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Analysis of $^{13}NH_4^+$ Efflux in Spruce Roots[1]

A Test Case for Phase Identification in Compartmental Analysis

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$^{13}NH_4^+$-efflux analyses were conducted with roots of intact *Picea glauca* (Moench) Voss. seedlings at external NH_4^+ concentrations of 100 μM and 1.5 mM. Three kinetically distinct phases were identified with half-lives of exchange of approximately 2 s, 30 s, and 14 min. The presumed identities of the subcellular compartments corresponding to these phases were confirmed by several techniques, including pretreatment of roots (a) at 75°C or with SDS, (b) with α-keto-glutarate or L-methionine-DL-sulfoximine, (c) at elevated levels of Ca^{2+}, and (d) at low pH or with Al^{3+} at low pH. Treatments a and b selectively influenced phase III without affecting phases I and II. Similarly, treatment c selectively perturbed phase II, and treatment d affected phases II and III. Based on these findings and the assumption of an in-series arrangement of root cell compartments, it was concluded that phase III corresponded to the cytoplasm, phase II corresponded to the Donnan free space, and phase I corresponded to a film of solution adhering to the root surface.

Efflux analysis is widely used to determine unidirectional ion fluxes, kinetic exchange constants of subcellular compartments, and ionic concentrations within compartments. In plants, compartmental analyses have been undertaken for a variety of ions, including Na^+, K^+, Mg^{2+}, Ca^{2+}, NH_4^+, NO_3^-, Pi, SO_4^{2-}, Cl^-, and Br^- (for refs., see Wang, 1994). The majority of efflux studies have been limited to nonmetabolized ions and were performed usually on excised tissues or suspension-culture systems, mainly because of the (presumed) absence of complicating factors such as metabolism and long-distance transport to the shoot (Pitman, 1963; Cram, 1968; Poole, 1971a, 1971b; Macklon, 1975a, 1975b; Macklon and Sim, 1976, 1981; Pfrüner and Bentrup, 1978; Macklon et al., 1990). For use with intact plant material, a detailed treatise on parameter extraction was presented by Jeschke and Jambor (1981) and Jeschke (1982). More recently, compartmental analysis has also been applied to metabolized ions, including SO_4^{2-} (Thoiron et al., 1981; Cram, 1983; Bell et al., 1994), Pi (Lefebvre and Clarkson, 1984; Macklon and Sim, 1992), NO_3^- (Presland and McNaughton, 1984; Lee and Clarkson, 1986; Macklon et al., 1990; Siddiqi et al., 1991; Devienne et al., 1994; Kronzucker et al., 1995a, 1995b), and NH_4^+ (Presland and McNaughton, 1986; Cooper et al., 1989; Macklon et al., 1990; Wang et al., 1993a; Kronzucker et al., 1995c).

Despite this widespread use of the technique, workers have typically neglected to conduct physiological tests to verify the subcellular identities of the phases revealed in efflux data. As a consequence of this omission, the assignment of particular kinetically defined phases to their corresponding subcellular compartments has not always been unequivocal (Macklon et al., 1990). Only in studies by Cram (1968), Lee and Clarkson (1986), and Siddiqi et al. (1991), as well as in a previous study of NO_3^- exchange in spruce (Kronzucker et al., 1995a), was the assignment of compartments substantiated. Usually phase assignment has been based on the assumption of an in-series arrangement of cell compartments, i.e. cell wall, cytoplasm, and vacuole (Pitman, 1963; Cram, 1968, 1975). Thus, the first (rapidly exchanging) phase has been assumed to represent the cell wall and the last (slowest exchanging) phase has been assumed to represent the vacuole. However, the derivation of flux components as well as of pool sizes from efflux data is valid only if subcellular compartments are assigned correctly to their corresponding efflux phases.

We have used a combination of strategies to analyze the efflux reported in the present study to distinguish between membrane-bound and metabolically dependent (intracellular) compartments and those that are nonmembrane bound and apparently independent of metabolism (extracellular). As a model system, we have used intact seedlings of white spruce (*Picea glauca* [Moench]), since detailed studies of both NO_3^- and NH_4^+ exchange have been performed previously in the same species using the same technique (Kronzucker et al., 1995a, 1995b, 1995c). As in these earlier studies, the tracer ^{13}N was used, since its low detection limits allow for excellent time resolution in short-duration ef-

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Abbreviations: α-KG, α-keto-glutarate; $[x]_o$, concentration of ion x in solution (not necessarily equivalent to the chemical activity); MSO, L-Met-DL-sulfoximine; $[NH_4^+]_{cyt}$, NH_4^+ concentration in the cytoplasm; $[NH_4^+]_{free\ space}$, NH_4^+ concentration in the Donnan free space; $[NH_4^+]_o$, NH_4^+ concentration in the external solution; ϕ, ionic flux; ϕ_{co}, efflux from the cytoplasm; ϕ_{net}, net flux; ϕ_{oc}, unidirectional influx; $\phi_{vac./ass.}$, combined fluxes to ammonium assimilation and to the vacuole; ϕ_{xylem}, flux of ^{13}N to the shoot; $t_{1/2}$, half-life of exchange.

flux experiments (Siddiqi et al., 1991; Wang et al., 1993a; Kronzucker et al., 1995b). We believe the combined use of perturbational and nonperturbational treatments in the present study provides good evidence that the compartments seen in this type of efflux analysis are a film of solution adhering to the root surface (I), the adsorptive component of the cell wall (the Donnan free space) (II), and the cytoplasm (III).

MATERIALS AND METHODS

Plant Culture

Several-month-old seedlings of white spruce (*Picea glauca* [Moench] Voss., provenance 29170, from the Prince George region in British Columbia, Canada) were used. Seedlings were grown for a minimum of 3.5 months in a peat:perlite (3:1) mixture in Styrofoam boxes in an outdoor nursery located on the University of British Columbia campus. Seedlings were then transported indoors and, after gentle removal of the rooting medium, transferred to hydroponic culture in 24-L Plexiglas tanks. The tanks contained one-tenth-strength N-free Johnson's solution mixed with analytical grade chemicals in distilled, deionized water (for a detailed description of growth conditions and for exact solution composition, see Kronzucker et al. [1995b]). Seedling roots maintained in hydroponic solution were nonmycorrhizal, as determined by microscopic examination. Prior to experiments, the seedlings were maintained in the tanks for a period of 3 weeks. NH_4^+ was added as $(NH_4)_2SO_4$ at the desired concentration (i.e. 100 μM or 1.5 mM) 4 d prior to efflux analyses to provide steady-state conditions with regard to NH_4^+. Steady-state conditions were also given for all other nutrient concentrations throughout experiments (i.e. throughout growth, pretreatment, preloading, loading, and elution). To buffer against acidification caused by plant NH_4^+ uptake, powdered $CaCO_3$ was added to the tanks (pH was kept constant at approximately 6.5).

In high-temperature perturbation experiments, plant roots were pretreated at 75°C for 20 min prior to loading. In experiments in which SDS was used, roots were immersed for 20 to 30 min in solutions containing 1% (w/v) SDS before the onset of the efflux experiment. In experiments in which α-KG and MSO were used, roots were pretreated with 1 mM α-KG or MSO for 6 h prior to the experiments; α-KG and MSO were also provided during loading and elution. In cation-variation experiments, $[H^+]_o$ was increased by decreasing pH to 3.6 with H_2SO_4, $[Al^{3+}]_o$ was altered by adding $Al_2(SO_4)_3$ (at pH 3.6), and $[Ca^{2+}]_o$ was modified with $CaSO_4$. Roots were pretreated for 30 min with the respective cations added to the solutions and were also exposed during loading and elution.

All seedlings were maintained in a 16-h/8-h photoperiod, 70% RH, and at 20 ± 2°C. Light was provided by fluorescent tubes with a spectral composition similar to sunlight. Photon flux was approximately 250 μmol m^{-2} s^{-1} at plant level.

Production of $^{13}NH_4^+$

^{13}N ($t_{1/2}$ = 9.96 min) was produced by proton irradiation of H_2O (Meeks, 1993) at the Tri-University Meson Facility cyclotron on the University of British Columbia campus in Vancouver, Canada. $^{13}NO_3^-$ generated in this irradiation procedure (Kronzucker et al., 1995b) was chemically converted to $^{13}NH_4^+$. The chemical purification and conversion procedure was a modification of that of Meeks et al. (1978) and Wang et al. (1993a) and was described in detail by Kronzucker et al. (1995c).

Efflux Analysis

Efflux experiments were performed essentially as described elsewhere (Kronzucker et al., 1995b). In brief, roots of intact spruce seedlings were equilibrated in nonlabeled preloading solution, the chemical composition of which was identical with the loading solution. Preloading was for 60 min in 1-L darkened plastic beakers when $[Ca^{2+}]_o$, $[Al^{3+}]_o$, or pH was varied and for 6 h in 4-L vessels in MSO and α-KG experiments. The respective controls were pretreated for the same periods. Steady-state conditions with regard to all nutrients and pretreatment agents (i.e. Ca^{2+}, Al^{3+}, pH, MSO, α-KG) were maintained throughout preloading, loading, and elution. After preloading, plants were transferred for 60 min to loading solution containing $^{13}NH_4^+$. Based on our preliminary experiments, which revealed that the half-life of the (presumed) cytoplasmic compartment was approximately 14 min, a 60-min loading period should bring the cytoplasmic specific activity to ≥94% that of the loading solution. Seedlings were then transferred to "efflux funnels" and the roots were eluted with 60- to 100-mL aliquots of nonradioactive solution after various time intervals. These intervals ranged from 5 s to 2 min, over an experimental duration of 22 min. Eluates from a total of 25 intervals were collected separately, and the radioactivities of 20-mL subsamples from each eluate were determined in a Packard (Meridan, CT) gamma-counter (Minaxi δ, Auto-γ 5000 series). After the final elution, roots and shoots were excised, introduced into scintillation vials, and also counted for γ-activity.

Treatment of Data

Treatment of efflux data was as described by Siddiqi et al. (1991) and was based on the theoretical considerations of Lee and Clarkson (1986) as pertaining to efflux analysis of metabolized ions. All experiments were performed using two replicates and were repeated at least three times. The regression procedure used to determine kinetically distinct phases in these efflux plots was as described by Siddiqi et al. (1991). Student's t testing (for comparison of two lines) and multiple range testing according to Newman-Keuls (for comparison of more than two lines) were used to examine statistical differences in slopes and y intercepts of regression lines between separate experiments, which were plotted together in overlay graphs. Representative experiments were chosen for the semilogarithmic plots of the rate of release of ^{13}N versus time of elution. All data displayed in tables represent the means of several

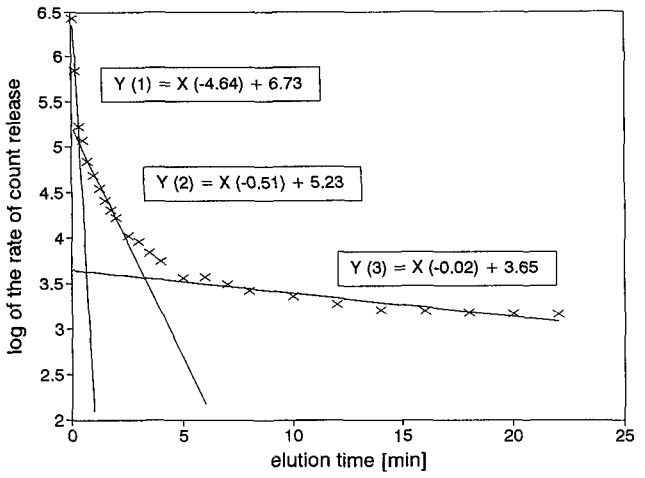

Figure 1. Representative semilogarithmic plot of the rate of release of ^{13}N [log (cpm released) g^{-1} min^{-1}] versus time of elution in roots of intact white spruce seedlings. Seedlings were maintained at 1.5 mM $[NH_4^+]_o$. The plot includes linear regression lines and equations for the three phases resolved in efflux analysis.

experiments (±SE, as indicated). Calculations of fluxes and compartmental NH_4^+ concentrations were as described for NO_3^- by Siddiqi et al. (1991). All fluxes are expressed in μmol NH_4^+ g^{-1} (root fresh weight) h^{-1}. Fluxes were obtained as follows: ϕ_{co} was obtained from the rate of ^{13}N release from the cytoplasm at time zero divided by the specific activity of the loading solution; ϕ_{net} was obtained directly from the accumulation of ^{13}N in the plants at the end of the elution period; ϕ_{oc} was calculated from $\phi_{net} + \phi_{co}$; ϕ_{xylem} was obtained directly from count accumulation in the shoot at the end of the elution period; and $\phi_{vac./ass.}$ was calculated as $\phi_{net} - \phi_{xylem}$. $[NH_4^+]_{cyt}$ was obtained from the quotient of the rate of $^{13}NH_4^+$ release divided by 5 times the $t_{1/2}$ of the cytoplasm and the proportion of efflux with respect to other fluxes removing $^{13}NH_4^+$ from the cytoplasm (Kronzucker et al., 1995b). The assumption was that the average tissue volume occupied by the cytoplasm was 5%. Similarly, the $[NH_4^+]_{free\ space}$ was calculated assuming that 10% of the average tissue volume was occupied by that compartment (Lee and Clarkson, 1986; Siddiqi et al., 1991). Since an independent determination of the exact respective tissue volumes has not been carried out in our system and since tissue heterogeneity in several-month-old conifer roots may be somewhat greater than in other more commonly used plant systems (Rüdinger et al., 1994; McKenzie and Peterson 1995a, 1995b), small errors in our reported estimates of $[NH_4^+]_{cyt}$ and $[NH_4^+]_{free\ space}$ cannot be ruled out (see also "Discussion"). However, since, especially for comparative purposes, concentration values are more meaningful than tissue contents (e.g. in μmol g^{-1}), the former expression was chosen over the latter.

RESULTS

Phase Regression and $t_{1/2}$

Three kinetically distinct efflux phases were distinguishable by linear regression of semilogarithmic plots of the ^{13}N efflux rate versus elution time. Figure 1 shows a representative plot obtained with intact spruce seedlings at 1.5 mM $[NH_4^+]_o$. The plot includes regression lines (r^2 = 0.91–0.99) for the three phases. Values of $t_{1/2}$ for these phases were determined from the slopes of the regression lines after data transformation to a natural logarithmic scale. Mean $t_{1/2}$ values were 2.4 s (phase I), 26.7 s (phase II), and 14.7 min (phase III). No significant difference was found in these $t_{1/2}$ values for seedlings grown at 100 μM or 1.5 mM $[NH_4^+]_o$ or after pretreatment with MSO, α-KG, or different levels of $[Ca^{2+}]_o$ or pH (Tables I–III). However, a significant decline in $t_{1/2}$ values, by as much as 60 to 80%, was noted for phase III following the treatments with SDS, 75°C (Table I), and Al^{3+} (Table III).

Flux Estimations

For seedlings grown and measured at 100 μM $[NH_4^+]_o$, ϕ_{oc} was determined to be approximately 1.9 μmol g^{-1} h^{-1} (Table IV) by compartmental analysis. Efflux of NH_4^+ from root tissue under those conditions was close to 25% of influx (see ϕ_{co}). No more than 3% of the total NH_4^+ taken up by the plants was translocated to the shoot within the time frame of the experiments (see ϕ_{xylem}), whereas as much as 97% of the incoming NH_4^+ was either channeled into metabolism or sequestered in the vacuole ($\phi_{vac./ass.}$). The denaturing treatment of plant roots at 75°C and with solutions containing 1% SDS led to substantial decreases in all parameters except ϕ_{co}, which was apparently unaffected by these treatments (Table IV). Exposure of seedling

Table I. *$t_{1/2}$ values for NH_4^+ of phases I, II, and III (assumed to represent surface film, Donnan free space, and cytoplasm, respectively) in roots of spruce seedlings grown at 100 μM $[NH_4^+]_o$ and following various (pre)treatments as indicated*

Data are means ± SE (n = 3–9).

(Pre)treatment	$t_{1/2}$		
	Phase I	Phase II	Phase III
	s	*s*	*min*
100 μM NH_4^+ (control)	2.22 ± 0.38	30.84 ± 2.5	15.08 ± 1.25
100 μM NH_4^+ + 1 mM MSO	2.31 ± 0.55	28.64 ± 2.71	12.72 ± 0.75
100 μM NH_4^+ + 1 mM α-KG	2.05 ± 0.67	24.7 ± 5.7	19.82 ± 4.11
100 μM NH_4^+ + 1% SDS	2.24 ± 0.49	43.73 ± 12.54	6.49 ± 2.78
100 μM NH_4^+ + 75°C	3.34 ± 0.78	29.68 ± 3.09	3.29 ± 2.99

Table II. *$t_{1/2}$ values for NH_4^+ of phases I, II, and III (assumed to represent surface film, Donnan free space, and cytoplasm, respectively) in roots of spruce seedlings grown at 100 μM $[NH_4^+]_o$ and (pre)treated with various $[Ca^{2+}]_o$*

Data are means ± SE (n = 3–9).

(Pre)treatment	$t_{1/2}$		
	Phase I	Phase II	Phase III
	s	*s*	*min*
100 μM NH_4^+ + 50 μM Ca^{2+}	2.26 ± 0.57	22.39 ± 6.6	15.48 ± 0.05
100 μM NH_4^+ + 500 μM Ca^{2+}	1.24 ± 0.83	14.27 ± 3.61	17.71 ± 3.39
100 μM NH_4^+ + 5 mM Ca^{2+}	2.48 ± 0.13	20.18 ± 2.5	15.12 ± 0.43

roots to 1 mM Gln synthetase/glutamate synthase inhibitor MSO for 6 h prior to loading led to a slight depression of ϕ_{oc} (about 20%), whereas ϕ_{co} was enhanced by almost 40%. ϕ_{net}, therefore, was significantly lower than in control plants. ϕ_{xylem} was almost 40 times lower in MSO-pretreated plants than in controls. Treatment of seedling roots with 1 mM α-KG as the carbon source for 6 h caused an even larger (about 40%) depression of ϕ_{oc} than was seen with MSO (Fig. 2). However, in this case, ϕ_{co} was depressed proportionately even more (about 60%) than ϕ_{oc}, which led to a higher ϕ_{oc} to ϕ_{co} ratio than in control plants (5.8 versus 4.1; with MSO treatment this ratio was 2.4). It is interesting that ϕ_{xylem} was also decreased by α-KG treatment, almost 3-fold compared to control plants. By contrast, in statistical analyses of slopes and y intercepts of efflux plots obtained in experiments on seedlings (pre)-treated with various $[Ca^{2+}]_o$, no significant differences were found for transmembrane flux parameters at the 0.05 level of probability (Table V). However, $^{13}NH_4^+$ efflux from the free space to bulk solution and estimates of cell-wall $[NH_4^+]$ were diminished as $[Ca^{2+}]_o$ was increased (Fig. 3).

Plants grown and measured at 1.5 mM $[NH_4^+]_o$ exhibited ϕ_{oc} values of approximately 6.6 μmol g^{-1} h^{-1} (Table VI). Treatment of these plants with 1 mM MSO for 6 h had no significant effect on ϕ_{oc}, but ϕ_{co} increased more than 2-fold (Fig. 4). Consequently, ϕ_{net} was decreased by about 35% with respect to control plants. ϕ_{xylem} was decreased by as much as 27-fold. Even more pronounced reductions in ϕ_{xylem} were observed after seedling roots were treated at pH 3.6 or at 1.5 mM Al^{3+} (which also decreased solution pH to 3.6). These latter treatments also effected substantial reductions in ϕ_{oc} and ϕ_{co}. ϕ_{oc} depression was approximately 86% at pH 3.6 and approximately 91% with Al^{3+} (Table VI). Because of differential effects on ϕ_{co} in the two treatments, ϕ_{net} was depressed to a similar extent in both cases, to a "residual" rate of approximately 0.3 μmol g^{-1} h^{-1}.

Compartmental Concentrations

Assuming 5% tissue volume for the average root cell cytoplasm and 10% for the tissue volume occupied by the cell wall free space (Kronzucker et al., 1995a, 1995b, 1995c), NH_4^+ concentrations for these two compartments were calculated from the $^{13}NH_4^+$ contents in phases II and III (Tables VII–IX). $[NH_4^+]_{cyt}$ for spruce seedlings grown at 100 μM $[NH_4^+]_o$ was approximately 13 mM, and $[NH_4^+]_{free\ space}$ was approximately 1.2 mM. It is interesting that (pre)treatment of seedling roots with either MSO or α-KG led to a decrease in $[NH_4^+]_{cyt}$ of about 30% (Fig. 2), whereas $[NH_4^+]_{free\ space}$ was not altered significantly (Table VII). Addition of 1% SDS or pretreatment at 75°C dramatically reduced the $^{13}NH_4^+$ effluxing from the presumed "cytoplasm" to 12% of control values in SDS-treated roots and as little as 7% after high-temperature treatment. By contrast, $[NH_4^+]_{free\ space}$ was not changed by these manipulations (Table VII). Increasing $[Ca^{2+}]_o$ had no significant effect on $[NH_4^+]_{cyt}$ but reduced estimated $[NH_4^+]_{free\ space}$ (Fig. 3) by approximately 30% when $[Ca^{2+}]_o$ was increased from 50 to 500 μM and by almost 80% at 5 mM $[Ca^{2+}]_o$ with respect to the control at 50 μM (Table VIII).

Plants grown at 1.5 mM $[NH_4+]_o$ accumulated NH_4^+ to levels of approximately 35 mM in the cytoplasm and about 8.8 mM in the free space (Table IX). An approximate 40% increase in $[NH_4^+]_{cyt}$ was observed in these plants after exposure of roots to 1 mM MSO for 6 h (Fig. 4). $[NH_4^+]_{free\ space}$ appeared to be slightly enhanced, but this increase was statistically insignificant. Lowering solution

Table III. *$t_{1/2}$ value for NH_4^+ of phases I, II, and III (assumed to represent surface film, Donnan free space, and cytoplasm, respectively) in roots of spruce seedlings grown at 1.5 mM $[NH_4^+]_o$ and following various (pre)treatments as indicated*

Data are means ± SE (n = 3–9).

(Pre)treatment	$t_{1/2}$		
	Phase I	Phase II	Phase III
	s	*s*	*min*
1.5 mM NH_4^+ (control)	2.66 ± 0.08	29.78 ± 0.19	10.92 ± 1.67
1.5 mM NH_4^+ + 1 mM MSO	3.1 ± 0.65	29.44 ± 2.79	14.65 ± 2.16
1.5 mM NH_4^+ + pH 3.6	2.31 ± 0.11	23.14 ± 12.54	10.42 ± 2.35
1.5 mM NH_4^+ + 1.5 mM Al^{3+}	1.99 ± 0.94	23.01 ± 5.7	6.19 ± 1.17

Table IV. *NH_4^+ fluxes as estimated from compartmental analysis*

Plants were maintained and measured at 100 μM $[NH_4^+]_o$. (Pre)treatments were as indicated. Data are means ± SE (n = 3–9).

(Pre)treatment	NH_4^+ Flux				
	ϕ_{oc}	ϕ_{co}	ϕ_{net}	$\phi_{vac./ass.}$	ϕ_{xylem}[a]
			μmol g^{-1} h^{-1}		
Control (100 μM)	1.89 ± 0.14	0.46 ± 0.05	1.42 ± 0.14	1.38 ± 0.13	44.8 ± 17.9
+ MSO	1.51 ± 0.11	0.64 ± 0.07	0.87 ± 0.11	0.87 ± 0.11	1.2 ± 0.3
+ α-KG	1.11 ± 0.12	0.19 ± 0.03	0.91 ± 0.09	0.9 ± 0.09	15.6 ± 3.1
+ SDS	0.51 ± 0.19	0.42 ± 0.06	0.09 ± 0.01	0.08 ± 0.01	9.7 ± 2.1
+ 75°C	0.59 ± 0.22	0.5 ± 0.06	0.09 ± 0.01	0.08 ± 0.01	10.1 ± 2.6

[a] ϕ_{xylem} is expressed in nmol g^{-1} h^{-1}.

pH from 6.5 to 3.6 decreased $[NH_4^+]_{cyt}$ by 92% and reduced free-space NH_4^+ binding by approximately 30% (Table IX). This presumed free-space binding was decreased even further (to less than one-fourth of the control value) by addition of 1.5 mM Al^{3+} to pretreatment and loading solutions. The Al^{3+} treatment decreased $[NH_4^+]_{cyt}$ to less than 2 mM (Table IX).

DISCUSSION

It is well recognized that data extraction from efflux analysis can have serious shortcomings (Cheeseman, 1986; Zierler, 1981). In addition to prerequisites that are common to all systems that have been explored by this methodology (see below), the conifer roots that we have used in the present study are characterized by somewhat greater tissue diversity than is typical of, for example, young cereal roots (Rüdinger et al., 1994; Kronzucker et al., 1995d; McKenzie and Peterson, 1995a, 1995b). In studies using single-celled organisms, the ideal situation is met and the parameters obtained from analysis represent means for relatively homogeneous cells. By contrast, when used to analyze compartmental characteristics in complex organs such as roots, derived parameters must reflect the means of several cell types. Yet, if time constants for efflux differed substantially among cell types, efflux curves would be expected to reflect this heterogeneity. A failure to distinguish additional phases in our analyses leads to the conclusion that time constants do not differ significantly among cell types (see also point e below) but only among the in-series compartments discussed. Estimates of kinetic constants and tissue concentrations should therefore be taken as representing average values for the system under study. In addition, the following prerequisites for efflux analysis should be satisfied universally: (a) The tissue must be at steady state for the duration of the experiment. (b) The specific activity of the tracer in the subcellular compartment(s) to be investigated must be the same as or very close to that of the outside solution prior to the onset of elution; loading with tracer must therefore occur for at least 4 to 5 times the $t_{1/2}$ values of the respective compartment(s) (Cram, 1968). (c) The tracer for the ion under study must be taken up at the same rate as the nonlabeled ion (i.e. isotope discrimination at the uptake step, as known for several tracers, must be taken into account; West and Pitman, 1967; Jacoby, 1975; Behl and Jeschke, 1982). (d) Incoming tracer must be well mixed in the compartment(s) under study to make calculations of compartmental concentrations possible. (e) If several compartments are under study, these must be arranged in series (Pitman, 1963; Cram, 1968, 1975), and their $t_{1/2}$ values must be sufficiently different to allow resolution by linear regression of a semilogarithmic efflux plot (Cheeseman, 1986). (f) If metabolized ions are to be investigated, metabolism needs to be taken into account, either by subtraction of the metabolized fraction when using a standard plot of tissue tracer content versus elution time (Bell et al., 1994) or by plotting the rate of tracer release from the tissue versus elution time (Lee and Clarkson, 1986). (g) The assignment of linear phases in semilogarithmic efflux plots to actual subcellular compartments must be tested.

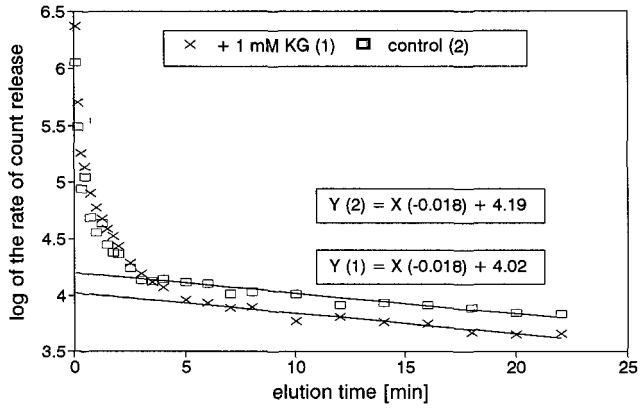

Figure 2. Combined semilogarithmic plots of the rate of release of ^{13}N [log (cpm released) g^{-1} min^{-1}] versus time of elution in roots of intact white spruce seedlings. Seedlings were maintained at 100 μM $[NH_4^+]_o$. Experiment 1 represents plants (pre)treated with 1 mM α-KG for 6 h, and experiment 2 represents control plants. Plots include linear regression lines and equations for the presumed cytoplasmic phases (phase III).

Although most of these issues are usually adequately addressed in efflux studies, the importance of testing for compartment identity (prerequisite g) has been largely ignored, with the exception of a few preliminary attempts (Cram, 1968; Lee and Clarkson, 1986; Siddiqi et al., 1991; Kronzucker et al., 1995b). An untested a priori approach has been taken in most studies, leading, in some cases, to

Table V. *NH_4^+ fluxes as estimated from compartmental analysis*

Plants were maintained and measured at 100 μM $[NH_4^+]_0$ and were (pre)treated at the indicated $[Ca^{2+}]_0$. Data are means ± SE (n = 3–9). See also Table IV.

(Pre)treatment	NH_4^+ Flux				
	ϕ_{oc}	ϕ_{co}	ϕ_{net}	$\phi_{vac./ass.}$	ϕ_{xylem}[a]
			μmol g⁻¹ h⁻¹		
+ 50 μM Ca^{2+}	1.82 ± 0.12	0.33 ± 0.06	1.5 ± 0.12	1.44 ± 0.12	58.3 ± 23.1
+ 500 μM Ca^{2+}	1.64 ± 0.13	0.53 ± 0.06	1.11 ± 0.11	1.08 ± 0.1	32.8 ± 7.2
+ 5 mM Ca^{2+}	1.6 ± 0.33	0.42 ± 0.08	1.18 ± 0.26	1.11 ± 0.24	71.7 ± 12.2

[a] ϕ_{xylem} is expressed in nmol g^{-1} h^{-1}.

considerable discrepancies in the literature concerning parameters derived from efflux analysis (see, for example, the summary of $t_{1/2}$ values for NO_3^- by Devienne et al. [1994] or for NH_4^+ by Wang et al. [1993a]). Yet it is clear that, if any of the derivative flux or pool size calculations are to be valid, a knowledge of compartment identity is imperative. For this reason, the purpose of the present study was to substantiate the assignment of logarithmic phases seen in $^{13}NH_4^+$-efflux data to the corresponding subcellular compartments. As in our earlier study of NH_4^+ exchange in white spruce (Kronzucker et al., 1995c), three kinetically distinct phases were found. These were tentatively assigned to a film of solution adhering to the root surface, to binding sites in the cell wall, and to the cytoplasm (Fig. 1). This interpretation was consistent with earlier studies from this laboratory (Siddiqi et al., 1991; Wang et al., 1993a; Kronzucker et al., 1995a, 1995b).

In the present study, we selected treatments designed to selectively influence NH_4^+ exchange in either the (presumed) cytoplasmic phase or the (presumed) cell-wall phase. For example, pretreating plant roots at 75°C or in 1% (w/v) SDS solution was anticipated to selectively reduce plasma membrane fluxes. The same rationale dictated our choice of MSO and α-KG exposures. In contrast, varying external cation concentrations could be expected to selectively affect cell-wall-exchange properties. These treatments included varying concentrations of Ca^{2+}, H^+, and Al^{3+}. The latter two treatments also affected cytoplasmic parameters. However, together with results from the Ca^{2+} treatments, confirmation of the cation-exchange nature of phase II was possible.

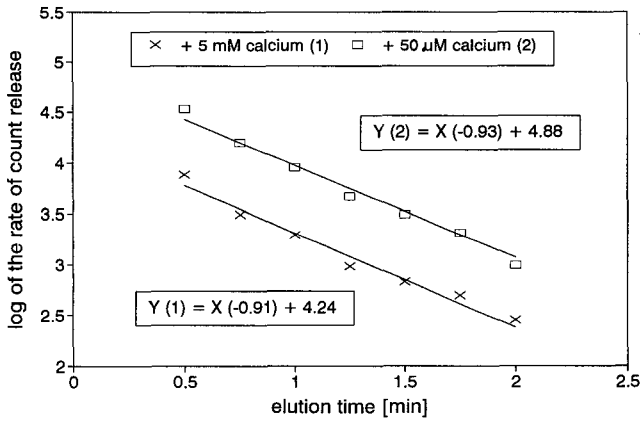

Figure 3. Combined semilogarithmic plots of the rate of release of ^{13}N [log (cpm released) g^{-1} min^{-1}] from phase II versus time of elution in roots of intact white spruce seedlings. Seedlings were maintained at 100 μM $[NH_4^+]_0$. Experiment 1 represents plants (pre)treated at 5 mM $[Ca^{2+}]_0$ for 6 h, and experiment 2 represents plants (pre)treated for 6 h at 50 μM $[Ca^{2+}]_0$. Plots were obtained after subtraction of counts eluting from phase III and include linear regression lines and equations for the presumed Donnan free space (phase II). Phase I is not included.

High-temperature pretreatment of plant roots and treatment with SDS have been previously used by Siddiqi et al. (1991) and by Kronzucker et al. (1995b) in $^{13}NO_3^-$-efflux studies of barley and spruce, respectively. In both of these studies a 22-min elution time was used, and a substantial decrease of ^{13}N release from phase III was reported; phases I and II remained unaffected. Since high-temperature and detergent treatments disrupt the lipid bilayer of the plasma membrane and denature or solubilize membrane proteins, respectively, it was concluded that phase III probably represented the cytoplasm. Similarly, in an early study of Cl^- exchange in carrot root tissue, Cram (1968) used chloroform-killing of cells to distinguish between "intracellular" and "extracellular" binding. In our study of NO_3^- exchange in spruce (Kronzucker et al., 1995b), we also treated seedlings with H_2O_2 and 2-chloro-ethanol to perturb a membrane-bound and metabolically dependent compartment. The results were in agreement with the assumption that phase III corresponded to the cytoplasm and that therefore, by elimination, phases I and II represented extracellular binding.

In our present study of NH_4^+ in spruce, we found a similar reduction in ^{13}N exchange with phase III following applications of high temperature or SDS and yet no effect on phases I and II. These results are consistent with a cytoplasmic identity for compartment III. More subtle explorations of the identity of compartment III were achieved by exposures to MSO and α-KG. MSO is a known inhibitor of Gln synthetase (Wedler and Horn, 1976; Meister, 1981; Monselise and Kost, 1993), the primary NH_4^+-assimilating enzyme located in the cytoplasm. Significant increases in cytoplasmic NH_4^+ concentrations (up to 90 mM) after MSO pretreatment in root cells of other plant systems have been observed by NMR (Lee and Ratcliffe, 1991) and inferred from labeling kinetics (Fentem et al., 1983a, 1983b). It has also been determined that MSO does not influence exchange characteristics with the cell wall (Lee and Ayling, 1993). Figure 4 illustrates that the effect of a 6-h MSO pretreatment of seedling roots was limited to phase III.

Table VI. *NH_4^+ fluxes as estimated from compartmental analysis*

Plants were maintained and measured at 1.5 mM $[NH_4^+]_o$. (Pre)treatments were as indicated (see also Table III). Data are means ± SE (n = 3–9). See also Table IV.

(Pre)treatment	NH_4^+ Flux				
	ϕ_{oc}	ϕ_{co}	ϕ_{net}	$\phi_{vac./ass.}$	ϕ_{xylem}[a]
			μmol g⁻¹ h⁻¹		
Control (1.5 mM)	6.63 ± 0.34	1.82 ± 0.08	4.81 ± 0.26	4.56 ± 0.26	246.4 ± 0.9
+ MSO	6.99 ± 0.15	3.84 ± 0.18	3.15 ± 0.2	3.14 ± 0.2	9.01 ± 2.8
+ pH 3.6	0.93 ± 0.27	0.62 ± 0.13	0.31 ± 0.04	0.3 ± 0.04	2.03 ± 0.8
+ Al^{3+}	0.58 ± 0.13	0.25 ± 0.1	0.33 ± 0.001	0.32 ± 0.002	1.6 ± 0.2

[a] ϕ_{xylem} is expressed in nmol g^{-1} h^{-1}.

However, whereas at 1.5 mM $[NH_4^+]_o$ an expected increase in $[NH_4^+]_{cyt}$ was observed after exposure to MSO (Table IX), $[NH_4^+]_{cyt}$ actually decreased by approximately 30% (Table VII) at 100 μM $[NH_4^+]_o$.

We explain this apparent discrepancy by a discrete effect of MSO not only at the enzyme level but also at the level of plasma membrane NH_4^+ transport in our plant system. Given the chemically analogous structure of the MSO molecule to that of Gln, a "recognition" of MSO as a Gln analog by either influx or efflux transport proteins is conceivable. Although an effect consistent with this assumption has normally not been observed (Lee and Ayling, 1993), both depression of NH_4^+ influx and enhancement of NH_4^+ efflux, apparently caused by direct MSO effects on NH_4^+ transport, have been documented in *Sorghum bicolor* L. (Feng et al., 1994). At the enzyme level, it is known that MSO can act as an inhibitor not only of Gln synthetase but also of glutamate synthase (Gauthier, 1983; Takashi et al., 1983) as well as Asn synthetase (Monselise and Kost, 1993).

Our results (Tables IV and VI) show that the rates of unidirectional influx and efflux of NH_4^+ were clearly altered by MSO. Efflux was significantly enhanced in plants maintained both at 1.5 mM $[NH_4^+]_o$ and at 100 μM $[NH_4^+]_o$. This is seen clearly in the marked increase of the y intercept of the cytoplasmic regression line in an MSO (pre)treatment experiment compared to a control experiment without MSO (Fig. 4). In addition to an enhancement of ϕ_{co}, ϕ_{oc} was depressed following MSO pretreatment in plants maintained at 100 μM $[NH_4^+]_o$, an effect not observed at 1.5 mM $[NH_4^+]_o$. This led to a marked increase in the ϕ_{co}/ϕ_{oc} ratio, and to the observed decrease in $[NH_4^+]_{cyt}$ in the roots of these plants (Table VII).

These findings, although initially surprising, are in keeping with the widely documented negative-feedback role of Gln upon NH_4^+ uptake (for refs., see Wang et al., 1993b; Kronzucker et al., 1995a), if MSO is able to mimic the feedback role of Gln on the transporter level. It is not inconsistent with these assumptions that the putative negative-feedback effect caused by this Gln analog be different at 100 μM than at 1.5 mM $[NH_4^+]_o$, since distinct high-affinity and low-affinity transport systems operate at these two concentrations (Wang et al., 1993b; H.J. Kronzucker, unpublished results). Such discriminating effects of MSO on separate systems of NH_4^+ uptake and on the concentration of free NH_4^+ in phase III, combined with the fact that the characteristics of phases I and II are not affected, provides strong support for the assumption that phase III indeed represents the cytoplasmic compartment.

Pretreatment of seedling roots with α-KG led to similar results in terms of phase identification. α-KG should alleviate possible carbon limitation to the assimilation of NH_4^+ and thereby alter $[NH_4^+]_{cyt}$ and plasmalemma fluxes of NH_4^+ while having no effect on the binding of NH_4^+ in the cell wall. The provision of α-KG failed to alter exchange characteristics of phase I or II but decreased the concentra-

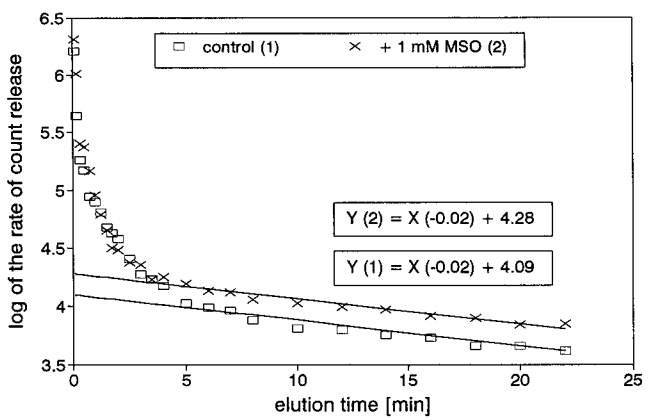

Figure 4. Combined semilogarithmic plots of the rate of release of ^{13}N [log (cpm released) g^{-1} min^{-1}] versus time of elution in roots of intact white spruce seedlings. Seedlings were maintained at 1.5 mM $[NH_4^+]_o$. Experiment 1 represents control plants, and experiment 2 represents plants (pre)treated with solutions containing 1 mM MSO for 6 h. Plots include linear regression lines and equations for the presumed cytoplasmic phases (phase III).

Table VII. *Compartmental concentrations of NH_4^+ in spruce root compartments as estimated by compartmental analysis at 100 μM $[NH_4^+]_o$ and after various (pre)treatments*

For details see text; see also Table I. Data are means ± SE (n = 3–9).

(Pre)treatment	Compartmental NH_4^+ Concentration	
	$[NH_4^+]_{free\ space}$	$[NH_4^+]_{cyt}$
	mM	
Control (100 μM)	1.22 ± 0.23	13.44 ± 1.51
+ MSO	1.51 ± 0.16	9.24 ± 1.35
+ α-KG	1.41 ± 0.18	9.91 ± 0.89
+ SDS	1.39 ± 0.34	1.59 ± 0.78
+ 75°C	1.22 ± 0.2	0.93 ± 0.47

Table VIII. *Compartmental concentrations of NH_4^+ in spruce root compartments as estimated by compartmental analysis at 100 μM $[NH_4^+]_o$ and at various $[Ca^{2+}]_o$*

For details, see text. Data are means ± SE (n = 3–9).

(Pre)treatment	Compartmental NH_4^+ Concentration	
	$[NH_4^+]_{free\ space}$	$[NH_4^+]_{cyt}$
	mM	
100 μM NH_4^+/50 μM Ca^{2+}	1.16 ± 0.2	13.52 ± 2.42
100 μM NH_4^+/500 μM Ca^{2+}	0.84 ± 0.19	11.35 ± 1.74
100 μM NH_4^+/5 mM Ca^{2+}	0.25 ± 0.02	11.63 ± 2.53

tion of NH_4^+ in phase III (Fig. 2), the putative cytoplasmic phase. Although this was most certainly attributable to enhanced rates of NH_4^+ assimilation, a depression of NH_4^+ influx also contributed to the effect (Table IV). It is well established that the availability of carbon skeletons to the roots, both endogenous in the roots and supplied via the shoot, affects rates of N uptake (Michael et al., 1970; Monselise and Kost, 1993; Rideout et al., 1994). In our study, an additional "regulatory" role of α-KG was evident in an effect on the partitioning of NH_4^+ assimilation products between root and shoot. Significantly reduced rates of ϕ_{xylem} (Table IV) indicate that transport of products of ^{13}N assimilation to the shoot was reduced under these conditions. Changes in the allocation of NH_4^+ assimilation products to root and shoot in response to changing carbon abundance in the roots have been documented by others (Talouizte et al., 1984; Champigny and Talouizte, 1986; Tolley-Henry and Raper, 1986; Henry and Raper, 1991). The observed effects on both ϕ_{oc} and ϕ_{xylem} in our study argue for a possible feedback role of α-KG on the uptake and assimilation of NH_4^+, as well as on the allocation pattern of assimilation products. It remains undetermined in the present study, however, whether the α-KG effect is, in fact, caused by α-KG directly or whether it is mediated via some downstream NH_4^+ assimilation product, such as glutamate or Gln. More importantly, however, from the perspective of compartment identification, the results of the α-KG experiments appear to substantiate our assignment of phase III to the root cell cytoplasm.

In our previous attempts to characterize compartment III as membrane bound and metabolically active, the identities of the remaining two phases were deduced by default to correspond to extracellular compartments. In the present study of NH_4^+ exchange, the assumptions regarding phases I and II were tested more directly, by varying the cation composition of the preloading and loading solutions, utilizing the different capacities for ionic binding to cation-exchange matrices exhibited by Al^{3+}, H^+, and Ca^{2+} compared to NH_4^+. Since our previous tentative interpretation of phase II in NH_4^+-exchange kinetics was that it was the Donnan free space in the cell wall, cations capable of competing with NH_4^+ for these binding sites were anticipated to selectively affect $^{13}NH_4^+$ exchange with phase II. According to the lyotropic series for cations (the Hofmeister series), the strength of adsorptive binding to nonspecific cation-exchange matrices should decrease in the order: $Al^{3+} \geq H^+ \geq Ca^{2+} \geq NH_4^+$ (Brady, 1974).

The above cations significantly reduced NH_4^+ binding in phase II. Figure 3 shows this effect on phase II for Ca^{2+} in an overlay graph, which was obtained after correction for specific activity and subtraction of counts eluting from phases I and III. Although phase I remained unaffected by all manipulations, treatment at low pH and with Al^{3+} also affected characteristics of phase III. This was evident in changes in the plasmalemma fluxes of NH_4^+ and in $[NH_4^+]_{cyt}$ (Tables VI and IX). The results from H^+ and Al^{3+} experiments are therefore useful only in combination with results obtained in experiments using Ca^{2+}, which, for the range of 50 μM to 5 mM, did not seem to perturb phase III (Tables V and VIII). Although the $t_{1/2}$ values for phase II remained statistically unchanged with the cation treatments (Tables II and III), significant differences in y intercepts indicated that the NH_4^+ content of that phase was significantly altered by cation competition. Displacement of NH_4^+ from phase II by Al^{3+}, H^+, and Ca^{2+} was exactly consistent with expectations of the lyotropic series. Furthermore, as $[Ca^{2+}]_o$ increased, the $[NH_4^+]$ of phase II decreased correspondingly (Table VIII). Thus, the data are in excellent agreement with the assumption that phase II represents a cation-exchange matrix. Together with the earlier findings that phase II was neither membrane bound nor metabolically dependent, our interpretation of phase II as the Donnan free space in the cell wall is supported substantially.

Phase I was affected neither by the earlier treatments designed to alter phase III nor by the cation variations. Rather, the NH_4^+ exchanging with that phase seemed to be exclusively dependent on the specific activity of the loading solution. This suggests, along with the very small $t_{1/2}$ for that phase, that it reflects a film of solution adhering to the root surface, possibly also including the water-free space of the cell wall. The absence of a phase that might be identified as the water-free space suggests that either the latter is kinetically indistinguishable from the Donnan free space or it is indistinguishable from the rapidly exchanging surface film. A priori, the former seems unlikely, whereas a half-life of 2 to 3 s for the water-free space may appear too short. However, Siddiqi et al. (1991) were able to detect $^{13}NO_3^-$ in the shoots of barley after root exposure to labeled solution for as few as 10 s. Such rapid (apparently apoplasmic) movements of ions may indicate that equilibration of the water-free space is more rapid than might have been formerly anticipated.

Table IX. *Compartmental concentrations of NH_4^+ in spruce root compartments as estimated by compartmental analysis at 1.5 mM $[NH_4^+]_o$ and after various (pre)treatments*

For details see text; see also Table III. Data are means ± SE (n = 3–9).

(Pre)treatments	Compartmental NH_4^+ Concentration	
	$[NH_4^+]_{free\ space}$	$[NH_4^+]_{cyt}$
	mM	
Control (1.5 mM)	8.82 ± 0.59	34.71 ± 1.99
+ MSO	10.47 ± 2.63	48.59 ± 6.84
+ pH 3.6	6.42 ± 1.67	2.86 ± 0.36
+ Al^{3+}	2.04 ± 1.31	1.73 ± 0.06

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