plants, the ratio of magnesium to manganese in the shoots has been shown to influence growth, although no critical toxic Mn^{2+} concentration was identified (Goss and Carvalho, 1992) In this work, protection against Mn^{2+} toxicity by a number of biologically-essential cations, including Mg^{2+} was investigated, and the interrelationship between intracellular levels of Mn and protective ions in determining Mn^{2+} toxicity was ascertained

1 11 4 Development of new protocol and assessment of procedures for extraction of soluble ion pools

Metal cation accumulation in yeast is a complicated and ion selective process Some bulk cellular ions like K^+ and Mg^{2+} are required at high concentrations, while a number of others are maintained at trace levels Inside the cell, the dependence of rates and directions of many metabolic processes on the presence of some inorganic ions suggests that their intracellular concentrations are under strict control Controlled changes in ion concentrations provide an important means for metabolic regulation Transport systems of the plasmalemma and tonoplast acting in conjunction with ion-complexing biomolecules maintain cytoplasmic ion levels at concentrations appropriate for normal metabolism As many ions, including inorganic phosphate and monovalent and divalent cations are preferentially located in vacuoles (Okorokov *et al*, 1980, Lichko *et al*, 1980, Gadd, 1993) intracellular ion gradients exist between the cytoplasm and vacuole Methods for extraction of subcellular ion pools enable qualitative assessment of subcellular cation compartmentation Cell fractionation protocols have inherent drawbacks, as it is not possible to eliminate in a straightforward manner the possibility that metal cations are mobilised during the operation and subsequently bind to new sites of higher affinity which are exposed during the disruption or fractionation process (Beveridge *et al*, 1997)

A number of protocols for differential extraction of subcellular ion pools are available in the literature Generally, cells are washed with buffer to remove loosely associated cations, followed by extraction of the cytoplasmic pool whilst maintaining vacuolar integrity This step is crucial to the overall protocol, as subsequent steps are based on the assumption that cytoplasmic contents have been removed Vacuoles are then lysed, and the vacuolar ion pool separated form the remaining cell debris Some early protocols subjected yeast cells to cytochrome *c* permeabilisation of the cell membrane, subsequently followed by osmotic shock and digestion of remaining cellular material in hydrochlorate to release cytoplasmic, vacuolar and bound ion pools, respectively (Okorokov *et al*, 1977, 1980, Lichko *et al*, 1980) Fluorescent dye staining of cells was used to assess the degree of cell membrane permeabilisation Joho *et al*, (1985a,b) incubated *S cerevisiae* cells with a chitosan solution (50 $\mu g\ ml^{-1}$) in order to permeabilise the plasmalemma for extraction of cytosolic contents In this instance,

release of UV-adsorbing material and cellular Mg^{2+} was used to assess permeabilisation However, the long incubation time (1 h) and release of more than 50% of cell Mg^{2+} suggested that vacuolar integrity may have been compromised as Okorokov *et al* (1980) determined a Mg^{2+} gradient of 1 15 existed between cytosolic and vacuolar compartments The membrane disruptive effect of Cu^{2+} ions were used to selectively permeabilise the yeast plasma membrane, releasing cytosolic contents with no apparent loss of vacuolar integrity (Ohsumi *et al*, 1988) Analysis of intracellular amino acids, specifically glutamate (cytosolic marker) and arginine (vacuolar marker) was used to quantify the separation DEAE-dextran (0 1 mg ml^{-1}) caused only slow release of intracellular components and required long incubation times (20-60 minutes) Theuvenet *et al* (1986) required a DEAE-dextran concentration of 3 mg ml^{-1} for 5 minutes to permeabilise yeast cells as assessed by K^+ efflux Here, in the absence of an isotonic sorbitol solution, total loss of cellular K^+ was observed (vacuolar and cytosolic pools), but only 30-40% of cell K^+ was lost in the presence of 0 7 M sorbitol (cytoplasmic pool) as sorbitol isotonically stabilises vacuoles DEAE-dextran lysis of spheroplasts was used to isolate intact vacuoles (Durr *et al*, 1975) utilising α-glucosidase activity and soluble arginine as cytoplasmic markers respectively However, the fact that some arginine is present in the cytoplasm (not totally localised to the vacuole) coupled with vacuole leakage may have led to some error in assessing degree of separation A vacuolar fraction containing 60% of cell arginine and less than 1% of α-glucosidase activity was isolated

By far the most popular method for differential extraction of subcellular ion pools is the modified method of Huber-Walchi and Wiemken (1979) and White and Gadd (1986) used by a number of researchers (Avery and Tobin, 1992, Garnham *et al*, 1992, Perkins and Gadd, 1993a,b) This technique utilises DEAE-dextran permeabilisation of the cell membrane followed by methanol treatment to rupture vacuoles However, assessment of degree of separation of subcellular pools is frequently not reported, and use of biochemical markers is difficult due to inactivation of biological activity by methanol treatment step In this work, a differential extraction protocol utilising soluble cytosolic and vacuolar marker enzymes to quantify separation was developed Difficulties due to incomplete DEAE-dextran membrane permeabilisation were overcome by partial spheroplasting of cells prior to treatment Treatment with

methanol was dispensed with in favour of glass bead disruption with enabled marker activity to be maintained Subcellular location of various metal cations by this method was compared to the originally used technique Results and implications are discussed

CHAPTER 2

MATERIALS AND METHODS

Accumulation and subcellular localisation of metal cations by Saccharomyces cerevisiae

Ph D Research Thesis by

Kevin J Blackwell B Sc

CHAPTER 2. MATERIALS AND METHODS

2.0 Organism, culture conditions and metal analysis

2.0.1 *Saccharomyces cerevisiae biomass*

A brewing strain of *Saccharomyces cerevisiae* supplied by a local brewery was cultured in liquid medium comprising (in grams per litre) the following KH_2PO_4, 2 72, K_2HPO_4, 3 98, $(NH_4)_2SO_4$, 2 0, $MgSO_4\ 7H_2O$, 0 5, $FeSO_4\ 7H_2O$, 0 0022, $ZnSO_4\ 7H_2O$, 0 004, $MnSO_4\ 4H_2O$, 0 004, $CuSO_4\ 5H_2O$, 0 004, D-glucose, 20 0, and yeast extract (Oxoid, Hampshire, England), 1 0 Cultures were grown at 25°C on an orbital shaker (150 rpm) Stock cultures were maintained on a solid medium of the above composition, achieved by the addition of 1 5% bacteriological agar (Cat no L11, Oxoid, Hampshire, England)

Medium (1 litre volume in a 2 litre conical flask) of the above composition was inoculated with 20 ml of a 24 h starter culture, and for experimental purposes cells from the early stationary phase (18 h) were harvested by vacuum filtration through Whatman No 1 filter paper (Whatman, Kent, England) The resulting cake was washed twice with distilled deionised water, and pressed dry with Whatman filter papers The cake was removed from the filter paper and retained for immediate use

2.0.2 *Dry weights*

Dry weights of live cells were determined by oven drying biomass in foil cups for 48 h at 55°C

2 0 3 *Metal analysis*

Cd^{2+}, Sr^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+} and K^{+} were analysed using a Perkin-Elmer 3100 atomic absorption spectrophotometer (Perkin Elmer, Norwalk, Ct , USA), 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, prepared from nitrate or sulphate salts dissolved in distilled, deionised water

All chemicals used were reagent grade Riedel-de-Haen (Seelze, Germany) products

2 0.4 *pH analysis*

pH values were measured using a Corning 220 pH meter (Corning, NY 14831, USA) fitted with a W T W E56 precision glass electrode (W T W , Wilheim, Germany) For continuous pH measurement, where required, a W T W (Wilheim, Germany) PMX 3000 ionmeter fitted with a E56 precision glass electrode was used

2 1 Effect of environment on metal accumulation

Accumulation of Cd^{2+} by live S cerevisiae was investigated under three sets of conditions, buffered, unbuffered and pH controlled, over a five hour period Supernatants were analysed for metal content, and the presence of Mg^{2+}, Ca^{2+} and K^{+} ions Cell viability was also assessed Cd^{2+} accumulation by live biomass was compared with that of metabolically inactivated (irradiated) cells to ascertain extent of metabolic uptake

2.1.1 *Metal uptake experiments*

A brief description of the experimental set-up follows Experiments were carried out under three sets of conditions (i) buffered, (ii) unbuffered and (iii) pH contiolled The buffered system consisted of an aqueous solution containing 10 mM 2-[-N-morpholine] ethanosulphonic acid (MES buffer), previously adjusted to pH 5 5 by using 1 M NaOH or HNO_3 MES buffer was chosen for its low metal binding properties (Good *et al* , 1966) The unbuffered system was distilled, deionised water, adjusted to pH 5 5 after addition of biomass The pH controlled system was similar to unbuffered, except that pH was constantly monitored at all stages of the experiment and maintained at pH 5 5 by constant, dropwise addition of 1 M NaOH or HNO_3 as necessary

General experimental protocol was as follows Biomass was cultured and harvested as described previously in section 2 0 1 Acid-washed 250 ml Erlenmyer flasks were prepared with 100 ml of solution (depending on which system was under investigation) Freshly harvested biomass was introduced to a final concentration of 0 1 g (dry weight) 100 ml^{1} and allowed to equilibrate for 90 minutes with rotary shaking at room temperature In the case of the pH controlled system, flasks were agitated with magnetic stirrers Where required, glucose (2% w/v) was added 10 minutes prior to addition of metal ion of interest (as nitrate salt) to the desired final concentiation At specified intervals, 5 ml samples were removed, and the biomass separated by

centrifugation (1200 × *g*, 5 minutes) The supernatants were retained for metal analysis as described in section 2 0 3 Pellets were washed twice with distilled deionised water, resuspended in 5 ml of distilled, deionised water and retained for viability measurement

2 1 2 *Viability measurement*

Cell viability was determined using the citrate methylene blue staining technique (Pierce, 1971) The staining solution was prepared in the following manner 0 01 g of methylene blue (BDH, Poole, England) was dissolved in 10 ml of distilled, deionised water, to which 1 0 g of tri-sodium citrate (BDH, Poole, England) was added A further 60-70 ml of distilled deionised water added, the solution mixed and filtered through Whatman no 1 filter paper (to remove any undissolved solids) and made up to 100 ml with distilled, deionised water

Cells for viability counting were stored at 4°C, and were tested within 24 h of sampling After suitable dilution to give a countable number of cells, 1 ml of the cell suspension was added to 1 ml of staining solution and allowed to incubate for 5 minutes prior to counting on an improved Neubauer haemocytometer Dead cells stained blue having taken up the stain, whereas viable cells appeared colourless A minimum of 250 cells were counted and the numbers of live cells to total number counted expressed as a percentage

2 1 3 *Preparation of irradiated biomass*

In order to ascertain the difference between surface bound and intracellularly accumulated metal, non-metabolising biomass was prepared by irradiation Freshly harvested cells were irradiated in a Faxitron series HP 43804N X-ray system for varying exposure times to alter applied radiation dose A control sample of cells was also placed in the irradiation tube but covered with lead shielding Percentage viability was assessed by the citrate-methylene blue technique

2.2 Accumulation and subcellular localisation of metals

For each of the ions Sr^{2+}, Cd^{2+} and Mn^{2+}, cellular accumulation was monitored over a five hour period at concentrations of 100 μM in the presence and absence of glucose Due to the toxic nature of Cu^{2+}, accumulation of Cu^{2+} was examined at a concentration of 5μM Cell samples exposed to metals for 1, 3, and 5 h were analysed to determine subcellular metal distribution, and consequent effects on subcellular Mg^{2+} and K^{+} levels

2.2.1 *Metal uptake experiments*

Biomass was cultured and harvested as described previously in section 2 0 1 200 ml of 10 mM MES buffer, previously adjusted to pH 5 5 by using 1 M NaOH or HNO_3 was pipetted into acid-washed 250 ml Erlenmyer flasks Freshly harvested biomass was introduced to a final concentration of 0 2 g (dry weight) 200 ml^{-1} and allowed to equilibrate for 90 minutes with rotary shaking at room temperature Where required, glucose (2% w/v) was added 10 minutes prior to addition of metal ion of interest (as nitrate salt) to the desired final concentration At specified intervals, 5 ml samples were removed, and the biomass separated by centrifugation (1200 × *g*, 5 minutes) The supernatants were retained for metal analysis Cell pellets were washed twice with distilled, deionised water and also retained for metal analysis Results were compared with appropriate metal-free controls

2.2.2 *Subcellular compartmentation of metal ions (**Method 1**)*

Cell pellets were obtained by centrifugation (1200 × *g*, 5 minutes) of 30 ml samples after 0, 1, 3, and 5 h of incubation with the metal of interest in the presence and absence of 2% (w/v) glucose Pellets were washed twice with distilled deionised water and stored at 4°C until required Generally, this original pellet was divided into three or six (an additional three for acid digestion to determine overall cellular ion levels in cases where separate samples had not been taken for this purpose) during the initial washing step of the extraction procedure

Differential extraction of soluble ion pools from cells followed a modification of the protocol used by Huber-Walchi and Wiemken (1979) and White and Gadd (1986) A wash fraction was obtained by washing pellets twice (1 minute each), 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, with 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 5 ml of 0 7 M sorbitol in 10 mM Tris-MES buffer, pH 6 0, at 0-4°C, with incubation for 1 minute at each wash Supernatants were removed and retained after centrifugation at each wash and were combined with that from the initial permeabilisation step to yield a cytosolic pool

The vacuolar membrane was permeabilised by suspending the remaining pellet in 60% (v/v) methanol at 1-4°C After incubation for 1 minute, cell fragments were centrifuged (8000 × g, 40 s) and the supernatant retained and combined with those from two further washes with 60% (v/v) methanol (1 ml each time) followed by 10 mM Tris-MES, pH 6 0 (three times, 1 ml each time), giving a vacuolar pool Suspensions were incubated for one minute at 0-4°C for each of these washes

The remaining cell debris (termed bound fraction) was digested in 0 5 ml of 6 M HNO_3 for 1 h at 100°C, as were samples for overall metal levels, and subsequently diluted by addition of 3 ml distilled deionised water

2.3 Influence of intracellular and extracellular magnesium concentrations on manganese uptake and toxicity.

The protective effect conferred by magnesium ions to Saccharomyces cerevisiae against manganese was investigated with respect to uptake and toxicity Manipulation of intracellular and extracellular magnesium levels resulted in marked differences in manganese toxicity and accumulation Other heavy metal cations were tested to assess if the observed protective effect was widespread

2 3 1 *Organism, medium and growth conditions*

Saccharomyces cerevisiae NCYC 1383 was routinely maintained on solid YEPD medium, comprising the following (g l^{-1}) neutralised bacteriological peptone (Oxoid, Hampshire, England), 10 0, yeast extract (Oxoid), 5 0, D-glucose, 10 0, and agar (technical no 3, Oxoid), 18 0 For experimental purposes, cultures were grown in 100 ml liquid medium of the same composition (but without agar), in 250 ml Erlenmeyer

flasks Cells from 48 h liquid starter cultures were used to inoculate experimental flasks to an initial OD_{550nm}~ 0 1 Cultures were grown at 25°C with rotary aeration at 120 rev minute ^{1}

2.3.2 *Growth experiments*

Individual flasks containing 100 ml of YEPD medium was supplemented with sterile $Mn(NO_3)_2$ to the required final concentration singularly or in combination with $Mg(NO_3)_2$ or $Ca(NO_3)_2$ (final concentration of 10 mM), and KCl (50 mM final concentration) Suitable controls, less $Mn(NO_3)_2$ were also set up Growth was assessed by performing cell counts with an improved Neubauer haemocytometer over a 24 h period At least 250 cells were counted each time Samples (3-10 ml volumes, depending on growth stage) were removed for metal analysis at intervals by centrifugation (1200 × *g*, 5 minutes) and washed twice with distilled deionised water

The subcellular compartmentation of magnesium in magnesium-supplemented and unsupplemented control flasks was determined using the differential extraction of soluble ion pools procedure described in section 2 2 2

Cellular magnesium levels were found be enhanced by growth in $Mg(NO_3)_2$ supplemented YEPD medium (see section 3 3 4) Cells which displayed an approximate 2-3 fold difference in intracellular magnesium levels were obtained by harvesting after 6 h growth in standard YEPD, at which this point, intracellular magnesium levels are at their lowest levels and at 16 h from unsupplemented and Mg-supplemented (30 mM) medium, to give cells with low, standard and high intracellular Mg levels, respectively

2.3 3 *Manganese uptake and toxicity in cell suspensions*

Cells from 16 h cultures (unless otherwise specified) were harvested by centrifugation (1,200 × g, 5 minutes) at room temperature and washed twice with distilled, deionised water The final cell pellet was resuspended in a few millilitres of distilled, deionised water 1 ml of the suspension was added to 100 ml of 10 mM MES buffer, adjusted to pH 5 5 using NaOH and HNO_3, to give a final cell concentration of approximately 2 × 10^7 ml ^{1} After 30 minute pre-equilibration with shaking (120 rev minute ^{1}) at 25°C, Mn^{2+} was added to the desired final concentration For metabolism-dependent uptake studies, glucose was added (2%, w/v) 15 minutes prior to the addition of Mn^{2+} For

metal analysis, samples were removed at specified intervals, washed twice with distilled, deionised water having being pelleted by centrifugation (1,200 × g, 5 minutes) at each step.

For metabolism-independent uptake studies, the procedure was as above except that cells were incubated in a range of Mn^{2+} concentration (0-5 mM) and glucose was not added to the cell suspension prior to addition of test metal. Furthermore, the contact time was reduced to 10 minutes after which samples were taken for metal analysis in the usual manner.

On observing the differences in Mn^{2+} accumulation that arose between magnesium-supplemented and unsupplemented cells at similar concentrations, it was thought that these differences in levels of metal accumulation could be used as an assay to screen other metal cations (both mono and divalent) to determine any magnesium protective effect. Metal cations tested were Cd^{2+} (20-1000 µM), Co^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}(all at 50 µM), Sr^{2+}(500 µM), Cu^{2+}(10 µM), Ag^{+} (5 µM) and Cs^{+} (50 and 5000 µM). The concentrations used were to minimise any potential toxic effects of the metal ions themselves, and the experimental protocol was as outlined above. Sr^{2+} and Cu^{2+} analysis was carried out using a JY70C combination inductively-coupled-plasma-emission spectrophotometer; all other cations were analysed by atomic absorption spectrophotemetry.

Cs^{+} uptake experiments involved working with the Cs^{137} radioactive isotope (Amersham, UK). Briefly, the experimental procedure was as follows; a cell suspension of approximately 2×10^{7} ml^{-1} was made up with 10 mM MES containing 2% glucose to 16 ml in 50 ml acid-washed Erlenmeyer flasks, which were allowed to equilibrate with rotary shaking for 15 minutes. At t_o (initiation of experiment) 1 ml was removed for cell counting and a minimum volume of prepared radioactive Cs^{+} stock was added to give the desired concentration. At specified time intervals, 200 µl samples were removed from each flask and layered into 500 µl microcentrifuge tubes containing 200 µl of a mixture of 20% di-isonyl-pthalate and 80% silicone oil (Dow Corning, USA). The tubes were spun (8000 × *g*, 30 s) in a microcentrifuge, after which the lower portion of the tube containing the pelleted cells was cut off into a scintillation vial to which 4 ml of Ecosint A scintillation fluid (National Diagnostics, UK) was added. Samples were counted in a 2250CA Packard Tri-carb scintillation analyser.

2 3 4 *Measurement of net magnesium uptake*

Cells were grown for 16 h in magnesium supplemented and unsupplemented medium and harvested as previously outlined (section 2 3 1) Cells were introduced to 100 ml of 10 mM MES buffer in acid-washed 250 ml Erlenmeyer flasks to give a suspension of approximately 2×10^7 cells ml^{-1} Mg^{2+} ions were added to the desired final concentration and the suspension allowed to equilibrate with rotary shaking for 25 minutes 2% glucose was added to initiate metabolism-associated uptake At frequent intervals, 1 5 ml samples were taken and spun (8000 × *g*, 30 s) in a microcentrifuge 1 ml of supernatant was removed to a test tube containing 1 ml of distilled, deionised water and retained for analysis Samples were also taken during the equilibration period, and results were compared with Mg^{2+} free controls

2.3.5 *Metal analysis*

Samples of cell suspensions were centrifuged (1,200 × g, 5 minutes) and washed twice with distilled, deionised water Final cell pellets were digested for 1 h at 100°C in 0 5 ml of 6 M HNO_3 After addition of 3 ml distilled deionised water, the metal content of digests was determined using a Baird alpha-2 atomic absorption spectrophotometer with reference to appropriate standard solutions

2 3.6 *Cell viability measurements*

In this section, cell viability was defined as the ability to produce colony-forming-units after exposure to Mn^{2+} For these experiments, Mn^{2+} uptake experimental protocol, as described above was altered to maintain aseptic conditions Alterations included aseptic harvesting of cells, and the preparation and use of sterile buffer, metal and glucose stocks

For viability measurement 100 µl samples were removed from experimental flasks after varying lengths of exposure to Mn^{2+} and following appropriate dilution with sterile water were plated in triplicate on YEPD agar Colonies were enumerated after 3 days incubation at 25°C No new colonies developed during further incubation after this time Viability was defined as the ratio of experimental to control counts, expressed as a percentage

2.3 7 *Assay for measurement of surface charge*

The alcian blue retention assay as described by Rapoport and Beker (1985) was used to determine the negative surface charge of whole cultures Cells were washed three times in sterile 0 02 M acetate buffer, pH 4 0 and suspended to 1 25 × 10^7 cell ml^{-1} in 20 ml of sterile 0 02 M acetate buffer, pH 4 0 containing 50 µg ml^{-1} alcian blue dye (Sigma) The suspension was divided into 1 3 ml aliquots in 12 microcentrifuge tubes and incubated at 25°C at 120 rev minute $^{-1}$ on an orbital shaker for 30 minutes The cell suspensions were then centrifuged (8000 × g, 5 minutes), and absorbance at 607nm of the supernatants was determined by spectrophotometric analysis The extent of alcian blue retention at the cell surface was determined from a standard curve of known alcian blue concentrations in 0 02 M acetate standards Alcian blue dye retention was expressed as mg (10^7 cells) $^{-1}$

2.4 Development of new protocol and assessment of procedures for extraction of soluble ion pools

A novel protocol for the differential extraction of soluble ion pools from Saccharomyces cerevisiae was developed, and the steps involved in the development of this new technique are outlined Enzymatic markers for cytoplasm and vacuolar pools were used to assess the degree of separation of respective fractions, and the metal levels present in each fraction were analysed

2 4.1 *Marker enzymes*

The biochemical markers chosen for the cytoplasmic and soluble vacuolar contents were glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) and carboxypeptidase Y (CpY) respectively These enzymes occur exclusively in the cytoplasm and vacuole of *S cerevisiae* and have convenient assays that can be used to track yeast organelles during cell fractionation (Schekman, 1982)

2 4.2 *G-3-PDH assay* (Krebbs, 1955)

As a component of the glycolytic pathway, G-3-PDH catalyses the phosphorylation of glyceraldehyde phosphate (GAP) in the presence of the co-enzyme nicotinamide adenine dinucleotide (NAD) and phosphate (P) as follows,

$$GAP + NAD^+ + P \leftrightarrow 1,3\text{-Diphosphoglycerate} + NADH + H^+$$

Principle The rate of increase in absorbance at 340 nm is a measure of NADH production as a consequence of reduction of nicotinamide adeniene dinucleotide (NAD^+) resulting from glyceraldehyde phosphate (GAP) oxidation

2.4.2.1 *Reagents*

All reagents were supplied by the Sigma Chemical Co London, UK and were made up with distilled, deionised water

Glyceraldehyde phosphate (GAP) solution, 7 6 mM

Nicotinamide adenine dinucleotide (NAD^+) solution, 7 6 mM

0 03 M pyrophosphate - 0 006 M cysteine buffer, pH 8 5 Prepared immediately before use by diluting 0 3 M cysteine hydrochloride solution 1 50 with 0 03 M sodium pyrophosphate solution and adjusting pH to 8 5 with HCl

Sodium arsenate solution, 0 17 M

2.4.2.1 *Procedure*

Test tubes containing incomplete reaction mixtures were prepared as follows

2 5 ml of pyrophosphate-cysteine

0 2 ml NAD^+

0 1 ml of enzyme solution (in this case, samples of subcellular ion pools)

Incubate at room temperature for 30 minutes to ensure activation of enzyme by cysteine

A reference cell (less enzyme solution) was used The initial absorbance of the reaction mixture was measured at 340 nm in a cuvette with a 1 cm light path by a Shimadzu UV-160A recording spectrophotometer, and the final component, 0 3 ml of a 1 1 mixture of GAP and arsenate is added and the tubes mixed A final reading is taken after 25 minutes and corrected for any initial absorbance

2.4.2.3 *Activity*

One unit of activity is defined as 1 µmole of product formed ($NADH + H^+$) per minute, assuming a molar absorbance (E_{NADH}) of 6220 The total activity of each fraction can be determined by multiplying calculated activity by total fraction volume

2.4.3 *CpY assay* (Jones, 1990)

CpY will catalyse cleavage of esters, amides, and peptides An assay based on its amidase activity can be employed for kinetic analyses or modified to a fixed time point assay (below)

Principle CpY will catalyse the hydrolysis of N-benzoyl-L-tyrosine *p*-nitroanilide (BTPNA) to give the yellow *p*-nitroaniline Production can be followed by measuring absorbance at 410 nm

2 4 3.1 *Reagents*

All reagents were supplied by the Sigma Chemical Co London, UK and were made up in distilled, deionised water

BTPNA solution (6 mM) 2 43 mg dissolved in 1 ml of dimethylformamide (DMF)

0 1 M Tris-HCl, pH 7 6

1 mM $HgCl_2$

20% Lauryl sulphate (SDS) in 0 1 M Tris-HCl, pH 7 6

2 4 3 2 *Procedure*

Tubes containing 0 8 ml of 0 1 M Tris-HCl, pH 7 6, and 0 2 ml of 6 mM BTPNA at 37°C were prepared, to which was added 0 2 ml of enzyme solution (in this case, samples from soluble ion pools) A reference cell (less enzyme) is used The tubes were mixed and incubated for 60 minutes at 37°C, after which 3 0 ml of $HgCl_2$ was added to stop the reaction A further addition of 0 2 ml of SDS is made, and after vortexing, the tubes are incubated at 70°C until solubilisation of protein, as evidenced by clearing, ensues Absorbance was read at 410 nm in a cuvette of 1 cm light path by a Shimadzu UV-160A recording spectrophotometer

2.4.3 3 *Activity*

One unit of activity corresponds to 1 µmole of *p*-nitroaniline produced per minute, assuming a molar absorbance (E_{pNA}) of 8800 The total activity of each fraction can be determined by multiplying calculated activity by total fraction volume

2 4.4 *Cell disruption methods employed*

The various methods that were employed in developing a protocol to extract subcellular ion pools are outlined below

2.4 4.1 *Sonication*

30 ml of culture broth was spun down (1200 × g, 5 minutes) and the pellet washed twice with distilled deionised water The resulting pellet was resuspended in 10 ml of 10 mM MES buffer, and subjected to 6 × 30 s sonication cycles, at top speed with a Braun, Labsonic U sonicator The sample was continually cooled on ice throughout, with 1 minute between cycles to dissipate any heat energy produced The sonicated extract was centrifuged (1200 × g, 5 minutes) and the supernatant retained for enzymatic analysis (stored at 4°C)

2 4 4.2 *Triton X-100/dimethylformamide (DMF)*

10 mls of culture broth was pelleted in a universal container (1200 × g, 5 minutes), and the resulting pellet washed twice with 0 1 M Tris-HCl The pellet was then resuspended in either 0 2% Triton X-100 (Sigma Chemical Co , London, UK) or 20% DMF, and incubated on ice for 1 h After a further centrifugation step (1200 × g, 5 minutes), the supernatant was retained for enzymatic analysis

2.4.4.3 *Cell disruption with glass beads*

Cell samples for disruption were pelleted (1200 × g, 5 minutes) in a universal and resuspended in 1 ml of 10 mM Tris-MES, pH 6 0 at 4°C Glass beads, size 40 mesh (Sigma Chemical Co , London, UK) were added to the level of the liquid, and the suspension vortexed for 6 × 30 s cycles Samples were kept on ice between cycles The suspension was then transferred to a 10 ml polyproplyene Pierce column containing a foam sinter (Pierce Chemical Co , Rockford, IL, USA) and centrifuged (1200 × g, 5 minutes) into an Eppendorf tube The Eppendorf tube was spun at high speed (8000 × g, 10 minutes) to pellet solid cellular material The clear supernatant was removed to a fresh Eppendorf tube and retained for enzymatic analysis

2 4.4.4 *Freeze-thaw disruption*

1 ml aliquots of a concentrated cell suspension in 10 Tris-MES buffer containing 0 7 M sorbitol were placed in Eppendorf tubes and subjected to a number of freeze-thaw cycles Cells were held at -80° C for 10 minutes and then transferred to a 30° C water bath for a further 10 minutes during each cycle 10 minutes was sufficient time to freeze

and thaw samples After the desired number of cycles, solid cellular material was spun down (8000 × g, 1 minute) and the supernatant retained The pellet was washed twice with 0 5 ml of 10 mM Tris-MES buffer containing 0 7 M sorbitol, centrifuged each time and all supernatants combined The remaining pellet was subjected to glass bead disruption as previously outlined

2.4.5 *Formation of spheroplasts*

Spheroplast formation followed the method of Alexandre *et al* (1994) Briefly, the experimental protocol was as follows Cells were washed twice with distilled, deionised water, resuspended in 4 ml of pretreatment buffer (consisting of 10 mM Tris-HCl, 10 mM dithiotreitol, 5 mM EDTA adjusted to pH 8 0) and incubated at 37°C for 30 minutes with gentle shaking Cells were centrifuged (1200 × g, 5 minutes) and washed three times (4 ml each time) with 50 mM phosphate buffer, pH 7 5, and resuspended in 10 mM Tris-MES buffer, pH 6 0 containing 1 M sorbitol 10 mg ml^{-1} of Novozym 234 (Novo Biolabs) was added and the suspension incubated at room temperature with gentle shaking for up to 3 h The hydrolysis of yeast cell walls was monitored spectrophotometrically at 610 nm (lysis test) 0 1 ml sample of suspension was diluted with 4 ml of distilled, deionised water and the absorbance measured The absorbance decrease from t_0 is related to spheroplast formation and is expressed as a percentage (Rose and Veazey, 1992) Protoplasts were harvested by centrifugation (1200 × g, 5 minutes), washed twice with 10 mM Tris-MES buffer containing 1 mM sorbitol and resuspended in same

2.4.6 *Partial spheroplasting technique for recovery of subcellular ion pools* (**Method 2**)

This technique comprises of a partial spheroplasting step followed by DEAE dextran permeabilisation of the weakened yeast cells The final step involves disruption with glass beads The experimental protocol was as follows

Cell samples were initially treated as outlined for preparation of spheroplasts (see above) The supernatants of pretreatment buffer and three subsequent washes were combined and retained for further analysis (termed Pre-wash fraction) Cells were resuspended in 1 7 ml of spheroplasting buffer (10 mM Tris-MES, 1 M sorbitol) and 20

mg of the cell wall degrading enzyme Novozym 234 was added The reaction was allowed to proceed until approximately 15-20% conversion occurred, as determined photometrically (see above) This took approximately 12-15 minutes for control cells and up to 10 minutes longer for metal-loaded cells Samples were pelleted by centrifugation (4000 × g, 2 minutes) and the supernatant retained (termed Enz fraction) Pellets were rinsed twice (1 ml each time) with 10 mM Tris MES buffer, pH 6 0 containing 1 0 M sorbitol, samples were gently inverted each time and the supernatants from each spin (4000 × g, 2 minutes) combined (Wash fraction) The remaining pellet was very gently resuspended with a thin glass rod and gentle inversion, in 0 5 ml of 10 mM Tris-MES buffer containing 0 7 M sorbitol prior to addition of 60 µl of DEAE dextran (10 mg ml ^{1}) After 1 minute, samples were centrifuged (8000 × g, 1 minute) and washed five times (1 minute, 0 5 ml each time) with 10 mM Tris-MES-0 7 M sorbitol buffer Samples were gently shaken by hand during the first two washes and resuspended with a glass rod thereafter All supernatants were combined and termed fraction 1 (F1) The remaining pellet was resuspended in 1 ml of 10 mM Tris-MES buffer and disintegrated with glass beads as previously outlined in section 2 4 4 3 The supernatant from this fraction was termed fraction 2 (F2) and the remaining cell debris was digested in 6 M HNO_3 to release bound cations and termed Pellet fraction All fractions were assayed for metal content and enzyme activity where appropriate

CHAPTER 3

RESULTS

Accumulation and subcellular localisation of metal cations by Saccharomyces cerevisiae

Ph D. Research Thesis by

Kevin J Blackwell B.Sc.

CHAPTER 3: RESULTS

3.1 Effect of environment on metal accumulation· results

3.1.1 *Cd^{2+} accumulation by S cerevisiae under different environmental conditions*

Accumulation of Cd^{2+} by a brewing strain of *S cerevisiae* was examined under three different environmental circumstances, (i) an unbuffered system consisting of a cell suspension in distilled, deionised water adjusted to pH 5 5, (ii) a buffered system comprising of a cell suspension in 10 mM MES buffer (adjusted to pH 5 5), and (iii) a pH controlled system identical to (i) except that pH was maintained at pH 5 5 by constant addition of 5 M NaOH or HNO_3 Cd^{2+} to a final concentration of 100 and 200 µM was added to each system and uptake monitored over 5 h (see Figures 3 1 1 1 and 3 1 1 2) Incubation in 100 µM Cd^{2+} (Figure 3 1 1 1) underlined the differences in Cd^{2+} accumulation between each system In the presence of glucose, the buffered and pH controlled systems accumulated similar levels of Cd^{2+}, 92 and 89 µmol g^{-1} respectively after 5 h incubation (Figure 3 1 1 1, b and c) Accumulation was gradual in the buffered system with the rate of accumulation decreasing with time of incubation However in the pH controlled system, a greater proportion of overall Cd^{2+} was taken up during the early stages of contact (40 µmol g^{-1} within 5 minutes compared with 16 µmol g^{-1} during the same time in the buffered system) and consequently, metal uptake levels remained consistently higher in the pH controlled system in comparison to the buffered system until the 3 h time point, by which time rates of metal accumulation in the pH controlled system had slowed dramatically In contrast, metal uptake in the unbuffered was the lowest of the three systems with only 58 µmol g^{-1} accumulated after 5 h, some 35% lower than in the buffered or pH controlled systems (Figure 3 1 1 1,a) Here, accumulation was slow and gradual after an initial period of rapid uptake (18 µmol g^{-1} after 5 minutes)

In the absence of glucose, similar uptake levels were achieved in each system, approximately 33 µmol g^{-1} after 5 h However, uptake did not remain constant as was expected In the unbuffered system, initial uptake after 5 minutes was determined to be 10 µmol g^{-1} and remained less than 20 µmol g^{-1} up to 1 h Thereafter, uptake steadily increased to 32 µmol g^{-1} at 5 h In the buffered system, initial uptake was higher at 21 µmol g^{-1} after 5 minutes, and increased steadily thereafter during the next 5 h Initial

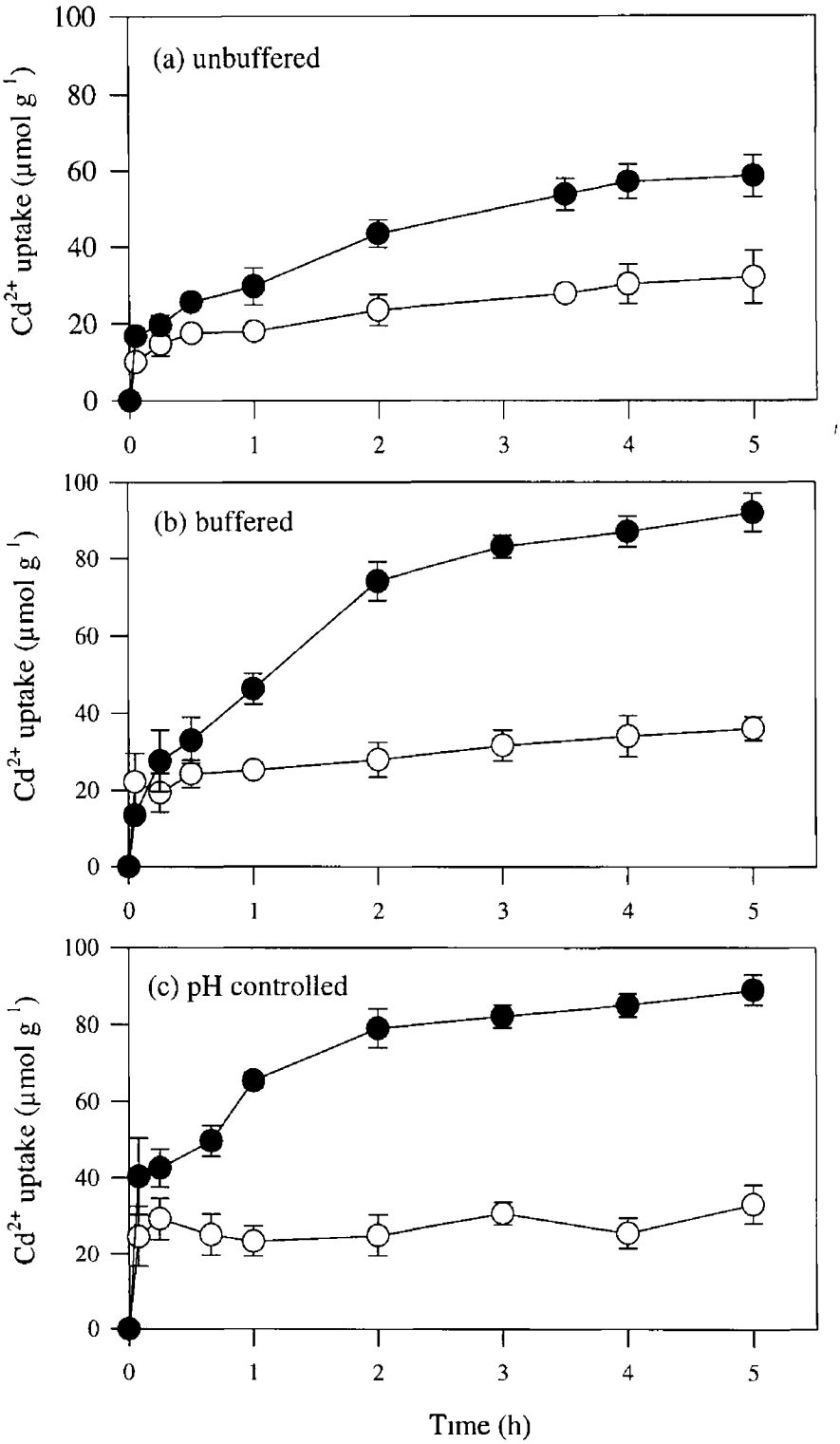


Figure 3 1 1 1 Cd^{2+} uptake from a 100 µM solution by *Saccharomyces cerevisiae* in (a) unbuffered, (b) buffered and (c) pH controlled environment The graphs show levels of Cd^{2+} removed from solution (average value from duplicate flasks, error bars indicate individual values) m the presence (closed symbols) or absence (open symbols) of 2% glucose

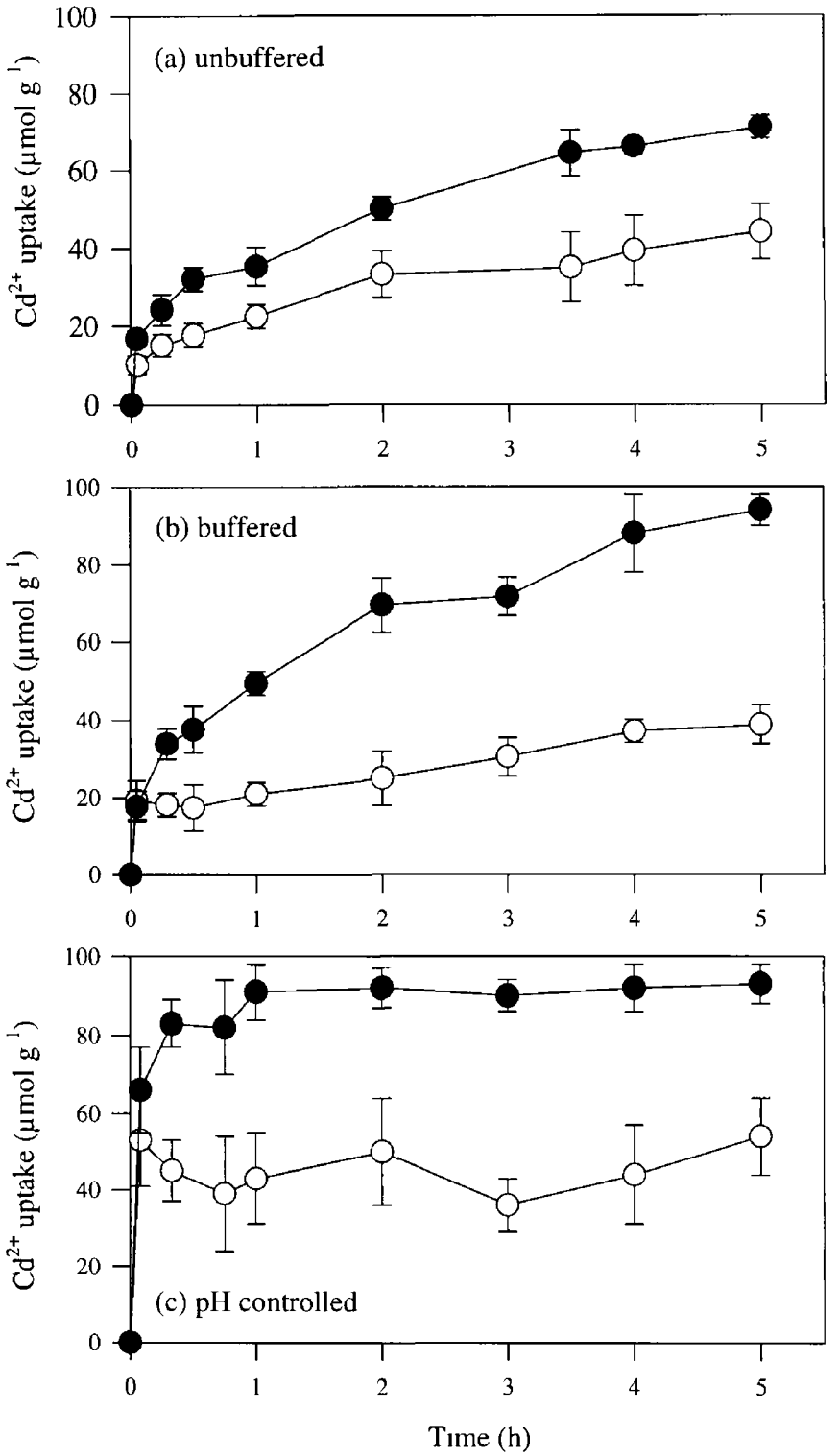


Figure 3 1 1 2 Cd^{2+} uptake from a 200 µM solution by *Saccharomyces cerevisiae* in (a) unbuffered, (b) buffered and (c) pH controlled environment The graphs show levels of Cd^{2+} removed from solution (average values from duplicate flasks, error bars indicate individual values) in the presence (closed symbols) or absence (open symbols) of 2% glucose

uptake in the pH controlled system was higher again at 24 µmol g^{-1}, although subsequent metal removal levels were erratic during prolonged incubation

When the initial concentration of Cd^{2+} was 200 µM, these trends were again apparent (Figure 3 1 1 2) Generally metal uptake in the unbuffered system were observed to increase (compared with observations at 100 µM Cd^{2+}), but in the presence of glucose remained some 20% less than in the other systems In the absence of glucose, the gradual time-dependent uptake previously observed was more marked with 44 µmol g^{-1} Cd^{2+} accumulated after 5 h, a 35% increase on uptake levels observed at the lower Cd^{2+} concentration Final levels of Cd^{2+} removal in the buffered and pH controlled systems in the presence of glucose were similar at 94 µmol g^{-1} after 5 h However, uptake in the buffered system was gradual over the 5 h incubation period but much more rapid in the pH controlled system Here, 66 µmol g^{-1} Cd^{2+} was taken up within 5 minutes with no further Cd^{2+} accumulation occurring after 1 h In the absence of glucose, the buffered system continued to exhibit time-dependent metal accumulation, with final levels 25% higher than observed at the lower Cd^{2+} concentration, at 38 µmol g^{-1} after 5 h By contrast, metal uptake in the absence of glucose in the pH controlled system was approximately double that observed at the lower Cd^{2+} concentration Over the 5 h incubation period, Cd^{2+} uptake levels varied between 40 and 55 µmol g^{-1}, but showed no clear increase or decrease

3.1.2 *Ion exchange during Cd^{2+} accumulation*

The effect of Cd^{2+} accumulation on extracellular K^+ and Mg^{2+} in each system was investigated In metal-free controls, levels of K^+ and Mg^{2+} were remarkably similar for each system in the absence of glucose, with extracellular Mg^{2+} levels remaining constant at approximately 10 µmol g^{-1} over 5 h and extracellular K^+ levels slowly increasing from 105 µmol g^{-1} at 0 h to 160 µmol g^{-1} at 5 h (data not shown) When glucose was added to metal-free controls in a buffered and pH controlled environment, previously released extracellular K^+ and Mg^{2+} were taken up by the biomass within 30 minutes and extracellular ion levels remained constant at approximately 20 µmol g^{-1} for K^+ and < 5 µmol g^{-1} for Mg^{2+} during the following 4 5 h However in the unbuffered system, glucose had only a modest, transient (10-15 minute) effect on accumulation of

previously released K^+ ions K^+ continued to leak from the cells in a fashion similar to that observed in the absence of glucose (data not shown)

No net release of Mg^{2+} occurred in any system during Cd^{2+} uptake as evidenced by extracellular Mg^{2+} levels which were very similar to metal-free control values However, considerable amounts of K^+ were released which differed in each system (see Figure 3 1 2) The highest levels of extracellular K^+ were observed in the pH controlled system followed by the buffered system with the lowest levels of release in an unbuffered environment, in both the presence or absence of glucose In the absence of glucose, K^+ release in each system was slow and gradual, whereas in the presence of glucose, K^+ profiles were characterised by substantial release during the first hour of contact with rates of K^+ efflux decreasing during the subsequent 4 h After 5 h incubation with 100 µM Cd^{2+} in the absence of glucose, extracellular K^+ levels had reached 191, 225 and 239 µmol g^{-1} in the unbuffered, buffered and pH controlled systems, respectively By comparison, uptake of Cd^{2+} in the presence of glucose brought about release of 316, 430, and 485 µmol g^{-1} K^+ after 5 h in each particular environment (Figure 3 1 2) Increasing the Cd^{2+} concentration to 200 µM served to increase cellular K^+ efflux in each system, particularly in the buffered environment Here, in the absence of glucose, K^+ release increased 50% to 340 µmol g^{-1} and by 20% to 510 µmol g^{-1} in the presence of glucose In the unbuffered and pH controlled systems, K^+ efflux increased 25% in the absence of glucose to 240 and 295 µmol g^{-1} respectively and by approximately 15% in the presence of glucose to 365 and 550 µmol g^{-1}, respectively (data not shown)

3.1.3 *Uptake of Cd^{2+} by non-metabolising biomass*

Due to the observed time-dependent nature of Cd^{2+} uptake in the absence of glucose, it was decided to determine what fraction of uptake could be attributed to non-metabolic processes Irradiation was chosen as the method of biomass inactivation in order to minimise heat denaturation effects associated with oven dried cells Irradiated biomass was prepared as outlined in section 2 1 3 and contacted with 100 µM Cd^{2+} An assay of cell viability with citrate methylene blue staining prior to addition of Cd^{2+} confirmed that less than 2% of cells remained viable From Figure 3 1 3, it can be clearly seen that uptake by irradiated biomass stabilised within 15 minutes at 25 µmol g^{-1} and remained

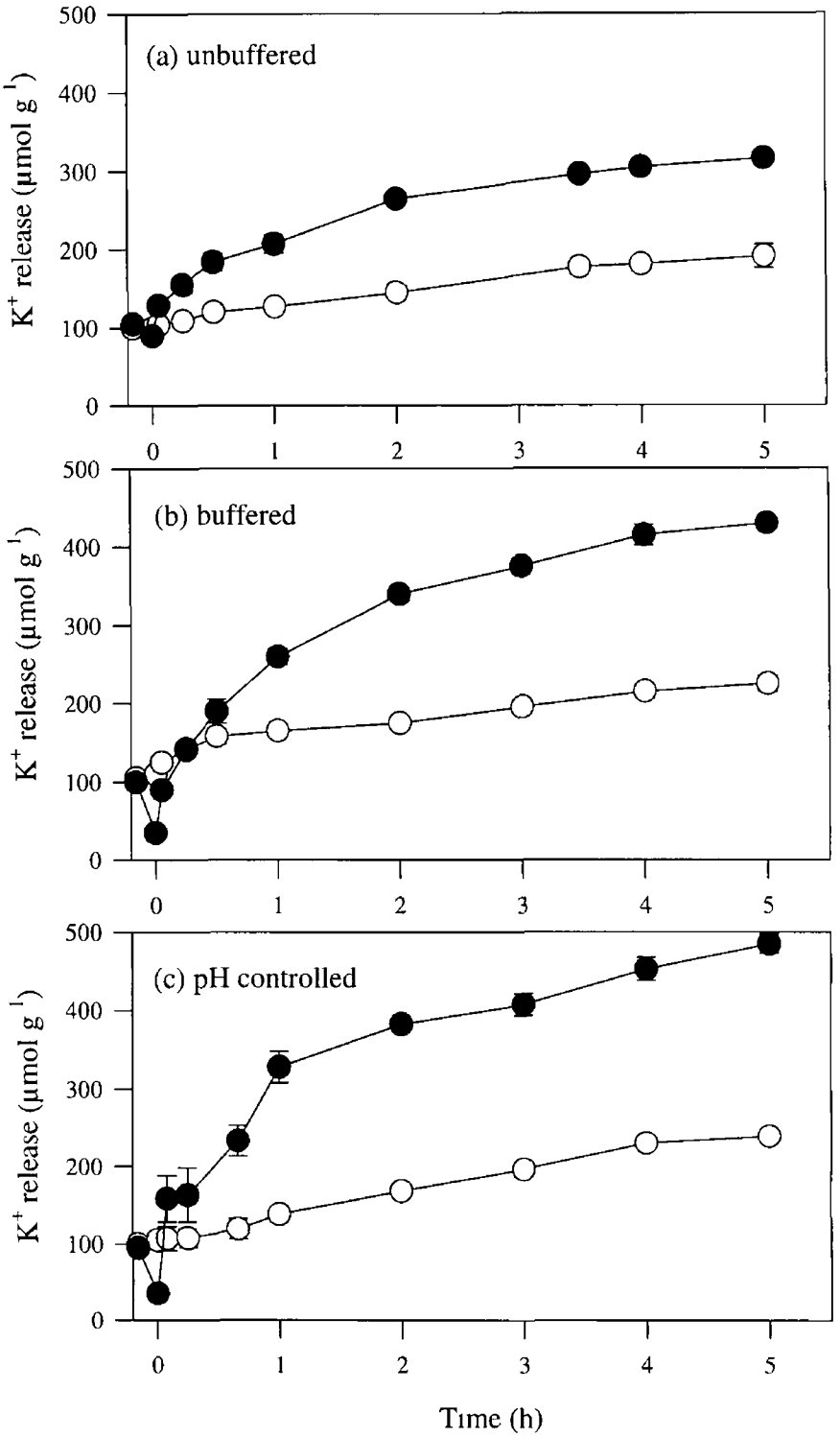


Figure 3 1 2 K^+ release from *Saccharomyces cerevisiae* in the presence of 100 µM Cd^{2+} in (a) unbuffered, (b) buffered and (c) pH controlled environment The graphs show extracellular K^+ levels (average values from duplicate flasks, error bars indicate individual values) in the presence (closed symbols) and absence (open symbols) of 2% glucose

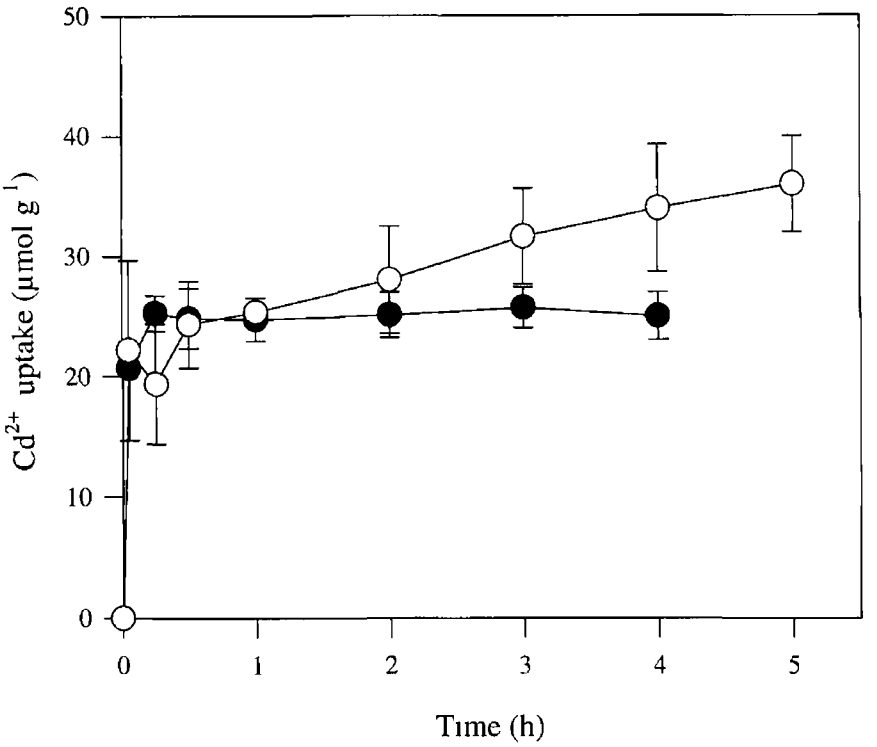


Figure 3 1 3 Cd^{2+} uptake by live (open symbols) and irradiated (closed symbols) *Saccharomyces cerevisiae* biomass from a 100 µM solution The graph shows levels of Cd^{2+} removed from solution (average values from duplicate flasks, error bars indicate individual values) in the absence of a metabolisable energy source

constant thereafter during prolonged incubation The addition of glucose prior to addition of Cd^{2+} did not enhance uptake in any way For comparison, Cd^{2+} uptake in the buffered system from a 100 µM solution is shown and the time dependent nature of uptake is clear

Levels of Mg^{2+} release, not recorded previously were observed on addition of Cd^{2+} ions Immediate Mg^{2+} release of 35 µmol g^{-1} was evident within 5 minutes and by 15 minutes had reached 40 µmol g^{-1}, and remained unchanged at this level for the remainder of the experiment

3.1.4 *pH fluctuations during Cd^{2+} accumulation*

In the unbuffered and buffered systems, extracellular pH was measured throughout Cd^{2+} uptake (see Figure 3 1 4a,b) There were considerable changes in extracellular pH in the unbuffered environment, particularly in the presence of glucose (as expected) but additionally, pH variations were detected in the buffered system This was surprising and may have been due to the fact that the buffering capacity of the environment was overcome by the high levels of H^+ ions produced during metabolism of glucose In the unbuffered system (Figure 3 1 4a), extracellular pH levels rose slowly from pH 5 5 to pH 6 1 in the absence of glucose The presence or absence of Cd^{2+} ions did not appear to affect this process In the presence of glucose, its rapid metabolisation led to a reduction from pH 5 5 to approximately pH 4 4 within the 10 minutes prior to addition of Cd^{2+} ions It is likely that this lowering of pH contributed to the lower levels of Cd^{2+} accumulation observed in this system through competitive inhibition of uptake It was also observed that pH values in the presence of Cd^{2+} ions were higher than in metal-free controls, indicating the adverse nature of Cd^{2+} ions on cellular metabolism

In a buffered environment, pH values remained constant in the absence of glucose (Figure 3 1 4b) However in the presence of glucose, changes in pH did occur In metal-free control flasks pH values had fallen to pH 5 25 within 1 h of glucose addition and continued to decrease in a linear fashion during the following 4 h incubation When Cd^{2+} ions were present, the decrease in pH was still evident but was much reduced Within 5 h, pH values had decreased to pH 5 15 in the presence of 100 µM Cd^{2+} At increased Cd^{2+} concentration of 200 µM, pH values exhibited similar trends The effect of Cd^{2+} ions on cellular metabolism is more visible here than observed in the unbuffered system This may have facilitated by masking of initial H^+

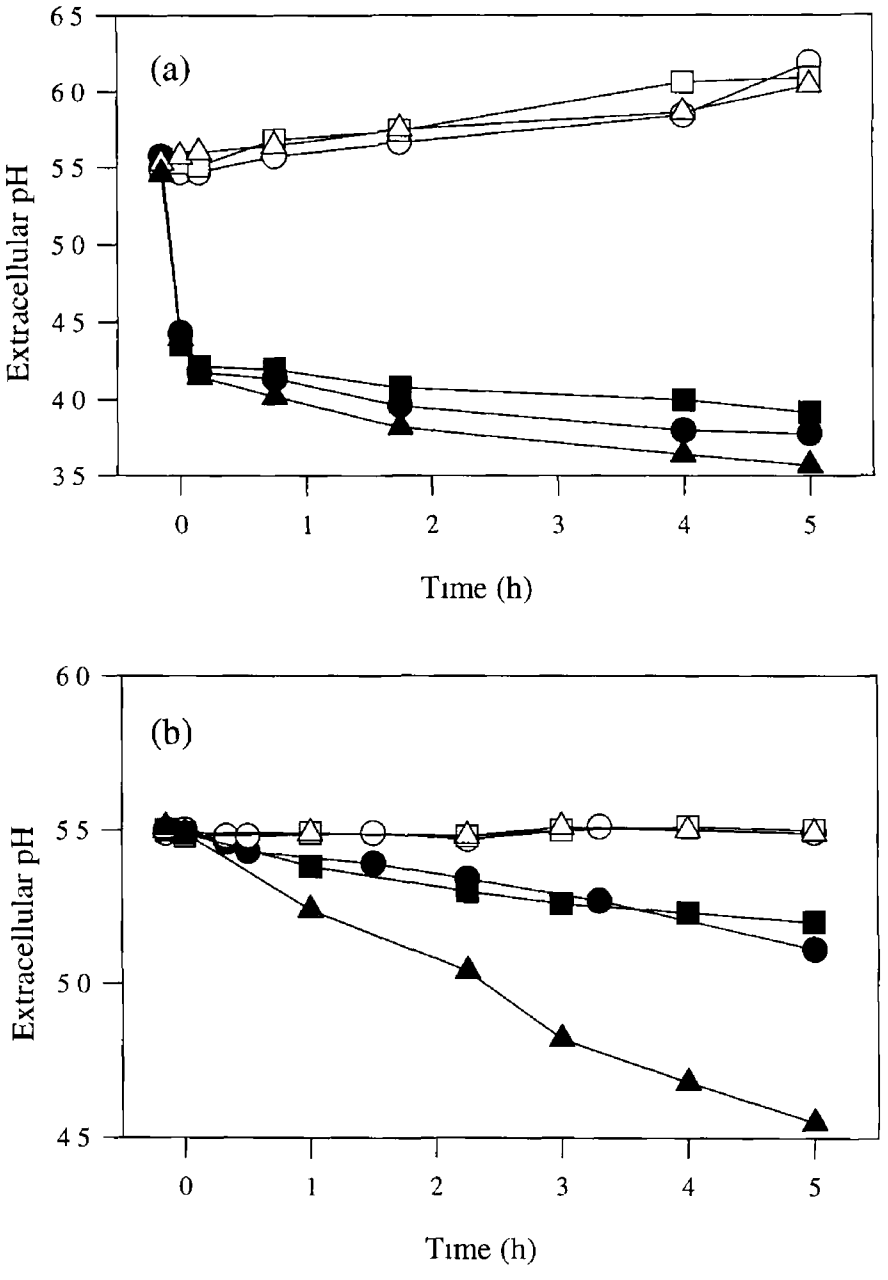


Figure 3 1.4 Changes in extracellular pH during Cd^{2+} accumulation by *Saccharomyces cerevisiae* in (a) unbuffered and (b) buffered environment The graphs show variations in extracellular pH in the presence of 0 (Δ , ▲), 100 (□ , ■) and 200 (○ , ●) μM Cd^{2+} in the presence (closed symbols) and absence (open symbols) of 2% glucose

efflux by MES buffer, and possibly influenced by the more rapid Cd^{2+} accumulation observed in this type of environment

3.1.5 *Cell viability during Cd^{2+} uptake*

Cell samples taken during uptake of 100 μM Cd^{2+} under three different environmental conditions were assayed for viability using citrate-methylene blue staining and the results are shown in Figure 3 1 5 Where viability loss occurred, the majority takes place during the initial moments of contact with Cd^{2+} ions (within 10 minutes) and thereafter decreases at a slow rate during the remainder of incubation period The presence or absence of glucose does not appear to effect loss of viability

From the graph it can be seen that viability loss is influenced by the system in which uptake occurs In the buffered, unbuffered and pH controlled systems, cell viabilities remained between 80-90%, 70-80% and 60-70% respectively during Cd^{2+} accumulation The buffered system, designed to minimise effects of extracellular H^+ changes on the biomass provided the best protection against Cd^{2+} toxicity Greater loss of viability was observed in the unbuffered system even though Cd^{2+} accumulation here was the lowest recorded of the three systems The greatest decrease in cell viability observed in the pH controlled system may have resulted from more rapid Cd^{2+} uptake coupled with rapidly changing pH values, albeit over a narrow range, that may have increased cellular susceptibility to Cd^{2+} toxicity Metal-free controls in a pH controlled environment did not show any loss of viability due to pH adjustment alone Indeed, no appreciable viability loss was recorded in any of the control experiments (buffered, unbuffered or pH controlled systems) with cell viabilities remaining > 90% at all times

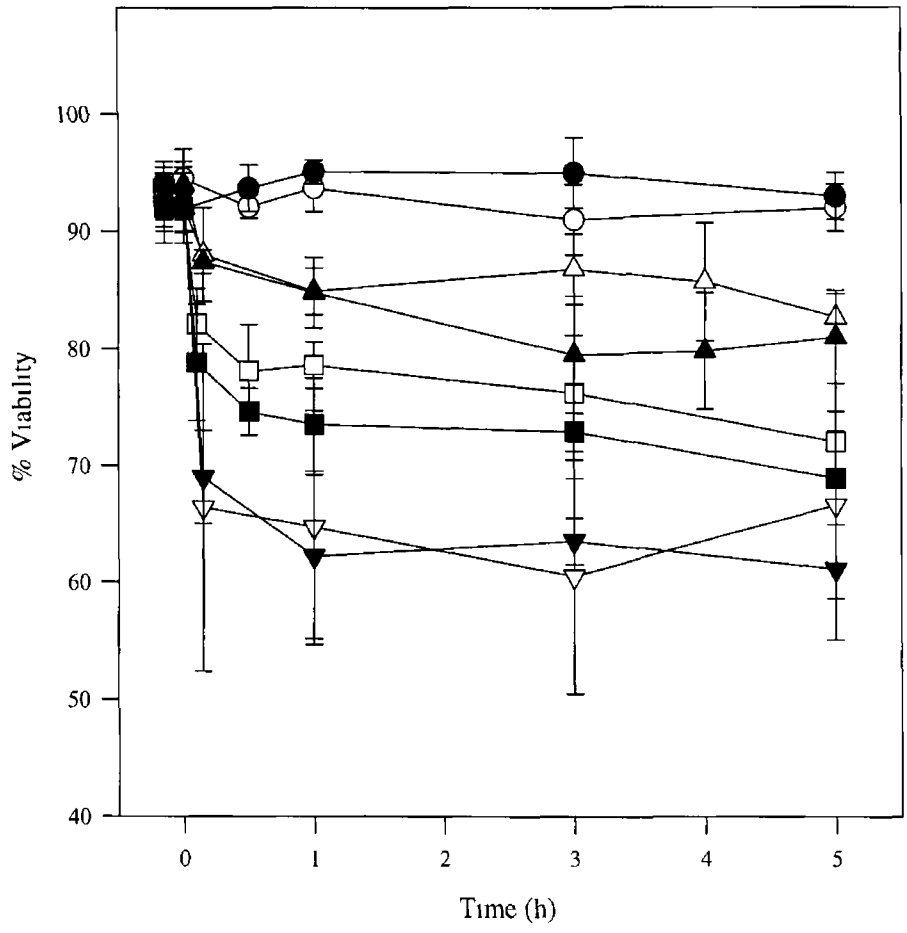


Figure 3 1 5 % viability of *Saccharomyces cerevisiae* during exposure to 100 μM Cd^{2+} The graph shows unstained cells as a percentage of the total (average values from duplicate flasks, error bars indicate individual values) in the presence (closed symbols) and absence (open symbols) of glucose Typical control values (○ , ●), unbuffered system (□ , ■), buffered system (Δ , ▲), pH controlled system (▽ , ▼)

3.2 Accumulation and subcellular localisation of metals: results

3.2.1 *Metal uptake profiles and consequent ion release.*

Metal uptake by cells of *S. cerevisiae* incubated for 5 h in 100 μM Cd^{2+}, Mn^{2+}, Sr^{2+} or 5 μM Cu^{2+} in the presence and absence of 2% glucose were monitored continuously (by measuring remaining extracellular metal levels, except in the case of Cu^{2+} where cellular metal levels were determined), and the results displayed in Figure 3.2.1.1. In each case, excluding that of Cu^{2+}, the presence of glucose resulted in increased levels of metal sequestration. Cd^{2+} accumulation in the presence of glucose was rapid within the first hour of contact (60 μmol g^{-1} after 60 minutes) but thereafter rates of accumulation slow appreciably (Figure 3.2.1.1,a). By 5 h, virtually all the Cd^{2+} present at 0 h had been removed from solution. In the absence of glucose, Cd^{2+} uptake levels remained at approximately 20 μmol g^{-1} up to 1 h, but increased at a steady rate of approximately 5 μmol g^{-1} h^{-1} during the following 4 hours, resulting in final Cd^{2+} levels of 40 μmol g^{-1} after 5 h.

Differences in levels of metal accumulation between glucose-supplemented and unsupplemented cells were most apparent during Mn^{2+} accumulation. Glucose-supplemented cells removed approximately five-fold more Mn^{2+} from solution compared with unsupplemented cells (Figure 3.2.1.1,b). Mn^{2+} accumulation in the presence of glucose was characterised by a steady rate of accumulation after initial metal binding (first time point, 5 min after metal addition) up to 3 h of contact at a rate of approximately 25 μmol g^{-1} h^{-1}. Between 3 and 5 h, the remaining Mn^{2+} in solution was taken up by the cells. At 5 h, extracellular Mn^{2+} concentration had decreased to < 4 μM. In the absence of glucose, Mn^{2+} uptake levels remained consistently low over the 5 h incubation period. Initial surface binding after 5 minutes accounted for 9 μmol g^{-1} of eventual Mn^{2+} uptake. During the following 5 h, levels of Mn^{2+} removed from solution slowly increased, effectively doubling to 19 μmol g^{-1} after 5 h.

Sr^{2+} accumulation was not enhanced by glucose to the same extent as for Cd^{2+} and Mn^{2+}. Here, uptake was rapid with 70% of final Sr^{2+} levels accumulated within the first hour of incubation (26 μmol g^{-1}), but by 5 h, cells had only sequestered 38 μmol g^{-1} Sr^{2+} (Figure 3.2.1.1c) resulting in large amounts of Sr^{2+} remaining unbound in solution. In the absence of glucose, Sr^{2+} uptake was approximately 2-fold lower, and was

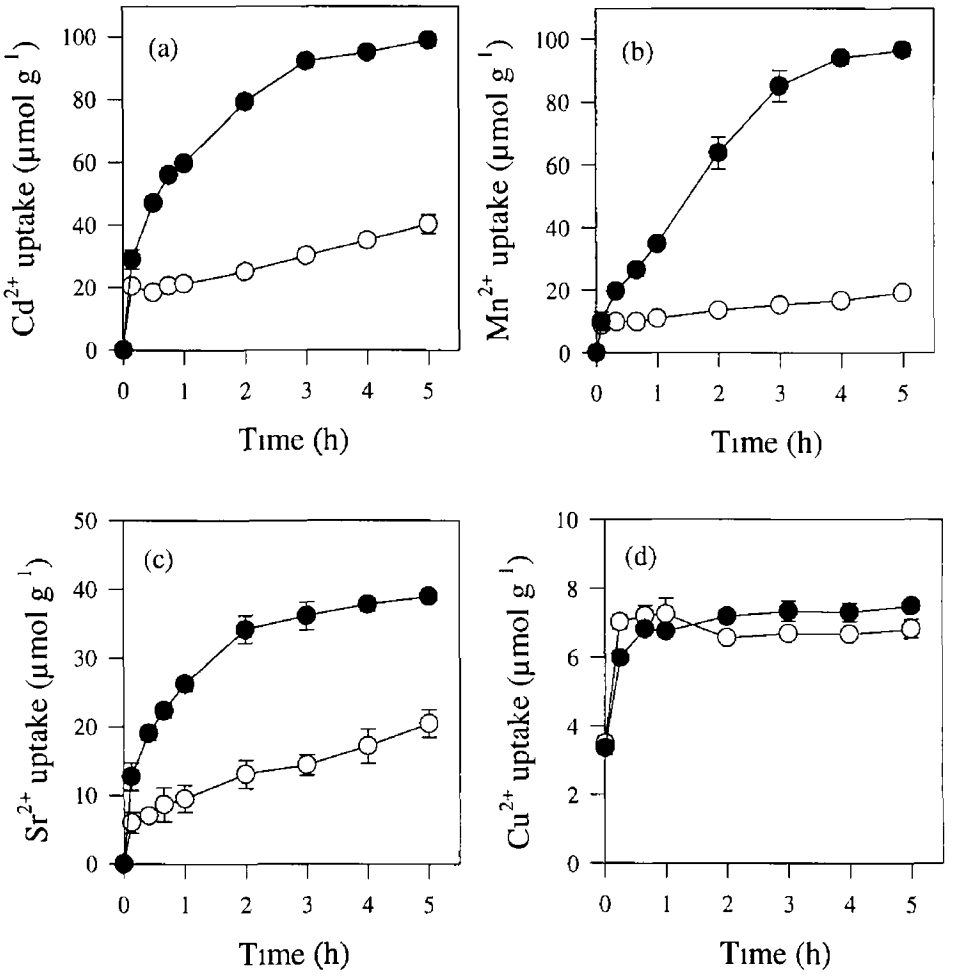


Figure 3.2 1 1 Metal cation accumulation by *Saccharomyces cerevisiae* over 5 h in the presence (closed symbols) and absence (open symbols) of 2% glucose The graphs show uptake of (a) Cd^{2+}, (b) Mn^{2+} and (c) Sr^{2+} from 100 µM solutions, and (d) Cu^{2+} from a 5 µM solution Mean values ± standard error of the mean from three replicate determinations are shown

characterised by a slow, steady increase after initial binding Sr^{2+} uptake increased 3-fold from 6 µmol g^{-1} after 10 minutes to 20 µmol g^{-1} within 5 h

Uptake of Cu^{2+} was virtually identical in the presence and absence of glucose Cells initially contained approximately 3 4 µmol g^{-1} (due to presence of Cu^{2+} in growth medium) and additional extracellular Cu^{2+} was rapidly taken up (Figure 3 2 1 1,d) In the absence of glucose, cellular Cu^{2+} levels remained effectively constant after 15 minutes In the presence of glucose, a slight increase in cellular Cu^{2+} was observed between the 15 min and 2 h time points but thereafter remained constant at values similar to those obtained in the absence of glucose Analysis of supernatants at each time point (15 min - 5 h) revealed the presence of only trace levels (< 0 5 µM) of Cu^{2+}, indicating the rapid and permanent nature of the uptake process

Release of cellular Mg^{2+} and K^+ as a result of metal uptake were investigated concurrently with metal uptake Metal-free controls indicated that Mg^{2+} release remained constant at approximately 10 µmol g^{-1} over 5 h incubation in the absence of glucose Upon addition of glucose, extracellular Mg^{2+} levels decreased to < 5 µmol g^{-1} within 15 minutes and remained at this low level for the duration of the experiment (data not shown) During uptake of Cd^{2+}, Mn^{2+} and Sr^{2+}, levels of extracellular Mg^{2+} ions were similar to those observed in metal-free controls both in the presence and absence of glucose However, during Cu^{2+} uptake in the presence of glucose, it was observed that extracellular Mg^{2+} did not decrease, but slowly increased to 20 µmol g^{-1} by 5 h Similarly, in the absence of glucose, some Mg^{2+} release did occur, with 15 µmol g^{-1} released after 5 h (data not shown)

The pattern of K^+ release varied from metal to metal, and was influenced by the presence or absence of glucose Typical K^+ release profiles in metal-free controls are shown in Figure 3 2 1 2, Cu^{2+}(triangular symbols) During the 1 5 h equilibration period, cells released approximately 110 µmol g^{-1} of intracellular K^+ In the absence of glucose, this slow release continued during subsequent incubation over 5 h Addition of glucose resulted in rapid (within 20-30 minutes) accumulation of previously released K^+ to an effectively constant extracellular level of approximately 20 µmol g^{-1} In the absence of glucose, each cation tested induced K^+ efflux in excess of control levels, and all except Sr^{2+} interfered with K^+ uptake in the presence of glucose No stoichiometric balance between K^+ loss and accumulated test ion was observed

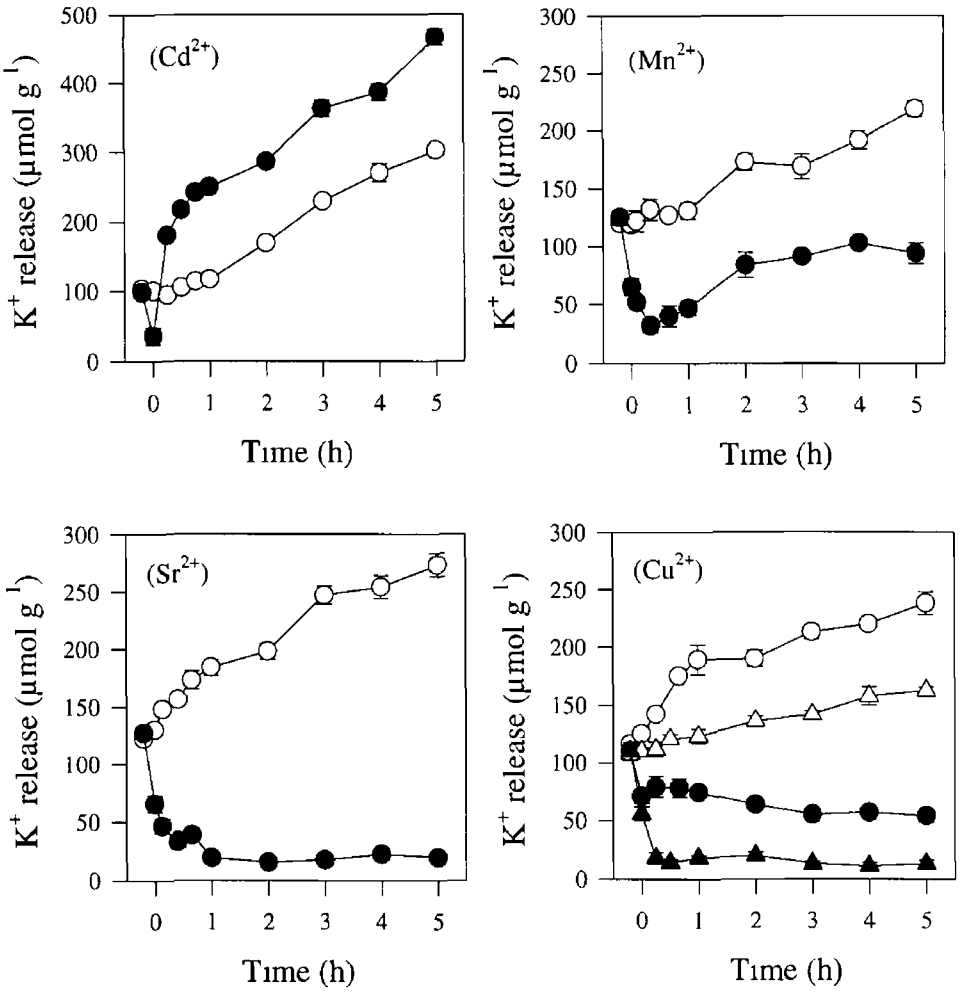


Figure 3 2 1 2 K^+ release from *Saccharomyces cerevisiae* over 5 h during accumulation of a range of metal cations in the presence (closed symbols) and absence (open symbols) of 2% glucose The graphs show extracellular K^+ levels during incubation with 100 µM Cd^{2+}, Mn^{2+} and Sr^{2+}, and 5 µM Cu^{2+} Extracellular K^+ control values (Δ , ▲) are shown on the Cu^{2+} plot Mean values ± standard error of the means from three replicate determinations are shown

Cd^{2+} was the only ion tested to induce rapid release of cellular K^+ in the presence of glucose K^+ release was most apparent within 1 h of contact by which time approximately 230 µmol g^{-1} of K^+ had been released (Figure 3 2 1 2, Cd^{2+}) and corresponded to the most rapid period of Cd^{2+} accumulation During the subsequent 4 h, a further 200 µmol g^{-1} of K^+ was released In the absence of glucose, K^+ release was similar to control levels during the first hour of contact with Cd^{2+}, but thereafter increased rapidly with > 250 µmol g^{-1} of K^+ released during the next 4 h Again, this period of K^+ release coincided with increased Cd^{2+} accumulation

Incubation of cells with Mn^{2+} in the absence of glucose induced K^+ loss similar to control levels during the first hour of incubation, but thereafter, K^+ loss increased up to 5 h as observed with Cd^{2+}, although levels of K^+ released were not as high (Figure 3 2 1 2, Mn^{2+}) In the presence of glucose, extracellular K^+ was taken up by cells in a fashion similar to metal-free controls during the initial 20 minutes of contact with Mn^{2+} but ceased with extracellular K^+ levels at 30 µmol g^{-1} For the following 1 5 h, cells gradually released approximately 55 µmol g^{-1} K^+ Between 2 and 5 h, release of K^+ had slowed as to be effectively constant

Addition of Sr^{2+} induced sustained K^+ release in the absence of glucose over and above control levels K^+ loss was in excess of 25 µmol g^{-1} h^{-1} and resulted in levels of release second only to those induced by Cd^{2+} over a similar time period In presence of glucose, addition of Sr^{2+} did not affect eventual uptake of K^+, although it appeared to slow K^+ accumulation, resulting in 1 h elapsing (as compared with 15-20 minutes) before extracellular K^+ levels reached levels observed in metal-free controls

Cu^{2+} accumulation had an effect on extracellular K^+ levels similar to that observed with Mn^{2+} In the presence of glucose, addition of Cu^{2+} arrested K^+ uptake, but within 1 h, K^+ uptake recommenced at a reduced rate However, extracellular K^+ levels did not fall below 50 µmol g^{-1} during the 5 h incubation period In the absence of glucose, Cu^{2+} induced the release of 70 µmol g^{-1} K^+ within 1 h, but only 50 µmol g^{-1} during the following 4 h The major part of K^+ release was exhibited within the first hour and corresponded with the period of rapid Cu^{2+} uptake

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

After 0, 1, 3, and 5 h of incubation with 100 µM Cd^{2+} in both the presence and absence of glucose, cells were fractionated (Method 1) into cytoplasmic, vacuolar and bound pools for analysis of Cd^{2+}, Mg^{2+} and K^+ ions Ca^{2+} levels were found to be negligible in native biomass, probably resulting from the lack of Ca^{2+} in growth medium, and consequently were not further analysed In each case it was found that Cd^{2+} ions were present in all four subcellular fractions examined irrespective of glucose addition, and that specific levels of Cd^{2+} present in each fraction increased with time (Table 3 2 2 1) 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, both in the presence and absence of glucose The percentage Cd^{2+} in each fraction was approximately 4-5, 2-3, 20-23 and 68-73 % for wash, cytoplasmic, vacuolar and bound fractions respectively A mass balance between overall cellular Cd^{2+} levels and the sum of Cd^{2+} levels present in each subcellular fraction was observed, whereby the sum of individual fractions agreed to within 10% of whole cell Cd^{2+} levels In general, small quantities of Cd^{2+} ions were detected in the wash fraction although these levels did increase over time, but did not exceed 5% of total cellular Cd^{2+} Cytoplasmic Cd^{2+} levels were the lowest of the four fractions with some 40-70% higher levels in the cytoplasmic pool of cells incubated with glucose The influence of glucose was most apparent in compartmentation of Cd^{2+} to the vacuolar and bound fractions Vacuolar Cd^{2+} compartmentation in cells incubated in the absence of glucose was apparent within 1 h and continued to increase over the next 4 h In comparison, vacuolar Cd^{2+} levels of cells incubated with glucose were 3-fold greater at 1 h and approximately 2 5-fold greater at successive time points The bound fraction at each time point was consistently observed to contain 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 2-2 5 times that observed without glucose at successive time points

Determination of intracellular Mg^{2+} and K^+ levels revealed that Cd^{2+} sequestration had a greater impact on K^+ than Mg^{2+} Although little net extracellular Mg^{2+} release was observed (<5 µmol g^{-1} in both the presence and absence of glucose), there were changes to Mg^{2+} levels in subcellular compartments (Table 3 2 2 2) Overall recovered cellular Mg^{2+} levels (sum of fractions) agreed to within 12% of whole cell Mg^{2+} levels The

Intracellular Cd^{2+} levels [µmol g^{-1}]												
Time	**0 h**		**1 h**				**3 h**				**5 h**			
2% glucose	-	+	-		+		-		+		-		+	
				(%)		(%)		(%)		(%)		(%)		(%)
Wash	-	-	1 3 ± 0 1	(5)	0 8 ± 0 2	(1)	1 8 ± 0 1	(5)	3 3 ± 0 1	(4)	2 6 ± 0 3	(5)	6 4 ± 0 1	(6)
Cytosolic	-	-	0 6 ± 0 1	(3)	0 9 ± 0 1	(1)	1 1 ±0 1	(3)	1 5 ± 0 1	(2)	1 5 ± 0 1	(3)	2 6 ± 0 1	(3)
Vacuolar	-	-	5 7 ± 0 1	(23)	15 2 ± 0 3	(24)	7 1 ± 0 2	(21)	17 0 ± 0 5	(19)	9 6 ± 0 5	(20)	21 8 ± 0 5	(22)
Bound	-	-	16 7 ± 0 2	(69)	47 0 ± 0 8	(74)	24 5 ± 0 2	(71)	66 9 ± 0 7	(75)	34 7 ± 0 3	(72)	67 0 ± 1 2	(69)
Sum	-	-	24 3±0 4	(100)	63 9±1 3	(100)	34 5±0 6	(100)	88 7± 1 4	(100)	48 4±1 2	(100)	97 8±1 9	(100)
Whole cells	-	-	25 6 ± 0 3		60 5 ± 1 7		36 8 ± 0 3		92 2 ± 0 3		45 0 ± 3 1		98 9 ± 1 8	

Table 3 2 2 1 Levels of Cd^{2+} in subcellular pools of *Saccharomyces cerevisiae* incubated with 100 µM Cd^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean from three replicate determinations are shown

Intracellular Mg^{2+} levels during Cd^{2+} accumulation [µmol g^{-1}]												
Time	**0 h**				**1 h**				**3 h**			
2% glucose	-		+		-		+		-		+	
		(%)		(%)		(%)		(%)		(%)		(%)
Wash	1 4 ± 0 1	(1)	1 7 ± 0 1	(1)	2 3 ± 0 1	(2)	2 1 ± 0 1	(1)	5 9 ± 0 1	(3)	10 3 ± 0 1	(7)
Cytosolic	1 9 ± 0 1	(1)	2 1 ± 0 1	(1)	2 3 ± 0 1	(2)	2 8 ± 0 4	(2)	3 0 ± 0 1	(2)	3 8 ± 0 2	(2)
Vacuolar	84 0 ± 0 2	(57)	85 3 ± 1 0	(58)	77 4 ± 0 3	(54)	80 2 ± 1 0	(53)	84 1 ± 0 4	(55)	77 5 ± 0 7	(50)
Bound	59 2 ± 0 2	(41)	57 6 ± 0 7	(40)	60 6 ± 0 5	(42)	65 4 ± 1 1	(43)	61 1 ± 0 6	(40)	63 6 ± 0 4	(41)
Sum	146 5±0 6	(100)	146 7±1 9	(100)	142 6±1 0	(100)	150 5±2 6	(100)	154 1±1 2	(100)	155 2±1 4	(100)
Whole cells	165 5 ± 2 5		164 7 ± 2 0		161 7 ± 0 8		167 8 ± 2 5		163 9 ± 2 4		169 1 ± 0 3	

Intracellular Mg^{2+} levels during Cd^{2+} accumulation [µmol g^{-1}]												
Time	**5 h**											
2% glucose	-		+									
		(%)		(%)								
Wash	7 3 ± 0 3	(5)	26 8 ± 0 2	(19)								
Cytosolic	3 6 ± 0 1	(2)	9 5 ± 0 1	(7)								
Vacuolar	82 0 ± 0 6	(56)	60 9 ± 0 3	(43)								
Bound	53 0 ± 0 6	(36)	44 8 ± 0 5	(32)								
Sum	145 9±1 6	(100)	142 0±1 1	(100)								
Whole cells	158 2 ± 9 9		153 9 ± 3 7									

Table 3 2 2 2 Levels of Mg^{2+} in subcellular pools of *Saccharomyces cerevisiae* during incubation in 100 µM Cd^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

quantity of Mg^{2+} ions in wash fractions increased with time in the presence of Cd^{2+} and was complemented by small decreases in vacuolar and bound Mg^{2+} of approximately similar magnitude, which was particularly noticeable at the later time points in the presence of glucose Small increases in cytoplasmic Mg^{2+} in the presence of Cd^{2+} ions in both the presence and absence of glucose were also observed Vacuolar Mg^{2+} did show a small decrease in the presence of glucose (no change in the absence of glucose) and bound Mg^{2+} was also observed to decline both in the presence and absence of glucose particularly at the final 5 h time point, possibly as a result of a transfer of Mg^{2+} to the wash fraction in the presence of Cd^{2+} ions In comparison, control experiments in the absence of Cd^{2+} did not indicate similar changes in subcellular Mg^{2+} levels at the later stages of incubation values obtained were comparable to those presented at 0 h here

Cd^{2+} ions induced substantial K^+ efflux in both the presence and absence of glucose, resulting in reduced cellular levels Net extracellular K^+ release was greater from cells incubated with glucose than from those without A mass balance on total K^+ present in subcellular fractions agreed to within 10% of whole cell K^+ levels at each time point Large amounts of K^+ were detected in the wash fraction (21-102 μmol g^{-1}) in the presence Cd^{2+} ions, which increased with incubation time, particularly in the presence of glucose (Table 3 2 2 3) Cytoplasmic K^+ levels were the highest of the three ions examined, and were observed to decrease upon addition of Cd^{2+} in the presence or absence of glucose Bound K^+ was minimal (less than 1% of total cellular K^+), suggesting that K^+ is predominantly a soluble ion rather than associated with solid cellular material Cd^{2+} ions had a negligible effect on K^+ levels here, even though the majority of Cd^{2+} was localised in this fraction The major part of cell K^+ was localised in the vacuole, approximately 480 μmol g^{-1} at 0 h (90% of total cellular K^+) and it was here that the greatest changes were observed Glucose-free control cells exhibited a slow, sustained decrease in vacuolar K^+ over 5 h resulting in increased extracellular K^+, while over the same time period in the presence of glucose, Cd^{2+}-free control cells conserved vacuolar K^+ at levels similar to those observed at 0 h In contrast, the presence of Cd^{2+} ions induced substantial K^+ loss from cell vacuoles which was most apparent in the presence of glucose For example, in the absence of glucose, vacuolar K^+ levels fell from 459 μmol g^{-1} at 0 h to 225 μmol g^{-1} after 5 h incubation with Cd^{2+} ions By comparison in the presence of glucose,

Intracellular K^+ levels during Cd^{2+} accumulation [µmol g^{-1}]												
Time	**0 h**				**1 h**				**3 h**				**5 h**			
2% glucose	-		+		-		+		-		+		-		+	
		(%)		(%)		(%)		(%)		(%)		(%)		(%)		(%)
Wash	16 9 ± 0 3	(3)	33 5 ± 0 8	(6)	21 4 ± 0 5	(5)	28 1 ± 4 0	(8)	52 2 ± 0 9	(15)	102 4±0 2	(34)	39 4 ± 2 1	(14)	80 3 ± 5 7	(44)
Cytosolic	28 9 ± 0 9	(6)	25 9 ± 1 0	(5)	15 4 ± 0 3	(4)	20 1 ± 3 0	(6)	11 6 ± 0 3	(3)	10 0 ±0 6	(3)	12 0 ± 0 7	(4)	12 4 ± 0 3	(7)
Vacuolar	459 2±13 5	(90)	498 0±9 7	(89)	404 4±10 9	(91)	308 2±8 7	(86)	292 8±2 7	(81)	180 7±12 8	(61)	225 6 ± 7 7	(81)	86 9 ± 2 1	(48)
Bound	3 8 ± 0 6	(1)	4 5 ± 0 2	(1)	3 4 ± 0 4	(1)	3 6 ± 0 3	(1)	2 9 ± 0 3	(1)	4 7 ± 0 7	(2)	2 7 ± 0 3	(1)	2 5 ± 1 0	(1)
Sum	508 8±15 3	(100)	561 9±11 7	(100)	444 2±12 1	(100)	360 0±16 0	(100)	359 5±4 1	(100)	297 8±14 3	(100)	279 7±10 8	(100)	182 1±9 1	(100)
Whole cells	553 2 ± 6 8		545 8 ± 9 5		480 4 ± 4 4		360 3 ± 6 9		370 3 ± 4 0		297 8 ± 12 0		286 7 ± 16 7		180 6 ± 8 7	

Table 3 2.2 3 Levels of K^+ in subcellular pools of *Saccharomyces cerevisiae* during incubation in 100 µM Cd^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

Table 3.2.2.4 Comparison of vacuolar K^+ loss with extracellular K^+ levels

	Net extracellular K^+ increase		Net vacuolar K^+ loss	
2% glucose	-	+	-	+
Time (h)	**µmol g^{-1}**		**µmol g^{-1}**	
0	0	0	0	0
1	25 ± 5	215 ± 17	55 ± 23	290 ± 19
3	134 ± 8	327 ± 25	167 ± 16	318 ± 23
5	207 ± 10	431 ± 24	234 ± 21	411 ± 13

a greater decrease from 498 µmol g^{-1} to 86 µmol g^{-1} occurred over the same time period This vacuolar loss of K^+ was approximately equivalent to the observed net extracellular increase at each time point (Table 3 2 2 4)

3 2.3 *Subcellular compartmentation of Mn^{2+} and its effect on intracellular levels of Mg^{2+} and K^+*

Mn^{2+} ions were detected in each subcellular pool, irrespective of glucose addition, although the presence of glucose substantially increased overall cellular Mn^{2+} levels In the presence of glucose, 3- and 6-fold differences in cellular Mn^{2+} levels were observed at 1 and 3 h compared with cells incubated without glucose (Table 3 2 3 1) At 5 h, a 4 5-fold difference was observed, and may be explained by a virtual cessation of Mn^{2+} uptake in the presence of glucose, whilst accumulation continued in the absence of glucose at a slow rate The percentage distribution of Mn^{2+} between subcellular pools was different to, and more dynamic than that observed with Cd^{2+} The presence or absence of glucose influenced the subcellular percentage distribution of Mn^{2+} and unlike Cd^{2+}, no uniform subcellular partitioning was observed Mn^{2+} levels recovered by fractionation were in good agreement (within 10%) with overall cellular Mn^{2+} levels as determined by acid-digestion of whole cells The wash fraction contained levels of Mn^{2+} that changed little over 5 h in both the presence and absence of glucose Due to lower accumulation in the absence of glucose, the wash fraction contained an appreciable percentage of overall cellular Mn^{2+} It varied from 24% at 1 h to 14% at 5 h, even though the absolute value of Mn^{2+} present actually increased Similarly, in the presence of glucose, the fractional distribution

Intracellular Mn^{2+} levels [µmol g^{-1}]												
Time	**0 h**		**1 h**				**3 h**				**5 h**			
2% glucose	-	+	-		+		-		+		-		+	
				(%)		(%)		(%)		(%)		(%)		(%)
Wash	-	-	2 2 ± 0 1	(23)	2 7 ± 0 1	(9)	2 5 ± 0 1	(20)	2 9 ± 0 1	(4)	2 6 ± 0 1	(14)	3 6 ± 0 1	(4)
Cytosolic	-	-	1 1 ± 0 1	(12)	1 1 ± 0 1	(3)	1 1 ± 0 1	(8)	1 2 ± 0 1	(2)	1 4 ± 0 1	(8)	1 7 ± 0 1	(2)
Vacuolar	-	-	3 0 ± 0 1	(32)	16 0 ± 0 2	(51)	4 7 ± 0 1	(37)	42 5 ± 0 6	(51)	8 0 ± 0 1	(44)	40 9 ± 0 3	(50)
Bound	-	-	3 1 ± 0 1	(33)	11 7 ± 0 1	(37)	4 4 ± 0 1	(35)	36 3 ± 0 6	(43)	6 3 ± 0 1	(34)	35 9 ± 0 4	(44)
Sum	-	-	9 4 ± 0 4	(100)	31 5 ± 0 5	(100)	12 7 ± 0 4	(100)	82 9 ± 1 4	(100)	18 3 ± 0 4	(100)	82 1 ± 0 9	(100)
Whole cells		-	8 1 ± 0 1		35 0 ± 2 5		13 9 ± 0 1		79 1 ± 1 1		20 8 ± 0 2		84 7 ± 1 1	

Table 3 2 3 1 Levels of Mn^{2+} in subcellular pools of *Saccharomyces cerevisiae* incubated with 100 µM Mn^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

decreased from 8% to 4% between 1 and 5 h, irrespective of the fact that Mn^{2+} levels were higher, and exhibited a greater increase over the 5 h period than did values obtained in the absence of glucose Cytoplasmic Mn^{2+} levels were the lowest of all fractions and although small increases were observed with increased incubation time, cells incubated with glucose exhibited no more than a 15% increase compared with glucose-free cells Vacuolar and bound fractions between them contained the bulk of cellular Mn^{2+}, 65-80% of cellular Mn^{2+} in the absence of glucose and >90% for cells incubated with glucose In contrast with Cd^{2+}, the vacuolar fraction sequestered the highest level of Mn^{2+} in the presence and absence of glucose and increased with time In the absence of glucose, vacuolar levels almost tripled within 5 h, and the fractional distribution rose from 32% at 1 h to 37% and 44% at 3 and 5 h respectively In the presence of glucose, the vacuolar fraction sequestered 50% of cellular Mn^{2+} at each time point, and absolute values increased approximately 3-fold over the 5 h incubation period Bound levels of Mn^{2+} remained low in the absence of glucose and accounted for 32-35% of cell Mn^{2+} at each time point In contrast, in the presence of glucose bound Mn^{2+} was higher and as a percentage of total cellular Mn^{2+}, increased from 37% to 44% between 1 and 5 h

Extracellular Mg^{2+} release was limited to < 5 μmol g^{-1} at each time point indicating that overall cellular Mg^{2+} remained constant There was good agreement between whole cell Mg^{2+} and that recovered from subcellular fractions at all time points (Table 3 2 3 2), and generally cell Mg^{2+} levels were comparable to those previously observed for cells loaded with Cd^{2+} The percentage subcellular distribution of Mg^{2+} remained relatively unaffected by Mn^{2+} accumulation in the absence of glucose, whereas the presence of glucose did induce changes to the vacuolar and bound fractions Mg^{2+} levels in the wash and cytosolic fractions remained between 2-4% and 1-2% of cellular Mg^{2+} respectively in both the presence and absence of glucose The absolute Mg^{2+} levels in these fractions increased slightly with time and were generally higher in the presence of glucose at each time point (Table 3 2 3 2) The majority of cellular Mg^{2+} was localised in the vacuolar fractions, and as a percentage of subcellular Mg^{2+} remained constant at approximately 60-62% in the absence of glucose, even though small changes in absolute levels of Mg^{2+} present were observed In the presence of glucose, vacuolar Mg^{2+} levels increased with time during incubation with Mn^{2+} from 58% of cellular Mg^{2+} at 1 h to 67% after 5 h During the same period, bound Mg^{2+} decreased from 36% to 28%, but in

Intracellular Mg^{2+} levels during Mn^{2+} accumulation [µmol g^{-1}]												
Time	**0 h**				**1 h**				**3 h**				**5 h**			
2% glucose	-		+		-		+		-		+		-		+	
		(%)		(%)		(%)		(%)		(%)		(%)		(%)		(%)
Wash	6 9 ± 0 2	(4)	7 7 ± 0 1	(4)	3 3 ± 0 1	(2)	4 7 ± 0 1	(3)	3 8 ± 0 1	(2)	4 3 ± 0 1	(2)	4 0 ± 0 1	(2)	4 4 ± 0 1	(2)
Cytosolic	4 3 ± 0 1	(2)	4 7 ± 0 1	(2)	2 4 ± 0 1	(1)	2 7 ± 0 1	(2)	2 8 ± 0 1	(2)	2 9 ± 0 1	(2)	2 6 ± 0 1	(2)	2 9 ± 0 1	(2)
Vacuolar	111 4 ± 1 1	(60)	112 5 ± 9 5	(58)	110 9 ± 6 7	(63)	102 2 ± 0 5	(60)	105 6 ± 0 2	(62)	116 7 ± 0 6	(66)	105 9 ± 3 9	(62)	121 9 ± 0 6	(67)
Bound	64 4 ± 0 9	(34)	68 9 ± 0 7	(36)	60 5 ± 1 2	(34)	59 3 ± 0 8	(35)	57 2 ± 0 5	(34)	52 1 ± 1 8	(30)	59 1 ± 1 1	(34)	51 9 ± 0 5	(29)
Sum	187 0±2 3	(100)	193 8±10 4	(100)	177 1±8 1	(100)	168 9±1 5	(100)	169 4±0 9	(100)	176 0±2 6	(100)	171 6±5 2	(100)	181 1±1 3	(100)
Whole cells	168 1 ± 1 4		163 1 ± 1 8		165 1 ± 3 6		166 5 ± 1 5		166 5 ± 2 1		172 0 ± 0 7		166 7 ± 1 1		173 9 ± 0 8	

Table 3 2 3 2 Levels of Mg^{2+} in subcellular pools of *Saccharomyces cerevisiae* during incubation in 100 µM Mn^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

the absence of glucose remained constant at 34% These changes to Mg^{2+} levels in the vacuolar and bound fractions in the presence of glucose were similar to those observed during incubation with Cd^{2+} at equivalent time points

Changes to subcellular K^+ levels were not as dramatic as observed with Cd^{2+} as cells generally retained approximately 75% of their initial K^+ level In the absence of glucose, levels of K^+ recovered from fractions decreased with time of incubation (Table 3 2 3 3), mirroring the rise in extracellular K^+ over the same period By comparison in the presence of glucose, overall recovered K^+ levels were similar at 0 h and 1 h but decreased at 3 and 5 h Wash levels of K^+ were generally higher than observed with Cd^{2+} and decreased with time in the absence of glucose by approximately 50%, but remained consistently high in the presence of glucose (Table 3 2 3 3) Cytosolic levels of K^+ in the presence and absence of glucose did decrease by approximately one third between 0 h and 1 h time points and effectively remained constant thereafter Levels of K^+ in the vacuolar fractions decreased slowly with time in the absence of glucose and when taken together with decreases observed in wash fractions, may account for the observed rise in extracellular K^+ In the presence of glucose, vacuolar levels were variable and exhibited a small increase of 15 µmol g^{-1} between 0 h and 1 h samples, but decreased between 1 h and 3 h samples, possibly accounting for the observed fluctuations in extracellular K^+ levels over the same period

3 2.4 *Subcellular compartmentation of Sr^{2+} and its effect on intracellular levels of Mg^{2+} and K^+*

Sr^{2+} recovered from subcellular fractions was in good agreement (within 10%) with that recovered from whole cells at each time point However, these levels of uptake were significantly different from those observed when remaining supernatant metal concentrations at a particular time point were used to determine uptake (as was done in Figure 3 2 1 1c) Here, cellular metal levels were 50% and 65% less in the presence and absence of glucose respectively, when compared with data in Figure 3 2 1 1c (see Table 3 2 4 1) In the absence of glucose, cellular Sr^{2+} levels increased from 3 5 to 6 3 µmol g^{-1} between 1 and 5 h, and from 12 2 to 17 3 µmol g^{-1} in the presence of glucose over the same period Wash fractions in the absence of glucose were observed to contain higher levels of Sr^{2+} at 3 h and 5 h and due to the low overall cellular Sr^{2+} levels, these fractions contained 45-60% of total recovered cellular Sr^{2+} In the presence of glucose, wash

Intracellular K^+ levels during Mn^{2+} accumulation [µmol g^{-1}]								
Time	**0 h**		**1 h**		**3 h**		**5 h**	
2% glucose	-	+	-	+	-	+	-	+
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Wash	112 2 ± 8 9 (20)	112 2 ± 8 7 (19)	65 1 ± 2 8 (13)	116 1 ± 1 6 (19)	62 6 ± 2 5 (12)	117 3 ± 3 4 (21)	54 0 ± 4 3 (11)	86 0 ± 3 4 (16)
Cytosolic	30 6 ± 0 3 (5)	29 6 ±0 7 (5)	18 9 ± 0 6 (4)	23 2 ± 0 5 (4)	20 5 ± 0 6 (4)	23 5 ± 0 3 (4)	20 3 ± 0 4 (4)	22 1 ± 0 7 (4)
Vacuolar	424 6 ± 4 4 (74)	428 4±17 1 (75)	429 8 ± 3 0 (83)	444 7 ± 3 7 (76)	415 7 ± 6 7 (83)	400 7 ± 4 5 (74)	397 7 ± 3 8 (84)	413 7 ±7 9 (79)
Bound	5 7 ± 0 6 (1)	4 4 ± 0 4 (1)	3 3 ± 0 5 (1)	3 1 ± 0 2 (1)	2 6 ± 0 2 (1)	2 3 ± 0 1 (1)	2 6 ± 0 3 (1)	2 9 ± 0 3 (1)
Sum	573 1±14 2(100)	574 6±26 9(100)	517 1±6 9 (100)	587 0±6 0 (100)	501 4±10 0(100)	543 8±8 3 (100)	474 6±8 8 (100)	524 7±12 3(100)
Whole cells	478 9 ± 7 1	476 1 ± 9 6	470 7 ± 11 4	479 7 ± 1 5	440 5±10 8	438 0 ± 4 1	405 3 ± 5 6	458 2 ± 12 6

Table 3 2 3 3 Levels of K^+ in subcellular pools of *Saccharomyces cerevisiae* during incubation in 100 µM Mn^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

Intracellular Sr^{2+} levels [µmol g^{-1}]												
Time	**0 h**		**1 h**				**3 h**				**5 h**			
2% glucose	-	+	-		+		-		+		-		+	
				(%)		(%)		(%)		(%)		(%)		(%)
Wash	-	-	2 2 ± 0 1	(61)	2 4 ± 0 1	(19)	2 8 ± 0 2	(48)	2 0 ± 0 1	(12)	2 8 ± 0 1	(44)	1 9 ± 0 1	(11)
Cytosolic	-	-	0 7 ± 0 1	(19)	0 7 ± 0 5	(6)	0 6 ± 0 1	(11)	0 8 ± 0 1	(6)	0 7 ± 0 1	(11)	0 8 ± 0 1	(5)
Vacuolar	-	-	0 2 ± 0 1	(6)	3 3 ± 0 1	(27)	0 6 ± 0 1	(11)	3 0 ± 0 2	(19)	0 6 ± 0 1	(10)	2 8 ± 0 2	(16)
Bound	-	-	0 5 ± 0 1	(14)	6 0 ± 0 1	(48)	1 7 ± 0 1	(30)	9 9 ± 0 2	(63)	2 2 ± 0 1	(35)	11 7 ± 0 3	(68)
Sum	-	-	3 6 ± 0 4	(100)	12 4 ± 0 8	(100)	5 7 ± 0 5	(100)	15 7 ± 0 6	(100)	6 3 ± 0 4	(100)	17 2 ± 0 7	(100)
Whole cells	-	-	3 52 ± 0 1		10 7 ± 0 2		4 9 ± 0 2		14 5 ± 0 1		5 0 ± 0 3		16 2 ± 0 2	

Table 3 2 4 1 Levels of Sr^{2+} in subcellular pools of *Saccharomyces cerevisiae* during incubation in 100 µM Mn^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

fractions contained slightly lower levels of Sr^{2+} but due to greater cellular Sr^{2+} accumulation (compared with levels obtained in the absence of glucose) only represented some 10-20% of cellular Sr^{2+} In both the presence and absence of glucose, the importance of Sr^{2+} in wash fractions as a percentage of overall cellular Sr^{2+} decreased with time as Sr^{2+} was sequestered to other subcellular compartments In the absence of glucose, cytoplasmic Sr^{2+} levels (though variable) were found to be greater than or equal to vacuolar levels at each time point, which was not observed with any of the other metal cations tested At 1 h, the cytosolic fraction contained 18% of total Sr^{2+} compared with 5% in the vacuole At the later time point of 5 h, Sr^{2+} levels had increased in the vacuolar fraction and represented 9% of cellular Sr^{2+} in comparison to 11% in the cytosol In the presence of glucose, cytosolic Sr^{2+} increased with time, but remained only approximately 5% of cellular Sr^{2+} Absolute vacuolar levels declined in real terms over the incubation period, and consequently, as a fraction of overall cellular Sr^{2+}, decreased from 26% at 1 h to 16% at 5 h As noted with Cd^{2+} localisation, the major difference in Sr^{2+} levels between glucose-supplemented and unsupplemented cells was observed in the bound fractions Sr^{2+} levels increased from 0 5 to 2 2 µmol g^{1} (14 to 35% of cellular Sr^{2+}) between 1 h and 5 h in the absence of glucose, but with glucose-supplemented cells, Sr^{2+} levels rose for 6 0 to 11 7 µmol g^{1} in the same period

Sr^{2+} accumulation had little influence on the distribution of subcellular Mg^{2+} and total Mg^{2+} levels recovered from fractions were similar to those obtained in experiments with Cd^{2+} and Mn^{2+} Wash fraction Mg^{2+} levels (lowest of the four fractions) were variable but appeared to decrease in the presence of glucose, but exhibited small increases in its absence (Table 3 2 4 2) Cytosolic Mg^{2+} levels were also variable but showed no clear increase or decrease during the course of the experiment Vacuolar Mg^{2+} levels remained constant during Sr^{2+} accumulation in the absence of glucose, but were observed to decrease from 97 µmol g^{1} at 0 h to 81 µmol g^{1} after 5 h in the presence of glucose During the same period, bound Mg^{2+} levels rose by approximately an equivalent amount in the presence of glucose, but remained constant in its absence

Cellular K^{+} levels were observed to fall in the absence of glucose and rise in its presence (total sum of fractions and whole cell measurements) reflecting the K^{+} profiles observed in Figure 3 2 1 2,Sr^{2+} (see Table 3 2 4 3) The observed changes in extracellular K^{+} may be attributable to vacuolar K^{+} fluctuations From Table 3 2 4 3, vacuolar K^{+}

Intracellular Mg^{2+} levels during Sr^{2+} accumulation [µmol g^{-1}]																
Time	**0 h**				**1 h**				**3 h**				**5 h**			
2% glucose	-		+		-		+		-		+		-		+	
		(%)		(%)		(%)		(%)		(%)		(%)		(%)		(%)
Wash	3 9 ± 0 4	(3)	4 2 ± 0 2	(3)	2 6 ± 0 2	(2)	2 6 ± 0 1	(2)	3 8 ± 0 1	(2)	3 5 ± 0 2	(2)	5 3 ± 0 1	(3)	1 9 ± 0 1	(1)
Cytosolic	4 1 ± 0 2	(3)	3 9 ± 0 1	(3)	4 1 ± 0 3	(3)	4 1 ± 0 2	(3)	3 6 ± 0 1	(2)	3 9 ± 0 6	(2)	3 8 ± 0 2	(2)	3 2 ± 0 4	(2)
Vacuolar	102 3 ± 0 8	(67)	97 9 ± 1 6	(66)	102 1 ± 1 4	(67)	97 9 ± 0 3	(63)	109 6 ± 2 6	(66)	85 8 ± 0 9	(56)	103 2 ± 1 2	(66)	81 1 ± 1 5	(53)
Bound	41 4 ± 0 5	(27)	42 1 ± 0 2	(28)	43 7 ± 0 5	(29	49 7 ± 1 1	(32)	48 6 ± 1 1	(29)	58 8 ± 0 5	(39)	45 1 ± 0 9	(29)	68 3 ± 0 9	(44)
Sum	151 7±1 9	(100)	148 1±2 1	(100)	152 5±2 3	(100)	154 3±1 7	(100)	165 6±3 9	(100)	152 0±2 2	(100)	157 4±2 4	(100)	154 5±2 9	(100)
Whole cells	158 3 ± 0 6		142 9 ± 9 3		156 1 ± 1 4		157 4 ± 1 8		161 4 ± 0 7		158 8 ± 0 9		162 0 ± 0 4		162 8 ± 1 2	

Table 3.2 4 2 Levels of Mg^{2+} in subcellular pools of *Saccharomyces cerevisiae* during incubation in 100 µM Sr^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

Intracellular K^+ levels during Sr^{2+} accumulation [µmol g^{-1}]								
Time	**0 h**		**1 h**		**3 h**		**5 h**	
2% glucose	-	+	-	+	-	+	-	+
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Wash	64 8 ± 3 2 (12)	86 3 ± 2 9 (15)	56 6 ± 4 8 (12)	59 7 ± 8 4 (9)	56 3 ± 1 5 (12)	66 8 ± 3 5 (10)	62 6 ± 4 9 (15)	39 5 ± 6 8 (6)
Cytosolic	28 3 ± 1 0 (5)	30 9 ± 0 1 (6)	28 1 ± 1 1 (6)	37 9 ± 1 9 (6)	25 9 ± 0 4 (5)	27 5 ± 1 0 (4)	24 1 ± 1 3 (6)	25 6 ± 1 9 (4)
Vacuolar	451 4 ± 4 9 (82)	438 3 ± 3 9 (78)	398 6 ± 8 8 (81)	551 6±28 4 (83)	377 3±20 4 (80)	580 3 ±4 9 (85)	328 5±11 4 (77)	551 6 ± 5 9 (88)
Bound	6 9 ± 2 1 (1)	6 9 ± 0 7 (1)	6 9 ± 1 9 (1)	10 5 ± 1 9 (2)	12 8 ± 5 1 (3)	8 9 ± 2 2 (1)	9 1 ± 0 2 (2)	9 5 ± 1 1 (2)
Sum	551 4±11 2(100)	562 4±7 6 (100)	490 2±16 4(100)	659 7±40 6(100)	472 3±27 4(100)	683 5±11 6(100)	424 3±17 8(100)	626 2±15 7(100)
Whole cells	522 4 ± 1 9	538 8 ± 38 4	483 8 ± 8 9	593 3 ± 6 7	431 5 ± 8 8	604 9 ± 11 7	417 5 ± 10 5	617 2 ± 10 9

Table 3 2 4 3 Levels of K^+ in subcellular pools of *Saccharomyces cerevisiae* during incubation in 100 µM Sr^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

levels decreased from 451 µmol g^{-1} at 0 h, (82% of recovered cellular K^+) to 328 µmol g^{-1} after 5 h (76% of recovered cellular K^+) in the absence of glucose, which compares closely with a 150 µmol g^{-1} rise in extracellular K^+ levels over the same period (Figure 3 2 1 1,Sr^{2+}) Conversely, in the presence of glucose vacuolar K^+ levels increased from 438 µmol g^{-1} at 0 h (78% of recovered cellular K^+) to 551 µmol g^{-1} after 5 h (88% of recovered cellular K^+) whilst extracellular K^+ concentrations decreased by approximately an equivalent amount (~ 100 µmol g^{-1}) during the same time In other subcellular compartments such as the wash fraction, K^+ levels remained constant in the absence of glucose (approximately 12% of subcellular K^+) but exhibited a slight decrease in its presence (from approximately 17% to 6% of recovered K^+ over 5 h) Cytosolic and bound K^+ were variable (represented <8% of recovered cellular K^+ combined) but showed no clear increase or decrease

3 2.5 *Subcellular compartmentation of Cu^{2+} and its effect on intracellular levels of Mg^{2+} and K^+*

As cells were grown in medium containing trace amounts of Cu^{2+}, and low concentrations were used for uptake studies, it was decided to determine subcellular Cu^{2+} levels prior to uptake studies Whole cells were found to contain approximately 3400 nmol Cu^{2+} g^{-1} when harvested, but total Cu^{2+} recovered from subcellular fractions at 0 h was approximately 30% lower than this value Initial subcellular Cu^{2+} levels were relatively evenly distributed between the four fractions, with approximately 27, 18, 32, and 23% of cellular Cu^{2+} in the wash, cytoplasmic, vacuolar and bound fractions respectively (Table 3 2 5 1) Upon incubation with 5 µM Cu^{2+}, Cu^{2+} ions were primarily sequestered to the vacuolar and bound fractions Within 1 h, in the absence of glucose, vacuolar Cu^{2+} levels increased from 770 nmol g^{-1} to 1021 nmol g^{-1} and bound Cu^{2+} levels increased 6-fold from 528 nmol g^{-1} to 3428 nmol g^{-1} During the following 4 h incubation, vacuolar Cu^{2+} levels increased by a further 150 nmol g^{-1} but bound Cu^{2+} remained effectively unchanged In the presence of glucose, vacuolar Cu^{2+} levels doubled during the first hour of contact from 770 nmol g^{-1} to 1458 nmol g^{-1} and continued to increase for the remainder of the experiment Bound Cu^{2+} also increased 5-fold during the first hour, but decreased by approximately 25% over the following 4 h, possibly as a result of transfer to the vacuolar pool During the 5 h experimental period, wash and cytosolic Cu^{2+} levels

Intracellular Cu^{2+} levels [nmol g^{-1}]																
Time	**0 h**				**1 h**				**3 h**				**5 h**			
2% glucose	-		+		-		+		-		+		-		+	
		(%)		(%)		(%)		(%)		(%)		(%)		(%)		(%)
Wash	656 ± 88	(28)	635 ± 100	(26)	635 ± 109	(11)	572 ± 51	(10)	416 ± 47	(8)	385 ± 17	(7)	616 ± 135	(11)	437 ± 85	(7)
Cytosolic	381 ± 37	(16)	459 ± 48	(19)	625 ± 88	(11)	386 ± 10	(7)	355 ± 62	(7)	277 ± 46	(5)	286 ± 46	(5)	286 ± 43	(5)
Vacuolar	770 ± 95	(33)	770 ± 95	(32)	1021 ± 95	(18)	1458 ± 72	(25)	1184 ± 54	(23)	2021 ± 130	(35)	1187 ± 150	(22)	2772 ± 216	(47)
Bound	528 ± 32	(23)	550 ± 43	(23)	3428 ± 75	(60)	3331 ± 28	(58)	3245 ± 49	(62)	3008 ± 77	(53)	3352 ± 57	(62)	2404 ± 37	(41)
Sum	2355 ± 252	(100)	2414 ± 286	(100)	5709 ± 367	(100)	5747 ± 161	(100)	5200 ± 212	(100)	5691 ± 270	(100)	5441 ± 388	(100)	5899 ± 381	(100)
Whole cells	3486 ± 223		3354 ± 186		7239 ± 464		6746 ± 110		6685 ± 75		7332 ± 185		6820 ± 263		7485 ± 134	

Table 3 2 5 1 Levels of Cu^{2+} in subcellular pools of *Saccharomyces cerevisiae* during incubation in 5 µM Cu^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

generally remained at levels similar to, or less than those observed at 0 h The only exception was a 244 nmol g^{-1} increase in cytosolic Cu^{2+} after 1 h in the absence of glucose, but by 3 h, levels had fallen below 0 h values

Cellular Mg^{2+} levels were generally conserved but subcellular distributions altered during incubation with Cu^{2+}, most notably in the absence of glucose Here, Mg^{2+} concentrations in wash fractions increased with time of incubation from 15 µmol g^{-1} at 0 h (representing 10% of total cellular Mg^{2+}) to 49 µmol g^{-1} after 5 h (representing 35% of total cellular Mg^{2+}) In addition, cytosolic levels increased 3-fold and vacuolar levels declined by 50% during the same period (Table 3 2 5 2) The net decease in vacuolar Mg^{2+} between 0 h and successive time points closely resembles the net increase in wash and cytoplasmic Mg^{2+} during the same period Bound Mg^{2+} levels also steadily decreased at a slow rate during incubation with Cu^{2+} and may have been partially responsible for increases in wash Mg^{2+}, or may account for the aforementioned increases in extracellular Mg^{2+} (section 3 2 1) In the presence of glucose, Mg^{2+} levels exhibit small fluctuations in each subcellular fraction but no clear trends were apparent Therefore, it was unclear which subcellular fractions contributed to observed increase in extracellular Mg^{2+} levels

Overall K^+ levels recovered from subcellular fractions reflect extracellular K^+ profiles previously observed (Figure 3 2 1 2,Cu^{2+}), whereby notable decreases in recovered K^+ in the absence of glucose over 5 h were recorded (Table 3 2 5 3) In the presence of glucose, recovered K^+ levels were variable but changed little between 0 h and 5 h time points Whole cell K^+ and that recovered from fractions at each time point differed by approximately 10% but exhibited similar trends (Table 3 2 5 3) In the absence of glucose, wash K^+ remained unchanged, but in the presence of glucose, high wash K^+ levels observed at 0 h and 1 h decreased considerably at the latter time points of 3 h and 5 h Initial cytoplasmic K^+ levels decreased with time during incubation with Cu^{2+} in both the presence and absence of glucose Vacuolar K^+ levels were variable in both the presence and absence of glucose In the absence of glucose, vacuolar K^+ levels decreased by approximately 100 µmol g^{-1} within 3 h, possibly accounting for the observed rise in extracellular K^+ of similar magnitude during the same period In the presence of glucose, vacuolar K^+ levels slowly increased over time which may have been attributable to transfer from wash fractions, coupled with slow accumulation of previously released extracellular K^+

Intracellular Mg^{2+} levels during Cu^{2+} accumulation [µmol g^{-1}]																
Time	**0 h**				**1 h**				**3 h**				**5 h**			
2% glucose	-		+		-		+		-		+		-		+	
		(%)		(%)		(%)		(%)		(%)		(%)		(%)		(%)
Wash	15 5 ± 0 2	(10)	11 8 ± 0 1	(8)	21 7 ± 0 3	(15)	8 5 ± 0 2	(5)	35 5 ± 0 2	(24)	6 9 ± 0 1	(4)	49 5 ± 0 4	(35)	6 9 ± 0 3	(4)
Cytosolic	4 4 ± 0 7	(3)	3 8 ± 0 1	(3)	5 6 ± 0 1	(3)	3 5 ± 0 1	(2)	7 7 ± 0 2	(5)	2 9 ± 0 2	(2)	12 7 ± 0 1	(9)	2 7 ± 0 1	(2)
Vacuolar	78 3 ± 0 7	(51)	85 8 ± 1 4	(56)	71 4 ± 2 6	(48)	90 8 ± 0 6	(58)	55 9 ± 0 1	(38)	90 5 ± 0 8	(58)	36 3 ± 0 4	(26)	86 1 ± 0 3	(57)
Bound	56 0 ± 0 7	(36)	50 4 ± 2 1	(33)	50 9 ± 0 1	(34)	56 1 ± 0 4	(35)	47 2 ± 0 4	(32)	55 1 ± 0 6	(36)	43 1 ± 0 3	(30)	56 0 ± 0 9	(37)
Sum	154 2±2 3	(100)	151 8±3 7	(100)	149 6±3 1	(100)	158 9±1 3	(100)	146 1±0 9	(100)	155 4±1 7	(100)	141 6±1 2	(100)	151 7±1 6	(100)
Whole cells	162 0 ± 0 9		165 4 ± 2 3		143 2 ± 2 6		148 9 ± 8 3		144 7 ± 11 8		169 5 ± 1 6		157 7 ± 3 0		157 9 ± 9 0	

Table 3 2.5 2 Levels of Mg^{2+} in subcellular pools of *Saccharomyces cerevisiae* during incubation in 5 µM Cu^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

Intracellular K^+ levels during Cu^{2+} accumulation [µmol g^{-1}]								
Time	**0 h**		**1 h**		**3 h**		**5 h**	
2% glucose	-	+	-	+	-	+	-	+
	(%) (%)		(%) (%)		(%) (%)		(%) (%)	
Wash	39 6 ± 1 8 (8)	73 6 ± 1 2 (13)	38 1 ± 2 8 (9)	60 3 ± 0 7 (11)	35 7 ± 2 0 (10)	31 8 ± 0 6 (6)	33 0 ± 1 9 (10)	18 2 ± 0 5 (3)
Cytosolic	44 8± 0 5 (9)	58 4 ± 0 9 (11)	48 5 ± 3 6 (11)	57 3 ± 1 1 (11)	31 7 ± 2 0 (10)	30 3 ± 1 1 (6)	22 2 ± 0 1 (7)	29 3 ± 1 3 (6)
Vacuolar	396 1 ± 1 9 (82)	406 6 ±5 7 (75)	342 8 ± 4 2 (79)	414 8 ± 5 0 (77)	270 5 ± 3 9 (79)	453 3 ± 5 9 (87)	276 5 ± 7 0 (82)	462 8±13 5 (90)
Bound	2 5 ± 0 5 (1)	3 5 ± 0 1 (1)	2 4 ± 0 3 (1)	3 9 ± 0 5 (1)	3 2 ± 0 2 (1)	4 5 ± 0 4 (1)	3 0 ± 0 6 (1)	2 1 ± 0 2 (1)
Sum	483 0±4 7 (100)	542 1±7 9 (100)	431 8±10 9(100)	536 3±7 3 (100)	341 1±8 1 (100)	519 9±8 0 (100)	334 7± 9 6(100)	512 4±15 5(100)
Whole cells	502 0 ± 6 3	539 8 ± 11 2	428 9 ± 9 8	474 4 ± 2 9	387 8 ± 5 5	511 4 ± 7 9	385 8 ± 9 5	543 2 ± 6 3

Table 3.2 5 3 Levels of K^+ in subcellular pools of *Saccharomyces cerevisiae* during incubation in 5 µM Cu^{2+} in the presence and absence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

3.3 Influence of intracellular and extracellular magnesium concentrations on manganese uptake and toxicity results

3 3 1 *Influence of Mn^{2+} on growth of S cerevisiae NCYC1383*

S cerevisiae was grown in YEPD medium, supplemented with a range of Mn^{2+} concentrations ranging from 0 1 to 5 mM and monitored over a 24 h period (Figure 3 3 1) At the lowest concentration of Mn^{2+} tested (0 1 mM), growth was unaffected by the presence of Mn^{2+} ions and continued at a similar rate to cells grown in unamended medium However, all other Mn^{2+} concentrations examined were found to inhibit growth, and the degree of inhibition was increased with Mn^{2+} concentration For example, cells grown in medium supplemented with 0 5 mM Mn^{2+} continued to grow as normal up to mid to late exponential phase (9 h), but thereafter growth slowed appreciably resulting in an approximate 40% reduction in the final cell yield At the higher concentrations of 2 and 5 mM Mn^{2+} growth was arrested during early exponential phase (3-4 h) giving larger reductions (84% and 90% respectively) in final cell yield

3 3 2 *The effect of other cations on Mn^{2+} toxicity*

In order to investigate the influence of other cations on Mn^{2+} toxicity, *S cerevisiae* was grown in YEPD medium containing 2 mM Mn^{2+} and supplemented with either 10 mM $Ca(NO_3)_2$, 10 mM $Mg(NO_3)_2$ or 50 mM KCl (to give at least a five-fold molar excess of competing cations over Mn^{2+}) The Mg and K contents of unsupplemented YEPD medium were determined to be approximately 150 µM and 8 9 mM, respectively Ca^{2+} and K^+ did not discernibly alter the growth inhibitory effects of 2 mM Mn^{2+} (Figure 3 3 2), and initially, growth levels during the first 6 h were similar to those observed for 2 mM Mn^{2+} alone Between 6 and 9 h, growth in cultures supplemented with K^+ had slowed to such an extent as to have effectively ceased, whereas in cultures supplemented with Ca^{2+}, cell numbers continued to increase at a very slow rate up to 18 h Growth in control flasks containing equivalent concentrations of K^+ and Ca^{2+}, but lacking Mn^{2+}, was similar to that observed in unsupplemented medium (data not shown), confirming that growth inhibition was specific to Mn^{2+} In contrast to Ca^{2+} and K^+, supplementation of medium with 10 mM Mg^{2+} completely eliminated the toxic effects of 2 mM Mn In the presence of excess Mg^{2+}, growth was comparable to that of control cultures (unsupplemented YEPD, lacking

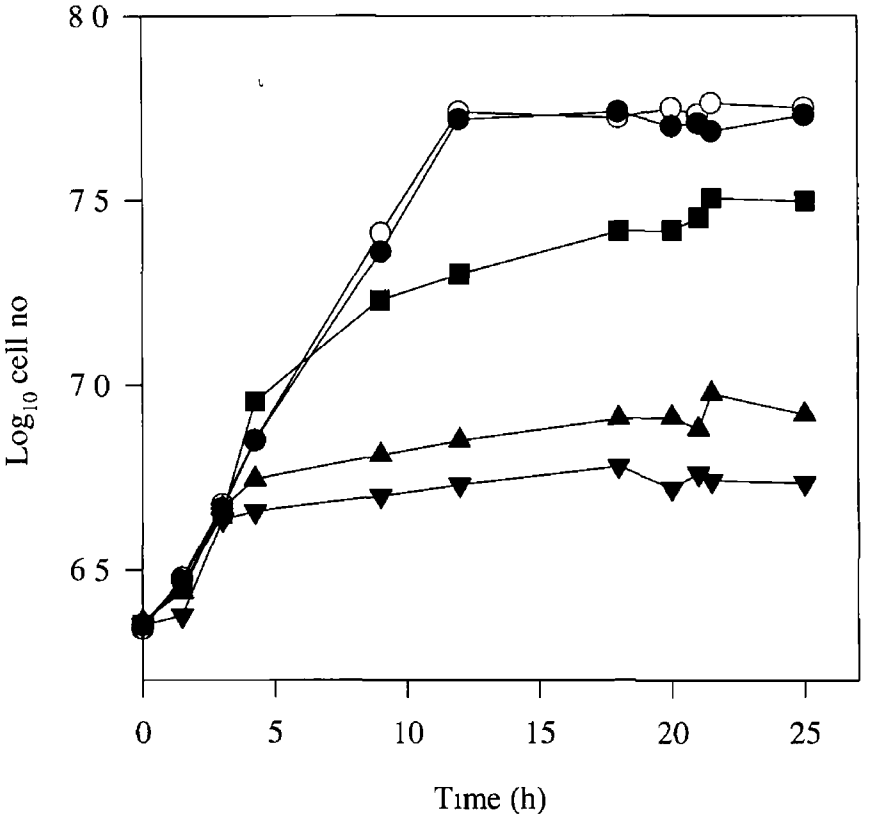


Figure 3.3.1 Growth of *Saccharomyces cerevisiae* in $Mn(NO_3)_2$-supplemented YEPD medium Cells were initially suspended to OD_{550nm}~0 1 and flasks were incubated with shaking at 120 rev min^{-1} at 25° C The graph shows cell numbers in medium supplemented with 0 (O), 0 1 (●), 0 5 (■), 2 0 (▲) and 5 0 (▼) mM $Mn(NO_3)_2$ SEM (n=3) were smaller than dimensions of symbols

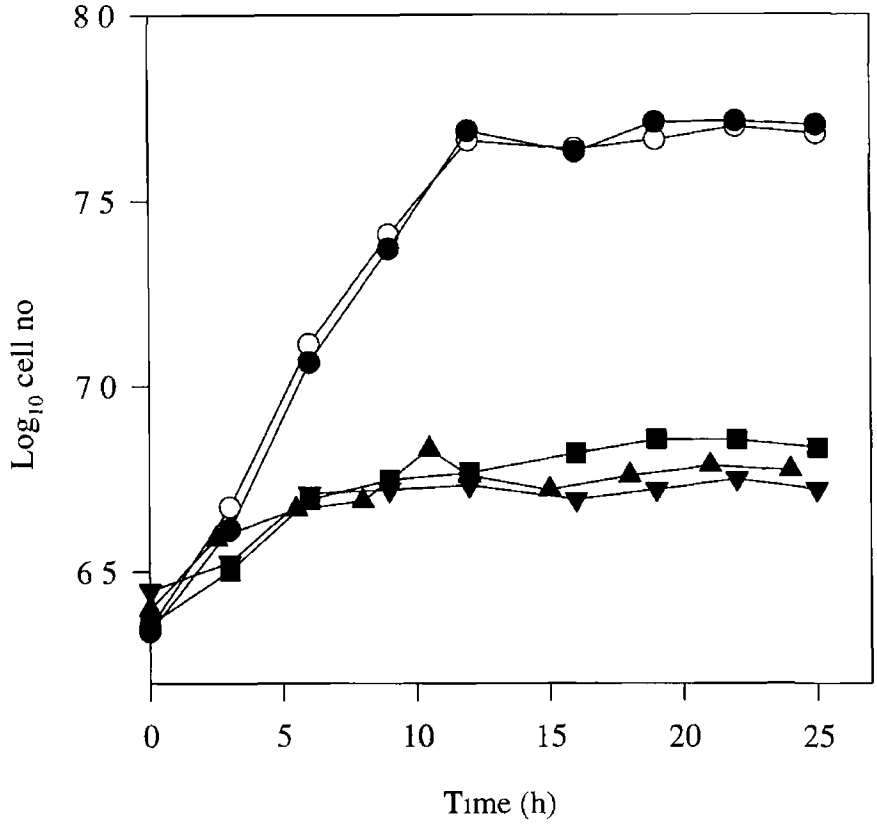


Figure 3 3 2 Growth of *Saccharomyces cerevisiae* in YEPD medium containing 2 0 mM $Mn(NO_3)_2$ supplemented with Mg, Ca and K The graph shows cell numbers in medium containing 2 0 mM Mn^{2+} (▲), 2 0 mM Mn^{2+}/10 mM Mg^{2+} (●), 2 0 mM Mn^{2+}/10 mM Ca^{2+} (■), 2 0 mM Mn^{2+}/50 mM K^{+} (▼) or YEPD medium containing no supplements (○) Cells were initially suspended to OD_{550nm}~0 1 and flasks were incubated with shaking at 120 rev min^{-1} at 25° C SEM (n=3) were smaller than dimensions of the symbols

Mn^{2+}), and exhibited no lag phase or reduction in growth rate prior to entry into stationary phase (after approximately 12 h, Figure 3 3 2)

3.3.3 *The influence of medium Mg supplementation on cellular Mn levels*

Cells grown in YEPD medium containing 2 mM Mn^{2+}, with and without a 10 mM Mg^{2+} supplement were analysed for cellular Mn levels over a 24 h period Differences in cellular Mn levels were observed, which were influenced by the presence or absence of a Mg^{2+} supplement (Figure 3 3 3) During the early exponential phase (0-3 h), cells in both the presence and absence of a Mg^{2+} supplement began to accumulate Mn^{2+} and after 3 h, cellular Mn levels were 396 and 612 nmol $(10^9$ cells$)^{-1}$ in Mg-supplemented and unsupplemented medium respectively As growth proceeded (from 3 h), cells grown in the absence of a Mg^{2+} supplement continued to accumulate Mn^{2+} at a rate of approximately 160 nmol $(10^9$ cells$)^{-1}$ h^{-1} until the end of the exponential phase (12 h) The rate of accumulation slowed in early stationary phase (12-15 h), and from the 15 h time point cellular Mn levels were variable but showed no clear increase in medium lacking a Mg^{2+} supplement

In contrast, cells grown in the presence of 10 mM Mg^{2+} began to show reduced Mn levels over a 6 h period from maximal levels at 3 h (396 nmol $(10^9$ cells$)^{-1}$) to levels of approximately 100 nmol $(10^9$ cells$)^{-1}$ at 9 h Cellular Mn levels remained constant at this level over the next 15 h The presence of a five-fold excess of Mg^{2+} apparently enabled the cell to actively discriminate against Mn^{2+} uptake from an early stage (circa 3 h) and thereafter maintain cellular Mn at non-toxic levels

3 3 4 *Influence of Mg-supplementation and growth stage on intracellular Mg in S cerevisiae*

Initial results showed that cells grown for 16 h in Mg-supplemented medium possessed higher intracellular Mg levels relative to cells grown in unsupplemented medium The Mg concentration of standard YEPD medium was determined to be approximately 150 µM, but manipulation of the intracellular Mg content of *S cerevisiae* was achieved by culturing in $Mg(NO_3)_2$-supplemented (30 mM Mg) medium

During exponential growth (0-10 h, Figure 3 3 4a) in the presence of either 150 µM or 30 mM Mg, an overall decline in cellular Mg was evident (Figure 3 3 4b)

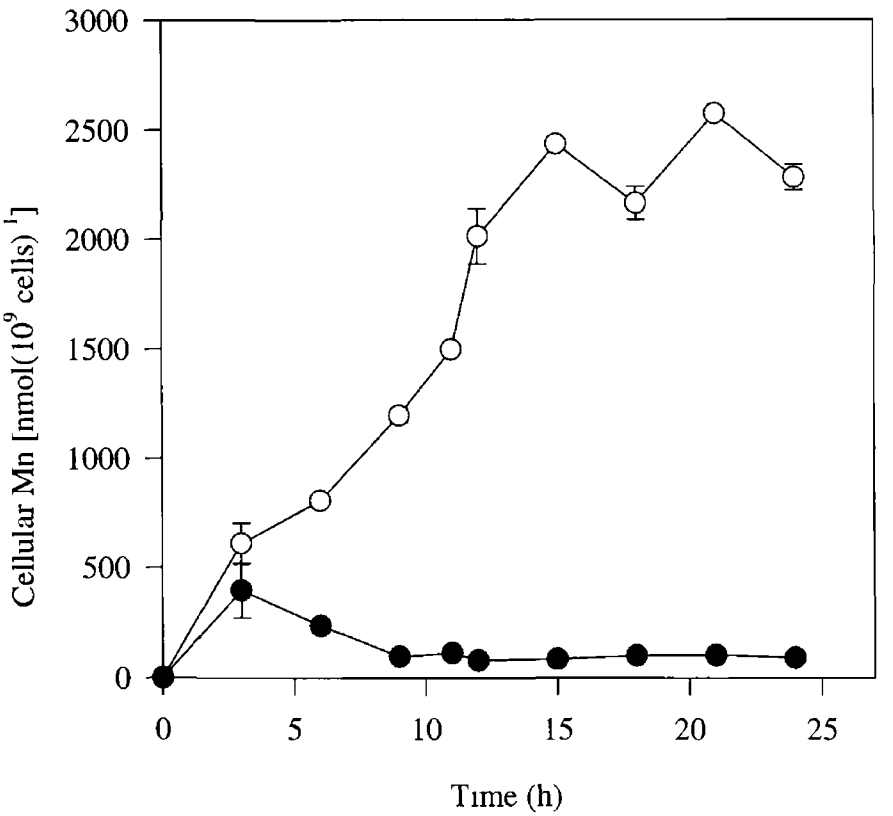


Figure 3 3 3 Intracellular Mn^{2+} levels of *Saccharomyces cerevisiae* during growth in unsupplemented or Mg-supplemented medium Cells were grown in YEPD medium supplemented with 2 0 mM Mn^{2+} (○), or 2 0 mM Mn^{2+}/10 mM Mg^{2+} (●) Flasks were incubated at 25° C with shaking (120 rev min^{-1}) Mean values ± SEM from three replicate determinations are shown where these exceed the dimensions of the symbols

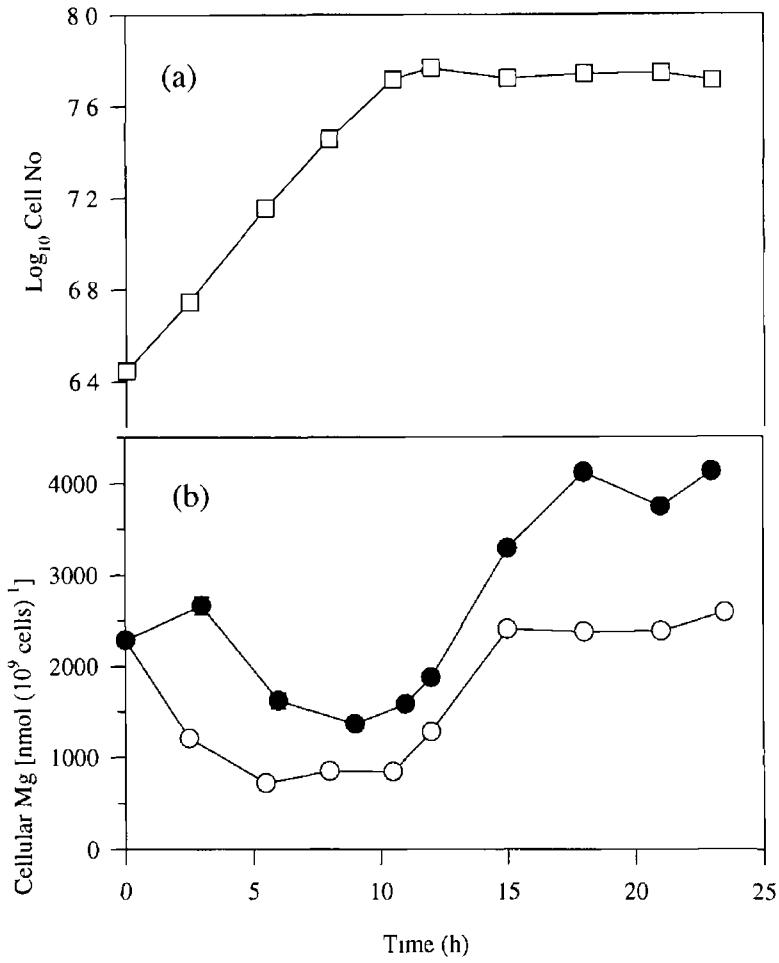


Figure 3.3.4a,b Mg content and growth of *Saccharomyces cerevisiae* in unsupplemented (150 μM Mg) and Mg-supplemented (30 mM Mg) YEPD medium Cells were initially suspended to OD_{550nm}~0 1 and flasks were incubated with shaking at 120 rev min^{-1} at 25° C **(a)** Cell numbers (□) [identical in the presence or absence of a Mg supplement], **(b)** cellular Mg during growth in unsupplemented (○) and Mg-supplemented (●) medium Growth rates were unaffected by Mg supplementation Mean values from three replicate determinations are shown, ± SEM where these exceed the dimensions of the symbols

However, this reduction was more marked in cells grown in unsupplemented medium The resultant difference in cellular Mg levels was approximately maintained as cells, grown in the presence and absence of 30 mM Mg, subsequently accumulated Mg^{2+} during the early stationary phase After 15 h, the Mg content of cells grown in unsupplemented and Mg-supplemented media were approximately 2,300 and 3,300 nmol (10^9 cells) ^{1}, respectively Little change in cellular Mg was observed between 15 and 24 h (Figure 3 3 4b) Growth in medium supplemented with up to 100 mM Mg resulted in no further increase in cellular Mg content and was found to have no effect on the growth rates or final cell yield of *S cerevisiae*

The enhancement of intracellular Mg levels was not found to be a transient phenomenon Magnesium enhanced cells subsequently incubated in 10 mM MES buffer retained their elevated Mg levels over a 5 h period Differential extraction of subcellular ion pools revealed marked differences in the subcellular distribution of accumulated Mg between Mg-supplemented and unsupplemented cells The study revealed approximate 88%, 72% and 10% increases in the Mg content of cytosolic, vacuolar and bound fractions, respectively, during growth in Mg-supplemented medium (Table 3 3 4 1) Because approximately 70% of total cellular Mg was localised in vacuolar pool, increased intracellular Mg was primarily accounted for by vacuolar Mg accumulation

Table 3 3.4 1 Subcellular magnesium distribution in *Saccharomyces cerevisiae* grown for 16 h in unsupplemented and Mg-supplemented medium Mean values ± SEM from three replicate determinations are shown

Cellular compartment	**Mg content [nmol (10^9 cells) ^{1}]**	
	Unsupplemented cells	**Mg^{2+} supplemented cells**
Cytosolic	35 ± 2	66 ± 2
Vacuolar	1734 ± 25	2984 ± 55
Bound	944 ± 14	1046 ± 21
Sum	2713 ± 41	**Sum** 4096 ± 21
Whole cells	2435 ± 19	3906 ± 41

Absolute intracellular Mg levels did have a tendency to vary, moreso with Mg-supplemented cultures than with unsupplemented cultures But for any given experiment, there was at least an approximate 20% difference in cellular Mg levels between Mg-supplemented and unsupplemented cultures

3 3.5 *Metabolism-dependent manganese uptake by unsupplemented and Mg-supplemented S cerevisiae*

Mn^{2+} accumulation by metabolising (in the presence of 2% w/v glucose) *S cerevisiae* during uptake experiments increased with time of incubation (up to 6 h), although the rate of Mn^{2+} accumulation decreased during this time (Figure 3 3 5) However, there was a marked difference in the amounts of Mn^{2+} accumulated by each cell type under similar conditions It was observed that cells previously grown in Mg-supplemented medium exhibited a considerably reduced ability to accumulate Mn^{2+} in these experiments which was independent of Mn^{2+} concentration, and could not be ascribed to metal toxicity as similar metal uptake profiles were observed at toxic and non-toxic Mn^{2+} levels After 6 h incubation in the presence of 50 μM $Mn(NO_3)_2$, a non-toxic concentration, cellular Mn was approximately 1,200 and 750 nmol $(10^9$ cells$)^{-1}$ in unsupplemented and Mg-supplemented cells, respectively (Figure 3 3 5,a) This difference was more marked at the toxic concentration of 5 mM Mn^{2+}, where final cellular Mn levels were 2-fold higher (approximately 1,700 compared with 900 nmol $(10^9$ cells$)^{-1}$) in cells previously grown in unsupplemented medium compared to those grown in the presence of 30 mM Mg (Figure 3 3 5,b) Similar results were obtained for Mn^{2+} concentrations intermediate to these data

Intracellular Mg levels were conserved by both cell types, differing by not more than 5% during the course of Mn^{2+} uptake However, noticeable differences were evident with respect to cellular K^+ release by each cell type K^+ release mirrored Mn^{2+} uptake in that cells that accumulated greater levels of Mn^{2+}, (unsupplemented cells) released greater levels of K^+ At a given Mn^{2+} concentration, K^+ release by unsupplemented cells was 1 5-2 fold greater than for Mg-supplemented cultures (Figure 3 3 5 2) Indeed, it appeared that Mg-supplemented cells were better able to retain K^+ and limit release to levels similar to those observed in control flasks at the lower Mn^{2+} concentration and during the first 2 h of uptake at 5 mM Mn^{2+} (Figure 3 3 5 2b,d)

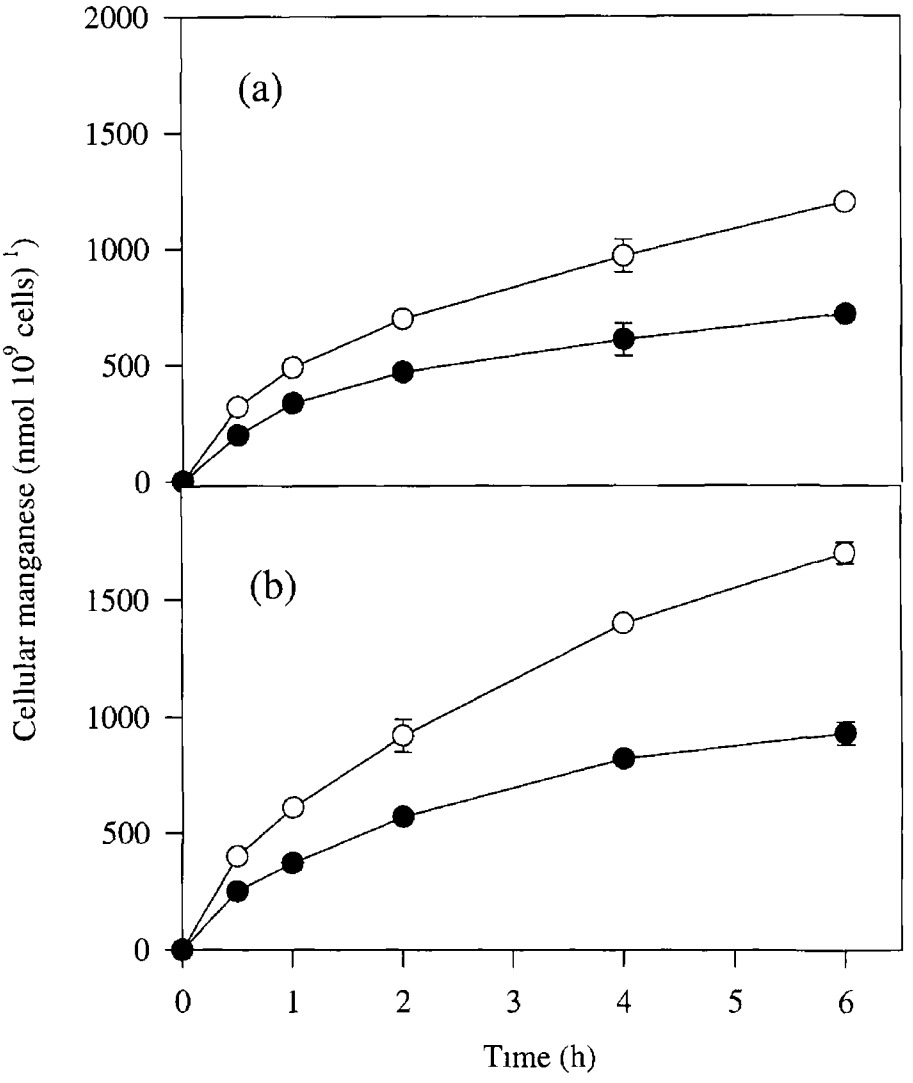


Figure 3 3.5 1a,b Metabolism-dependent Mn^{2+} uptake by *Saccharomyces cerevisiae* Cells were previously grown for 16 h in unsupplemented (○) or Mg-supplemented (●) medium Mn^{2+} uptake was monitored during incubation in 10 mM MES buffer, pH 5 5, with 2% glucose in the presence of **(a)** 50 μM or **(b)** 5 mM Mn^{2+} Mean values from three replicate determinations are shown ± SEM where these exceed the dimensions of the symbols

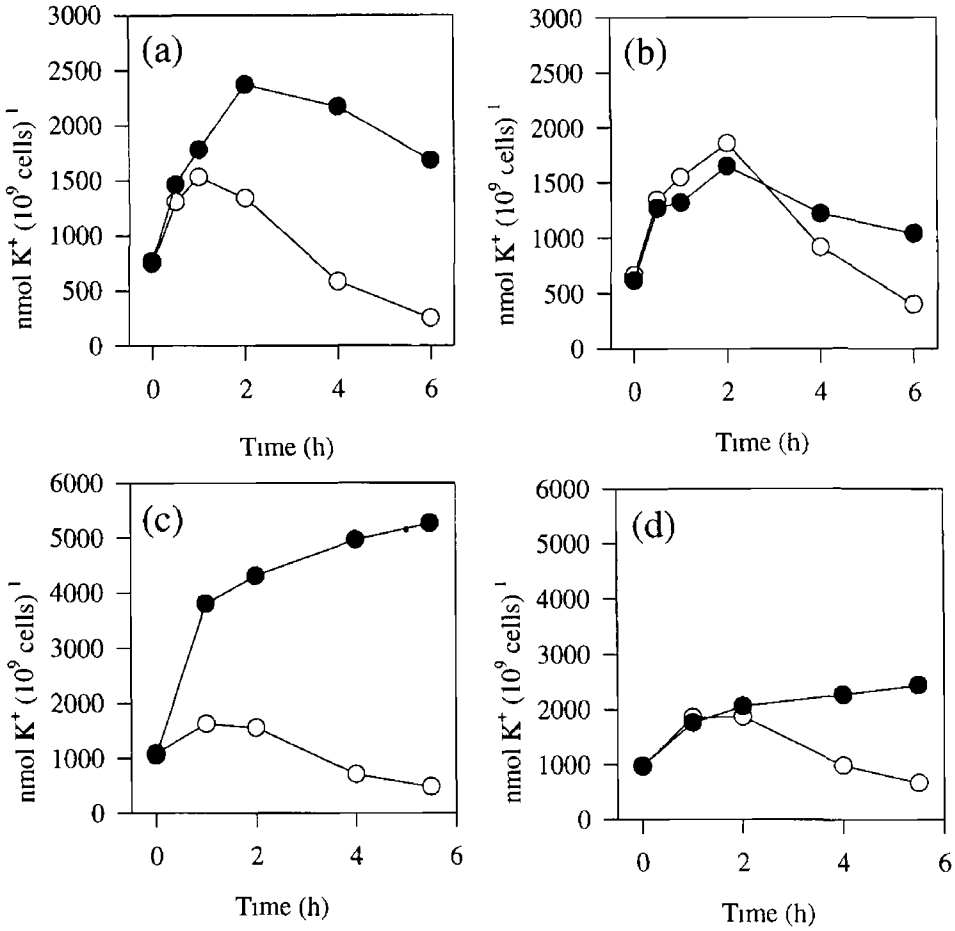


Figure 3 3 5.2 K^+ release from *Saccharomyces cerevisiae* during metabolism-dependent Mn^{2+} accumulation The graphs show K^+ release from unsupplemented (a, c) and Mg-supplemented (b, d) cells incubated with 50 μM (a, b) or 5 mM (c, d) Mn^{2+} (closed symbols) Mn^{2+}-free control values (open symbols) are shown for comparison Mean values from three replicate determinations are shown ± SEM where these exceed the dimensions of the symbols

Unsupplemented cells began to lose K^+ in excess of control levels as soon as Mn^{2+} accumulation commenced, and was further accentuated by the higher Mn^{2+} concentration (Figure 3 3 5 2a,c)

3.3.6 *Net Mg^{2+} uptake by Mg-supplemented and unsupplemented S cerevisiae*

In order to investigate whether cell Mg^{2+} transport systems were influencing Mn^{2+} uptake, Mg^{2+} transport rates were measured for unsupplemented and Mg-supplemented, cultures Figure 3 3 6 shows net uptake of Mg^{2+} ions from a 50 μM solution, as possible Mg^{2+} efflux could not be quantified by the technique employed Mg-supplemented cells, despite their already enhanced Mg levels, displayed a higher rate of Mg^{2+} uptake than unsupplemented cells Rates of uptake were measured at 4 24 and 5 17 nmol Mg^{2+} (10^9 cells)$^{-1}$ min^{-1} respectively for unsupplemented and Mg-supplemented cells, which represented a 21% increase in the rate of Mg^{2+} for cells previously grown in Mg-supplemented medium

3.3.7 *Metabolism-independent manganese uptake and surface-charge of S cerevisiae*

To ascertain whether differences in Mn^{2+} uptake were attributable to differences in initial cell-surface binding of Mn^{2+}, Mn^{2+} uptake was examined in non-metabolising cells (in the absence of glucose) over a range of Mn^{2+} concentrations Metabolism-independent Mn^{2+} uptake increased with extracellular Mn^{2+} concentration for both unsupplemented and Mg-supplemented cells Uptake was generally slightly lower in cells that had previously been grown in Mg-supplemented medium (Figure 3 3 7) at external Mn^{2+} concentration of 1 mM or greater For example, at 1 and 5 mM Mn^{2+}, Mn^{2+} uptake was approximately 1 3-fold higher in cells grown in the presence of 150 μM Mg than in those grown in the presence of 30 mM Mg At Mn^{2+} concentrations lower than 1 mM, differences in levels of accumulated Mn^{2+} by each cell type diminished and appeared to be approximately equal at the lowest concentrations examined (50 and 100 μM Mn^{2+}) However, these differences were smaller than those observed during metabolism-dependent Mn^{2+} accumulation (Figure 3 3 5 1)

Measurements of cell surface charge as determined by cellular-retention of the cationic dye alcian blue revealed small differences between the surface charge of cells grown in Mg-supplemented and unsupplemented media Cultures grown in the absence

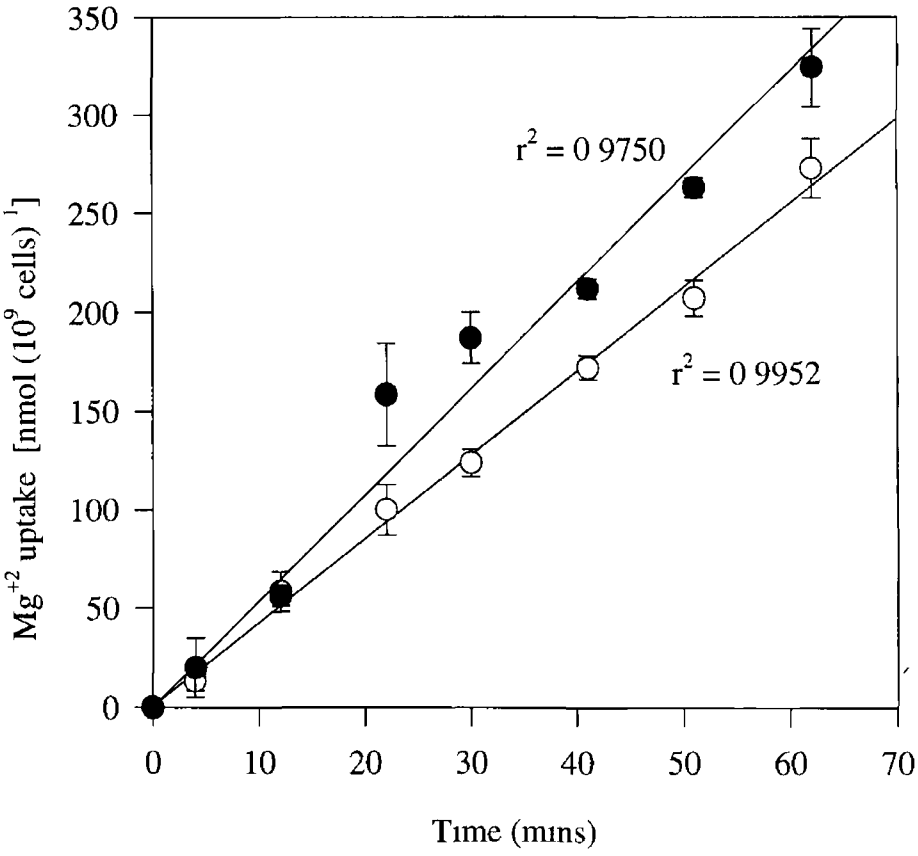


Figure 3 3 6 Net Mg^{2+} uptake by Mg-supplemented (closed symbols) and unsupplemented (open symbols) cells The graph shows uptake of Mg^{2+} from a 50 µM solution in the presence of 2% glucose Mean values ± standard error of the mean of three replicate determinations are shown

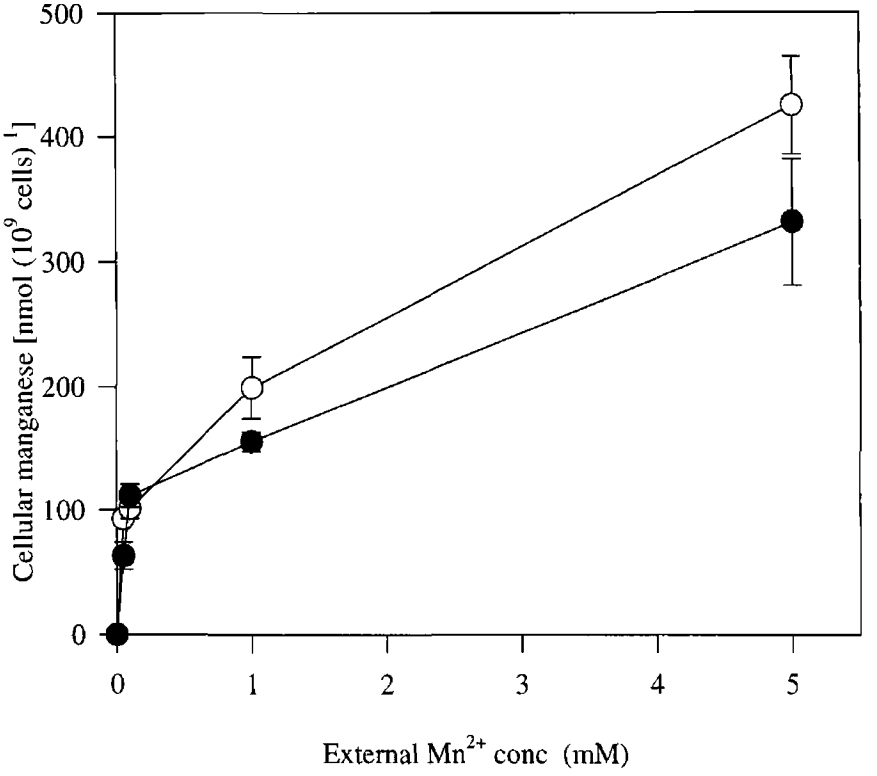


Figure 3.3 7 Metabolism-independent Mn^{2+} uptake by Mg-supplemented and unsupplemented *Saccharomyces cerevisiae* Cells were previously grown for 16 h in unsupplemented (○) or Mg-supplemented (●) medium Mn^{2+} was measured after 10 min incubation in 10 mM MES in the presence of various concentrations of $Mn(NO_3)_2$ Mean values ± SEM from three replicates are shown where these exceed the dimensions of the symbols

of a magnesium supplement retained less dye [954 μg dye $(10^9$ cells$)^{-1}$] than their Mg-supplemented counterparts [1,174 μg dye $(10^9$ cells$)^{-1}$]. Thus, surface charge actually appeared to be slightly more negative in Mg-supplemented cells.

3.3.8 *Manganese toxicity towards unsupplemented and Mg-supplemented S. cerevisiae*

In order to elucidate whether cellular Mg status also influenced cellular tolerance to manganese, cells were harvested at growth-stages determined from Figure 3.3.4b to give the most marked differences in cellular Mg (low, standard and high magnesium cells), and incubated with 5 mM Mn^{2+}. Cell viability (measured as the ability to produce colony-forming-units) was found to decrease with time of exposure to Mn^{2+} (Figure 3.3.8). This decline was most marked in cells with low initial Mg levels. Thus, within 2 h of Mn^{2+} addition, only 40% of cells harvested during early-exponential growth in unsupplemented medium [cells contained 1,420 nmol Mg $(10^9$ cells$)^{-1}$] remained viable. In contrast, approximately 84% and 88% of cells harvested during stationary phase in unsupplemented [cellular levels of 2663 nmol Mg $(10^9$ cells$)^{-1}$] and Mg-supplemented [cells contained 3346 nmol Mg $(10^9$ cells$)^{-1}$] media, respectively, were viable at this stage. After 7 h incubation in the presence of Mn^{2+}, the proportion of viable cells from unsupplemented stationary phase cultures had declined to 32%. By comparison almost 78% of cells with the highest initial cellular Mg levels still retained their capacity to form colonies on agar (Figure 3.3.8).

3.3.9 *Importance of the intracellular Mg:Mn ratio in determining manganese toxicity*

Cells with low, standard and high intracellular Mg levels were exposed to a range of Mn^{2+} concentrations (0-50 mM) over a 6 h period. Mn^{2+} accumulation and resultant toxicity were monitored, and results are shown in Table 3.3.9.1. After 2 h incubation, low-magnesium cells had sequestered the highest levels of Mn followed by standard-magnesium cells, with high-magnesium cells accumulating the lowest levels of Mn. These results were consistent with previous observations on effects of cellular Mg on Mn^{2+} uptake. However, after 6 h incubation at the higher Mn^{2+} concentrations (2-20 mM), Mn^{2+} accumulation by standard-magnesium cells had surpassed that of low-magnesium cells. For all three cell types, intracellular Mn levels varied no more than 2-fold over the 0.25-50 mM concentration range at either time point.

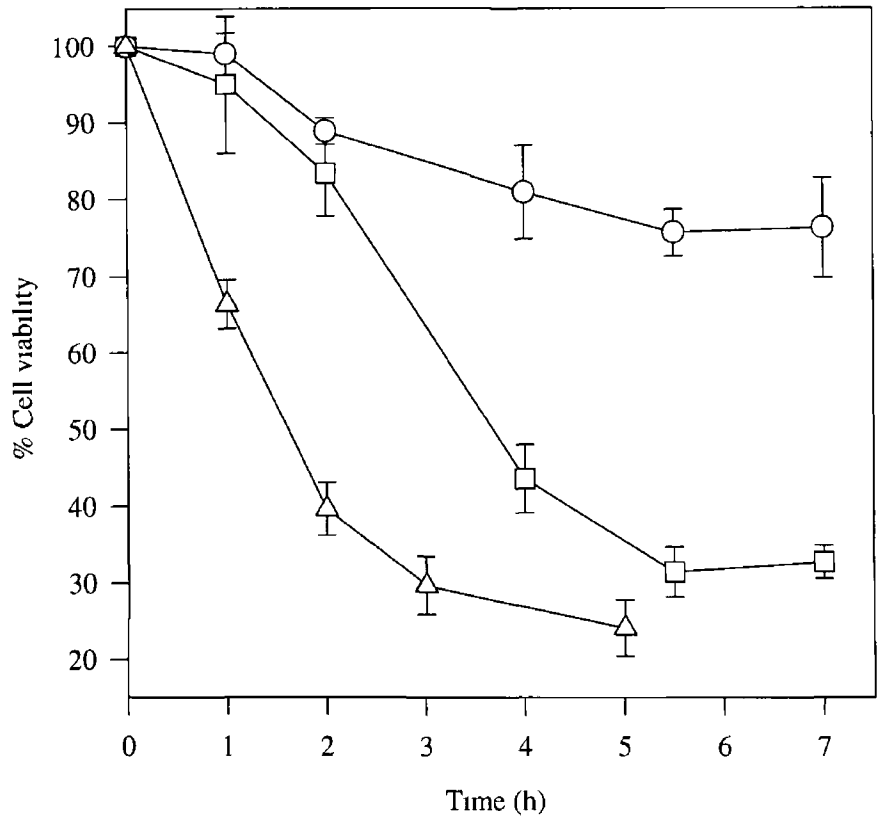


Figure 3 3 8 Influence of cellular Mg on Mn^{2+} toxicity in *Saccharomyces cerevisiae* Cells were harvested after 16 h growth in Mg-supplemented (○) or unsupplemented (□) medium, or after 6 h growth in unsupplemented medium (Δ) Cellular Mg levels were determined to be 3346 ± 46, 2663 ± 14, and 1420 ± 55 nmol (10^9 cells)$^{-1}$ respectively Cells were incubated in 10 mM MES buffer with 2% glucose in the presence of 5 mM Mn^{2+} At intervals, samples were removed for viability measurements Mean values from three replicate determinations are shown ± SEM where these exceed the dimensions of the symbols

Culture type	Sampling time (h)	Initial Mn^{2+} (mM)	Intracellular Mg (nmol/10^9 cells)	Intracellular Mn (nmol/10^9 cells)	% Viability	Intracellular Mg Mn ratio
6 h unsupp	2 h	0 25	990 ± 14	561 ± 8	56 8 ± 4 0	1 76
		0 5	1189 ± 74	619 ± 29	55 7 ± 9 3	1 92
		1 0	881 ± 139	516 ± 90	51 7 ± 3 6	1 70
		2 0	1292 ± 6	848 ± 21	62 5 ± 10 7	1 52
		5 0	1377 ± 76	976 ± 72	39 8 ± 1 7	1 41
		20 0	1133 ± 156	1200 ± 108	52 3 ± 7 9	0 94
	6 h	0 25	994 ± 66	1223 ± 65	31 8 ± 1 4	1 23
		0 5	743 ± 135	1047 ± 98	23 6 ± 1 4	1 40
		1 0	1099 ± 44	1100 ± 13	25 5 ± 1 4	1 00
		2 0	1249 ± 119	1283 ± 37	25 5 ± 2 0	1 02
		5 0	1251 ± 79	1143 ± 27	16 4 ± 2 3	0 91
		20 0	1542 ± 110	1296 ± 30	21 8 ± 2 2	0 84
16 h unsupp	2 h	0 25	2338 ± 18	413 ± 6	96 6 ± 2 5	5 66
		0 5	2441 ± 2	555 ± 8	99 7 ± 5 6	4 39
		1 0	2622 ± 15	664 ± 7	93 8 ± 1 2	3 94
		2 0	2438 ± 20	688 ± 4	90 5 ± 3 7	3 54
		5 0	2618 ± 12	737 ± 11	100 9 ± 3 9	3 55
		20 0	2740 ± 4	836 ± 11	95 1 ± 1 7	3 27
		50 0	2682 ± 60	834 ± 12	99 7 ± 4 1	3 21
	6 h	0 25	2288 ± 9	855 ± 14	82 0 ± 2 2	2 67
		0 5	2061 ± 10	1000 ± 2	74 5 ± 1 7	2 06
		1 0	2303 ± 49	1181 ± 28	85 1 ± 4 3	1 95
		2 0	2436 ± 18	1477 ± 16	79 8 ± 5 2	1 64
		5 0	2445 ± 37	1468 ± 53	74 3 ± 1 2	1 66
		20 0	2707 ± 23	1707 ± 15	74 8 ± 2 6	1 58
		50 0	2656 ± 44	1598 ± 10	77 7 ± 1 9	1 66
16 h Mg	2 h	0 25	2554 ± 10	435 ± 5	92 8 ± 1 0	5 87
supp		0 5	2425 ± 13	526 ± 5	90 9 ± 1 9	4 61
		1 0	2538 ± 94	535 ± 27	98 1 ± 4 2	4 74
		2 0	2490 ± 19	595 ± 5	94 4 ± 2 8	4 18
		5 0	2658 ± 41	612 ± 11	83 7 ± 2 8	4 34
		20 0	2581 ± 214	628 ± 12	93 5 ± 3 8	4 11
		50 0	2836 ± 34	698 ± 16	78 5 ± 1 6	4 06
	6 h	0 25	2597 ± 10	640 ± 8	84 9 ± 8 2	4 05
		0 5	2776 ± 33	858 ± 108	82 8 ± 5 8	3 23
		1 0	2595 ± 56	774 ± 25	87 3 ± 1 9	3 35
		2 0	2740 ± 31	1041 ±22	78 5 ± 3 0	2 63
		5 0	2791 ± 11	1071 ± 3	90 3 ± 7 9	2 60
		20 0	2888 ± 96	1071 ± 39	88 8 ± 2 6	2 69
		50 0	2752 ± 75	1110 ± 18	85 4 ± 4 5	2 47

Table 3 3 9 1 The effect of intracellular Mg on Mn accumulation and toxicity in *S cerevisiae* Mean values ± standard error of the mean from three replicate determinations are shown

When percentage viability data from Table 3 3 9 1 were plotted against cellular Mn content, no simple relationship was evident as cells with similar Mn^{2+} contents exhibited significant differences in viability (Figure 3 3 9a) Generally, low-magnesium cells were more susceptible to Mn^{2+} toxicity than standard and high-magnesium cells, even when actual cellular levels of Mn were similar (Table 3 3 9 1) For example, after 2 h incubation, low, standard and high-magnesium cells incubated with 0 25-1 0 mM Mn^{2+} exhibited comparable intracellular levels of Mn [400-650 nmol Mn^{2+} (10^9 cells) ^{1}], but the viability of low-magnesium cells (52-56 %) was approximately half that of standard and high-magnesium cells (90-100%) Similarly, after 6 h incubation, low-magnesium cells that had sequestered 1000-1300 nmol Mn^{2+} (10^9 cells) ^{1} (at 0 25-20 0 mM external Mn^{2+}) showed further reductions in viability to 16-30 %, whereas, despite similar levels of Mn accumulation, standard-magnesium (incubated at 0 25-1 0 mM Mn^{2+}) and high-magnesium (incubated at 2 0-50 0 mM Mn^{2+}) cells retained 75-90% viability For 16 h unsupplemented cells, incubated for 2 h with 5 0 mM Mn^{2+}, the average experimental count slightly exceeded the average control count at that time point, resulting in a viability value >100% when the experimental count was expressed as a percentage of control levels

When intracellular Mg levels were taken into consideration, by plotting percentage viability against cellular Mg Mn ratio (Figure 3 3 9b), a good correlation was apparent Cells with an internal Mg Mn ratio greater than approximately 3 0 retained 90% viability or greater Decreased viability was evident with decreases in the Mg Mn ratio Viability showed a sharp decline at cellular Mg Mn ratios below 2 0, which was identified as the critical ratio for detemining toxicity For example, cells that contained approximately equivalent levels of Mg and Mn (Mg Mn ~ 1 0) displayed only approximately 20% viability, whereas cells possessing Mg levels approximately double those of Mn (Mg Mn ~ 2 0) retained approximately 70% viability

3.3 10 *Investigation of magnesium protective effect with other metals*

Utilising differences in metal accumulation as an assay to investigate the effects of enhanced cellular magnesium levels, a range of metals (both mono and divalent) were contacted with Mg-supplemented and unsupplemented cells and the results illustrated in Figure 3 3 10 There were considerable differences in the levels of metal accumulated, Cs^{+} and in particular Sr^{2+} (even at a high external concentration) were accumulated to

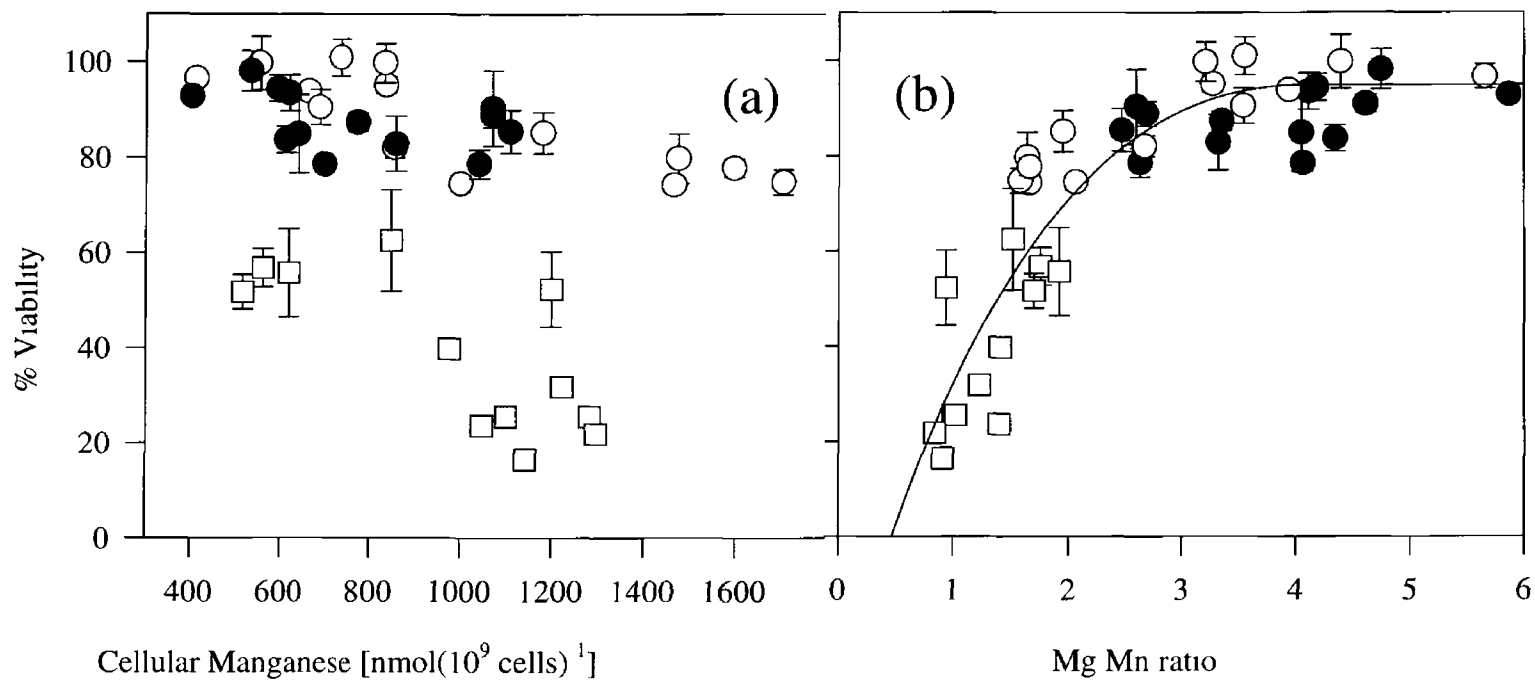


Figure 3.3 9 Relationship between cellular Mg^{2+} and Mn^{2+} levels and cell viability (a) cell viability plotted against cellular Mn^{2+} levels of *Saccharomyces cerevisiae*, (b) cell viability plotted against cellular Mg Mn ratio Cells had previously been grown in unsupplemented medium for 6 h (□) or 16 h (○), or in 30 mM Mg-supplemented medium (●) for 16 h, to yield low-, standard-, and high-magnesium cells respectively Cells were incubated in 10 mM MES buffer with 2% glucose in the presence of a range of Mn^{2+} concentrations Samples were removed for viability measurements after 2 h and 6 h Mean values from three replicate determinations are shown ± SEM where these exceed the dimensions of the symbols

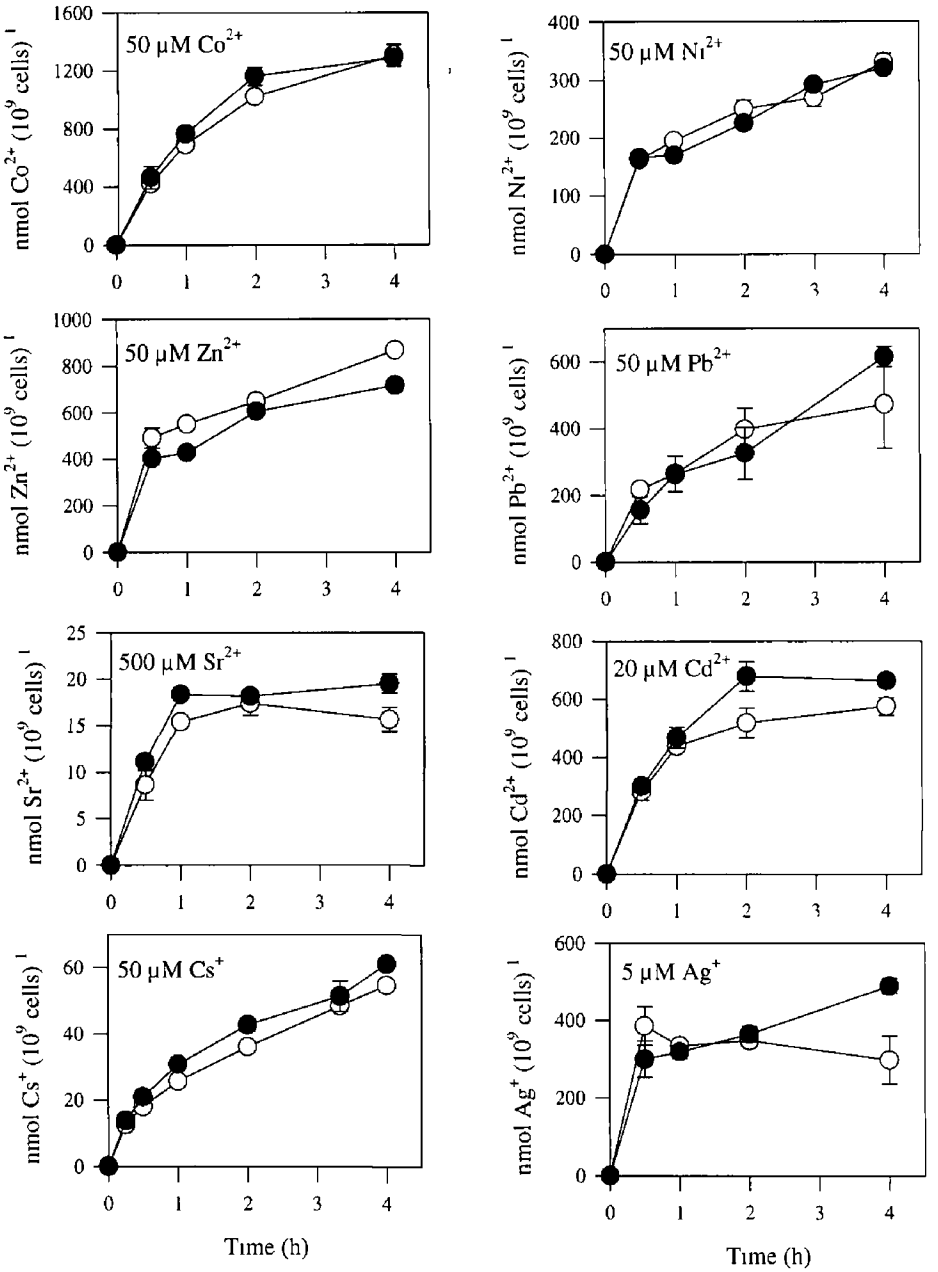


Figure 3 3 10 Metabolism-dependent accumulation of a range of metal cations by Mg-supplemented and unsupplemented *Saccharomyces cerevisiae* The graphs show uptake of metal ions at specified concentrations by Mg-supplemented (closed symbols) and unsupplemented (open symbols) cells in the presence of 2% glucose Mean values ± SEM are shown where theses exceed the dimensions of the symbols

the lowest levels, whereas Co^{2+}, Zn^{2+} and Cd^{2+} were taken up to greatest extent Cells possessed a high affinity for Ag^{+}, even at a very low concentration However, none of these metals displayed differences in accumulation similar to those previously observed between Mg-supplemented and unsupplemented cells with Mn^{2+} The only metal whose accumulation was influenced by intracellular Mg levels was Cu^{2+}, and here, the situation was the converse of that observed with Mn^{2+} (Figure 3 3 11) Both cell types initially possessed low levels of Cu^{2+} after growth in YEPD medium, but on exposure to 10 μM Cu^{2+}, Mg-supplemented cells accumulated 2-fold more Cu^{2+} than their unsupplemented counterparts Increased accumulation by Mg-supplemented cells during the first 15 minutes of contact was mainly responsible for the overall differences in Cu^{2+} levels, Mg-supplemented cells accumulated 22 as compared with 13 nmol Cu^{2+} (10^9 cells)$^{-1}$ Thereafter Cu^{2+} accumulation was quite slow, but noticeably higher in Mg-supplemented cells, during the following 1 h 45 minutes Mg-supplemented cells accumulated 11 nmol (10^9 cells)$^{-1}$ compared with 6 nmol (10^9 cells)$^{-1}$ by unsupplemented cells

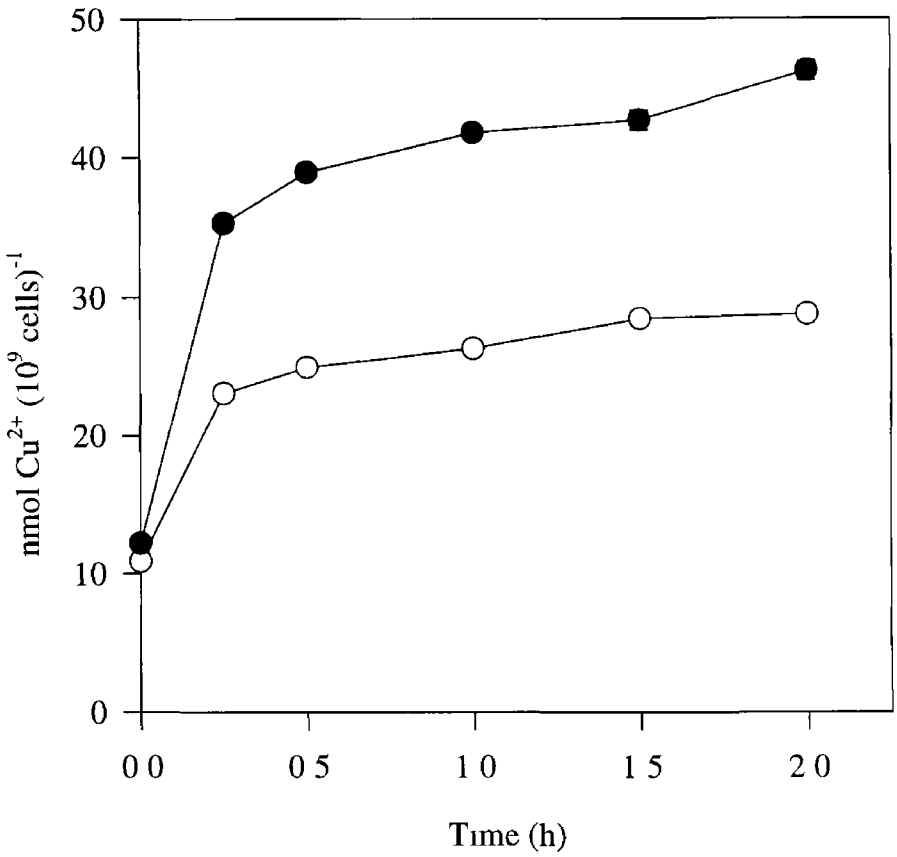


Figure 3 3 11 Metabolism-dependent accumulation of 10 μM Cu^{2+} by Mg-supplemented and unsupplemented *Saccharomyces cerevisiae* The graph shows uptake of Cu^{2+} by Mg-supplemented (closed symbols) and unsupplemented (open symbols) cells m the presence of 2% glucose Mean values from three replicate determinations are shown ± SEM where these exceed the dimensions of the symbols

3.4 Development of new protocol and assessment of procedures for extraction of soluble ion pools' results

3.4.1 *Enzyme assay validation*

Glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) and carboxypeptidase Y (CpY) which were chosen as marker enzymes for cytoplasmic and soluble vacuolar contents respectively, are routinely used for this purpose (Schekman, 1982) The performance of both G-3-PDH and CpY enzyme assays were assessed with crude cell preparations (prepared by glass bead disintegration of whole cells) and the formation of product over time (as measured by absorbance increase) was recorded (Figure 3 4 1) Both enzymes functioned well with CpY and G-3-PDH producing a linear and non-linear response respectively, over the time period investigated This was as expected and consistent with relevant assay literature (Krebbs, 1955, Jones, 1990) Subsequent fixed time point assays required a measuring point prior to each enzyme reaching maximum production In the case of G-3-PD, the 25 minute time point was chosen For CpY, 60 minutes was chosen as this enzyme possessed a slower reaction rate than G-3-PDH

Both enzyme assays are suitable for use with crude cell preparations in the manner outlined above Cells permeabilised with 0 1-0 2% Triton X-100 or 10-20% DMF have been previously used as an enzyme source for CpY (Jones, 1990) In the case of G-3-PDH assay, other reactions (triose isomerase and glycerophosphate dehydrogenase) may interfere (Krebbs, 1955), but as these enzymes are also resident in the cytoplasm, they do not conflict with the outlined application

3.4.2 *Measurement of enzyme activity in subcellular fractions*

Cells exposed to 100 µM Cd^{2+} for 1 h were subjected to differential extraction of subcellular pools as outlined in section 2 2 2 (Method 1, M 1) In addition, a further cell sample was subjected to a modified extraction procedure whereby a glass bead disintegration step (section 2 4 4 3) was substituted for the 60% methanol extraction step (Modified Method 1, MM 1) Metal-free control samples were also fractionated and the resulting fractions analysed for G-3-PDH and CpY activity The results are shown in Table 3 4 2 1 and Table 3 4 2 2 and show total enzyme activity in each faction A total cellular enzyme activity control is also shown

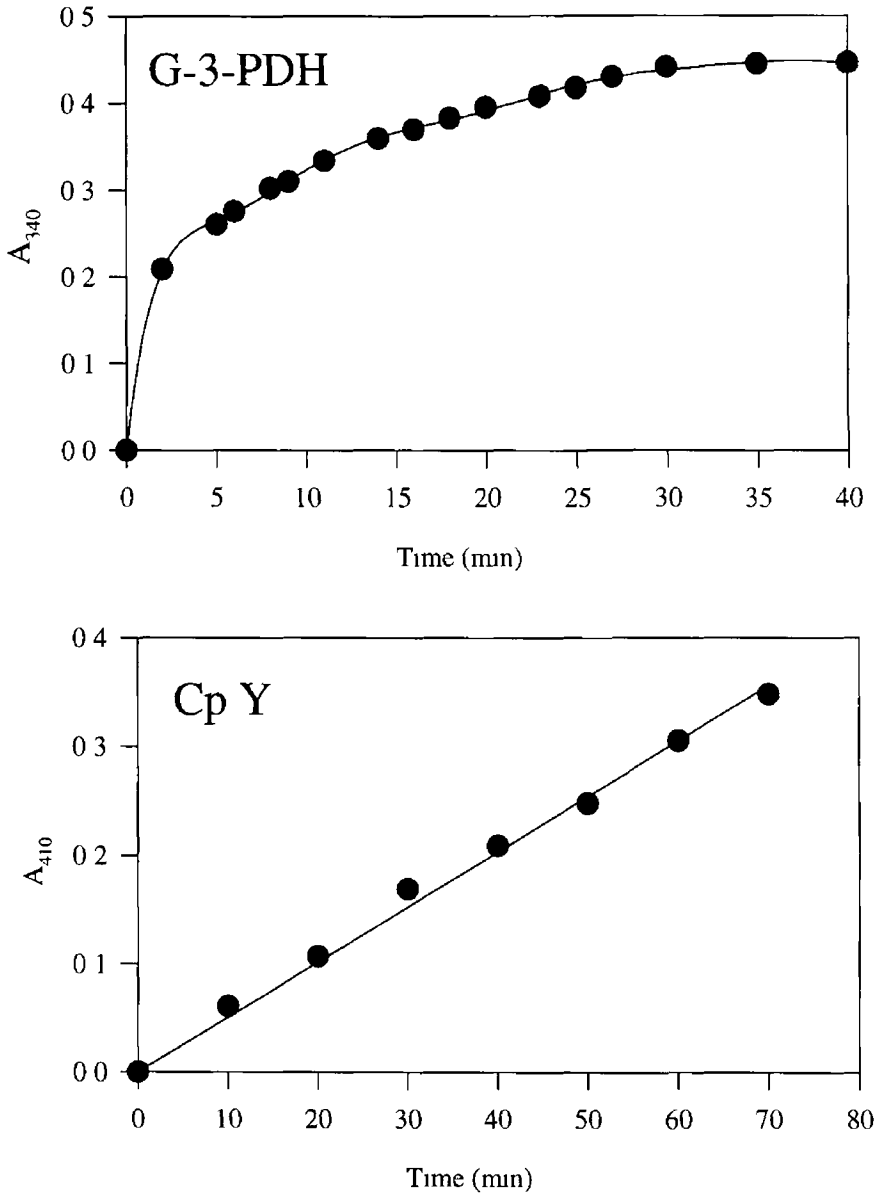


Figure 3 4 1 Enzyme assay validation plots for yeast G-3-PDH and CpY activity The assays were conducted in accordance with the procedures of Krebbs (1955) and Jones (1990) with a crude cell extract prepared by disintegration with glass beads

Table 3 4 2 1 G-3-PDH activity in subcellular pools of *Saccharomyces cerevisiae* Mean values ± SEM from four replicate determinations are shown

	Total G-3-PDH activity [µmol (min) $^{-1}$]			
Cell fraction	**M 1, Control**	**M 1, Cd^{2+}**	**MM 1, Control**	**MM 1, Cd^{2+}**
Surface	1 21 ± 0 78	0 98 ± 0 59	1 03 ± 0 27	1 28 ± 0 34
Cytosolic	2 00 ± 0 63	0 49 ± 0 20	1 33 ± 1 14	2 10 ± 0 45
Vacuolar				
(i) MeOH	2 32 ± 1 22	1 41 ± 0 26	-	-
(ii) Glass beads	-	-	39 87 ± 0 23	36 29 ± 0 20
Whole cell extract (Control)	49 83 ± 0 78			

M 1 refers to Method 1, MM 1 refers to modification of Method 1 by inclusion of glass bead step

Table 3 4.2 2 CpY activity in subcellular pools of *Saccharomyces cerevisiae* Mean values ± SEM from four replicate determinations are shown

	Total CpY activity [µmol (min) $^{-1}$]			
Cell fraction	**M 1, Control**	**M 1, Cd^{2+}**	**MM 1, Control**	**MM 1, Cd^{2+}**
Surface	0 082 ± 0 055	0 018 ± 0 010	0 000 ± 0 000	0 031 ± 0 022
Cytosolic	0 059 ± 0 026	0 006 ± 0 006	0 138 ± 0 129	0 000 ± 0 000
Vacuolar				
(i) MeOH	0 000 ± 0 000	0 018 ± 0 018	-	-
(ii) Glass beads	-	-	3 351 ± 0 122	3 355 ± 0 180
Whole cell extract (Control)	3 091 ± 0 236			

M 1 refers to Method 1, MM 1 refers to modification of Method 1 by inclusion of glass bead step

From the data presented, it can be seen that total cellular G-3-PDH activity is at least one order of magnitude greater than CpY activity for equal numbers of cells Recovery of cellular enzyme activity was greater with the modified extraction method when compared to controls This was due to the fact that both G-3-PDH and CpY express very low levels of activity in fractions extracted with 60% methanol For example compared with controls, only 3-5% of G-3-PDH and even less CpY activity was expressed in fractions isolated with methanol The vast majority of both enzyme activities (> 90%) was observed in those fractions produced through cell disintegration with glass beads

The majority of CpY activity was observed in vacuolar pool fractions as expected (Table 3 4 2 2), with little or no activity in the preceding fractions However, those subcellular pools extracted with 60% methanol exhibited very low activity, if any This can be attributed to the inhibitory influence of high methanol levels present The expression of G-3-PDH activity (Table 3 4 2 1) mirrored that of CpY In this instance, low levels (< 5%) of activity were recorded in the surface and cytosolic pools with the remainder in fractions isolated with glass beads Activity in fractions isolated with methanol was again dramatically reduced With less than 5% of cytosolic marker activity located in the cytosolic pool fraction and the bulk of G-3-PDH activity in the succeeding vacuolar fraction, it appeared that the DEAE-dextran permeabilisation step did not release contents of the cytosolic pool

Incubation of cells in 100 μM Cd^{2+} prior to fractionation had no effect on expression of G-3-PDH or CpY activities Levels of activity and distribution between subcellular pools were similar to controls Subsequent experiments determined that levels of expression and partition of enzyme activity remained unchanged in cells previously incubated for between 1 and 5 h in 100 μM Cd^{2+}

3 4.3 *Investigation of DEAE-dextran permeabilisation step*

The efficacy of DEAE-dextran permeabilisation was assessed utilising a number of staining solutions and measuring G-3-PDH activity The stains used were trypan blue (Sigma T8154, a 0 4% solution in 0 85% NaCl), citrate methylene blue and methyl green

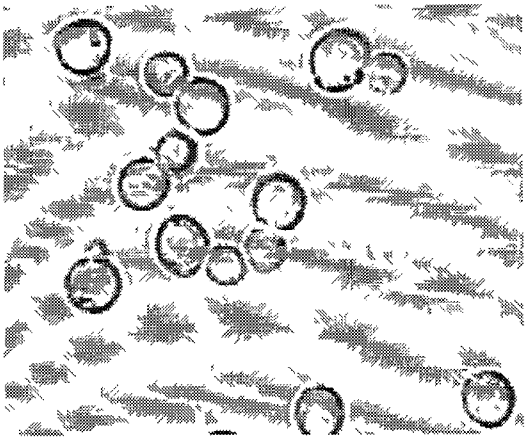
A quantity of cells was resuspended in 1 ml of 10 mM Tris-MES containing 0.7 M sorbitol and treated with 40 µl of 10 mg ml^{-1} DEAE-dextran (0.4 mg ml^{-1} final concentration). 0.2 ml of trypan blue stain was added and the suspension observed under the microscope. After 1 minute, all cells remained colourless. By 2 minutes some cells had taken up the stain but > 95% remained colourless. After 10 minutes a greater number of cells appeared blue but the percentage remaining colourless was > 80%. Controls (less dextran) exhibited a similar rate of colouration and after 10 minutes, 92% of cells remained colourless. Increasing dextran concentration to 2 mg ml^{-1} did not result in an increased number of coloured cells. Similarly, with citrate methylene blue staining, less than 5 % of cells stained blue in the presence or absence of DEAE-dextran (0.4 mg ml^{-1}).

Methyl green had been previously used to determine optimum DEAE-dextran concentrations for permeabilisation (White and Gadd, 1986). In the present work, 0.1 and 0.5% solutions of methyl green were contacted with cells in the presence of dextran. In both cases, cell take-up of the dye was so faint as to be indistinguishable from control cells (no DEAE-dextran added).

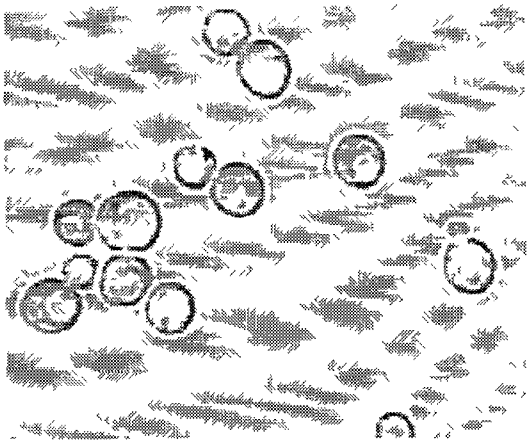
Due to the poor quality and resolution of dye testing, an attempt was made to measure G-3-PDH activity of cells incubated in a range dextran concentrations. To 1 ml samples of cell suspension in 10 mM Tris-MES buffer containing 0.7 M sorbitol, 100 µl of DEAE-dextran solution (2-10 mg ml^{-1} final concentration) was added. After 2 and 20 minutes, cells were removed by centrifugation, and the pellet washed three times (0.5 ml each time) with 10 mM Tris-MES, 0.7 M sorbitol buffer. The supernatants were combined and assayed for G-3-PDH activity. No activity was observed at any DEAE-dextran concentration at either time point.

3.4.4 *Microscopy of cells undergoing differential extraction of subcellular pools*

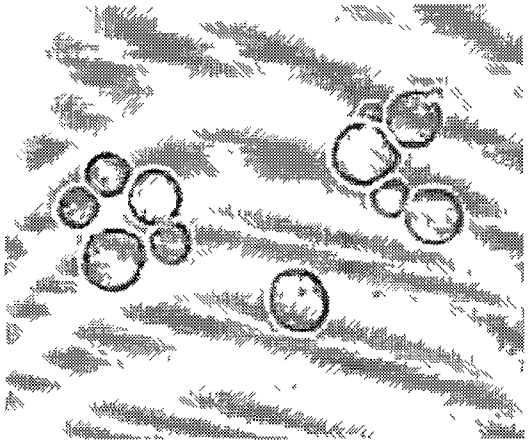
Microscopic examination of cells at different stages of the extraction procedure revealed the effects of each successive treatment (Figure 3.4.2). Washing with MES buffer had no effect, as cells retained the similar shape, size and intracellular organellar definition as control cells (Figure 3.4.2 (i) and (ii)). Following treatment with DEAE-dextran, no change in morphology was observed. Cell shape and size remained unchanged, but internal cell definition became less clear. Cells took on a more grainy appearance, but



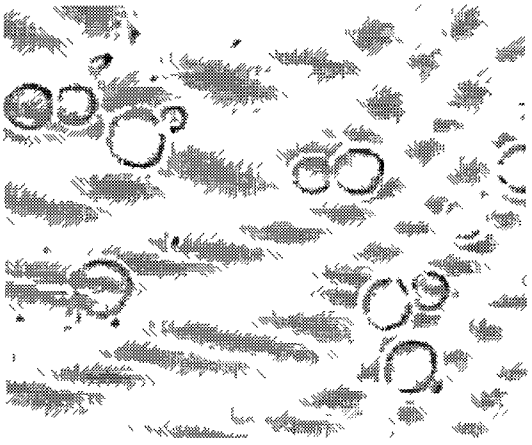
(i) untreated cells (control)



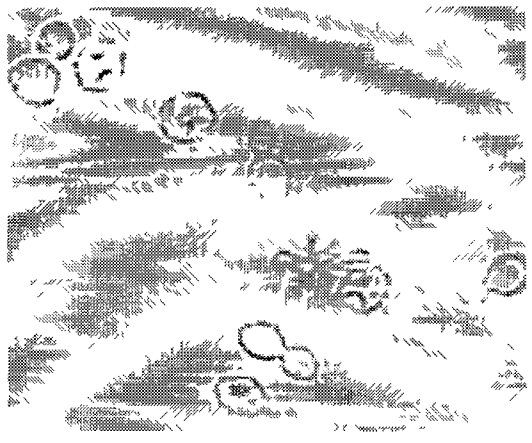
(ii) cells after wash step



(iii) cells after DEAE-dextran treatment



(iv, a) cells after methanol treatment



(iv, b) cells after treatment with glass beads

Figure 3 4 2 Visual effect of differential extraction of subcellular ion pools on *Saccharomyces cerevisiae* (40X magnification) Following DEAE-dextran treatment (iii), cells were subjected to either methanol extraction (iv, a) or disintegration with glass beads (iv, b), but not both

vacuolar outlines were still visible (Figure 3 4 2 (iii)) Methanol treatment had a noticeable effect on cells Cell size was unchanged, but cell shape appeared slightly less rounded, although cells themselves appeared essentially intact There was a complete loss of intracellular definition, no structures that could be identified as cytoplasm or vacuoles were visible (Figure 3 4 2 (iv, a)) In contrast, cells disrupted with glass beads after DEAE-dextran treatment lost their shape and size Cracked cell envelopes, smaller in size than whole cells along with cellular debris were visible (Figure 3 4 2 (iv, b)), completely lacking any internal definition Not all cells were completely disrupted and those that appeared to remain intact appeared darker and similar in size to control cells (top left hand corner of Figure 3 4 2 (iv, b)) Cell counts revealed that < 4% remained in this state after treatment with glass beads, and there was no change in cell numbers between successive steps at any of the stages described above

3 4.5 *Further attempts to extract subcellular pools*

A number of cell disruption techniques were investigated as to their possible use or adaptation for the purpose of differential extraction of subcellular pools The techniques examined included cell permeabilisation with 0 2% Triton X-100 or 20% DMF, freeze-thaw fracturing, and sonication of cells Subsequent measurement of G-3-PDH and CpY activity was used to assess each method

Cell permeabilisation with Triton X-100 and DMF was found to be unsuitable Following the treatment, the supernatant was found to contain no G-3-PDH or CpY activity, possible as a consequence of interference from the reagents involved Freeze-thaw fracturing of cells proved to be similarly disappointing Analysis of supernatants (fraction 1) and pellets (fraction 2, after glass bead disintegration) following 1-5 freezing and thawing cycles, revealed no G-3-PDH activity in any of the supernatants, all enzyme activity was localised in fraction 2 This, coupled with microscopic observations suggested that the procedure did not crack the cells Sonication yielded more positive results Initial results revealed G-3-PDH activity in supernatants but no CpY Subsequent experiments determined that 70% of G-3-PDH activity was localised in the supernatant with the remaining 30% observed to be pellet associated However, no CpY activity was recorded in either supernatant or pellet fractions, possibly as a result of denaturation during the sonication procedure

3.4.6 *Use of spheroplasts for differential extraction of subcellular pools*

Spheroplasts were relatively easy to generate, but required careful handling due to their delicate nature Approximately 1 h was required in order to achieve maximum conversion (~90% when tested for lysis spectrophotometrically) Microscopy confirmed spheroplasts to be perfectly round, single cells A sample of spheroplasts was subjected to washing with isotonic buffer, DEAE-dextran treatment followed finally by glass bead disintegration, and the distribution of G-3-PDH activity measured It was observed that as a percentage of total recovered activity, the original suspension buffer, wash, cytosolic and glass beaded fraction contained 15, 20, 60 and 5% activity respectively Therefore, release of cytosolic contents of spheroplasts could be achieved with DEAE-dextran After treatment with DEAE-dextran, microscopic observations revealed spheroplasts lost their round shape and clumped together in large aggregates that could not be separated, even with vigorous vortexing The separation of the cytosolic pool obtained with spheroplasts confirmed the hypothesis that the cell wall was the main obstacle to extraction of subcellular pools

Cells that had been incubated for 1 and 2 5 h with 100 μM Cd^{2+} were also subjected to spheroplasting and extraction as outlined above with metal-free cells In this instance, spheroplasting efficiency was reduced by approximately 15 and 20% compared with metal-free cells, with the greater decrease observed for cells incubated for 2 5 h in Cd^{2+} In addition, the distribution of G-3-PDH activity was altered, with a small increase in activity in the wash fraction to 25% of total activity recovered and consequent decrease in cytosolic activity to 53% Therefore, due to the delicate nature of spheroplasts and the decreased efficiency of spheroplasting metal-loaded cells, it was decided to attempt differential extraction with cells that had undergone only partial spheroplasting

A technique of partial spheroplasting followed by DEAE-dextran and glass bead disintegration based on the method outlined in section 2 4 6 was carried out on a sample of cells The spheroplasting reaction was allowed to proceed for 15 and 30 min before subsequent extraction of subcellular pools into three fractions The resulting split of G-3-PDH activity was approximately 65% in the cytosolic fraction (F 1) with the remainder distributed between wash and glass beaded fractions (F 2) This was an encouraging result, and attempts were made to refine the technique further

Experiments were conducted varying the length of the spheroplasting reaction between 10 min and 1 h, with differing consequences on enzyme distribution A link between percentage conversion of cells to spheroplasts was established, with 15-20% conversion best for optimum separation of G-3-PDH and CpY activities into the desired fractions Percentage conversion exceeding this level often resulted in an 50 50 split of G-3-PDH activity between fractions F 1 and F 2, although CpY activities remained relatively unaffected with approximately 70% activity present in F 2 fraction

A consistent problem that arose was the significant levels of G-3-PDH and CpY activity in the wash fraction, which could be up to 15% of total recovered G-3-PDH activity, and 25% for CpY activity Osmolarity of the wash buffer was varied between 0 7 and 1 2 M sorbitol in an attempt to reduce enzyme release through cell lysis, but met with little success It appeared inevitable that a number of cells would lyse during the spheroplasting and wash steps regardless of how mild the treatment (*i e* pellets washed by gentle inversion in buffer) Therefore when calculating enzyme distribution between subsequent fractions (F 1 and F 2), that activity present in the wash fraction was neglected (providing it did not exceed the tolerances stated above) and total cellular enzyme activity was taken to be the sum of activities recovered in F 1 and F 2 fractions

Partially spheroplasted cells were sensitive to shearing at all stages of the extraction protocol Hence, cells were resuspended by gentle inversion only during the wash step, as resuspension by vortexing, pipetting or agitation with a glass rod resulted in undesirably high levels of both enzymes in this fraction During extraction of F 1 fraction with DEAE-dextran, complete resuspension of the pellet was kept to a minimum Vigorous resuspension, or further additions of DEAE-dextran solution resulted in undesirable partitioning of CpY activity into this fraction In arriving at the protocol in section 2 4 6, the main objective was to maximise G-3-PDH activity and minimise CpY activity in the F 1 fraction, coupled with minimisation of G-3-PDH activity levels in F 2 fraction

3 4.7 *Comparison of subcellular metal distributions in S cerevisiae fractionated by Method 1 and Method 2*

Experiments were carried out to determine if G-3-PDH and CpY activities were inhibited or reduced in cells exposed to Mn^{2+}, Sr^{2+} or Cu^{2+} Cells preloaded with these metals (1 h incubation) were disintegrated with glass beads and the supernatants assayed for enzyme activity No differences in either enzyme activity were observed between experimental and control cells The effect of different metals on the efficacy of Novozym 234 was tested by monitoring spheroplast formation of cells previously incubated in 100 µM Mn^{2+}, Sr^{2+}, Cd^{2+} and 5 µM Cu^{2+} for 1 h Spheroplasting proceeded at a slower rate than observed with metal-free controls for all samples except those preloaded with Mn^{2+}, where > 80% conversion was achieved within 1 h After a similar incubation time, at least 50% conversion was achieved with cells preloaded with Sr^{2+} and Cd^{2+}, and 30% conversion Cu^{2+} loaded cells Therefore, obtaining the required 15-20% conversion prior to extraction of subcellular pools was not problematic, and metal-loaded cells were fractionated as outlined in section 2 4 6 Fractions were analysed for metal content and enzyme activity where appropriate Enzyme activities and metal distributions are shown in Tables 3 4 7 1-3 4 7 8 In addition, metal-loaded cells were subjected to fractionation by Method 1 and analysed for metal content as a comparison

Tables 3 4 7 1 and 3 4 7 2 show distribution of G-3-PDH and CpY activity in subcellular pools of cells loaded with Sr^{2+}, Mn^{2+} and Cd^{2+} (data for cells loaded with Cu^{2+} are shown separately in Tables 3 4 7 6-3 4 7 8) With respect to G-3-PDH activity (Table 3 4 7 1) similar levels of activity were expressed in corresponding fractions irrespective of metal loading Enzyme activity in the wash fractions was less than 15% of the total recovered, with the bulk of activity located in the F 1 fraction (corresponds to cytosolic pool) The distribution of G-3-PDH activity between F1 and F2 fraction (excluding activity in the wash fraction) was approximately 70 30 in percentage terms for control and metal loaded cells Recovered CpY activity (Table 3 4 7 2) was more variable, with Cd^{2+} loaded cells exhibiting a 45% increase in recovered enzyme activity compared with metal-free controls Activity in the wash fraction varied between 15-25% for experimental and control samples, but the majority of CpY activity was observed in F 2 (corresponds to vacuolar pool), with an approximate percentage split between F 1 and F2 of in excess of 30 70 From the G-3-PDH and CpY data, it was concluded that

Table 3 4.7.1 Distribution of G-3-PDH activity in subcellular pools of cells exposed to Sr^{2+}, Mn^{2+} and Cd^{2+} The values shown are the average of two replicate determinations ± error from the mean

Fraction	**G-3-PDH activity** (µmol min ^{1})			
	Control	**Sr^{2+}**	**Mn^{2+}**	**Cd^{2+}**
Wash	16 29 ± 0 11	14 74 ±0 46	18 26 ± 2 02	12 76 ± 0 17
F 1	105 50 ± 1 34	95 21 ± 1 77	106 50 ± 1 34	103 21 ± 1 40
F 2	54 78 ± 0 82	51 08 ± 0 57	41 66 ± 0 15	50 09 ± 0 46
% F1.F2	66 34	65 35	72 28	67 33

Table 3.4.7 2 Distribution of CpY activity in subcellular pools of cells exposed to Sr^{2+}, Mn^{2+} and Cd^{2+} The values shown are the average of two replicate determinations ± error from the mean

Fraction	**CpY activity** (µmol min ^{1})			
	Control	**Sr^{2+}**	**Mn^{2+}**	**Cd^{2+}**
Wash	0 288 ± 0 031	0 416 ± 0 019	0 310 ± 0 020	0 359 ± 0 017
F 1	0 419 ± 0 038	0 426 ± 0 038	0 144 ± 0 020	0 383 ± 0 013
F 2	0 910 ± 0 065	0 812 ± 0 030	0 765 ± 0 009	1 616 ± 0 025
% F1.F2	31 69	34 66	16 84	19 81

an acceptable differentiation of cytosolic and vacuolar pools was obtained, with at least 70% of each marker enzyme localised in the relevant fraction

Comparison of subcellular metal cation distributions of metal-loaded cells (1 h incubation) fractionated by both Method 1 and 2 provided for an assessment of the accuracy of each fractionation procedure Measurements of subcellular levels of Sr^{2+}, Mn^{2+} and Cd^{2+} are shown in Tables 3 4 7 3-3 4 7 5 respectively Generally, levels of metal recovered from cells subjected to Method 2 (partial spheroplasting technique) were less than those obtained for cells subjected to Method 1 fractionation by approximately 20% for Sr^{2+} and Mn^{2+}, and 10% for Cd^{2+} With respect to Method 2, considerable amounts of all three metals were located in the Pre-wash fraction, and this may be attributable to stripping of surface-bound ions by the metal chelator EDTA which was a component of the pre-treatment buffer In addition, the Enz fraction would contain a proportion of metal ions previously bound by the cell wall As a consequence metal levels recovered in the Pellet fraction were reduced compared to metal levels in the Bound fraction of Method 1

Subcellular Sr^{2+} (Table 3 4 7 3) as determined from fractions isolated by Method 1 was mostly localised to the Bound fraction (40%) Soluble Sr^{2+} levels were high in the Cytosolic and Vacuolar fractions accounting for 7% and 29% of cellular Sr^{2+} respectively By comparison, the bulk of Sr^{2+} was localised in the Pre-wash fraction (36%) of cells fractionated by Method 2 However, it was noted that soluble Sr^{2+} in F 1 and F 2 fractions was approximately 5% and 19% respectively of total recovered Sr^{2+}, which was similar to Sr^{2+} partitioning in equivalent fractions by Method 1 The low levels of Sr^{2+} recovered in the Pellet fraction may be due to loss of structurally bound Sr^{2+} to the Pre-wash and Enz fractions during the fractionation protocol

Cellular Mn^{2+} was localised mainly to the Vacuolar (41%) and Bound (55%) fractions following fractionation by Method 1 Here, Vacuolar Mn^{2+} was approximately 10% lower than was previously observed after an equivalent incubation period (see section 3 2 3 and Table 3 2 3 1), but the sequestration of high levels of Mn^{2+} to cell vacuoles was underlined This was supported by the fact that F 2 levels of Mn^{2+} were the highest of any fractions isolated by Method 2 protocol, representing 34% of recovered Mn^{2+} Levels of Mn^{2+} bound by insoluble cellular material (Pellet fraction) were low, possibly for similar reasons outlined above in the case of Sr^{2+} However,

Table 3 4 7 3 Sr^{2+} levels in subcellular pools after 1 h incubation in 100 µM Sr^{2+}
Average of two replicate determinations is shown ± error from the mean

Sr^{2+} per fraction [nmol (10^9 cells)$^{-1}$]					
Method 1			**Method 2**		
		(%)			(%)
			Pre-wash	22 94 ± 6 88	(36)
			Enz	9 56 ± 5 26	(15)
Wash	18 77 ± 3 62	(24)	***Wash***	8 68 ± 1 15	(14)
Cytosolic	5 78 ± 2 87	(7)	***F 1***	2 99 ± 63	(5)
Vacuolar	23 20 ± 6 88	(29)	***F 2***	12 05 ± 4 52	(19)
Bound	31 90 ± 0 15	(40)	***Pellet***	6 69 ± 2 00	(11)
Total fractions	78 65 ± 9 90	(100)	***Total fractions***	62 91 ± 10 66	(100)

Table 3.4.7.4 Mn^{2+} levels in subcellular pools after 1 h incubation in 100 µM Mn^{2+}
Average of two replicate determinations is shown ± error from the mean

Mn^{2+} per fraction [nmol (10^9 cells)$^{-1}$]					
Method 1			**Method 2**		
		(%)			(%)
			Pre-wash	76 85 ± 23 34	(13)
			Enz	88 88 ± 21 39	(15)
Wash	19 17 ± 3 88	(3)	***Wash***	24 72 ± 1 29	(4)
Cytosolic	10 24 ± 1 62	(1)	***F 1***	38 53 ± 1 00	(6)
Vacuolar	300 60 ± 3 93	(41)	***F 2***	210 77 ± 7 84	(35)
Bound	404 96 ± 49 92	(55)	***Pellet***	166 81 ± 13 61	(27)
Total fractions	734 97±59 40	(100)	***Total fractions***	606 56±68 54	(100)

Table 3 4 7.5 Cd^{2+} levels in subcellular pools after 1 h incubation in 100 µM Cd^{2+}
Average of two replicate determinations is shown ± error from the mean

Cd^{2+} per fraction [nmol (10^9 cells)$^{-1}$]					
Method 1			**Method 2**		
		(%)			(%)
			Pre-wash	47 74 ± 1 86	(8)
			Enz	17 54 ± 0 35	(3)
Wash	9 22 ± 4 21	(1)	***Wash***	5 16 ± 0 72	(1)
Cytosolic	6 31 ± 1 94	(1)	***F 1***	6 21 ± 0 71	(1)
Vacuolar	172 29 ± 1 60	(25)	***F 2***	45 27 ± 2 82	(7)
Bound	502 ± 1 41	(73)	***Pellet***	499 85 ± 13 86	(80)
Total fractions	690 04±14 95	(100)	***Total fractions***	621 77±20 37	(100)

when Mn^{2+} levels present in the Pre-wash and Enz fractions were considered (these fractions contain metal ions previously bound by the cell wall) along with Pellet Mn^{2+} levels, the resulting proportion of recovered Mn^{2+} (55%) was similar to the proportion of recovered Mn^{2+} in the Bound fraction of Method 1 (55%).

With respect to Cd^{2+} partitioning by either fractionation protocol, it was observed that the major part of cellular Cd^{2+} was bound by insoluble cellular material. Approximately 76% of recovered Cd^{2+} was localised to the Bound fraction (Method 1) compared with 80% in the Pellet fraction (Method 2). The bulk of remaining cellular Cd^{2+} was present in the Vacuolar fraction (Method 1) or partitioned between the Pre-wash and F 2 fractions (Method 2).

With Cu^{2+}-loaded cells, a 60:40% split in G-3-PDH activity was obtained between F 1 and F 2 (Table 3.4.7.6). Levels of activity between experimental and control samples were similar in wash and F 1 fractions, but Cu^{2+}-loaded cells possessed greater activity in the F 2 fraction. Similarly with CpY, Cu^{2+}-loaded cells exhibited considerably increased activity (>250%) in the F 2 fraction compared with control samples, and resulted in an improved distribution ratio between F 1 and F 2 of 15:85.

Analysis of subcellular metal distributions was complicated by the fact that the growth medium contained approximately 15 µM Cu^{2+}(subcellular Cu^{2+} distribution in control cells is shown in Table 3.4.7.7). Discrepancies arise concerning overall Cu^{2+} levels and its subcellular distribution. Overall Cu^{2+} levels as determined by Method 2 were 28% lower than those determined by Method 1 (Table 3.4.7.7). It was apparent that subcellular Cu^{2+} partitioning in control cells was quite different depending on the method of fractionation used. However, the observation that soluble Cu^{2+} was predominantly partitioned to fractions representing cell vacuoles (Vacuolar fraction for Method 1, F 2 fraction for Method 2) rather than the cytosol was common to both protocols. Following further incubation with Cu^{2+} for 1 h, it was generally observed that the major part of cell Cu^{2+} was bound by insoluble cellular material. This corresponded to approximately 70% in the Bound fraction (Method 1) compared with approximately 73% for Method 2 (sum of Cu^{2+} distribution in Pre-wash, Enz and Pellet fractions).

From the trends outlined above, it was concluded that both Method 1 and Method 2 facilitate estimation of subcellular metal distributions. The biochemical markers used in Method 2 allowed for characterisation of the separation of cytosolic and

Table 3.4.7 6 Distribution of G-3-PDH and CpY activity in subcellular pools of cells incubated with 5 μM Cu^{2+} The values shown are the average of two replicate determinations ± error from the mean

Fraction	G-3-PDH activity (μmol min $^{-1}$)		CpY activity (μmol min $^{-1}$)	
	Control	Cu^{2+}	Control	Cu^{2+}
Wash	33 45 ± 0 51	41 72 ± 1 61	0 239 ± 0 058	0 947 ± 0 032
F 1	90 57 ± 4 42	99 04 ± 1 88	0 434 ±0 031	0 579 ± 0 020
F 2	37 40 ± 0 10	64 73 ± 66	0 925 ± 0 060	3 312 ± 0 149
% F1 F2	70 30	61 39	32 68	15 85

Table 3 4 7.7 Cu^{2+} levels in subcellular pools of control cells Average of two replicate determinations are shown ± error from the mean

Cu^{2+} per fraction [nmol (10^9 cells) $^{-1}$]					
Method 1			Method 2		
		(%)			(%)
			Pre-wash	16 88 ± 1 70	(36)
			Enz	5 80 ± 1 40	(12)
Wash	8 36 ± 1 21	(13)	*Wash*	8 35 ± 2 04	(18)
Cytosolic	5 72 ± 0 31	(9)	*F 1*	1 17 ± 0 12	(3)
Vacuolar	24 24 ± 0 75	(37)	*F 2*	8 19 ± 1 60	(18)
Bound	26 38 ± 2 66	(41)	*Pellet*	5 66 ± 0 18	(13)
Total fractions	64 70 ± 4 93	(100)	*Total fractions*	46 59 ± 5 34	(100)

Table 3 4 7.8 Cu^{2+} levels in subcellular pools of cells incubated for 1 h with 5 μM Cu^{2+} Average of two replicate determinations are shown ± error from the mean

Cu^{2+} per fraction [nmol (10^9 cells) $^{-1}$]					
Method 1			Method 2		
		(%)			(%)
			Pre-wash	28 88 ± 2 01	(27)
			Enz	7 16 ± 1 64	(7)
Wash	11 36 ± 2 45	(5)	*Wash*	7 97 ± 1 93	(7)
Cytosolic	7 28 ± 1 72	(4)	*F 1*	6 51 ± 0 12	(6)
Vacuolar	41 48 ± 1 29	(21)	*F 2*	13 93 ± 0 31	(14)
Bound	141 00 ± 3 47	(70)	*Pellet*	39 99 ± 0 35	(39)
Total fractions	201 12 ± 8 93	(100)	*Total fractions*	104 44 ± 4 35	(100)

vacuolar pools The trends in accompanying subcellular metal distribution appeared to generally confirm the trends previously obtained with Method 1 (see results section 3 2)

CHAPTER 4

DISCUSSION

Accumulation and subcellular localisation of metal cations by Saccharomyces cerevisiae

Ph D Research Thesis

Kevin J Blackwell B.Sc

CHAPTER 4 DISCUSSION

4.1 Effect of environment on metal uptake. discussion

4 1.1 *Cd^{2+} accumulation under different environmental conditions*

The particular environment in which Cd^{2+} uptake experiments were conducted, was observed to affect levels of Cd^{2+} removed from solution in both the presence and absence of glucose (Figure 3 1 1 1 and 3 1 1 2) Where glucose was present, its metabolism led to acidification of the extracellular medium, which was particularly rapid in the unbuffered system (pH decreased from 5 5 to 4 4 prior to addition of Cd^{2+} ions) It is evident from the results presented that where pH changes were minimised, such as in the buffered and pH controlled systems, levels of Cd^{2+} uptake were greater compared with the unbuffered system pH controlled systems have been used to assess effects of differing external pH on metal uptake (Matheickal *et al* , 1997), but have not been previously compared with buffered systems, despite the fact that the reason for using either system were similar *i e* to minimise extracellular pH fluctuations In the present work, similarities were observed between levels of metal uptake from buffered and pH controlled systems with an initial concentration of 100 µM Cd^{2+} At the higher Cd^{2+} concentration (200 µM) in the pH controlled system (Figure 3 1 1 2,c), Cd^{2+} removal from solution was more erratic and may have been as a result of transient localised precipitation of Cd^{2+} due to addition of concentrated NaOH to counteract pH changes Volesky and May-Phillips (1995) did compare the effect of potassium biphthalate buffer on the equilibrium biosorption of selected metals (no glucose present) with an unbuffered solution These authors reported a small depression of Zn^{2+} and Cu^{2+} uptake in the presence of buffer compared to its absence, but no differences in uranyl ion uptake were detected These findings correspond to Cd^{2+} uptake in the absence of glucose in the present work, where no differences in uptake levels were apparent after 5 h between systems It was only when glucose was present that levels of Cd^{2+} removed from solution differed in each system Decreased Cd^{2+} accumulation in the unbuffered system in the presence of glucose as compared with the buffered and pH controlled systems may be attributed to the increased presence of H^+ ions H^+ ions are known to compete with metal cations for cellular binding sites and reduce potential metal

interactions with cells (Gadd, 1993, Sag *et al*, 1995) Extracellular H^+ levels are reduced in the buffered and pH controlled systems by complexation and neutralisation, respectively

In each system, a time-dependent component of Cd^{2+} uptake was observed in the absence of glucose, which was more pronounced at the higher Cd^{2+} concentration This was unexpected and may be attributed to some degree of cellular activity as cells inactivated by irradiation exhibited no time-dependent Cd^{2+} accumulation (Figure 3 1 3) It is known that cation transport is dependent on the electrochemical proton gradient that is the result of plasma membrane H^+-ATPase activity and that cation accumulation can continue in the absence of energy metabolism provided that a transmembrane gradient still exists (Jones and Gadd, 1990) In the present work, it is possible that such a gradient continued to exist, resulting in the observed slow cation accumulation A further possibility may involve time-dependent Cd^{2+} deposition on the cell surface as a result residual cellular metabolism Such a phenomena has been observed previously during Pb^{2+} (Swift and Forciniti, 1997) and uranium uptake (Volesky and May-Phillips, 1995, Hafez *et al*, 1997)

4 1.2 *Perturbation in extracellular K^+ and H^+ levels, and viability*

Release of cellular cations upon exposure to Cd^{2+} was confined to K^+, as extracellular Mg^{2+} levels remained consistent with metal-free controls, and cellular Ca^{2+} levels were negligible due to the low levels present in growth medium Conservation of cellular Mg^{2+} levels is indicative of cellular integrity, as Mg^{2+} ions have a role in stabilisation of the cell envelope and other cellular biomolecules (Walker, 1994) K^+ release in the presence of Cd^{2+} ions has been reported in the past (Norris and Kelly, 1977, Gadd and Mowll, 1983, Belde *et al*, 1988, Assmann *et al*, 1996) but in the present study, levels of K^+ loss were different in each system Extracellular K^+ levels increased in the order, unbuffered system < buffered system < pH controlled system, which was in line with increased levels of Cd^{2+} uptake in each system In the absence of glucose, K^+ loss was gradual over 5 h, but in the presence of glucose, K^+ release was greatest during the first hour of contact where Cd^{2+} uptake was maximal, particularly in the buffered and pH controlled systems This increased dissipation of the cellular K^+ gradient in the buffered and pH controlled systems may have contributed to the higher levels of Cd^{2+} transport

and accumulation observed here (Borbolla *et al.*, 1985; Okorokov *et al.*, 1985). Assmann *et al.* (1996) reported loss of K^+ from energised, but not from deenergised cells, suggesting that Cd^{2+} must accumulate within the cells before causing a detectable effect. By contrast, the present results show K^+ loss in the absence of glucose (where reduced intracellular Cd^{2+} uptake occurred as compared with that in the presence of glucose - see section 3.2.2). As Cd^{2+} ions are also reported to damage yeast plasma membrane integrity as a consequence of external binding (Gadd and Mowll, 1983; Kessels *et al.*, 1987; Assmann *et al.*, 1996), this may have contributed to the observed K^+ release in the absence of glucose.

The degree of extracellular pH control was the main difference between the three systems. Extracellular pH was maintained at 5.5 in the pH controlled system by continuous addition of concentrated acid or base but these additions produced frequent small pH changes of approximately 0.1 units either side of the set point. This constant variation, particularly in the presence of glucose, may have perturbed membrane cation transport systems, resulting in increased Cd^{2+} removal from solution. In the unbuffered system, extracellular H^+ concentrations were dependent on cellular activity, resulting in a gradual increase in extracellular pH in the absence of glucose and a sharp reduction in the presence of glucose. The observed reduction in Cd^{2+} accumulation here, compared with the buffered and pH controlled systems may have been due to the reduction in pH of approximately 1.1 units prior to Cd^{2+} addition, as pH reduction is acknowledged to inhibit metal cation uptake through competitive interactions (Gadd and White, 1985; Fourest and Roux, 1992). The enzymatically-mediated process of H^+ efflux as a result of plasma membrane-bound proton pumping ATPase activity in the presence of glucose may be used as an indicator of toxicity (White and Gadd, 1987b; Karamushka and Gadd, 1994). Here, the presence of Cd^{2+} ions inhibited medium acidification in the buffered and unbuffered systems when compared with metal-free controls (Figure 3.1.4), which was indicative of its toxic nature. Viability staining with citrate-methylene blue indicated that viability in the presence of Cd^{2+} ions was best maintained in a buffered system, with the greatest decrease in viability observed in the pH controlled system. Viability loss in all three systems occurred within minutes of contact with the test ion (Figure 3.1.5), but stabilised thereafter. However, the methylene blue method measures the presence and activity of particular enzymes rather than the ability to

produce daughter cells (Enari, 1977) Therefore, it may not be as reliable as other methods of viability assessment such as spread plating In the present work, measured viability levels were higher than reported by other workers at similar Cd^{2+} concentrations (Gadd and Mowll, 1983)

On the basis of the above findings, it was decided to utilise a buffered system (10 mM MES) for all subsequent work

4 2 Accumulation and subcellular localisation of metals discussion

4.2.1 *Metal uptake profiles in the presence and absence of glucose*

Stimulation of Cd^{2+}, Mn^{2+} and Sr^{2+} uptake by *S cerevisiae* in the presence of glucose was clearly evident The addition of glucose in advance of test ions stimulated cellular metabolism enabling rapid uptake of metal ions within the first hour of contact (Figure 3 2 1 1), and may be due to biosynthesis of transport proteins that were repressed during pre-experimental starvation and/or plasma membrane ATPase activity (Failla *et al*, 1976, Jones and Gadd, 1990, Lin *et al*, 1993b) Cd^{2+} and Mn^{2+} ions were accumulated to a greater extent compared with Sr^{2+} which is likely due to differing affinities of these cations for cellular transport systems It is known that the rate limiting step of metabolically mediated metal cation accumulation is transport of metal ions across the cell membrane (Theuvenet *et al*, 1986) and that transporters involved possess varying affinities for different cations (Mowll and Gadd, 1984, Melhorn, 1986, White and Gadd, 1987a) However, some authors have attributed the larger accumulation of Mn^{2+} compared with Sr^{2+} by *S cerevisiae* to the operation of an efflux pump which possessed a higher affinity for Sr^{2+} than for Mn^{2+} (Nieuwenhuis *et al*, 1981, Theuvenet *et al*, 1986)

In the absence of glucose, a time-dependent component of metal uptake was observed which considerably enhanced metal cation removal from solution Between 1 h and 5 h, metal removal from solution by the biomass increased approximately 2-fold for Cd^{2+} and Mn^{2+}, and 3-fold for Sr^{2+} Norris and Kelly (1977) reported a time-dependent component of Cd^{2+} and Co^{2+} uptake by *S cerevisiae*, as did White and Gadd (1989) for thorium accumulation by live biomass in the absence of a metabolisable energy source Extended contact times, up to a number of days have produced similar

findings (Volesky and May-Philips, 1995, Suh *et al*, 1998) In the present work, a period of starvation was included in experimental protocol prior to addition of metal ions for the purposes of depleting cellular energy reserves This starvation period, coupled with the absence of a metabolisable substrate would lead to the expectation that metal uptake could not be metabolically mediated However, the fact that a considerable portion of cellular metal was sequestered to the vacuole suggested that some form of active ion transport process occurred Ion translocation may take place when cellular energy metabolism has ceased, provided that a transmembrane proton gradient is maintained (Jones and Gadd, 1990) Electrochemical energy released as a consequence of dissipation of the cellular K^+ gradient may also be used for transport of charges solutes (Borbolla *et al*, 1985) The extensive loss of the K^+ gradient coupled with the membrane permeabilising effects of Cd^{2+} (Gadd and Mowll, 1983, Kessels *et al*, 1987) may have been responsible for the observed increased Cd^{2+} accumulation in the absence of glucose Furthermore, it is possible that diffusional processes (Gadd and White, 1989) coupled with time-dependent precipitation within the cell wall (Swift and Forcinti, 1997) may also contribute to metal uptake

Accumulation of Cu^{2+} was similar in the presence and absence of glucose As the concentration of Cu^{2+} added was low (5 µM), it is possible that metal uptake may have been mostly due to cell wall binding This was supported by the findings of Lin *et al*, (1993a,b) at similar Cu^{2+} concentrations Consequently, little Cu^{2+} can be accumulated by metabolic means during the initial contact period, but time-dependent intracellular partitioning of Cu^{2+} is not precluded (Lin *et al*, 1993b)

4 2 2 *Release of intracellular ions during metal accumulation*

Extracellular levels of Mg^{2+} in the presence of Cd^{2+}, Mn^{2+} and Sr^{2+} were similar to those observed in metal-free controls, remaining at 10 µmol g^{-1}, or less in the presence of absence of glucose Clearly, no displacement of cellular Mg^{2+} for accumulated Cd^{2+}, Mn^{2+} or Sr^{2+} ions occurred Norris and Kelly (1977) observed unchanged levels of cellular Mg^{2+} during Co^{2+} and Cd^{2+} uptake by *S cerevisiae* where as Avery and Tobin (1993) reported Mg^{2+} and Ca^{2+} release of less than 5 µmol g^{-1} due to biosorption of a range of cations, up to concentrations of 50 µM By contrast, Cu^{2+} at a concentration of 200 µM induced release of 60% of cellular Mg^{2+} within 1 h (Brady and Duncan, 1994b)

In literature reports, Mg^{2+} exchange for accumulated metal cations is more prevalent with other types of fungal biomass, many of which are denatured in some fashion (Akthar *et al*, 1996, Brady and Tobin, 1994, 1995) Where substantial loss of Mg^{2+} by live *S cerevisiae* was observed, it was correlated with rapid viability loss during accumulation of Cd^{2+} ions (Gadd and Mowll, 1983)

Loss of K^+ was the main cellular response upon addition of heavy metal cations, but was not always a feature of metal uptake (see Figure 3 2 1 2) During Cd^{2+} accumulation in the absence of glucose, release of K^+ in excess of control levels coincided with substantial time-dependent Cd^{2+} uptake (1-5 h) By comparison, in the presence of glucose, rapid efflux of K^+ was observed within the first hour of contact where Cd^{2+} uptake was greatest Internalisation of Cd^{2+} ions may have been necessary to induce K^+ efflux (Assmann *et al*, 1996) In the absence of glucose, Cd^{2+} internalisation occurred at a slower rate, possibly accounting for absence of substantial K^+ efflux within the first hour of contact By contrast, K^+ release during Mn^{2+} accumulation in the presence of glucose exhibited only small differences compared to controls, even though levels of metal uptake similar to those obtained with Cd^{2+} were observed Sr^{2+} accumulation in the presence of glucose had no effect on extracellular K^+ levels These observation suggest that K^+ release is not related to levels of test ion accumulation, but to their differing toxic effects Cd^{2+} (Gadd and Mowll, 1983, Kessels *et al*, 1987) and Cu^{2+} (Ohsumi *et al*, 1988) ions are known to be capable of increasing plasma membrane permeabilisation, of which K^+ loss is an indicator (Gadd *et al*, 1986) The changes in K^+ profiles induced by Cd^{2+} and Cu^{2+} ions were likely to reflect these toxic effects Concentrations of Cu^{2+} at levels used in the present work, although low, were capable of inducing K^+ loss through membrane permeabilising effects (Avery *et al*, 1996) The minimal effects of Mn^{2+} and Si^{2+} on K^+ efflux in the presence of glucose may be due to their more efficient subcellular sequestration in a non-toxic form In the absence of glucose, K^+ loss observed in the presence of these ions may be due to cellular attempts to maintain ionic balance (Ramos *et al*, 1985) although no stoichiometric relationship was evident here K^+ efflux by yeast in response to metal cations is a complex and poorly understood process which includes both transport and membrane damaging effects Factors which influence K^+ efflux in a given situation may include the

different metal cations used in the study, their concentrations and the microbial strains involved

4.2 3 *Subcellular localisation of accumulated metal ions*

Each of the four ions investigated was compartmented throughout *S cerevisiae* in both the presence and absence of glucose Approximately 70% of cellular Cd^{2+} was localised in the bound fraction, with 20% in the vacuolar fraction at each time point (see Table 3 2 2 1) This partitioning suggested some level of active cellular compartmentation in cells in the absence of glucose, with the lower levels of Cd^{2+} sequestered implying that such a mechanism was operating at a reduced rate compared to cells incubated with glucose The presence or absence of glucose did not affect percentage subcellular Cd^{2+} compartmentation (major part of cellular Cd^{2+} was localised to the bound fraction in both instances) Cd^{2+} binding to solid cellular material appears related to its toxic effects as strains of *S cerevisiae* that possessed some degree of tolerance exhibited reduced Cd^{2+} levels in bound fraction, with increased sequestration to subcellular soluble pools (Joho *et al* , 1985a,b, White and Gadd, 1986) However, it is possible that the reported affinity of Cd^{2+} binding to insoluble cellular material may be an artefact of the fractionation protocol employed as Volesky *et al* (1993) reported that less invasive electron micrographs of Cd^{2+}-loaded cells revealed only vacuolar cadmium phosphate deposits following incubation in the absence of a metabolisable substrate This suggested a metabolically driven Cd^{2+} deposition process as no Cd^{2+} was detected on the cell wall or in the cytosol

In contrast to the observed subcellular Cd^{2+} distribution, Mn^{2+} was predominantly located in the vacuolar fraction (approximately 40-50% of cell Mn^{2+}) with a lower proportion (approximately 35-45%) localised to the bound fraction, both in the presence and absence of glucose (Table 3 2 3 1) Other soluble fractions (wash and cytosolic fraction) contained the remainder of cellular Mn^{2+} and in the absence of glucose, constituted some 25-35% of accumulated cell Mn^{2+} The tendency for Mn^{2+} compartmentation to soluble subcellular fractions may be a consequence of its chemical similarity to Mg^{2+} which is primarily a soluble ion, and its affinity for the Mg^{2+} transport system through which Mn^{2+} ions may be taken up (Jasper and Silver, 1977) Okorokov *et al* (1977) reported that the cell wall and other insoluble cellular material accounted

for 55% of accumulated Mn^{2+}, with approximately 40% and 5% localised in vacuolar and cytosolic pools Mn^{2+} uptake by *Chlorella* species was mostly accounted for cell wall (42%) and other insoluble material (21%), with the remainder located in cytosolic and vacuolar pools (Garnham *et al*, 1992) Similarly, Farcasanu *et al* (1995) observed rapid precipitation of Mn^{2+} ions upon entering the cell, and partitioning in the ratio 60 40 between soluble and bound pools Kihn *et al* (1988) also reported Mn^{2+} precipitation and/or complexation by phosphate moieties in the cytosol, but only approximately 10% of cell Mn^{2+} was determined to be bound by the cell wall The high levels of Mn^{2+} ions present in soluble subcellular fractions underline its important role in yeast metabolism (Loukin and Kung, 1995)

Subcellular Sr^{2+} distribution was markedly influenced by the presence or absence of glucose In the present work, it was observed that when Sr^{2+} loaded cells were washed with distilled, deionised water during sampling prior to fractionation, measured Sr^{2+} levels were considerably lower (Table 3 2 4 1) than those estimated from external Sr^{2+} levels in time course experiments (Figure 3 2 2 1,c) It is likely that Sr^{2+} released by washing of cells represented mobile ions that were taken up into water-filled spaces in the cell wall (Avery and Tobin, 1992) or that cells may possess mechanisms to actively extrude accumulated cellular Sr^{2+} during water-washing prior to fractionation (Nieuwenhuis *et al*, 1981, Theuvenet *et al*, 1986) In the absence of glucose, the bulk of cellular Sr^{2+} was localised in soluble fractions cytoplasmic Sr^{2+} was in excess of vacuolar levels and wash Sr^{2+} constituted some 45-60% of cellular Sr^{2+} (Table 3 2 2 1) Levels of bound Sr^{2+} increased from 15% to 35% between 1 and 5 h By contrast in the presence of glucose, bound Sr^{2+} accounted for 50-68% of accumulated cellular Sr^{2+} and vacuolar Sr^{2+} levels were 4-5 fold higher than in cytosolic pools This suggested more vigorous compartmentation of Sr^{2+} in the presence of glucose with the emphasis on complexation by cellular structural components rather than compartmentation to a soluble pool Other researchers have focused on the soluble nature of subcellular Sr^{2+} (Nieuwenhuis *et al*, 1981, Avery and Tobin, 1992) but did not attempt to characterise bound Sr^{2+} The levels of Sr^{2+} bound by insoluble cellular material in the present study, particularly in the presence of glucose, are consistent with the cellular response of reducing soluble free metal levels by immobilising surplus metal ions in a non-toxic, less inhibitory fashion

Cu^{2+} accumulated during growth was predominantly localised in soluble fractions (less than 25% of cell Cu^{2+} was in the bound fraction) and may have been associated with a number of Cu-complexing molecules such as copper metallothionein, superoxide dismutase or glutathione (Lin *et al*, 1993a) Following further uptake of 5 μM Cu^{2+}, accumulated Cu^{2+} ions were mostly sequestered to the bound fraction (60% of cell Cu^{2+}) within 1 h in the presence or absence of glucose (Table 3 2 5 1) This binding of Cu^{2+} by insoluble cellular material may be indicative of an initial cellular response to rapidly changing extracellular metal levels prior to slower redistribution over an extended time period In the absence of glucose, subcellular Cu^{2+} distribution changed little during further incubation but in the presence of glucose, a shift of cellular Cu^{2+} from bound to vacuolar pools was observed Lin *et al* (1993a) reported that cell wall bound Cu^{2+} was exchangeable and could be mobilised for efflux As the Cu^{2+} translocation pathway was highly specific for Cu^{2+} at low concentrations and an apparent threshold of cellular Cu^{2+} was required before Cu^{2+} entered the cell (Lm *et al*, 1993b), it is possible in the present study that once cell wall Cu^{2+} attained this critical threshold, active transport systems internalised Cu^{2+} ions and sequestered them to the vacuole In the absence of glucose, it would be expected that these transport systems would be operating at a much reduced rate, if at all, which may account for the absence of this shift in Cu^{2+} distribution during prolonged incubation

4.2 4 *Perturbation of subcellular Mg^{2+} and K^{+} distributions*

As reflected by negligible induced Mg^{2+} loss (by all cations tested, except Cu^{2+}), the effects of metal uptake and sequestration on subcellular Mg^{2+} distribution were minimal During Cd^{2+} accumulation, wash Mg^{2+} concentrations were observed to increase with incubation time, particularly in the presence of glucose This was accompanied by decrease in bound Mg^{2+} and may be as a result of displacement of structural Mg^{2+} ions by the high levels of Cd^{2+} present Mn^{2+} binding had no effect, except that small increases in vacuolar Mg^{2+} occurred at the expense of bound Mg^{2+} in the presence of glucose only Similarly, during Sr^{2+} accumulation in the presence of glucose, there was a shift of Mg^{2+} from the vacuolar to the bound fraction These changes may reflect displacement by actively accumulated Mn^{2+} and Sr^{2+} of Mg^{2+} ions from biomolecules as a precursor for their sequestration in these fractions By contrast, increased wash Mg^{2+}

levels were only observed in the absence of glucose during Cu^{2+} accumulation and may be as a result of membrane permeabilising effects of Cu^{2+} (Ohsumi *et al*, 1988) Another study to consider subcellular Mg^{2+} distribution (Avery and Tobin, 1992) observed small decreases in vacuolar Mg^{2+} levels (less than 10% decrease compared with levels prior to metal addition) during Sr^{2+} accumulation These changes were recorded only with actively metabolising cells and correlated with increased vacuolar Sr^{2+} sequestration It appears that cellular Mg^{2+} levels are under tight control, especially when compared with cell K^+ levels (see below) Where appreciable loss of Mg^{2+} occurs, it has been attributed to disintegration of subcellular components, resulting in loss of cellular function (Webster and Gadd, 1996) and viability (Gadd and Mowll, 1983) However, in the present study, little if any, cellular Mg^{2+} release was observed, but perturbation in its subcellular distribution may indicate disruption of normal cellular metabolism by accumulated metal cations

Loss of cellular K^+ by *S cerevisiae* following exposure to heavy metal cations is well documented (Norris and Kelly, 1977, de Rome and Gadd, 1987, Brady and Duncan, 1994b) but few studies have investigated its altered subcellular distribution as a consequence of metal uptake Uptake studies with monovalent Cs^+ and Li^+ have reported direct replacement of vacuolar K^+ in a stoichiometric (1 1) fashion (Avery *et al*, 1993, Perkins and Gadd, 1993a,b) but the effect of divalent cation uptake on intracellular K^+ levels has not been previously reported Prior to addition of test ions, *S cerevisiae* cells possessed high vacuolar concentration of K^+, with relatively little K^+ in the cytosol and a negligible amount bound to insoluble cellular material During accumulation of Mn^{2+}, Sr^{2+} and Cu^{2+}, perturbations of subcellular K^+ were observed in the absence of glucose, whereas during Cd^{2+} uptake, subcellular K^+ levels were affected in both the presence and absence of glucose No changes in K^+ subcellular distribution were recorded in the presence of glucose (uptake of Mn^{2+}, Sr^{2+} and Cu^{2+}), despite the fact that greater levels of metal cations, particularly Mn^{2+} and Sr^{2+} were accumulated This may be as a result of efficient intracellular complexation and/or sequestration of accumulated metal cations in actively metabolising cells by biomolecules specific for that purpose, which rendered them less damaging to cellular structures As the vacuole is the main compartment of K^+ ions in yeast (Okorokov *et al*, 1980, Lichko *et al*, 1980), it was from this pool that the bulk of K^+ loss occurred as a consequence of metal

cation uptake Vacuolar K^+ decreases did not correlate stoichiometrically with vacuolar levels of sequestered metal cations and may have resulted from metal damage to organellar membranes or to systems concerned with maintenance of transmembrane ion gradients In the case of Cd^{2+} uptake, cytosolic K^+ levels decreased by half both in the presence and absence of glucose, in addition to substantial vacuolar loss, which was particularly marked in the presence of glucose It has been reported that Cd^{2+} must accumulate within cells to induce K^+ loss (Gadd and Mowll, 1983, Kessels *et al* , 1987, Assmann *et al* , 1996) and it seems likely that the differential in Cd^{2+} accumulation between cells incubated in the presence and absence of glucose in this work was responsible for the substantial K^+ loss in the piesence of glucose

4.2.5 *Application of hard and soft theory*

The cations used in the present study were chosen as they possessed varying degrees of class A and B character Sr^{2+} is a class A (hard) metal ion, whereas Mn^{2+}, Cd^{2+} and Cu^{2+} are classified as borderline ions (Nieboer and Richardson, 1980) Mn^{2+} is the borderline cation possessing the most class A character, whereas Cd^{2+} and Cu^{2+} are of distinctly greater class B character Covalent index and class B character (softness) increase in the order $Sr^{2+} < Mn^{2+} < Cd^{2+} < Cu^{2+}$, and in genei al, the greater the covalent index and class B character of a metal ion, the higher its potential to form covalent bonds with biological ligands (Brady and Tobin, 1994) On considering the results of accumulation studies of Sr^{2+}, Mn^{2+} and Cd^{2+} (100 µM applied concentration) it was evident that in the absence of glucose, the harder cations Sr^{2+} and Mn^{2+} were accumulated to a lesser extent than the softer ion Cd^{2+} This may be due to a lower proportion of oxygen-containing sites (the preferred binding sites of class A cations) as compared with nitrogen- or sulphur-containing sites (the preferred binding sites of class B cations) on the cell surface As a consequence of reduced surface binding, metal cations transporters (which would already be operating at a ieduced rate) may not have had access to sufficient "substrate" *i e* metal ions, for transport Alteinatively, softer cations such as Cd^{2+}, have been reported to be more toxic than thei hard counterparts (Avery and Tobin, 1993) This may have resulted in exposure of furthei binding sites, through toxic interactions at the cell surface after prolonged incubation, thereby increasing overall accumulation Brady and Tobin (1995) reported a correlation between metal uptake and covalent index

(a measure of class B character) Previous work has correlated metal uptake and ionic radii of cations (Tobin *et al*, 1984), and is pertinent because ionic radius is a factor in the computation of covalent index values

In the presence of glucose, close similarities exist between metal uptake profiles of Mn^{2+} and Cd^{2+} but Sr^{2+} uptake remained less than that of either Cd^{2+} or Mn^{2+} Here, the affinity of each cation for actively functioning transporters may have determined ultimate cellular levels of these ions In addition, efflux systems specific for certain cations may have played an influential role in final levels of accumulation It is possible that other effects such as decreasing metal availability and metabolic effects may have masked the effects of metal chemistry Metal uptake by actively metabolising biomass clearly involves more complex mechanisms than uptake by non-active biomass (Avery and Tobin, 1992,1993) and the impact of metal chemistry is not as clear

Displacement of H^+ ions has been interpreted to indicate covalent bonding, with release of Ca^{2+} and Mg^{2+} ions denoting ionic interactions (Crist *et al*, 1990, Avery and Tobin, 1993, Brady and Tobin, 1995) However, in the present work use of a buffered system precluded estimation of H^+ release In addition, displacement of Mg^{2+} was slight, if present at all, and occurred at the subcellular level mostly in the presence of glucose, suggesting some degree of ionic interaction in certain subcellular pools However, this could not be correlated with class A or B character of accumulated metal ions In this work, K^+ release was greatest during accumulation of the soft ion Cd^{2+} However, the hard ion Sr^{2+} induced K^+ release in excess of that observed during incubation with the borderline ion Mn^{2+} Therefore, no simple correlation linking K^+ loss with the hard or soft nature of applied metal cations was found

Studies that have successfully linked metal chemistry and uptake have primarily been concerned with cation binding at the cell surface (Crist *et al*, 1990, Avery and Tobin, 1992, 1993, Brady and Tobin, 1994, 1995) Intracellular localisation of metal cations is more dependent on a number of cellular transport systems than surface binding effects, and as such is a more complex phenomenon Varying degrees of ionic and covalent interactions may well have occurred prior to transport but do not appear to play a major role during subcellular compartmentation of accumulated metal ions in the present work

4 3 Influence of intracellular and extracellular magnesium concentrations on manganese uptake and toxicity discussion

4.3.1 *Influence of Mn^{2+} on growth of S cerevisiae and the effect of competing cations on Mn^{2+} toxicity*

Mn^{2+} was observed to inhibit cell division at a concentration of 0 5 mM or higher during growth in standard medium 0 1 mM Mn^{2+} had no discernible effect on cell division, although this concentration was considerably higher than the reported optimum (2 - 10 μM Mn^{2+}) for yeast growth and fermentation (Jones and Gadd, 1990) This contrast, and the ability of the present strain to grow normally up to late-exponential phase in the presence of 0 5 mM Mn^{2+}(Figure 3 3 1), may be due to complexation of Mn^{2+} by medium components (e g anionic ligands), or inhibition of Mn^{2+} uptake by cationic species in the medium used here (Singleton and Simmons, 1996, Gadd, 1993) Cell division was not immediately arrested at the higher Mn^{2+} concentrations, as some growth occurred during the initial 3-4 h of incubation It seems that Mn^{2+} toxicity may only be manifested above a certain intracellular threshold concentration, attained as growth progresses The results presented here suggest a threshold of approximately 500-700 nmol Mn^{2+} (10^9 cells)$^{-1}$, although (as discussed below) cellular Mn^{2+} concentration is clearly not the sole determinant of Mn^{2+} toxicity

Medium supplementation with Ca^{2+} and K^+ did not alleviate Mn^{2+} toxicity, indicating that these ions do not compete with Mn^{2+} ions for binding and/or transport in *S cerevisiae* (Figure 3 3 2) The absence of a protective effect of K^+ is consistent with the existence of distinct monovalent and divalent cation transport systems in *S cerevisiae* (Jones and Gadd, 1990) By contrast to the present work, 0 5 mM Ca^{2+} alleviated (but did not entirely eliminate) the toxic effects of 1 0 mM Mn^{2+} on growth and pigment production in the green alga *Kirchneriella lunaris* (Issa *et al* , 1995) Ca^{2+} supplementation (up to 5 0 mM) also reduced the growth inhibitory effects of 1 5 mM Ni^{2+} towards yeast by about 20 % (Joho *et al* , 1991) A ten-fold molar excess of Ca^{2+} (0 5 mM) completely negated the toxic effects of 50 μM Cu^{2+} on yeast and considerably reduced the toxicity of up to 150 μM Cu^{2+} (Karamushka and Gadd, 1994) In the present work, the absence of competitive interactions with Ca^{2+} was unexpected It can be inferred that greatly elevated Ca^{2+} concentrations are required for inhibition of Mn^{2+} uptake in *S cerevisiae*, and that

uptake of Ca^{2+} occurs via pathways having more in common with those for metals such as Ni^{2+} and Cu^{2+} than with Mn^{2+} In contrast to Ca^{2+}, a five-fold excess of Mg^{2+} was sufficient to eliminate the inhibitory effects of Mn^{2+} The protective effect of extracellular Mg^{2+} against Mn^{2+} was consistent with Mg^{2+} protection from several other heavy metal cations reported previously (Gadd and Laurence, 1996), including Cu^{2+} (Karamushka and Gadd, 1994), Ni^{2+} (Joho *et al* , 1991), Cd^{2+} (Kessels *et al* , 1985), Co^{2+} (Norris and Kelly, 1979) and Al^{3+} (MacDiarmuid and Gardner, 1996) In many of these cases, protection was considered to result principally from inhibition (generally competitive) of metal uptake

4.3 2 *Influence of medium Mg-supplementation on cellular Mn levels*

The protective effect of exogenous Mg^{2+} against Mn^{2+} toxicity during growth was simultaneous with a reduction in Mn^{2+} uptake by the cells, suggesting a competitive protection mechanism In the absence of an extracellular Mg-supplement, cellular Mn^{2+} accumulation continued until stationary phase (Figure 3 3 3), while the decline in cellular Mn levels evident after 3 h in Mg-supplemented cultures was likely due to dilution of the initial Mn pool during cell division and possible exchange for extracellular Mg^{2+} It should be noted that Mn^{2+} efflux systems have been reported (Farcasanu *et al*, 1995, Nieuwenhuis *et al* , 1981) and these may be influenced or controlled by cellular Mg levels (as appears to be the case for Mn^{2+} uptake systems, as discussed below) Mg is essential for the stabilisation of cell structures and biomolecules, and is known to facilitate cellular damage-repair mechanisms (*e g* following metal exposure) (Aoyama *et al* , 1986) The marked reduction in Mn^{2+} accumulation in the presence of Mg^{2+} suggests that Mn^{2+} uptake occurred via a relatively low-specificity Mn^{2+} transport system (Gadd and Laurence, 1993) Corroborative evidence is supplied by the fact that Mn^{2+} accumulation can be readily inhibited by other metal cations such as Zn^{2+} (Garnham *et al* , 1992) and Co^{2+} (Parkin and Ross, 1985, Gadd and Laurence, 1996) In *S cerevisiae*, at least two concentration-dependent Mn^{2+} transport systems have been identified, with differing specificities and affinities (Gadd and Laurence, 1996)

4.3.3 *Effect of Mg supplementation on intracellular Mg levels and subsequent* Mn^{2+} *accumulation*

The Mg content of *S cerevisiae* was found to vary over a 5-fold range, and was dependent on the composition of the growth medium and the cells growth stage (Figure 3 3 4) Mg concentrations that support yeast division at half the maximal rate are generally in the micromolar range (Walker, 1994, Jasper and Silver, 1977) This high microbial requirement for Mg^{2+} is reflected in growth-related demands (Walker, 1994), and in this study dilution of the initial cellular Mg pool during early-exponential growth was followed by Mg^{2+} accumulation during the late-exponential and stationary phases During these latter phases, cellular Mg levels were larger than those required to support division (the rate of division did not increase with Mg accumulation) and excess Mg was largely localised in vacuoles, which act as the primary regulators of intracellular ion levels (Lichko *et al*, 1982) The additional Mg accumulated during growth in Mg-supplemented medium was modest compared to the 200 fold increase in extracellular Mg supplied The tight regulation of cellular Mg levels implied by these results corresponds to earlier reports where only a 3-fold variation in Mg content of *S cerevisiae* resulted from a 2,500-fold variation in exogenous Mg (Lichko *et al*, 1982, Okorokov *et al*, 1980)

Cells enriched with Mg displayed lower rates of Mn^{2+} uptake (Figure 3 3 5) This observation does not appear to be related to increased susceptibility of the plasma membranes in unsupplemented cells to permeabilisation followed by passive metal influx (Avery *et al*, 1996) as intracellular Mg^{2+} levels were conserved and the differences in uptake were evident at both toxic (5 mM) and non-toxic (50 μM) Mn^{2+} concentrations The observed K^+ loss at 50 μM Mn^{2+} likely reflects the cells response to maintain ionic balance for accumulated Mn^{2+} (White and Gadd, 1987b, Ramos *et al*, 1995) However, the high level of K^+ release observed with unsupplemented cells (at 5 0 mM Mn^{2+}) (see Figure 3 3 5 2) correlates with high Mn^{2+} accumulation which may have resulted in membrane damage and/or loss of viability (Gadd and Mowll, 1983) Whereas the protection conferred by extracellular Mg^{2+} against metal toxicity has in many previous cases been attributed to direct competition between Mg^{2+} and metal ions for transport (Gadd and Laurence, 1993, Karamushka and Gadd, 1994, Garnham *et al*, 1992, Kessels *et al*, 1985, Bianchi *et al*, 1981, Rothstein *et al*, 1958), here reduced

Mn^{2+} uptake was observed in Mg-enriched cells that had been removed from the Mg-supplemented medium

4.3.4 *Cell surface effects and net Mg^{2+} accumulation*

Surface-effects could also be discounted as a possible explanation as differences in the surface charge or cell-surface Mn^{2+} binding of Mg-enriched cells were only small compared with unsupplemented cultures It is, therefore, probable that the observed differences in Mn^{2+} accumulation between Mg-enriched and unsupplemented cells were related to altered divalent-cation transport activity The maintenance of intracellular Mg^{2+} (or any other essential ion) at a concentration which is optimal for growth and metabolism is dependent on relative rates of uptake and efflux of the ion (Walker, 1994, Jones and Gadd, 1990) Thus, in order to compensate for any change in intracellular Mg^{2+} above or below the optimum, the relative activities of the transport systems would be expected to change accordingly As Mg^{2+} and Mn^{2+} share one or several uptake systems in yeasts (Hughes and Poole, 1989, Jasper and Silver, 1977), it follows that a dependence of Mg^{2+} uptake on intracellular Mg levels will also be reflected in the rate of cellular Mn^{2+} uptake, as was evident here However, it was observed that Mg-supplemented cells possessed higher rates of Mg^{2+} transport compared with unsupplemented cells (Figure 3 3 6), which contrasts with their reduced Mn^{2+} accumulating capacity and it is therefore unlikely that Mn^{2+} accumulation occurs via the Mg^{2+} transporter in this instance But it is nonetheless possible that what ever the nature of transporter responsible for Mn^{2+} accumulation, of which at least two are known (Gadd and Laurence, 1996), it is subject to some level of control by overall cellular Mg levels That these effects are mediated primarily at the plasma membrane is suggested by evidence from other studies that plasmalemma-transport is the rate-limiting step for divalent cation uptake by *S cerevisiae* (Theuvenet *et al* , 1986), although alterations in vacuolar transporters can also result in reduced whole-cell Mn^{2+} accumulation (Bode *et al* , 1995)

4.3.5 *Mn^{2+} toxicity towards unsupplemented and Mg-supplemented cells*

In the present study, Mn^{2+} toxicity has been correlated with initial cellular Mg content (Figure 3 3 8) The relationship between Mn^{2+} uptake and toxicity in *S cerevisiae* was

recently linked by Farcasanu *et al* (1995) to cellular protein phosphatase 2B (PP2B) levels, which appeared to be involved in the regulation of Mn^{2+} uptake Indeed, these researchers speculated that PP2B-mediated Mn^{2+} homeostasis occurred through activation of the enzyme only when cellular Mn reached a certain concentration The present results are the first demonstration of a link between intracellular Mg and the susceptibility of *S cerevisiae* to Mn^{2+} toxicity Previous studies with higher plants have demonstrated the importance of the internal Mg Mn ratio in determining Mn toxicity, although the effect of altered cellular Mg on Mn^{2+} uptake was not investigated (Goss and Carvalho 1992) It is known that elevated cellular Mg exerts a general protective effect on microorganisms against various environmental stresses (Walker 1994) However, in the present study protection against Mn^{2+} has been specifically related to reduced ion uptake by Mg enriched cells

The variation in viability of cells displaying similar intracellular Mn levels is noteworthy (Figure 3 3 9 and Table 3 3 9) The results obtained here suggest that Mn^{2+} toxicity depends directly on the cellular ratio of magnesium to manganese, rather than on the absolute cellular Mn level A study of Mn^{2+} toxicity towards wheat did not identify a critical toxic concentration of Mn^{2+}, but found that growth was reduced when the ratio of manganese to magnesium in the shoots fell below a critical value (Goss and Carvalho 1992) High-magnesium plants also displayed an increased ability to discriminate against Mn^{2+} ions in translocation from roots to shoots In the present study, a possible influence of differences in growth-stage on Mn^{2+} toxicity cannot be discounted, a growth-stage dependence of Co^{2+} accumulation by *S cerevisiae* has been reported (Aoyama *et al*, 1986) However, the good correlation that was obtained only when cellular Mg levels were also taken into account (Fig 3 3 9b) was strongly indicative of a critical role for intracellular competition with Mg^{2+}, independent of any growth stage effects

4 3 6 *Application of Mg-protective effect towards other metal cations*

However, it appears that the observed protective effect of elevated cellular Mg levels was confined to Mn^{2+} ions only Mg-supplemented and unsupplemented cells challenged with a range of mono- and divalent cations did not generally exhibit differences in metal uptake similar to those observed for Mn^{2+} (Figure 3 3 10) Previously, Mg-limited cultures of *Klebsiella pneumoniae* were observed to be more

sensitive to growth inhibition by Co^{2+} and Ni^{2+} ions, but levels of ion uptake were not reported (Ainsworth *et al*, 1980) It is possible that increased cellular Mg levels may have effects that are not related to differences in cation accumulation, such as altered metal tolerances or viability, but these traits were not examined However, differences were observed in relation to Cu^{2+} accumulation by Mg-supplemented and unsupplemented cells (Figure 3 3 11) Mg-supplemented cells accumulated higher levels of Cu^{2+} compared with unsupplemented cultures, which may be related to the previously observed differences in surface charge, Mg-enriched cells were more negatively charged, possibly accounting for their higher Cu^{2+} uptake within the early stages of contact It is also possibly that the altered rates of Mg^{2+} transport between the two cell types (Mg-enriched cell possessed a higher rate of net Mg^{2+} uptake) may have contributed to the observed differences in Cu^{2+} accumulation Addition of exogenous Mg^{2+} ions is known to reduce Cu^{2+} toxicity by competitive and stabilising interactions at the cell surface, as well as through its physiological functions (Karamushka and Gadd, 1994) but in the present work, cellular Mg levels appear to influence levels of Cu^{2+} accumulation

4 4 Development of new protocol and assessment of procedures for extraction of soluble ion pools discussion

4 4.1 *Use of biochemical markers to characterise separation of cytosolic and vacuolar pools*

G-3-PDH and CpY were convenient markers for cytosolic and soluble vacuolar contents respectively Assays were specific, simple to conduct, of relatively short duration and could be used on crude cell extracts (Schekman, 1982, Jones, 1990) Utilising these markers, it was found that DEAE-dextran treatment, whose efficiency and action is crucial for success of the protocol, did not release cytosolic marker enzymes Both cytosolic and vacuolar markers were located in the same fraction following cell disruption with glass beads Little or no activity of either marker was observed in fractions isolated with 60% methanol, presumably due to denaturing effects of the methanol This is in contrast to a number of studies that report release of cytoplasmic contents at DEAE-dextran concentrations similar to those used here, approximately 0 4 mg ml^{-1} (White and Gadd,

1986, Avery and Tobin, 1992, Perkins and Gadd, 1993a b) However, some of these reports contain no reference as to the degree of permeabilisation (Avery and Tobin, 1992, Perkins and Gadd, 1993a,b) whereas White and Gadd (1986) assessed permeabilisation microscopically using uptake of methyl green dye as an indicator of cell permeability In the present study, utilisation of the same dye proved inconclusive, as its uptake by cells was very poor Similarly, DEAE-dextran treated and untreated cells did not stain differentially when treated with methylene blue or trypan blue Theuvenet *et al* (1986) utilised DEAE-dextran concentrations of 3 0 mg ml^{-1} to permeabilise the plasma membrane and release cytosolic ions Vacuolar integrity was assessed by analysis of partial K^+ loss by DEAE-dextran treated cells in the presence or absence of buffered sorbitol In the present work, increasing DEAE-dextran levels up to 10 mg ml^{-1} did not result in any expression of cytosolic marker activity in supernatant fractions In the original protocol, Huber-Walchi and Wiemken (1979) measured arginine as a percentage of released cellular amino acids to quantify separation of cytoplasmic and vacuolar pools This required cells to be grown in medium rich in a range of amino acids including arginine, and the fact that a small amount of arginine is present in the cytoplasm (arginine is not strictly localised to the vacuole), together with some inevitable vacuolar leakage may have led to error in assessing separation of cytosolic and vacuolar fractions (Durr *et al* , 1975) No reports utilising specific marker enzymes were found in the literature

In this work, DEAE-dextran treatment did not permeabilise the plasma membrane to the extent required to release marker enzymes, which calls into question its efficacy in releasing accumulated cytosolic cations which in turn would affect measured vacuolar ion levels Microscopic evidence was provided (Figure 3 4 2) which illustrated a physical lack of cell disruption following treatment with 60% methanol with possible consequences for release of intracellular ion pools, when compared to that observed after treatment with glass beads The loading of cells with Cd^{2+} ions did not influence the partitioning of enzyme activity

Attempts to permeabilise the cell membrane with Triton X-100 and DMF did not meet with success, although these methods had been previously reported to release intracellular contents (Jones, 1990) Physical treatments such as freeze/thawing were not successful but sonication did result in result in release and adequate separation of G-3-PDH activity between a soluble, presumably cytosolic pool and remaining cell debris

However, the lack of vacuolar marker activity in either fraction, possibly as a result of enzyme denaturation during the sonicating procedure did not allow for determination of partitioning between cytosolic and vacuolar pools

4 4 2 *Development of alternative differential extraction technique*

Treatment of spheroplasts with DEAE-dextran was observed to release the bulk of cellular G-3-PDH activity, which proved that DEAE-dextran could disrupt the plasma membrane This, together with earlier observations on the lack of DEAE-dextran permeabilisation of whole cells, suggested that the cell wall acted as a selective permeability barrier (Stratford, 1994) to DEAE-dextran, inhibiting its permeabilising effects The action of the cell wall degrading enzyme Novozyme 234 was only moderately inhibited by cellular loading with metal cations This observation, coupled with the fragility of cells lacking the structural support of a cell wall (Kilis, 1994) suggested that partial removal of the cell wall may enable extraction of the cytosolic pool whilst maintaining a degree of cell mechanical strength to conserve vacuolar integrity It was found that 15-20% conversion of cells to spheroplasts resulted in good partitioning of cytoplasmic and vacuolar pools, as determined from measurement of enzyme activities Metal-loaded cells were subjected to differential extraction of soluble subcellular pools with this method Measurement of enzyme activities confirmed acceptable separation of cytoplasmic and vacuolar pools with at least 70% of each marker enzyme present in the relevant fractions for all ions tested (Tables 3 4 7 1, 3 4 7 2 and 3 4 7 6)

4 4 3 *Comparison of subcellular metal ion distributions obtained by two fractionation protocols*

The biochemical markers used in Method 2 allow for characterisation of the separation of subcellular cytosolic and vacuolar pools Trends in accompanying subcellular metal cation distribution facilitate estimation of the accuracy of Method 1 as a protocol for the differential extraction of subcellular ion pools Comparison of the two methods and interpretation of results is difficult as only certain fractions are directly comparable These are the Cytoplasmic (Method 1) and F 1 (Method 2) fractions and Vacuolar (Method 1) and F 2 (Method 2) fractions The Bound fraction of Method 1 was approximately comparable to the sum of Enz, Wash and Pellet fractions of Method 2 as metal levels in

these fractions of Method 2 were derived mainly from the breakdown of the cell wall, which would be expected to be present in the Bound fraction of Method 1 The Wash (Method 1) and Pre-wash (Method 2) fractions were thought not to be comparable as use of EDTA, a strong chelator of metal ions (Ainsworth *et al*, 1980, Matheickal *et al*, 1997) in the Pre-wash buffer solution in Method 2 may have resulted in removal of some surface bound metal ions that would not otherwise be removed by washing with Tris-MES (Method 1) The objective of this work was to assess the efficacy of Method 1 as a fractionation protocol for isolation of subcellular ion pools by comparison with the newly developed Method 2

As total recovered metal levels are consistently lower with Method 2 than for Method 1, comparison of methods was facilitated by examination of metal levels in each fraction as a percentage of total recovered metal By this process, although discrepancies arise between absolute metal levels, certain trends became apparent which enabled an assessment of the accuracy of each fractionation method In the Sr^{2+} studies, it was evident that cytosolic Sr^{2+} (Cytosolic and F 1 fractions for Methods 1 and 2, respectively) was lower than vacuolar (Vacuolar and F 2 fraction for Methods 1 and 2, respectively) levels with both fractionation methods (Table 3 4 7 3) Similarly with Mn^{2+}, high vacuolar compartmentation (approximately 41% and 34% of total cellular Mn^{2+} for Method 1 and 2, respectively) and similar proportions of recovered Mn^{2+} bound by insoluble cellular material were visible with both Methods 1 and 2 (Table 3 4 7 4) With Cd^{2+} and Cu^{2+} sequestration, it was determined from each fractionation protocol that the bulk of cellular metal was bound by solid cellular material with only small amounts sequestered to the soluble fractions (cellular vacuolar and cytosolic pools) Levels of Cd^{2+} bound by solid cellular material accounted for 72% of cellular Cd^{2+} (Bound fraction) for Method 1, compared with 80% of cellular Cd^{2+} (Pellet fraction) for Method 2 (Table 3 4 7 5) Appreciable levels of cellular Cu^{2+} were present due to low levels of Cu^{2+} in the growth medium (Table 3 4 7 7) Cu^{2+} bound by solid cellular material accounted for the largest portion of growth-accumulated cellular Cu^{2+} 41% for Method 1 (Bound fraction) compared to 60% (Pellet + Pre-wash + Enz fractions) for Method 2 However, there were considerable differences between Cu^{2+} levels in cytosolic and vacuolar pools isolated by each method Following uptake of 5 µM Cu^{2+} (Table 3 4 7 7) it was determined with Method 2 that 72% of total cellular Cu^{2+} was bound to solid cellular material (Pellet + Pre-

wash + Enz fractions) compared with 70% as determined by Method 1 Consequently, only approximately 20-25% of cellular Cu^{2+} was localised to the cytosolic and vacuolar pools as determined by either fractionation method

Method 2 provides evidence as to the level of separation between cytosolic and vacuolar pools making use of specific biochemical markers Despite the fact that Method 1 does not release cytosolic marker enzymes, the similarities between it and Method 2 in terms of trends in subcellular metal localisation patterns suggest that Method 1 provides a good estimation of subcellular metal ion distribution It is possible that some proportion of metal ions isolated in the 60% methanol fraction may originate from the cytosol Indeed, metal ions bound by cytoplasmic proteins comparable in size to G-3-PDH or larger, would not be released by DEAE-dextran permeabilisation, based on evidence presented here DEAE-dextran treatment alone did release some portion of intracellularly accumulated metal ions (heavy metal cations were detected in all Cytoplasmic fractions from Method 1), possibly corresponding to unbound free ions in the cytosol Both methods used in the present study have inherent disadvantages as it is not possible to easily eliminate or quantify binding by cations mobilised in the fractionation procedure to previously unexposed sites (Beveridge *et al*, 1997) Working within these limitations, existing techniques have been combined in a novel fashion to develop a fractionation protocol enabling differential extraction of subcellular ion pools in a manner that can be characterised by reference to biochemical markers Further refinement of the protocol is possible and may indeed be essential for other species of biomass It was concluded that both methods outlined do give an estimation of subcellular compartmentation of accumulated metal ions, and provide researchers with a valuable tool towards investigation of subcellular sites for metal toxicity

CHAPTER 5

CONCLUSION

Accumulation and subcellular localisation of metal cations by Saccharomyces cerevisiae

Ph D Research Thesis

Kevin J Blackwell B.Sc.

CHAPTER 5· CONCLUSION

5.1 Concluding remarks

The experimental environment in which metal uptake studies were conducted had a significant impact on levels of metal cations removed from solution Cd^{2+} removal from solution by live biomass of a brewing strain of *Saccharomyces cerevisiae* was observed to be greater in systems where extracellular pH fluctuations were minimised *i e* in the buffered and pH controlled systems However, these differences were only observed during active metabolic accumulation of metal cations in the presence of glucose, and were attributed to reduced competition effects from extracellular H^+ ions Levels of metal uptake by resting, non-metabolising biomass were not dissimilar between systems, but displayed time-dependent Cd^{2+} removal from solution during the 5 h incubation period, which was likely due to residual metabolic activity as inactivated biomass did not exhibit time-dependent Cd^{2+} removal In general, K^+ release reflected Cd^{2+} uptake, in that K^+ release was greater in systems where Cd^{2+} uptake was high, and cell viability was observed to be least affected during Cd^{2+} accumulation in the buffered system This, coupled with the disadvantages of the unbuffered and pH controlled systems led to utilisation of the buffered system for subsequent metal accumulation studies

The present work is the first study to investigate sequestration of a range of metal cations (which are chemically different in terms of their preference for ionic and/or covalent interactions with ligands) to subcellular compartments in both the presence and absence of glucose The high level of vacuolar compartmentation in the absence of glucose, most notably of Cd^{2+} and Mn^{2+} ions was unexpected and was concluded that cellular ion translocation mechanisms continued to function for some considerable time after harvesting, relying on cellular energy reserves accumulated during growth The subcellular distribution of Cd^{2+} and Mn^{2+} ions was similar in the presence and absence of glucose, with approximately 70% of cell Cd^{2+} bound by insoluble cellular material and the bulk of the remainder (20-25%) in the vacuolar compartment Mn^{2+} was localised predominantly in soluble pools, particularly the vacuolar fractions (35-50%), with less that 45% of cell Mn^{2+} bound by solid cellular material By comparison, the subcellular distribution of Sr^{2+} and Cu^{2+} was influenced by

the presence or absence of glucose Sr^{2+} sequestration in the absence of glucose was mainly to soluble fractions (wash, cytosolic and vacuolar), but the major part of cellular Sr^{2+} in the presence of glucose was associated with the bound fraction (50-70%) Subcellular Cu^{2+} distributions in the presence and absence of glucose were similar after 1 h but there appeared to be a transfer of Cu^{2+} ions from the bound fraction to the vacuolar compartment following prolonged incubation in the presence of glucose

Subcellular Mg^{2+} distributions were unperturbed in the absence of glucose during accumulation of Cd^{2+}, Mn^{2+} and Sr^{2+} ions However, in the presence of glucose, changes in subcellular Mg^{2+} distributions were observed, which were attributed to increased metal cation accumulation Loss of Mg^{2+} from the vacuolar and bound fractions during metal cation accumulation was balanced by Mg^{2+} increases in other cellular compartments as no extracellular Mg^{2+} release was recorded By contrast, Cu^{2+} uptake in the absence of glucose resulted in loss of vacuolar and bound Mg^{2+} to the cytosolic and particularly the wash fractions, but no major changes were observed in the presence of glucose This may be attributed to more efficient sequestration of Cu^{2+} ions in a non-damaging form in the presence of glucose K^+ release was observed in the absence of glucose during accumulation of all four cations and was lost primarily from the vacuolar compartment as it contained the highest levels of cellular K^+ In the presence of glucose, substantial vacuolar K^+ loss occurred only during Cd^{2+} accumulation Uptake of Mn^{2+}, Sr^{2+} and Cu^{2+} did not unduly perturb subcellular K^+ distributions to a similar extent although some changes to K^+ levels in other subcellular compartments was observed

The growth inhibitory effects of Mn^{2+} were alleviated by supplementation with excess Mg^{2+} but not with Ca^{2+} or K^+ Growth in Mg-supplemented medium allowed for manipulation of intracellular Mg levels, and it was observed that Mg-enriched cells accumulated less Mn^{2+} compared with their unsupplemented counterparts This was not attributable to altered surface Mn^{2+}-binding, surface charge or reduced activity of Mg^{2+} transporters Cells possessing low levels of intracellular Mg were more susceptible to Mn^{2+} toxicity which was dependent on the cellular Mg Mn ratio rather than absolute levels of accumulated Mn^{2+} This Mg effect was not observed for a range of other cations except for Cu^{2+} Here, Mg-enriched cells exhibited enhanced Cu^{2+} uptake (in direct contrast to that observed with Mn^{2+}) which may be due to more rapid

accumulation of Cu^{2+} via the Mg^{2+} transporter. In view of the variability in environmental Mg concentration and in the physiological (and thus Mg) status of microorganisms, the present results may be of considerable relevance in assessing manganese toxicity towards microorganisms in natural ecosystems.

A new method of differential extraction of subcellular ion pools was developed with reference to biochemical markers to assess the original fractionation protocol (Method 1). Following partial removal of the cell wall with digestive enzymes, the major part of cytosolic marker activity was released by DEAE-dextran with the bulk of vacuolar marker activity present in the succeeding fraction. Metal-loaded cells were subjected to this partial spheroplasting technique (Method 2), with approximately 70% of cytosolic and vacuolar marker activity localised to the relevant fractions, but differences in levels of metal recovered were observed compared with Method 1 protocol. However, similar trends in subcellular metal localisation patterns were common to each method and it was concluded that both protocols provided a good estimation of subcellular metal cation distributions. It is acknowledged that further fine-tuning of Method 2 is desirable which may lead to increased accuracy in terms of enzyme separation and metal recovered.

5.2 Direction for future work

This study made use of cell fraction techniques that per se cannot eliminate the possibility that metal cations mobilised during the fractionation protocol bound to new sites exposed during the disruption process. Application of non-invasive techniques would be advantageous for the study of subcellular metal cation localisation. Techniques involving scanning and transmission electron microscopy coupled with energy dispersive X-ray microanalysis permit examination of metal sequestration within individual cells. Used in conjunction with image processors, it may be possible to estimate metal deposition rates within various cellular organelles and quantify metal binding moieties.

Refinement of Method 2 could be undertaken in an attempt to further improve the separation of cytosolic and vacuolar pools. Steps such as optimisation of cell wall degradation in terms of percentage conversion to spheroplasts whilst maintaining cell mechanical strength, gentle cell handling and use of different osmolarity buffers may

serve to enhance cytosolic and vacuolar pool separation Removal of EDTA from pre-treatment buffer would also enhance metal levels in cellular compartments This technique could possibly be combined with radioactive metal isotopes to assess and minimise the degree of secondary metal binding during the separation protocol

Further characterisation of the mechanisms regulating Mg^{2+} transport and homeostasis in *S cerevisiae* is required, although currently such research is hampered by the short half-life of magnesium radioisotopes (Walker, 1994) and the unreliability of Mg^{2+} specific fluorescent probes (Morelle *et al* , 1994) With respect to cations other than Mn^{2+}, it is possible that differences in metal uptake between Mg-supplemented and unsupplemented cells may not be as comprehensive an indicator of protective effects and other indicators such as cell viabilities should be investigated Such a study would provide and overview of whether or not cellular Mg status has a bearing on metal toxicity in general

REFERENCES

Ainsworth, M.A., Tompsett, C P , Dean, A.C. (1980) Cobalt and nickel sensitivity and tolerance in *Klebsiella pneumoniae* Microbios, **27**, 175-184

Aksu, Z., Kutsal, T (1987) The usage of *Chlorella vulgaris* in waste water treatment containing heavy metal ions Proc 4th Eur Conf Biotechnol **2**, 80-83

Akthar, N., Sastry, S., Mohan, P (1995) Biosorption of silver ions by processed *Aspergillus niger* biomass Biotech Lett **17**, 551-556

Alexandre, H , Berlot, J P , Charpentier, C (1994) Effect of ethanol on membrane fluidity of protoplasts from *Saccharomyces cerevisiae* and *Kloeckera apiculata* grown with or without ethanol, measured by fluorescence anisotropy Biotech Tech **8**, 295-300

Aoyama, I., Kudo, A., Veliky, I A (1986) Effect of cobalt-magnesium interactions on growth of *Saccharomyces cerevisiae* Tox Assess **1**, 211-226

Assmann, S., Sigler, K., Hofer, M (1996) Cd^{2+}-induced damage to yeast plasma membrane and its alleviation by Zn^{2+} studies on *Schizosaccharomyces pombe* cells and reconstituted plasma membrane vesicles Arch Microbiol **165**, 279-284

Avery, S.V., Codd, G A , Gadd, G M (1992a) Replacement of cellular potassium by caesium in *Chlorella emersonii* differential sensitivity of photoautotrophic and chemoheterotrophic growth J Gen Microbiol **138**, 69-76

Avery, S.V., Codd, G A , Gadd, G.M (1992b) Caesium transport in the cyanobacterium *Anabaena variabilis* Kinetics and evidence for uptake via ammonium transport system(s) FEMS Micro Lett **95**, 253-258

Avery, S V., Codd, G A., Gadd, G M (1993) Transport kinetics, cation inhibition and intracellular location of accumulated caesium in the green microalga *Chlorella salina* J Gen Micro **139**, 827-834

Avery, S.V., Howlett, N G , Radice, S (1996) Copper toxicity towards *Saccharomyces cerevisiae* dependence on plasma-membrane fatty acid composition Appl Environ Microbiol **62**, 3960-3966

Avery, S.V , Tobin, J M. (1993) Mechanisms of adsorption of hard and soft metal ions to *Saccharomyces cerevisiae* and influence of hard and soft anions Appl Environ Microbiol **59**, 2851-2856

Avery, S.V., Tobin, J M. (1992) Mechanisms of strontium uptake by laboratory and brewing strains of *Saccharomyces cerevisiae* Appl Environ Microbiol **58**, 3883-3889

Azab, M S , Peterson, P.J , Young, T.W.K. (1990) Uptake of cadmium by fungal biomass Microbios, **62**, 23-28

Baes, C.F., Mesner, R E (1976) The hydrolysis of cations John Wiley and Sons Inc, New York

Belde, P.J.M., Kessels, B G F , Moelans, I.M., Borst-Pauwels, G W F H (1988) Cd^{2+} uptake, Cd^{2+} binding and loss of K^{+} by a Cd-sensitive and a Cd-resistant strain of *Saccharomyces cerevisiae* FEMS Microbiol Lett **49**, 493-498

Beveridge, T., Hughes, M., Lee, H., Leung, K., Poole, R , Savvaidis, I , Silver, S , Trevors, J. (1997) Metal-microbe interactions contemporary approaches Adv Micro Physiol **38**, 177-243

Bianchi, M.E., Carbone, M L , Lucchini, G (1981) Mn^{2+} and Mg^{2+} uptake in Mn-sensitive and Mn-resistant yeast strains Plant Sci Lett **22**, 345-352

Blackwell, K.J., Singleton, I , Tobin, J M. (1995) Metal cation uptake by yeast a review Appl Microbiol Biotechnol **43**, 579-584

Blackwell, K J , Tobin, J.M (1996) The influence of buffering on Cd^{2+} uptake by *Saccharomyces cerevisiae* Abstracts from the proceedings of the Fifth Environmental Researchers Colloquium, University College Cork, January 1995 Biology and Environment, **95B**, 123-163

Blackwell, K.J., Tobin, J.M., Avery, S.V (1997) Manganese uptake and toxicity in magnesium-supplemented and unsupplemented *Saccharomyces cerevisiae* Appl Microbiol Biotechnol **47**, 180-184

Blackwell, K.J., Avery, S V , Tobin, J M (1998) Manganese toxicity towards *Saccharomyces cerevisiae* Dependence on intracellular and extracellular magnesium concentrations Appl Microbiol Biotechnol **49**, 751-757

Bode, H.P., Dumschat, M., Garotti S., Fuhrmann, F. (1995) Iron sequestration by the yeast vacuole A study with vacuolar mutants of *Saccharomyces cerevisiae* Eur J Biochem **228**, 337-342

Borbolla, M., Pena, A , Pardo, J P (1985) Characteristics of Ca^{2+} transport in yeast *In* I S Kulaev, E A Dawes, and D W Tempest (eds) Environmental regulation of microbial metabolism, Academic Press, London

Borst-Pauwels, G. (1981) Ion transport in yeast Biochim Biophys Acta **650**, 88-127

Borst-Pauwels, G., Severens, P. (1984) Effect of the surface potential upon ion selectivity found in competitive inhibition of divalent cation uptake A theoretical approach Physiol Plant **60**, 86-91

Borst-Pauwels, G., Theuvenet, A (1984) Apparent saturation kinetics of divalent cation uptake in yeast caused by a reduction in the surface potential Biochim Biophys Acta, **771**, 171-176

Brady, D., Duncan, J.R (1994a) Bioaccumulation of metal cations by *Saccharomyces cerevisiae* Appl Microbiol Biotechnol **41**, 149-154

Brady, D., Duncan, J R. (1994b) Cation loss during accumulation of heavy metal cations by *Saccharomyces cerevisiae* Biotech Lett **10**, 543-548

Brady, D., Duncan, J.R. (1994c) Binding of heavy metals by the cell walls of *Saccharomyces cerevisiae* Enzyme Microb Technol **16**, 633-638

Brady, D , Galum, D , Duncan, J R (1994a) Copper tolerance in *Saccharomyces cerevisiae* Lett Appl Micro **18**, 245-250

Brady, D , Rose, P D , Duncan, J R (1994b) The use of hollow fiber cross-flow microfiltration in bioaccumulation and continuous removal of heavy metals from solution by *Saccharomyces cerevisiae* Biotechnol Bioeng **44**, 1362-1366

Brady, D , Stoll, A.D., Starke, L , Duncan, J.R. (1994c) Chemical and enzymatic extraction of heavy metal binding polymers from isolated cell walls of *Saccharomyces cerevisiae* Biotechnol Bioeng **44**, 297-302

Brady, J.M., Tobin, J M (1994) Adsorption of metal ions by *Rhizopus arrhizus* biomass characterisation studies Enzyme Microb Technol **16**, 671-675

Brady, J.M., Tobin, J M (1995) Binding of hard and soft metal ions to *Rhizopus arrhizus* biomass Enzyme Microb Technol **17**, 791-796

Brennan, R J , Schiestl, R H (1996) Cadmium is an inducer of oxidative stress in yeast Mut Res **356**, 171-178

Butt, T., Ecker, D (1987) Yeast metallothionein and applications in biotechnology Micro Revs **51**, 351-364

Cabral, J P.S (1992) Limitations of the use of an ion-selective electrode in the study of the uptake of Cu^{2+} by *Pseudomonas syringae* cells J Microbiol Methods, **16**, 149-156

Capasso, C , Nazzaro, F , Marulli, F , Capasso, A , La Cara, F , Parisi, E (1996) Identification of high-molecular weight cadmium-binding proteins in copper-resistant *Bacillus acidocaldarius* cells Res Microbiol **147**, 287-296

Cervantes, C , Corona, F G (1994) Copper resistance mechanisms in bacteria and fungi FEMS Micro Revs **14**, 121-138

Collins, Y., Stotzky, G. (1989) Factors affecting the toxicity of heavy metals to microbes *In* Beveridge, T , Doyle, **R** (eds) Metal Ions and Bacteria, John Wiley and Sons

Chaney, R.L , Malik, M , Li, Y M , Brown, S L , Brewer, E P., Angle, J S., Baker A. (1997) Phytoremediation of soil metals Curr Opin Biotechnol **8**, 279-284

Crist, R.H , Martin, J.P., Guptill, P W., Eslinger, J.M (1990) Interactions of metals and protons with algae 2 Ion exchange in adsorption and metal displacement by protons Environ Sci Technol **24**, 337-342

Cunningham, K , Fink, G (1994) Ca^{2+} transport in *Saccharomyces cerevisiae* J Exp Biol **196**, 157-166

Davidova, E.G , Kasparova, S G. (1992) Adsorption of metals by yeast cell walls Translation from Mikrobiologiya, **61**, 1018-1022

de Rome, L , Gadd, G.M (1987) Measurement of copper uptake in *Saccharomyces cerevisiae* using a Cu^{2+}- selective electrode FEMS Microbiol Lett **43**, 283-287

Dedyukhina, E G , Eroshin, V K (1991) Essential metal ions in the control of microbial metabolism Process Biochem **26**, 31-37

Durr, M., Boller, T., Wiemken, A (1975) Polybase induced lysis of yeast spheroplasts A new gentle method for preparation of vacuoles Arch Microbiol **105**, 319-327

Eccles, H. (1995) Removal of heavy metal from effluent streams-why select a biological process Inter Biodeterior Biodegrad 5-16

Enari, T M. (1977) European Brewery Convention, Analytica Microbiologica J Inst Brew **83**, 109-118

Engl, A., Kunz, B. (1995) Biosorption of heavy metals by *Saccharomyces cerevisiae* effects of nutrient conditions J Chem Tech Biotechnol **63**, 257-261

Enzminger, J D (1991) Metal finishing and processing Research Journal Water Pollution Control Federation, **63**, 489-494

Failla, M.L., Benedict, C.D., Weinberg, E D (1976) Accumulation and storage of Zn^{2+} by *Candida utilis* J Gen Microbiol **94**, 23-36

Farcasanu, I C , Hirata, D., Tsuchiya, E , Nishiyama, F , Miyakawa, T (1995) Protein phosphatase 2B of *Saccharomyces cerevisiae* is required for tolerance to manganese in blocking the entry of ions into the cells Eur J Biochem **232**, 712-717

Farcasanu, I , Ohta, N , Miyakawa, T (1996) The fate of Mn^{2+} ions inside *Saccharomyces cerevisiae* cells seen by electron paramagnetic resonance Biosci Biotech Biochem **60**, 468-471

Fodor, E., Szabo-Nagy, A., Erdei, L. (1995) The effects of cadmium on the fluidity and H^+-ATPase activity of plasma membrane from sunflower and wheat roots. J. Plant Physiol. **147**, 87-92.

Ford, T., Mitchell, R. (1992) Microbial transport of toxic metals. *In*: Environmental Microbiology, Wiley-Liss Inc. 83-101.

Fourest, E., Roux, J.C. (1992) Heavy metal biosorption by fungal mycelial by-products: mechanisms and influence of pH. Appl. Microbiol. Biotechnol. **37**, 399-403.

Fuhrmann, G.F., Rothstein, A. (1968) The transport of Zn^{2+}, Co^{2+} and Ni^{2+} into yeast cells. Biochim. Biophys. Acta, **163**, 325-330.

Gadd, G.M. (1990a) Biosorption. Chem. Ind. **13**, 421-426.

Gadd, G. M. (1990b) Heavy metal accumulation by bacteria and other microorganisms. Experientia, **46**, 834-840.

Gadd, G.M. (1992) Metals and microorganisms: a problem of definition. FEMS Microbiol. Lett. **100**, 197-204.

Gadd, G M. (1993) Interaction of fungi with toxic metals. New Phytol. **124**, 25-60.

Gadd, G.M., Laurence, O.S. (1996) Demonstration of high-affinity Mn^{2+} uptake in *Saccharomyces cerevisiae*: specificity and kinetics. Microbiol. **142**, 1159-1167.

Gadd, G.M., Mowll, J.L. (1983) The relationship between cadmium uptake, potassium release and viability in *Saccharomyces cerevisiae*. FEMS Micro. Lett. **16**, 45-48.

Gadd, G.M., Mowll, J.L. (1985) Copper uptake by yeast-like cells, hyphae, and chlamydospores of *Aureobasidium pullulans*. Exp. Mycol. **9**, 230-240.

Gadd, G M., Mowll, J.L , White, C , Newby, P J (1986) Methods for assessment of heavy metal toxicity towards fungi and yeasts Tox Assess **1**, 169-185

Gadd, G.M., Stewart, A , White, C , Mowll, J L (1984) Copper uptake by whole cells and protoplasts of a wild type and copper-resistant strain of *Saccharomyces cerevisiae* FEMS Micro Lett **24**, 231-234

Gadd, G M , White, C (1985) Copper uptake by *Penicillium ochro-chloron* Influence of pH on toxicity and demonstration of energy-dependent copper influx using protoplasts J Gen Microbiol **131**, 1875-1879

Gadd, G M , White, C. (1989) Uptake and intracellular compartmentation of thorium in *Saccharomyces cerevisiae* Environ Poll **61**, 187-197

Garnham, G.W., Codd, G A., Gadd, G M (1992) Kinetics of uptake and intracellular location of cobalt, manganese and zinc in the estuarine green alga *Chlorella salina* Appl Microbiol Biotechnol **37**, 270-276

Geddie, J.L., Sutherland, I.W. (1993) Uptake of metals by bacterial polysaccharides J Appl Bacteriol **74**, 467-472

Golab, Z., Breitenbach, M (1995) Sites of copper binding in *Streptomyces pilosus* Water Air Soil Poll **82**, 713-721

Good, N.E , Winget, G.D , Winter, W , Connolly, T N , Izawa, S , Singh, R M M (1966) Hydrogen buffers for biological research Biochemistry, **5**, 467-474

Gooday, G.W. (1993) Cell envelope diversity and dynamics in yeasts and filamentous fungi J Appl Bactriol Symposium Supplement, **74**, 12-20

Goss, M.J., Carvalho, M J (1992) Manganese toxicity the significance of magnesium for the sensitivity of wheat plants Plant and Soil, **139**, 91-98

Gruzina, T G , Balakina, M N , Karamushka, V I , Stepura, L G , Ul'berg, Z R (1997) The bacterial plasma membrane ATPase in assessment of heavy metal toxicity Microbiol **66**, 8-11

Hafez, N., Abdel-Razek, A S , Hafez, M B (1997) Accumulation of some heavy metals on *Aspergillus flavus* J Chem , Tech Biotechnol **68**, 19-22

Huang, C , Huang, P. (1996) Application of *Aspergillus oryzae* and *Rhizopus oryzae* for Cu(II) removal Wat Res **30**, 1985-1990

Huang, C., Huang, P., Morehart, A. (1990) The removal of Cu(II) from dilute aqueous solutions by *Saccharomyces cerevisiae* Wat Res **24**, 433-439

Huber-Walchi, V., Wiemken, A. (1979) Differential extraction of soluble pools from the cytosol and the vacuoles of yeast (*Candida utilis*) using DEAE-dextran Arch Microbiol **120**, 141-149

Hughes, M.N., Poole, R K (1989) Metals and micro-organisms Chapman and Hall, London

Hughes, M N., Poole, R.K (1991) Metal speciation and microbial growth - the hard (and soft) facts J Gen Microbiol **137**, 725-734

Issa, A.A., Abdel-Basset, R , Adam, M S. (1995) Abolition of heavy metal toxicity on *Kirchneriella lunaris* (Chlorophyta) by calcium Ann Bot **75**, 189-192

Jamieson, D J. (1995) The effect of oxidative stress on *Saccharomyces cerevisiae* Redox Report, **1**, 89-95

Jasper, P , Silver, S (1977) Magnesium transport in microorganisms *In* Weinberg, E D (ed) Microorganisms and Minerals, Marcel Dekker, New York, pp 7-47

Joho, M., Imai, M., Murayama, T (1985a) Different distributions of Cd^{2+} between Cd-sensitive and Cd-resistant strains of *Saccharomyces cerevisiae* J Gen Microbiol **131**, 53-56

Joho, M , Fujioka, Y., Murayama, T (1985b) Further studies on the subcellular distribution of Cd^{2+} in Cd-sensitive and Cd-resistant strains of *Saccharomyces cerevisiae* J Gen Micro **131**, 3185-3191

Joho, M., Sukenobu, Y , Egashira, E , Murayama, T. (1983) The correlation between Cd^{2+} sensitivity and Cd^{2+} uptake in the strains of *Saccharomyces cerevisiae* Plant Cell Physiol **24**, 389-394

Joho, M., Tarumi, K , Inouhe, M , Tohoyama, H , Murayama, T (1991) Co^{2+} and Ni^{2+} resistance in *Saccharomyces cerevisiae* associated with a reduction in the accumulation of Mg^{2+} Microbios **67**, 177-186

Jones, E.W (1990) Vacuolar proteases in yeast *Saccharomyces cerevisiae In* Goeddel, D V (ed) Gene expression technology, Methods in Enzymology, Volume 185, Academic Press

Jones, R P , Gadd, G M. (1990) Ionic nutrition of yeast - physiological mechanisms involved and implications for biotechnology Enzyme Micro Technol **12**, 1-17

Junghans, K , Straube, G (1991) Biosorption of copper by yeasts Biol Metals, **4**, 233-237

Kanazawa, S., Mori, K. (1996) Isolation of cadmium-resistant bacteria and their resistance mechanisms Soil Sci Plant Nutr **42**, 731-736

Kaplan, D , Heimer, Y , Abeliovich, A , Goldsbough, P (1995) Cadmium toxicity and resistance in *Chlorella* sp Plant Sci **109**, 129-137

Karamushka, V.I., Gadd, G.M. (1994) Influence of copper on proton efflux from *Saccharomyces cerevisiae* and the protective effect of calcium and magnesium. FEMS Microbiol. Lett. **122**, 33-38.

Karamushka, V.I., Sayer, J.A., Gadd, G.M. (1996) Inhibition of H^+ efflux from *Saccharomyces cerevisiae* by insoluble metal phosphates and protection by calcium and magnesium: inhibitory effects a result of soluble metal cations. Mycol. Res. **100**, 707-713.

Karna, R.R., Sajani, L.S., Mohan, P.M. (1996) Bioaccumulation and biosorption of Co^{2+} by *Neurospora crassa*. Biotech. Lett. **18**, 1205-1208.

Kessels, B., Belde, P., Borst-Pauwels, G. (1985) Protection of *Saccharomyces cerevisiae* against Cd^{2+} toxicity by Ca^{2+}. J. Gen. Microbiol. **131**, 2533-2537.

Kessels, B., Theuvenet, A., Peters, P., Dobbelmann, J., Borst-Pauwels, G. (1987) Changes in ^{45}Ca and ^{109}CD uptake, membrane potential and cell pH in *Saccharomyces cerevisiae* provoked by Cd^{2+}. J. Gen. Micro. **133**, 843-848.

Kihn, J.C., Dassargues, C.M., Mestdagh, M.M. (1988) Preliminary ESR study of Mn(II) retention by the yeast *Saccharomyces*. Can. J. Microbiol. **34**, 1230-1234.

Klis, F.M. (1994) Cell wall assembly in yeast. Yeast, **10**, 851-869.

Koch, U., Glatzle, D., Ringenbach, F., Dunz, T., Steger-Hartmann, T., Wagner, E. (1995) Measurement of ion leakage from plant cells in response to aquatic pollutants. Bull. Environ. Contam. Toxicol. **54**, 606-613.

Kramer, U., Cotter-Howells, J.D., Charnock, J.M., Baker, A., Smith, A. (1996) Free histidine as a metal chelator in plants that accumulate nickel. Nature, **379**, 635-638.

Krebbs, E G (1955) Glyceraldehyde-3-phosphate dehydrogenase from yeast *In* Methods in Enzymology, Volume 1, Academic Press

Kujan, P., Votruba, J., Kamenik, V (1995) Substrate dependent bioaccumulation of cadmium by growing yeast *Candida utilis* Folia Microbiol **40**, 288-292

Lapinskas, P J , Lin, S.J., Culotta, V C (1996) The role of the *Saccharomyces cerevisiae* CCC1 gene in the homeostasis of manganese ions Mol Microbiol **21**, 519-528

Lichko, L.P., Okorokov, L A , Kulaev, I S (1980) Role of vacuolar ion pool in *Saccharomyces carlsbergensis* potassium efflux from vacuoles is coupled with manganese or magnesium influx J Bacteriol **144**, 666-671

Lichko, L.P., Okorokov, L A., Kulaev, I S. (1982) Participation of vacuoles in regulation of levels of K^+, Mg^{2+} and orthophosphate ions in cytoplasm of the yeast *Saccharomyces cerevisiae* Arch Microbiol **132**, 289-293

Lin, C M , Crawford, B F , Kosman, D.J (1993a) Distribution of ^{64}Cu in *Saccharomyces cerevisiae* cellular locale and metabolism J Gen Micro **139**, 1605-1615

Lin, C.M., Crawford, B F , Kosman, D J. (1993b) Distribution of ^{64}Cu in *Saccharomyces cerevisiae* kinetic analyses of partitioning J Gen Micro **139**, 1617-1626

Loaec, M , Olier, R , Guezennec, J (1997) Uptake of lead, cadmium and zinc by a novel bacterial exoploysaccharide Wat Res **31**, 1171-1179

Loukin, S , Kung, C. (1995) Manganese effectively supports yeast cell-cycle progression in place of calcium J Cell Biol **131** 1025-1037

MacDiarmid, C W , Gardner, R.C (1996) Al toxicity in yeast A role for Mg Plant Physiol **112**, 1101-1109

Matheickal, J.T., Yu, Q , Feltham, J (1997) Cu(II) binding by *E radiata* biomaterial **18**, 25-34

McMurrough, I., Rose, A H. (1967) Effect of growth rate and substrate limitation on the composition and structure of the cell wall of *Saccharomyces cerevisiae* Biochem J **105**, 189-203

Mehlhorn, R J (1986) The interaction of inorganic species with biomembranes In Bernhard, M , Brinckman, F , Sadler, P (eds) The importance of chemical speciation in environmental processes Springer-Verlag, Berlin

Meikle, A J , Gadd, G.M , Reed, R H. (1990) Manipulation of yeast for transport studies critical assessment of cultural and experimental procedures Enzyme Microb Technol **12**, 865-872

Moradas-Ferreira, P , Costa, V , Piper, P , Mager, W. (1996) The molecular defences against reactive oxygen species in yeast Mol Micro **19**, 651-658

Morelle, B., Salmon, J S , Vigo, J , Viallet, P (1994) Are intracellular ionic concentrations accessible using fluorescent probes? The example of Mag-indo-1 Cell Biol Toxicol **10**, 339-344

Mowll, J.L , Gadd, G M. (1983) Zinc uptake and toxicity in the yeasts *Sporobolomyces roseus* and *Saccharomyces cerevisiae* J Gen Micro **129**, 3421-3425

Mowll, J.L., Gadd, G M. (1984) Cadmium uptake by *Aureobasidium pullulans* J Gen Micro **130**, 279-284

Murray, A.D., Kidby, D.K. (1975) Sub-cellular location of mercury in yeast grown in the presence of mercuric chloride J Gen Micro **86**, 66-74

Nakajima, A , Sakaguchi, T (1993) Accumulation of uranium by basidiomycetes Appl Microbiol Biotechnol **38**, 574-578

Nieboer, E , Richardson, D H S (1980) The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions Environ Pollut Ser B **1**, 3-26

Nies, D H , Silver, S. (1995) Ion efflux systems involved in bacterial metal resistances J Ind Micro **14**, 186-199

Nieuwenhuis, B , Weijers, C., Borst-Pauwels, G (1981) Uptake and accumulation of Mn^{2+} and Sr^{2+} in *Saccharomyces cerevisiae* Biochim Biophys Acta, **649**, 83-88

Nisbet, E.G., Fowler, C.M. (1995) Is metal disposal toxic to deep oceans? Nature, **375**, 715-716

Norris, P R., Kelly, D P. (1977) Accumulation of cadmium and cobalt by *Saccharomyces cerevisiae* J Gen Micro **99**, 317-324

Norris, P.R , Kelly, D P (1979) Accumulation of metals by bacteria and yeasts Developments in Industrial Microbiology, **20**, 299-308

Ohsumi, Y , Kitamoto, K., Anraku, Y (1988) Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion J Bacteriol **170**, 2676-2682

Okorokov, L.A. (1985) Main mechanisms of ion transport and regulation of ion concentration in the yeast cytoplasm *In* Kulaev, I S , Dawes, E A , Tempest, D W (eds) Environmental regulation of microbial metabolism Academic Press

Okorokov, L.A., Kulakovskaya, T.V., Lichko, L.P., Polorotova, E.V. (1985) H^+/ion antiport as the principle mechanism of transport systems in the vacuolar membrane of the yeast *Saccharomyces carlsbergensis*. FEBS Lett. **192**, 303-306.

Okorokov, L.A., Lichko, L.P., Kadomtseva, V.M., Kholodenko, V.P., Totovsky, V.T., Kulaev, I.S. (1977) Energy-dependent transport of manganese into yeast cells and distribution of accumulated ions. Eur. J. Biochem. **75**, 373-377.

Okorokov, L.A., Lichko, L.P., Kulaev, I.S. (1980) Vacuoles: main compartments of potassium, magnesium and phosphate in *Saccharomyces carlsbergensis* cells. J. Bacteriol. **144**, 661-665.

Okorokov, L., Lichko, L., Andreeva, N. (1983) Changes of ATP, polyphosphate and K^+ contents in *Saccharomyces carlsbergensis* during uptake of Mn^{2+} and glucose. Biochem. Inter. **6**, 481-488.

Omar, N.B., Merroun, M.L., Gonzalez-Munoz, M.T., Arias, J.M. (1996) Brewery yeast as a biosorbent for uranium. J. Appl. Bacteriol. **81**, 283-287.

Ono, B., Ohue, H., Ishihara, F. (1988) Role of cell wall in *Saccharomyces cerevisiae* mutants resistant to Hg^{2+}. J. Bacteriol. **170**, 5877-5882.

Parkin, M.J., Ross, I.S. (1985) Uptake of copper and manganese by the yeast *Candida utilis*. Microbios Lett. **29**, 115-120.

Pearson, R.G. (1963) Hard and soft acids and bases. J. Am. Chem. Soc. **85**, 3533-3539.

Perkins, J., Gadd, G.M. (1993a) Accumulation and intracellular compartmentation of lithium ions in *Saccharomyces cerevisiae*. FEMS Micro. Lett. **107**, 255-260.

Perkins, J., Gadd, G.M. (1993b) Caesium toxicity, accumulation and intracellular localisation in yeasts. Mycol. Res. **97**, 717-724.

Pierce, J S. (1971) Recommended methods of analysis J Inst Brew **77**, 181-227

Ramos, S , de la Pena, P , Valle, E (1985) Coupling of protons and potassium gradients in yeast *In* Kulaev, I S , Dawes, E A , Tempest, D W (eds) Environmental regulation of microbial metabolism Academic Press, London

Rapoport, A L., Beker, M E (1985) Changes in the surface charge of yeast cells during their dehydration and rehydration Translated from Mikrobiologiya, **54**, 450-453

Raskin, I., Smith, R D , Salt, D E (1997) Phytoremediation of metals using plants to remove pollutants from the environment Curr Opin Biotechnol **8**, 221-226

Remacle, J (1990) The cell wall and metal binding *In* Volesky, B (ed) Biosorption of Heavy Metals, CRC Press, Boca Raton

Riordan, C , Bustard, M , Putt, R , McHale, A P (1997) Removal of uranium from solution using residual brewery yeast combined biosorption and precipitation Biotech Lett **19**, 385-387

Roomans, G., Theuvenet, A , Van Den Berg, T , Borst-Pauwels, G (1979) Kinetics of Ca^{2+} and Sr^{2+} uptake by yeast Effects of pH, cations and phosphate Biochim Biophys Acta, **551**, 187-196

Rose, A., Veazey, F.J. (1992) Membranes and lipids of yeast *In* Campbell, I , Duffus, J H (eds) Yeast, a pratical approach IRL Press, Oxford

Ross, I S (1977) Effect of glucose on copper uptake and toxicity in *Saccharomyces cerevisiae* Trans Br Mycol Soc **69**, 77-81

Rothstein, A , Hayes, A D (1956) The relationship of the cell surface to metabolism XIII The cation-binding properties of the yeast cell surface Arch Biochem Biophys **63**, 87-99

Rothstein, A , Hayes, A., Jennings, D , Hooper, D (1958) The active transport of Mg^{2+} and Mn^{2+} into the yeast cell J Gen Physiol **41**, 585-594

Sag, Y., Kutsal, T (1996a) The selective biosorption of chromium (VI) and copper (II) ions from binary metal mixtures by *R arrhizus* Process Biochem **31**, 561-572

Sag, Y , Kutsal, T. (1996b) Fully competitive biosorption of chromium (VI) and iron (III) ions from binary metal mixtures by *R arrhizus* use of the competitive langmuir model Process Biochem **31**, 573-585

Sag, Y , Ozer, D., Kutsal, T (1995) A comparative study of the biosorption of lead (II) ions to *Z ramigera* and *R arrhizus* Process Biochem **30**, 169-174

Schekman, R (1982) Biochemical markers for yeast organelles *In* Strathern, J N , Jones, E W , Broach, J R (eds) The molecular biology of the yeast *Saccharomyces* Cold Spring Harbor Laboratory

Scott, J.A., Palmer, S J. (1990) Sites for cadmium uptake in bacteria Appl Microbiol Biotechnol **33**, 221-225

Silver, S., Phung, L.T. (1996) Bacterial heavy metal resistance new surprises Annu Rev Microbiol **50**, 753-789

Simmons, P , Singleton, I. (1996) A method to increase silver biosorption by an industrial strain of *Saccharomyces cerevisiae* Appl Microbiol Biotechnol **45**, 278-285

Simmons, P, Tobin, J M, Singleton, I. (1995) Considerations on the use of commercially available yeast biomass for the treatment of metal-containing effluents J Ind Microbiol **14**, 240-246

Singleton, I., Simmons, P. (1996) Factors affecting silver biosorption by an industrial strain of *Saccharomyces cerevisiae* J Chem Tech Biotechnol **65**, 21-28

Sloof J., Viragh, A, Van Der Veer, B (1995) Kinetics of cadmium uptake by green algae Water Air Soil Poll **83**, 105-122

Spears, D.R, Vincent, J B (1997) Copper binding and release by immobilised transferrin a new approach to heavy metal removal and recovery Biotechnol Bioeng **53**, 1-9

Starling, A P, Ross, I.S. (1990) Uptake of manganese by *Penicillium notatum* Microbios, **63**, 93-100

Stohs, S.J, Bagchi, D (1995) Oxidative mechanisms in the toxicity of metal ions Free Radical Biology and Medicine, **18**, 321-336

Stoll, A, Duncan, J R (1996) Enhanced heavy metal removal from waste water by viable, glucose pretreated *Saccharomyces cerevisiae* cells Biotech Lett **18**, 1209-1212

Stoll, A., Duncan, J.R (1997) Comparison of heavy metal sorptive properties of three types of immobilised, non-viable *Saccharomyces cerevisiae* biomass Process Biochem **32**, 467-472

Stranberg, G.W., Schumate, S.E., Parrott, J.R. (1981) Microbial cells as biosorbents for heavy metals accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa* Appl Environ Micro **41**, 237-245

Stratford, M (1994) Another brick in the wall? Recent developments concerning the yeast cell envelope Yeast, **10**, 1741-1752

Suh, J H , Kim, D.S , Yun, J W , Song, S K (1998) Process of Pb^{2+} accumulation in *Saccharomyces cerevisiae* Biotechnol Lett **20**, 153-156

Supek, F , Supekova, L , Nelson, H., Nelson, N (1996) A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria Proc Natl Acad Sci USA, **93**, 5105-5110

Swift, D.T , Forciniti, D (1997) Accumulation of lead by *Anabaena cylindrica* Mathematical modeling and an energy dispersive x-ray study Biotechnol Bioeng **55**, 408-417

Theuvenet, A., Nieuwenhuis, B , van de Mortel J, Borst-Pauwels, G (1986) Effect of ethidium bromide and DEAE-dextran on divalent cation accumulation in yeast Evidence for an ion-selective extrusion pump for divalent cations Biochim Biophys Acta, **855**, 383-390

Ting, Y.P., Teo, W.K. (1994) Uptake of cadmium and zinc by yeast effects of co-metals ion and physical/chemical treatments Bioresource Technol **50**, 113-117

Tobin, J.M , Cooper, D J., Neufeld, R J. (1984) Uptake of metal ions by *Rhizopus arrhizus* biomass Appl Environ Microbiol **47**, 821-824

Tobin, J.M , Cooper, D.G , Neufeld, R J (1987) Influence of anions on metal adsorption by *Rhizopus arrhizus* biomass Biotechnol Bioeng **30**, 882-886

Tobin, J.M , Cooper, D G., Neufeld, R J (1990) Investigation of the mechanism of metal uptake by denatured *Rhizopus arrhizus* Enzyme Miciobe Technol **12**, 591-595

Tobin, J M , White C , Gadd, G.M (1994) Metal accumulation by fungi applications in environmental biotechnology J Ind Microbiol **13**, 126-130

Tohoyama, H., Inouhe, M., Joho, M., Murayama, T (1995) Production of metallothionein in copper- and cadmium-resistant strains of *Saccharomyces cerevisiae* J Ind Micro **14**, 126-131

Tomsett, A B. (1993) Genetics and molecular biology of metal tolerance in fungi In Jenmngs, D H (ed) Stress tolerance of fungi Marcel Dekker, New York

Trevors, J T (1987) Silver resistance and accumulation in bacteria Enzyme Microb Technol **9**, 331-333

Trevors, J T , Stratton, G.W., Gadd, G M (1986) Cadmium transport, resistance and toxicity in bacteria, algae and fungi Can J Microbiol **32**, 447-464

Tsezos, M., Remoundaki, E., Angelatou, V. (1996) A study of the effects of competing ions on the biosorption of metals Inter Biodeter Biodeg 19-29

Tsezos, M. Volesky, B. (1981) Biosorption of uranium and thorium Biotechnol Bioeng **23**, 583-604

Tynecka, Z., Gos, Z., Zajac, J (1981) Reduced cadmium transport determined by a resistance plasmid in *Stapphylococcus aureus* J Bacteriol **147**, 305-312

Urrutia Mera, M , Kemper, M , Doyle, R , Beveridge, T J (1992) The membrane-induced proton motive force influences the metal binding ability of *Bacillus subtilis* cell walls Appl Environ Microbiol **58**, 3837-3844

Van Der Rest, M , Kamminga, A , Nakano, A , Anraku, Y., Poolman, B , Konings, W (1995) The plasma membrane of *Saccharomyces cerevisiae* structure, function, and biogensis Microbiol Revs **59**, 304-322

Veglio, F , Beolchini, F , Gasbarro,' A (1997) Biosorption of toxic metals an equilibrium study using free cells of *Arthobacter* sp Process Biochem **32**, 99-105

Volesky, B. (1990) Biosorption by fungal biomass *In* B Volesky (ed), Biosorption of heavy metals, CRC Press, Boca Raton, Fla

Volesky, B , Holan, Z.R. (1995) Biosorption of heavy metals Biotechnol Prog **11**, 235-250

Volesky, B , May, H , Holan, Z.R (1993) Cadmium biosorption by *Saccharomyces cerevisiae* Biotechnol Bioeng **41**, 826-829

Volesky, B , May-Phillips, H A (1995) Biosorption of heavy metals by *Saccharomyces cerevisiae* Appl Microbiol Biotechnol **42**, 797-806

Vulpe, C D., Packman, S. (1995) Cellular copper transport Annu Rev Nutr **15**, 293-322

Walker, G M. (1994) The roles of magnesium in biotechnology Crit Rev Biotechnol **14**, 311-354

Webster, E.A., Gadd, G.M (1996a) Perturbation of monovalent cation composition in *Ulva lactuca* by cadmium, copper and zinc Biometals, **9**, 51-56

Webster, E.A , Gadd, G M. (1996b) Stimulation of respiration in *Ulva lactuca* by high concentrations of cadmium and zinc evidence for an alternative respiratory pathway Environ Toxicol Water Quality, **11**, 7-11

White, C , Gadd, G M. (1986) Uptake and cellular distribution of copper, cobalt and cadmium in strains of *Saccharomyces cerevisiae* cultured on elevated concentrations of these metals FEMS Micro Ecol **38**, 277-283

White C , Gadd, G.M. (1987a) The uptake and cellular distribution of zinc in *Saccharomyces cerevisiae* J Gen Microbiol **133**, 727-737

White, C , Gadd, G M (1987b) Inhibition of H^+ efflux and K^+ efflux in yeast by heavy metals Tox Assess **2**, 437-447

Wilhelmi, B S , Duncan, J.R (1995) Metal recovery from *Saccharomyces cerevisiae* biosorption columns Biotech Lett **17**, 1007-1012

Wong, P , Fung, K. (1997) Removal and recovery of nickel ion (Ni^{2+}) from aqueous solution by magnite-immobilised cells of *Enterobacter* sp 4-2 Enzyme Microbe Technol **20**, 116-121

Wood, J.M , Wang, H. (1983) Microbial resistance to heavy metals Environ Sci Technol **12**, 582-590