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Al-toxicity studies in yeast using gallium as an aluminum analogue

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Abstract Aluminum (Al) is normally present in soils as the insoluble, harmless Al_2O_3. The highly toxic Al^{3+} and $AlOH^{2+}$ monomeric cations are formed in acid soils but there is little consensus on the physiological basis of Al toxicity in plants. A major factor that has retarded progress in understanding aluminum toxicity in vascular plants is the lack of a *convenient* radioisotope for Al. Yeast and vascular plants share similar membrane transport mechanisms and so yeast (*Saccharomyces cerevisiae*) provides a convenient model system for studies of Al-toxicity. Al and gallium (Ga) have closely similar toxic effects on the yeast cells ($K_i \approx 100$ mmol m^{-3}) and Ga^{3+} and Al^{3+}, respond similarly to pH and are both reversible by a chelation agent (citric acid). We tested the feasibility of using ^{67}Ga radioisotope as a tracer for Al transport with the view of using it to investigate the mechanism of Al uptake and toxicity in plants. The clinically available ^{67}Ga citrate is unsuitable to use as an aluminum analogue because the chelated form is not toxic. Arrangements need to be made for it to be supplied as $^{67}GaCl_3$. Large amounts of ^{67}Ga rapidly bind to the cell wall of yeasts with a $t_{1/2}$ of ≈ 1 s. There is a very slow net uptake of ^{67}Ga into a second phase, presumably the cytoplasm. Uptake into the slow phase has a V_{max} of only $\approx 16 \pm 4$ pmol m^{-2} s^{-1} ($n = 16$). The K_m of ^{67}Ga uptake could not be precisely determined but is below 100 mmol m^{-3} (45 ± 42 mmol m^{-3}, $n = 16$).

Keywords *Saccharomyces cerevisiae* · Aluminum · Al-toxicity · Gallium · Ga-toxicity · Allotropy · Analogue tracer

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Introduction

Al-toxicity is widespread in acidified agricultural soils and is of great economic importance in much of tropical and subtropical Asia and Australia, particularly for cereal crops (Marschner 1995; Atwell et al. 1999; Kochian et al. 2004), but is one of the least understood toxic reactions of vascular plants (Delhaize and Ryan 1995; Kochian 1995; Rengel and Zhang 2003, Kochian et al. 2004). Two major factors that have retarded progress in understanding aluminum (Al) toxicity in plants: (a) the complicated chemistry of Al ions in solution and (b) the lack of a *convenient* radioisotope for Al that could be used as a tracer in membrane transport studies.

Yeast (*Saccharomyces cerevisiae* strain CM-52) was used in the present study as a model eukaryotic unicellular plant cell, because it shows an Al-toxicity syndrome comparable to that found in vascular plants (MacDiarmid and Gardner 1996) and is very easy to grow reproducibly under acid conditions. Furthermore, growth of this organism can be monitored

using automated methods such as a titre plate reader to select for Al-tolerant strains.

Radioactive loading experiments with yeast can be done using the silicone-oil microfuge technique (Ritchie and Gibson 1987; Ritchie 1998; Wood et al. 1998). Yeast has been used extensively to identify genetic factors that influence metal toxicity for a long time. As in the case of vascular plants, *Saccharomyces* has been shown to exhibit Al toxicity symptoms only if grown in acid pH (MacDiarmid and Gardner 1996). Ezaki et al. (1999, 2005) have shown that Al-tolerance genes from vascular plants will confer Al-tolerance in yeast. Hamilton et al. (2001) have shown that the vacuolar H^+ATPase plays a critical role in Al-resistance in yeast and hence probably also in vascular plants.

The chemistry of Al ions in solution is complex (Greenwood and Earnshaw 1984; Delhaize and Ryan 1995; Kochian 1995; Marschner 1995). At low pH (below pH 4) the trivalent Al^{3+} cation (more accurately $Al(H_2O)_6^{3+}$) dominates: this is replaced by the $Al(OH)^{2+}$ and $Al(OH)_2^+$ ions as the pH increases. At near neutral pH the insoluble Al $(OH)_3$ or gibbsite is formed. The Al^{3+} cation is toxic to plants at micromolar external concentrations (Kinraide 1991; Kinraide et al. 1992; Ryan et al. 1992; Delhaize and Ryan 1995). The polynuclear form of Al cation $(AlO_4Al_{12}(OH)_{24}(H_2O)_{12})^{7+}$, commonly called Al13, which forms from the hydrolysis of Al^{3+}, is also highly toxic (Kinraide 1991; Kochian 1995). Formation of Al_{13} is catalysed by plant cell walls (Masion and Bertsch 1997): the 7+ charge on Al_{13} binds it strongly to the fixed negative charges of plant cell walls. Other Al-ions (such as $Al(OH)^{2+}$, $Al(OH)_2^+$ and $Al(OH)_4^-$) are considered non-toxic or only mildly toxic. Reid et al. (1996) in their study of the toxicities of Al, scandium and gallium in the giant-celled alga, *Chara* used the GEOCHEM PC program (Parker 2003) to estimate the relative abundance of the different forms of Al ions at different pH (Kinraide and Ryan 1991; Kinraide et al. 1992; Kinraide 1994, Reid et al. 1996).

Al^{3+} ions form stable complexes with a range of compounds (including phosphate, sulphate, fluorides and some di- and tri-carboxylates such as malate and citrate). Excretion of chelating compounds such as malate and citrate, which reverse Al-toxicity, have been used as a screening criterion for the development of Al-resistant varieties of cereals (Ryan et al. 1992, 1995a, b; Kochian 1995; Jones and Kochian 1996; Jones et al. 1996a, b; Rengel and Zhang 2003; Kochian et al. 2004). Plants known to accumulate Al such as hydrangeas do so as Al-citrate (Ma et al. 1997). Chelation effects can lead to misinterpretation of experiments and difficulties in comparing the results found in different laboratories, for example: (a) there may be unsuspected secretion of chelation agents by the experimental organisms themselves, (b) the presence of chelation agents in most trace element mixes is often overlooked and (c) the chelating effects of some commonly used buffers.

The only radioisotope of aluminum with an experimentally useable half-life is ^{26}Al, which has a half-life of 740,000 year. It is not routinely available and is very costly to synthesize. It is available only at very low specific activity, making it difficult to count using a γ-ray or scintillation counter even though it emits γ and β radiation that would otherwise make it a simple radiotracer to count. Exceptionally, Priest et al. (1995, 1996) had access to enough ^{26}Al to be able to use a γ-ray counter to count blood samples.

Taylor et al. (2000) were able to obtain enough ^{26}Al to use it as a tracer for Al uptake by the giant-celled alga, *Chara*. They used an AMS (accelerator mass spectrometer) to detect the ^{26}Al. They measured ^{26}Al-uptake in artificial pond water with a pH_o of around 5 to simulate typical pH conditions found in acidified soils. Nearly all the label was bound to the cell wall but a very small, but detectable, amount was taken up by the cytoplasm and moved from there to the vacuole. The uptake rate across the cytoplasmic membrane was very slow, 30–250 pmol m^{-2} s^{-1} from a 50 mmol m^{-3} solution. Nevertheless, this is much greater than available estimates of net uptake fluxes in *Chara* based on AAS analysis of cell sap (Reid et al. 1996). Taylor et al. (2000) found that detectable amounts of ^{26}Al crossed the tonoplast into the vacuole of *Chara.*

During transport from the bulk electrolyte to inside the vacuole of a plant it is likely that Al changes chemical form several times. At the near neutral conditions in the cytoplasm ($pH_c \approx 7.3$), the dominant form would be the insoluble Al $(OH)_3$ and the partially soluble Al $(OH)_4^-$ and chelated forms such as Al-citrate and Al-malate (Ma et al. 1997; Kochian et al. 2004). The concentration of Al^{3+} in the cytoplasm must be very low. It is important to also consider the complex ion, $(AlO_4Al_{12}(OH)_{24}(H_2O)_{12})^{7+}$ commonly called

Al_{13}. The very large size and high valency of Al_{13} makes it unlikely that it penetrates into the cytoplasm (Kinraide 1991; Kochian 1995; Reid et al. 1996) but it is possible that it forms in the cytoplasm.

One of the crucial problems in studies of Al-toxicity has been the problem of whether Al-toxicity is primarily a surface effect due to damage to the cell membrane and its proteins and consequent interference with membrane transport functions or whether the Al ions need to penetrate the cytoplasm for all the symptoms of Al-toxicity to appear (Kochian et al. 2004). Apoplasmic binding (binding to the fixed negative charges of the cell wall and membrane surface) for trivalent cations, such as Al^{3+} and Ga^{3+}, can reach several 1,000-fold compared to the bulk electrolyte but this is very dependent upon the composition of the bathing solution, the pH and the total ionic strength of the bulk electrolyte (Kinraide and Ryan 1991; Kinraide et al. 1992; Kinraide 1994; Rengel 1996; Rengel and Zhang 2003).

Transmembrane fluxes of Al in eukaryotic plants are known to be very low (Reid et al. 1996; Ryan et al. 1994, 1997; Rengel and Reid 1997; Taylor et al. 2000). Rao and Easwaran (1997) used ^{27}Al-NMR and dysprosium shift reagent to demonstrate that aluminum definitely does enter yeast cells, taking about 4 h to reach an equilibrium state. In principle it should be possible to make more detailed measurements of the time course and kinetics of aluminum uptake using NMR methods. ^{27}Al-NMR methods could potentially be used to identify the chemical forms of Al actually inside living cells. This is a critical question complicated by the recent finding by Ezaki et al. (2005) recently that vesicular transport mechanisms play an important role in conferring Al-resistance in yeast.

Even where ^{26}Al is available, it is generally not available at a high enough specific activity to measure fluxes across plasma membranes of cells. ^{26}Al is not of great practical use in investigating the physiology of Al toxicity in plants, particularly in screening investigations where large numbers of samples need to be assayed. Development of a radioactive tracer technique for studies of Al-toxicity and transport in plants would greatly help progress in understanding the mechanism of Al-toxicity.

This project aimed to validate using the readily available radioisotope of gallium (^{67}Ga) as an analogue tracer for Al transport in plant systems. The chemistry of Ga is very similar to that of Al but its abundance in the environment is very low compared to Al (Bailar et al. 1973; Greenwood and Earnshaw 1984). Gallium is a rare element that has no known biological role and is not environmentally important, however a high-specific activity radioisotope is readily available. The ionic radius of Ga^{3+} is 62×10^{-12} m compared to 53.5×10^{-12} m for Al^{3+}. Like ^{27}Al (Rao and Easwaran 1997), it is possible to detect ^{71}Ga using NMR techniques (Bradley et al. 1990, 1993). The behaviour of Ga ions in solution is poorly documented compared to that of Al (Greenwood and Earnshaw 1984; Brown 1989, Bradley et al. 1990). Like Al, Ga in solution forms an almost insoluble oxide (Ga_2O_3) near neutral pH and an anion $Ga(OH)_4^-$ under alkaline conditions. Under the acid conditions where Al is toxic Ga forms Ga^{3+}, (more correctly $Ga(6H_2O)^{3+}$), $Ga(OH)^{2+}$ and $Ga(OH)_2^+$. The pK_a for $Ga^{3+}/Ga(OH)^{2+} + H^+$ is ≈ 2.6 (Greenwood and Earnshaw 1984) or ≈ 3.16 according to Brown (1989) compared to a $pK_a \approx 5$ for Al^{3+} (Greenwood and Earnshaw 1984; Reid et al. 1996). Although gallium is toxic to plants (Reid et al. 1996), under the pH conditions where Al toxicity is apparent in soils (pH_o 4–5), the abundance of Ga^{3+} is very low compared to Al^{3+} at the same pH_o. The predominant species present are $Ga(OH)^{2+}$ and $Ga(OH)_2^+$, which, by analogy with the equivalent Al-ions, are probably not very toxic. Gallium, like Al, forms complexes with various common anions such as sulphate, phosphate and with malate and citrate. Gallium cations also hydrolyse at pH values below about pH 4_o to form a polynuclear cation analogous to Al_{13}, (GaO_4-$Ga_{12}(OH)_{24}(H_2O)_{12})^{7+}$ or Ga_{13} (Bradley et al. 1990, 1993). Ga_{13} is just as phytotoxic as Al_{13} (RJ Reid "personal communication"). ^{67}Ga (as Ga citrate) is a readily available, high-specific activity, radioisotope because it is used in clinical work as a tracer for Fe^{3+} in humans. Ferrotransferrin is important in the iron metabolism of vertebrates. Ga^{3+} also chelates with transferrin to form a gallium-labelled analogue (Ga-transferrin). It is hence used in routine clinical work for a purpose unrelated to its use in the present study.

The most direct test for the efficacy of an analogue tracer is to perform double-labelling experiments. A few clinical studies have attempted to measure Al uptake using ^{26}Al in mammalian systems (humans: Priest et al. 1995, 1996; mice: Radunovic et al. 1997a; rats: Radunovic et al. 1997b; Zafar et al. 1997, 1999, review: Gupta et al. 2005). Some of these studies attempted to use ^{67}Ga as a tracer for Al

and had the resources to be able to do $^{26}Al/^{67}Ga$ double labelling experiments. Radunovic et al. (1997a) did $^{59}Fe/^{67}Ga$ double labelling experiments to investigate Ga substitution for Fe in the Fe-transferrin system of rats. They concluded that $^{67}Ga^{3+}$ behaving as both a Fe^{3+} and Al^{3+} analogue in mammals was a major complicating factor in interpreting the results of ^{67}Ga-labelling studies in mammalian systems (Radunovic et al. 1997a, b).

Very little information is available on Ga uptake by vascular plants. Ferrotransferrin is not found in plants but there are many other Fe^{3+} binding proteins in plants that might bind $^{67}Ga^{3+}$ as a Fe^{3+} analogue. Blaylock et al. (1989) has shown in barley (*Hordeum vulgare*) that Ga^{3+} was taken up by vascular plants but did not compete with Fe^{3+} in uptake, nor would it substitute for iron even under severe iron deficiency stress. No published information is apparently available on gallium toxicity in *Saccharomyces*. It appears that ^{67}Ga is unlikely to be recognized by the iron uptake mechanism of yeast or vascular plants but this needs to be tested experimentally.

Materials and methods

Laboratory chemicals and radiochemicals

l-Asparagine, Biotin, Ca pantothenate, Folic acid, Inositol, Niacin, *p*-Amino Benzoic Acid, Pyridoxine hydrochloride, Riboflavin, Thiamine HCl, Fluorinert FC-77 were from Sigma-Aldrich. Silicone oils (AR20 and AR200, Wackerchemie, Germany) were gifts from Drs G.D. Price and M. Badger (Research School of Biological Sciences, ANU, Canberra) and Assoc Prof John Patrick (University of Newcastle, NSW, Australia). 400 µl Microfuge tubes were from Bio-Rad Laboratories, Hercules, CA, USA or Sarstedt Aktiengesellschaft and Co, Numbrecht, Germany. [^{3}H]Inulin was from Amersham Life Sciences, Amersham, UK. $^{67}GaCl_3$ in HCl (rather than the Ga-citrate form usually used clinically) was specially supplied by ANSTO (Lucas Heights, NSW, Australia).

Experimental material

Common yeast (*Saccharomyces cerevisiae*) is a completely sequenced eukaryotic cell (*Saccharomyces* Genome Database) and shares many of its transport mechanisms with vascular plants (MacDiarmid and Gardner 1996, *Saccharomyces* Genome Database, KEGG (Kyoto Encyclopaedia of Genes and Genomes). *Saccharomyces cerevisiae* CM-52 was kindly provided by Dr. R.C. Gardner (University of Auckland, New Zealand).

Culture medium

Saccharomyces was grown in a modified version of Wickerham's chemically defined medium (Difco Laboratories 1984; Rose and Harrison 1971) as described by MacDiarmid and Gardner (1996). The awkward chemistry of Al in solution dictates that only a fully defined medium should be used for Al-toxicity studies in yeast. MacDiarmid and Gardner (1996) emphasized that Al-toxicity is manifested in yeast only if it is grown under specific conditions. The standard defined medium for yeast is Wickerham's medium but this must be modified by reducing the $[Mg^{2+}]$ to 50 mmol m^{-3} and $[PO_4]$ to 25 mmol m^{-3} for Al-toxicity to be demonstrable. In the present study, the medium was further simplified by omitting the minor amino acids, histidine, methionine and tryptophan. The trace element and vitamin mixes were not modified. Chelation agents such as EDTA, citric acid, malic and glutamic acid need to be avoided because they reduce or eliminate the toxicity of both Al and Ga

Table 1 Modified Wickerham's chemically defined medium

Compound	Concentration
Glucose	50.5 mol m^{-3}
$(NH_4)_2SO_4$	14.8 mol m^{-3}
l.Asparagine	10 mol m^{-3}
KCl	7.35 mol m^{-3}
KH_2PO_4	25 mmol m^{-3}
NaCl	2.0 mol m^{-3}
$MgSO_4 \cdot 7H_2O$	50 mmol m^{-3}
$CaCl_2 \cdot 2H_2O$	0.25 mol m^{-3}
Trace element mix	As for Wickerham's medium
Vitamins	As for Wickerham's medium

Major Ions (including contributions from trace element mix)

NH_4^+ 29.6 mol m^{-3}, Na^+ 2.0 mol m^{-3}, K^+ 7.38 mol m^{-3}, Mg^{2+} 50 mmol m^{-3}, Ca^{2+} 250 mmol m^{-3}, Cl^- 9.85 mol m^{-3}, SO_4^{2-} 15.05 mol m^{-3}, $H_2PO_4^-$ mmol m^{-3}; Ionic strength = 56 mol m^{-3}

ions. Table 1 sets out the composition of the major components of the modified Wickerham's medium used in the present study. The table includes a summary of the major ionic composition used for calculations of the relative abundances of Al ions using the GEOCHEM PC program (see Parker 2003).

Saccharomyces was grown routinely in 100 ml aliquots of Wickerham's medium with the pH adjusted to 3 at 30°C on an orbital shaker set at 100 rpm under aerobic conditions. Growth of *Saccharomyces* was monitored by measuring A_{600} over time using a SPECTRONIC 20D+ spectrophotometer (Bausch and Lomb, USA). Preliminary studies showed that cell numbers, determined by hemocytometer counts were directly proportional to A_{600} up to $A_{600} \approx 1.3$. The usual doubling time for cultures started at an optical density of about 0.1 was about 3–4 h. If cells were treated with either Al or Ga, similar growth inhibition was observed (Fig. 2). The exponential growth constant ($k\ h^{-1}$) was determined by non-linear least squares fitting and its asymptotic error determined by matrix inversion (Johnson and Faunt 1992).

Kinetics of growth

The Michaelis–Menten model was used to calculate the inhibition constant (K_i) for both aluminum and gallium under a wide variety of conditions (Neame and Richards 1972). Equation 1 was fitted to inhibition curves using non-linear least squares methods and the errors of K_i and V_{max} calculated by matrix inversion (Johnson and Faunt 1992).

$$V = V_{max} - \frac{V_{max} \cdot [I]}{K_i + [I]}$$

which simplifies to:

$$V = \frac{V_{max} \cdot K_i}{K_i + [I]} \quad (1)$$

where, V is the growth rate, V_{max} is the maximum growth rate in the absence of the inhibitor, [I] is the concentration of the inhibitor, K_i is the Michaelis–Menten type inhibition constant.

^{67}Gallium as a tracer for Aluminum

^{67}Ga is readily available because it is used in routine clinical diagnoses (Priest et al. 1995, 1996; Radunovic et al. 1997a, b). It is a cheap and readily available isotope in Australia (ANSTO, Lucas Heights, NSW) and elsewhere. It is used clinically in tumour detection and is normally supplied as gallium citrate in physiological saline ($[Ga_3(OH)_4citrate_2]$) but in this form it is unsuitable to use as an aluminum analogue. ^{67}Ga is synthesized as $^{67}GaCl_3$ and then converted to the chelated form for clinical use. ANSTO kindly supplied ^{67}Ga in HCl as a special preparation.

^{67}Ga has a 1/2-life of 3.261 d: this convenient half-life minimizes contamination problems. It emits low energy γ radiation (0.093, 0.184, 0.3 and 0.393 MeV). Perspex or 2 cm lead shielding provided adequate protection. The ready availability and relatively low hazard of ^{67}Ga means that it could be used for screening for Al transport activity in plants in any laboratory suitable for low hazard, non-volatile radionuclides. A γ-ray counter was not readily available and so in this study ^{67}Ga was counted using a Packard Tricarb 1600 TR scintillation counter using the standard ^{14}C-channel and StarScint® scintillation fluid (as previously for $^{201}Tl^+$, Ritchie and Larkum 1998). The specific activity used was about 100×10^9 Bq mol^{-1}.

Surface area and volumes of cells

The mother cells of the budding yeast, *Saccharomyces cerevisiae* CM52, have a prolate spheroidal shape; buds are basically spherical. Only log-phase cultures used in growth experiments were used for determination of cell sizes and determination of mitotic indices. The cells were measured at 1,000× magnification under oil immersion. The average of length and breadths of the cells were measured and used to calculate the surface area and volumes of the cells in the Appendix. Estimates of cell dimensions were based on a total of 600 measurements from 10 different cultures in exponential growth phase. The mitotic index (proportion of Colony Forming Units, CFU with buds) was also estimated on several different samples of eight different exponentially growing cultures. The surface area and volumes of the CFU could then be calculated and are presented in the Appendix.

Surface areas and volumes of cells in a cell suspension

A relationship between the absorbance of a culture (A_{600}) and the number of cells of the CM-52 yeast

strain was worked out using more than 10 different cultures. Strictly speaking the cell counts used here were "colony forming units" CFU because a cell with a bud was counted as 1 unit. Cells were counted every hour using a hemocytometer. A graph was plotted with number of cells along the Y-axis and the optical density at A_{600} along the X-axis. This empirical relationship is only valid for the spectrophotometer used in the present study. The relationship between the CFU numbers and the optical density was plotted and found to be linear up to $A_{600} \approx 1.3$. The relationship between numbers of CFU and A_{600} could then be used to calculate the number of CFU and hence their volumes and surface areas present in a cell suspension.

Silicone oil gradients

Silicone oil gradient methods were used to estimate uptake of ^{67}Ga by yeast cells (adapted from Ritchie and Gibson 1987; Ritchie 1998). 400 µl Microfuge tubes were used. A 1:2 mixture of AR200 and AR20 (density 1.013) was used as the silicone oil layer (100 µl) and 10 µl of Fluorinert was used as a seat for the pellet. Tubes were centrifuged using a Beckman 152 Microfuge (Palo Alto, California) was used ($\approx$7,000 rpm or 3,300*g*) for about 10–20 s. The extracellular water volume of yeast cell pellets was measured using [^{3}H]Inulin and used to correct for the ^{67}Ga-labelling in the bulk electrolyte carried through with the cells. The extracellular volumes calculated on various bases are shown in the Appendix based upon four separate experiments with eight replicates in each experiment.

Statistics

All values quoted in the present paper are presented as means ±95% confidence limits with the number of separate experiments (a) and the total number of observations (b) quoted in brackets (a, b).

Results

Determination of the effects of Al and Ga upon growth

Growth of the yeast was measured by following the optical density at 600 nm over time (Fig. 1). If the

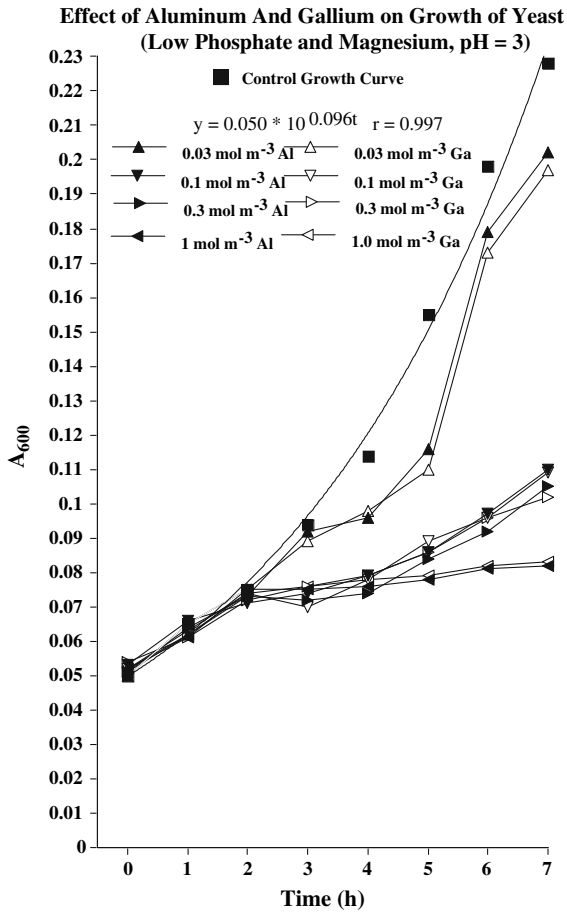

Fig. 1 Growth was followed using optical density at 600 nm (but if the A_{600} was greater than one the cell sample was diluted and the density of the culture calculated from the diluent). Growth of a control culture was included in each experiment. Experimentally treated cells were first preincubated for 2–3 h and growth followed to check that they behaved like the controls before the experimental treatment was added. Growth was then followed for at least 5 h. In the example shown, the effect of aluminum and gallium upon exponential growth of yeast was followed. Exponential growth constants were determined by non-linear least squares fitting. Cells were grown in modified Wickerham's medium at pH 3_o (MacDiarmid and Gardner 1996)

A_{600} was greater than 1.0 the cell sample was diluted and the density of the culture calculated from the diluent. Growth of a control culture was included in each experiment. Experimentally treated cells were first preincubated for 2–3 h and growth followed to check that they behaved like the controls before the experimental treatment was added. Growth was then followed for at least 5 h. In the example shown, the effect of aluminum and gallium upon exponential

growth of yeast was determined at pH_o 3. Figure 1 shows that progressive increases in added Al or Ga had almost identical inhibitory effects upon growth. 1.0 mol m^{-3} Al or Ga almost halted growth. Inhibitory effects were noticeable within 1 h of exposure. There appeared to be no recovery of growth capacity even after 5 h exposure to Al or Ga. There did not appear to be any acclimation to the toxic metals.

Exponential growth constant could be calculated using non-linear least squares fitting methods using the data shown in Fig. 1. The growth constants (h^{-1}) were: control, 0.221 ± 0.0166 (98); 0.03 mol m^{-3} Al, 0.208 ± 0.0680 (6); 0.1 mol m^{-3} Al, 0.0882 ± 0.0234 (6); 0.3 mol m^{-3} Al, 0.0766 ± 0.0380 (6); 1 mol m^{-3} Al, 0.0201 ± 0.00792 (6); 0.03 mol m^{-3} Ga, 0.198 ± 0.0698 (6); 0.1 mol m^{-3} Ga, 0.0862 ± 0.0380 (6); 0.3 mol m^{-3} Ga, 0.0722 ± 0.0155 (6); 1 mol m^{-3} Ga, 0.0233 ± 0.00384 (6). The inhibitory effects of aluminum and gallium were not significantly different at each concentration used. Fitting the growth constants to Equation the inhibition constant for Al and Ga (K_i) was 100 ± 34 mmol m^{-3} ($n = 56$, $r = 0.8965$).

Figure 2 shows the exponential constants determined in an experiment similar to that shown in Fig. 1 plotted against the concentration of aluminum and gallium ions. The inhibition constant (K_i) for aluminum and gallium was determined using non-linear least squares fitting. A student's *t*-test showed that they were not significantly different. The overall mean K_i was 102 ± 33 mmol m^{-3} ($n = 90$, $r = 0.8632$).

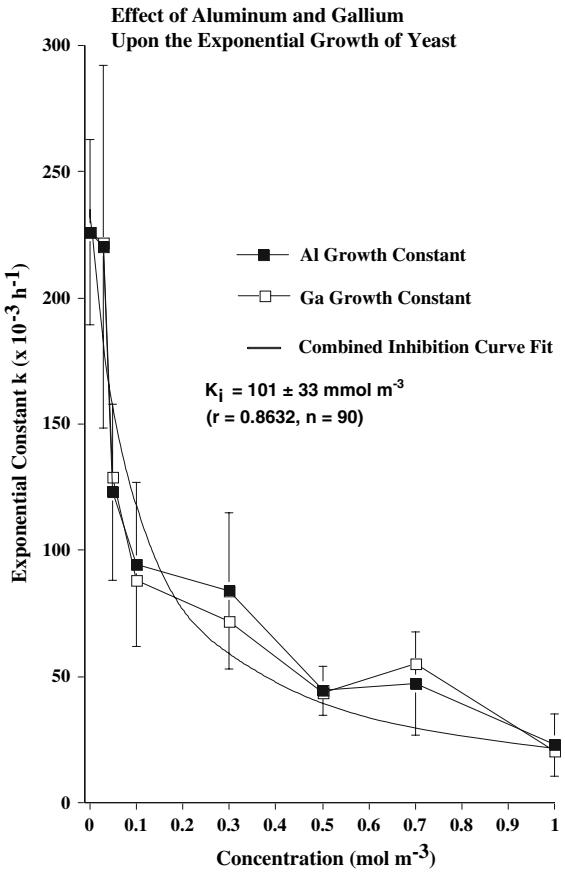

Fig. 2 Effect of aluminum and gallium upon exponential growth of yeast at pH_o 3. Growth constants are based on growth at 6 time points. The inhibition constant (K_i) for aluminum and gallium were not significantly different and so an overall mean K_i could be calculated

Reversal of Al and Ga toxicity using citric acid

The toxicity of both aluminum and gallium ions can be reversed using citric acid (a chelation agent) (Fig. 3). Three lots of cells were incubated in modified Wickerham's medium at pH_o 3. During the first 3 h the cells were incubated in modified Wickerham's medium and all three cultures grew at a similar rate. Addition of 100 mmol m^{-3} aluminum and 100 mmol m^{-3} gallium strongly inhibited the growth of the cells. There appeared to be no significant difference in the effect of the two metals. After 6 h 1 mol m^{-3} citrate was added to the cultures with the Al and Ga added. Citrate relieved the inhibitory effects of both metals. Figure 3 shows that both Al and Ga inhibit growth of yeast in less than 30 min of exposure. Addition of excess citric acid rapidly relieves inhibition.

Effect of chelates upon Al and Ga toxicity

Figures 4 and 5 are a comparison of the toxicity of 100 mmol m^{-3} aluminum and gallium over a range of pH_o in the presence and absence of citric acid (a chelation agent). Figure 4 shows that both aluminum and gallium are strongly inhibitory at acid pH_o but their toxicity decreases in media that is less acidic. Gallium and aluminum toxicity are essentially equivalent and respond to pH_o in a similar way. The control shows that pH_o has little effect upon the growth of yeast. Figure 5 shows that citric acid

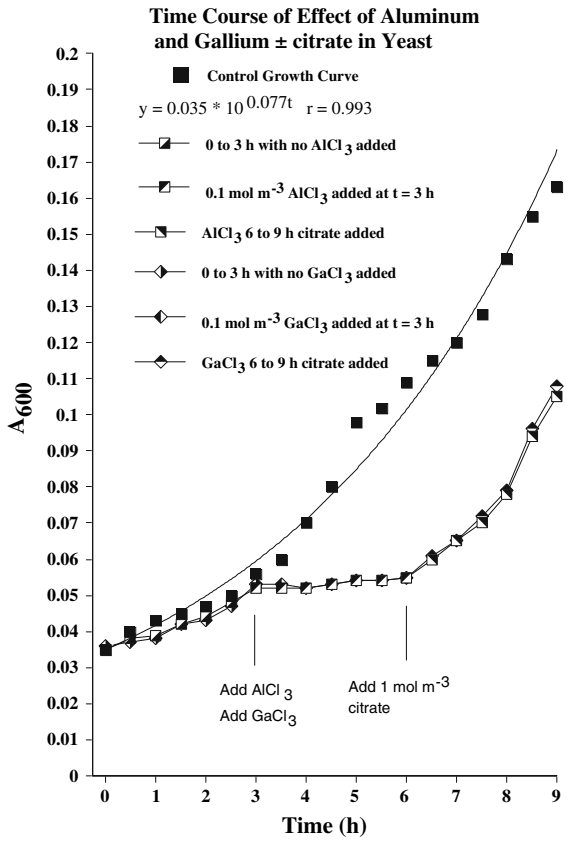

Fig. 3 The effect of aluminum and gallium (100 mmol m^{-3}) upon the growth of yeast in the presence and absence of citrate (1 mol m^{-3}). Three lots of cells were incubated in modified Wickerham's medium at pH_o 3. During the first 3 h the cells were incubated in modified Wickerham's medium and grew at a rate similar to the control cells. Addition of 100 mmol m^{-3} aluminum and 100 mmol m^{-3} gallium strongly inhibited growth of the cells. There appeared to be no significant difference in the effect of the two metals. After 6 h 1.0 mol m^{-3} citrate was added. This chelating agent relieved the inhibitory effects of both metals

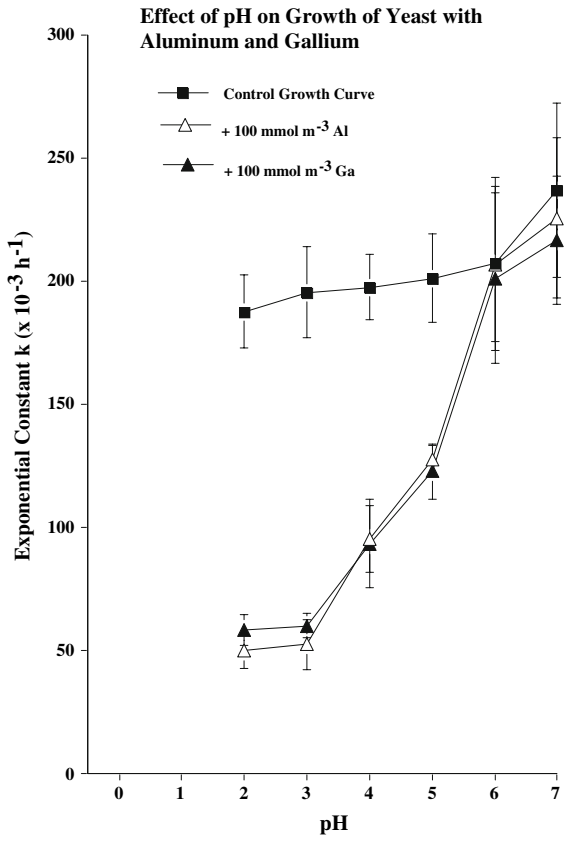

Fig. 4 Comparison of the toxicity of 100 mmol m^{-3} aluminum and gallium over a range of pH_o in the presence and absence of citric acid (a chelation agent). Growth constants are based on growth at 8 time points. Both aluminum and gallium are strongly inhibitory at acid pH_o but their toxicity decreases in media that is less acidic. Gallium and aluminum toxicity are essentially equivalent. The control shows that pH_o has little effect upon the growth of yeast

(1 mol m^{-3}) has no significant effect upon the control cells but citrate completely abolishes both aluminum and gallium toxicity. In the experiment shown in Fig. 5 an excess of citric acid (1 mol m^{-3}) was used to reverse the toxicity of Al and Ga.

Other experiments demonstrated that there was a progressive reversal of Al and Ga with increasing concentration of citric acid at pH_o 3. Aluminum and gallium concentrations were held at 100 mmol m^{-3}. The responses of gallium and aluminum toxicities to increasing concentrations of citric acid were essentially equivalent. It takes about 2–3 moles of citrate/mole of the toxic metal to reverse toxicity.

Binding of ^{67}Ga to cells

A large proportion of ^{67}Ga labelling of yeast cells appears to be extracellularly bound to the cells. If cells are incubated in 100 mmol m^{-3} Ga at $pH_o = 3$, the $t_{1/2}$ for the process is about 1.24 ± 0.29 (3, 24) s. The rate of binding is not affected by the presence or absence of Al ions. The rate of binding is 72 ± 18 nmol m^{-2} s^{-1} and the size of the bound pool is 110 ± 18 nmol m^{-2} on a cell surface area bases. Figure 6 shows an experiment

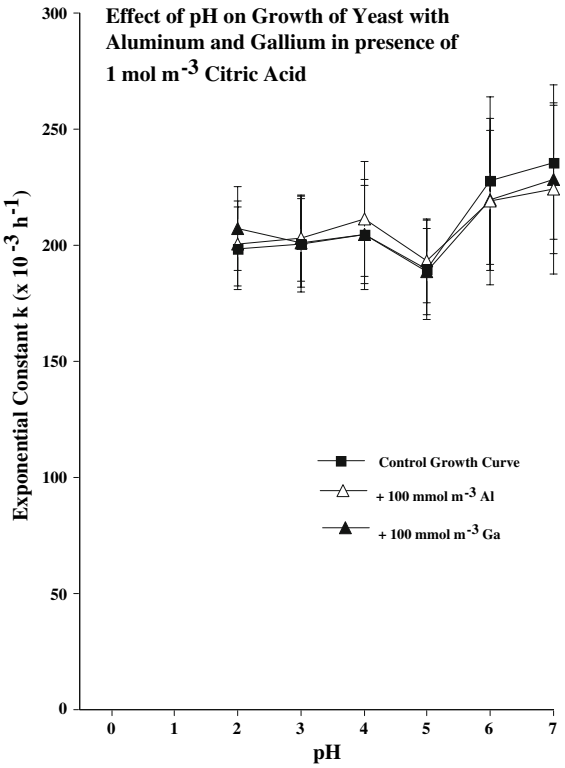

Fig. 5 Comparison of the toxicity of 100 mmol m^{-3} aluminum and gallium over a range of pH_o in the presence and absence of citric acid (a chelation agent). Growth constants are based on growth at 8 time points. Citric acid (1 mol m^{-3}) has no significant effect upon the control cells but citrate completely abolishes both aluminum and gallium toxicity

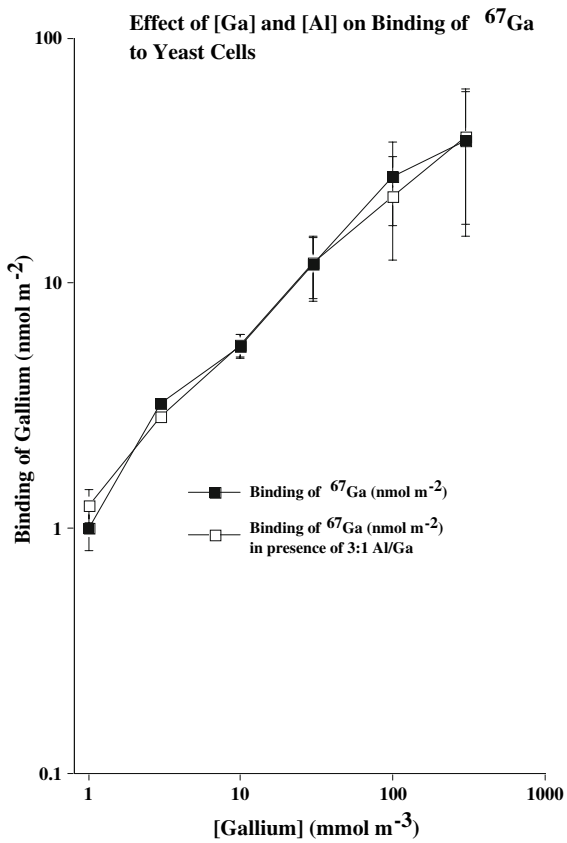

Fig. 6 Binding of gallium to yeast cells at an external pH_o of 3 using a silicone oil gradient separation. Binding was corrected for the extracellular volume of the cell pellets using [^{3}H]Inulin volume as a measure of the volume of bulk electrolyte carried through with the cells. Binding is expressed as mol per unit cell surface area ($n = 8$). Binding was also measured in the presence of Al; molar abundance 3Al:Ga

where cells were labelled with ^{67}Ga at concentrations of 1–300 mmol m^{-3} Ga for about 30 s then spun through silicone oil. Binding of ^{67}Ga appears to be a simple adsorption isotherm where binding is directly proportional to $[Ga]_o$. Figure 6 also shows a similar experiment where binding of ^{67}Ga was measured in the presence of an excess of Al (molar ratio 3Al:Ga). It appears that Al and Ga ions are interchangeable.

As would be expected, pH_o has a substantial effect upon the rapid phase binding of ^{67}Ga (100 mmol m^{-3} [Ga]) to yeast cells. Above pH 5_o the apparent binding is a constant; about 170 ± 15 nmol m^{-2}. Binding decreases as the pH_o decreases and is half-maximum at about pH_o 3 (Fig. 7). When compared to Fig. 4 showing the toxicity of gallium it can be concluded that binding to the surface of the cells is highest under near-neutral pH conditions where gallium is not toxic. Hence, the amount of surface binding is not directly related to toxicity.

Attempts to measure the rate of intracellular uptake of ^{67}Ga

Attempts were made to measure ^{67}Ga uptake by cells by incubating them in ^{67}Ga for a given time and then spinning them through silicone oil. If yeast cells are incubated for up to 1 h in ^{67}Ga a very slow apparent uptake of ^{67}Ga is observed after the initial rapid binding phase (complete after less than 1 min). The slow uptake phase was approximately linear with time and the rate was estimated by linear regression. The uptake rate is very slow; in 100 mmol m^{-3} [Ga] the uptake rate is about 25 ± 8 pmol m^{-2} s^{-1} (5,134) which is close to the limits of detection using the specific activity of ^{67}Ga used for the present study. The bound ^{67}Ga resulted in a high

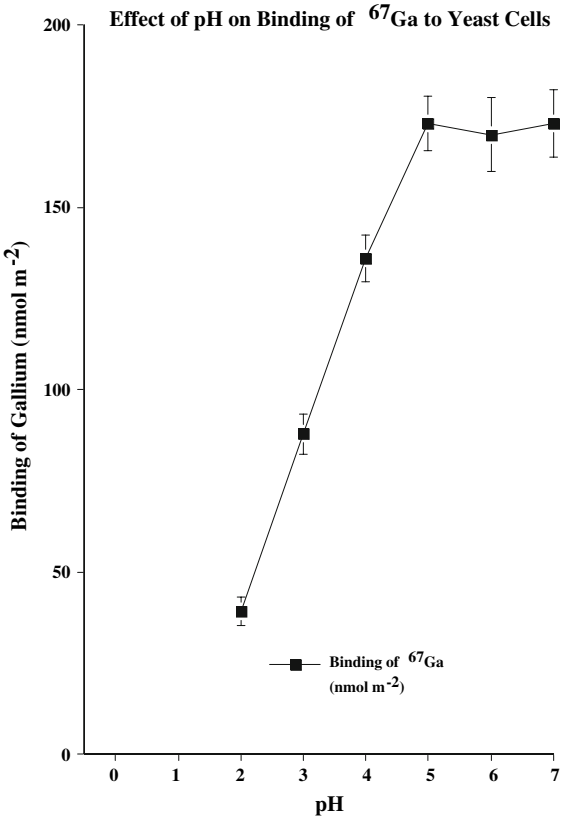

Fig. 7 Binding of gallium to yeast cells at a range of pH_o from 2 to 7 using silicone oil gradient techniques. As for Fig. 6, binding was corrected for ^{67}Ga present in extracellular space of the pellets and calculated on a cell surface area basis ($n = 8$)

"background" which made it difficult to measure the intracellular uptake rate.

The very slow intracellular uptake rate for ^{67}Ga makes it technically very difficult to measure short-term uptake rates needed to estimate K_m and V_{max} using the Michaelis–Menten equation. The Michaelis–Menten equation was fitted using non-linear least squares fitting methods (Ritchie and Prvan 1996) and the asymptotic errors of K_m and V_{max} calculated by matrix inversion (Johnson and Faunt 1992). Preliminary data gives an estimate of K_m to be 45 ± 42 mmol m^{-3} (16) and a V_{max} of 16 ± 4 pmol m^{-2} s^{-1} (16) ($r = 0.79$, $P \ll 0.001$). These results establish that the K_m for uptake of Ga is below 100 mmol m^{-3} but K_m could not be estimated precisely enough for it to be feasible to attempt to measure the discrimination ratio between Ga and Al. Preliminary experiments showed that 100 mmol m^{-3} Al decreased ^{67}Ga uptake from 100 mmol m^{-3} Ga by about 50% suggesting that the discrimination ratio is about 1:1 (consistent with the K_i data based upon the effects of Al and Ga upon growth (Fig. 2).

A more precise method for measuring rates of intracellular uptake of ^{67}Ga needs to be developed to characterise the kinetics of intracellular uptake of Ga. Unsuccessful attempts were made to measure the intracellular uptake rate for ^{67}Ga by filtering cells, at 5 or 10 min intervals, onto polycarbonate membrane filters, then washing the cells with a leachate containing unlabelled Ga to remove extracellular ^{67}Ga. The ^{67}Ga bound to the yeast cells in the first few seconds of labelling (Figs. 6 and 7) was not readily removable by replacement with unlabelled Ga and the intracellular uptake rate could not be estimated.

Discussion

Aluminum and gallium toxicity in yeast appears to be the same syndrome as Al-toxicity. In yeast, Al/Ga toxicity can be conveniently measured by the effects of these metals on growth. A disadvantage of using growth to monitor effects however, is that rapid responses of cells to a toxic ion (time scale of seconds) cannot be measured easily as is the case where electrophysiological or fluorescence methods are used. The K_i of Al and Ga are both about 100 mmol m^{-3}; toxicities of both metals can be reversed by citric acid and neutral pH_o.

It is thought in vascular plants that only the Al^{3+} form is toxic (Kinraide 1991; Kochian 1995). Toxicity in plants is reversible by chelation by organic acids such as malic, glutamic, oxalic and citric acids (Jones and Kochian 1996; Jones et al. 1996a, b; Ryan et al. 1997b; Rengel and Zhang 2003; Ma et al. 1997, 1998; Kochian et al. 2004). Our results show a strong effect of pH_o on both Al-toxicity and Ga-toxicity (Fig. 4) and the observation that toxicity is reversed by the presence of chelation agents such as citric acid (Figs. 3 and 5) is in agreement with previous findings by MacDiarmid and Gardner (1996).

Al-resistant varieties of corn (*Zea mays*) are known to secrete citric acid (Ryan et al. 1995a, b; Rengel and Zhang 2003). In the present study, attempts were also made to demonstrate reversal of Al-toxicity in yeast using malic acid. Preliminary experiments showed that malic acid was highly toxic ((1 mol m^{-3}) to yeast. We found that yeast grew equally well on glutamic acid as

with asparagine (Table 1) but glutamate-grown yeast was found to be insensitive to Al and Ga when tested in a modified Wickerham's medium where asparagine was replaced by glutamate (data not shown). Hence, at least one dicarboxylic acid (glutamate) will reverse the Al-toxicity syndrome in yeast. Figures 3–5 show that the effect of pH_o and chelators upon both Al and Ga toxicity is essentially equivalent but this observation does not agree with the known differences in the acid/base behaviour of aluminum and gallium ions. The pKa for Al^{3+} is well documented ($pK_a \approx 5$, Greenwood and Earnshaw 1984; Reid et al. 1996) but the pK_a for Ga^{3+} is less well known but is about 2.6–3.2 (Greenwood and Earnshaw 1984; Brown 1989). Experimentally, we have found that both Al and Ga inhibit yeast cells with the same K_i (Fig. 2) and pH affects toxicity in the same way (Fig. 4). This is a surprising result because the weak acid behaviour of Al and Ga ions is different. Gallium should become innocuous at a much lower pH_o than aluminum (Henderson–Hasselbach Equation). Al and Ga toxicities in yeast are not a simple function of the abundance of the trivalent forms of these two metal ions in the bulk medium.

There has long been argument about whether Al ions need to enter the cytoplasm to manifest toxicity or whether the Al-toxicity syndrome is primarily a toxic effect upon the cell membrane of plants (Kinraide 1991, 1994; Kinraide and Ryan 1991; Kinraide et al. 1992, 1994; Ryan et al. 1992; Rengel 1996; Reid et al. 1996; Taylor et al. 2000; Rengel and Zhang 2003; Kochian et al. 2004). The very rapid electrical effects of very low concentrations of Al-ions under acid conditions (Kinraide et al. 1992; Kinraide 1994; Ryan et al. 1992, 1994, 1997a, b) and the rapid efflux of protective organic acids (Ryan et al. 1995a, b; Jones and Kochian 1996; Ryan et al. 1997a, b) would appear to be good arguments in favour of a primary surface effect. Interactions between Al-toxicity and calcium can also be accounted for in terms of interactions of aluminum ions with cell surface proteins that require calcium for proper function (Kinraide et al. 1992, 1994; Ryan et al. 1994; Reid et al. 1995; MacDiarmid and Gardner 1996; Ryan et al. 1997a; Rengel and Zhang 2003). Rengel and Zhang (2003) also conclude that interference with intracellular Ca^{2+}-homeostasis would account for most of the known intracellular effects associated with Al-toxicity.

^{67}Ga experiments show that ^{67}Ga rapidly binds to the cell surface ($t_{1/2} \approx 1$ s) in relatively large amounts ($\approx 110 \pm 18$ nmol m^{-2}). ^{67}Ga uptake into the cell is very slow (26 ± 8 pmol m^{-2} s^{-1} in 100 mmol m^{-3} [Ga]). However, even at this very slow uptake rate it can be calculated that it would take only 26 ± 8 s for the average intracellular [Ga] to build up to 1 mmol m^{-3}. The low rates of uptake of Al and Ga into the cytoplasm of cells is not a water-tight argument against the idea that much of the toxicity of Al is due to Al that actually enters the cytoplasm of cells. Rengel and Zhang (2003) point out that the free Ca^{2+} activity inside the cytoplasm of plant cells is about 0.2 mmol m^{-3} and so only minute amounts of Al or Ga would need to enter a cell to cause major upset of the calcium balance of a cell. NMR techniques (Bradley et al. 1993) would be a useful adjunct to ^{67}Ga studies to determine the chemistry of Ga inside cells and whether Ga-toxicity is truly analogous to aluminum-toxicity.

Binding of ^{67}Ga to the surface of the cells appears to be dependent on both concentration of Ga (Fig. 6) and pH_o (Fig. 7). At pH_o 3, where Ga is highly toxic (Fig. 4), adsorption appears to be directly proportional to the concentration of Ga in the incubation medium but if adsorption is measured over a wide range of pH_o, it is found that maximum adsorption occurs above pH 5_o where Ga and Al are both not very toxic (Fig. 4). Hence the degree of binding of ^{67}Ga is not directly related to the relative toxicity of the ion. The half maximum binding is at about pH_o 3, which is similar to the pK_a of the chitin, pectin and pectin-like compounds responsible for the fixed negative charges found in plant and fungal cell walls.

The very slow intracellular uptake rate for ^{67}Ga makes it technically very difficult to measure uptake rates over a range of concentrations to estimate K_m and V_{max}. Preliminary data gives an estimate of K_m to be 45 ± 42 mmol m^{-3} (16) and a V_{max} of 16 ± 4 pmol m^{-2} s^{-1} (16). This very slow maximum uptake rate is comparable to the Al uptake flux found in *Chara* by Reid et al. (1996) using AAS and Taylor et al. (2000) using ^{26}Al. Using electrical methods, the Al uptake flux in vascular plants is also known to be very low (Ryan et al. 1992). The K_m for ^{67}Ga uptake is not inconsistent with the observed K_i for the effect of gallium upon growth of yeast (Fig. 2). The data at hand hence suggests that there is a saturable mechanism for uptake of ^{67}Ga and that the

K_m for this process at least appears to be similar to the K_i observed in growth experiments (Figs. 2–5).

Both aluminum and gallium form complex hydroxides in solution, the relative abundances of which are pH-dependent (Kochian et al. 2004). The abundances of the various aluminum ions that exist in a complex medium such as the modified Wickerham's medium used in the present study (Table 1) can be calculated using the program GEOCHEM PC. Figure 8 shows the abundances of different aluminum ions and complexes as a function of pH_o calculated using the GEOCHEM PC program for 100 mmol m^{-3} total added Al. Al^{3+} ions only appear in significant concentrations below pH_o 4.5. Large amounts of ^{67}Ga label ($\approx$170 mmol m^{-2}) adhere to yeast cell walls above pH_o 5 where virtually all gallium would be present as insoluble

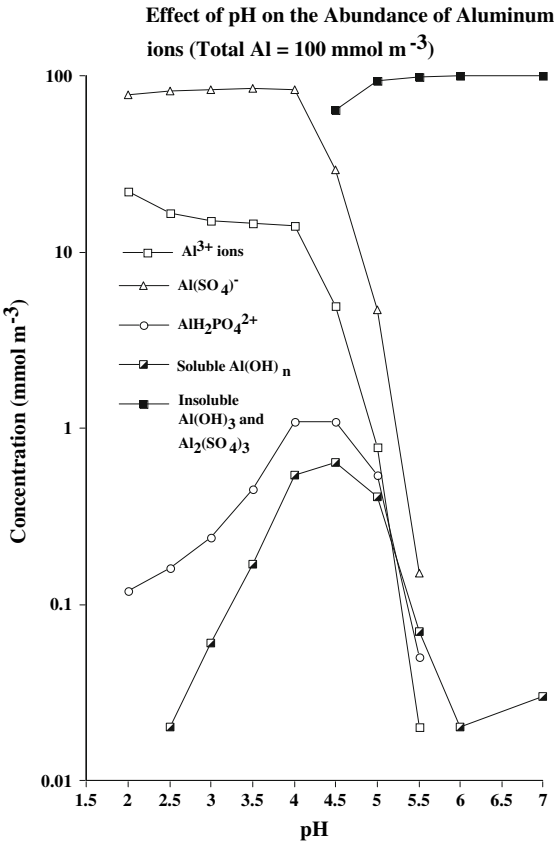

Fig. 8 Abundances of different aluminum ions and complexes as a function of pH_o calculated using the GEOCHEM PC program for the modified Wickerham's defined yeast medium. Al^{3+} ions only appear in significant concentrations below pH_o 4.5. Insoluble forms account for nearly all Al above pH_o 4. An Al–sulphate complex anion accounts for a large proportion of total Al below pH_o 4.5

$Ga(OH)_3$ (Greenwood and Earnshaw 1984). In principle, GEOCHEM PC could also be used to calculate the abundances of gallium ions and complexes at a range of pH_o however; the necessary equilibrium constants are not well documented.

An Al-sulphate complex accounts for a large proportion of total Al below pH_o 4.5 (Fig. 8). The actual concentration of Al^{3+} present at pH_o 3 is therefore only about 10% of the total Al ions present. Studies of electrical responses of vascular plant roots to aluminum ions at acid pH_o routinely report drastic effects at concentrations in the low micromolar (mmol m^{-3}) range (Kinraide 1991, Ryan et al. 1995a, b; Jones and Kochian 1996, Jones et al. 1996a, b; Rengel and Zhang 2003; Kochian et al. 2004). Such experiments are usually run in very simple solutions containing very low concentrations of mineral ions and little or no sulphate. It was expected that Al-toxicity in yeast would be manifested at similarly low concentrations rather than the rather higher concentrations found in the present study ($K_i \approx 100$ mmol m^{-3}), but this was before the significance of the high sulphate concentrations in Wickerham's medium was appreciated (Fig. 8).

The apparent K_i of the effects of both aluminum and gallium ions at pH_o 3 was about 100 mmol m^{-3} and the provisional estimate of the K_m of ^{67}Ga uptake was also about 100 mmol m^{-3}. Figure 7 shows that at pH_o 3 a large proportion of aluminum in solution in Wickerham's medium is actually present as an Al-sulphate complex. We have good evidence from the present study and from the work of MacDiarmid and Gardner (1996) and Ezaki et al. (1999, 2005) that Al-toxicity in yeast is the same syndrome as that found in vascular plants. We propose that in yeast, although the K_i for Al-toxicity is about 100 mmol m^{-3} if expressed on the basis of total Al-ions, the K_i of the toxic Al^{3+} form is actually of the order of 10 mmol m^{-3}. The high concentrations of sulphate present in the experimental medium used in this study and by MacDiarmid and Gardner (1996) partially protected the cells from Al-toxicity. MacDiarmid and Gardner (1996) pointed out that the yeast cells manifested no toxicity effects of aluminum unless Wickerham's medium was modified to contain very little phosphate. The chemistry of the experimental media used in Al-studies need to be examined very carefully in order to make cross-comparisons between the results of different studies.

We conclude that ^{67}Ga shows great promise as an analogue tracer for Al. ^{67}Ga is readily available at high

specific activity, has a $t_{1/2}$ of 3 days and appears to be useable as an Al-tracer analogue to investigate the mechanisms of transport and binding of Al. Although the pK_a of Ga^{3+} and Al^{3+} are substantially different, the toxicity responses of yeast to aluminum and gallium ions at various pH_o are almost identical. Yeast does not appear to discriminate between aluminum and gallium under any of the experimental conditions used in the present study. When better techniques are developed to measure uptake fluxes of ^{67}Ga it will be crucially important to conclusively demonstrate competitive inhibition of intracellular ^{67}Ga uptake by Al. It would also be very interesting to perform $^{67}Ga/^{45}Ca$ double labelling experiments to investigate possible interactions between Al and Ca transport. An alternative approach would be to investigate the effects of these ions upon intracellular Ca-florescence dyes.

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Appendix: Surface area and volume of yeast cells

*All error bars are ±95% confidence limits.

Mitotic Index (MI)

$$MI = 0.362 \pm 0.041 \ (n = 81)$$

Mother cells (Prolate Spheroid)

$$\text{Cell Length } (L_m) = 5.01\ (\pm 0.0692) \times 10^{-6}\ \text{m}\ (n = 600)$$

$$\text{Cell Width } (W_m) = 3.86\ (\pm 0.0637) \times 10^{-6}\ \text{m}\ (n = 600)$$

$$\text{Surface Area } (SA_m) = 55.0\ (\pm 1.31) \times 10^{-12}\ \text{m}^2\ \text{CFU}^{-1})$$

$$\text{Volume } (V_m) = 39.1\ (\pm 1.40) \times 10^{-18}\ \text{m}^3\ \text{CFU}^{-1})$$

Bud cells (Spheres)

$$\text{Bud Cell Diameter } (r_b) = 3.01\ (\pm 0.041) \times 10^{-6}\ \text{m}\ (n = 600)$$

$$\text{Surface Area } (SA_b) = 28.5\ (\pm 0.776) \times 10^{-12}\ \text{m}^2\ \text{CFU}^{-1})$$

$$\text{Volume } (V_b) = 14.3\ (\pm 0.584) \times 10^{-18}\ \text{m}^3\ \text{CFU}^{-1})$$

Surface Area and Volume of Colony Forming Units (CFU)

$$\text{Surface Area } (SA_{cfu}) = SA_m + MI(SA_b) = 65.3\ (\pm 1.80) \times 10^{-12}\ \text{m}^2\ \text{CFU}^{-1})$$

$$\text{Extracellular Volume } ([^3H]\text{Inulin})/\text{CFU Surface Area} = V_{ecv}/SA_{cfu} = 281\ (\pm 12.8) \times 10^{-9}\ \text{m}^3\ \text{m}^{-2} (4,32)$$

$$\text{Volume } (V_{cfu}) = V_m + MI(V_b) = 44.2\ (\pm 1.53) \times 10^{-18}\ \text{m}^3\ \text{CFU}^{-1}$$

$$\text{Extracellular Volume } ([^3H]\text{Inulin}) = V_{ecv} = 18.3\ (\pm 0.821) \times 10^{-18}\ \text{m}^3\ \text{CFU}^{-1} (4,32)$$

$$\text{Extracellular Volume } ([^3H]\text{Inulin})/\text{CFU Volume} = V_{ecv}/V_{cfu} = 0.414\ (\pm 0.0191)\ \text{m}^3\ \text{m}^{-3} (4,32)$$

Relationship between A_{600} and Total Surface Area and Volume of Cells in a cell suspension for Spectronic 20D+ spectrophotometer.

$$N_{cfu} = 51\ (\pm 1.33) \times 10^6\ \text{CFU}\ A_{600}^{-1}\ \text{ml}^{-1}\ (n = 112)$$

therefore,

$$\text{Surface Area } (SA_{toal}) = SA_{cfu} N_{cfu} = 3.33\ (\pm 0.126)\ \times 10^{-3}\ \text{m}^2\ A_{600}^{-1}\ \text{ml}^{-1})$$

$$\text{Volume } (V_{total}) = V_{cfu}\ .\ N_{cfu}) = 2.25(\pm 0.098) \times 10^{-9}\ \text{m}^3\ A_{600}^{-1}\ \text{ml}^{-1}$$

$$\text{Extracellular Volume } ([^3H]\text{Inulin}) = V_{ecv}.N_{cfu} = 934\ (\pm 36)\ \times\ 10^{-12}\ \text{m}^3 A_{600}^{-1}\ \text{ml}^{-1}\ (4, 32)$$

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