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Association of nickel versus transport of cadmium and calcium in tonoplast vesicles of oat roots

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Abstract. The plant vacuole has long been suspected of being a site for accumulation of Ni in plant roots, but testing this hypothesis directly by vacuole isolation is technically difficult and has not been reported. Here, we have attempted to determine if Ni can be transported into isolated oat (*Avena sativa* L.) root tonoplast vesicles as an alternative approach toward understanding the importance of the vacuole in Ni accumulation in roots. We found that, in contrast to Ca and Cd, Ni did not affect the proton gradient of vesicles (MgATP energized or artificially created), and further, that Cd/H antiport activity was not affected by the presence of Ni. Nickel was associated with vesicles, but relative rates of accumulation/association of metals with vesicles were Ca > Cd ≫Ni. Protonophores and the potential Ni ligands citrate and histidine, and nucleoside triphosphates or PPi did not stimulate Ni association with vesicles. Comparison of Ni versus Ca and Cd associated with vesicles using various membrane perturbants indicated that while Ca and Cd are rapidly and principally antiported to the vesicle sap, Ni is only slowly associated with the membrane in a not-easily dissociated condition. Our results indicate the absence of an Ni/H antiport or Ni-nucleotide-dependent pump in oat root tonoplasts, and support the contention that the vacuole is not a major compartment for Ni accumulation in oat roots.

Key words: *Avena* (root, tonoplast) – Calcium – Cadmium – Nickel – Tonoplast – Transport (ion)

Introduction

Plants readily take up Ni from soil, particularly under acid soil conditions (Farago and Cole 1988). Nickel is considered to be essential for plants (Brown et al. 1987). It is a component of several bacterial enzymes, including urease and certain dehydrogenases, and may be a transcriptional regulator in bacteria (Kim and Maier 1990). Nickel deficiency has been described for several plants, including oats, the plant studied here (Brown et al. 1987; Farago and Cole 1988).

Phytotoxicity occurs in many plant species after exposure to moderate levels of Ni. However, Ni tolerance is found in certain plants, particularly those inhabiting Ni-rich soils, e.g. serpentine soils. The majority of these serpentinophytes appear to limit Ni^{2+} uptake; however, 145 plant species are thus far known to accumulate Ni^{2+} in above-ground parts (Baker 1989). In some plants Ni^{2+} is accumulated in leaves to levels of $\geq 10\,000\ \mu g \cdot g^{-1}$ (dry weight basis) where the metal is speculated to be sequestered in the vacuole as Ni-citrate (see Farago and Cole 1988). But, there is little direct experimental evidence regarding the subcellular site of Ni in either leaves or roots of serpentinophytes, or in plants which are not capable of high Ni accumulation/tolerance. Recently, Brune et al. (1995) compared the metal contents of mesophyll protoplasts and vacuoles isolated from primary leaves of barley. They found that unlike Cd, Zn and Mo, which were found to be mostly vacuolar, Ni was primarily extravacuolar. In their study, roots accumulated more Ni than leaves (seedlings exposed to 100 μM $NiCl_2$), but roots were not examined for subcellular metal distributions, presumably because of the technical difficulty in obtaining root protoplasts and vacuoles in quantity/condition useful for such analyses.

We attempted to isolate useful quantities of protoplasts and vacuoles from oat roots using methods previously applied to vacuole/extravacuole distribution studies (Krotz et al. 1989; Vogeli-Lange and Wagner 1990) but failed. So, here we have taken a different approach to questioning the importance of root vacuoles in Ni accumulation. We tested the possibility that Ni is transported across the tonoplast by an Ni/H antiport mechanism like that shown for Ca (Blumwald and Poole 1986; Shumaker and Sze 1986; Blackford et al. 1990), Na

Abbreviations: CCCP = carbonylcyanide *m*-chlorophenylhydrazone; DMSO = dimethyl sulfoxide; FCCP = carbonylcyanide *p*-(trifluoromethoxy) phenylhydrazone

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(Garbarino and DuPont 1988; Barkla and Blumwald 1991), Mg (Amalou et al. 1992), and Cd (Salt and Wagner 1993) transport. We also investigated the possibility that Ni might be transported as a complex with citrate or histidine or by a nucleotide-dependent membrane pump. Citrate accumulation has been correlated with Ni accumulation in serpentinophytes (Farago and Cole 1988) and histidine was recently shown to be elevated in the xylem sap of an Ni hyperaccumulator and to increase Ni accumulation in histidine-exposed roots (Kramer et al. 1996). The distribution of Ni between the membrane and sap of vesicles was also studied to differentiate between transport to the sap and membrane association.

We have used oat roots in this study for several reasons. First, membrane vesicles from this tissue have been used extensively for investigating the transport properties of tonoplasts. Second, oats appear to tolerate relatively high concentrations of Ni in their tissues compared with other grasses (Farago and Cole 1988), and related to this, observations from root elongation studies indicate that oat roots are little affected when grown in ≤ 50 μM Ni (Gries 1994).

Materials and methods

Plant material, seed germination and seedling growth. Oat (*Avena sativa* L.; 250 g) seed was surface-sterilized for 5 min in 1 L of 5% sodium hypochlorite, rinsed three times with distilled water and spread onto one layer of cheesecloth covering a mesh (52 cm × 41 cm) that was supported by a plastic frame. The frame was set on supports so that the mesh was approx. 1.5 cm above the surface of an aerated 0.5% $CaCl_2$ solution. Roots were harvested after 5 d of germination and growth in the dark at room temperature (about 23 °C). Unrolled oat seed was purchased from Southern States Cooperative, Lexington, Ky., USA.

Isolation of sealed tonoplast-enriched vesicles. The procedure of Randall and Sze (1987) (Churchill and Sze 1983; Schumaker and Sze 1985; Salt and Wagner 1993) was used with some modifications. All steps were conducted at 4 °C and, where used, DTT and phenylmethylsulfonylfluoride (PMSF) were added just before use. Oat roots (20–55 g) were soaked in homogenization buffer for about 10 min and cut into pieces of about 2 cm length before homogenization with a mortar and pestle using a buffer-to-tissue ratio of 1.5 mL to 1 g. Homogenization buffer contained 250 mM mannitol, 6 mM EGTA, 0.1% BSA, 0.1 mM PMSF, 20 mM Hepes adjusted with Bistris propane (BTP) to pH 7.4 and 1 mM DDT. After filtration of the brie through four layers of cheesecloth, the retentate was again homogenized in 1.5 mL homogenization buffer per gram of original tissue weight and the product was filtered. The resulting suspension was centrifuged for 5 min at 1000 *g*, then 10 min at 12 000 *g*. The resulting supernatant was centrifuged for 30 min at 60 000 *g*. Pellets (total microsomal membranes) were gently suspended in 1 mL suspension buffer, containing 250 mM mannitol, 0.1% BSA, 0.1 mM PMSF, 2.5 mM Hepes-BTP at pH 7.2 and 0.1 mM DTT, and pooled. The crude microsomal suspension (6 mL) was layered over 10 mL of a 6% (w/v) dextran cushion containing 6% dextran (w/v) (D-4751; Sigma Chemical Co., St. Louis, Mo., USA, averaging MW 74 000), 2.5 mM Hepes-BTP at pH 7.2 and 250 mM mannitol (2-cm-diameter tube). Gradients were centrifuged for 2 h at 70 000 *g*. A visible band at the mannitol-dextran interface was collected and is henceforth referred to as ''tonoplast-enriched vesicles'' or ''vesicles''. The collected interface was diluted tenfold with resuspension buffer and centrifuged at 90 000 *g* for 30 min. Pelleted vesicles were resuspended in resuspension buffer to a volume of 200–350 μL giving a concentration of 5–10 mg protein · mL^{-1}.

Formation of a pH gradient across the tonoplast. Two methods were used to generate a pH gradient across the tonoplast of tonoplast-enriched vesicles. When the V-type ATPase was employed, vesicles were supplied with 3 mM MgATP (Salt and Wagner 1993) and 28 mM chloride as BTP-Cl to stimulate the V-type ATPase. All assays with MgATP-energized vesicles were performed at 15 °C to maintain the generated ΔpH.

For artificial generation of ΔpH, vesicles were loaded with potassium followed by dilution into K^+-free medium and addition of nigericin (see Salt and Wagner 1993). Assays were conducted at 4 °C to reduce H^+ permeability in K^+/nigericin-energized vesicles. In experiments containing carbonylcyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), carbonylcyanide *m*-chlorophenylhydrazone (CCCP), gramicidin, or nigericin, these were added from stock solutions in ethanol to give final concentrations of 40 μM, 10 μM, 3 μM and 5 μM, respectively. Ethanol concentration in transport assays was 1% (v/v).

Assay of proton translocation activity: methylamine accumulation. A pH gradient was formed (acid inside the vesicles) using either MgATP-dependent H^+-ATPase or by addition of nigericin to K^+-loaded vesicles. Formation of a ΔpH was measured as [^{14}C]methylamine accumulation in vesicles by monitoring 60 to 100-μl aliquots at specified time points using the direct vesicle filtration assay (Churchill and Sze 1983). The MgATP-dependent formation of ΔpH in vesicles was initiated by addition of 3 mM $MgSO_4$ to a reaction mixture containing 25 mM Hepes-BTP at pH 7.0, 20 μM [^{14}C]methylamine (37 $kB_2 \cdot mL^{-1}$), and vesicles (80–150 μg protein · mL^{-1}). Results are presented as nanomoles of methylamine per milligram of protein.

Transport of Ca, Cd and Ni. Uptake of Ca^{2+}, and Cd^{2+} and Ni^{2+} were measured using about 1.1×10^6 dpm ^{45}Ca, ^{109}Cd and ^{63}Ni per ml assay (10 mM total metal). A ΔpH was developed using either the MgATP-dependent or K^+/nigericin methods described above. At specified time points, 60- to 100-μl aliquots were assayed using the direct vesicle filtration method. All additions were made in 2.5 mM Hepes-BTP at pH 7.0. Results are presented as nanomoles Ca^{2+}, Cd^{2+} or Ni^{2+} per milligram of protein.

Direct filtration assay. The procedure was after that of Churchill and Sze (1983) with the modification that Millipore filters (Millipore HATF type HA, 0.45 μm; Millipore Corp., Bedford, Mass., USA) were presoaked prior to use with ice-cold resuspension medium (250 mM mannitol, 0.1% BSA, 0.1 mM PMSF, 2.5 mM Hepes-BTP at pH 7.2 and 0.1 mM DTT; Salt and Wagner 1993).

Membrane perturbation experiments. After vesicles were incubated with a reaction mixture including 3 mM MgATP and 10 μM metal for 30 min at 15 °C, aliquots were transferred into Eppendorf tubes for further chemical or physical treatments. Treatments included the addition of the chelating agent EDTA, the detergent Triton X-100, various ionophores (A-23187, Glyceollin I, Alamethicin), other potential leak-inducing compounds [Hemolysin, Streptolysin O, Filipin, dimethyl sulfoxide (DMSO), *n*-propanol], sucrose to plasmolyze, and physical treatments (osmotic shock, boiling). When Streptolysin O was used, it was preincubated with 4 mM DTT at room temperature for 15–20 min to activate this polypeptide. Plasmolysis of the vesicles was accomplished by adding solid sucrose to the reaction mixture to achieve 500 mM concentration. After thorough mixing, aliquots were taken for the direct filtration assay.

To osmotically shock vesicles, an aliquot of reaction mixture was diluted sixfold into water and aliquots were filtered 5 min after diluting. Where an increase of volume occurred due to the addition of a reagent, this was compensated for in the control. In experiments containing Triton X-100, DMSO, *n*-propanol, Hemolysin, Streptolysin O, and EDTA, perturbants were added from stock

solutions in 2.25 mM Hepes-BTP (pH 7.0) to give final concentrations in assays of 0.01–0.1% (v/v), 0.5 or 5% (v/v), 0.5 or 5% (v/v), 100 or 500 hemolytic units, 100 or 500 hemolytic units and 3.1 mM, respectively. Stock solutions in 96% ethanol were prepared for the agents Filipin, Alamethicin, A-23187, and Glyceollin I to give final concentrations in the assays of 100 or 500 μM, 25 or 100 μM, 2 or 25 μM and 100 or 500 μM, respectively. Final ethanol concentrations were <1% (v/v). The treatments were made after 30 min incubation of MgATP-energized vesicles with ^{63}Ni. Parallel experiments were performed with MgATP-energized vesicles in the presence of ^{45}Ca or ^{109}Cd.

Determination of protein concentration and preparation of ^{3}H-choline-labeled vesicles. Protein concentration was estimated using method of Bensadoun and Weinstein as modified for membrane proteins (see Salt and Wagner 1993). Vesicles labeled with [^{3}H]choline were prepared by growing oat seedlings for 3 d in 0.5 mM $CaSO_4$ then transferring seedlings (about 50) to a vessel containing [methyl 1-^{3}H]choline chloride (2960 $GB_2 \cdot \mu mol^{-1}$; New England Nuclear, Boston, Mass., USA) in 0.5 mM $CaSO_4$. After 2 d in the dark, roots were washed and used to prepare vesicles as above. Separation of an aliquot by isopycnic centrifugation indicated that ^{3}H was only associated with tonoplast-like vesicles equilibrating at 1.1 $g \cdot mL^{-1}$ (not shown).

Reagents. Chemicals were purchased from Sigma Chemical Company, including [^{14}C]methylamine (2035 $MB_2 \cdot mmol^{-1}$). Pierce BCA protein assay was obtained from Pierce, Rockford, IU., USA, ^{45}Ca (411 $MBq_2 \cdot mg^{-1}$) was purchased from ICN Radiochemicals, Irvine, Calif., USA, ^{109}Cd (78.1 $MBq_2 \cdot mg^{-1}$) and ^{63}Ni (74–185 $MB_2 \cdot mg^{-1}$) from NEN-DuPont, Wilmington, Del., USA. Glycellin I was a gift from Dr. G.O. Spessard, St. Olaf College, Northfield, Minn., USA.

Results and discussion

Effect of Ni on the proton gradient. We tested the possibility of an H^+/Ni^{2+} antiport mechanism using oat root tonoplast vesicles. Proton gradients (acid inside vesicles) were formed by activating the MgATP-dependent proton pump (Fig. 1A) or artificially by addition of nigericin to K^+-loaded vesicles (Fig. 1B). Addition of MgATP caused a rate of proton accumulation (monitored using [^{14}C]methylamine) that was similar to that reported previously by ourselves and others using this system (see Salt and Wagner 1993). Subsequent addition of $NiCl_2$ (100–330 μM) did not cause efflux of methylamine (Fig. 1A). Similar results were obtained with 10–1000 μM $NiCl_2$ and MgATP-energized vesicles using the acridine-orange fluorescence-quench method (data not shown). These observations indicated that either Ni^{2+} was not exchanged (antiported) with H^+ or only a very low level of exchange took place. In contrast to the absence of an Ni^{2+} effect (up to 330 μM Ni^{2+}), clear evidence for H^+ exchange upon addition of Ca^{2+} and Cd^{2+} to MgATP-energized vesicles occurs at <10 μM Ca^{2+} and ≥ 10 μM Cd^{2+}, respectively (Shumaker and Sze 1986; Salt and Wagner 1993). To rule out any effect of the presence of Ni on MgATPase, experiments were conducted using preparations in which the proton gradient was artificially created by addition of nigericin to K^+-loaded vesicles. As shown in Fig. 1B, 83–1670 μM $NiCl_2$ provided no evidence for H^+/Ni^{2+} exchange. Therefore, while low levels of Ca^{2+} or Cd^{2+} are shown to cause concentration-dependent proton

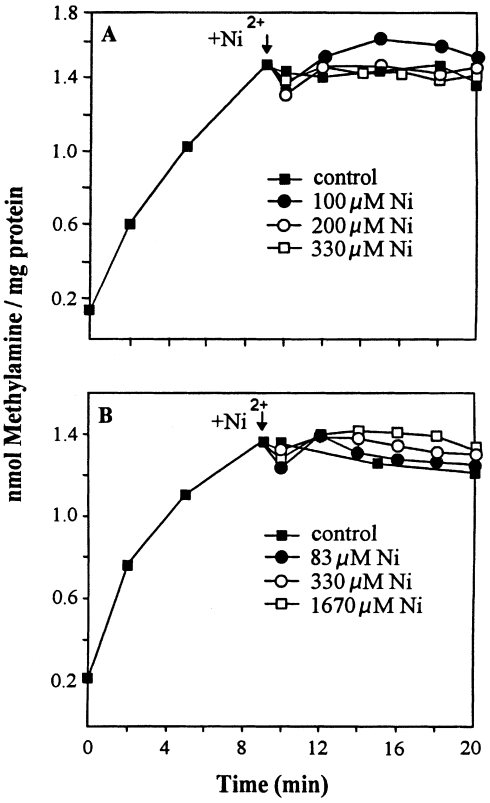

Fig. 1A,B. Effects of Ni^{2+} on the proton gradient in oat root tonoplast-enriched vesicles. **A** MgATP-energized vesicles, after establishment of a steady-state proton gradient, were exposed to no(-■-), 100 μM(-●-), 200 μM(-○-), or 330 μM(-□-) $NiCl_2$. **B** K^+/nigericin-energized vesicles were exposed to no(-■-), 83(-●-), 330(-○-), or 1670 μM(-□-) $NiCl_2$

exchange under the conditions used, up to 1670 μM $NiCl_2$ resulted in no such effect.

Since Ni^{2+} is known to have affinity for histidine residues of proteins and these are crucial in a number of enzymatic reactions, it was possible that the lack of H^+/Ni^{2+} exchange in vitro was due to modification of tonoplast transport proteins by Ni^{2+}. To test this, K^+/nigericin-energized vesicles were exposed to 100 μM $NiCl_2$ for several minutes followed by addition of 50 μM $CdCl_2$. Addition of Cd^{2+} resulted in a rate of H^+ efflux (Fig. 2A) comparable to that observed in the absence of Ni^{2+} pretreatment (Fig. 2B). This result shows directly that 100 μM $NiCl_2$ treatment (and its continued presence) does not interfere with H^+/Cd^{2+} antiport activity. The concentration dependence of $2H^+/Cd^{2+}$ activity observed (Fig. 2B) was similar to that reported earlier (Salt and Wagner 1993).

Ionophores and NH_4Cl were used to further exclude the possibility that the vacuole proton gradient energizes Ni^{2+} association with tonoplast vesicles. First, $^{63}NiCl_2$ was shown to associate with vesicles (see below). Addition of FCCP (40 μM), CCCP (10 μM), gramicidin (3 μM) or NH_4Cl (250 μM) prior to addition of Mg^{2+} to activate the MgATP-dependent proton pump resulted in $^{63}Ni^{2+}$ associations of 86 ± 7%, 73 ± 18%, 102 ± 12% and 84 ± 8%, respectively, of that found with no ionophore or NH_4Cl addition. Values are the means of

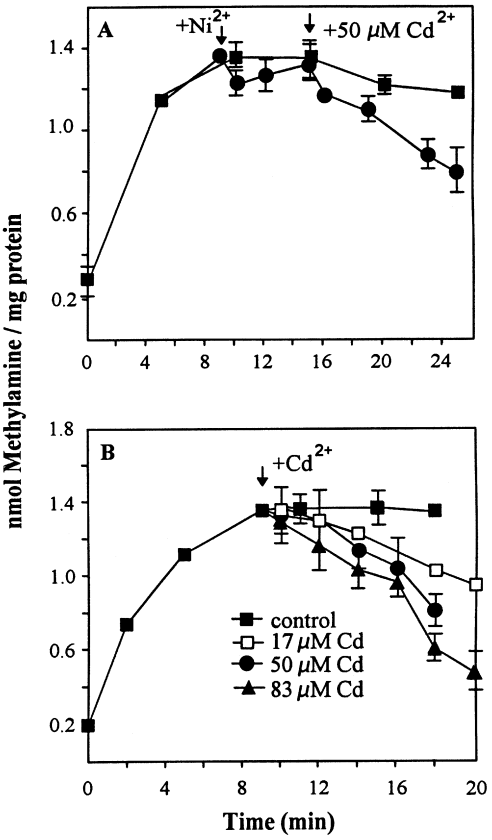

Fig. 2A,B. Effects of pretreating K^+/nigericin-energized vesicles with 100 μM $NiCl_2$ on $Cd^{2+}/2H^+$ antiport activity. **A** Addition of 100 μM $NiCl_2$ at 8 min followed by no further addition (-■-), or addition of 50 μM $CdCl_2$ at 15 min(-●-). **B** $CdCl_2$ concentration-dependent depletion of the proton gradient: 0 Cd(-■-), 17 μM(-□-), 50 μM(-●-), 83 μM(-▲-) $CdCl_2$

three to five experiments, each with three replications. Taken together, the above results indicate that an H^+/Ni^2 antiport mechanism does not operate in oat root tonoplast vesicles.

It is unlikely the Ni^{2+} is transported across the tonoplast via a membrane potential because of its positive charge and the potential poise of this membrane in vivo. However, if Ni^{2+} were present in the cytoplasm as a complex with net negative charge (i.e. as Ni-citrate, 1:1), such a species might be transported by a positive-inside membrane potential. This is a particularly important possibility since Ni-citrate is considered to be the principal Ni^{2+}-ligand pair accumulated in Ni hyperaccumulators (Farago and Cole 1988). We tested the effects of the presence of citrate in $^{63}Ni^{2+}$ transport assays (MgATP-energized vesicles) using ratios of Ni to citrate of 3:1 and 1:1. No clear evidence was found for marked stimulation of Ni association by citrate under conditions of 3 mM ATP, 3 mM MgATP, 3 mM Mg or no ATP or Mg addition (data not shown). We also tested effects of adding histidine because this amino acid is an efficient Ni^{2+}-complexing agent (Martin and Mariam 1979) and was recently found to be elevated in the xylem fluid of an Ni^{2+} hyperaccumulator (Kramer et al. 1996). Histidine (10 μM) in a ratio of 1:1 with $NiCl_2$ had no clear simulatory effect on $^{63}Ni^{2+}$ association with vesicles (data not shown).

Impact of nucleotide triphosphates on Ni association with vesicles. The transport or membrane association of $^{63}NiCl_2$ with tonoplast vesicles is shown in Fig. 3. As shown, a time-dependent increase in $^{63}Ni^{2+}$ association with vesicles occurred in the absence of ATP and Mg, and in the presence of ATP or MgATP. Higher association was found without ATP, indicating the lack of importance of ATP and the possible chelation of Ni^{2+} (10 μM) by ATP (3 mM). Complexation of Ni^{2+} by ATP has been reported (Martin and Mariam 1979). A continued linear increase was observed up to 60 min either using MgATP-energized vesicles (Fig. 3), or K^+/nigericin-energized vesicles (not shown). In contrast to Ni^{2+}, transport of Ca and Cd is apparently saturated in ≤ 20 min using MgATP-energized vesicles (Salt and Wagner 1993) and in < 5 min when the proton gradient is established artificially (Ca^{2+} and Cd^{2+} [Salt and Wagner 1993], and Mg^{2+} [Amalou et al. 1992]). Higher $^{63}Ni^{2+}$ association in the presence of MgATP versus ATP alone may reflect competition between 10 μM Ni^{2+} and 3 mM Mg for ATP. To test $^{63}Ni^{2+}$ association with vesicles under different conditions, K^+/nigericin-energized vesicles were exposed to $^{63}NiCl_2$ in the presence and absence of nigericin and the protonophore FCCP or the P-type ATPase inhibitor vanadate (50 μM). In no case was a clear difference in ^{63}Ni association with time observed and apparent saturation was not observed after 20–30 min. Gramicidin at 3 μM also had no effect. Also, under these conditions ^{63}Ni association was found to increase linearly when vesicles were exposed to ^{63}Ni, 10 to 100 μM total $NiCl_2$ (specific radioactivity of Ni^{2+} held constant). These results support the conclusion that $^{63}Ni^{2+}$ association with vesicles was not energized by the proton gradient and was not due to transport via an MgATP-dependent pump such as the Ca^{2+} ATPase of the plasmalemma (Giannini et al. 1987).

ABC-type transporters are reported to move various solutes, including ions (see Ortiz et al. 1995; Salt and Rauser 1995). These transporters are characterized as

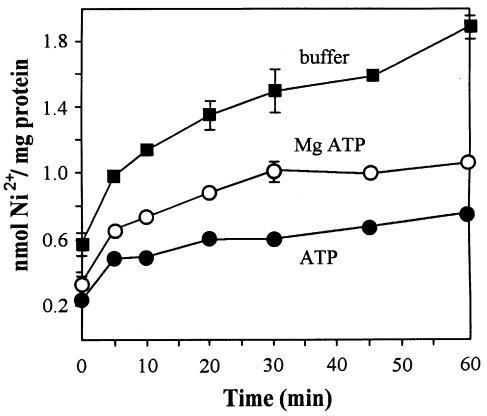

Fig. 3. Association of $^{63}Ni^{2+}$ with MgATP-energized vesicles in the presence or absence of MgATP or ATP

Table 1. The effect of nucleotide triphosphates and PPi in the presence and absence of Mg on $^{63}Ni^{2+}$ association with oat root, tonoplast-enriched vesicles

Nucleotides and PPi	No Addition of Mg^{2+} (nmol Ni^{2+} · (mg protein)$^{-1}$)	Addition of 3 mM Mg^{2+} (nmol Ni^{2+} · (mg protein)$^{-1}$)
ATP (3 mM)	0.24, 0.39	0.46, 0.50
GTP (3 mM)	0.35, 0.32	0.47, 0.46
UTP (3 mM)	0.36, 0.42	0.40, 0.41
PPi (0.5 mM)	0.51, 0.58	0.44, 0.45
None	0.52, 0.56	–

having high activity with GTP as well as ATP. We monitored $^{63}Ni^{2+}$ association with vesicles as affected by various nucleotide triphosphates and PPi to test the possibility that a pump with unusual energization was involved. As shown in Table 1, similar $^{63}Ni^{2+}$ association occurred in the presence of ATP, GTP, UTP and PPi. The PPi and no-addition conditions gave comparable values that were higher than values obtained with nucleotide triphosphates. Table 1 data support the conclusion reached from Fig. 3 data, namely that nucleotide triphosphates complex $^{63}Ni^{2+}$ under the conditions tested. Lower $^{63}Ni^{2+}$ association in the presence of MgPPi than PPi alone may be due to competition of Mg (3 mM) for Ni^{2+} (10 μM).

Relative uptake/association of Ni, Ca and Cd. The level of Ca^{2+} and Cd^{2+} transport, and Ni^{2+} association were compared in MgATP-energized vesicles under conditions of exposure to 10 μM metal (Fig. 4). Nickel association (after 10 min) with vesicles was about 170 times and about 70 times less, respectively, than that of Ca^{2+} and Cd^{2+} accumulated. On a vesicle-volume basis (assuming 10 μl · mg^{-1} vesicle protein), we calculate the accumulation of Ca, Cd and Ni to be 4, 4 and 0.03 nmol metal · μl^{-1}. Thus, we observed a low rate of Ni^{2+} association with oat root tonoplast vesicles compared with Ca^{2+} and Cd^{2+} transport. We note that in preliminary experiments with putative tonoplast vesicles prepared from leaf-derived suspension cells of *Alyssum murale* (an Ni hyperaccumulator), Ni association (protein basis) was about five- to sixfold higher than that observed with oat root vesicles under the same conditions. Suspension cells were prepared using standard methods and putative tonoplast vesicles were obtained following procedures used for oat roots. Since it is difficult to prepare sufficient amounts of vesicles from these slow-growing cells for continuous experimentation, experiments using this system are proceeding more slowly. Further study is needed to determine if vacuolar association of Ni in *A. murale* is correlated with Ni tolerance in this plant.

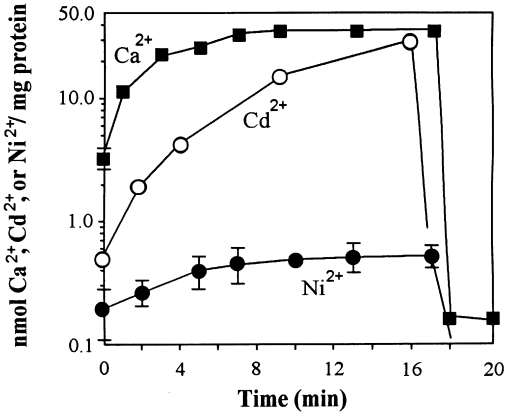

Fig. 4. Relative accumulation of Ca^{2+} and Cd^{2+} versus association of Ni^{2+} with time in MgATP-energized vesicles. At about 16 min, 0.03% (v/v) Triton X-100 was added

Distribution of Ni, Cd and Ca between membrane and sap of vesicles. To assess if $^{63}Ni^{2+}$ associated with oat root vesicles was in the vesicle sap or in the tonoplast, various membrane perturbants and treatments were used with MgATP-energized vesicles. The same conditions and agents were used in parallel experiments to assess the sap versus membrane location of $^{45}Ca^{2+}$ and $^{109}Cd^{2+}$ accumulated in vesicles (Table 2). The divalent ion chelator EDTA at 3 mM had little effect after accumulation or association of all three ions. This result indicates that Ni^{2+} (like Ca^{2+} and Cd^{2+}) is not simply associated with the outside surfaces of vesicles. We note that when vesicles having associated $^{63}Ni^{2+}$ were washed with 1 mM unlabeled $NiCl_2$, no loss of $^{63}Ni^{2+}$ occurred, again indicating that $^{63}Ni^{2+}$ was not easily exchanged from the surface of vesicles. Triton X-100 is often used (commonly at about 0.03%, v/v) to purportedly leak sap ions in vesicle filtration studies such as those reported on here. We found that 0.03% Triton X-100 reduced vesicle Ca^{2+} and Cd^{2+} to low levels while $^{63}Ni^{2+}$ association was reduced to only 41% of control levels. Addition of 0.1% Triton X-100 resulted in complete loss of associated Ni. Dimethyl sulfoxide has been used to permeabilize plant cells (Delmer 1979) so we tested this agent. At 5% v/v, 66% of $^{63}Ni^{2+}$ was retained while retention of $^{45}Cd^{2+}$ was 28 and 44% (two experiments) and retention of ^{45}Ca was 9 and 14% (two experiments). With 5% v/v propanol, retentions of $^{63}Ni^{2+}$, $^{45}Cd^{2+}$ and $^{109}Cd^{++}$ were 90, 83 and 12%, respectively.

The physical treatments, plasmolysis (Ortiz et al. 1995), osmotic shock, and boiling prior to filtration yielded interesting results. While $^{109}Cd^{2+}$ and especially $^{45}Ca^{2+}$ were largely lost from vesicles, $^{63}Ni^{2+}$ was substantially retained, showing its tight association with the membrane. In the case of osmotic shock, increased membrane $^{63}Ni^{2+}$ above the control level may be due to exposure of additional Ni^{2+}-binding sites upon exposure of the inside surface of the membrane, or alteration of membrane ligands by this treatment.

Of the pore-formers tested – Filipin (Milhaud 1992), Hemolysin, Streptolysin O (Ahnert-Hilger et al. 1989)

Table 2. Use of various membrane perturbants to assess vesicle sap versus membrane association of Ni^{2+} versus Ca^{2+} and Cd^{2+} in MgATP-Energized oat root, tonoplast-enriched vesicles; n.d. = not determined

Treatment	Incubation time (min)	Incubation temperature (°C)		Metal retention with vesicles (%)		
		Ni	Cd, Ca	Ni	Cd	Ca
EDTA (3 mM)	5	23	23	96	97, 100	87, 90
0.01% Triton	5	23		93	n.d.	n.d.
0.03% Triton	5	23	23	41	5	−5
0.1% Triton	5	23		−7	n.d.	n.d.
0.5% DMSO	5	23		74	n.d.	n.d.
5% DMSO	5	23	23	66	28, 44	9, 14
0.5% Propanol	5	23		93	n.d.	n.d.
5% Propanol	5	23	23	90	83	12
Plasmolysis	5	23	23	78	33	9
Osmotic shock	5	23	23	131, 130	23	8
Boiling	15/25	100		93	−35	−3.3
100 μM Filipin	30	23		75	n.d.	n.d.
500 μM Filipin	30	23	15	46	43	28
100 μM Hemolysin	30	23		96	n.d.	n.d.
500 μM Hemolysin	30	23	15	94, 103	87	46
100 μM Streptolysin	30	23		91, 103	n.d.	n.d.
500 μM Streptolysin	30	23	15	99, 105	113	96
25 μM Alamethicin	10	23		83	n.d.	n.d.
25 μM Alamethicin	20	23		90	n.d.	n.d.
100 μM Alamethicin	5		23	n.d.	−4, 2.2	n.d.
100 μM Alamethicin	20/25	23	15	50	−16	−5
100 μM Glyceollin I	5	23		100	n.d.	n.d.
500 μM Glyceollin I	5	23	23	100	105, 111	93, 96
2 μM A-23187	10	23		87	n.d.	n.d.
2 μM A-23187	30	23		164	n.d.	n.d.
25 μM A-23187	10/5	23	23	394	88, 111	23, 35
25 μM A-23187	30/25	23	15	618	156	18

and Alamethicin (Woolley and Wallace 1992) – only the last (after prolonged exposure to 100 μM Alamethicin) was interesting in that it resulted in essentially total loss of $^{45}Ca^{2+}$ and $^{109}Cd^{2+}$ but only 50% loss of $^{63}Ni^{2+}$. The phytoalexin Glyceollin II has been reported to cause proton leakage from tonoplast and plasma-membrane vesicles (Giannini et al. 1991). At 500 μM, it had little effect on $^{63}Ni^{2+}$, $^{45}Ca^{2+}$ or $^{109}Cd^{2+}$ release. Negative values in Table 2 result from subtracting metal associated with vesicles prior to addition of Mg to activate the MgATPase.

The Ca^{2+} ionophore A-23187 is also an Ni^{2+} chelator (Pfeiffer and Lardy 1976). At 25 μM and after 5–30 min exposure to A23187, $^{45}Ca^{2+}$ was largely depleted while $^{109}Cd^{2+}$ was either retained (5–10 min exposure), or somewhat increased after longer exposure. In contrast, $^{63}Ni^{2+}$ association increased substantially with time. We interpret this result to indicate that A-23187 chelates Ni (and Cd) and the complexes accumulate, increasing membrane Ni^{2+} or Cd content.

Overall, the results of treatments described in Table 2 indicate that Ni^{2+} association with vesicles prepared and assayed as described here was due substantially to metal bound in or on the membrane in a manner that is not easily exchanged. This is in contrast to Ca^{2+} and Cd^{2+} that are antiported into the vesicle sap and are largely soluble there. Further study is needed to explain instances of higher retention of Cd^{2+} than Ca^{2+}, as in the case of propanol treatment, and to extend these somewhat preliminary findings.

We investigated further the effect of Triton X-100 on Ni^{2+} retention because this agent appeared to show critical concentration-dependent depletion of Ni, like no other treatment or agent tested. Figure 5 shows the effects of increasing Triton X-100 concentration on Ni^{2+}, ^{3}H from [^{3}H]choline-labeled tonoplast membranes, and light scattering. Nickel ions and ^{3}H were reduced to 50 and 30%, respectively, by 0.03% Triton X-100, a detergent concentration often used as a control to define vesicle sap location and to deplete sap ions. Substantial loss of ^{3}H indicates partial dissolution of

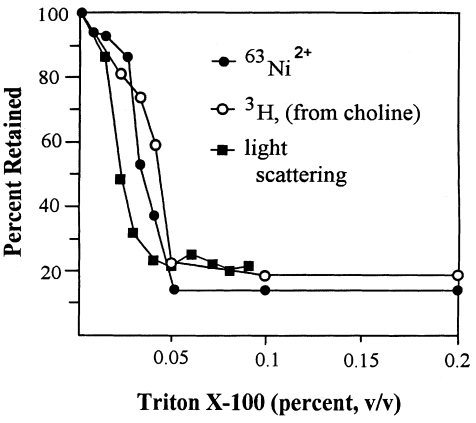

Fig. 5. Triton X-100 concentration-dependent reduction of filterable $^{63}Ni^{2+}$(-●-), ^{3}H from [^{3}H]choline membrane labeling (-○-), and light scattering (A_{550}; -■-)

vesicle membrane under these conditions. Similar loss of Ni^{2+} at a similar level of Triton X-100 supports the conclusion that Ni^{2+} is tightly associated with the vesicle membrane. Also, as shown in Fig. 5, light scattering (as a measure of vesicle integrity) was lost at low Triton X-100 concentrations. We conclude that the apparent depletion of Ni^{2+} by 0.1% Triton X-100 (Table 2) was due to dissolution of the membrane and that no evidence exists for substantial sap location of associated Ni^{2+} under the conditions of the assay used.

In summary, oat root tonoplast vesicles capable of transporting Ca^{2+} and Cd^{2+} by a $2H^+$/ion antiport mechanism do not show an H^+/Ni^{2+} antiport activity even above 1600 μM Ni. Nickel is, however, associated with vesicles after incubation in the presence or absence of a proton gradient, nucleotide triphosphates, PPi or protonophores, and Ni^{2+} association is not nucleotide dependent or specific, and does not readily reach apparent saturation. The level of Ni^{2+} association relative to that of Ca^{2+} and Cd^{2+} in the presence of a proton gradient was low. While Ca and Cd were apparently located largely in the vesicle sap, Ni^{2+} was not. Results are consistent with only a low-level vacuolar association of Ni^{2+} with oat root tonoplast vesicles by an as yet unknown mechanism.

Our results indicate that oat root vacuoles do not actively accumulate Ni and are therefore not likely to be a major compartment for Ni accumulation in this tissue. While our approach to the question of the subcellular location of Ni in oat roots was, by necessity indirect, our results are consistent with the findings of Brune et al. (1995) who concluded that the vacuole is not a major Ni-accumulating compartment in barley leaves, another Ni non-hyperaccumulator plant.

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