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Ljerka Kunst
University of Stockholm, Sweden

Godfried M. Roomans
University of Stockholm, Sweden

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INTRACELLULAR LOCALIZATION OF HEAVY METALS IN YEAST BY X-RAY MICROANALYSIS

Ljerka Kunst* and Godfried M. Roomans

Wenner-Gren Institute, University of Stockholm,
Norrtullsgatan 16, S-11345 Stockholm, Sweden

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Abstract

The intracellular localization of heavy metals in yeast cells was studied by means of energy-dispersive X-ray microanalysis. The yeast, Saccharomyces cerevisiae, was pretreated with phosphate and then loaded with different metal ions, by suspending the cells in salt solutions (Ni, Zn, Cd, Pb, Al and Cr). For the analysis, the cells were embedded in gelatin, rapidly frozen, and thin cryosections were cut on a dry knife.

A considerable uptake of divalent cations by the yeast cells was found to occur. The cations were bound to the polyphosphate granules localized in or close to the cell vacuoles. Immediately after phosphate loading, the polyphosphate granules were predominantly located in the cytoplasm, but as the incubation progressed, they migrated to the vacuole. As for trivalent cations, Cr was taken up and also stored in the polyphosphate granules, but Al could not be demonstrated with certainty in the cells, only in the cell walls. Incubation of the cells with zinc, cadmium or lead ions caused a significant decrease of the relative size of the vacuole.

Key words: yeast, X-ray microanalysis, cryoultramicrotomy, heavy metals, aluminum, polyphosphate, vacuole, cell wall, ion transport, toxicity.

* Address for correspondence:
Ljerka Kunst, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824
Phone No. (517) 353-8764

Introduction

Uptake of divalent cations by yeast is dependent on the intracellular phosphate concentration. The rate of uptake of divalent cations by phosphate-rich yeast is several times that by phosphate-deficient yeast (Rothstein et al. 1958, Jennings et al. 1958, Roomans et al. 1979). Roomans (1980a) showed, using X-ray microanalysis, that Ca and Sr accumulated by phosphate-rich yeast was localized in cytoplasmic polyphosphate granules. This finding was in apparent conflict with results obtained with other techniques, according to which divalent cations were localized in the yeast cell vacuole (Okorokov et al. 1977, 1980, Lichko et al. 1982, Nieuwenhuis et al. 1982).

The divalent cation uptake system does not only transport Ca and Sr ions, but also other divalent cations such as Ni, Zn, and Cd (Fuhrmann and Rothstein 1968, Ponta and Broda 1970, Norris and Kelly 1977, Gadd and Mowll 1983, Mowll and Gadd 1984).

In the present study, the localization of a number of divalent cations in phosphate-rich yeast was investigated by X-ray microanalysis and it was attempted to resolve the conflict between cytoplasmic and vacuolar localization. In addition, the subcellular localization of the trivalent cations Al and Cr was investigated.

Materials and Methods

Yeast cells

Baker's yeast, Saccharomyces cerevisiae L. (Jästbolaget, Sollentuna, Sweden) was starved under aeration in distilled water overnight. Phosphate enrichment of the starved cells was carried out by resuspending the cells (2% w/v) in 45 mM Tris-citrate buffer pH 5.0 with 3% glucose, 10 mM NaH_2PO_4 and 0.1 mM $MgCl_2$ for 1 h at 25°C under aerobic or anaerobic (N_2) conditions. The addition of Mg ions to the incubation

medium is necessary to prevent depletion of intracellular Mg and the consequent inhibition of metabolism (Borst-Pauwels 1967, Roomans et al. 1979). After phosphate enrichment, the cells were centrifuged and resuspended in 5% glucose in distilled water to which one of the following salts to a final concentration of 2 mM was added: nickel chloride, zinc sulfate, cadmium chloride, lead acetate, aluminum chloride, or chromium chloride. No salts were added to the control. The cells were incubated for 4 h at 25°C under aerobic or anaerobic conditions.

Preparation for X-ray microanalysis

The yeast cells were centrifuged and resuspended in a small volume of liquid 20% gelatin in distilled water (Roomans 1980a). Small pieces of the solidified gelatin-yeast mixture were placed onto silver pins and frozen in Freon 13 subcooled by liquid nitrogen as described by Barnard (1982). Thin (about 100 nm) cryosections were cut on an LKB Ultrotome III equipped with an LKB CryoKit at a specimen temperature of -110°C and a knife temperature of -100°C. The sections were cut on a dry knife and collected with a hair probe onto Formvar-film covered titanium or copper grids (Roomans and Sevéus 1976, Roomans et al. 1982). The sections were dried in the cryochamber at -100°C for several hours and slowly brought to room temperature. In a dry box, the sections were covered with a second Formvar film, which subsequently was coated with a thin conductive carbon layer. The grids were then immediately transferred to the electron microscope for analysis.

X-ray microanalysis

Energy-dispersive X-ray microanalysis was carried out with a Kevex 7000 spectrometer system in combination with a JEOL 100C electron microscope with ASID-4B scanning attachment. A carbon specimen holder (Liljesvan and Roomans 1976) was used. The sections were viewed in the scanning transmission mode and analyzed at 80 kV with a stationary spot, for 120 sec.

Quantitative analysis

Quantitative analysis was carried out according to the principle outlined by Hall (1971). The relative peak intensity for element x (R_x) was calculated from:

$$R_x = P_x / (W - W_e) \qquad (1)$$

where P_x is the characteristic intensity of element x, determined after frequency filter background removal, W is the total background in an appropriate peak-free region of the spectrum, chosen between 4 and 6 keV, and W_e is the extraneous background in the same region of the spectrum, calculated according to:

$$W_e = W_{film} + (G - G_{film}) * (W_{grid}/G_{grid}) \qquad (2)$$

(Gupta and Hall 1979, Roomans and Kuypers 1980). In equation (2) W_{film} is the background determined on a part of the grid covered by the Formvar film, and W_{grid} the background determined on a naked grid. G is the characteristic intensity of the grid material in the spectrum from the specimen, G_{film} and G_{grid} are the grid material intensities in spectra from the film or the naked grid, respectively. The measurements on the film and the naked grid were carried out under the same instrumental conditions as those used for analysis of the specimen.

A standard was used consisting of a matrix of 20% gelatin and 5% glycerol containing mineral salts in known concentrations (Roomans and Sevéus 1977). This standard was frozen, sectioned and analyzed in the same way as the specimen. The relative peak intensities in specimen and standard were compared by:

$$C_{x,sp} = C_{x,st} * (R_{x,sp}/R_{x,st}) * (Z^2/A_{sp}/Z^2/A_{st}) \qquad (3)$$

where C_x is the dry weight concentration of element x, and Z^2/A is the weighted mean of Z^2/A for all elements present in standard (st) or specimen (sp) (Roomans 1980b).

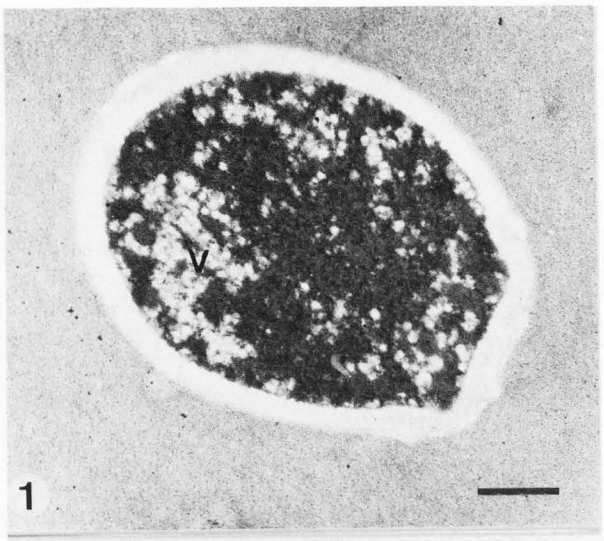
For those elements not present in the standard, sensitivity factors (f) were calculated to be inserted in the above equations according to:

$$C_{x1} / C_{x2} = f_{x1,x2} * (P_{x1} / P_{x2}) \qquad (4)$$

(Russ 1974, Roomans 1980b). The sensitivity factors were calculated from the analysis of dried microdroplets of binary salts, obtained by spraying a salt solution onto a film-covered grid (Morgan et al. 1975).

Electron microscopy and morphometry

For conventional electron microscopy, the yeast-gelatin mixture was fixed in 2% formaldehyde - 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, overnight, and postfixed in 1% osmium tetroxide in the same buffer. Alternatively, the cells were fixed in 2% $KMnO_4$ in distilled water for 1 h. After dehydration in a graded ethanol series, the specimens were embedded in Spurr's epoxy resin (Spurr 1969). Ultrathin sections, poststained with uranyl acetate and lead citrate were examined



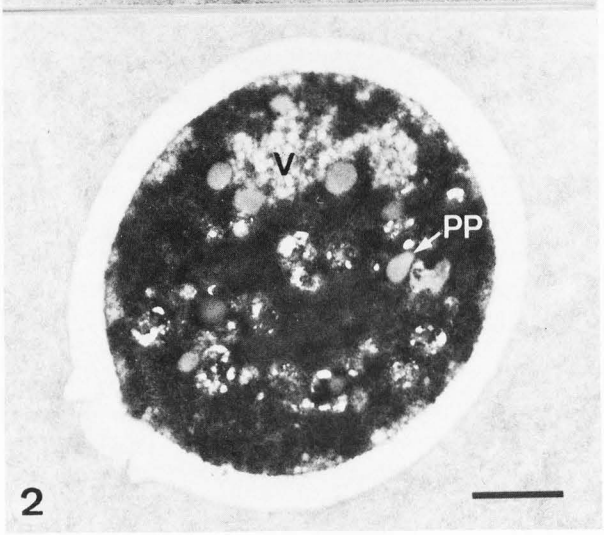
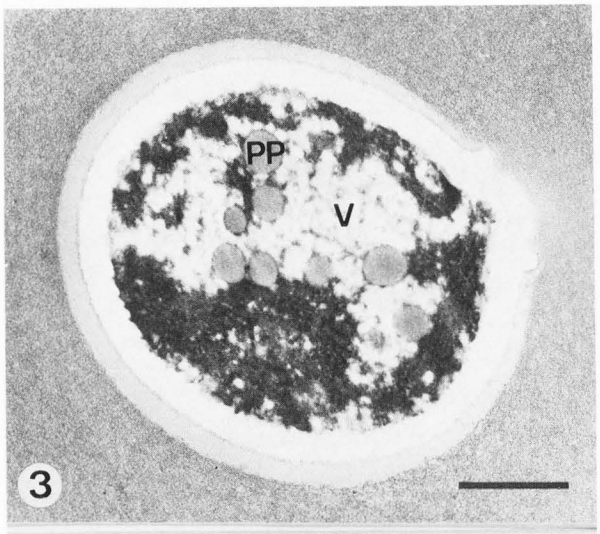


Fig. 1: Yeast cell after overnight starvation in distilled water. Several small vacuoles (V) are present but no polyphosphate granules. Aldehyde fixation. Bar = 1 µm.

Fig. 2: Yeast cell after 1 h phosphate loading under aerobic conditions. Polyphosphate granules (PP) are present in the cytoplasm and in the vacuole (V). Aldehyde fixation. Bar = 1 µm.



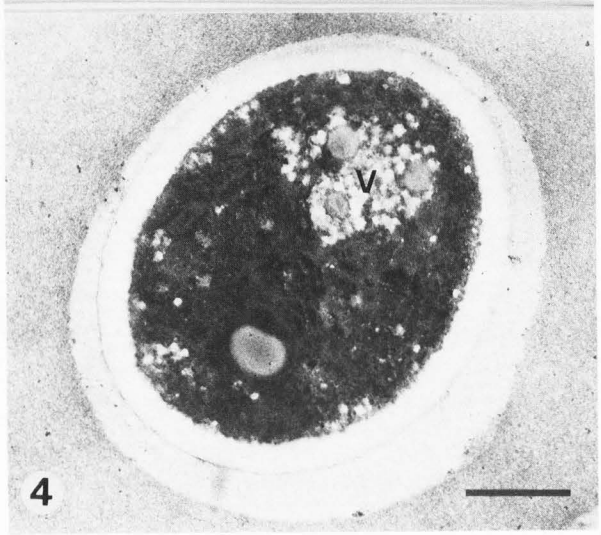


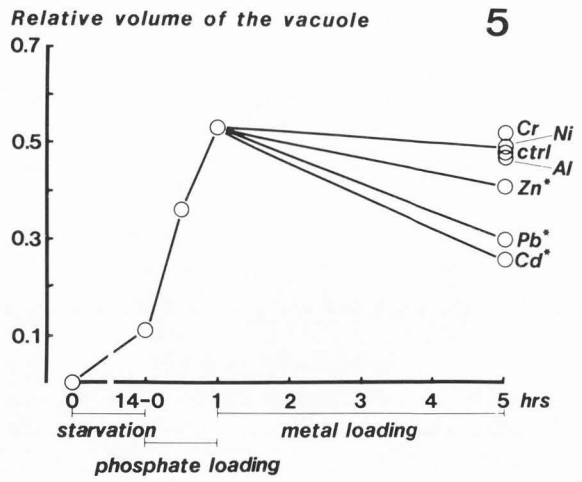
Fig. 3: Phosphate-rich yeast cell after control incubation (4 h in the absence of metal) under aerobic conditions. Most of the polyphosphate granules (PP) are now in the vacuole (V). Aldehyde fixation. Bar = 1 µm.

Fig. 4: Phosphate-rich yeast cell after aerobic incubation in the presence of 2 mM lead acetate. The vacuole (V) is much smaller than in the control. Aldehyde fixation. Bar = 1 µm.

in a JEOL 100S electron microscope. The volume fraction (V_V) of the vacuole was determined from the area fraction (A_A) on micrographs, using a HIPAD (Houston, Texas) digitizer board in combination with an LSI-11 (Digital) computer.

Results

In resting yeast cells, no vacuole was present. During starvation, several small vacuoles developed (Fig. 1). During phosphate loading the size of the vacuoles increased and the small vacuoles merged into a larger vacuole (Fig. 2). Continued incubation with glucose, but without phosphate, and in the absence of added cations, did not change



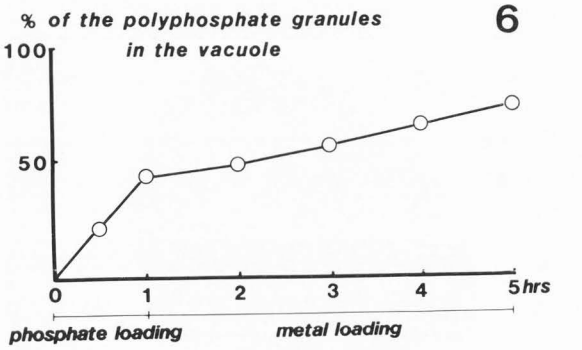


Fig. 5: Relative volume of the vacuole during the experiment (aerobic conditions). Values are means of 20-25 cells for each point. The data denoted by asterisks are significantly different ($p<0.05$) from the control incubation (ctrl).

Fig. 6: Distribution of polyphosphate granules between vacuole and cytoplasm during the experiment (aerobic conditions). Values are means of 15-20 cells for each point.

the relative volume of the vacuole (Fig. 3). Incubation with zinc, cadmium or lead salts caused a significant decrease of the relative volume of the vacuole (Fig. 4). The changes in the relative volume of the vacuole during the incubation are summarized in Fig. 5.

Polyphosphate granules develop during the phosphate loading step (Fig. 2) and, initially, occur mainly in the cytoplasm. Gradually, the granules migrate towards the vacuole and at the end of the experiment about 75% of the granules are located in the vacuole (Fig. 3). The changes in the distribution of the polyphosphate granules during the incubation are summarized in Fig. 6.

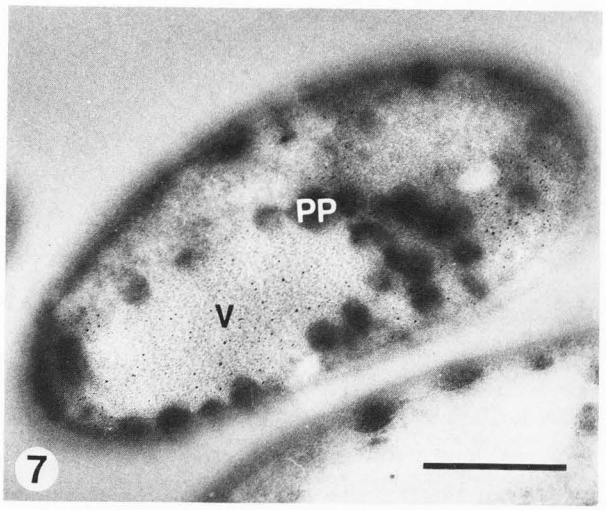


Fig. 7: Uncontrasted freeze-dried cryosection of a phosphate-rich yeast cell loaded with Ni. The vacuole (V) and the polyphosphate granules (PP) can be easily identified. Bar = 1 μ m.

Both Fig. 5 and Fig. 6 represent data from experiments carried out under aerobic conditions. Results obtained under anaerobic conditions were not significantly different.

The polyphosphate granules could be identified without difficulty in uncontrasted ultrathin cryosections of the yeast cells (Fig. 7). After metal loading the polyphosphate granules contained significant quantities of the metal added to the incubation medium (Fig. 8), except in the case of Al, for which the concentration in the polyphosphate granules did not differ significantly from zero (Table 1). Quantitative analysis showed that metal loading generally resulted in a decreased concentration of Mg and K in the polyphosphate granules (Table 1). In addition, all metals, including Al, could be demonstrated in the cell wall (Fig. 9).

Discussion

In an earlier study (Roomans 1980a) it was found that conventional preparation for electron microscopy caused loss of cations from the polyphosphate granules of the yeast cell. Rapid freezing and cryosectioning were therefore considered to be essential for successful localization of metal ions in yeast cells. Embedding the cells in gelatin prior to freezing not only facilitates the handling of the specimen during this

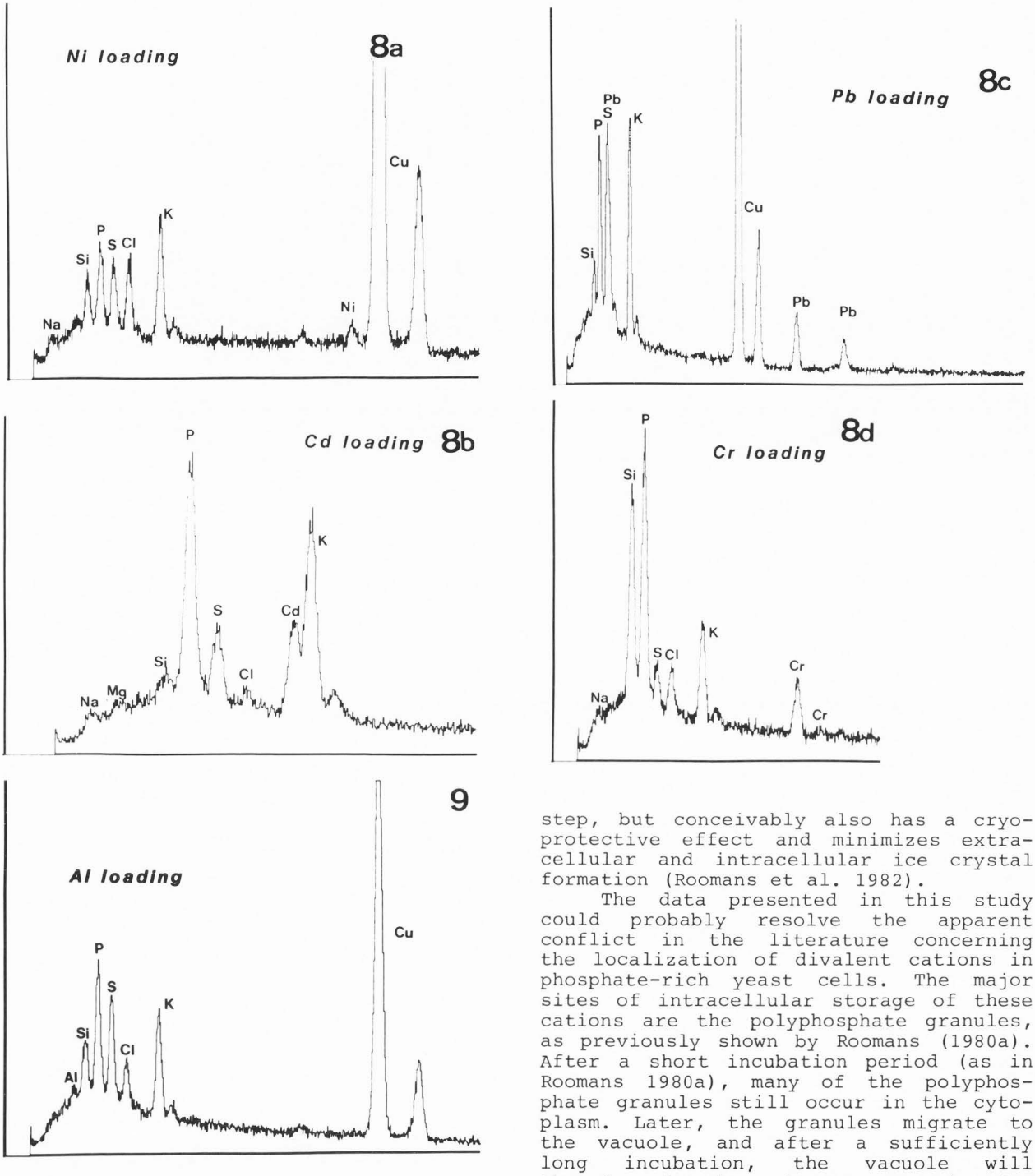


Fig. 8: Energy-dispersive X-ray spectra of polyphosphate granules after loading with (a) Ni, (b) Cd, (c) Pb and (d) Cr.

Fig. 9: Energy-dispersive X-ray spectrum of the cell wall after Al loading.

step, but conceivably also has a cryoprotective effect and minimizes extracellular and intracellular ice crystal formation (Roomans et al. 1982).

The data presented in this study could probably resolve the apparent conflict in the literature concerning the localization of divalent cations in phosphate-rich yeast cells. The major sites of intracellular storage of these cations are the polyphosphate granules, as previously shown by Roomans (1980a). After a short incubation period (as in Roomans 1980a), many of the polyphosphate granules still occur in the cytoplasm. Later, the granules migrate to the vacuole, and after a sufficiently long incubation, the vacuole will therefore be the major storage site for divalent cations, as found by Okorokov et al. (1977, 1980), Lichko et al. (1982) and Nieuwenhuis et al. (1982). These studies used biochemical techniques of cell fractionation, without ultrastructural correlation. The exact location of the divalent cations within the vacuoles could therefore not be investigated. On the other hand, X-ray microanalysis in the electron micro-

TABLE 1

X-RAY MICROANALYSIS OF POLYPHOSPHATE GRANULES AFTER METAL LOADING

	Mg	P	S	K	metal
ctrl	102 ± 15	667 ± 48	380 ± 39	564 ± 39	0
Ni	67 ± 13	507 ± 60	415 ± 28	506 ± 67	39 ± 5
Zn	81 ± 10	851 ± 118	484 ± 45	575 ± 82	35 ± 3
Cd	68 ± 12	728 ± 63	297 ± 7	381 ± 32	24 ± 4
Pb	91 ± 5	632 ± 44	599 ± 10	521 ± 33	52 ± 4
Al	90 ± 5	709 ± 56	394 ± 15	466 ± 31	9 ± 9
Cr	73 ± 7	731 ± 42	152 ± 12	428 ± 39	39 ± 4

Data in mmol/kg dry weight; mean and standard error of the mean of 15-20 measurements

scope allowed the demonstration of the association of the metals with vacuolar or cytoplasmic polyphosphate granules.

Under control conditions, potassium and magnesium appear to be the cations associated with the polyphosphate granules. Uptake of (heavy) metal cations and binding of these cations to the granules leads to a decrease of the K and Mg concentration, as was also found by Lichko et al. (1982). A decrease of cellular K after exposure of yeast cells to cadmium ions has also been shown in other studies (Norris and Kelly 1977, Gadd and Mowll 1983, Mowll and Gadd 1984).

Polyphosphate granules are also found in other fungi, algae and higher plants, and, under normal conditions contain K, Mg and Ca (Sicko-Goad et al. 1975, Tillberg et al. 1979, White and Brown 1979, Pedersén et al. 1981, Wei et al. 1981). The high affinity of the granules for heavy metal cations is well known, and can e.g. be used for cytochemical demonstration of polyphosphate granules (Vorisek et al. 1982).

The binding of heavy metals to the polyphosphate granules may mitigate their toxic effect on the cells (Sicko-Goad and Stoermer 1979). Nevertheless, exposure to relatively high concentrations of zinc, cadmium and lead (but not of nickel, aluminum and chromium) resulted in significant ultrastructural effects. The mechanism and biological significance of the reduction of the vacuolar volume in yeast by these metals is not known. In blue-green algae, Zn appears to increase the vacuolization of the cell (Lazinsky and Sicko-Goad 1983).

The finding of Cr in the polyphosphate granules shows that also chromium ions are taken up by yeast cells. Uptake of trivalent cations by yeast cells has to our knowledge not been shown previously. Whether there is a separate uptake mechanism for trivalent cations, or whether chromium ions have affinity for the divalent cation transport system remains to be investigated. In contrast to Cr, of which the presence in polyphosphate granules could be unequivocally demonstrated, the concentration of Al in the granules appeared to be below the detection limit under the experimental conditions used. No evidence could therefore be obtained for transport of aluminum ions across the yeast cell membrane.

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Discussion with Reviewers

G. T. Cole: What was the condition of the yeast cells just prior to initiation of starvation? Did you begin the experiments with a homogeneous population of cells?
Authors: The aim of the starvation period is to bring the cells in the same condition. The yeast used was a commercial preparation and may not have been a homogeneous population before starvation.

D. M. R. Harvey: Bearing in mind the chemistry of Al_{3-} to what extent are you certain that Al^{3+} exists as such in your external medium?
Authors: The conditions under which the loading step was performed (distilled water, i.e. pH < 7, no complexing ions) would favour the occurrence of free Al^{3+} ions, although we do not claim that all Al in the medium was ionized. A further decrease of the medium pH could have increased the ionization of Al. However, from experiments with Ca and Sr it is known that uptake of divalent cations is reduced at low pH (Roomans et al. 1979). If aluminium ions would be transported by the same mechanism a low pH would, despite the increase in ionized Al, have adverse effects on uptake. It should also be mentioned that under the same experimental conditions we have observed uptake of Al by a blue-green algae (to be published).

D. M. R. Harvey: Can you please comment on the possible action of gelatin as a cryoprotectant: do you believe it is non-penetrating, or that, for example it enters the cells by endocytosis? Do you know what the effects of gelatin on yeast physiology/ion relationships are likely to be?
Authors: In view of the size of the molecule, the short time the cells were exposed to the gelatin, and the fact that the gelatin solidified nearly immediately after addition to the yeast cells, we think it highly unlikely that it would be able to pass the cell wall, let alone enter the cell, under the conditions used in the present study. We consider gelatin to be a non-penetrating cryoprotectant, in contrast to penetrating cryoprotectants such as glycerol and dimethylsulfoxide (DMSO). Other 'non-penetrating' high-molecular weight cryoprotectants may enter animal cells by means of endocytosis (Roomans et al. 1982), but no data are available on plant cells. High-molecular weight cryoprotectants have been shown to be able to withdraw water from plant cells (Andrén et al. 1983), without, however, changing the ion concentrations related to dry weight. The withdrawal of water is due to the high apparent osmolarity of the cryoprotectant solution. We do not know, however, whether this also would occur with a substance that forms a gel. Since yeast cells can be cultured on gelatin, we think it unlikely that gelatin has adverse effects on cell function.

D. M. R. Harvey: Can you please describe the peak deconvolution routine that you used to separate Cd L_α and K K_α for quantitative analysis?
Authors: The program used for the determination of the net peak intensities by the frequency filter method is standard Kevex software. This program separated the K and Cd peaks.

D. M. R. Harvey: I would have thought that Cu grids were not a particularly good choice of grid material for the detection of Ni (Fig. 8) since their peaks are close together. Perhaps e.g. Ti would have been better.
Authors: The resolution of our spectrometer is 146 eV and the distance between Cu and Ni is thus sufficient for accurate analysis.

L. S. Goad: Have you completed more detailed morphometric studies that would indicate what cellular components are increasing relative to the vacuole reduction? Studies that we have conducted in our lab indicate that changes in vacuole volume are usually accompanied by changes in vacuole content, i.e., changes in amounts of storage products such as lipids or polyphosphate in the vacuole. Since you have observed that there is a migration of polyphosphate to the vacuole, it is not unreasonable to suggest that the vacuole volume (i.e. the volume contained by the vacuole membrane) is probably constant and if examined in the T.E.M. the sum of polyphosphate volume and vacuole would account for the "reduction" in vacuole volume.
Authors: We have not carried out more detailed morphometric studies, but from a qualitative assessement of the micro-

graphs it does not seem very likely that your suggestion would be relevant to this particular case. Nevertheless, this might well be subject of further study.

G. T. Cole: Is there evidence that distribution of divalent cations in yeast cells may influence resistance as suggested in the case of bacteria (e.g. Stewart et al., J. Bacteriol. 147: 670-674, 1981)?
Authors: Heavy metal resistance in yeast cells has generally been associated with the presence of specific binding proteins, such as a copper-binding protein. The idea that polyphosphate bodies may have some role in metal resistance is, however, attractive.

Additional Reference

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