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ROLE OF THE VACUOLE IN METAL ION TRAPPING
AS STUDIED BY IN VIVO ^{31}P -NMR SPECTROSCOPY

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INTRODUCTION

^{31}P -NMR spectroscopy has been extensively used to study the energetic profiles in intact plant tissues (Roberts and Jardetzsky, 1981; Roberts, 1984; Loughman and Ratcliffe, 1984). These reports clearly demonstrate that this technique can be used to measure energy status, ATP/ADP ratio (Roberts et al., 1985), changes in intracellular pH (Roberts et al., 1981), effects of hypoxia (Roberts et al., 1984), phosphate movement (Rebeille et al., 1983), and aluminium ion toxicity (Pfeffer et al., 1986).

In a number of cases Mn^{2+} has been demonstrated to produce toxic effects in growing plants (Pfeffer et al., 1986). For example, in cotton Mn^{2+} lowers ATP concentrations and respiration rates as well as altering the activity of certain enzymes and hormones (Sirkar and Armin, 1979). In some cases healthy roots have been shown to reduce Mn^{2+} toxicity by precipitating oxidized Mn as MnO_2 on root surfaces (Foy, 1984). Plants such as maize may protect itself from such toxicity by entrapment of relatively high concentrations of the metal ion in the vacuole (Foy, 1973). A similar observation has been proposed for Cu^{2+} as well (Woodhouse and Walter, 1981).

Paramagnetic Mn^{2+} is a useful probe for examining the movement of a divalent cation via the ^{31}P -NMR spectrum of intact plant tissue (Loughman and Ratcliffe, 1984; Pfeffer et al., 1986). This is accomplished because the metal ion induces paramagnetic broadening on the ^{31}P resonances and this can be used to monitor the location of the metal as it migrates from one intracellular compartment to another (Loughman and Ratcliffe, 1984; Pfeffer et al., 1986).

In this report, we present our observations of 161.7 MHz ^{31}P -NMR studies of corn root tip tissue in which we have examined the movement of the divalent paramagnetic cation Mn^{2+} in and out the meristem tissue under different energetic conditions.

MATERIAL AND METHODS

Plant Tissue

Maize (*Zea mays* L. var FRB-73, Illinois Foundation Seeds) were germinated in a growth chamber at 28°C for 72 h as previously described (Pfeffer et al., 1986). Each experiment required approximately 700-900, 3-5 mm root tips which were generally examined by ^{31}P -NMR in the perfused state within one hour of excision.

Experimental Solutions

All solutions at pH 4 were unbuffered and contained from 0.1 to 10 mM calcium sulfate, 50 mM glucose, 25 mM sucrose or 5 mM 2-deoxyglucose with the addition of 1 mM MnCl_2 . The pH of these perfusate solutions were monitored and adjusted throughout the experiments. Solutions at pH 6.0 were buffered with 10 mM Mes. All solutions were continually saturated with either, O_2 or N_2 gas and circulated through the root tissue at a rate > 45 ml/min. Prior to the connection between the perfusion tubes and the NMR tube, a 30 sec vacuum evacuation of the tube was carried out to remove trapped gas bubbles from between the roots. This procedure was important to prevent heterogeneous broadening of the spectra. A change from one perfusate solution to another was accomplished by means of a two way double stopcock assembly that connected two separate reservoirs to the peristaltic pump and NMR tube assembly. Prior to changing from one solution to another a 100 ml flush of the new perfusate through the system was carried out to minimize contamination.

NMR Experiments

A narrow bore (54 mm) JEOL GX-400 NMR spectrometer operating at 21-22°C was used to obtain the 161.7 MHz ^{31}P spectra of 700-900 excised (3-5mm) root tips as described previously (Pfeffer et al., 1986). In order to safely carry out perfusion/NMR experiments (without leakage hazards) for extended periods of time (~ 48 h) inside the magnet, an additional external suction tube was set into the reservoir of the NMR tube spinner housing to prevent overflow of liquid (in the event of a leak at the NMR cap) onto the probe and shim coil insert. In all other respects, the perfusion system design and operation was as previously described (Pfeffer et al., 1986). A reference capillary containing 120 mM HMPA (hexamethylphosphoramide) was used to give a satisfactory size reference peak for each spectrum. HMPA exhibited a resonance at 13.78 δ downfield from MDP (methylene diphosphoric acid). All chemical shifts were referenced relative to MDP which was assigned a value of 0.0 δ .

The concentrations of mobile phosphorus compounds in the root tissue samples, averaged over the total sample volume within the detector coils were determined by comparing the area of the signal from the HMPA resonance (observed in the tissue spectra) to the area response given by the phosphorus resonances for 1 mM standard solutions of ATP, glucose-6-phosphate and P_i in the same sample volume. Both the tissue and the standard solution spectra were acquired under quantitative conditions. Adjustments were then made for differences in signal responses (see above) to establish a direct relationship between the area of the resonances obtained under the fast and slow acquisition regions. A typical spectrum obtained from approximately 800 tips exhibited a concentration profile within the coil volume of the probe (1-2 ml) of approximately 1.0 - 2.0 mM sugar phosphates, 0.6 - 0.7 mM cytoplasmic P_i , 1.0 - 3.0 mM vacuolar P_i , 0.3 - 0.5 mM NTP, and 0.3 - 0.5 mM UDPG + NAD depending upon the age and size of the tips used. Since the data collection process for the experiment often last overnight, it was important to access the possible cellular aging effect on NMR signal characters. Spin lattice relaxation times (T_1) were determined by the inversion recovery method (180 - 90°) with 16 s repetition times for the non-nucleotide resonances and 4 s for the nucleotide resonances between scans, respectively. Relaxation values were calculated

using a two-parameter exponential fit. The T_1 values, as reported earlier (Pfeffer et al., 1986) for both nucleotide and non-nucleotide resonances at the initiation and completion of a 34 h sequence of experiments were found to be identical within experimental error.

Estimation of pH

Cytoplasmic and vacuolar pH were estimated from standard calibration curves for P_i that have been described earlier (Pfeffer et al., 1986). Only very small changes, < 0.1 pH unit, were observed in the cytoplasmic pH over the duration of experiments up to 34 h. Because of the insensitivity of the chemical shift of the vacuole P_i at pH 5.5 no measurable change in the vacuolar pH could be detected throughout these experiments.

RESULTS AND DISCUSSION

Extensive use of ^{31}P -NMR in plant tissue studies has made it possible to evaluate the pH values, P_i content and NTP/ADP ratios of the cytosol and vacuole and to some extent pH changes in the vacuole. Fig. 1 shows the standard ^{31}P -NMR profiles of maize root tips (~ 800) under perfusion conditions using rapid (A) and short (B) instrument recycling times. The assignment of each well established resonance (Roberts, 1984; Loughman and Ratcliffe, 1984) is given in the figure. Although the rapid pulsing-technique (A) gives rise to distortion in the ratio of nucleotides to the other phosphates, it can still be used to enhance the former

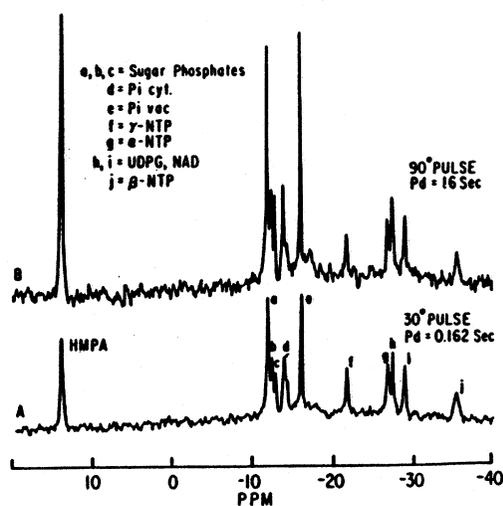


Figure 1

Intensity distortion due to rapid acquisition
 A 161.7 MHz ^{31}P spectrum of approximately 800 excised corn root tips (3-5 mm)
 taken with (A) rapid acquisition parameters
 and with (B) slow acquisition parameters as indicated above

Each spectrum was obtained with a spectral width of 16,000 Hz and 2,000 data points zero filled to 16,000.

so that better quantitation of relative changes can be assessed. In order to evaluate the real concentrations of the various components, conversion for relaxation differences has been established to standardize area of resonance peaks. Typically, the concentration of compounds represented in the spectra approximately 0.60.7 mM cytoplasmic P_i , 1.0 - 3.0 mM vacuolar P_i , 0.3 - 0.5 mM NTP and 0.3 - 0.5 mM UDPG and NAD based on the resonance areas compared with the standard HPMA (hexamethylphosphoramide) at 13.78 δ and standard solutions of the corresponding compounds.

Effects of Mn^{2+}

Introduction of 1 mM Mn^{2+} into the perfusion medium containing a sufficient supply of glucose (50 mM) under aerobic conditions caused a rapid broadening of the NTP resonances (Fig. 2). This broadening is due to the paramagnetic properties of Mn^{2+} and its subsequent shortening of the relaxation properties of the ^{31}P nuclei of the strongly binding compounds. After 90 min of perfusion we also observed that P_i and sugar phosphates in the cytoplasm had undergone strong broadening. By 150 min, significant broadening of the vacuolar P_i had occurred indicating that

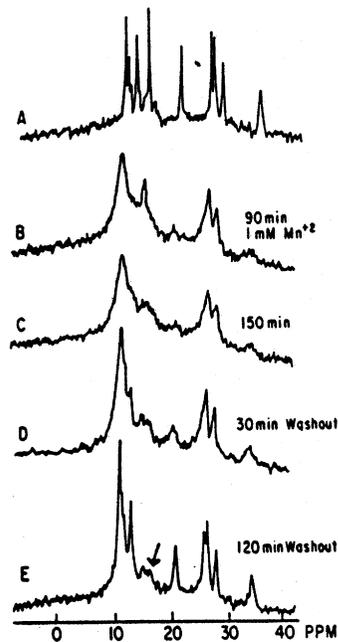


Figure 2

Mn^{2+} uptake in vacuole

- A. ^{31}P spectra of approximately 800 excised corn root tips after perfusion for 2 hr with 0.1 mM $CaSO_4$, 50 mM glucose and 10 mM Mes buffer at pH 6.0 with O_2 .
- B. Same as in A after the addition of 1 mM $MnCl_2$ for period indicated.
- C. Same as B after period indicated.
- D. Washout following C for 30 minutes; washout after 120 minutes.

migration of Mn^{2+} across the tonoplast and into the vacuole became prominent. Exchange of the perfusate to one not containing Mn^{2+} caused a partial reversal of the broadening effects on all resonances representing components within the cytoplasm, however continual perfusion up to 5 hr (not shown) gave no regeneration of the vacuole P_i signal as in Fig. 2-E. Based on binding studies of Mn^{2+} , ATP and P_i as given in Fig. 3 we noted that the full broadening of the P_i resonance by Mn^{2+} required 6.4 times more Mn^{2+} at pH 5.5 than at pH 7.5. Consequently, in order to effect comparable line broadening of the vacuolar P_i resonance, the vacuolar (Mn^{2+}) must be 6.4 times higher than that of cytoplasm. On a per phosphorus basis, ATP requires about 5.7 times the concentration of Mn^{2+} to undergo comparable linebroadening as cytoplasmic P_i . However, the ATP- Mn^{2+} complex is formed preferentially because of its tighter binding constant defined as :

$$\frac{ATP}{P_i} \frac{M_n}{M_n} \frac{K_{stab}}{K_{stab}} = 158$$

Considering the effective concentration of mobile phosphorus compounds observed in the spectra we estimate that there is approximately 0.015 mM Mn^{2+} associated with the P_i representing the vacuolar compartment when broadening is complete and 0.03 mM Mn^{2+} associated with the P_i and nucleotide in the cytoplasm.

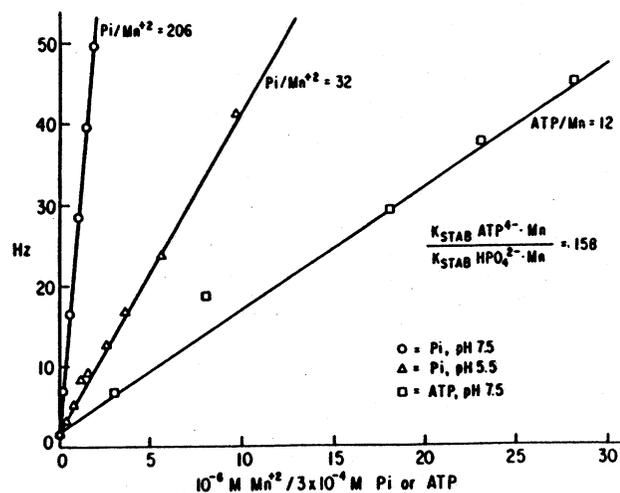


Figure 3

Phosphate and ATP linebroadening with Mn^{2+}

Mn^{2+} Migration as a Function of Carbohydrate Supply and Metabolism

In order to understand the effects of the carbohydrate supply on the influx of Mn^{2+} we examined its relative rate of uptake in the absence of exogenous glucose. Omission of glucose in the perfusion medium followed by the introduction of Mn^{2+} showed a marked slowing of the migration and subsequent broadening of the vacuolar P_i resonance (90 min with glucose, 135 min without glucose). These results indicate that at a somewhat lowered energy state, the Mn^{2+} migration across the tonoplast membrane is inhibited. Movement, however, into the cytoplasm as evidenced by

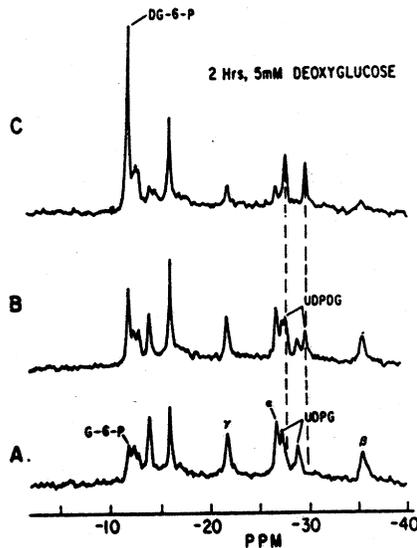


Figure 4

Incubation with 2-deoxy-glucose

^{31}P spectra of corn root tips (A) before addition of 5 mM 2-deoxy-glucose, (B) after 30 minutes treatment with 5 mM 2-deoxy-glucose, (C) after 2 hr treatment with 5 mM 2-deoxy-glucose.

rapid cytoplasmic resonance linebroadening was insignificantly affected. To evaluate this phenomenon more directly we decided to look at the response of the metal ion movement by inhibiting the glycolytic pathway directly. Fig. 4 shows the effect of the initial incubation of excised root tips with 5 mM 2-deoxyglucose in the absence of any other exogenous carbohydrate source. After 0.5 hr we noted the buildup of the 2-deoxyglucose-6-phosphate in the cytoplasm due to its lack of conversion to fructose-6-phosphate in the glycolytic pathway. Also we observed the production of two new resonances at ~ 27 and 29 ppm corresponding to the 2-deoxyglucose derivatives of UDPG. Following 2 hr of perfusion, NTP levels decreased by $2/3$ (due to the decrease of substrate-level and oxidative phosphorylation) and UDPG was replaced by UDP-2-deoxy-G. In this state, treatment of the tips in the normal manner with 1 mM Mn^{2+} for 2 hr showed diminished Mn^{2+} broadening or invasion into the vacuole (Fig. 5). After 5.5 hr of washings, little if any, broadening of the vacuolar P_i resonance was observed. Thus in the presence of a minimal level of NTP only a marginal amount of metal ion could be moved effectively and trapped in the vacuole.

Effects of Hypoxia (N_2) on Metal Ion Trapping

It has been well established that during hypoxia, maize root tips can be sustained by anaerobic metabolism with much reduced levels of NTP and production of lactate and ethanol (Roberts et al., 1984). In this altered state the pH of the cytoplasm is maintained at pH 6.7-6.9 as opposed to 7.5 to 7.6 in the aerobic state (Fig. 6). Also note (in Fig. 6-B) that under hypoxia we observed the presence of NDP, an increased concentration of cytoplasmic P_i and only 35 - 40% of the original NTP levels. Following 108 min of exposure to Mn^{2+} we observed the complete loss

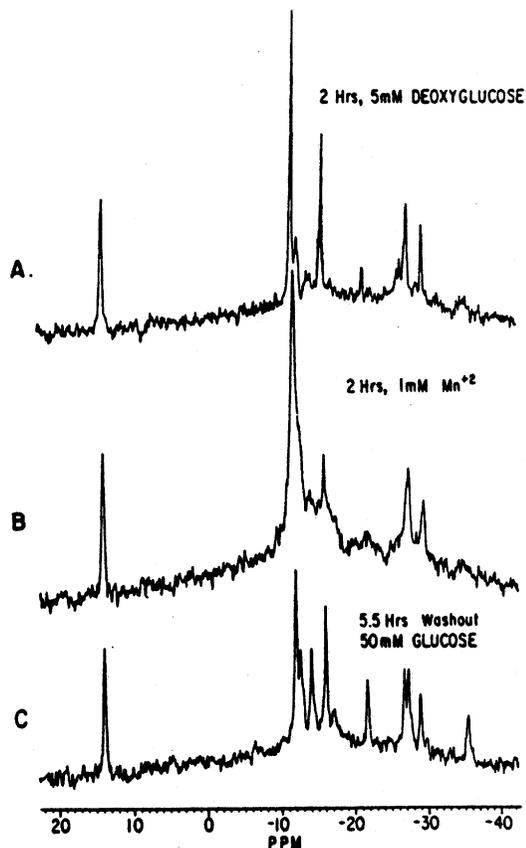


Figure 5

Suppression of Mn^{2+} migration with 2-deoxy-glucose

^{31}P spectra showing suppression of Mn^{2+} migration into the vacuole following a 2 hr treatment with 5 mM 2-deoxy-glucose. A. Spectrum prior to treatment with 1 mM $MnCl_2$; B. Spectrum following 2 hr treatment with 1 mM $MnCl_2$; C. Spectrum resulting from 5.5 hr wash-out with 10 mM Mes, pH 6.0, 50 mM glucose and 0.1 mM $CaSO_4$ perfusate.

of the ATP resonances, however no change in the observed line widths corresponding to the other cytoplasmic compounds. This suggests that only a very minimal amount of Mn^{2+} migration across the plasmalemma has taken place. Efforts to wash out this small amount of Mn^{2+} from the cytoplasm under an N_2 atmosphere was unsuccessful. With the resumption of an O_2 atmosphere, however, Mn^{2+} washout was complete and full generation of the narrow line width spectrum was complete.

Competition of Ca^{2+} with Mn^{2+} for Migration in Root Tips

It is well established that the application of Ca^{2+} to the soil decreases the effect of toxic metal ions (Lund, 1970). Yet, it is not known whether this is a direct consequence of Ca^{2+} competing more favorably than other metal ions for movement

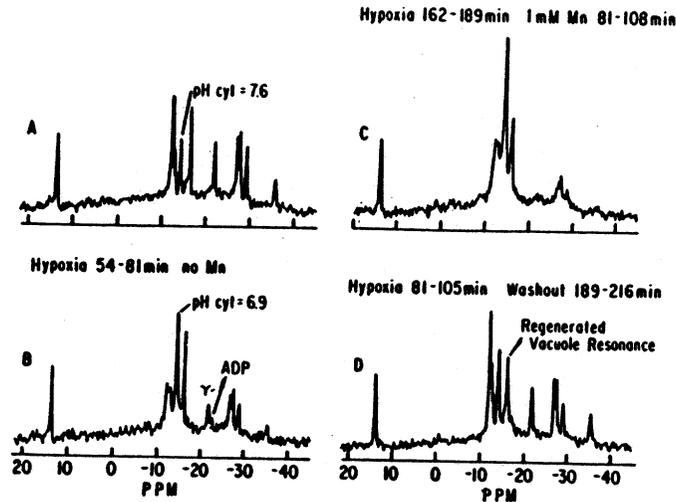


Figure 6

Suppression of Mn^{2+} migration to vacuole under hypoxia

^{31}P spectrum of corn root tips at pH 6.0, 10 mM Mes, 0.1 mM $CaSO_4$ and 50 mM glucose. A. With O_2 ; B. Following 54-81 minutes of hypoxia (N_2); C. Spectrum during 162-189 minutes of hypoxia and 81-108 minutes perfusion with medium containing 1 mM $MnCl_2$; D. Final washout under O_2 after 189-216 minutes with medium that does not contain Mn^{2+} .

across the plasmalemma. Treatment of maize root tips with a perfusate containing 10.0 mM Ca^{2+} , 5.0 mM glucose at pH 6.0 produced a significantly less broadened spectrum following 150 min of exposure to Mn^{2+} (Fig. 7). Subsequent washout of the Mn^{2+} containing perfusate gave a spectrum which showed a slight indication of the presence of Mn^{2+} in the vacuole with no indication of its presence in the cytoplasm. Clearly, under the employed conditions, Mn^{2+} movement across the plasmalemma in both directions is free of restrictions. However, in the presence of high concentration of Ca^{2+} , less Mn^{2+} can reach the cytoplasm within the limited 150 min of perfusion. Presumably, Mn^{2+} has to compete with Ca^{2+} for the same transport mechanism associated with the plasma membrane. Consequently, the secondary migration of a diminished concentration of Mn^{2+} across the tonoplast is significantly suppressed. We have also observed a similar effect with Cd^{2+} , except in this instance Ca^{2+} was capable of preventing the cytoplasmic "poisoning" induced by Cd^{2+} invasion (unpublished results).

Divalent Cation Transport Mechanism

The data obtained in this study demonstrate that the uptake of metal ions, e.g. Mn^{2+} , is regulated by the energetic status ($O_2 + \text{exogenous glucose} > O_2 > O_2 + 2\text{-deoxy-glucose} > N_2 + \text{glucose}$) of the root cells. Furthermore, the uptake of different divalent cations may compete for the same membrane apparatus. Based on the accepted general concepts (Racker, 1979) of ion transport developed mainly from research in non-plant systems, a mechanism, as depicted in Fig. 8, is proposed to account for the observations mentioned in this report.

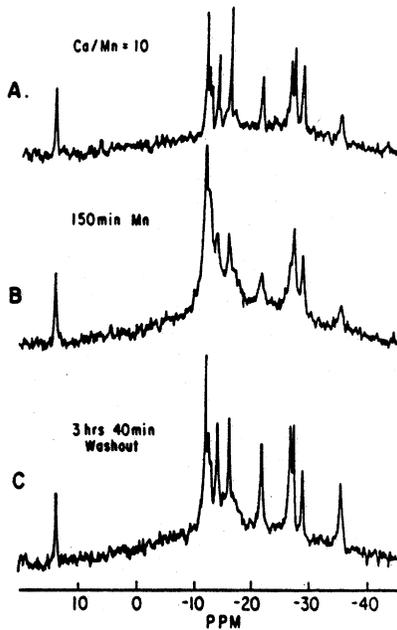


Figure 7

Ca suppression of Mn passage

A. ^{31}P spectrum of corn root tips at pH 6.0, 10 mM Mes, 10.0 mM MnSO_4 , 50 mM glucose, O_2 atmosphere; B. Following 150 min treatment with same medium containing 1 mM MnCl_2 ; C. Following 3 hr. and 40 minutes of washout with medium as in A except containing 0.1 mM CaSO_4 .

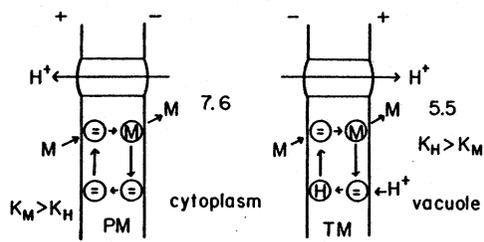


Figure 8

Proposal for transport mechanism of $\text{M}(2+)$

Our basic assumptions are the following :

- (1) The proton pumping processes associated with the plasma membrane (PM) and the tonoplast membrane (TM) generate transmembranous proton electrochemical potentials in the directions as shown in Fig. 8 (Sze, 1985) (active H^+ -pumping outward from cytoplasm, membrane potential negative in cytoplasm).

- (2) The actual passage of metals (M) through the membranes is facilitated by certain negatively-charged membrane carriers which can bind with either protons or metal ions.

- (3) The affinities of carriers to protons (K_H) or metal ions (K_M) are regulated by pH.

Thus, under the conditions of O_2 + exogenous glucose, the added metal ion, e.g. Mn^{2+} , binds with the PM carrier to form a natural complex which diffuses through PM and releases the metal. The negatively charged carrier (the alkaline cytoplasmic pH ~ 7.8 precludes the protonation of PM carrier) is then driven back to the exterior surface of the membrane for the next loading of metal ion. Although the transport is diffusion in nature (along a concentration gradient), the process is facilitated by the energy input in the form of a membrane potential to enhance the returning of the negatively charged carrier to the exterior surface of PM. The transport of M from the cytoplasm to vacuole follows a similar mechanism. However, the low pH of the vacuole region tends to protonate the carrier on the inner surface of TM. The protonated carrier can only slowly diffuse back to the cytoplasmic side (no facilitated movement) when the protons are released. Since the motion of the TM carrier is not facilitated in either direction, the uptake across TM is slower than that associated with PM. During the washout process, the concentration gradient of M is reversed and the carrier mediated back diffusion through the PM takes place. However, the back diffusion across TM is hindered because the vacuolar metal ion can not compete with protons for the TM carrier. Thus, metal ions are trapped.

Under hypoxia conditions, the cytoplasmic pH decreases to ~ 6.9 or lower. This acidic condition promote the protonation of both PM and TM carriers at cytoplasmic surfaces. Consequently, the facilitated motion of the PM carrier is hindered and the uptake across PM is by diffusion only. The transport across TM becomes unnoticeable because the protonated TM carrier cannot release its protons. The inefficient washout of M from cytoplasm under hypoxia is expected because the relatively acidic cytoplasm may trap M by the similar mechanism for vacuolar M trapping.

The experimental results obtained under the conditions of either the omission of exogeneous glucose or the use of 2-deoxy-glucose are also expected since the cells are in intermetant energy states between O_2 + exogeneous glucose and N_2 + glucose.

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