Zinc Uptake in *Neocosmospora vasinfecta*

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SUMMARY

Mycelium of *Neocosmospora vasinfecta* harvested in mid-logarithmic phase absorbs Zn^{2+} from dilute solutions in the absence of growth. Zn^{2+} uptake involves two phases: a rapidly established phase 1 believed to represent adsorption to negatively charged groups in the hyphal surface-membrane and a slowly established phase 2 which represents transport into the cytoplasm. Phase 1 is not influenced by low temperature, NaN_3 or anaerobiosis applied for short periods, conforms to the Langmuir adsorption equation and is reduced in the presence of various other divalent cations. Phase 2 is strongly inhibited by low temperature, NaN_3 and anaerobiosis and exhibits carrier-type kinetics with an apparent K_m for Zn^{2+} of 0.2 mM. Mn^{2+} competitively inhibits phase 2 zinc uptake: Mg^{2+} acts as a 'mixed-type' inhibitor. Electron microscopy of unstained material indicates that part at least of phase 2 Zn is deposited in the cytoplasm and nucleus. A model for the metabolic uptake of Zn^{2+} involving phase 1 binding as a requisite preliminary process is suggested.

INTRODUCTION

Fungi, like other organisms, require for growth a number of polyvalent metal ions (Bowen, 1966). Detailed study of their uptake in fungi has been confined to *Saccharomyces cerevisiae* (van Steveninck & Booij, 1964; Rothstein, 1965; Fuhrmann & Rothstein, 1968; Ponta & Broda, 1970), though both ascospores (Lowry, Sussman & von Boventer, 1957) and growing mycelium (Venkateswerlu & Sastry, 1970) of *Neurospora crassa* have received attention. As with other organisms, the uptake of di- or polyvalent cations by fungi includes a rapid and easily reversible phase (assumed to be localization of the metal ion at the cell or hyphal surface), which may or may not be associated with a slower, apparently irreversible phase (assumed to represent transfer into the cytoplasm). Further information on both of these phases and their interrelationships is desirable, particularly for those elements essential for growth. Possible linkages between metabolism and uptake are most simply investigated using non-growing material but for vegetative fungal material this has so far been done only with yeast. Mycelium of *Neocosmospora vasinfecta* from submerged culture absorbs Zn^{2+} in the absence of growth and without the necessity for prior mycelial conditioning. The properties of Zn^{2+} uptake in this mycelium therefore were investigated further (Paton, 1971).

METHODS

Organism and growth. The organism and method of growth in liquid shaken culture were as described earlier (Budd, 1969*a*) but using medium G1 (Budd, 1969*b*). The Zn content of this medium was not less than 25 μM . For some experiments the Zn content was varied by using a trace element solution lacking Zn and adding the desired amount of ZnSO_4 . Mycelium was harvested by filtration in mid-exponential phase and washed by resuspension in

demineralized water, and samples for experimental work were prepared by serial dilution.

Uptake of Zn^{2+} . Mycelia were incubated with Zn^{2+} solutions with vigorous aeration in glass or polyethylene containers, previously coated with a silicone compound ('Siliclad': Clay Adams, Parsippany, New Jersey, U.S.A.) to prevent adsorption of Zn^{2+} ions to the vessel walls. The density of mycelial suspension did not exceed 200 mg dry wt/l. Anaerobic conditions, where employed, were attained by bubbling O_2 -free N_2 through the suspension for 30 min before adding Zn^{2+} and during the course of the experiment. Buffers used (at 2 to 10 mM) were $KH_2PO_4 + KOH$ and KH phthalate + KOH (Clark & Lubs, 1916); PIPES (piperazine-*N, N'*-bis (2-ethane sulphonic acid)) + KOH (Good *et al.* 1966). Alternatively, a pH meter PHM 26c and titrator TTT 11b (Radiometer, Copenhagen, Denmark) with a miniature combination pH probe (E. H. Sargent & Co., Toronto, Canada) regulated the pH within 0.05 unit by automatic addition of 0.1 N- KOH or HCl .

Zn^{2+} was supplied as $ZnSO_4$ or $^{65}ZnSO_4$ (sp. act. 0.1 to 0.2 mCi/mmol). The initial Zn^{2+} concentration varied between 0.01 and 10.0 mM. Total volume and mycelial suspension-density were adjusted so that depletion of external Zn^{2+} never exceeded 5 % of the total in the course of an experiment. Incubation was stopped by rapidly filtering with suction through Whatman no. 54 paper and washing the mycelium with demineralized water. The mycelium was then either extracted or subjected to desorption to remove reversibly bound Zn. Except where stated, desorption was by aerating for 15 min at room temperature in excess 10 mM-KH phthalate buffer, pH 4.0, containing 0.1 or 1.0 mM- $ZnSO_4$, then filtering and washing as above.

Zn and other metals were extracted from the mycelium by wet combustion with conc. HNO_3 . Determinations of Zn and other metals in the diluted digest were made by atomic absorption or emission spectrophotometry using a Unicam SP90A atomic absorption spectrophotometer. ^{65}Zn in the digest was counted with a solid scintillation counter (Nuclear Chicago model DS-202) to 1 % counting error.

α -Amino nitrogen in the desorption medium, concentrated *in vacuo* at 35 °C, was determined by the method of Yemm & Cocking (1955).

Electron microscopy. For electron microscopy, mycelium was fixed for 16 h at 4 °C in 3 % glutaraldehyde buffered with 0.1 M-phosphate to pH 7.4, and fixed for 2 h in 1 % or 2 % OsO_4 in the same buffer. The fixed material was dehydrated using ethanol and propylene oxide and embedded in Epon 812 (Luft, 1961), and sections were cut with a Dupont diamond knife and Huxley ultramicrotome. Micrographs were made with an RCA EMU-3G electron microscope.

Chemicals. ^{65}Zn was purchased as the carrier-free isotope in 0.5 M- HCl from New England Nuclear (Canada) Ltd. Zn standards for atomic absorption spectrophotometry were prepared from metallic Zn (99.7 % pure, w/w; J. T. Baker Chemical Co., Phillipsburg, New Jersey, U.S.A.) and analytical grade HNO_3 . Glutaraldehyde was obtained from Ladd Research Industries, Burlington, Vermont, U.S.A., and OsO_4 from Stevens Metallurgical Corporation, New York, N.Y., U.S.A. Epon 812 was supplied by Fisher Scientific Co., Fair Lawn, New Jersey, U.S.A. PIPES and actidione were supplied by Sigma Chemical Co., St. Louis, Missouri, U.S.A. Demineralized water used in this work had a specific resistance in excess of 1 $M\Omega\ cm^{-3}$. All other chemicals were of analytical grade.

RESULTS

Washed mycelium harvested from the normal growth medium (25 μM - Zn^{2+}) contained between 0.7 and 1.3 μ equiv. Zn/100 mg dry matter. The growth rate was the same with

Fig. 1. The effect of external ZnSO₄ on desorption of ⁶⁵Zn from mycelia previously incubated with 1.0 mM-⁶⁵ZnSO₄ in 5 mM-phosphate buffer at 25 °C. Zinc determined by atomic absorption spectrophotometry (a); and by radioactivity (b). Desorption into 10 mM-phthalate buffer, pH 4.0, at 25 °C alone (O) or with 0.1 mM-ZnSO₄ added (●); desorption into 5 mM-phosphate buffer, pH 6.5, containing 0.1 mM-ZnSO₄ (×).

Table 1. The effects of different desorption treatments, following exposure to ZnSO₄ solution, on mycelial zinc content

Mycelium was incubated in 0.1 mM-ZnSO₄ in 5 mM-phosphate buffer, pH 6.5, for 10 min at 25 °C, then filtered off and transferred to the above solutions. Initial Zn content was 88.0 μequiv./100 mg dry wt.

Treatment	Zn ²⁺ content (% initial value) after		
	15 min	30 min	60 min
Demineralized water, 25 °C	92.0	83.0	68.2
Phthalate buffer, pH 4.5, 25 °C	27.3	39.8	47.7
Phthalate buffer, pH 4.0, 25 °C	4.8	5.7	7.1
Phthalate buffer, pH 3.0, 25 °C	4.7	4.9	4.8
Phthalate buffer, pH 4.0, 0 °C	4.7	4.5	4.7

Fig. 2. The progress of total zinc uptake (upper curves) and of uptake into non-desorbed form (lower curves) at 25 °C. Mycelium incubated with 0.1 mM-ZnSO₄ in 5 mM-PIPES+KOH buffer at pH 6.5 (○) or with the pH-stat at pH 6.5 (●). Desorption for 15 min in 10 mM-KH phthalate, pH 4.0, at 25 °C.

10.0 mM-Zn²⁺, but at 100 mM-Zn²⁺ growth was inhibited by 90 %. After 48 h of growth in Zn-deficient medium (2 μM-Zn²⁺), the mycelium was halved in yield and did not grow when inoculated into fresh Zn-deficient medium. These experiments show that Zn has low toxicity towards *Neocosmospora vasinfecta* and is required for growth and that the mycelium used was not Zn-deficient.

Absorption of Zn by the mycelium from solutions containing only Zn²⁺ was variable and accompanied by a rapid decrease in pH. Reproducible absorption was obtained by stabilizing the pH to 6.5. In all cases an initial rapid uptake of Zn took place (see Fig. 2) which was greatest when phosphate buffers were employed. Subsequent transfer to Zn-free solutions (Table 1) resulted in desorption of Zn from the mycelium, which increased at lower pH values. Desorption was maximal after 15 min at 25 °C and pH 4.0. Although desorption at pH 3.0 and at pH 4.0 at 0 °C showed no subsequent resorption, they were considered physiologically undesirable. At pH 3.0, and 25 °C over 50 % of mycelial K⁺ was lost within 15 min, as compared to 1.7 % at pH 4.0 at this temperature. Loss of α-amino N to phthalate buffer at pH 4.0 was the same as that to water for the first 30 min at 25 °C, but loss to both was almost fivefold higher at 0 °C. For determination of total non-desorbed Zn the preferred desorption procedure was therefore to hold at pH 4.0 for 15 min at 25 °C. Desorption of ⁶⁵Zn²⁺, incorporated by previous uptake, was followed by mass and radioactivity in the presence and absence of non-radioactive Zn²⁺ (Fig. 1). At pH 4.0, loss of total Zn was greatest in Zn²⁺-free buffer (Fig. 1a) whereas loss measured as ⁶⁵Zn²⁺ (Fig. 1b) was greatest in the presence of non-radioactive Zn²⁺, presumably because of isotopic exchange.

Fig. 3. Application of the Langmuir equation to the anaerobic uptake of Zn^{2+} . On the ordinate, external zinc concentration is in mm and zinc uptake is expressed as $\mu\text{equiv./100 mg dry wt.}$ Mycelium incubated 2 min at 25°C , with pH maintained at 6.5 by pH-stat. For explanation, see text.

Table 2. *The effects of metabolic inhibition on phase 1 zinc uptake during 2 min exposure to $^{65}\text{Zn}^{2+}$*

Mycelium incubated 15 or 30 min in the appropriate treatment before addition of Zn^{2+} . Uptake was from 0.1 mM-ZnSO_4 in 5 mM-PIPES+KOH , pH 6.5. Desorption as given in Methods. All values averages of duplicates.

Inhibition treatment	Zinc uptake ($\mu\text{-equiv./100 mg dry wt}$)	
	Control	+ Treatment
Anaerobiosis	8.58	8.60
NaN_3 , 1.0 mM	8.35	8.70
NaN_3 , 0.1 mM	8.35	8.51
Low temperature (3°C)	8.89	8.57

Non-radioactive Zn^{2+} at pH 6.5 was relatively ineffective in removing $^{65}\text{Zn}^{2+}$ (Fig. 1*b*) and total Zn within the mycelium actually increased (Fig. 1*a*).

Uptake of $^{65}\text{Zn}^{2+}$ into mycelium, with and without subsequent desorption, enabled incorporation to be classed as desorbable uptake (phase 1) and non-desorbable uptake (phase 2), respectively (Fig. 2). Phosphate buffer was replaced by PIPES + KOH in this and all later experiments (see Discussion) which at 5 mM was without effect on mycelial O_2 uptake (not shown), and almost exactly duplicated the pattern of Zn uptake found with the pH-stat. The increase in phase 1 Zn was complete within 30 min, but phase 2 Zn continued to increase for at least 3 h. Phase 1 Zn was smaller than when phosphate buffer was used (Fig. 1). Further experiments examined the properties of the two phases of Zn^{2+} uptake separately.

Fig. 4. The effect of pH on phase I zinc uptake. pH adjusted upward from pH 4.0 with KOH by means of the pH stat. Mycelium was incubated anaerobically; samples were withdrawn 2 min and 5 min after stabilization at each new pH value and phase I determined by extrapolation to time 0 at the new pH.

Phase I of Zn²⁺ uptake

Incorporation of ⁶⁵Zn²⁺ into phase I was complete within 2 min. Anaerobiosis, low temperature and NaN₃ had no effect on this (Table 2). However, incubation for 3 h in anaerobic conditions or NaN₃ or at 3 °C reduced subsequent phase I Zn uptake by 25 % to 30 %, whereas aeration at normal temperatures for similar periods caused the amount of phase I Zn to increase slightly.

The relationship between external Zn²⁺ concentration and phase I uptake was examined using anaerobic conditions to suppress phase 2 incorporation (see below). The results can be expressed in the form of a Langmuir plot (Fig. 3). According to this treatment (see Langmuir, 1918):

$$y = \frac{abx}{1 + bx} \quad (1)$$

where y = amount of solute adsorbed per unit mass of adsorbent; x = concentration of solute; a = adsorption capacity of adsorbent at saturation; b = a constant for the system.

Equation (1) rearranges to the form

$$\frac{x}{y} = \frac{x}{a} + \frac{1}{ab} \quad (2)$$

Table 3. *The effect of sodium azide and low temperature on phase 2 zinc uptake*

Mycelium incubated in 0.1 mM-ZnSO₄ in 5 mM-PIPES+KOH at pH 6.5. Temperature 25 °C except as indicated. Pretreatment with NaN₃ as above but without ZnSO₄. Values are averages of close duplicates.

Pretreatment	Alteration to uptake conditions	Zn ²⁺ uptake (% control) after		
		30 min	60 min	120 min
None	+ 1 mM-NaN ₃	—	22.8	16.2
None	+ 0.1 mM-NaN ₃	—	17.0	27.5
0.1 mM-NaN ₃ , 15 min	—	—	54.8	56.8
None	3 °C	32.8	30.9	26.5

that is, the graph of x/y against x is a straight line with slope of $1/a$ and intercept $1/ab$. Fig. 3 shows that the experimental points gave a good fit to a straight line, indicating that phase 1 represents adsorption to the mycelial surface. The constant 'a', the exchange capacity for Zn²⁺ under the conditions used, was 12.1 μ equiv./100 mg dry matter from the slope of the line in Fig. 3. Half-saturation of phase 1 uptake under the conditions of Fig. 3 occurred at 0.1 to 0.2 mM-Zn²⁺.

The relationship between external pH and phase 1 uptake was also examined using anaerobic conditions for exposure to Zn²⁺ (Fig. 4). Phase 1 Zn uptake increased by approximately one-third between pH 4.0 and pH 6.8. Above pH 6.8 there was a sharp increase, which may represent the precipitation of insoluble Zn(OH)₂ at the mycelial surface.

Uptake of Zn into phase 1 decreased in the presence of certain other divalent cations. At pH 6.5, Cu²⁺ and UO₂²⁺, at the same molarity as Zn²⁺ (0.1 mM), both reduced phase 1 Zn²⁺ uptake by about 70 %. Similarly, Co²⁺, Mn²⁺, Ni²⁺ and Mg²⁺ decreased phase 1 Zn uptake by 39 % to 26 % whereas iron (Fe³⁺ and Fe²⁺) increased it by 12 %. K⁺ at 1 mM had no significant effect. During the establishment of phase 1 in ZnSO₄, with the pH held at 6.5 by the pH-stat, uptake of Zn²⁺ was almost equivalent to the combined losses of Mg²⁺, K⁺ and H⁺ from the mycelium, with Mg²⁺ loss equivalent to 80 % of Zn²⁺ absorbed. These observations are consistent with phase 1 being an ion-exchange process.

When mycelium was grown with limiting phosphate (0.9 mM instead of 7.3 mM), maximum phase 1 Zn uptake was reduced by 10 % to 15 %.

Phase 2 of Zn²⁺ uptake

The endogenous Zn of freshly harvested mycelium is not exchanged for Zn²⁺ taken up subsequently, as was shown by growing mycelium on ⁶⁵Zn²⁺ and transferring to non-radioactive Zn²⁺ under normal uptake conditions. The standard desorption procedure with non-radioactive Zn²⁺ following a period of ⁶⁵Zn²⁺ uptake also shows the phase 2 Zn is non-exchangeable. Consequently, Zn²⁺ uptake into phase 2 as measured by ⁶⁵Zn²⁺ is identical with Zn²⁺ influx.

Phase 2 Zn²⁺ uptake was strongly but not completely inhibited by NaN₃ at 0.1 mM and 1.0 mM (Table 3). The effect of 0.1 mM-NaN₃ did not appear to be fully reversible. At 3 °C, phase 2 Zn²⁺ uptake was also reduced. Anaerobiosis produced the strongest inhibition of phase 2 Zn²⁺ uptake, amounting to 88 % after 2 h (Fig. 5). However, in this case, uptake was fully restored on returning to aerobic conditions, even after 230 min of anaerobiosis.

Phase 2 uptake was influenced by external pH (Table 4). The data suggest a bimodal relationship with the major peak at around pH 6.5, the pH at which most experiments were performed.

Fig. 5. The effect of anaerobiosis on phase 2 zinc uptake. Mycelium incubated at 25 °C in 0.1 mM- ZnSO_4 in 5 mM-PIPES+KOH at pH 6.5. Upper curve, incubated with aeration; lower curve, incubated in a stream of oxygen-free N_2 , with prior deoxygenation by this gas-stream for 30 min before exposure to ZnSO_4 . After 200 min the anaerobic mycelium was switched to normal aeration for a further 3 h.

Fig. 6. The relationship between zinc uptake into phase 2 and ZnSO_4 concentration in the presence (○) and absence (●) of MnSO_4 (5×10^{-4} M), plotted by the method of Lineweaver & Burk (1934). Mycelium incubated in 5 mM-PIPES+KOH at pH 6.5 and 25 °C for up to 15 min.

Table 4. *The effect of external pH on phase 2 zinc uptake during two different incubation periods*

Mycelium incubated in 0.1 mM- ZnSO_4 at 25 °C;
pH controlled by pH-stat.

External pH	Zn^{2+} uptake (μ -equiv./100 mg dry wt) after	
	30 min	180 min
4.5	1.45, 1.55	3.95, 3.85
5.5	1.28, 1.22	3.45, 3.25
6.5	1.93, 1.87	4.78, 4.52
7.5	1.23, 1.17	2.70, 2.60

Fig. 7. Transverse section of hypha from mycelium incubated aerobically for 30 min at 25 °C in 1.0 mM-ZnSO₄ in PIPES+KOH, pH 6.5, followed by desorption at pH 4.0 in zinc-free buffer. CW = cell wall; Mi = mitochondrion; D = electron-dense deposit presumed to include Zn. Unstained.

Kinetic studies of Zn²⁺ influx were carried out making use of the observation that at pH 6.5 the rate of phase 2 Zn incorporation was linear for the first 10 min of exposure to the highest Zn²⁺ concentration tested (1.0 mM). At lower concentrations, uptake was linear for longer periods (see Fig. 5, control curve). During exposures of up to 15 min, phase 2 uptake was a hyperbolic function of Zn²⁺ concentration, as shown by the control (–Mn²⁺) curve of Fig. 6. The apparent K_m for phase 2 Zn²⁺ uptake, determined by extrapolation of this curve to the abscissa, was 0.20 mM: the mean from six other kinetic experiments was 0.19 mM. Mn²⁺ behaved as a competitive inhibitor of phase 2 uptake (Fig. 6), whereas inhibition by Mg²⁺ (not shown) was of the 'mixed' type, increasing the apparent K_m for Zn²⁺ and decreasing the rate of phase 2 uptake except at the highest Zn²⁺ concentrations, where the rate was actually increased. Kinetic analyses of the effects of other cations were not done. However UO₂²⁺ decreased phase 2 uptake by over 80 % when present at the same molarity (0.1 mM) as Zn²⁺; Cu²⁺, Ni²⁺ and Co²⁺ were less inhibitory, and Fe²⁺ with Fe³⁺ stimulated phase 2 uptake by 40 %.

Phosphate buffer (5 mM, pH 6.5) compared with 5 mM-PIPES+KOH inhibited phase 2 Zn²⁺ uptake by 30 % during 1 h. However, treating the mycelium for 15 min in phosphate buffer, followed by washing it until free of phosphate had no effect on subsequent phase 2 Zn²⁺ uptake from PIPES buffer. Glucose inhibited phase 2 uptake from 1 mM-Zn²⁺ at all concentrations tested from 0.1 mM to 90 mM during 20 min. Inhibition was curvilinear with respect to glucose concentration and amounted to 46 % at 90 mM-glucose. Treating

Fig. 8. Transverse section of hypha from mycelium treated as in Fig. 7.
Nu = nucleus; other symbol as in Fig. 7. Unstained.

the mycelium for 15 min in 1.0 mM-glucose slightly (18 %) inhibited subsequent phase 2 uptake: if 5 mM-phosphate at pH 6.5 was also present during preincubation, this inhibition fell to 10 %. In mycelium grown on limiting phosphorus (see above), phase 2 uptake was reduced by 20 % during 40 min.

Actidione (10 μ g/ml) had no influence on phase 2 uptake during 2 h, indicating that protein synthesis was not essential for phase 2 uptake.

Localization of phase 2 Zn. Preliminary information on the intrahyphal location of Zn was obtained by electron microscopy of unstained mycelium. Local concentrations of Zn are sufficiently electron-dense to be observed in such material. Fig. 7 and 8 show the appearance of mycelium incubated aerobically for 30 min in ZnSO_4 ; phase 1 Zn was removed by desorption at pH 4.0 in the absence of ZnSO_4 . Two types of electron-dense deposit occur. The first type (Fig. 7) is large and irregular and located apparently just inside the hyphal membrane. The second type (Fig. 8) consists of small, discrete sports concentrated mainly in the nucleus. Both deposits were observed to 'bubble' in the electron-beam, and must therefore decompose or melt at relatively low temperatures.

DISCUSSION

Zn can exist in soluble form as Zn^{2+} , as a complex anion, or as chelates with numerous organic ligands. Uptake of Zn^{2+} was ensured in this work by maintaining the pH at or below 6.5, and by using a non-chelating buffer (Good *et al.* 1966) and excluding other possible ligands. At pH values of 6.8 or above, insoluble $\text{Zn}(\text{OH})_2$ forms (Jurinak & Inouye, 1962)

and seems to precipitate at the mycelial surface. Precipitation of $\text{Zn}_3(\text{PO}_4)_2$ at the hyphal surface also seems to occur when phosphate is present at pH 6.5. The slow exchange with unlabelled Zn^{2+} at pH 6.5 (Fig. 1) indicates that in this case phase 1 uptake is mostly insoluble. Its availability for transfer into the cytoplasm should therefore be reduced, which may explain the inhibition of phase 2 uptake by phosphate at pH 6.5.

Phase 1 uptake. This reaches equilibrium with the external solution within 2 min, in agreement with observations on the binding of divalent cations to the surface of yeast cells (Rothstein, Hayes, Jennings & Hooper, 1958; van Steveninck & Booij, 1964). The reversibility of phase 1 uptake by isotopic exchange, and its close fit to the Langmuir adsorption equation, agree with a surface localization. The fluctuations in phase 1 Zn uptake following several hours of treatment under anaerobiosis or in air show that the zinc-binding sites are metabolically turned over, which suggests that this zinc is associated with the cell membrane rather than the cell wall.

Phase 1 Zn is decreased in phosphorus-deficient mycelium, and in normal mycelium it is decreased after holding for 3 h in anaerobic conditions but is increased after a similar period in air. These observations are parallel to those of van Steveninck & Booij (1964), who concluded that divalent cations are bound to polyphosphate in the yeast-cell membrane.

Phase 2 uptake. Fig. 7 and 8 support the view that this phase represents transfer of Zn across the surface membrane. The kinetics of Zn^{2+} influx (Fig. 6) are those of carrier-mediated transfer, as described for Zn^{2+} and other divalent cations in yeast (Fuhrmann & Rothstein, 1968; Ponta & Broda, 1970), but the apparent K_m for Zn^{2+} influx of 0.2 mM is considerably lower than that (1.0 to 1.3 mM) in yeast (Ponta & Broda, 1970). Mn^{2+} , but not Mg^{2+} , appears to be transported via the same site on the Zn^{2+} carrier. The mixed-type inhibition by Mg^{2+} indicates both inhibitory and stimulatory effects on Zn^{2+} uptake, suggesting that Mg^{2+} acts as a co-factor in phase 2 uptake.

Phase 2 uptake is strongly dependent on aerobic metabolism (Table 3; Fig. 5). Respiratory energy probably is required to transport Zn^{2+} into the cell, either because this transport is thermodynamically 'uphill', or more likely because the hyphal surface membrane is impermeable to Zn^{2+} .

Interrelationships between phase 1 and phase 2. Several factors suggest a close relationship between the surface binding of divalent cations and their transport across the cell membrane. Firstly, phase 1 Zn seems to be held at the surface membrane, where it would be ideally situated for access to a phase 2 carrier system. Secondly, the half-saturation concentrations of Zn^{2+} for phase 1 and phase 2 are similar (0.1 to 0.2 mM). Thirdly, several factors have parallel effects on both phases; for example, the growth of mycelium under phosphorus-limiting conditions, the inhibition by UO_2^{2+} and the anomalous effects of iron. Therefore, it is proposed that phase 1 binding is prerequisite to phase 2 transport. Phase 1 Zn, we propose, is bound to polyphosphate in the surface membrane and released during phase 2 transport by enzymatic breakdown of this compound, which provides energy for the translocation. This is similar to one mechanism proposed for hexose uptake in yeast (van Steveninck & Booij, 1964; van Steveninck, 1968) and it is noteworthy that glucose inhibits phase 2 Zn^{2+} uptake in *Neocosmospora vasinfecta*. Continued phase 2 transport is assumed to require resynthesis of polyphosphate in the vicinity of the carrier, using ATP generated by aerobic respiration. Both Zn and phosphate would be simultaneously translocated into the cytoplasm and $\text{Zn}_3(\text{PO}_4)_2$ would accumulate. The enzyme acting on polyphosphate would be an integral part of the carrier system. Although speculative, the above model accounts for the basic features of total Zn^{2+} uptake in *Neocosmospora vasinfecta*, and is under further investigation.

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