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Interactions of fungi with toxic metals

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CONTENTS

Summary		V. Mycorrhizal interactions with toxic metals	45
I. Introduction	25	VI. Accumulation of metals by macrofungi	45
II. Definition and classification of metals	26	VII. Biotechnological aspects of fungal metal accumulation	46
III. Fungitoxicity of metals	28	1. General aspects	46
1. Mechanisms of toxicity	28	2. Mechanisms of heavy metal and radionuclide accumulation by fungi of biotechnological relevance	47
2. Resistance and tolerance	29	3. Metal removal by induced, excreted or derived biomolecules	48
3. Environmental influence on metal toxicity towards fungi	29	4. Metal recovery	49
4. Fungi in polluted habitats	31	5. Biosorption of radionuclides by fungal biomass	49
IV. Interactions between toxic metals and fungi	32	6. Industrial considerations	50
1. Extracellular precipitation and complexation	32	Acknowledgements	51
2. Metal binding to cell walls	33	References	51
3. Transport of toxic metal cations	35	Note added in proof*	60
4. Intracellular fate of toxic metals	39		
(a) Metal binding proteins and peptides of filamentous fungi and yeasts	39		
(b) Vacuolar compartmentation	43		
5. Metal transformations	44		

SUMMARY

This review details the major interactions of fungi with toxic metal species. Physico-chemical and biological aspects are discussed including definitions and classification of metals in biological contexts, mechanisms of metal fungitoxicity, resistance and tolerance, environmental influences and the occurrence of fungi in metal-polluted habitats. Cellular interactions include extracellular precipitation and complexation, binding to cell walls, transport of monovalent and divalent metal cations, intracellular fates of toxic metal species (including metal-binding proteins and peptides, and vacuolar compartmentation), and metal transformations including organometal(loid) synthesis. Significant interactions between metals and mycorrhizal fungi and macrofungi are summarized with reference to the amelioration of plant phytotoxicity and metal accumulation respectively. Biotechnological aspects of metal–fungal interactions relate to the biological treatment of metal-contaminated effluents and waste streams for metal removal/recovery and environmental protection.

Key words: Fungi, metals, toxicity, biosorption, transport.

I. INTRODUCTION

Fungi are ubiquitous in natural environments and important in industrial processes. A range of morphologies are found from unicellular yeasts to poly-

morphic and filamentous fungi, many of which have complex macroscopic fruiting bodies. Their most important roles are as decomposers of organic material, with concomitant nutrient cycling, as pathogens and symbionts of animals and plants, and as spoilage organisms of natural and synthetic materials, e.g. wood, paint, leather, food and fabrics.

* Sections to which ‘Noted added in proof’ refer are marked with an asterisk.

They are also utilized as producers of economically important substances, e.g. ethanol, citric acid, antibiotics, polysaccharides, enzymes and vitamins.

The study of the interaction between toxic metals and fungi has long been of scientific interest. This is because metal toxicity has been, and remains the basis of many fungicidal preparations used in the control of plant pathogens and to preserve natural and synthetic materials (Horsfall, 1956; Ross, 1975). Early work often had a phytopathological perspective with many studies relating to assessment of toxicity (Somers, 1961). Subsequent observations on the ability of fungi to resist and adapt to toxic metals stimulated further work on the physiological, biochemical and genetical explanations for these phenomena (Ashida, 1965; Ross, 1975; Gadd 1986*a*). Research has been stimulated particularly by the continuous refinement in biochemical and molecular techniques and by the general appreciation of filamentous fungi and yeasts as model eukaryotic cells systems. In an environmental context, accelerating pollution of the natural environment by toxic metals, metalloids, radionuclides and organo-metal(loid)s has also led to increased interest because of the ubiquitous and sometimes dominant presence of fungi in metal-polluted habitats, the uptake and translocation of toxic metals and radionuclides to fruit bodies of edible fungi, and the significance of mycorrhizal fungi in polluted habitats (Gadd, 1986*a*; Brown & Hall, 1990). While amelioration of metal phytotoxicity by mycorrhizal fungi is relevant to land reclamation (Colpaert & van Assche, 1987), another area of applied interest is the use of fungal (and other microbial) biomass for the detoxification of metal/radionuclide-containing industrial effluents (Gadd, 1986*b*, 1990*a*, 1992*a*).

The objective of this review is to outline the major interactions of fungi with toxic metals, with reference to those physical and chemical properties of metal species and the fungal environment which determine toxicity and cellular response. Physico-chemical, biochemical and genetical aspects of cellular interactions are included, mainly with reference to 'microfungi' and yeasts, though mention is also made of mycorrhizas and macrofungi in polluted habitats. Biotechnological discussion relates only to the use of fungal biomass and products for environmental protection. Several important topics such as the roles of essential metal ions in fungal metabolism and differentiation (Jones & Gadd, 1990), yeast flocculation (Kuriyama, Umeda & Kobayashi, 1991), iron acquisition and function (Winkelmann, Van der Helm & Neilands, 1987; Winkelmann, 1992), interactions of metals with lichens (Nieboer, Richardson & Tomassini, 1978; Nash, 1990) and (organo)metal-containing fungicides and their effects (Cooney & Wuertz, 1989) are not covered in detail.

II. DEFINITION AND CLASSIFICATION OF METALS

Metals are directly and/or indirectly involved in all aspects of fungal growth, metabolism and differentiation. While many metals are essential, e.g. K, Na, Mg, Ca, Mn, Fe, Cu, Zn, Co and Ni, many others have no apparent essential function, e.g. Rb, Cs, Al, Cd, Ag, Au, Hg and Pb. However, all these elements can interact with fungal cells and be accumulated by physico-chemical mechanisms and transport systems of varying specificity (Gadd, 1988). Most essential and inessential metals exhibit toxicity above a certain concentration, which will vary depending on the organism, the physico-chemical properties of the metal and environmental factors. This may necessitate expression of a detoxification mechanism if the organism is to survive (Fig. 1). Although the connotations of toxicity and bioaccumulation are most often linked with inessential metals, the potential toxicity of many essential metals should not be overlooked. A good example is provided by calcium, an important intracellular second messenger in fungal growth and differentiation, which can also be highly toxic within cells owing to precipitation of phosphates. Fungi, like other eukaryotic cells, maintain cytosolic free $[Ca^{2+}]$ at around $0.1 \mu M$ by means of a variety of transport systems located on the plasma and vacuolar membranes which effect influx/efflux and vacuolar compartmentation; cytosolic free $[Ca^{2+}]$ may also be modulated by interaction with calcium-binding proteins (Miller, Vogg & Sanders, 1990; Gadd & Brunton, 1992).

Many attempts have been made to define metals in relation to biological effects using their physical, chemical and biological properties. Metals and metalloids can be considered to include all the elements except the noble gases and H, B, C, N, O,

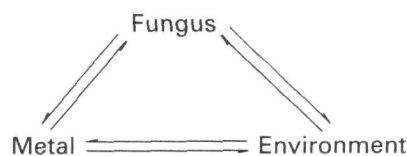


Figure 1. Diagrammatic representation of metal-fungal interactions. Each arrow can represent multiple effects which emphasizes the complexity of interaction between cellular, physical and chemical phenomena. The environment (natural, synthetic or laboratory) may affect fungal growth, metabolism and differentiation and the availability and speciation of metal(s). Metal species may positively or negatively affect fungal growth, metabolism and differentiation depending on the element(s) concerned and concentration, and alter the physico-chemical characteristics of an environment. Fungi may remove metals from solution by physico-chemical and biological processes and/or transform them into other forms, including organometal(loid)s. Fungi may alter the environment by metabolic activities and growth.

Table 1. Classification of metal ions into type-A, transition metal, and type-B metal cations (adapted, with permission, from Morgan & Stumm, 1991) and according to the hard and soft acid scheme, with ligand preferences and stability sequences (adapted from Pearson, 1963; Morgan & Stumm, 1991). Referral to other related works (e.g. Nieboer & Richardson, 1980; Ochiai, 1987; Fergusson, 1990; Hughes, 1990; Hughes & Poole, 1989, 1991; Gadd, 1992c) is recommended

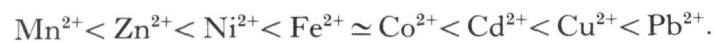
Type-A metal cations	Transition metal cations	Type-B metal cations
Electron configuration of inert gas	1–9 outer shell electrons	Electron number corresponds to Ni ⁰ , Pd ⁰ and Pt ⁰ (10 or 12 outer shell electrons)
Low polarizability 'Hard spheres' (H ⁺ , Li ⁺ , Na ⁺ , K ⁺ , Be ²⁺ , Mg ²⁺ , Ca ²⁺ , Sr ²⁺ , Al ³⁺ , Sc ³⁺ , La ³⁺ , Si ⁴⁺ , Ti ⁴⁺ , Zr ⁴⁺ , Th ⁴⁺)	Not spherically symmetric V ²⁺ , Cr ²⁺ , Mn ²⁺ , Fe ²⁺ , Co ²⁺ , Ni ²⁺ , Cu ²⁺ , Ti ³⁺ , V ³⁺ , Cr ³⁺ , Mn ³⁺ , Fe ³⁺ , Co ³⁺	Low electronegativity High polarizability 'Soft spheres' Cu ⁺ , Ag ⁺ , Au ⁺ , Ga ⁺ , Zn ²⁺ , Cd ²⁺ , Hg ²⁺ , Pb ²⁺ , Sn ²⁺ , Tl ³⁺ , Au ³⁺ , In ³⁺ , Bi ³⁺
Hard acids	Borderline	Soft acids
All type-A metal cations plus Cr ³⁺ , Mn ³⁺ , Fe ³⁺ , Co ³⁺ , UO ²⁺ , VO ²⁺ In addition species such as BF ₃ , BCl ₃ , SO ₃ , RSO ₂ ⁺ , RPO ₂ ⁺ , CO ₂ , RCO ⁺ , R ₃ C ⁺	All divalent transition metal cations plus Zn ²⁺ , Pb ²⁺ , Bi ³⁺ SO ₂ , NO ⁺ , B(CH ₃) ₃	All type-B metal cations minus Zn ²⁺ , Pb ²⁺ , Bi ³⁺ All metal atoms, bulk metals I ₂ , Br ₂ , ICN, I ⁺ , Br ⁺
Preference for ligand atom N ≫ P O ≫ S F ≫ Cl		P ≫ N S ≫ O I ≫ F
Stability sequence Cations: Stability ∝ (charge/radius)	Cations Irving-Williams series: Mn ²⁺ < Fe ²⁺ < Co ²⁺ < Ni ²⁺ < Cu ²⁺ > Zn ²⁺	
Ligands F > O > N = Cl > Br > I > S OH ⁻ > RO ⁻ > RCO ₂ ⁻ CO ₃ ²⁻ ≫ NO ₃ ⁻ PO ₄ ³⁻ ≫ SO ₄ ²⁻ ≫ ClO ₄ ⁻		Ligands S > I > Br > Cl = N > O > F

F, P, S, Cl, Br, I and At. Metalloids are Si, Ge, As, Se, Sb and Te. Groups Ia and IIa of the Periodic Table, the 's-block' metals, form monovalent cations (alkali metals) and divalent cations (alkaline earth metals) respectively. Groups IIb to VIb contain the 'p-block' metals (Morgan & Stumm, 1991). The transition elements are those whose ions have incompletely filled *d* orbitals (Hughes, 1990). The three transition series of the Periodic Table occupy rows 4, 5 and 6 and elements of the first and subsequent transition series can exhibit a wide variety of oxidation states, except for group IIb metals which have the III oxidation state only. The actinides and lanthanides comprise an 'inner' transition series (Morgan & Stumm, 1991).

In Table 1 metal ions are classified as 'type-A' and 'type-B' metal cations (governed by the number of outer shell electrons) and transition metal cations (Morgan & Stumm, 1991) as well as the hard and soft acid classification (Pearson, 1963; Hughes & Poole, 1989). Many schemes used in a biological context are derived from such parameters, particularly ligand preferences (Sillen & Martell, 1971)

and polarizing power (charge/radius ratio) since such properties can determine biological activity (Nieboer & Richardson, 1980; Ochiai, 1987; Hughes & Poole, 1989, 1991; Morgan & Stumm, 1991). Oxygen-donating ligands are hard whereas sulphur donors are soft. In general, hard ligands bind to hard metal ions and soft ligands bind to soft metal ions. In a mixture of hard and soft metal ions, soft metal ions may outcompete hard metal ions for binding ligands (Hughes & Poole, 1991). Hard cations, e.g. Na⁺, K⁺ and Mg²⁺, are generally small, exhibit high electronegativity and low polarizability and tend to participate in electrostatic bonding. Many essential metal ions, e.g. Fe³⁺, Ca²⁺, Mg²⁺ and K⁺, are hard whereas inessential toxic metals, e.g. Ag⁺, Cd²⁺, Hg²⁺ and Sn²⁺, are soft. However, biologically-inessential hard metals include Rb⁺, Sr²⁺ and Al³⁺ while a borderline category includes Cu²⁺, Ni²⁺, Zn²⁺ and Co²⁺ (Hughes & Poole, 1991). The need for 'borderline' categories, which reflects gradually varying properties of metal ions rather than absolute distinctions (Morgan & Stumm, 1991), emphasizes the difficulty of rigorous definition (Gadd, 1992c, d).

Borderline metal ions do not show a clear preference for the above metal-binding donor atoms and ligands, affinities which depend on a variety of factors. While 'type-A' metal ions are sharply separated from borderline metals, the distinction between 'type-B' and borderline categories is not so clear. Among borderline metal ions, class B character increases in the order:



Metal ions compete with H^+ for sites on ligands and H^+ may, in fact, be regarded as a borderline ion (Nieboer & Richardson, 1980). An increase in acidity will therefore result in protonation of the ligand anion and an increase in the concentration of the free metal ion (Hughes & Poole, 1991). The above classifications of metals may be useful in defining or predicting certain interactions with organisms, as well as in assessing metal speciation in environmental samples or growth media (Hughes & Poole, 1991).

Other definitions may be based on toxicity and environmental impact although this can be highly variable and dependent on, e.g. the organism, element, speciation, concentration, physico-chemical factors and anthropogenic activities (Babich & Stotzky, 1985; Gadd, 1990*b*). In fungi, metal effects can vary between organisms, strains, the stage of growth and even different vegetative and reproductive forms of the same organism (Gadd & Mowll, 1985; Sabie & Gadd, 1990). A given organism may exhibit several mechanisms by which potentially toxic metal species are detoxified, both dependent and independent of metabolism and all affected by environmental factors. Furthermore, other elements not considered to be true metals according to chemical definitions may have some metallic properties and exhibit varying degrees of toxicity as well as accumulation by fungi. These include metalloids (see above), and the actinides and lanthanides, e.g. uranium and thorium. The alkali metals are not usually included in discussions on metal toxicity but nevertheless exhibit a dramatic range of biological properties as exemplified by K^+ , Na^+ and Cs^+ as respectively representing an essential non-toxic, an essential but potentially-toxic, and an inessential potentially-toxic metal ion. Caesium, and many actinides, are of current concern because of environmental contamination, accumulation by the biota, including fungi, and transfer to other organisms including humans (Gadd, 1992*a*).

Current definitions of the term, 'heavy metal', are particularly variable. These are often defined as a group of approximately 65 metallic elements, of density greater than 5, with the general ability to exert toxic effects on microbial and other life forms (Gadd & Griffiths, 1978; Nieboer & Richardson, 1980). However, this imprecise definition includes elements with diverse physical, chemical and biological properties. A further complication is that

'heavy elements' may be defined as Pb, As, Cd, Hg, Sb, Se, Tl, In, Bi, and Te (Fergusson, 1990). This is again a diverse group and, in fact, As, Sb, Bi, Se and Te have elemental structures more typical of non-metals (Fergusson, 1990; Morgan & Stumm, 1991).

Organometallic compounds, simply defined as a compound containing at least one metal-carbon bond (Thayer, 1988), frequently exhibit enhanced toxicity, hence their use as fungicides. When such compounds contain metalloid elements, the term organo-metalloid may be used. Such substances should also be included in discussions of metal interactions with fungi because of their significance as environmental pollutants, as fungicides and because some may be synthesized by fungi as a result of exposure to, and transformation of, the 'parental' metal species.

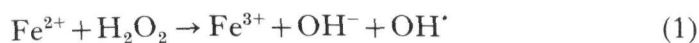
III. FUNGITOXICITY OF METALS

1. Mechanisms of toxicity*

Toxic metals can exert harmful effects in many ways but principally as a result of their strong coordinating abilities (Ochiai, 1987). Toxic effects include the blocking of functional groups of biologically important molecules (e.g. enzymes and transport systems for essential nutrients and ions), the displacement and/or substitution of essential metal ions from biomolecules and functional cellular units, conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organellar membrane integrity (Ochiai, 1987). Because of the wide spectrum of potentially toxic interactions between metals and fungi, almost every aspect of their metabolism, growth and differentiation may be affected, depending on the organism, metal species and concentration, and physico-chemical factors (Gadd, 1986*a, c*; Gadd & White, 1989*a*). Thus, toxic symptoms may vary widely between different fungi and for different metal species. A prerequisite for direct toxic interactions is contact between the active metal species and cellular components (Gadd & White, 1989*a*). The cell membrane is an obvious initial site of action for a toxic metal species and membrane damage can result in loss of mobile cellular solutes, e.g. K^+ , and increased permeability of the cell to external materials (Norris & Kelly, 1977; Kuypers & Roomans, 1979; Mowll & Gadd, 1983; White & Gladd, 1987*a, b*; Laurence, Cooney & Gadd, 1989).

Indirect mechanisms of metal toxicity may involve free radicals, which are deleterious to cells as they can take part in chain reactions which involve the breakdown of biological macromolecules. Consequently, aerobic organisms possess protective enzymes such as superoxide dismutases, which are metalloenzymes containing either Mn, Fe or Cu/Zn (Greco *et al.*, 1990; Galiazzi *et al.*, 1991), which eliminate the radicals produced by normal metabolism. Major targets in cells are membranes, where

lipid peroxidation, whereby the alkyl chains of lipids are converted to peroxyalkyl radicals and fatty acid hydroperoxides, is initiated (Mehlhorn, 1986). Lipid-soluble complexes of transition elements such as Fe(II) may undergo the Fenton reaction [(1), (2)] with the hydroperoxides and accelerate this process (McCord & Day, 1978).



Complexes and free ions of these cations may also undergo this reaction in aqueous solution. In animal systems, metal ions such as Hg^{2+} , Cd^{2+} , Pb^{2+} and Ag^{2+} induce free radical toxicity as a result of their reactions with thiols or enzymes which normally protect against these reactive species (Mehlhorn, 1986). Little work on this aspect has been carried out with fungi except for Cu^{2+} and *Saccharomyces cerevisiae* (Greco *et al.*, 1990).

Organometallic compounds are of increasing environmental significance because of their use in the chemical and petroleum industries and as biocides. Organometals are generally more toxic towards fungi than corresponding free metal ions and the toxicity of their compounds varies with the number and with the identity of the organic groups (Blunden, Hobbs & Smith, 1984; Cooney & Wuertz, 1989). Major effects of organotins and organoleads are disruption of mitochondrial membranes and action as Cl^-/OH^- ionophores. In this way, they depolarize electrochemical gradients and consequently interfere with energy conservation (Blunden *et al.*, 1984; Cooney & Wuertz, 1989). In fact, organotin-resistant mutants of *S. cerevisiae* can exhibit increased respiration rates (Dupont *et al.*, 1990) and modification of the organotin-binding site on the inner mitochondrial membrane (Lancashire & Griffiths, 1975). Organometals may also damage membranes by the production of free radicals since the carbon-metal bond readily reacts with available radicals to produce peroxyalkyl radicals which can result in lipid peroxidation (Mehlhorn, 1986). As well as the mitochondrial membrane, organometallic compounds may also exert a disruptive effect on cell membranes and cause a loss of K^+ (Cooney *et al.*, 1989).

2. Resistance and tolerance

Fungal survival in the presence of toxic metals mainly depends on intrinsic biochemical and structural properties, physiological and/or genetical adaptation, including morphological changes, and environmental modification of metal speciation, availability and toxicity, the relative importance of each often being difficult to determine (Gadd & Griffiths, 1978; Gadd, 1990b, 1992b). Arbitrary terms such as 'resistance' and 'tolerance' which are used rather loosely and, often, interchangeably in the

literature are generally based on the ability to grow on a certain metal concentration in laboratory media (Trevors, Stratton & Gadd, 1986; Gadd, 1992b, c). It is probably more appropriate to define 'resistance' as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned, e.g. synthesis of metallothioneins or γ -glutamyl peptides (Mehra & Winge, 1991) (see later). 'Metal tolerance' may be defined as the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity (Gadd, 1992b, c). Intrinsic properties that can determine survival include possession of impermeable pigmented cell walls, extracellular polysaccharide and metabolite excretion, especially where this leads to detoxification of the metal species by, e.g. binding or precipitation (Gadd, 1990b). However, in many cases distinctions are difficult because of the involvement of several direct and indirect physico-chemical and biological mechanisms in survival in field and laboratory. Biological mechanisms implicated in fungal survival (as distinct from environmental modification of toxicity) include extracellular precipitation, complexation and crystallization, transformation of metal species by, e.g. oxidation, reduction, methylation and dealkylation, biosorption to cell walls, pigments and extracellular polysaccharide, decreased transport or impermeability, efflux, intracellular compartmentation and precipitation and/or sequestration (Fig. 2) (Ross, 1975; Gadd & Griffiths, 1978; Gadd, 1988, 1990a, b, 1992a-c; Brown & Hall, 1990; Mehra & Winge, 1991). A particular organism may directly and/or indirectly rely on several survival strategies. For example, metallothionein synthesis is a mechanism of Cu^{2+} resistance in *S. cerevisiae* (see later) yet Cu^{2+} binding or precipitation around the cell wall and intracellular transport are also components of the total cellular response (Gadd & White, 1989a). Detailed accounts of metal resistance and tolerance of fungi can be found elsewhere (Ross, 1975; Gadd, 1986a, 1990b, 1992b; Mehra & Winge, 1991).

3. Environmental influence on heavy metal toxicity towards fungi*

The physico-chemical properties of a given environment, or growth medium, determine metal speciation and therefore biological availability and toxicity, as well as other essential and inessential metal-organism interactions (Fig. 1). Where such factors as pH, E_h , the presence of other anions and cations, and of particulate and soluble organic matter, and clay minerals decrease biological availability, toxicity may be reduced (Gadd & Griffiths, 1978). It is therefore clear that the concentration of free metal ion, defined as $\text{pM} = -\log [\text{M}]$ (Hughes & Poole, 1991), may be an important consideration in

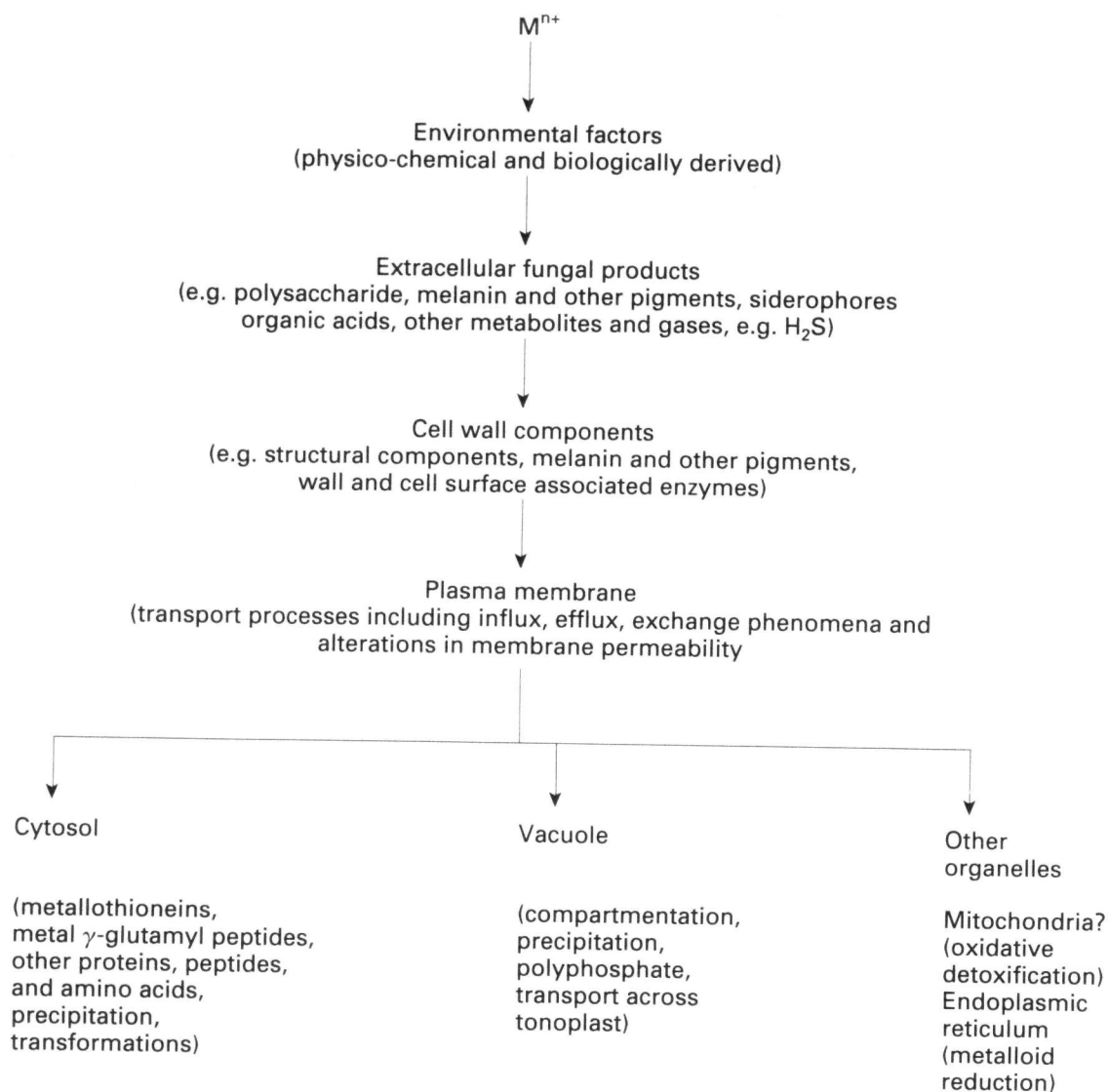


Figure 2. Diagrammatic representation of metal interactions with fungi. The metal species is represented in cationic form. All the interactions shown may be involved in fungal survival and/or metal detoxification.

many cases, although toxicity may still be exerted by many inorganic and organic metal complexes in the absence of free metal cations, M^{n+} . In simple terms, if excess metal ion (M) is in the presence of a strongly binding ligand (L), $[M_{\text{free}}] = [M_{\text{total}}] - [L_{\text{total}}]$. If the ligand is in excess, and unprotonated, $L_t = ML + L$, $[ML] \sim [M_t]$ and the pM may be calculated:

$$K_1 = [ML]/[M][L] = [M_t]/[M][L_t - M_t],$$

$$\text{pM} = -\log [M] = \log K_1 + \log ([L_t - M_t]/[M_t]).$$

If the ligand is protonated, the calculation becomes $L_t = ML + L + HL + H_2L \dots H_nL$ (Hughes & Poole, 1991). In relation to the complex situation which occurs in the environment and in growth media, the multiplicity of metal-ligand binding interactions that can occur may mean that calculation of pM is not possible.

The pH can affect metal-fungal responses by effects on metal speciation and mobility and indirectly by influencing other aspects of cell physiology and metabolism. Increasing pH can result in the formation and precipitation of metal hydroxides or oxides. In aqueous solution, divalent metal cations form multiple hydroxylated species, some forming

precipitates (Collins & Stotzky, 1989; Hughes & Poole, 1991).



At higher pH values, some precipitates redissolve giving anionic hydroxometallate complexes, e.g. $Zn(OH)_4^{2-}$ (Hughes & Poole, 1991). The pH values at which hydroxylated species form varies between different metals while different hydroxylated forms possess differing toxicities (Collins & Stotzky, 1989). The formation of hydrolysis products may also favour biosorption (see later).

Decreasing pH may increase the concentration of free metal ions in solution although, as mentioned previously, H^+ may compete with metal ions for cellular binding sites and reduce potential interactions with cells. External pH may also affect the speciation of metal-binding ligands and therefore metal complexation. Many toxic interactions of heavy metals with fungal cells can be interpreted in terms of pH effects on metal speciation and availability (Newby & Gadd, 1986; Gadd, 1986a). Fungitoxicity of Cd may increase with increasing pH

(pH 5–9), possibly as a result of formation of CdOH^+ which may enter cells more easily than Cd^{2+} , particularly as less H^+ will be present to compete with CdOH^+ at higher pH values. With certain other metals, e.g., Ni, toxicity may decrease as the pH increases, possibly as a result of formation of hydroxylated species or pH effects on transport. A reduction in metal toxicity at low pH may result from H^+ competition and also pH effects on intracellular uptake. This has been described for those fungi, e.g. *Penicillium ochro-chloron*, that are capable of growing in saturated CuSO_4 and high concentrations of other heavy metal salts (Gadd *et al.*, 1984a). With increasing pH, intracellular Cu accumulation dramatically increased in *P. ochro-chloron* and at pH 6 and above, this fungus was sensitive to quite low concentrations of Cu (Gadd & White, 1985).

Oxidation-reduction potential (E_h) may affect heavy metal availability and toxicity. In a reducing environment (negative E_h), insoluble metal sulphides may form which exhibit little or no toxicity; indeed, sulphide precipitation is a detoxification mechanism found in several fungi (see later). The E_h may also effect speciation, e.g. chromium exists as Cr^{6+} or Cr^{3+} depending on the E_h ; Cr^{6+} exhibits the greater toxicity towards hyphal growth and sporulation in several fungi (Collins & Stotzky, 1989).

Inorganic anions may affect toxicity by forming inorganic complexes, as mentioned previously, and by precipitation of, e.g. carbonates and phosphates (Gadd & Griffiths, 1978). Increasing Cl^- , for example, decreased Cd toxicity towards several filamentous fungi, possibly as a result of the formation of negative Cd–Cl coordination complexes (Babich & Stotzky, 1982; Collins & Stotzky, 1989). Other cations may affect fungitoxicity of heavy metals by competing for binding sites on cell surfaces, including membranes, and for transport mechanisms. Heavy metal toxicity towards fungi was also reduced in hard water, an effect attributed to CO_3^{2-} (Babich & Stotzky, 1981). Many heavy metal salts are of low solubility, e.g. sulphides and phosphates, and so readily precipitate out of solution. Silver chloride is highly insoluble and the concentration of free Ag^+ in solution may be negligible in many cases, particularly in growth media (Hughes & Poole, 1991).

Clay minerals can adsorb heavy metal cations and reduce their potential toxicity (Gadd & Griffiths, 1978; Babich & Stotzky, 1980). In general, minerals with a high cation exchange capacity (CEC) are more effective in reducing toxicity than minerals with a lower CEC (Babich & Stotzky, 1977a, b; Bewley & Stotzky, 1983). Montmorillonite was more effective than kaolinite, with a lower CEC, in protecting fungi from Cd, Ni and Pb toxicity (Babich & Stotzky, 1978, 1979, 1982), such effects also being influenced by soil pH (Collins & Stotzky, 1989).

Dissolved and particulate organic matter in the environment, and in growth media, can influence metal toxicity by complexation and binding, generally reducing toxicity. Examples of such organic materials include natural and synthetic chelating agents, including plant, fungal and other microbial exudates and metabolites, humic acids and soil colloids, and soluble organic chemicals and mixtures. It is commonly observed that metal toxicity towards fungi is reduced in complex media compared to simple defined media, such effects also being influenced by pH for the reasons outlined previously (Gadd & Griffiths, 1978; Collins & Stotzky, 1989).

4. Fungi in polluted habitats

A range of fungi from all major taxonomic groups may be found in metal-polluted habitats and the ability to survive and grow in the presence of potentially toxic concentrations is frequently encountered (Ross, 1975; Gadd, 1986a; Baldi, Vaughan & Olson, 1990; Turnau, 1991). In general terms, toxic metals are believed to affect fungal populations by reducing abundance and species diversity and selecting for a resistant/tolerant population (Babich & Stotzky, 1985; Duxbury, 1985). However, the effect of toxic metals on microbial abundance in natural habitats varies with the metal species and organisms present and with environmental factors (Gadd & Griffiths, 1978; Duxbury, 1985).

General reductions in fungal numbers have often been noted in soils polluted with Cu, Cd, Pb, As and Zn (Babich & Stotzky, 1985). Along a steep gradient of Cu and Zn in soil towards a brass mill, fungal biomass decreased by ~75% (Nordgren, Bååth & Söderström, 1983) while fungal numbers were reduced in glucose-supplemented soil by Cd or Zn, the former metal being of greater toxicity (Bewley & Stotzky, 1983). However, numerical estimates alone which have built-in inadequacies, may provide little meaningful information unless possible changes in fungal groups and species are considered. There is evidence that such changes can occur in response to metal exposure.

In Cu- and Zn-polluted soil, *Geomyces* and *Paecilomyces* spp. and some sterile forms increased with increasing pollution, whereas *Penicillium* and *Oidiodendron* spp. declined at polluted sites (Nordgren *et al.*, 1983). *Trichocladium asperum*, *Trichoderma hamatum*, *Zygorrhynchus moelleri* and *Chrysosporium pannorum* were isolated more frequently from an organomercurial-treated golf green than from untreated locations, whereas *Chaetomium*, *Fusarium*, *Penicillium* and *Paecilomyces* spp. were greatly reduced (Williams & Pugh, 1975). Several other studies have indicated sensitivity of *Penicillium* spp. towards heavy metals (Duxbury, 1985). However, some of the best examples of microbial metal

tolerance are also found in the genus *Penicillium*, which underlines the fact that metal responses may be strain specific. As noted above, *P. ochro-chloron* can grow in saturated CuSO_4 and it is frequently isolated from industrial effluents (Stokes & Lindsay, 1979), whereas *P. lilacinum* comprised 23 % of all fungi isolated from soil polluted by mine drainage (Tatsuyama *et al.*, 1975). In soil polluted with cadmium dust, *Strobilurus tenacellus*, *Mycena ammoniaca*, *Auriscalpium vulgare* and *Armillaria lutea* were the most common Basidiomycetes (Turnau, 1991).

Heavy metal pollution of plant surfaces is widespread and, while there may be significant decreases in total microbial numbers (including bacteria) on phylloplanes, numbers of filamentous fungi and non-pigmented yeasts are generally little affected (Bewley, 1979, 1980; Bewley & Campbell, 1980; Mowll & Gadd, 1985) and, if metal-supplemented isolation media are used, only fungi may be isolated (Bewley, 1980). On polluted oak leaves, *Aureobasidium pullulans* and *Cladosporium* spp. were the most numerous organisms and a greater proportion were metal-tolerant when compared with control isolates (Bewley & Campbell, 1978; Bewley, 1980). In fact, numbers of *A. pullulans* showed a good positive correlation with lead concentrations, whether derived from industrial or vehicular sources and it often became the dominant organism, in some cases comprising up to 97 % of the leaf surface population on a numerical basis (Bewley & Campbell, 1980; Mowll & Gadd, 1985). However, adaptation was unnecessary for growth of *A. pullulans* on polluted leaf surfaces and the ability to tolerate high concentrations of lead occurred in isolates from the unpolluted site (Gadd, 1984; Mowll & Gadd, 1985).

In contrast, the numbers of the ballistospore-producing yeast, *Sporobolomyces roseus*, and heterotrophic bacteria were very low or absent from polluted samples while numbers of *S. roseus* showed a significant negative correlation with increasing lead concentrations (Mowll & Gadd, 1985). Smith (1977) also studied the effect of heavy metals on phylloplane fungi and found that *A. pullulans*, *Epicoccum* sp. and *Phialophora verrucosa* were tolerant, *Gnomia platani*, *Cladosporium* and *Pleurophomella* spp. were of intermediate tolerance while *Pestalotiopsis* and *Chaetomium* spp. were sensitive. Mercury-tolerant fungi have been isolated from the surfaces of seeds treated with mercury compounds. These include *Pyrenophora avenae*, *Penicillium crustosum*, *Cladosporium cladosporioides*, *Syncephalastrum racemosum* and *Ulocladium atrum* (Ross & Old, 1973; Greenaway, Cripps & Ward, 1974).

It seems, therefore, that elevated concentrations of heavy metals can affect the qualitative and quantitative composition of fungal populations though it must be stressed that it may be difficult to separate

metal effects from those of other environmental components. Phylloplane mycoflora may be subject to the influence of other potential toxicants, e.g. SO_2 (Babich & Stotzky, 1980), while polluted soils may be nutrient-poor, of variable pH and also contain additional toxicants (Gadd & Griffiths, 1978). These factors may affect fungal populations. Furthermore, there are well known difficulties in obtaining meaningful assessments of fungal numbers and diversity which cannot be ignored. Despite this, it is less apparent that certain fungi can exhibit considerable tolerance towards toxic metals and can become dominant microorganisms in some polluted habitats. However, while species diversity may be reduced in certain cases, resistance/tolerance can be exhibited by fungi from both polluted and non-polluted habitats. Fungal numbers were reduced and there were alterations in species composition in polluted soil near a zinc smelter (Jordan & Lechevalier, 1975). However, there was little difference in the zinc tolerance of fungi isolated from either site and most could exhibit 50 % growth at $700 \mu\text{M Zn}^{2+}$. At control sites, Zn-tolerant genera included *Bdellospora*, *Verticillium* and *Paecilomyces* with *Penicillium*, *Torula* and *Aureobasidium* at polluted sites. In another study, fungal populations in soil contaminated with Ni, Cu, Fe and Co were not significantly different from those at control sites (Freedman & Hutchinson, 1980). Cu- and Ni-tolerant fungi (defined as being capable of growth at approximately 1.6 mM Cu^{2+} and/or Ni^{2+}) were isolated from both control and contaminated sites, the predominant genera being *Penicillium* (60 %) followed by *Trichoderma*, *Rhodotorula*, *Oidiodendron*, *Mortierella* and *Mucor*. Examination of microfungi isolated from unpolluted and copper-polluted forest soil confirmed that species from the polluted site were usually copper-tolerant although there was little evidence for adaptation in isolates from sites with short or long histories of pollution (Arnebrant, Bååth & Nordgren, 1987). Such studies indicate that, in many cases, survival must be dependent on intrinsic properties of the organisms, rather than adaptive changes (which are generally studied under laboratory conditions), and physico-chemical properties of that environment. These will include changes associated with the metal pollution, which may modulate toxicity and affect species composition (Gadd, 1986a, 1990b).

IV. INTERACTIONS BETWEEN TOXIC METALS AND FUNGI

1. Extracellular precipitation and complexation

Many extracellular fungal products can complex or precipitate heavy metals. Citric acid can be an efficient metal-ion chelator and oxalic acid can interact with metal ions to form insoluble oxalate crystals around cell walls and in the external

Table 2. *Macromolecular constituents of fungal cell walls (adapted from Peberdy, 1990)*

Skeletal elements	
Chitin:	β -1-4-linked homopolymer of <i>N</i> -acetyl-D-glucosamine
β -glucans:	β -1,3-glucan homopolymer comprised of D-glucose units with β -1,3 and β -1,6-glucosidic bonds (R-glucan)
Cellulose:	β -1,4-linked homopolymer of glucose
Matrix components	
α -glucan:	α -1,3-homopolymer of glucose (S-glucan) α -1,3- and α -1,4-linked glucan (nigeran)
Glycoproteins	
Mannoproteins	
Miscellaneous components	
Chitosan:	β -1,4-polymer of D-glucosamine
D-galactosamine polymers	
Polyuronides	
Melanins	
Lipids	

medium (Murphy & Levy, 1983; Sutter, Jones & Walchli, 1983). The production of H₂S by yeasts can result in precipitation of metals as insoluble sulphides predominately in and around cell walls (Ashida, Higashi & Kikuchi, 1963; Minney & Quirk, 1985). For strains of *S. cerevisiae* growing on a copper-containing medium, resultant colonies may appear dark in colour owing to formation of copper sulphide (Ashida *et al.*, 1963).

Iron is of fundamental importance to living cells and many filamentous fungi and yeasts release high affinity Fe-binding molecules called siderophores (see Winkelmann *et al.*, 1987; Winkelmann, 1992). The externally-formed Fe³⁺ chelates may subsequently be taken up into the cell (Raymond, Muller & Matzanke, 1984; Adjimani & Emery, 1987). In several fungi, the excretion of such iron-binding molecules is markedly stimulated by Fe deficiency and such compounds may also bind Ga (Adjimani & Emery, 1987). *Debaryomyces hansenii* produced riboflavin, or a related compound, when grown in Fe-deficient media or in the presence of copper, cobalt and zinc. Polarographic analysis showed that the pigment was capable of Fe³⁺ binding (Gadd & Edwards, 1986).

2. Metal binding to cell walls

The wall is the first cellular site of interaction with metal species and metal removal from solution may be rapid although rates will depend on factors such as type of metal ion and biomass, concentration of metal and environmental factors. Metabolism-independent association of metal species to fungal walls may include ion exchange, adsorption, complexation, precipitation and crystallization (Mullen *et al.*, 1992). ‘Biosorption’ is a term that is frequently used to describe such non-directed physico-chemical interactions between metals (including radionuclide) species and microbial biomass,

particularly in a biotechnological context (Shumate & Strandberg, 1985).

The fungal cell wall thus has important protective properties and so may act as a barrier, controlling the uptake of solutes into the cell including potentially-toxic metal species (Gadd & Griffiths, 1980*a*; Gadd, 1986*a, b*; Ono, Ohue & Ishihara, 1988), and also indirectly affects the intracellular ionic composition by restricting cellular water (Peberdy, 1990). The wall is mainly composed of polysaccharides, some of which may have associated protein, with other components including lipids and melanins (Table 2). All filamentous fungi, with the exception of the Oomycetes, contain chitin with (1-3)- β -glucan as skeletal components. The Oomycetes, together with Hyphochytridiomycetes possess cellulosic walls (Peberdy, 1990). In yeasts, skeletal components consist of (1-3)- β -D-glucans with (1-6)- β -linkages at branch points, the degree of branching determining crystallinity (Bartnicki-Garcia, 1973). Matrix components in yeasts comprise a mannan-protein complex, while (1-3)- α -glucan normally occurring at the outer surface of the wall, is the major matrix polymer in filamentous fungi (Peberdy, 1990). Heterogeneous glycoproteins are also found in filamentous fungal walls (Peberdy, 1990).

It is clear, therefore, that metal biosorption in fungal cell walls may be complex, involving different components and mechanisms, and variable, depending on wall structure and composition, the latter itself being affected by the presence of toxic metals (Gadd & Griffiths, 1980*a*; Newby & Gadd, 1987; Venkateswerlu & Stotzky, 1989; Venkateswerlu, Yoder & Stotzky, 1989). A variety of potential sites may be involved in metal sequestration including carboxyl, amine, hydroxyl, phosphate and sulphhydryl groups although their relative significance is usually difficult to resolve (Strandberg, Shumate & Parrott, 1981). However, primary interactions probably involve binding to carboxyl and phosphate groups which may be enhanced by

electrostatic attraction to other negatively charged functional groups (Tobin, Cooper & Neufeld, 1990). Metabolism-independent biosorption is frequently rapid and unaffected over moderate ranges of temperature, e.g. 4–30 °C (Norris & Kelly, 1977; De Rome & Gadd, 1987*a, b*; Junghans & Straube, 1991). In *Rhizopus arrhizus*, biosorption was related to ionic radius for, e.g. La^{3+} , Mn^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} , Ba^{2+} , Hg^{2+} , Pb^{2+} , UO_2^{2+} and Ag^+ , but not Cr^{3+} or the alkali metal cations, Na^+ , K^+ , Cs^+ and Rb^+ , which were not taken up by the cell walls (Tobin, Cooper & Neufeld, 1984). Low external pH often decreases biosorption of, e.g., Cu^{2+} , Cd^{2+} , Zn^{2+} , Mn^{2+} and Co^{2+} (Fuhrmann & Rothstein, 1968; Paton & Budd, 1972; Roomans *et al.*, 1979; Gadd & Mowll, 1985; De Rome & Gadd, 1987*a, b*; Venkateswerlu & Stotzky, 1989; Gadd, 1990*a*; Huang, Huang & Morehart, 1990) while other anions and cations may have the same effect by, e.g. precipitation by phosphates, and removal of metal from solution or by competition for binding sites, e.g., Mg^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} and Mn^{2+} (Tobin, Cooper & Neufeld, 1987, 1988; Gadd, 1990*a*). The latter effects may be dependent on the relative concentrations and chemical behaviour of the different metal species present. Biomass concentration may also effect fungal biosorption. At a given equilibrium concentration, the uptake of metals when expressed on a unit dry weight or cell basis, is generally greater at lower cell densities than at high ones (Itoh, Yuasa & Kobayashi, 1975; De Rome & Gadd, 1987*a*; Junghans & Straube, 1991). Since growth conditions affect the structure and composition of fungal cell walls, their manipulation offers potential for biosorbency for specific purposes (Volesky, 1990).

Chitin and chitosan have received attention as significant metal-biosorbing substances in cell walls of fungi. Chitin is a polymer of *N*-acetyl-D-glucosamine; chitosan is deacetylated chitin (Volesky, 1990). A variety of metal ions are readily bound by chitin although alkali metals, NH_4^+ , Ca^{2+} and Mg^{2+} are not sequestered (Muzzarelli, Bregani & Sigon, 1986). In *R. arrhizus*, coordination of uranium to the amine nitrogen of chitin and simultaneous adsorption in the cell wall chitin structure are followed by slower precipitation of uranyl hydroxide (Tsezos & Volesky, 1982*a*). Accumulation of hydrolysis products continues until final equilibrium (Tsezos, 1983). The mechanism of thorium deposition involved localization at the outer region of the wall in *R. arrhizus*. Coordination to amine nitrogen still occurred but the hydrolysis of Th^{4+} to $\text{Th}(\text{OH})_4$ at pH 4 resulted in deposition at the chitin surface. The formation of hydrolysis products favours biosorption; at pH 2, where Th^{4+} is the main thorium species, lower biosorption occurred with a general dispersal of Th^{4+} through the wall (Tsezos & Volesky, 1982*b*). The chitin–chitosan content of fungal walls varies between species, e.g. *Neurospora*

crassa (2.6 % of the wall dry weight) and *R. nigricans* (53 %), and this may change during growth (Volesky, 1990). Purified chitin and chitosan derivatives may also have a biosorptive ability although this may be influenced by the extraction method employed. Isolated chitin from *R. arrhizus* showed decreased uranium biosorption in comparison with intact biomass (Tsezos, 1986). Chitin phosphate and chitosan phosphate were able to remove metal and actinide species from solution, both substances showing a markedly greater affinity for UO_2^{2+} than other metal species including Cu^{2+} , Co^{2+} , Cd^{2+} , Mn^{2+} , Zn^{2+} , Mg^{2+} , Ni^{2+} and Ca^{2+} (Sakaguchi & Nakajima, 1982). Non-phosphorylated chitin and chitosan were not as efficient biosorbents (Sakaguchi & Nakajima, 1982). Insoluble chitosan–glucan complexes and glucans possessing amino- or sugar acid groups from *Aspergillus niger* exhibit biosorptive properties and may efficiently remove transition metal ions from solution (Muzzarelli *et al.*, 1986). Synthesized chitosan derivatives, e.g. *N*-[2-(1,2-dihydroxyethyl)tetra-hydrofuryl] chitosan, can remove uranium from brines at an order of magnitude greater than intact biomass of *R. arrhizus* (Muzzarelli *et al.*, 1986; Macaskie & Dean, 1990*a*). Hydroxide treatment of fungi, to ‘expose’ chitin/chitosan, may also improve biosorptive abilities (Wales & Sagar, 1990).

Melanins are important fungal pigments which enhance the survival of many species in response to environmental stress (Bell & Wheeler, 1986). Fungal melanins are located in and/or exterior to cell walls where they may appear as electron-dense deposits and granules on electron micrographs. These granules may be released into the external medium and be termed ‘extracellular melanin’, although this is generally of identical composition to wall-associated melanin. ‘Extracellular melanins’ are more correctly defined as melanins synthesized exterior to cells by secretion of phenol oxidases which oxidize phenolics or secretion of phenols that are subsequently oxidized (Bell & Wheeler, 1986). Fungal phenolic polymers and melanins contain phenolic units, peptides, carbohydrates, aliphatic hydrocarbons and fatty acids and therefore possess many potential metal-binding sites (Saiz-Jimenez & Shafizadeh, 1984; Senesi, Sposito & Martin, 1987; Sakaguchi & Nakajima, 1987). Oxygen-containing groups in these substances, including carboxyl, phenolic and alcoholic hydroxyl, carbonyl and methoxyl groups may be particularly important in metal binding (Gadd, 1988). The order of binding ability of fungal phenolic polymers and melanins followed the sequence $\text{Cu} > \text{Ca} > \text{Mg} > \text{Zn}$ (Zunino & Martin, 1977).

A variety of melanin types occur in fungi. Although it is apparent that many fungi can form extracellular DOPA (3,4-dihydroxyphenylalanine)-melanin via tyrosinase-mediated oxidation of tyrosine, there is

still no firm evidence for the existence of DOPA-melanin in fungal cell walls (Bell & Wheeler, 1986). Melanins in cell walls of Basidiomycetes are derived from γ -glutaminy-3,4-dihydroxybenzene (GDHB) or catechol. In Ascomycetes and Deuteromycetes, wall melanins are generally synthesized by the pentaketide pathway via 1,8-dihydroxynaphthalene (DHN) as the immediate precursor. Other extracellular dark pigments may be referred to as 'heterogeneous' melanins (Bell & Wheeler, 1986). A variety of heavy metals can induce or accelerate melanin production in fungi and melanized cell forms, e.g. chlamydospores, can have high capacities to adsorb metals with virtually all the metal being located in the cell wall (Mowll and Gadd, 1984). Melanin from *Aureobasidium pullulans* can bind significant amounts of Cu^{2+} and Fe^{3+} (Gadd, 1984; Senesi *et al.*, 1987; Gadd & De Rome, 1988) as well as organometallic compounds, e.g. tributyltin chloride (Gadd, Gray & Newby, 1990).

3. Transport of toxic metal cations*

The plasma and vacuolar membranes are the main transport membranes of fungi and the basic energetics, as well as detailed kinetic analysis, have been established for a variety of solutes, particularly carbon and/or nitrogen sources (Sanders, 1988, 1990). Most work on metal ion transport in fungi has concerned K^+ and Ca^{2+} , largely because of their great importance in fungal growth, metabolism and differentiation. The transport of toxic metal species is still poorly understood.

Transport systems in fungal (and plant) cell membranes are usually classed as either carrier or channel systems. In the former, conformational changes in the transport protein are believed to result in alternate exposure of the transport binding site(s) on each side of the membrane. Carriers include all metabolically-coupled and H^+ -gradient driven transport systems. Fluxes through such systems saturate with respect to ligand concentration and, if a current is carried, with respect to the membrane potential ($\Delta\psi$) (Sanders, 1990). Ion channels are a class of proteins that function as gated pores in the plasma membrane allowing the flow of ions down electrical and/or chemical gradients (Gustin *et al.*, 1986). Channels have higher turnover rates than carriers, 10^{-7} s $^{-1}$ compared to 10^{-4} s $^{-1}$ respectively (Sanders, 1990).

To date, the main primary transport systems which have been characterized in fungi derive energy from ATP hydrolysis and pump H^+ electrogenically from the cytosol, creating a transmembrane electrochemical H^+ gradient ($\Delta\tilde{\mu}_{\text{H}^+}$), negative and alkaline inside. Such an electrochemical gradient ($\Delta\tilde{\mu}_{\text{H}^+}$) has an electrical component, the membrane potential ($\Delta\psi$), and a chemical component, the pH

gradient (ΔpH) which are interconvertible and which can drive the transport of ionizable substances across membranes. Such secondary gradient-coupled transport systems exist for a variety of inorganic and organic solutes which are energized by coupling with passive reflux of H^+ ; proton and solute fluxes may be in the same (symport) or opposing (antiport) directions (Sanders, 1990).

In fungi and yeasts, three main classes of H^+ -pumping ATPases have been identified in mitochondrial, vacuolar and plasma membranes (Serrano, 1984, 1985) though it is now suggested that transport ATPases include a Ca^{2+} -ATPase located on the endoplasmic reticulum (Goffeau *et al.*, 1990). The main function of the mitochondrial ATPase is ATP synthesis via the mitochondrial respiratory chain. It is the plasma membrane and vacuolar ATPases that are associated with ion transport and intracellular compartmentation (see later) and regulation of intracellular pH (Jones & Gadd, 1990).

The transport of monovalent cations is linked to the action of the plasma membrane H^+ -ATPase that expels protons creating the transmembrane electrochemical proton gradient ($\Delta\tilde{\mu}_{\text{H}^+}$) previously described. K^+ uniport in fungi appears to be electrically coupled with H^+ efflux. Under usual cellular and environmental conditions, H^+ is extruded in an apparent 1:1 ratio to K^+ uptake with H^+ able to be extruded against 50:1 concentration gradients and K^+ to be taken up against 1000:1 such gradients (Borst-Pauwels, 1981). However, it is now known that, under K^+ -deficiency, K^+ influx may be coupled with H^+ influx in a H^+ - K^+ symport system. It has been suggested that observations of stoichiometric K^+/H^+ exchange arise because the H^+ pump maintains electroneutrality during K^+ uptake by efflux of one equivalent of H^+ per equivalent of charge flow mediated by symport (Sanders, 1988); the net inflow of positive charge is approximately twice the net K^+ uptake (Rodriguez-Navarro, Blatt & Slayman, 1986). One kind of K^+ uniport is via channels in the fungal plasma membrane (Gustin *et al.*, 1986). Since such channels are not active at the hyperpolarizing (negative) potentials found in intact cells, their physiological role is unclear (Sanders, 1988). It now appears that the plasma membrane of *Saccharomyces cerevisiae* may have at least 2 classes of K^+ -selective channels, the first of which is voltage gated (Gustin *et al.*, 1986) and the second possibly operated via second messengers and sensitive to blocking by divalent cations (Van de Mortel, Theuvenet & Borst-Pauwels, 1990).

The electrophoretic uptake of monovalent cations, e.g. K^+ , Na^+ and Rb^+ , is believed to be driven by the electrical component of $\Delta\tilde{\mu}_{\text{H}^+}$, the membrane potential ($\Delta\psi$) but, as mentioned above, there is still a possibility that these cations are taken up with protons inferring involvement of ΔpH (Serrano, 1984; Ramos *et al.*, 1985; Borst-Pauwels, Boxman &

Theuvenet, 1985). Most information is available for K^+ since this ion is preferentially taken up by the monovalent cation transport system (Conway & Duggan, 1958; Borst-Pauwels, 1981). Early work described this system as 'the physiological K^+ carrier' although other monovalent ions including Rb^+ , Na^+ , Cs^+ , Li^+ and NH_4^+ may be transported with lower affinities (Jennings, Hooper & Rothstein, 1958; Armstrong, 1964). Ca^{2+} and Mg^{2+} can also be taken up via the K^+ transport system though the affinity for these cations is very low (Borst-Pauwels, 1981). The relative order of affinities is:



Thus, Na^+ is generally excluded from cells and is present at much lower internal concentrations than K^+ . However, a considerable proportion of cellular K^+ can be replaced by Na^+ by repeated culture in Na^+ -rich medium containing little or no K^+ . In Na^+ -loaded cells, K^+ uptake is balanced by both Na^+ and H^+ efflux (Borst-Pauwels, 1981). Cellular K^+ can also be partially replaced by Rb^+ , Cs^+ , Li^+ and NH_4^+ by cultural manipulations (Armstrong, 1964; Borst-Pauwels, 1981, 1989).

The K_m for uptake of K^+ is approximately $500 \mu M$ with maximum rate occurring at $1.6 mM$ in the absence of other M^+ ions (Suomalainen & Oura, 1971). The K_m for Na^+ is about 25 times lower than that of K^+ (Conway & Duggan, 1958). K^+ and Rb^+ are competitive inhibitors of the uptake of all other M^+ with these other ions giving non-competitive inhibition of K^+ or Rb^+ uptake. For example, at a $pH > 5.0$, a Na^+/K^+ ratio of 20:1 will give 70% inhibition of K^+ uptake. As the pH is progressively decreased below $pH 7.0$, the selectivity for K^+ and Rb^+ over all other ions is increased. In a 1:1 mixture of K^+ and Na^+ at $pH 4.0$, the combined effects of competitive inhibition of K^+ against Na^+ and the effect of low pH against Na^+ mean that Na^+ uptake becomes negligible (Armstrong & Rothstein, 1964).

It should also be noted that the kinetics of K^+ transport can alter depending on growth and assay conditions. In arginine-containing medium lacking Na^+ and NH_4^+ , *S. cerevisiae* grew maximally at $5 \mu M$ K^+ . Cells grown under these conditions absorbed K^+ with an apparent K_m of $24 \mu M$ whereas, at a high medium K^+ concentration, the apparent K_m was $2 mM$ K^+ (Rodriguez-Navarro & Ramos, 1984). Such results may be explained by the existence of two K^+ transport systems or one system operating in two different ways. In addition, the Na^+/K^+ selectivity of yeast depends on the stage of growth, a greater proportion of K^+ being accumulated in the logarithmic phase than in the stationary phase (Rodriguez-Navarro & Ramos, 1984; Jones & Gadd, 1990).

The kinetics of monovalent cation transport are complex, with more than one binding site involved in translocation, and may be affected by the surface

and membrane potential (Borst-Pauwels, 1981; Jones & Gadd, 1990). Kinetic deviations in K^+ uptake are found at low pH and also at high pH in the presence of other monovalent and divalent ions, e.g. Li^+ , Na^+ , Cs^+ , Mg^{2+} and Ca^{2+} . In addition, there can be surface potential effects and the K_m for cation uptake may increase with increasing substrate concentration which will also give rise to deviations from Michaelis-Menten kinetics (Theuvenet & Borst-Pauwels, 1983). This effect is more likely to occur at low pH . Furthermore, when the cell membrane is depolarized by, e.g. dinitrophenol, proton symport or at high substrate cation concentrations, the dissociation constants of the cations increase. Thus, deviations from Michaelis-Menten kinetics may result from a decrease in the negative membrane potential, a decrease in the surface potential and interactions with the modifier site (Borst-Pauwels, 1981).

Both the electrical component ($\Delta\psi$) and the chemical component (ΔpH) of the proton gradient ($\Delta\tilde{\mu}_{H^+}$) can vary with the external and intracellular pH and the external ion concentration thus affecting transport (Jones & Gadd, 1990). In yeast cells a ($\Delta\tilde{\mu}_{H^+}$) of approximately $-150 mV$ at $pH 4.5$ to $-90 mV$ at $pH 7.0$ is maintained (De la Pena *et al.*, 1982). Inhibition of K^+ uptake by high external K^+ concentrations can be explained by its ability to depolarize the plasma membrane as $\Delta\tilde{\mu}_{H^+}$ decreases at pH values between 4.5 and 7.0 at an external K^+ concentration of $200 mM$ (De la Pena *et al.*, 1982), the main component affected being $\Delta\psi$. Since the internal K^+ concentration in yeast may be 100 – $200 mM$ or above (Boxman *et al.*, 1984), K^+ uptake cannot efficiently occur at external concentrations of K^+ similar to these; the effect of increasing external K^+ is to decrease $\Delta\tilde{\mu}_{H^+}$ (De la Pena *et al.*, 1982). As described, $\Delta\tilde{\mu}_{H^+}$ and $\Delta\tilde{\mu}_{K^+}$ are closely related in yeast cells, probably through the common component of $\Delta\psi$ (De la Pena *et al.*, 1982).

Potentially toxic metal ions, e.g. Cu^{2+} , Co^{2+} , Ni^{2+} , Cd^{2+} , Mn^{2+} , mercurials and other organometallic compounds, can inhibit the yeast plasma membrane ATPase by means of various binding interactions, both specific and non-specific (Ochiai, 1987; White & Gadd, 1987*a, b*). Such deleterious effects lead to a reduced ability in maintenance of the electrochemical gradients described. For H^+ efflux, heavy metal inhibition generally increased with increasing metal concentration with a toxicity sequence of $Cd^{2+} > Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}$, Mn^{2+} . These metals also inhibited K^+ uptake with a toxicity sequence of $Cd^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+}$, $Mn^{2+} > Zn^{2+}$. K^+ uptake was considerably more affected by these metals than was H^+ efflux which suggested that the inhibition of K^+ uptake was not solely a result of inhibition of the H^+ -ATPase and other factors such as increased membrane permeability to K^+ may have contributed (White & Gadd, 1987*a, b*).

Many divalent cations, such as Mg^{2+} , Ca^{2+} , Cu^{2+} , Zn^{2+} and Mn^{2+} , which are essential for growth and metabolism need to be accumulated from the external environment. However, above certain concentrations, many are toxic and may cause impairment of cell metabolism, including ionic nutrition, and ultimately cell death (see Section III). Inessential metals may also be accumulated, e.g. Cd^{2+} and Hg^{2+} . Some divalent cations, such as Ca^{2+} and Mg^{2+} , may enter *S. cerevisiae* as low-affinity substrates of the monovalent cation transport system but this is probably of little significance in relation to the systems that will now be described.

In filamentous fungi and yeasts, energy-dependent transport of many divalent cations has been demonstrated, the apparent affinity series being Mg^{2+} , Co^{2+} , $\text{Zn}^{2+} > \text{Mn}^{2+} > \text{Ni}^{2+} > \text{Ca}^{2+} > \text{Sr}^{2+}$ (Fuhrmann & Rothstein, 1968). However, differences in accumulation may not be due to differences in affinities for the transport mechanism as some reductions in the net rate of Ca^{2+} and Sr^{2+} uptake, for example, may be due to increased leakage or efflux of these cations. In addition, depending on the metal and its concentration, there may be effects on the structural integrity of the membrane (Gadd & Mowll, 1983; White & Gadd, 1987a). As with monovalent cations, uptake of divalent cations is inhibited or halted by metabolic inhibitors, low temperatures and the absence of energy-yielding substrates (Gadd & White, 1989a; Sabie & Gadd, 1990; Starling & Ross, 1990). It follows that uptake thus depends on the metabolic state of the cell and may vary with different growth media and conditions. For maximal uptake rates, cells require adequate K^+ and phosphate (Fuhrmann & Rothstein, 1968; Okorokov *et al.*, 1975; Roomans *et al.*, 1979) and the phosphate requirement may be indirect and related to the energetic state of the cell as shown for Mn^{2+} (Borst-Pauwels, 1981).

Divalent cation transport is dependent on plasma membrane H^+ -ATPase activity and a similar affinity sequence of divalent cations for stimulation of the ATPase as for transport (see above) has been demonstrated (Fuhrmann, 1974). Diethylstilboestrol (DES) is an effective *in vitro* and *in vivo* inhibitor of the plasma membrane H^+ -ATPase (Bowman *et al.*, 1978; Serrano, 1985) and also inhibits the uptake of divalent cations (Borst-Pauwels, 1981; White & Gadd, 1987a). However, a direct role of the H^+ -ATPase has been discounted for several reasons. Initial transport rates of Mn^{2+} and Sr^{2+} in *S. cerevisiae* were similar, yet Mn^{2+} strongly stimulated the plasma membrane H^+ -ATPase whereas Sr^{2+} had no effect (Roomans *et al.*, 1979; Nieuwenhuis, Weijers & Borst-Pauwels, 1981). Even though influx rates of these two ions were similar, much more Mn^{2+} was subsequently accumulated than Sr^{2+} which suggested efflux systems and vacuolar Mn^{2+} compartmentalization (Nieuwenhuis *et al.*, 1981) (see later). Calcium ions enter cells via ligand- or

voltage-gated channels. These are membrane proteins which, when in the open position, allow passive flux of Ca^{2+} down the electrochemical gradient, $\Delta\tilde{\mu}_{\text{Ca}^{2+}}$ (Miller *et al.*, 1990). Influx of Ca^{2+} may be difficult to distinguish because of its energy-dependent accumulation in fungal vacuoles (Eilam *et al.*, 1985a).

It appears that the major role of the plasma membrane H^+ -ATPase in the uptake of divalent cations is one of energizing the cell membrane by creating the electrochemical gradient previously mentioned. Transport depends on the membrane potential (Boutry, Foury & Goffeau, 1977; Borst-Pauwels, 1981; Eilam & Chernichovsky, 1987; White & Gadd, 1987a; Budd, 1988, 1989) and uptake is inhibited by substances, such as protonophoric uncouplers and high concentrations of external K^+ , which depolarize the cell membrane (Roomans *et al.*, 1979; Borbolla & Pena, 1980; Gadd & White, 1989a). Conversely, divalent cation uptake may be enhanced under conditions where the membrane potential is increased, e.g. by enhanced K^+ efflux (Eilam, 1984; Eilam, Lavi & Grossowicz, 1985b; Gadd & Mowll, 1985; Eilam & Chernichovsky, 1987). For *S. cerevisiae* treated with high concentrations of ATPase inhibitors, e.g. DCCD, the resulting hyperpolarization of the membrane caused increased Ca^{2+} uptake. The increase in $\Delta\psi$ balanced the decrease in pH so that the overall value of $\Delta\tilde{\mu}_{\text{H}^+}$ remained almost constant (Eilam, Lavi & Grossowicz, 1984). However, changes in $\Delta\psi$ only partly account for glucose-stimulated Ca^{2+} influx in this yeast with intracellular acidification also being involved (Eilam, Othman & Halachmi, 1990). Furthermore, it should be stressed that inhibitor studies should be treated with caution since the enhancement of M^{2+} uptake in yeast induced by several plasma membrane ATPase inhibitors was due to increased cation permeability of the membrane rather than hyperpolarization (Borst-Pauwels, 1988). Similar conditions may apply to interactions of yeast with those metal cations or xenobiotics which are potentially toxic and induce K^+ efflux (Gadd & Mowll, 1983; Gadd, 1986a, c; Theuvenet *et al.*, 1987; Borst-Pauwels, 1988; Belde *et al.*, 1988).

Divalent cation uptake, as measured in short-term experiments, is frequently accompanied by efflux of monovalent cations, usually K^+ , but H^+ for Ca^{2+} and Mg^{2+} since uptake can be by means of the monovalent cation transport system. Such efflux may be involved in the maintenance of electroneutrality and a $1\text{M}_{\text{in}}^{2+}:2\text{K}_{\text{out}}^{+}$ stoichiometry is sometimes observed (Fuhrmann & Rothstein, 1968). Coupled M^{2+} uptake and K^+ efflux may occur without a $2\text{K}_{\text{out}}^{+}:1\text{M}_{\text{in}}^{2+}$ relationship in *S. carlsbergensis* where K^+ efflux may be linked to other energy utilizing processes such as polyphosphate synthesis (Okorokov *et al.*, 1983a; Okorokov, Lichko & Andreeva, 1983b). Divalent cation transport in *S. carlsbergensis* is complex with

uptake of Mn^{2+} , Mg^{2+} and Zn^{2+} being accompanied by K^+ efflux. However, uptake of Co^{2+} or Ni^{2+} does not result in K^+ efflux in this yeast which may indicate two different transport systems for these cations. It has been suggested that the first group of cations discharges the K^+ gradient across the plasma membrane and uses its energy for transport whereas the transport system for Co^{2+} and Ni^{2+} is independent of the K^+ gradient (Okorokov *et al.*, 1983*a*; Okorokov, 1985). Abolition of the K^+ gradient by candicydin, for example, inhibits Mn^{2+} uptake (Lichko, Okorokov & Kulaev, 1980). Inhibition of Mn^{2+} uptake by reagents that abolish both the H^+ and K^+ gradients indicate the involvement of both these gradients in the energization of Mn^{2+} (Mg^{2+} and Zn^{2+}) uptake. Uptake of Co^{2+} and Ni^{2+} is commonly energized by the H^+ gradient (Okorokov, 1985) and that of the former is insensitive to inhibitors of the plasma membrane H^+ -ATPase, such as DES and DCCD (up to 200 μM) whereas Mn^{2+} uptake and H^+ efflux are completely blocked. However, 1 mM DNP inhibits Co^{2+} uptake by up to 80 %. The insensitivity of Co^{2+} uptake to DES and DCCD but sensitivity to the protonophore (DNP) suggest the existence of an additional H^+ pump in the plasma membrane that energizes Co^{2+} uptake when the H^+ -ATPase is blocked (Okorokov, 1985).

During Mn^{2+} uptake by *S. carlsbergensis*, the $\text{K}_{\text{out}}^+:\text{Mn}_{\text{in}}^{2+}$ stoichiometry varies from 0 to 7 and is equal to 7 and 5.5 after 10 and 20 min respectively. Such effluxes of K^+ exceed values for electroneutral exchange (Okorokov *et al.*, 1983*b*). However, the excess of the K^+ efflux is used for the synthesis of high-molecular-weight polyphosphates (HPP), i.e. K^+ efflux results in H^+ influx through the polyphosphatase or pyrophosphatase with resulting synthesis of HPP or pyrophosphate on the plasma membrane (Okorokov, 1985). Thus, the K^+ gradient can act as an energy buffer coupled with the H^+ gradient (Okorokov *et al.*, 1983*a, b*; Okorokov, 1985). More recently, an energy-dependent K^+ efflux system has been demonstrated in *S. cerevisiae* which was stimulated by several cationic organic molecules and also Al^{3+} (Pena & Ramirez, 1991).

However, K^+ release may not always be associated with divalent cation uptake or may show no simple stoichiometry (Gadd & White, 1989*a*). It is possible that K^+ efflux may not be detected if K^+ is re-taken into cells by means of the monovalent cation transport system (Borst-Pauwels, 1981). However, in many instances, and particularly where potentially-toxic metal ions are concerned, the kinetics of metal uptake and its relationship with K^+ efflux are obscured by toxic effects (Gadd & White, 1989*a*). As already mentioned, toxic metal ions can repress the plasma membrane H^+ -ATPase and inhibit H^+ efflux and K^+ uptake (White & Gadd, 1987*a, b*) and K^+ efflux can also result from treatment with ATPase inhibitors (Borst-Pauwels *et al.*, 1983). K^+ efflux

induced by Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} and Cd^{2+} showed two phases, reversible at low concentrations ($\leq 5 \mu\text{M}$) and irreversible at high concentrations (5–100 μM depending on the metal examined) which also caused complete inhibition of K^+ uptake (White & Gadd, 1987*a, b*). K^+ loss from *S. cerevisiae* in response to toxic agents may be an all-or-none effect for individual cells (Kuypers & Roomans, 1979; Borst-Pauwels, Theuvenet & Stols, 1983; Theuvenet *et al.*, 1983, 1987; Belde *et al.*, 1988; Borst-Pauwels, 1988). When a population of this yeast consisting of Cd^{2+} -sensitive and Cd^{2+} -resistant cells were exposed to Cd^{2+} , the Cd^{2+} -resistant cells subsequently absorbed the K^+ lost by the sensitive cells (Belde *et al.*, 1988). Since batch populations will be heterogeneous with respect to metal sensitivity, this phenomenon provides one explanation for reversible K^+ loss. Such observations also mean that measured net K^+ efflux from yeast cells exposed to xenobiotics is less than K^+ efflux from the affected cells where an all-or-none process occurs (Borst-Pauwels, 1988). Assays where K^+ release is used as an index of toxicity should therefore be cautiously interpreted and preferably in conjunction with alternative estimations of viability (Mowll & Gadd, 1983; Borst-Pauwels *et al.*, 1983; Theuvenet *et al.*, 1987; Borst-Pauwels, 1988). In addition, where a potentially-toxic divalent cation induces K^+ efflux by increasing membrane permeability, the uptake of other cations may be increased. Thus, Cd^{2+} stimulated Ca^{2+} uptake in yeast by increasing the cation permeability of the cell membrane (Kessels, Belde & Borst-Pauwels, 1987). That such permeabilization can lead to enhanced cation uptake is shown by the fact that DEAE-dextran, which permeabilizes the plasma membrane without affecting the vacuolar membrane, also enhances Ca^{2+} uptake (Theuvenet *et al.*, 1986).

Divalent cation transport is concentration-dependent and frequently shows saturation with increasing external concentration. Michaelis–Menten kinetics may be obeyed (Fuhrmann & Rothstein, 1968; Norris & Kelley, 1977; Borst-Pauwels, 1981) although there is extensive variation in reported kinetic parameters (Borbolla & Pena, 1980; Borst-Pauwels, 1981; White & Gadd, 1987*a*; Gadd & White, 1989*a*). One reason for this could be the existence of two or more transport systems of differing affinities (Norris & Kelly, 1977). Other possibilities include the existence of a 2-site translocation process or deviations resulting from the reduction in surface potential owing to cation binding at negatively-charged sites in the cell wall or membrane (Roomans *et al.*, 1979; Borst-Pauwels, 1981; Borst-Pauwels & Theuvenet, 1984). In fact, the concentration dependence of the rate of divalent cation uptake can be described by a simple diffusion process after accounting for the effects of surface potential on the M^{2+} concentration near the cell membrane (Borst-Pauwels & Theuvenet, 1984).

More mundane reasons for variation in kinetic parameters include differences between strains and experimental protocols, effects of binding or complexation involving wall materials, components of the buffer or suspending medium which may reduce the free ion concentration and toxic effects. The last is likely to be significant for cations such as Zn^{2+} , Cu^{2+} , Co^{2+} and Cd^{2+} and other heavy metals. Even low concentrations can affect the structural integrity of the cell membrane which can alter the kinetics of uptake. Progressive inhibition of H^+ extrusion could result in de-energization of the plasma membrane while increased membrane permeability can result in K^+ efflux (Kuypers & Roomans, 1979; White & Gadd, 1987*a, b*). For uptake of Zn^{2+} by *S. cerevisiae*, an apparent second phase of low-affinity transport coincided with such effects which suggested that altered kinetics were a result of increased membrane permeability (White & Gadd, 1987*a*). In fact, if affinity constants are compared for a range of potentially toxic divalent cations, e.g. Zn^{2+} , Cu^{2+} , Cd^{2+} , Ni^{2+} and Co^{2+} , it seems that, where the concentration range used is high, the apparent affinity of the transport system is low (Gadd & White, 1989*a*). Thus, the results of kinetical analyses must be treated with caution, particularly where toxic symptoms are manifest. However, at low, non-toxic, concentrations, it now seems clear that high affinity uptake systems exist for, e.g. Mn^{2+} and Zn^{2+} in filamentous fungi and yeasts (Parkin & Ross, 1985, 1986*a, b*; White & Gadd, 1987*a*; Hockertz, Schmid & Auling, 1987; Starling & Ross, 1990; Pilz *et al.*, 1991).

For potentially-toxic divalent metal cations, reduced transport into yeast cells is a frequently observed mechanism of resistance, e.g. in metal-resistant strains of *S. cerevisiae* (Joho *et al.*, 1983, 1991; Joho, Imada & Murayama, 1987; Gadd *et al.*, 1984*b*; Gadd, 1986*a*). However, a Mn^{2+} -resistant strain accumulated more Mn^{2+} than the wild-type strain, possibly by means of a more efficient internal sequestration system (Bianchi, Carbone & Lucchini, 1981*a*; Bianchi *et al.*, 1981*b*). Those external factors that can reduce uptake of toxic cations may also result in protection, e.g., a low external pH reduces the influx and toxicity of many metal ions (Gadd, 1986*a*; 1988; Gadd & White, 1989*a*) while protection of yeast cells by Ca^{2+} from Cd^{2+} toxicity was due to a reduction in Cd^{2+} uptake in the presence of Ca^{2+} (Kessels, Belde & Borst-Pauwels, 1985). For Li^+ , a low net entry into cells results from an electrogenic H^+/Li^+ antiport (Rodriguez-Navarro, Sancho & Perez-Lloveres, 1981). Extrusion pumps have also been demonstrated for divalent cations, e.g. Ca^{2+} and Sr^{2+} (Nieuwenhuis *et al.*, 1981; Theuvenet *et al.*, 1986). Earlier evidence indicated the existence of a $\text{Ca}^{2+}/\text{H}^+$ antiport on the plasma membrane although it is now proposed that Ca^{2+} efflux is via a $\text{H}^+/\text{Ca}^{2+}$ -ATPase with a stoichiometric

ratio of at least $2\text{H}^+/\text{Ca}^{2+}$ in *Neurospora crassa* (Miller *et al.*, 1990).

As noted above, a variety of fungi, including yeasts, secrete iron-chelating siderophores which remove iron from insoluble ferric forms and facilitate its transport into cells (Raymond *et al.*, 1984). Another strategy found in certain plants involves maintenance of iron in a soluble form by the secretion of protons and/or reducing agents (Olsen & Brown, 1980). In *S. cerevisiae*, Fe^{2+} is taken up faster than Fe^{3+} and uptake is strongly inhibited by the Fe^{2+} -trapping agent, ferrozine. Iron was physiologically reduced by an iron-induced *trans*-plasma membrane redox system and it seems that uptake of Fe^{2+} in *S. cerevisiae* is via a similar mechanism as for other divalent cations (Lesuisse, Raguzzi & Crichton, 1987; Lesuisse & Labbe, 1989; Lesuisse, Crichton & Labbe, 1990).

4. Intracellular fate of toxic metals*

(a) Metal-binding proteins and peptides of filamentous fungi and yeasts

(i) *Classification.* Metal-binding proteins are important in the modulation of intracellular concentrations of both potentially-toxic and essential metal ions. The super-family of proteins called metallothioneins, first discovered in mammalian systems, may achieve these roles by binding metal ions to cysteine thiolate groups (Hamer, 1986). It is now clear that a variety of metal-binding polypeptides are found in animals, plants and fungi and there has been some progress in devising a coherent nomenclature (Rausser, 1990). Polypeptides are designated 'metallothioneins' if they have properties which include low molecular mass, high metal content, high cysteine (Cys) content, lack aromatic amino acids and histidine, possess abundant Cys-X-Cys sequences (where X is an amino acid other than Cys), spectroscopic properties characteristic of metal thiolates, and metal-thiolate clusters, all features characteristic of equine renal metallothionein. Subdivision into three classes has been recommended (Rausser, 1990):

Class I: Polypeptides with locations of cysteine closely related to those in equine renal metallothionein.

Class II: Polypeptides with locations of cysteine only distantly related to those in equine renal metallothionein.

Class III: Atypical, non-translationally synthesized metal thiolate polypeptides.

Class I includes most vertebrate forms as well as some fungal metallothioneins, e.g. those of *Neurospora crassa* and *Agaricus bisporus* (Lerch & Beltramini, 1983; Munger & Lerch, 1985). Class II metallothioneins are found in *Saccharomyces cerevisiae* (Steffens, 1990). Algae, plants and certain

fungi, e.g. *Schizosaccharomyces pombe* and *Candida glabrata*, produce class III metallothionein (Rauser, 1990). The last occur as metal-binding complexes which behave like entities of relative molecular mass (M_r) 10000–13800 in gel filtration (Rauser, 1990). However, these complexes are aggregates of a heterogeneous group of polypeptides for which no single name is suitable. In this article, the term metal γ -glutamyl peptide will be used (Winge *et al.*, 1989) although, in the context of plants, this may be inappropriate because of the diversity of γ -glutamyl di- and tripeptides which may occur (Rauser, 1990).

(ii) *Metallothioneins*. Metallothioneins are small, cysteine-rich polypeptides that can bind essential metals, e.g. Cu and Zn, as well as inessential metals such as Cd. Copper resistance in *S. cerevisiae* can be mediated by the induction of a 6573 Da cysteine-rich protein, copper-metallothionein (Cu-MT) (Hamer, 1986; Butt & Ecker, 1987; Fogel, Welch & Maloney, 1988). This protein normally functions to maintain low concentrations of intracellular copper ions and thus prevent futile transcription of the *CUP1* structural gene (Wright, Hamer & McKenney, 1988). Although yeast metallothionein can also bind Cd and Zn *in vitro*, it is not transcriptionally induced by these ions and apparently does not protect against them (Butt *et al.*, 1984; Winge *et al.*, 1985).

S. cerevisiae contains a single Cu-MT gene which is present in the *CUP1* locus located on chromosome VIII (Fogel & Welch, 1982; Karin *et al.*, 1984). Copper-resistant *S. cerevisiae* strains (*CUP1*^r) contain 2–14 or more copies of the *CUP1* locus, which are randomly repeated, and may grow on media containing ≥ 2 mM copper (Fogel, Welch & Karin, 1983; Karin *et al.*, 1984). Copper-sensitive strains (*cup1*^s) do not grow at concentrations ≥ 0.15 mM (Butt & Ecker, 1987). Continuous subculture of *S. cerevisiae* in the presence of increasing copper concentrations can select for hyper-resistant strains (White & Gadd, 1986) which appear disomic for chromosome VIII. The disomic chromosomes exhibit differential patterns of *CUP1* gene amplification which indicates that the copper resistance mechanism involves not only amplification of the *CUP1* locus on the chromosomes but also disomy or aneuploidy of chromosome VIII (Fogel *et al.*, 1983; Butt & Ecker, 1987). The DNA fragments of the *CUP1* locus that confer copper resistance have been cloned (Fogel & Welch, 1982; Butt *et al.*, 1984; Henderson, Cox & Tubb, 1985) and evidence that the *CUP1* locus was present as multiple copies in *CUP1* strains was obtained after restriction enzyme analysis of the cloned DNA (Butt & Ecker, 1987; Fogel *et al.*, 1988). The basic repeating unit '*CUP1* locus' is composed of 2.0 kb DNA fragments containing, with respect to restriction enzymes, a unique *Xba* I site and two sites for *Kpn* I and *Sau*3A. The full nucleotide sequence of the *CUP1*

locus has been determined (Karin *et al.*, 1984).

Copper resistance in *S. cerevisiae* depends on amplification of the *CUP1* locus (Butt & Ecker, 1987; Fogel *et al.*, 1988). In simple terms, the gene amplification model suggests that, because of the proposed homology of DNA sequences at the junction of *CUP1* repeats, one or more units are looped out. If the loop is replicated, the copy number increases (amplification) but if the loop is degraded, the copy number decreases (deamplification) (Fogel *et al.*, 1983; Butt & Ecker, 1987; Fogel *et al.*, 1988). For more precise accounts of this model, readers should consult the detailed reviews available (Hamer, Thiele & Lemontt, 1985; Thiele & Hamer, 1986; Hamer, 1986; Butt & Ecker, 1987; Fogel *et al.*, 1988). It should be noted that Cu-MT is not directly involved in Cu²⁺ uptake in *S. cerevisiae* (Lin & Kosman, 1990).

The mechanism by which copper ions induce transcription of the Cu-MT gene has received detailed attention (Thiele & Hamer, 1986; Butt & Ecker, 1987; Thiele, 1988; Fürst *et al.*, 1988; Fürst & Hamer, 1989; Culotta *et al.*, 1989; Welch *et al.*, 1989; Hu, Fürst & Hamer, 1990). It is now known that a *trans*-acting regulatory protein, encoded by the *ACE1* locus (Thiele, 1988; Fürst *et al.*, 1988) and also known as the *CUP2* locus (Welch *et al.*, 1989; Buchman *et al.*, 1989), activates transcription of the Cu-MT gene in response to excess copper (or silver) ions (Hu *et al.*, 1990). Although the *ACE1* gene is constitutively expressed in the absence or presence of copper, the apoprotein cannot bind DNA (Fürst *et al.*, 1988). However, in the presence of Cu(I) [or Ag(I)], the amino-terminal portion of the ACE1 protein (amino acids 1–122) undergoes a conformational change which allows the protein specifically to bind to the *CUP1* upstream activator sequence (UAS) (Fürst *et al.*, 1988; Huibregtse, Enkelke & Thiele, 1989). This metal-dependent DNA-binding domain is rich in cysteines and basic amino acids, and bears structural similarities to yeast Cu-MT itself (Fürst *et al.*, 1988; Fürst & Hamer, 1989; Hu *et al.*, 1990; Dameron *et al.*, 1991). It is proposed that the cysteine residues of ACE1 form a polynuclear Cu(I)–S core in which cysteine thiols coordinate multiple Cu(I) ions and Cu(I) ions which are bound by multiple sulphur bonds (Hu *et al.*, 1990). Wrapping the protein around this Cu(I)–S scaffold would lead to the formation of positively charged loop structures that are postulated specifically to bind DNA and activate transcription (Fürst *et al.*, 1988). It therefore appears that the role of Cu in yeast Cu-MT regulation is to allow ACE1 to bind to the control sequences of the metallothionein gene (Fig. 3) (Hu *et al.*, 1990); the Cu cluster organizes and stabilizes the conformation of the N-terminal domain of ACE1 for specific DNA binding (Dameron *et al.*, 1991).

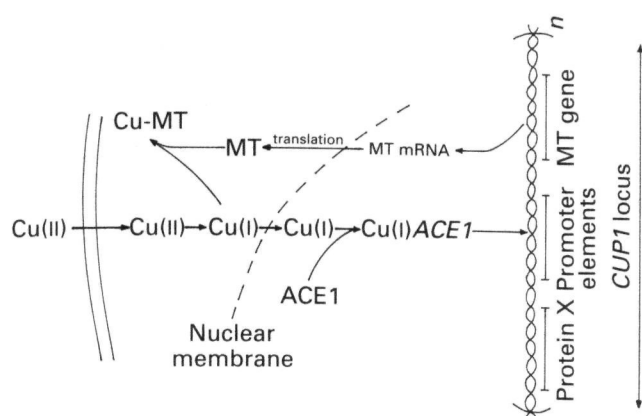


Figure 3. Metalloregulation of the *CUP1* locus in *Saccharomyces cerevisiae*. The Cu-ACE1 metalloprotein complex interacts with upstream sequences from the *CUP1* coding sequences and facilitates transcription of the MT gene with the *CUP1* locus. Translation of the MT mRNA yields MT protein which acts to regulate the cytosolic concentration of copper ions (adapted, with permission, from Mehra & Winge, 1991).

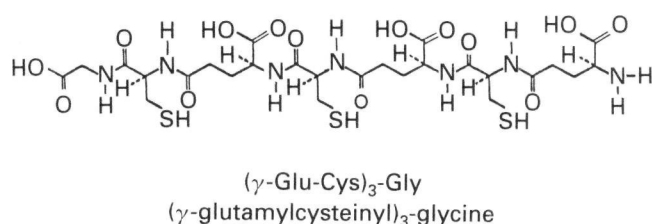
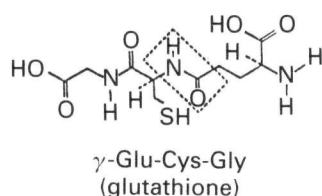


Figure 4. Structures of glutathione and the metal-binding polypeptide, $(\gamma\text{-glutamylcysteinyl})_3\text{-glycine}$. Peptide bonds between glutamate and cysteine utilize the side chain, or γ , carboxylate of glutamate (outlined) rather than the α -carboxylate characteristic of proteins whose synthesis is ribosome dependent (adapted from Steffens, 1990).

This metal-regulated system provides a powerful tool in biotechnology. Metal-regulated DNA sequences are efficient elements for heterologous gene expression and a variety of proteins have been expressed in the *CUP1* expression system including human serum albumin, human ubiquitin gene, *E. coli galK* gene and human hepatitis virus antigen (Gorman *et al.*, 1986; Butt & Ecker, 1987). Yeast Cu-MT may also be of potential in metal recovery since it can bind other metals besides Cu, e.g. Cd, Zn, Ag, Co and Au, although these metals do not induce MT synthesis (Butt & Ecker, 1987). The Cu-MT gene has also been used to transform brewing strains of *S. cerevisiae*, which are not very amenable to classical genetic studies, and copper selection has proved a useful tool for the genetical manipulation of such strains and product improvement (Butt *et al.*,

1984; 1988; Henderson *et al.*, 1985; Butt & Ecker, 1987). When *CUP1* was cloned into *Escherichia coli*, the bacterial cells acquired a new inducible ability to sequester toxic metals by metallothionein synthesis (Berka *et al.*, 1988).

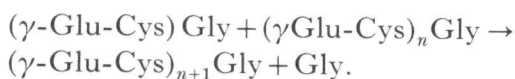
It should be mentioned that a Cd-resistant strain of *S. cerevisiae* synthesized a cytoplasmic Cd-binding protein when grown in Cd-containing medium (Joho, Imai & Murayama, 1985a; Joho, Fujioka & Murayama, 1985b; Joho, Yamanaka & Murayama, 1986). This protein (9 kDa) exhibits the characteristics of a metallothionein, being rich in cysteine (18 mol %) and having a high Cd content ($63 \mu\text{g Cd (mg protein)}^{-1}$). There was a high similarity in amino acid composition between Cd- and Cu-MT of *S. cerevisiae* (Inouhe *et al.*, 1989). The Cd-MT of Cd-resistant *S. cerevisiae* is also encoded by the *CUP1* gene and transcription of the *CUP1* gene occurs in the absence of added metal ions, unlike the inducible transcription in the Cu-resistant strain. Cadmium and copper further increase the rate of transcription of the *CUP1* gene in the Cd-resistant strain (Tohoyama *et al.*, 1992).

Genetical analyses of metal-regulated genes from some other fungi are also available. The Cu-MT gene of *Neurospora crassa* has been cloned and the nucleotide sequence determined; this gene codes for a 26 amino acid protein (Munger, Germann & Lerch, 1985; 1987). It contains 7 different amino acids and 28 % is composed of cysteine; it appears that copper is present in the Cu(I) form and all the cysteines are ligated to form Cu(I) complexes with 6 copper molecules per mol of protein (Lerch, 1980; Lerch & Beltramini, 1983). The isolated protein from *S. cerevisiae* contains 8 mol of copper [Cu(I)] ligated to 12 cysteines per mol protein (Winge *et al.*, 1985; George, Byrd & Winge, 1988). There is little detailed genetical information available for other fungi. While, e.g. Cd- and Cu-resistant mutants of *Aspergillus nidulans* (Cooley, Haslock & Tomsett, 1986; Tomsett *et al.*, 1989; Phelan, Thurman & Tomsett, 1990) and Cd-resistant mutants of *N. crassa* (Levine & Marzluf, 1989) have been isolated and characterized, resistance mechanisms have not been elucidated. While metal-binding proteins or peptides are an obvious suggestion (Tomsett *et al.*, 1989), it seems that altered uptake and morphological changes may also be involved (Levine & Marzluf, 1989; Phelan *et al.*, 1990).

(iii) *Metal γ -glutamyl peptides*. In addition to cysteine-rich polypeptides of the metallothionein family, another group of metal-binding molecules synthesized by fungi are short, cysteine-containing γ -glutamyl peptides, encompassed by the trivial name 'phytochelatins' (Winge *et al.*, 1989; Rauser, 1990). They are also sometimes referred to as 'cadystins' (Mutoh, Kawabata & Hayashi, 1991). These γ -glutamyl peptides are the main metal

detoxification mechanism in algae and plants (Grill *et al.*, 1988; Gekeler *et al.*, 1988) as well as in a range of filamentous fungi and yeasts. They have the general formula $(\gamma\text{Glu-Cys})_n\text{-Gly} [(\gamma\text{-EC})_n\text{G}]$ (Fig. 4) (Grill, Winnacker & Zenk, 1986). Peptides of $n = 2\text{--}5$ appear to be the most common (Reese *et al.*, 1988) and metal ions are bound within a metal-thiolate cluster composed of an oligomer of peptides (Winge *et al.*, 1989). Phytochelatins are apparently not synthesized by *S. cerevisiae* but *Schizosaccharomyces pombe* synthesises at least seven different homologous phytochelatins in response to metals (Grill *et al.*, 1986).

The similarity in structure between glutathione and γ -glutamyl peptides indicates that biosynthesis of these peptides shares common features with that of glutathione (Steffens, 1990) and, in fact, they may be viewed as linear polymers of the γ -glutamyl-cysteine ($\gamma\text{-Glu-Cys}$) portion of glutathione (Grill *et al.*, 1989). Because of the repetitive γ -glutamic acid bonds, they cannot be regarded as primary gene products and must be formed by a ribosome-independent enzyme reaction (Steffens, 1990). Linkage of glutathione metabolism with that of γ -glutamyl peptides has been kinetically demonstrated *in vivo* (Grill, Winnacker & Zenk, 1987; Steffens, 1990) and also by using mutants of *S. pombe* which lacked the glutathione biosynthetic enzymes, γ -glutamylcysteine synthetase and glutathione synthetase. These were unable to synthesise glutathione or $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ peptides and were hypersensitive to Cd (Mutoh & Hayashi, 1988). This work provided the first genetical evidence that γ -glutamyl peptides were essential for Cd-detoxification and clearly demonstrated biochemical linkage with glutathione metabolism. It is now known that γ -glutamyl peptides are synthesized by the enzyme γ -glutamyl-cysteine dipeptidyl transpeptidase ('phytochelatin synthase'). This catalyses the transfer of the γ -glutamylcysteine dipeptide moiety of glutathione to an acceptor glutathione molecule or a growing chain of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ oligomers, thus synthesizing metal-binding γ -glutamyl peptides (Grill *et al.*, 1989). The primary reaction can be expressed:



Cd^{2+} is the best activator of the enzyme followed by Ag^+ , Bi^{3+} , Pb^{2+} , Zn^{2+} , Cu^{2+} , Hg^{2+} and Au^+ and it appears that the reaction product (γ -glutamyl peptide) chelates the activating metal ions thus terminating the reaction (Grill *et al.*, 1989; Loeffler *et al.*, 1989). In cell-free systems of *S. pombe*, an additional pathway has also been discovered which involves γEC polymerization from $(\gamma\text{EC})_n$ and glutathione to $(\gamma\text{EC})_{n+1}$ followed by addition of glycine with glutathione synthetase (Hayashi *et al.*, 1991).

The originally discovered two γ -glutamyl peptides in *S. pombe*, cadystin A and B ($n = 2$ and 3,

respectively), were synthesized in response to Cd^{2+} (Murasugi, Wada & Hayashi, 1981; Kondo *et al.*, 1985) though other metals including Cu^{2+} , Pb^{2+} , Zn^{2+} and Ag^+ were also effective (Grill *et al.*, 1986; Hayashi, Nakagawa & Murasugi, 1986; Reese *et al.*, 1988), a situation analogous to that in vascular plants (Hayashi *et al.*, 1988; Rauser, 1990). However, the greatest amount of peptide synthesis occurs with Cd and it seems larger peptides bind Cd more strongly than smaller ones (Hayashi *et al.*, 1986, 1988). When *S. pombe* was exposed to Cu(II), a Cu- γ -glutamyl peptide was synthesized similar in structure to the Cd- γ -glutamyl peptide (Reese *et al.*, 1988). However, only one Cu- γ -glutamyl peptide complex which did not contain labile sulphur was observed; the metal was bound as Cu(I) (Reese *et al.*, 1988).

Cadmium-induced (but not copper-induced) γ -glutamyl peptides contain labile sulphur. Cadmium stimulates the production of S^{2-} in *S. pombe* and *Candida glabrata* and a portion of this may be incorporated within the Cd- γ -glutamyl peptide complexes (Mehra *et al.*, 1988; Reese & Winge, 1988; Winge *et al.*, 1989). The sulphide content is dependent on growth conditions and incubation time and concentrations of S^{2-} , ranging from 0.1 to 1.5 mol (mol peptide) $^{-1}$, have been demonstrated (Winge *et al.*, 1989). Incorporation of sulphide enhances the stability and metal content of the peptide complex (the molar sulphide:cadmium ratios ranged from < 0.06 in low sulphide forms to 0.8 in high sulphide forms) and this enhanced stability may increase the efficacy of the complex in maintaining a low intracellular concentration of Cd^{2+} (Winge *et al.*, 1989). Complexes with a S:Cd ratio of 0.7 contain a 20 Å diameter CdS crystallite coated with the γ -glutamyl peptides. These complexes are quantum particles exhibiting size-dependent electronic states (Dameron *et al.*, 1989; Dameron & Winge, 1990), a feature of relevance to solid state physics since the crystallites exhibit properties analogous to those of semiconductor clusters (Dameron *et al.*, 1989). The extracellular and cell-associated CdS colloid formed by interaction of Cd^{2+} with S^{2-} may also contribute to Cd^{2+} detoxification (see previous sections).

As mentioned, synthesis of $(\gamma\text{EC})_n\text{G}$ peptides is a predominant mechanism of Cd resistance in *S. pombe* although the genetics of Cd resistance in this organism is poorly understood. In order to clone genes conferring metal resistance in *S. pombe*, Yu *et al.* (1991) performed heterologous complementation by transformations of *S. cerevisiae* with a *S. pombe* cDNA expression library. The cDNA library was cloned into the *S. cerevisiae* expression vector pDB20 which expresses cDNAs via the constitutively-expressed promoter of the gene for alcohol dehydrogenase I (*ADH1*). Cd-resistant colonies of *S. cerevisiae* arose by recombination involving exchange of the *S. pombe* DNA with the chromosomal *ADH1*

gene. Concomitant overexpression of *ADH1* enhanced survival by formation of Cd-ADH (Yu, Macreadie & Winge, 1991).

Candida (Torulopsis) glabrata is unique in expressing both metallothionein and γ -glutamyl peptide synthesis for metal detoxification in a metal-specific manner (Mehra *et al.*, 1988, 1992*a, b*). *C. glabrata* synthesizes two distinct MT polypeptides in response to copper which exhibit repeats of the Cys-X-Cys sequence, common in mammalian MT, and coordinates copper in Cu(I)-thiolate clusters. The cysteine content of the two molecules is 30 mol % (*S. cerevisiae* Cu-MT = 22 mol %; *N. crassa* Cu-MT = 28 mol %) with the copper content being between 10 and 13 mol per mol protein for each (Mehra *et al.*, 1988). Two metallothionein genes (*MT-I* and *MT-II*) from *C. glabrata* have been cloned and sequenced. The former is present as a single copy but multiple (3–9) and tandemly arranged copies of one *MT-II* gene are present in different strains. *C. glabrata* strains hyper-resistant to Cu showed further stable chromosomal amplification (> 30 copies) of the *MT-II* gene (Mehra, Garey & Winge, 1990). Both genes were inducible by Ag but not by Cd, the latter reducing amounts of both *MT-I* and *MT-II* mRNAs (Mehra *et al.*, 1989). One *MT-II* gene, designated *MT-II_a*, was amplified in several wild-type strains of *C. glabrata* while another *MT-II_b*, occurs as a single copy (Mehra *et al.*, 1990). The amplified *MT-II_a* gene contains autonomously replicating sequences (ARS) (Mehra *et al.*, 1992*b*). The Cd- γ -glutamyl peptide of *C. glabrata* has $n = 2$ whereas peptides with $n = 3$ and 4 are more common in *S. pombe* and plants (Grill *et al.*, 1987; Reese *et al.*, 1988). Thus, *C. glabrata* can utilize two types of regulatory metal-binding molecules and the affinity and/or coordination preference of the molecules may determine whether MT or γ -glutamyl peptides are synthesized (Mehra *et al.*, 1988, 1992*a, b*).

Induction of γ -glutamyl peptides has so far been restricted to heavy metals and metalloids. However, other non-metallic inducing agents may induce their synthesis in yeasts. The antifungal agents, tetramethylthiuram disulphide (TMTD) or dimethyl dithiocarbamate (DMDTC), induce γ -glutamyl peptide synthesis to a lesser degree than CdCl₂ (but more than ZnCl₂ or CuSO₄) in *S. pombe* (Mutoh *et al.*, 1991). Such results may indicate biochemical functions other than that of metal detoxification including intracellular transport of essential metal ions, e.g. Zn²⁺ and Cu²⁺, detoxification of xenobiotics and scavengers of active oxygen (Mutoh, Kawabata & Hayashi, 1991). A detailed account of the involvement of metal-binding proteins and peptides in fungal resistance to metal ions is that by Mehra & Winge (1991). Related phenomena which have received relatively little attention are the possible roles of intracellular amino acids in metal resistance.

Changes in the amino acid pools of yeast can occur in response to exposure to Ni such that the formation of Ni-histidine complexes has been proposed as a survival mechanism (Joho *et al.*, 1988, 1990).

(b) *Vacuolar compartmentation*. The fungal vacuole is highly important organelle with functions including macromolecular degradation, storage of metabolites and cytosolic ion and pH homeostasis (Wiemken, Schellenberg & Urech, 1979; Davis, 1986; Wada *et al.*, 1987; Jones & Gadd, 1990). It is often considered analogous to the mammalian lysosome because hydrolytic enzymes are contained in an acidic compartment although, probably, it has greater similarity to vacuoles of plant cells (Matile, 1978; Achstetter & Wolf, 1985; Klionsky, Herman & Emr, 1990).

A vacuolar ATPase has been identified in several fungi, notably *S. cerevisiae*, *S. carlsbergensis* and *N. crassa* (Okorokov *et al.*, 1985; Bowman & Bowman, 1986; Anraku *et al.*, 1989) and is differentiated from mitochondrial and plasma membrane ATPases by means of pH optima, subunit composition and structure, and inhibitor sensitivity (Klionsky *et al.*, 1990). The vacuolar ATPase utilizes the energy arising from ATP hydrolysis to pump H⁺ into the vacuole, resulting in the generation of an electrochemical proton gradient of about 180 mV, inside positive, across the vacuolar membrane (tonoplast) (Kakinuma, Ohsumi & Anraku, 1981; Bowman & Bowman, 1986). In the presence of an energy source, a pH gradient of 0.5–1.5 units can be generated across the tonoplast (vacuolar pH generally ~ pH 6) (Jones & Gadd, 1990) and a membrane potential of ~75 mV has been recorded for *S. cerevisiae* and 25–40 mV for *N. crassa* (Klionsky *et al.*, 1990). The electrochemical proton gradient energizes transport of monovalent and divalent cations, as well as other substances including basic amino acids, into the vacuole (Jones & Gadd, 1990; Klionsky *et al.*, 1990). The main mechanism of transport appears to be proton antiport, although there is some evidence of a pyrophosphatase activity in *S. carlsbergensis* which may be responsible for a PP_i-dependent pH gradient (Klionsky *et al.*, 1990). The gradients of pH and electrical potential difference across the tonoplast may be regulated through the interactions of a membrane potential-dependent cation channel (Wada *et al.*, 1987; Bertyl & Slayman, 1990), chloride transport systems (Wada, Ohsumi & Anraku, 1986) and the vacuolar ATPase itself (Anraku *et al.*, 1989).

The fungal vacuole has an important role in the regulation of the cytosolic concentrations of metal ions, both for essential metabolic functions and the detoxification of potentially-toxic metal ions. Many ions, including inorganic phosphate (P_i) and monovalent and divalent metal cations, are preferentially located in vacuoles (Ohsumi, Kitamoto & Anraku,

1988). Ratios of [total ion]: [cytosolic ion] may be ~ 4 for K^+ , 4–28 for Mg^{2+} , > 70 for Mn^{2+} and ~ 10 for Ca^{2+} (Lichko, Ororokov & Kulaev, 1982; Jones & Gadd, 1990). The mechanism of ion transport across the tonoplast is by H^+ /ion antiporters and in *S. carlsbergensis*, apparent K_m values for the transport of Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} and P_i are 0.04, 0.3, 0.8, 0.055–0.17 and 1.5 mM, respectively. It is assumed that transport proceeds until cytosolic concentrations of ions approach these values (Okorokov *et al.*, 1985). These ionic substrates can stimulate vacuolar ATPase activity (Okorokov, 1985) although it is noteworthy that activity is inhibited by free Mg^{2+} at 0.4–0.5 mM (Borst-Pauwels & Peters, 1981).

Localization of metal ions in the vacuole enables low cytosolic concentrations of, for example, Ca^{2+} , to be maintained (Ohsumi & Anraku, 1983; Eilam *et al.*, 1985*a, b*; Okorokov *et al.*, 1985; Eilam & Chernichovsky, 1987; Cornelius & Nakashima, 1987; Ohsumi *et al.*, 1988). Cytosolic concentrations of Mg^{2+} and Mn^{2+} may remain relatively constant, even under considerable environmental perturbations (Lichko *et al.*, 1982; Kihn, Dassargues & Mestdagh, 1988). There is further evidence for compartmentation of Zn^{2+} , Co^{2+} and iron in yeast vacuoles, which may contribute to tolerant behaviour (White & Gadd, 1986, 1987*a*; Raguzzi, Lesuisse & Crichton, 1988). Mutants of *S. cerevisiae* defective in vacuolar function were sensitive to Ca^{2+} and other toxic metals (Kitamoto *et al.*, 1988). Polyphosphates, the only inorganic macromolecular anion found in the vacuole, have an important role in maintaining ionic compartmentation and their biosynthesis accompanies vacuolar accumulation of Mg^{2+} or Mn^{2+} (Okorokov *et al.*, 1977, Okorokov, Lichko & Kulaev, 1980; Lichko *et al.*, 1982; Okorokov *et al.*, 1983*a, b*; Miller, 1984; Kihn *et al.*, 1988). The significance of polyphosphate granules as a store for divalent cations has long been appreciated (Doonan *et al.*, 1979; Roomans, 1980). In *S. cerevisiae*, vacuolar inclusions of Ca^{2+} –polyphosphate complexes vary with the amount of available polyphosphate (Ohsumi *et al.*, 1988) although this was not the sole mechanism of Ca^{2+} ‘trapping’ in the vacuole (Ohsumi & Anraku, 1983). Vacuolar Zn^{2+} has also been associated with polyphosphate bodies in yeast (Bilinski & Miller, 1983) while intravacuolar deposits have also been observed in yeast grown in the presence of thorium (Gadd & White, 1989*b*). In the absence of an energy source, precipitation of Mn^{2+} within yeast cells has been demonstrated using electron spin resonance (ESR) spectroscopy (Kihn *et al.*, 1988).

5. Metal transformations

Fungi, as well as other microorganisms, can effect chemical transformations of metals by, e.g., oxidation, reduction, methylation and dealkylation (Gadd, 1992*a*) although, in contrast to bacteria,

detailed information is not available in several areas for fungi. Some enzymatic metal transformations may be involved in survival since certain transformed metal species are less toxic and/or more volatile than the original species. Plasmid-mediated reduction of Hg^{2+} to Hg^0 is a well-characterized bacterial metal resistance system and there is only a limited amount of evidence for an analogous process in filamentous fungi and yeasts (Vonk & Sijpesteijn, 1973; Brunker & Bott, 1974; Yannai, Berdicevsky & Duek, 1991). Other reductions carried out by fungi include Ag^+ to metallic Ag^0 which is deposited in and around cells (Kierans *et al.*, 1990) and the reduction of Cu(II) to Cu(I) by cell wall-associated materials in *Debaryomyces hansenii* (Wakatsuki, Iba & Imahara, 1988; Wakatsuki *et al.*, 1991*a, b*). A copper-reducing enzyme, copper reductase, catalyses Cu(II) reduction to Cu(I) with NADH or NADPH as e^- donor (Wakatsuki *et al.*, 1991*a, b*). The location of copper reductase appears to be the cell wall and this process may have a role in regulation of copper uptake (Wakatsuki *et al.*, 1991*b*). Fungal reductions of metalloids include reduction of tellurite to elemental tellurium, which can appear as a black deposit, located on the endoplasmic reticulum (Corfield & Smith, 1970; Smith, 1974) and the reduction of selenate or selenite to form amorphous selenium which imparts a red colour to fungal colonies owing to deposition, chiefly in walls and membranes (Konetzka, 1977).

Methylation of Hg, and other metals and metalloids can be catalyzed by several fungi, and may be viewed as a detoxification mechanism since methylated species are usually more volatile and may be lost from the environment (Landner, 1971; Yannai *et al.*, 1991; Gadd, 1992*a, b*). There is general agreement that methylation of Hg^{2+} involves methylcobalamin (vitamin B12) (Craig, 1986; Thayer, 1988).

Biomethylation of As by transfer of carbonium ions (CH_3^+) from S-adenosylmethionine (SAM) has also been demonstrated (Thayer, 1984, 1989; Andrae, 1986). Original work by Challenger (1945) first demonstrated that trimethylarsine could be produced by several fungi from arsenite. Biomethylation of Se appears to be by an analogous mechanism to that of arsenic, and several fungi can produce organoselenium compounds, e.g. dimethylselenide, from inorganic selenium ones (Chau & Wong, 1986; Thayer, 1988; Thompson-Eagle, Frankenberger & Karlson, 1989). Certain fungi may be capable of the production of volatile dimethyl telluride from tellurium salts (Konetzka, 1977; Chau & Wong, 1986). Apart from these examples, fungal involvement in the methylation of other metals and metalloids has not been examined in detail.

Organometallic compounds (which contain at least one metal-carbon bond) arise in the environment naturally, by abiotic and biotic means, and because

of accidental and deliberate introduction. The latter mainly results from the use of several organometallic and organometalloid compounds as biocides, e.g. organoarsenicals, organomercurials and organotins (Craig, 1986; Thayer, 1988; Cooney & Wuertz, 1989). Degradation involves breaking of the metal-C bonds though detailed information on such processes is rather sparse for fungi. It should be noted that metal-C bonds can be disrupted by physico-chemical means, although fungal activity may enhance the conditions necessary for abiotic attack by, e.g. alteration of pH or metabolite excretion. Organotin degradation involves sequential removal of organic groups from the tin atom, a process generally resulting in a decrease in toxicity (Blunden & Chapman, 1986). Degradation of tributyltin oxide and tributyltin naphthenate, used as wood preservatives, can be achieved by fungal action, breakdown products including mono- and di-butyltins (Barug, 1981; Orsler & Holland, 1982). Organomercurials may be detoxified by organomercury lyase, the resulting Hg^{2+} being then reduced to Hg^0 by mercuric reductase (Tezuka & Takasaki, 1988), a similar mechanism to that found in bacteria (see Gadd, 1992b). Trimethyl lead can be degraded by *Phaeolus schweinitzii*, a process with implications for fungal treatment of wastes (Macaskie & Dean, 1990b).

V. MYCORRHIZAL INTERACTIONS WITH TOXIC METALS

The responses of mycorrhizal fungi to toxic metals are of importance in view of interest in the reclamation of polluted sites and influence on plant growth and productivity. Although there appears to be a wide diversity in responses between different plant-fungal symbioses, the amelioration of metal phytotoxicity by mycorrhizal fungi has been widely demonstrated (Gildon & Tinker, 1981, 1983; Brown & Wilkins, 1985a, b; Jones & Hutchinson, 1986, 1988a, b; Dixon, 1988; Dixon & Buschena, 1988). In ericaceous plants, little or no growth occurs in mycorrhiza-free plants in the presence of copper and zinc (Bradley, Burt & Read, 1981, 1982). It was suggested that the hyphal complexes of the endophyte bound metals preventing metal translocation to the shoots and the resulting toxic symptoms (Bradley *et al.*, 1982). A similar explanation has been proposed for ectomycorrhizal fungi (Denny & Wilkins, 1987a). The importance of vesicular-arbuscular mycorrhizas varies between different hosts and fungal species. In some cases, protection from toxicity is apparent (Gildon & Tinker, 1983) whereas in some cases, increased metal uptake may reduce growth (Killham & Firestone, 1983). Two species of ectomycorrhizal fungi, *Amanita muscaria* and *Paxillus involutus*, increased zinc tolerance of

Betula sp. (Brown & Wilkins, 1985), the latter fungus also ameliorating aluminium toxicity towards Norway spruce when non-mycorrhizal and located in the rhizosphere (Wilkins & Hodson, 1989). Other ectomycorrhizal fungi capable of enhancing survival of *Betula papyrifera* are *Lactarius hibbardae*, *L. rufus*, *Laccaria proxima* and *Scleroderma flavidum* although overall effects cannot be generalized with respect to metal or mycobiont (Jones & Hutchinson, 1986; Jongbloed & Borst-Pauwels, 1990).

The isolation of metal-tolerant mycorrhizal fungi from polluted sites has been well documented (Gildon & Tinker, 1981; Brown & Wilkins, 1985a; Jones & Hutchinson, 1986; Colpaert & Van Assche, 1987) although considerable differences were encountered between effects of the different mycobionts on the plants inoculated. It has been suggested that tolerant behaviour of the mycobiont may be an important factor in conferring plant tolerance (Colpaert & Van Assche, 1987). However, there may be no correlation between metal tolerance of mycorrhizal fungi and the toxicity of the soil of origin (Brown & Wilkins, 1985b; Denny & Wilkins, 1987b; Darlington & Rauser, 1988). In addition, there was not necessarily a relationship between tolerance of ectomycorrhizal fungi, as measured in the laboratory, and their ability to increase the metal tolerance of *Betula papyrifera* (Jones & Hutchinson, 1988b). It is uncertain as to what extent the previously discussed difficulties in assessing and defining fungal resistance/tolerance in the laboratory and their relevance to the environment contribute to such observations (Gadd, 1983, 1986a, 1990a).

VI. ACCUMULATION OF METALS BY MACROFUNGI

Elevated concentrations of heavy metals and radionuclides often occur in the fruiting bodies of basidiomycete fungi when growing in polluted environments (Brown & Hall, 1990). This phenomenon is, as yet, poorly understood at the physiological level but is of significance because of the widespread consumption of edible wild fungi in many countries. In general, higher concentrations of Pb, Cd, Zn and Hg are found in macrofungi from urban or industrial areas although there are differences in uptake abilities between different species and different metals (Tyler, 1980; Bressa, Cima & Costa, 1988; Lepsova & Mejstrik, 1989; Brown & Hall, 1990). Cadmium appears to be accumulated to quite high values in macrofungi, averaging around $5 \mu\text{g g}^{-1}$ d.wt though concentrations of up to $40 \mu\text{g g}^{-1}$ d.wt have been recorded (Byrne, Ravník & Kosta, 1976). Average concentrations of As, Cu, Hg, Mn, Se and Zn in a range of up to 27 species were 1, 50, 2, 3, 30, 1.5 and $114 \mu\text{g g}^{-1}$ d.wt respectively (Byrne *et al.*, 1976). However, a wide range of accumulation values can occur depending on the species and location of

growth; caps of *Laccaria amethystina* exhibited total As concentrations of 100–200 $\mu\text{g g}^{-1}$ d.wt (Stijve, Vellinga & Hermann, 1990; Byrne *et al.*, 1991). Dimethylarsinic acid was a major arsenic compound in cap extracts, providing tentative evidence for biomethylation of this element (Byrne *et al.*, 1991). Accumulation of ^{110}Ag and ^{203}Hg was studied in *Agaricus bisporus* and concentration factors (metal concentration in mushroom:metal concentration in substrate) were found to be up to 40 and 3.7 respectively, with the highest Ag and Hg contents recorded being 167 and 75 $\mu\text{g g}^{-1}$ d.wt respectively. Silver–protein complexes were isolated which possibly corresponded with metallothioneins or analogous metal-binding proteins (Byrne & Tušek-Znidarič, 1990).

In recent years and following the accident of Chernobyl in 1986, there have been several studies on the accumulation of radiocaesium (mainly ^{137}Cs) by macrofungi. Saprotrophic and mycorrhizal basidiomycetes can accumulate radiocaesium (Haselwandter, 1978; Elstner *et al.*, 1987; Byrne, 1988; Dighton & Horrill, 1988; Haselwandter, Berreck & Brunner, 1988; Clint, Dighton & Rees, 1991; Dighton, Clint & Poskitt, 1991) and these organisms appear to have a slow turnover rate for Cs, forming a major pool of radiocaesium in soil (Clint *et al.*, 1991). Deviations in the ^{137}Cs : ^{134}Cs ratio attributable to Chernobyl have revealed that there was considerable accumulation of pre-Chernobyl Cs probably from weapons testing (Byrne, 1988; Dighton & Horrill, 1988). The ability of yeasts to accumulate Cs^+ is well known (Borst-Pauwels, 1981, 1989; Perkins & Gadd, 1991) although radiocaesium accumulation in basidiomycetes appears species-dependent, with influences exerted by soil properties. Some mushrooms appear to have little or no affinity for ^{137}Cs , e.g. *Agaricus campestris* and *A. bisporus* (Stijve & Poretti, 1990). Influx of ^{137}Cs into hyphae of several basidiomycetes showed considerable variation with saprotrophic species exhibiting highest rates and mycorrhizal species the lowest. Such observations may partly explain the wide differences in concentrations of radiocaesium encountered in fungal fruiting bodies after Chernobyl (Clint *et al.*, 1991; Heinrich, 1992). It has been concluded that the fungal component of soil can immobilize the total Chernobyl radiocaesium fallout received in upland grasslands (Dighton *et al.*, 1991) although grazing of fruit bodies by animals may lead to its transfer along the food chain (Bakken & Olsen, 1990).

accumulate metals and radionuclides from their external environment by means of the physico-chemical and biological mechanisms already discussed. Living and dead cells are capable of metal/radionuclide removal from solution as well as excreted or derived products, e.g. cell wall constituents, pigments, polysaccharides, proteins and siderophores (Table 3) (Gadd, 1988; Pighi, Pumpel & Schinner, 1989; Siegel, Galun & Siegel, 1990). The removal of radionuclides, metal or organo-metal(loid) species, compounds or particulates from solution by microbial biomass is now often referred to as 'biosorption', particularly where predominant interactions are physico-chemical. Biosorption is an area of increasing biotechnological interest since the removal of potentially toxic and/or valuable metals and radionuclides from contaminated effluents can be used to detoxify them prior to environmental discharge. Furthermore, appropriate treatment of loaded biomass can enable recovery of valuable metals for recycling or further containment of highly toxic and/or radioactive species. Detailed reviews of this research area are available (Gadd, 1988, 1990a, 1992a, b).

Although the basic features of metal accumulation are common to most microbial groups, fungi possess a number of unique attributes which reflect their morphological and physiological diversity. The majority of fungi exhibit a filamentous or hyphal growth form which enables efficient colonization of sub-

Table 3. Some examples of metal and actinide accumulation by fungi*

Organism	Element	Uptake (% dry weight)
<i>Phoma</i> sp.	Silver	2
<i>Penicillium</i> sp.	Uranium	8–17
<i>Rhizopus arrhizus</i>	Copper	1.6
	Cadmium	3
	Lead	10.4
	Uranium	19.5
	Thorium	18.5
	Thorium†	11.6
<i>Aspergillus niger</i>	Silver	5.4
	Mercury	5.8
	Thorium	18.5
	Thorium†	13.8
	Uranium	21.5
<i>Saccharomyces cerevisiae</i>	Uranium	10–15
	Thorium†	12
Yeast (14 strains)	Zinc	0.5
	Silver	0.05–1
Yeast (4 strains)	Copper	0.05–0.2

* Data adapted from several sources (see Gadd, 1988, 1990a). Consultation of original work is recommended for full experimental details, particularly metal and biomass concentration, suspension medium and time of incubation. Most of the values listed are derived from studies in a biotechnological context.

† Incubation in 1 M HNO_3 (Gadd & White, 1989b, c).

VII. BIOTECHNOLOGICAL ASPECTS OF FUNGAL METAL ACCUMULATION

1. General aspects

Fungi, in common with other microbial groups, can

strates. Hyphae exhibit apical growth and can produce lateral branches. There may be further structural and biochemical differentiation including the production of complex fruiting structures. Different cell forms may have different uptake capacities and a wide spectrum of sensitivities towards potentially toxic metals are encountered (Gadd, 1986a). There are important differences in uptake mechanisms between living and dead cells and, therefore, a variety of possible industrial applications. The use of dead biomass for metal binding circumvents nutrient supply and toxicity and subsequent recovery is often easier. However, living cells can accumulate metals intracellularly, often to higher concentrations than dead cells and may also precipitate them in and around cell walls or in the external medium by various metabolic products. In addition, many fungi can be extremely tolerant of toxic metals in comparison with other microbial groups (Gadd *et al.*, 1984a; Gadd, 1986a; Pighi *et al.*, 1989). Fungi are, in general, easy to grow and produce high yields of biomass. Furthermore several are amenable to genetical and morphological manipulation. Their use in a variety of industrial fermentations also means that fungal biomass is available as a waste product for which the biotechnology of metal removal may provide a good use. Biomass of *Aspergillus niger* arises in substantial quantities from citric acid production while *S. cerevisiae* is available from food and beverage industries. Other kinds of biomass arising from industrial fermentations include *Rhizopus arrhizus*, *Aspergillus terreus* and *Penicillium chrysogenum* (Tsezos & Volesky, 1981; Luef, Prey & Kubicek, 1991).

2. Mechanisms of heavy metal and radionuclide accumulation by fungi of biotechnological relevance

As described previously, a variety of mechanisms occur in fungal cells for the removal of metals and radionuclides from solution. These mechanisms range from physico-chemical interactions, such as adsorption, to processes dependent on cell metabolism, such as intracellular accumulation or extracellular precipitation of metals as a result of excreted metabolites. It therefore follows that there may be considerable differences in the mechanisms involved, depending on whether organisms are living or dead and, if living, whether growth takes place.

Metal uptake by living fungi can often be divided into two main phases. The first, which can also occur with dead cells, is the often rapid, metabolism-independent binding to cell walls and other external surfaces; the second is energy-dependent intracellular uptake across the cell membrane (Tsezos, Baird & Shemilt, 1986; Gadd, 1986a, b, 1988; Huang *et al.*, 1990). In some cases, particularly where toxicity is

evident, intracellular uptake may not be linked with metabolism but may be a consequence of increased membrane permeability and resultant exposure of metal-binding sites within the cell. These phases of uptake may not be clearly seen in all organisms. For certain radionuclides, e.g. actinides (see later), it appears that accumulation may primarily be a result of physico-chemical interactions with cell walls with little or no intracellular uptake whereas with others, e.g. caesium, most accumulation is intracellular. In growing cells, either or both phases of uptake may be obscured by additional aspects of metabolism, e.g. extracellular products which may complex or precipitate metals outside the cells.

Various treatments may be employed to increase the capacity of fungal biomass for metal and/or radionuclide adsorption. Certain killing treatments may increase the adsorption capacity and powdering of dried biomass exposes additional binding sites (Tobin *et al.*, 1984). Detergent treatment has also been used to increase the capacity of fungal biomass for adsorption, since this can result in disruption of cell components, such as membranes, and expose a wider variety of potential binding sites. Detergent treatment of *Penicillium spinulosum* greatly improved removal of Cu^{2+} from solution, especially at high Cu^{2+} concentrations (Ross & Townsley, 1986). Detergents also increased uptake of thorium by *Saccharomyces cerevisiae* and *Rhizopus arrhizus*, although the effect was not as significant as for *P. spinulosum* (Gadd & White, 1989c). However, the conditions for thorium uptake (in 1 M HNO_3) were markedly different. It was likely that the biomass was already substantially permeabilized prior to detergent treatment. In *S. cerevisiae*, uranium uptake was increased by HCHO and HgCl_2 (Strandberg *et al.*, 1981), again probably because of increased cell permeability.

In some circumstances, fungi can adsorb insoluble metal compounds, e.g. sulphides, and this may represent another area of potential biotechnological application. *Aspergillus niger* oxidized copper, lead and zinc sulphides to sulphates, the sulphide particles in the medium being adsorbed on to mycelial surfaces (Wainwright & Grayston, 1986). *Mucor flavus* could adsorb lead sulphide, zinc dust and $\text{Fe}(\text{OH})_3$ ('ochre') from acid mine drainage (Singleton, Wainwright & Edyvean, 1990). *A. niger*, *Fusarium solani* and *P. notatum* could also remove ochre from solution but not as efficiently as *M. flavus* (Wainwright, Grayston & De Jong, 1986). Intact, fresh mycelium was best for adsorption at 25 °C; the presence of a carbon source was unnecessary. When *M. flavus* was grown with PbS for 2–5 d, it converted small particles of the compound to a fine, even suspension which was then completely adsorbed after 7 d (Wainwright, Singleton & Edyvean, 1986). Magnetite-loaded fungal biomass was susceptible to a magnetic field which has implications for removal

of biomass from solution (Wainwright *et al.*, 1990; Dauer & Dunlop, 1991).

Metabolism-dependent intracellular uptake or transport of metal ions into cells [see Section IV(3)] may be a slower process than adsorption and is inhibited by low temperatures, the absence of an energy source (e.g. glucose), and by glucose analogues, metabolic inhibitors and uncouplers (Ross, 1977; Norris & Kelly, 1977, 1979; Borst-Pauwels, 1981; Gadd, 1986*a, b*; Starling & Ross, 1990). In certain fungi, especially yeasts, greater amounts of metal may be accumulated by such a process, in conjunction with internal sequestration, than by metabolism-independent processes (Ross, 1977; Norris & Kelly, 1977, 1979). Many metals are essential for fungal growth and metabolism, e.g. Cu, Fe, Zn, Co and Mn, and all organisms must have the ability to accumulate these intracellularly from low external concentrations so that physiological needs are satisfied. However, at the relatively high concentrations of metals frequently used in uptake studies, energy-dependent intracellular uptake may be difficult to characterize and may not be as significant a component of total uptake as general biosorption. This is particularly true for filamentous fungi where high values of biosorption mask low influx (Gadd & White, 1985; Gadd, White & Mowll, 1987). For such organisms, most published work suggests that general metabolism-independent processes such as adsorption and complexation make up the majority of total metal uptake by the biomass (Duddridge & Wainwright, 1980; Ross & Townsley, 1986). For uranium and thorium, it appears that little or none may enter cells (Shumate & Strandberg, 1985) although little detailed work on living cells has been carried out. However, yeasts may exhibit intracellular uptake of thorium (Gadd & White, 1989*b*). These organisms, as well as other fungi, may also precipitate metals around the cells as a result of metabolic processes, and are capable of synthesizing intracellular metal-binding proteins (see previous sections).

In actively growing fungal cultures, the phases of adsorption and intracellular uptake may be obscured by changes in the physiology and morphology of the fungus and the physical and chemical properties of the growth medium (Gadd, 1986*a*). A frequently observed phenomenon is that metal uptake by growing batch cultures is maximal during the lag period or early stages of growth and declines as the culture reaches the stationary phase. This has been shown for copper and *Aurobasidium pullulans* (Gadd & Griffiths, 1980*b*), *Debaryomyces hansenii* (Wakatsuki *et al.*, 1979) and *P. spinulosum*, *A. niger* and *Trichoderma viride* (Townsley & Ross, 1985, 1986; Townsley, Ross & Atkins, 1986*a*). Changes in the kinetics of Cu^{2+} uptake by *A. pullulans* were related to the fall in medium pH during growth and the alleviation of Cu^{2+} toxicity at low pH (Gadd &

Griffiths, 1980*b*). However, for several filamentous fungi, reduction of pH of the medium was not the only factor responsible for reduced Cu^{2+} uptake. A reduction could occur before any significant drop in the pH and in media where the pH was maintained at 5.5 (Townsley & Ross, 1985, 1986). Explanations for this include alterations in cell wall composition during growth and/or the release of metabolites, e.g. citric acid, that can control metal availability (Townsley & Ross, 1985, 1986). Complexation may be involved in uptake and may partly explain the differences in uptake capacities observed between non-growing and growing fungal cultures (Townsley & Ross, 1986). *Candida utilis* exhibits cyclical accumulation of Zn^{2+} in batch culture with highest uptake during the lag period and late exponential phase; it was suggested that there was regulation of Zn^{2+} uptake in this organism (Failla & Weinberg, 1977). At low (10 nM) external Mn^{2+} concentrations, intracellular $[\text{Mn}^{2+}]$ remained relatively constant in batch cultures of *C. utilis* and there appeared to be a specific, high affinity Mn^{2+} transport system (Parkin & Ross, 1986*a, b*).

As mentioned previously, many fungi have the ability to tolerate high concentrations of potentially-toxic metals which may be useful when employing living cells in recovery systems. However, tolerance may be related to decreased intracellular uptake and/or impermeability of the cells toward metal ions (Gadd, 1986*a*). A close connection between intracellular uptake and toxicity for Cu, Cd and Zn has been shown in *S. cerevisiae* (Ross, 1977; Ross & Walsh, 1981; Gadd & Mowll, 1983; Mowll & Gadd, 1983; Joho *et al.*, 1983, 1985*a, b*; Gadd *et al.*, 1984*b*; White & Gadd, 1986). Melanized chlamydospores of *A. pullulans* are impermeable to heavy metals and more tolerant than hyaline cell types (Gadd, 1984; Mowll & Gadd, 1984). In contrast to the above examples, a Mn-resistant mutant of *S. cerevisiae* accumulated more manganese than the sensitive parental strain, probably a result of an efficient internal sequestration system of greater efficiency in the resistant strain (Bianchi *et al.*, 1981*a, b*).

Where a reduction in intracellular uptake occurs at low external pH, a corresponding reduction in toxicity may also take place. *P. ochro-chloron* can grow in saturated CuSO_4 at pH 0.3–2.0, but is sensitive to $\geq 4 \times 10^{-5}$ M near neutrality (Gadd & White, 1985). For *P. ochro-chloron*, a constant rate of Cu^{2+} uptake occurs above approximately 16 mM at low pH but, at pH 6 and above, uptake markedly increases and is concomitant with toxicity (Gadd & White, 1985).

3. Metal removal by induced, excreted or derived biomolecules

Some metal-sequestering biomolecules may be induced by the presence of certain metals, e.g.

metallothioneins and phytochelatins (see previous sections) or by deprivation of an essential metal ion, e.g. Fe^{3+} , leading to siderophore production (Raymond *et al.*, 1984). Other molecules with significant metal-binding abilities may be overproduced as a result of exposure to potentially toxic metal concentrations, e.g. fungal melanins (Gadd, 1986*a*, 1988). However, the majority of metal-binding biomolecules found in fungi are synthesized or excreted as a result of 'normal' growth and/or are important structural components, particularly in cell walls (see previous sections) (Macaskie & Dean, 1990*a*). Metal-binding by such compounds may be fortuitous and relative efficiencies may largely depend on the metal species present and the chemical nature and reactivity of the metal binding ligands present (Volesky, 1990; Tsezos, 1990; Tsezos *et al.*, 1986). It should be noted that the macromolecular composition of a given fungal species can be altered by cultural and genetic manipulations, and there is diversity in chemical composition, particularly in relation to cell walls (see previous sections). As described earlier, many fungi have a high chitin content in cell walls and this polymer of *N*-acetyl glucosamine is a highly effective metal and radionuclide biosorbent (Tsezos, 1986, 1990; Macaskie & Dean, 1990*a*). For uranium, rapid coordination to the amine nitrogen of chitin and simultaneous adsorption in the cell wall chitin structure are followed by slower precipitation of uranyl hydroxide. Accumulation of hydrolysis products continues until a final equilibrium is achieved. Chitosan and other chitin derivatives also have significant biosorptive capability (Tsezos, 1986). Other biomolecules of probable interest from fungi include phenolic polymers, melanins, mannans, phosphomannans, other polysaccharides, proteins (including metallothioneins and other metal-binding proteins) and externally-excreted metal-binding molecules, including siderophores, and citric and oxalic acids (Gadd, 1986*a*, 1990*a*).

4. Metal recovery

Biotechnological exploitation of metal accumulation by microbial biomass, including fungi, may depend on the ease of metal recovery for subsequent reclamation, containment and biosorbent regeneration (Tsezos, 1984, 1986, 1990; Tsezos, McCready & Bell, 1989; Volesky, 1990; Gadd & White, 1992). The means of metal recovery may depend on the ease of removal from the biomass which in turn can depend on the metal species involved and the mechanism of accumulation. Metabolism-independent biosorption is frequently reversible by non-destructive methods whereas energy-dependent intracellular accumulation and compartmentation, binding to induced proteins, etc. is often irreversible, so requiring destructive recovery (Gadd, 1988). The

latter may be achieved by incineration or dissolution in strong acids or alkalis (Brierley *et al.*, 1985). Most work has concentrated on non-destructive desorption which should ideally be highly efficient, economical and result in minimal damage to the biosorbent. Dilute mineral acids (~ 0.1 M), e.g. HNO_3 , HCl or H_2SO_4 , can be effective for the removal of heavy metals and radionuclides from fungal biomass (Tsezos *et al.*, 1987; Luef *et al.*, 1991) although at higher concentrations (> 1 M), or with prolonged exposure, there may be irreversible damage which reduces the potential of the biomass to be re-used (Tsezos, 1984). Organic complexing agents may be effective, although relatively expensive, desorbers without affecting integrity of the biomass. EDTA treatment may also be useful in distinguishing surface complex formation from intracellular, unexchangeable metal (Galun *et al.*, 1983; Tsezos *et al.*, 1987).

Carbonates and/or bicarbonates are efficient desorption agents with potential for cheap, non-destructive metal recovery. Of several elution systems examined for uranium desorption from *R. arrhizus*, NaHCO_3 exhibited $> 90\%$ efficiency of removal and high uranium concentration factors (Tsezos, 1984). The solid:liquid ratios can exceed 120:1 (mg:ml) for a 1 M NaHCO_3 solution with eluate uranium concentrations of at least $1.98 \times 10^4 \text{ mg l}^{-1}$ (Tsezos, 1984). The solid:liquid (S/L) ratio, i.e. the ratio of weight of loaded biomass to the eluant volume required for complete recovery, needs to be maximized in any recovery process in order to yield an eluate of minimal volume yet containing the highest possible metal concentration (Tsezos, 1990).

5. Biosorption of radionuclides by fungal biomass

Radionuclide uptake has been examined in a range of filamentous fungi and yeasts, mainly from the perspective of environmental biotechnology and, while the mechanism(s) involved in uptake may vary between different elements and species, actinide accumulation appears mainly to comprise metabolism-independent biosorption (Tobin *et al.*, 1984). The main site of actinide uptake is the cell wall (Weidemann *et al.*, 1981; Tsezos *et al.*, 1986; Volesky, 1990), although permeabilization of cells with carbonates or detergents can increase uptake, indicating that intracellular sites are also capable of binding metals (Gadd & White, 1989*c*). The mechanism of biosorption varies between elements. Both adsorption and precipitation of hydrolysis products occurs with uranium (Tsezos & Volesky, 1982*a*) while coordination with cell wall nitrogen was the main mechanism for thorium (Tsezos & Volesky, 1982*b*, Tsezos, 1983). Precipitation or crystallization of radionuclides within or on cell walls may also be involved in some circumstances (Galun *et al.*, 1987).

In *S. cerevisiae*, uranium was deposited as a layer of needle-like fibrils on cell walls, reaching up to 50 % of the dry weight of individual cells (Strandberg *et al.*, 1981). Such precipitation has also been observed for thorium (Tsezos, 1986; Gadd & White, 1989*a*, *b*). Under some conditions, thorium may also accumulate intracellularly in *S. cerevisiae* with preferential localization in the vacuole (Gadd & White, 1989*c*).

Biosorption of uranium and thorium can be affected by the external pH. Initial rates of uranium uptake by yeast increase above pH 2.5 (Strandberg *et al.*, 1981). At pH values below 2.5, the predominant species is UO_2^{2+} but, at greater pH values, hydrolysis products include $(\text{UO}_2)_2(\text{OH})_2^{2+}$, $\text{UO}_2(\text{OH})^+$ and $(\text{UO}_2)_3(\text{OH})_5^+$. The resulting reduction in solubility favours biosorption (Tsezos & Volesky, 1982*a*, 1983). Similar phenomena occur with thorium where, at pH values below 2, Th^{4+} is the main species present. At higher pH values, $\text{Th}(\text{OH})_2^{2+}$ and other products form which are absorbed more efficiently than Th^{4+} (Tsezos & Volesky, 1982*b*). Most previous studies on thorium biosorption have been conducted at pH ranges above 2. This is representative of many natural waters but certain industrial process streams containing actinide elements are extremely acidic (pH < 1). Furthermore, other cations like Fe^{3+} , which may be present in waste liquors, can interfere with actinide uptake. Consequently, if biomass is used to remove actinides from these waste streams, it must be capable of performing at pH values < 1 and in the presence of potentially competing chemical components. Despite these difficulties, biomass from several fungal species removes thorium from solution in 1 M HNO_3 , pH 0–1 (Gadd & White, 1989*a*, *b*; White & Gadd, 1990). Thorium uptake was altered by the biomass concentration, the uptake per unit biomass being reduced at high biomass concentrations. Thorium uptake was also increased by detergent pretreatment and, in the case of filamentous fungi, varied with culture conditions. This implies that the characteristics of thorium uptake by fungal biomass can be manipulated to achieve optimal performance by these or similar means (Gadd & White, 1989*b*). Strains of fungi which gave the best performance as thorium biosorbents in batch culture under acidic conditions were examined in several bioreactor configurations (White & Gadd, 1990). Static or stirred-bed bioreactors removed thorium unsatisfactorily, probably because of poor mixing. However, an air-lift reactor removed approximately 90–95 % of the thorium supplied over extended time periods. This type of reactor provided efficient circulation and effective contact between the thorium solution and the fungal biomass. Of the species tested, *A. niger* and *R. arrhizus*, used as mycelial pellets, were the most effective with loading capacities of approximately 0.5 and 0.6 mmol g^{-1} respectively

(116 and 138 mg g^{-1} d. wt) at an inflow concentration of 3 mM thorium (as nitrate). The efficiency of thorium biosorption by *A. niger* was markedly reduced by other inorganic solutes while thorium uptake by *R. arrhizus* was relatively unaffected. Air-lift bioreactors containing biomass of *R. arrhizus* could remove thorium from acidic solution (1 M HNO_3) over a wide range of initial concentrations (0.1–3 mM) (White & Gadd, 1990). Thus, biosorption using fungal biomass is a technically feasible method for the removal of thorium from acidic solutions similar to those encountered in industrial process streams. However, the practicality of this approach is very much dependent on economic considerations. The availability of waste biomass or cheap growth substrates may favour such a process while others, such as the need for handling and transport of a bulky biosorbent or the need for on-site propagation, would mitigate against it. The balance of these factors may only be determined in individual cases by detailed costings.

6. Industrial considerations

As described previously, both living and dead fungi can accumulate heavy metals and radionuclides by a variety of mechanisms, some with obvious practical potential (Siegel, Galun & Siegel, 1990). A current perspective appears to be that using dead biomass has more advantages than using living material in that the former may be obtained inexpensively as waste, is not subject to metal toxicity or adverse operating conditions, nutrient supply is unnecessary and recovery of metals may be achieved by relatively simple nondestructive treatments which permit regeneration (Kelly, Norris & Brierley, 1979; Brierley *et al.*, 1985). Toxicity and susceptibility to adverse operating conditions may be a problem with living cells and, although it is easy to obtain tolerant strains, these may exhibit reduced uptake or impermeability. Living fungi may have possibilities in some circumstances but, so far, these possibilities have been largely unexplored. Metallothioneins, particulate metal accumulation, extracellular precipitation and complexation are all worthy of continued investigation and several postulated microbial methods for effluent detoxification involve some of these processes (Brierley *et al.*, 1985).

An important consideration, whether living or dead cells are used, is the form and kind of biomass to be used since, for industrial applications, biomass has some disadvantages. It is generally of low strength and density which can limit the choice of suitable reactors and make separation of biomass or effluent difficult (Tsezos, 1986). For use in packed bed or fluidized bed reactors, immobilized or pelleted biomass is of greater potential (Tsezos & Deutschmann, 1990). Immobilized biomass, whether within or on an inert matrix, has advantages

in that high flow rates can be achieved, clogging is minimized, particle size can be controlled and high biomass loadings are possible (Shumate *et al.*, 1980; Tsezos, 1986; Yakubu & Dudeney, 1986). Commercial processes using immobilized bacteria and algae already exist, particularly for recovery of valuable metals (Brierley *et al.*, 1986). Fungal examples of laboratory-scale immobilized systems include *A. oryzae* and *R. arrhizus* on reticulated foam particles (Kiff & Little, 1986; Lewis & Kiff, 1988) and *Trichoderma viride* packed in molochite and used for Cu removal from simulated effluents (Townsend, Ross & Atkins, 1986b), *S. cerevisiae* immobilized in a sand column and used for removal of Cu(II) (Huang *et al.*, 1990), and microfungal biomass incorporated into filters derived from paper and textile fibres (Wales & Sagar, 1990).

Filamentous fungi in culture may grow as pellets which have similar advantages to immobilized particles. Pellets (4 mm diameter) of *A. niger* have been used in a fluidized bed reactor for removal of uranium. A simple ion-exchange process predominated, where UO_2^{2+} ions replaced protons on amino-acid groups of proteins and glycoproteins within the cell wall. Such a system was 14 times more efficient than the commercial ion-exchange resin, IRA-400. However, fungal pellets may break up and cause increased resistance to liquid flow, and the similarity in specific gravity between cells and the liquid medium was found to be disadvantageous in continuous processes (Yakubu & Dudeney, 1986). However, metal-tolerant fungi proved successful for the removal of cadmium when grown in a continuous flow air-lift fermenter, with > 97% removed from the simulated effluent of 6 mg Cd l^{-1} (Campbell & Martin, 1990).

For industrial application, any biomass-related process must be economically competitive with existing technologies. The current consensus is that, for improved commercial use, immobilized or pelleted preparations should be used with recovery involving an inexpensive, recyclable stripping agent (Brierley *et al.*, 1986; Tsezos, 1986; Tsezos & Deutschmann, 1990). It is clear that, in relation to parameters of industrial relevance, fungi and fungal products can be highly efficient removal agents and exhibit high uptake capacities. Fuller discussion of economic considerations are given by Brierley, Goyak & Brierley (1986). Microbial biomass is particularly efficient at dilute external concentrations and, although it may not necessarily replace existing technologies, it may serve as a 'polisher' after an existing non-biological, inefficient treatment (Brierley *et al.*, 1986). It is also pertinent to point out the potential interaction of biosorption technology with microbial leaching of metal ions from ores and wastes. While the bulk of information on leaching is derived from the more important bacterial systems, it has been demonstrated that *Penicillium* spp. can

leach Zn from industrial wastes and mixtures of metal oxides (Schinner and Burgstaller, 1989; Franz, Burgstaller & Schinner, 1991). Further work is needed in several areas to establish or realize the full potential of fungal systems for environmental bioremediation.

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NOTE ADDED IN PROOF

(Sections below given same notation as in text)

III.1. *Mechanisms of toxicity.* Jungmann *et al.* (1993) have recently found that cadmium resistance in *Saccharomyces cerevisiae* is mediated in part by ubiquitin-dependent proteolysis suggesting that a significant part of cadmium toxicity involves formation of abnormal proteins. It is proposed that a fraction of these abnormal proteins are recognized and targetted for degradation by enzymes of the ubiquitin-dependent proteolysis system.

III.3. *Environmental influence on metal toxicity towards fungi.* The toxicity of some metals towards yeasts (and bacteria) may vary with pH because the binding of hydrolysed species, at higher pH values, alters the net charge of cells which, in turn, will affect physiological cell functions, as well as interactions with other cells and clay particles in the environment (Collins & Stotzky, 1992).

IV.3. *Transport of toxic metal cations.* The plasma membrane ferri-reductase activity of *S. cerevisiae* may have additional roles besides maintenance of cellular iron status. As well as being involved in reductive iron uptake, it also plays a more general role in adapting the redox potential of cells to growth conditions (Lesuisse & Labbe, 1992).

IV.4. Intracellular fate of toxic metals

(a) Metal binding proteins and peptides of filamentous fungi and yeasts

Metallothioneins. Recently, a hypothetical model has been presented for the structure of the metal cluster in the N-terminal DNA-binding domain of the ACE1 protein of *S. cerevisiae*, the regulatory gene product involved in transcriptional control of *CUP1*, the yeast metallothionein gene (Caras-Finet *et al.*, 1992).

Metal γ -glutamyl peptides. As well as toxic metals, wounds to the cell surface or incubation with chitosan induced synthesis of γ -glutamyl peptides in *Schizosaccharomyces pombe* which formed complexes with cellular Zn^{2+} with or without glutathione. Physiological roles in response to such alteration of membrane structure may include detoxification of superoxide or hydroxyl radicals, and/or in zinc homeostasis which may be part of the stress response (Hayashi *et al.*, 1992). Using a cadmium-sensitive mutant of *S. pombe*, deficient in the synthesis of a γ -glutamyl peptide, Ortiz *et al.* (1992) have isolated the gene, *hmt1* which complements this mutant. The deduced protein sequence of the *hmt1* gene shares identity with the family of ABC (ATP-binding cassette)-type transport proteins which are integral membrane proteins. Analysis of fractionated cell components indicated that the HMT1 polypeptide

was associated with the vacuolar membrane and it has been proposed that the vacuole plays a central role in γ -glutamyl peptide-mediated metal detoxification in *S. pombe* with S^{2-} being incorporated into the Cd- γ -glutamyl peptide in the vacuole (Ortiz *et al.*, 1992).

(b) *Vacuolar compartmentation.* Using laboratory and brewing strains of *S. cerevisiae*, Avery & Tobin (1992) have shown that intracellular accumulation of Sr^{2+} was predominantly in the vacuole and was accompanied by significant loss of vacuolar Mg^{2+} . Nickel has also been shown to be preferentially located in the vacuole of nickel-resistant strains of *S. cerevisiae* where it seems that the formation of intravacuolar nickel-histidine complexes reduced the vacuolar concentration of free Ni^{2+} (Joho *et al.*, 1992). Intracellular concentrations of Li^{+} were lower in Li^{+} -resistant strains of *S. cerevisiae* than in wild-type strains indicating some alteration of membrane transport in the resistant strain (Yagi, 1992). More recently, Perkins & Gadd (1993*a,b*) have demonstrated preferential localization of Li^{+} (and Cs^{+}) in the vacuole of *S. cerevisiae*, with significant exchange for K^{+} resulting on exposure to these monovalent cations.

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