A PHYSIOLOGICAL DISSECTION OF NITROGEN FLUXES AND POOLS IN HIGHER PLANT TISSUES

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Botany)

THE UNIVERSITY OF BRITISH COLUMBIA

February 2001

Abstract

Compartmental analysis by efflux (CAE) was used with the high-energy positron-emitting nitrogen (N) tracer ^{13}N to study: 1) fluxes and compartmentation of ammonium (NH_4^+) in leaf slices from rice (*Oryza sativa* L. cv. IR-72), wheat (*Triticum aestivum* L. cv. 'Max Red'), and tomato (*Lycopersicon esculentum* L. cv. 'Trust') seedlings; and 2) fluxes and compartmentation of ammonium and nitrate (NO_3^-) in roots of barley (*Hordeum vulgare* L. cv. CM-72, cv. 'Klondike') and rice seedlings. An equation set based on CAE kinetic data was developed to model underestimates in tracer influx measurements resulting from concurrent tracer efflux. Leaf-slice experiments showed that cellular fluxes and compartmentation of NH_4^+ in leaves were similar to those in roots. NH_4^+ efflux:influx ratios varied with plant species, in the order rice > wheat > tomato, corresponding to a declining gradient in NH_4^+ tolerance. Fluxes in barley (ammonium-intolerant) and rice (ammonium-tolerant) roots also showed pronounced interspecific differences, particularly that at high (10 mM) external ammonium concentrations ($[NH_4^+]_o$), NH_4^+ efflux, influx, and efflux:influx across the plasma membrane were 2.7, 1.9, and 1.4 times higher, respectively, in barley than in rice. Much greater futile cycling of NH_4^+ in barley was thus indicated under this condition. A Nernstian analysis of electrophysiological and compartmentation data indicated that the elevated efflux in barley was active, i.e., energy-dependent. While in barley this flux was associated with 41% higher oxygen consumption by intact barley roots at 10 mM $[NH_4^+]_o$, compared to 0.1 mM, no such difference was found in rice. Results are discussed in the context of NH_4^+ toxicity. Half-lives ($t_{½}$) of cytosolic ^{13}N efflux from

a wide variety of plants were independent of external [N] for a given nitrogen ion and a given plant species, and were restored within 5 min following ten-fold changes in $[NH_4^+]_o$. The evident steady-state constancy in cytosolic NH_4^+ and NO_3^- turnovers suggests a condition in which cytosolic [N] and influx are linearly related, in contrast to cytosolic potassium (K^+) concentrations, which are held constant as external [K^+] and K^+ influx change. These observations suggest the presence of fundamentally different control mechanisms for cytosolic pools of the two elements.

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Acknowledgements

I first wish to thank my supervisor, Professor ADM Glass, for his continuous intellectual and financial support, for his infectious enthusiasm for science, and for his patience with my prolonged graduate career. I am equally grateful for the instruction, laboratory assistance, and moral support given me by my two "unofficial" supervisors: Professor HJ Kronzucker, whose scientific insight and encyclopedic knowledge are inspiring; and Professor MY Siddiqi, whose understanding, eye for detail, and gift of compassion are profound.

This work could not have been done without the generous provision of ^{13}N tracer from the Tri-University Meson Facility (TRIUMF) at UBC, and without the help of the TRIUMF staff, especially of T Hurtado, M Adam, and T Ruth. ^{13}N experiments entailed extensive assistance in the Glass lab, and in this regard I wish to thank S Silim, M Sheriff, D Zhuo, X-J Min, J Vidmar, M Okamoto, A Kumar, and S Rawat.

Other (non- ^{13}N) work in and around the laboratory was made possible through assistance from S Silim, M Sheriff, M Ektefaie, and J Louie. Special thanks go to Professor JK Schjoerring for analysis of ^{15}N samples (which was not, unfortunately, included in this thesis) and to Professor RD Guy for equipment loans.

This work has been underpinned and encouraged by numerous discussions from the above-mentioned people, and from other inspiring individuals, including BA Bohm (who got me started on the research path), the members of my committee (Professors PJ Harrison, PA Jolliffe, and GHN Towers), and the

following: D Heim, C Brokaw, S Woodstock, G Lizee, B Green, S Ellis, N Huner, and G Kirk.

I also wish to thank my parents, whose backgrounds (my father a radiologist, my mother a botanist) may well have predisposed me to the pursuit of botanical tracer studies. Without their continued support and encouragement I may not have been able to complete this work.

Finally, I wish to thank my closest companion, Susan Cowan, whose love, encouragement and understanding have kept my body and soul together, in the face of at times daunting adversity.

Chapter 1. General Introduction

Nitrogen (N), an essential and often growth-limiting macronutrient for all plants, is a key constituent of nucleotides, enzymes, coenzymes, pigments, alkaloids, signaling molecules, and other essential biological compounds (Salisbury and Ross 1992, Marschner 1995). Plants extract N from the environment in various forms, including the two major inorganic N sources, the ions ammonium (NH_4^+) and nitrate (NO_3^-), which are found at widely varying concentrations among and, even within, different soils (Bormann *et al.* 1968, Chapin 1980, Vitousek *et al.* 1982, Jackson and Caldwell 1993, Wolt 1994). Ammonium and nitrate represent, respectively, the most negative and positive N oxidation states normally found in the biosphere: NH_4^+, with -3 and NO_3^-, with +5 (Pauling 1970).

This thesis examines the components involved in the membrane transport and subcellular compartmentation of inorganic nitrogen in higher plants, and strives to relate these functional components to one other and to supracellular levels of biological organization. Undoubtedly, this is a rather broad subject, and the work presented here ranges widely, but it is unified by the use of a technique known as compartmental analysis – or, more specifically, compartmental analysis by efflux (CAE; see Chapter 2 for a summary of the methodology). CAE fits exponential decay equations to changing rates of tracer release from components of labeled multi-compartment systems, and provides estimates of metabolite concentrations in each component compartment, based on specific and total tracer activity within it (Walker and Pitman 1976, Siddiqi *et al.* 1991,

Kronzucker *et al.* 1995c). In this work, the short-lived (half life = 9.98 min), positron-emitting, radioisotope ^{13}N (Meeks 1993) was used with this technique to isolate, or "dissect out", the cytosolic compartment of plant cells, and thereby to provide estimates of NH_4^+ transport through the cytosol, and of cytosolic NH_4^+ concentrations ($[NH_4^+]_c$), in a variety of plant species and tissues. The cytosol was of particular interest for three reasons: first, it is the cellular compartment that immediately interacts with the environment, via the plasma membrane; second, it is a major location of metabolic activity; and third, its pools provide materials to subcellular organelles for processing and storage, and to other parts of the plant via the vasculature.

In Chapter 3, CAE is used to develop a mathematical model that shows how efflux data can be applied to the analysis of influx. The influx of NH_4^+ is considered as a test case, but the conclusions of this chapter may be extended to other ion fluxes. Equations are developed which show how potentially serious errors in influx measurements, resulting from simultaneous efflux during the influx protocol, may be calculated. Complementary to these deliberations, data are presented that show that large changes in $^{13}NH_4^+$ efflux result from raising or lowering $[NH_4^+]_o$, relative to that provided during the preceding growth period, during tracer release experiments. This "perturbational" (as opposed to steady-state) efflux was found to be positively concentration-dependent, and the subsequent restoration of the kinetic constant for tracer efflux after the transition to a new state is discussed in terms of its possible significance for the understanding of cellular flux adjustments and the generation of influx isotherms.

In Chapter 4, CAE is used to examine the characteristics of NH_4^+ flux and compartmentation in leaf tissue of higher plants. While more conventional in approach than most of the work in this thesis, this study nevertheless has few precedents (Raven and Farquhar 1981, Nielsen and Schjoerring 1998). Leaf slices have been used to study ion transport and assimilation for several decades (Smith and Epstein 1964, Rains 1967, Jeschke 1976), but information about NH_4^+ membrane fluxes and subcellular pool sizes in leaves is scarce. Yet this subject is important because leaf cells consume large amounts of NH_4^+ via the glutamine synthetase-glutamate synthetase (GS-GOGAT) cycle and other assimilatory pathways, and release similar quantities via the photorespiratory nitrogen cycle, protein degradation, phenylpropanoid metabolism (i.e. deamination of phenylalanine), and other dissimilatory pathways (Joy 1988, Howitt and Udvardi 2000). In addition, NH_4^+ pools within leaves may be augmented by NH_4^+ sources from the root, via the transpiration stream (Mattsson and Schjoerring, 1996), and from the atmosphere, via gaseous NH_3 that becomes protonated in the leaf apoplast (Farquhar *et al.* 1980, Mattsson *et al.* 1997, Hanstein and Felle, 1999).

Chapter 4 presents the first application of CAE methodology to the observation of bi-directional fluxes in leaf tissues of terrestrial plants; the ammonium ion, being of major biochemical significance, is an appropriate subject for this novel application. Fluxes, efflux:influx ratios, and subcellular NH_4^+ pools in leaves of rice, wheat, and tomato (*Lycopersicon esculentum*) are shown to be highly responsive to external NH_4^+ conditions, and that many CAE-derived

parameters for leaf slices are surprisingly similar to those seen in intact roots, despite the invasive nature of the leaf slice investigation, and despite the functional and environmental differences between the two organs. A comparison of key flux parameters among these plant species is consistent with their differing abilities to tolerate NH_4^+ as a nitrogen source, and links are made between these findings and those presented in Chapter 5.

Chapter 5 develops the hypothesis that membrane transport of NH_4^+ plays a critical role in determining a plant's ability to utilize, without detriment, NH_4^+ as a sole nitrogen source. It explores the basis of the apparent preference among plant species for one form of nitrogen over another (Smirnoff and Stewart 1985, Klinka *et al.* 1989, Marschner 1995, Kronzucker *et al.* 1997). *Ammonophiles*, or plants that perform well on ammonium as a sole nitrogen source, in terms of key parameters such as growth, longevity, or fecundity, tend to be found in relatively undisturbed ecosystems or under flooded, hypoxic, or acidic conditions; these conditions tend to favour high ratios of NH_4^+ to other forms of available nitrogen (Ismunadji and Dijkshoorn 1971, Rice and Pancholy 1972, 1973, Vitousek *et al.* 1982, Marschner 1995, De Graaf *et al.*, 1998). Examples of ammonophiles include lowland rice (*Oryza sativa*), members of the Ericaceae (heather family), and late-successional conifers such as white spruce (*Picea glauca*) (Ismunadji and Dijkshoorn 1971, Ingestad 1973, Sasakawa and Yamamoto 1978, Peterson *et al.* 1988, Magalhaes and Huber 1989, Wang *et al.* 1993a, Kronzucker *et al.* 1997). *Nitrophiles*, on the other hand, appear to prefer NO_3^- as an N source, and such plants are often adapted to disturbed, oxidized sites where soil NO_3^- tends

to be high (Smirnoff and Stewart 1985, Klinka *et al.*, 1989). Nitrophiles include barley (*Hordeum vulgare*), members of the Rosaceae, (rose family) and Solanaceae (nightshade family), and several early-successional tree species such as trembling aspen (*Populus tremuloides*) and Douglas-fir (*Pseudotsuga menziesii*) (Polizotto *et al.* 1975, Smirnoff and Stewart 1985, Lewis *et al.* 1986, Klinka *et al.* 1989, Magalhaes and Huber 1989, Cao and Tibbits 1993, Min *et al.* 1999, 2000).

It is perhaps surprising that any plant should prefer NO_3^- over NH_4^+, since in order to be assimilated, it must first be reduced to NH_4^+ in an energy-requiring, multi-enzyme, multi-compartment process involving the transfer of eight electrons (Reisenauer 1978, Oaks and Hirel 1985, Bloom *et al.* 1992, Oaks 1994). Indeed, why some plants prefer the oxidized N form is not well understood, although it has been argued that its uptake may improve the anion-cation balance (and in turn the pH) of the plant, and improve the carbohydrate balance between root and shoot (Givan 1979, Davies 1986, Raven 1986, Van Beusichem *et al.* 1988, Gerendas and Sattelmacher 1990, Marschner 1995, Schubert and Yan 1996). Preference for NO_3^- may also be an effect of NH_4^+ toxicity, which can seriously diminish the growth of many nitrophiles upon exposure to even moderate concentrations of NH_4^+ as the sole N source (Bennett and Adams 1970, Reisenauer 1978, Givan 1979, Van Breeman *et al.* 1982, Van Breeman and Van Dijk 1988, Mehrer and Moore 1989, Gerendas and Sattelmacher 1990, Magalhaes *et al.* 1992, Pearson and Stewart 1993, Fangmeier *et al.* 1994, Gerendas *et al.* 1997, Kronzucker *et al.* 1997, De Graaf *et al.* 1998).

Ammonophiles may be able to take advantage of the moderate energetic savings that growing on a reduced N form may provide, but they need to tolerate the toxic effects of NH_4^+. Because the plasma membrane of the root cell is the surface of NH_4^+ entry from soil to plant (Kleiner 1981, Glass and Siddiqi 1995, von Wiren *et al.* 2000), transport systems at this biophysical boundary should be examined for evidence of adaptations that may confer NH_4^+ tolerance. However, while NH_4^+ fluxes across cell membranes, particularly the plasma membrane, have been characterized in great detail and in many plant species (see Glass *et al.* 1997, Howitt and Udvardi 2000, von Wiren *et al.* 2000 for recent reviews), they have not previously been studied in the context of its potential toxicity. Chapter 5 of this thesis begins to remedy this situation, through a comparison of two model species: rice, which is able to grow on NH_4^+ without suffering from toxicity (Magalhaes and Huber 1989), and barley, which is not adapted to NH_4^+ (Lewis *et al.* 1986). ^{13}N-based flux data are presented, demonstrating that these two grasses differ in key respects in terms of their membrane-flux adaptations to high (10 mM) external NH_4^+ concentrations ($[NH_4^+]_o$ – N.B. the subscript "o" refers to "outside" the plant). In brief, ammonium-tolerant rice roots, when compared to ammonium-sensitive barley roots, appear to more closely co-ordinate plasma membrane NH_4^+ influx with the assimilatory capacity of the plant. Evidence for this is seen in low ratios of unidirectional NH_4^+ fluxes (efflux to influx) across the plasma membrane in rice, versus very high ratios in barley, indicating in the latter case the presence of much higher NH_4^+ fluxes than required by the plant.

The lack of adaptation to high $[NH_4^+]_o$ at the membrane level is proposed to be not only a diagnostic for NH_4^+ sensitivity in barley, but also a direct cause of toxicity itself. In support of the latter idea, it is shown that the growth suppression of barley by high $[NH_4^+]_o$ is accompanied by a substantially increased root oxygen consumption (compared to low NH_4^+ treatment), a condition not seen in rice. Ammonium-stimulated respiration may be partly attributable to a "leak and pump" membrane transport condition (MacRobbie 1971, Nobel 1999) involving the futile cycling of NH_4^+ across the barley root plasma membrane. A thermodynamic (Nernstian) analysis in barley, using compartmentation and electrophysiological data, is presented, to indicate that the very high, apparently unregulated influx of NH_4^+ into the cytosol, probably via NH_4^+ channels (Tyerman *et al.* 1995, Nielsen and Schjoerring 1998), is "downhill", driven by a strong, inwardly-directed electrochemical potential gradient (hence, the term "leak"). By contrast, efflux from the cytosol to the external medium, which in barley can be as great as 95% of influx under high $[NH_4^+]_o$, is thermodynamically "uphill", that is, energy-requiring, and appears to involve an active NH_4^+ extrusion mechanism (the "pump"), as yet unknown at the molecular level. In Chapter 4 of this thesis, excessive bi-directional NH_4^+ flux capacity is also reported for leaf slices of wheat (*Triticum aestivum*) and tomato (*Lycopersicon esculentum*), the latter being an exceptionally NH_4^+ - intolerant species (Wilcox *et al.* 1977, Magalhaes and Wilcox 1983, 1984, Magalhaes and Huber 1989, Gerendas and Sattelmacher 1990). This phenomenon has also been reported (although not discussed in the context of toxicity) in seedling roots of two early-successional, NH_4^+-sensitive,

tree species, trembling aspen and Douglas-fir (Min *et al.* 1999, 2000), and might be generally applicable to plant species that suffer from NH_4^+ toxicity. Its investigation may therefore provide important insights into mechanisms of ecological succession (see Kronzucker *et al.* 1997).

Chapter 6 presents an interpretation of the remarkable constancy in half-life ($t_{½}$) values (equivalent to turnover rates) observed in CAE experiments for cytosolic ^{13}N efflux, occasionally alluded to in the plant ion transport literature (e.g. Kronzucker *et al.*, 1995c, 1995e), but not investigated in detail until now. As explained in Chapter 3, $t_{½}$ is a function of the magnitudes of fluxes moving traced material to and from a compartmented pool, and of the size of the pool. In the case of cytosolic NH_4^+ or NO_3^- pools, $t_{½}$ values can be derived from the exponential decline rates of $^{13}NH_4^+$ or $^{13}NO_3^-$ efflux from the cytosolic phase of labeled plant material. Data are presented in Chapter 6 which show how $t_{½}$ values for the cytosolic NO_3^- pools of barley roots are held constant over four orders of magnitude of external NO_3^- ($[NO_3^-]_o$), and even with plants uninduced for NO_3^- - assimilation. Information drawn from the literature, and from elsewhere in this thesis, shows that the constancy of NO_3^- turnover rates is not unique to cytosolic NO_3^- pools or to barley, but extends to cytosolic NH_4^+ pools, and to N turnover in a wide range of plant species, including other grasses, the genetic-model-system species *Arabidopsis thaliana*, and several gymnosperms. The perturbational $^{13}NH_4^+$ efflux data shown in Chapter 3 (see above) are revisited, since these suggest a rapid return, following perturbation, of previous turnover rates, demonstrating the apparent tenacity of this parameter. Inorganic N

turnover is contrasted with potassium (K^+) turnover, which does not display such constancy, but instead varies inversely with K^+ influx. This response results in the maintenance of cytosolic K^+ at a constant concentration of ~125 – 150 mM (Memon *et al.*, 1985), while cytosolic N concentrations are highly variable. Internal pool sizes of the two elements thus appear to be regulated in fundamentally different ways, and the terms "amplitude modulation" (for N) and "frequency modulation" (for K^+) are introduced to describe the two conditions.

In summary, this work is broadly based but unified by the theme of flux and compartmentation of nitrogen (particularly NH_4^+) in higher plant tissues. It should be noted that, while a number of novel ideas are advanced in this work, the evidence suggests many more unanswered questions and hence new directions for research, as will be discussed in the final chapter. Even in a field as well studied as plant-nitrogen relations, there is no shortage of new frontiers, particularly in the attempt to relate macroscopic phenomena, such as ecological succession and plant performance in the field, to cellular and subcellular plant function.

Chapter 2. Materials and Methods

Plant culture

Seeds of wheat (*Triticum aestivum* L. cv. 'Max Red'), barley (*Hordeum vulgare* L. cv. CM-72, and for experiments described in Chapter 6, cv. Midas) and rice (*Oryza sativa* L. cv. IR-72) were initially surface-sterilized with a 1% bleach solution and rinsed with deionized water. Subsequent treatments varied with grass species, due to the different germination and growth characteristics of each:

1) ***Wheat*** seeds, still dehydrated, were placed directly onto discs of plastic mesh (diameter of 3 cm, mesh size of 2 mm^2) that had been glued with silicone sealant to the inside of clear plastic cylinders (8 cm high). Seeds were covered with moist vermiculite, and the cylinders were placed in hydroponic tanks containing ¼-strength modified N-free Johnson's nutrient solution. This solution, referred to here as "solution 1", had the following composition:

- *Macronutrients:* KH_2PO_4 (0.5 mM); K_2SO_4 (0.5 mM); $MgSO_4$ (0.25 mM); $CaSO_4$ (0.2 mM).
- *Micronutrients:* KCl (50 μM); H_3BO_3 (25 μM); $MnSO_4$ (2 μM); $ZnSO_4$ (2 μM); $CuSO_4$ (0.5 μM); H_2MoO_4 (0.5 μM); Fe-EDTA (20 μM).

In the case of wheat, solution 1 was supplemented with 1 mM NH_4^+ as $(NH_4)_2SO_4$.

2) ***Barley*** seeds were hydrated in aerated, deionized water at 20°C for 20-30 min prior to placement in plastic cylinders with vermiculite, in tanks also

containing solution 1. This solution was supplemented with NH_4^+ or NO_3^- (as $(NH_4)_2SO_4$ or $Ca(NO_3)_2$, respectively), ranging from 0.01 to 10 mM (except for the NO_3^- - induction experiment, described in Chapter 6 and Table 5).

3) ***Rice*** seeds were hydrated and germinated in aerated, deionized water for 3 d at 30°C prior to placement on plastic cylinders with vermiculite, in tanks containing a second modified N-free Johnson's solution ("solution 2"), the composition of which was as follows:

- *Macronutrients:* NaH_2PO_4 (0.3 mM); $MgSO_4$ (2 mM); $CaCl_2$ (1 mM); K_2SO_4 (0.5 mM).
- *Micronutrients:* $MnCl_2$ (9 μM); $(NH_4)_6Mo_7O_{24}$ (5 μM); H_3BO_3 (20 μM); $ZnSO_4$ (1.5 μM); $CuSO_4$ (1.5 μM); Fe-EDTA (100 μM).

Solution 2 was supplemented with 0.1-10 mM NH_4^+ as $(NH_4)_2SO_4$. Rice seedlings intended for leaf-slice experiments were grown with 1 mM NH_4^+.

All solutions were buffered to a pH of 5.7 with excess $CaCO_3$, and were circulated continuously by pump (Lauda/Brinkman, Rexdale, ON, Canada) in the larger (24-48 L) tanks, or with compressed air, delivered by Pasteur pipette, in the smaller (8 L) tanks.

Plants were cultured under steady nutritional conditions for seven days (barley), ten days (wheat), or three weeks (rice), prior to experimentation. Wheat and barley plants, and rice plants intended for leaf-slice experiments, were cultured in a walk-in growth chamber under fluorescent lamps (Vita-Lite Duro-Test, Clifton, NJ, USA), with a 16h/8h photoperiod, a photon flux of approximately 200 μmol m^{-2} s^{-1}, a relative humidity (RH) of 70%, and a

temperature of 20°C. Rice plants grown for root experiments were cultured in a second growth chamber, also under fluorescent lamps (General Electric Watt-Miser 800, Burnaby, BC, Canada). In this chamber, the photosynthetic photon flux was approximately 650 μmol m^{-2} s^{-2} and the RH was 70%. Temperatures varied between 30°C during a 16-h light period, and 20°C during the subsequent 8-h dark period. All fluorescent lamps had a spectral composition similar to that of sunlight.

Tomato plants (*Lycopersicon esculentum* L. cv. 'Trust') were received as 4-week old seedlings and cultured in 10-cm pots containing a commercial soil preparation mixed with vermiculite. These were grown in the growth chamber used for wheat and barley and watered with bi-weekly applications of solution 1, supplemented with 1 mM NH_4^+ as $(NH_4)_2SO_4$. Tomato plants were used for leaf slice experiments at 6 weeks of age.

Preparation of leaf slices

Leaves of wheat and rice were excised above the basal sheath and sliced transversely on a sheet of glass, using frequently-changed razor blades, to produce segments 1-2 mm in width. Entire leaf laminae from tomato plants were sliced transversely and longitudinally into segments of approximately 2 mm^2. Batches of leaf slices were weighed and placed in a sidearm Erlenmeyer flask containing pretreatment solution, which was identical to the plants' growth solutions except that external NH_4^+ concentration ($[NH_4^+]_o$, provided as $(NH_4)_2SO_4$) varied according to subsequent ^{13}N-treatment (see Chapter 4). To diminish buoyancy, leaf slices were de-gassed by application of a vacuum, using

a tap aspirator for 10-15 s. After two washes with uptake solution, the slices were pretreated for 3 h prior to tracer experiments, or used immediately for depletion experiments. The pretreatment consisted of suspending the slices in non-labeled volumes of uptake solution in their originating growth chamber, with circulation provided by aeration. Solution volumes were chosen to prevent leaf slices from depleting the nutrient supply by more than 30%.

Depletion studies with leaf slices

Samples of sliced leaves (0.5-1.0 g) from wheat and rice seedlings were placed in 25-mL beakers containing 10-20 mL of uptake solution supplemented with 0.1 mM NH_4^+. Solutions were circulated by aeration. 1-mL aliquots of external solution were removed at 10-min intervals and analysed for NH_4^+ content by the phenol-nitroprusside method (Solorzano 1969). In shorter-term experiments, uptake solution was replaced every 30 min with undepleted solution, while for longer-term experiments, leaf slices were stored, between breaks in sampling, in 1-L volumes of uptake solution to maintain essentially constant $[NH_4^+]_o$. Slices were then transferred to 25-mL beakers for subsequent sampling at 10-min intervals.

Nutrient analysis

NH_4^+ in hydroponic tanks and depletion solutions was assayed every 1-3 days (Solorzano 1969), the frequency depending on tank volume and plant biomass. NO_3^- in tanks was assayed using the perchloric acid method (Cawse

1967). K^+ in tanks was assayed using flame spectrophotometry (Instrumentation Laboratory, Model 443, Lexington, MA, USA).

Isotope preparation

^{13}N was produced at the Tri-University Meson Facility (TRIUMF) on the UBC campus (Vancouver, BC, Canada) by proton bombardment of water. The following nuclear reaction was carried out in a CP42 cyclotron (Nordion International, Inc., Vancouver, BC, Canada):

$^{16}O + {}^{1}H \rightarrow {}^{13}N + {}^{4}He$ (Meeks 1993).

^{13}N was received from TRIUMF chiefly as nitrate ($^{13}NO_3^-$), and for experiments with this ion, three purification steps were taken: removal of ^{18}F (a by-product of the nuclear reaction) using an alumina-N ion exchange cartridge, oxidation of $^{13}NO_2^-$ with H_2O_2, decomposition of excess peroxide with catalase, and removal of ^{13}N-ammonium as $^{13}NH_3$ (g), as described in Kronzucker *et al.* (1995b). $^{13}NH_4^+$ was produced as described in Kronzucker *et al.* (1995c). Briefly, oxidized ^{13}N was reduced upon arrival in the laboratory, in a round-bottom flask containing Devarda's alloy (50% Cu, 45% Al, 5% Zn), at 70 – 80° C and at pH 11-12, resulting in the production of $^{13}NH_3$ gas according to the following reaction:

$$Na^{13}NO_3 + 4\ Zn + 3\ NaOH + 6\ H_20 \rightarrow 4Na[Zn(OH)_3] + {}^{13}NH_3 \quad .$$

The $^{13}NH_3$ gas was removed by distillation and trapped as $^{13}NH_4^+$ in a flask containing unbuffered experimental uptake solution (pH 1-2) acidified with H_2SO_4.

Compartmental analysis by efflux (CAE)

Please see pp. 17-18 for definitions and symbols of the basic terms of the analysis.

Intact roots. Compartmental analysis by efflux in plant roots was performed following the general procedures described in Siddiqi *et al.* 1991, and Kronzucker *et al.* 1995a, 1995b, and 1995e (see Fig. 1 for a flow chart summarizing CAE operations). Briefly, roots of 3-5 intact barley or rice seedlings were labeled in solutions chemically identical to growth solutions, except that they contained trace amounts of radioactive $^{13}NO_3^-$ or $^{13}NH_4^+$. Labeling time was usually 30 min for NO_3^- experiments and 1 h for NH_4^+ experiments, allowing for substantial labeling of cytosolic pools (Walker and Pitman 1976). When initial ^{13}N activity was low, shorter labeling periods were occasionally used, requiring the use of a correction factor, based on cytosolic nitrogen-exchange constants that were determined from tracer-efflux data (Fig. 1). Over the brief labeling periods used here, the vacuole was considered not to have accumulated sufficient tracer to significantly affect cytosolic washout curves, based on several lines of reasoning. First, the half-life of exchange for nitrate has been shown to be at least 100 times longer for the vacuole than for the cytosol (Belton *et al.* 1985, Lee and Clarkson 1986, Devienne *et al.* 1994a). ^{14}C-labeled methylammonium exchange studies indicate that the same may be true for ammonium (Ryan and

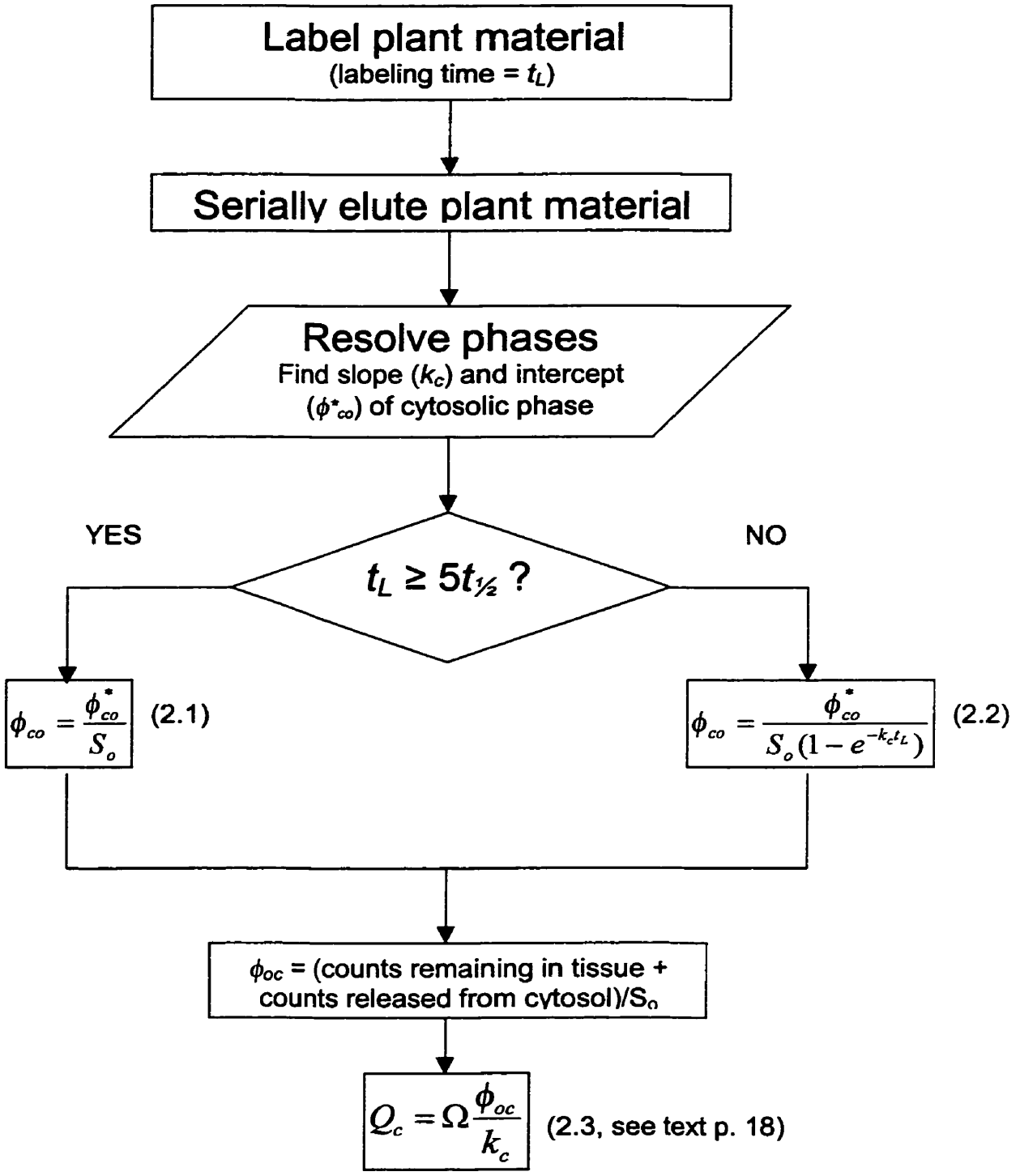

Figure 1. Flowchart of operations for compartmental analysis by efflux (CAE). See pp. 17-18 for definition of symbols.

Walker 1994). Second, in the present work, extrapolation of tracer efflux plots, over time periods sufficiently long to exhaust cytosolic tracer release (i.e. 5-6 times the half-life for this phase), indicates that the remaining counts available for release, presumably from a vacuolar space, are negligible. Third, direct influx measurements and estimates of influx from efflux analysis have been shown to agree closely (Wang *et al.* 1993a), indicating that flux distortions due to vacuolar efflux are insignificant. Although the use of curve-peeling procedures (Zierler 1981, Macklon *et al.* 1990) to subtract a vacuolar phase from a cytosolic one was impossible, due to the rapid decay of ^{13}N, the above arguments show that the error associated with a potential contribution to efflux from this phase was insignificant.

After labeling, each set of plants was transferred to an efflux funnel equipped with a clamped tygon spout to control elution. Periodic elution of tracer, from labeled roots into 20-mL aliquots of non-labeled growth solution, was then performed. A time series of elution was followed which declined in elution frequency during the 20- to 30-min periods of data collection (Kronzucker *et al.* 1995b; also see, e.g., Fig. 2). A typical time series was 10 s (3x), 15 s (6x), 30 s (4x), 1 min (2x), 2 min (10x). In steady-state experiments, tracer was eluted into growth solution (i.e. at constant [N]), while in perturbation experiments $[NH_4^+]_o$ was altered 10 min after the elution series began (see time course on p. 19). After the final elution, roots and shoots were divided, spun for 45 s to remove excess surface-bound solution and weighed. ^{13}N activity in roots, shoots, and efflux aliquots was determined using a gamma-counter (Packard Minaxi δ, Auto-γ

5000 Series, Mississauga, ON, Canada). The counter was programmed to correct for the rapid decay of ^{13}N, and the radiochemical purity of the samples was verified by consistency of radioactivity estimates in recounted samples. All experiments were performed using two replicates and repeated at least three times.

Calculations of component fluxes and compartmental concentrations were as described in Siddiqi *et al.* 1991 (also see below). All fluxes were expressed in μmol g^{-1} (fresh wt) h^{-1}. Symbols, and basic calculation methods, for fluxes are as follows:

ϕ_{co} = efflux from the cytosol, obtained from the rate of ^{13}N release from the cytosol at time zero, from the specific activity of the external solution ($S_o = {}^{13}N/({}^{14}N + {}^{13}N)$), and from the k_c value of the system (see below; also see Fig. 1 for the special case of short labeling times).

ϕ_{net} = net flux, obtained directly from the accumulation of ^{13}N in the plants at the end of the elution period, and from S_o.

ϕ_{oc} = unidirectional influx, calculated from $\phi_{net} + \phi_{co}$.

ϕ_{shoot} = flux of ^{13}N to the shoot, obtained directly from count accumulation there at the end of the elution period, and from S_o.

$\phi_{met/vac}$ = combined flux to assimilation and vacuole, resulting from $\phi_{net} - \phi_{shoot}$.

k_c values, representing cytosolic turnover rates (Kronzucker 1995e), were obtained from the slopes of regressed semi-logarithmic (phase III) tracer elution lines (see, e.g., Fig. 2). k_c values reported here are expressed on the basis of

decadal logarithms. Cytosolic half-lives of exchange ($t_{½}$) were calculated from the formula $t_{½} = (\log 2) / k_c$.

Cytosolic NO_3^- and NH_4^+ contents (Q_c) were calculated from the initial rate of ^{13}N release integrated over 5 times the half-life of cytosolic exchange, the ratio of efflux to all fluxes removing $^{13}NO_3^-$ or NH_4^+ from the cytosol, and the specific activity of the cytosol (S_c, estimated from labeling times in relation to the cytosolic nitrogen-exchange constants; see Siddiqi *et al.* 1991, and Fig. 1). An equivalent, but simpler, calculation method was built into a revision of the computer program used for CAE processing, based on the equation

$$Q_c = \Omega \frac{\phi_{oc}}{k_c} \qquad (2.3)$$

(MacRobbie 1971, Walker and Pitman 1976), where Ω is a proportionality constant, the value of which varies according to the units used. When ϕ_{oc} is in μmol g^{-1} (fresh wt) h^{-1}, k_c is in min^{-1}, Q_c is in mM, and concentration estimates using content values were based on the assumption that the cytosol occupies 5% of cell volume (Siddiqi *et al.* 1991), the value of Ω is 0.33. In the equivalent expression $Q_c = \Omega\ \phi_{oc}\ t_{½}$, $\Omega = 0.33/0.693 = 0.48$ since $t_{½} = 0.693/k_c$.

Perturbational experiments with barley plants followed a protocol similar to steady-state experiments, except that:

1) an initial elution series using aliquots of steady-state growth medium, containing 100 μM NH_4^+, was performed on a periodic schedule of 2, 4, 5, 6, 7, 8 min;

2) a more rapid elution series followed (to control for effects of plant handling on fluxes, see Aslam *et al.* 1996, Ter Steege *et al.* 1998), again with steady-state medium, on the schedule of 8.5, 9, 9.25, 9.5, 9.75, 10 min; and

3) the experiment was terminated with a final series of elution aliquots, containing solutions in which the 100 μM NH_4^+ was substituted by either 10 μM NH_4^+ or 10 mM NH_4^+; this series followed a schedule of 10.25, 10.5, 10.75, 11, 11.25, 11.5, 11.75, 12, 12.5, 13, 13.5, 14, 15, 16, 18, 20, 22, 24. 26, 28, 30, 32 min.

Because the specific activity of the pool that released tracer after a change in eluate [NH_4^+] was unknown, fluxes and pool sizes were not calculable in the case of perturbational experiments. These experiments were thus conducted principally for the observation of short-term concentration effects on k_c values.

Leaf slices. The procedure for leaf slices was similar in principle to that used with roots. Leaf-slice samples (1.00 g fresh wt. each) were transferred to radiotracer-containing uptake solutions for a 1-h labeling period. At the end of this period each tissue sample was placed in a 100 mL-capacity glass funnel, equipped with nylon mesh to prevent loss of plant material, and with a clamped tygon spout to control elution. Over the course of the experiment, 20-mL aliquots of an unlabeled washing solution chemically identical to the labeling solution were poured into the clamped funnel, released from it after specified, gradually increasing, time intervals, and immediately replaced with a subsequent aliquot. The schedule followed was 10 s (3x), 15 s (6x), 30 s (4x), 1 min (2x), 2 min (10x).

2-min washings were occasionally extended beyond 10x when tracer activity was sufficiently high, to check against premature truncation of tracer elution plots. Tracer captured in the eluates was determined by γ-emission, as was tracer remaining in the tissue after the final elution. Fluxes, exchange half-lives and compartment sizes were quantified as in experiments with roots.

Variations on this basic procedure with leaf slices included the following: 1) use of a cation exchange resin to determine the charge of the label in the eluate (see below); 2) treatment with 1% SDS during a 1 h pretreatment, as well as during labeling; 3) treatment with 1 mM MSX during a 2-3 h pretreatment, as well as during labeling and elution; 4) maintenance of leaf slices in the dark during a 2 h pretreatment, and during labeling and elution, with use of a green safe light (540 nm) during tracer labeling and elution.

Electrophysiology

Membrane electrical potential measurements were made using glass pipettes drawn into finely tipped (1 μm diameter) microelectrode probes by the heat and tension of a vertical pipette puller (David Kopf Instruments, model 700C, Tujunga, CA, USA). Impalement and reference microelectrodes were filled by syringe with a 3 M KCl electrolyte solution (pH 2), and mounted on gold-tipped contacts connected to an electrometer (World Precision Instruments, model FD 223, Sarasota, FL, USA) and a chart recorder. A compound microscope (Nikon model Alphaphot/YS, Tokyo, Japan), for the observation of microelectrode impalements, was affixed to a percussion-proof table at 90° to the microscope base, and a plastic chamber (160-mL capacity) was attached to the stage.

Microelectrode movement was controlled using a micromanipulator system (Narashige models M-152/M-203, Tokyo, Japan), also affixed to the table and to the microscope neck. A custom-built Faraday cage, to reduce electrical interferences, surrounded the table.

Membrane potential differences were measured as described in Wang *et al.* 1994. Briefly, intact barley roots were held between steel pins and pieces of silicone tubing in the plastic chamber, and impaled with the microelectrodes until a stable electrical potential difference between root and external solution was maintained for at least 2 min. Sixty-seven measurements were made on different plants for the 0.1 mM NH_4^+ condition, and 20 for the 10 mM NH_4^+ condition. Standard errors were < 3% of the means.

Growth Rates

Total plant (root + shoot) fresh weight of individual barley plants was monitored over days 1, 3, and 7 following germination. Surface water was removed from roots by brief blotting with filter paper. Plants were weighed and immediately returned to solution. Eight replicates were used for each of the two steady-state NH_4^+ growth concentrations.

^{13}N charge determination

A cation exchange resin (Dowex 50WX8-200, Na^+ form) was used to determine the charge of ^{13}N appearing in the eluates from radiolabeled leaf slices (see CAE section above; also see Siddiqi *et al.* 1991, Wang *et al.* 1993a, Kronzucker *et al.* 1995b). Eluate (5 mL) from an efflux time-series was passed

twice through a column containing approximately 5 g of resin. Eluates from the resins were γ-counted, as were the resins themselves.

Respiration measurements

Respiration measurements were made with intact roots of barley and rice, grown under steady-state $[NH_4^+]_o$ (0.1 or 10 mM). Roots were placed in rapidly stirred growth solutions in the 2.5-mL cuvette of a Clark-type polarographic electrode apparatus (model DW1, Hansatech Instruments Ltd, Norfolk, UK). The apparatus was set up as described in Walker (1987). Calculations were based on slopes of steady O_2 depletion lines generated over 3-10 min after which plants were blotted dry, divided into root and shoot, and weighed. In steady-state experiments, cuvettes were filled with freshly aerated growth solutions. Uncovered, air-saturated cuvettes without plants were noted to display no drift in O_2-dependent current, while cuvettes depleted of more than 30% O_2 showed a rise in O_2 after plants were removed; therefore all measurements were made before O_2 was depleted by more than 25%. In perturbational experiments, plants grown at 10, or 0.1, mM NH_4^+ were transferred to, and equilibrated in, beakers containing growth solution with 0.1, or 10, mM NH_4^+, respectively, for 30 min to 4 h prior to respiration assay. Pretreatments with 2 mM MSX lasted 3-6 h.

Chapter 3. How Can Unidirectional Influx Be Measured In Higher Plants? A Mathematical Approach Using Parameters from Compartmental Analysis

Introduction.

As the sequencing of the genomes of several representative higher plant species approaches completion, the problem of assigning function to the multitude of newly identified coding regions has grown. An inventory of the yeast genome suggests that several hundred proteins responsible for membrane transport are encoded (Paulsen *et al.* 1998), highlighting the central importance of transport functions and suggesting that this may also be true for higher plants. However, such impressive figures are based mainly upon sequence homologies to more or less well-defined reference points, rather than on actual demonstrations of function. Moreover, even functional assignments determined by experiment can be equivocal (Touraine and Glass 1997); uncertainties inherent in measuring transcript and protein abundance are compounded by the problems associated with measuring fluxes in systems as complex as intact plant roots. These latter problems, perhaps by virtue of being more mathematical and biophysical than biochemical in character, have been neglected in a number of recent analyses of this sort (see, e.g., Tsay *et al.* 1993, Krapp *et al.* 1998, Liu *et*

al. 1999), ultimately to the detriment of a clear understanding of the molecular basis of physiological processes.

A less casual approach to flux determination is therefore essential to the assignment of an expressed gene to a specific, unidirectional transport function. However, it has been claimed that even with exceptionally precise isotope methodology (^{13}N-tracing), unidirectional influx may not be accurately measurable under ordinary conditions (Lee and Ayling 1993). To date, this claim has not been refuted. In this chapter, kinetic parameters obtained from the method of compartmental analysis by efflux (CAE) are used to show why the claim is incorrect, by allowing the exact determination of errors caused by ionic counterfluxes under steady-state conditions (see beginning of Results and Discussion for a definition of "steady state"). Central to this analysis is the kinetic constant for cytosolic ion exchange (k_c), a term often misunderstood but critical to the understanding of the interrelated flux processes occurring simultaneously at the cellular level. This constant is most directly estimated by CAE, which, by providing values for additional kinetic parameters such as unidirectional influx and efflux, can be used to predict the extent of distortion inherent in assessments of influx at or close to the steady state.

A comprehensive and pragmatic approach to the design of ion transport experiments in plants is developed from a CAE-based approach, revising or eliminating unnecessary complexities from much-published classical models (see below). Results from steady-state flux measurements, however, while being useful for the estimation of efflux and pool sizes with CAE, are limited in

application to the study of concentration-dependent influx. This is because, in most practices, the plant's influx systems are only measured at steady state with respect to a single external concentration of the ion in question, the growth concentration. All other measurements involve some degree of perturbation of the root's nutrient availability and associated flux processes. Therefore, a departure from standard CAE protocols was taken here, with perturbational experiments performed to monitor efflux under non-steady-state conditions. The surprising outcome was that following a brief transitional state, the co-ordination of cytosolic flux processes was rapidly established following an NH_4^+ concentration shift, characterized by a restoration of the pre-perturbational (steady-state) k_c value. As will be discussed, when influx measurements are conducted subsequent to the establishment of this transitional state, an estimate of unidirectional influx should be possible with reasonable accuracy. In summary, procedural recommendations may be derived from CAE results, which may resolve some of the problems stated above.

Materials and Methods

Please refer to Chapter 2.

Results and Discussion

1. Steady-State Conditions

Before a quantitative discussion of the errors associated with influx determinations is possible, some key concepts underlying tracer studies need to

be reiterated. Most fundamentally, if chemical fluxes and pool sizes remain constant in the plant over the duration of flux measurement, a condition referred to throughout this work as *steady state* is achieved. At the steady state, the time course of exchange, or turnover, of a solute pool in a cell compartment is governed by first-order kinetics (MacRobbie and Dainty 1958, Pitman 1963, Pallaghy and Scott 1969, MacRobbie 1971, Pitman 1971, Poole 1971, Cram 1973, Walker and Pitman 1976, Jeschke and Jambor 1981, MacRobbie 1981, Behl and Jeschke 1982). The exponentiality of the compartmental labeling process in the steady state must be assumed *a priori* rather than derived *a posteriori* (*cf.* Rescigno 1999) as a best fit to data from washout experiments (*cf.* Lee and Clarkson 1986, and see below). As such, when a solute pool within the cytosol of a plant cell is labeled directly from an essentially non-changing pool of established specific activity (s_o), the specific activity of the cytosolic pool (s_c) will increase, at least initially, according to a kinetic constant k (or its inverse, the half-life of exchange, $t_{½} = 0.693/k$ when natural logarithms are used) describing the time dimension of pool turnover. As described in Walker and Pitman (1976), the rise in s_c during a labeling experiment can be modeled using this parameter in the following exponential equation

$$s_c = s_o(1 - e^{-kt}) \qquad (3.1)$$

where s_c is the tracer content after a labeling time t, and s_o is asymptotically approximated after labeling time in excess of 5 half-lives of exchange (also see Siddiqi *et al.* 1991). As will be discussed below, this simple equation does not

consider delivery fluxes of unlabeled ions from other (e.g. vacuolar) pools within the plant, which, if present, will cause a deviation from a simple exponential rise towards s_o (see below). In such cases, tracer content of the compartment will increase in a compoundly exponential manner, as the internal delivery fluxes become labeled.

When labeled tissue is next exposed to non-labeled external solution, the decline in tracer content of the cytosol over time can be described in the equation

$$s_c = s_{c,i}\, e^{-kt} \qquad (3.2)$$

where $s_{c,i}$ is the cytosolic specific activity at the start of elution. Again, however, this simple decline holds only in the absence of other compartments sufficiently labeled that they back-deliver significant amounts of tracer to the cytosol. If tracer is delivered from additional pools within the plant, the elution process is no longer governed by a single exponential (see below), entailing the application of curve-peeling procedures (Zierler 1981, Macklon *et al.* 1990).

Depending on the ion in question, the fluxes that contribute to cellular turnover may include metabolic activities and long- and short-distance transport processes. In some cases, the flux component is essentially irreversible, as in that of NO_3^- translocation from root to shoot or of nitrogen fluxes to metabolism (Siddiqi *et al.* 1991), while in others the opposing flux terms are nearly balanced, as in the case of efflux and influx of NH_4^+ across root plasma membranes under high external [NH_4^+] (Min *et al.* 1999, also see Chapter 5), or in the case of fluxes

across the tonoplast under steady-state conditions. Importantly, the magnitudes and directions of all the fluxes contributing to a compartmental pool are subsumed in the *k* term for that compartment. In the case of a cytosolic pool labeled by an external tracer source, variations in the kinetics of specific activity build-up in the cytosol as a result of fluxes from initially unlabeled, predominantly vacuolar, pools inside the plant, are entirely accounted for in the cytosolic *k* term (k_c). However, as emphasized above, and discussed further below, the rise in specific activity in the cytosol in the presence of such fluxes is a compoundly exponential process, involving not only k_c but also the *k* term for the vacuole (and all other delivery pools; see below). In such a case, deviations from eq. 3.2 are to be expected.

Tracer efflux is directly proportional to tracer activity within a source compartment, and therefore it must follow the same kinetics of rise or decline. The exponential nature of this decline is verified by the observation (Fig. 2) that the serial elution of tracer from a labeled root system follows a compoundly exponential decay pattern (Lee and Clarkson 1986, Siddiqi *et al.* 1991, Wang *et al.* 1993a). When transformed semi-logarithmically, this pattern may be dissected to resolve individual phases of tracer release, which in turn must be tested for correspondence to known source compartments within the multiphasic system (Kronzucker *et al.* 1995c). If such a correspondence is established, the kinetic constant *k* for turnover within the source compartment can be found directly from

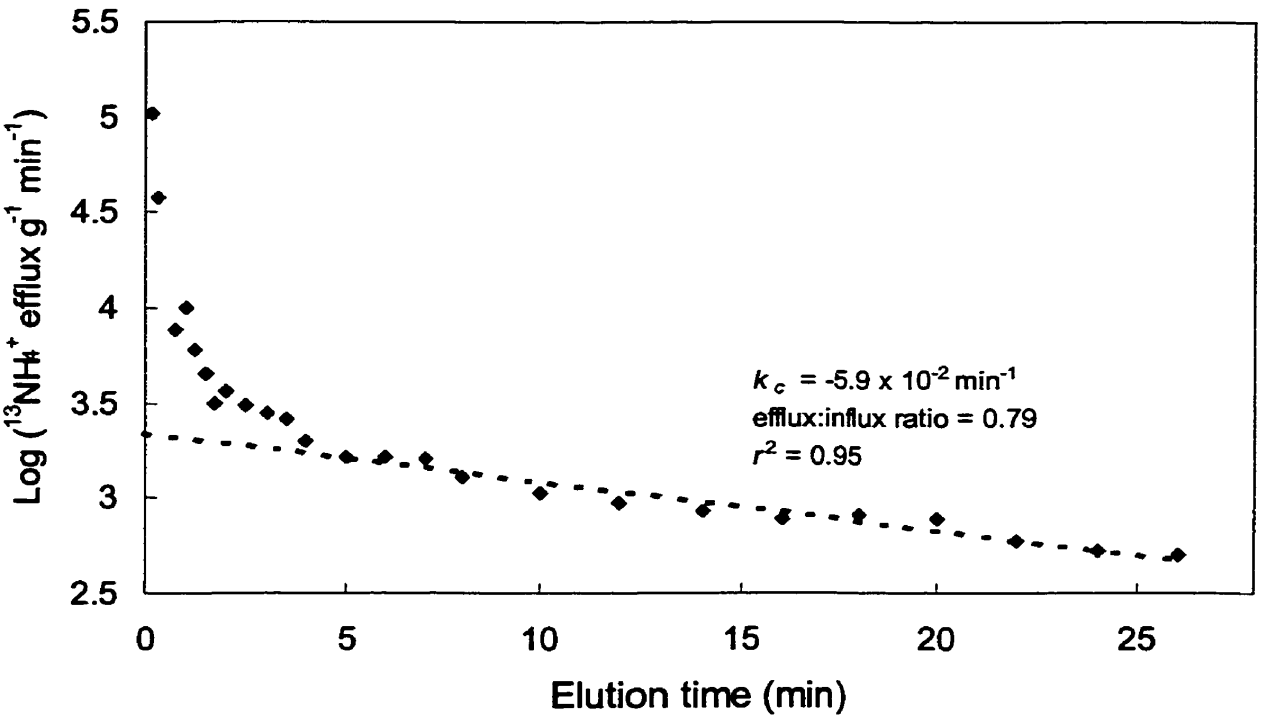

Figure 2. Representative plot of $^{13}NH_4^+$ efflux from roots of intact barley (*Hordeum vulgare* L., cv. CM-72) seedlings, grown and measured at 10 mM $[NH_4^+]_o$. Seedlings were labeled in $^{13}NH_4^+$-containing solution for 60 min and then washed during a 26-min elution protocol as described in Materials and Methods. The logarithm of the rate of release of radioactivity from root tissue was plotted vs. time of tracer elution, and linear regression on the semi-logarithmic plots was used to resolve separate phases. The broken line represents tracer elution from the root cytosol (Kronzucker *et al.* 1995c). The kinetic exchange constant (k_c), efflux:influx ratio, and the coefficient of determination (r^2) for this phase are as indicated.

the slope of the appropriate segment of the tracer-efflux plot[1], as long as *k* values corresponding to different segments are sufficiently distinct to be reproducibly resolved (Zierler 1981, Cheeseman 1986).

The *k* values for the various compartments are crucial to a mathematical appraisal of steady-state fluxes at the cellular level. Any plasmalemma influx measurement protocol must, at minimum, take into account k_c and also the magnitude of efflux from this compartment relative to influx. In addition, the presence of cell walls in plant systems necessitates the introduction of a desorption step following labeling, to remove tracer adsorbed in the apparent free space (Epstein 1972, Kronzucker *et al.* 1995c). Tracer efflux from the cytosol during this desorption is a further (and potentially more serious) source of error in most influx measurements and therefore must be incorporated into a comprehensive model. Interestingly, the kinetics of this very release during desorption forms the basis of efflux analysis.

Although k_c and efflux values often vary considerably between plant species, ion species, and external ion concentrations, the following model provides a generalised means of estimating steady-state unidirectional influx by taking into account the effect of efflux during labeling and desorption. It is important to note that this simplified equation applies only to a plant system in

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[1] In the case of non-metabolised ions, tracer retention in tissue after varying periods of elution can be used to characterise turnover, whereas for metabolised ions, the rate of tracer release must be monitored (Lee & Clarkson 1986) and the chemical identity of the eluate must be confirmed (Siddiqi et al. 1991).

which fluxes delivering unlabeled quanta of the measured ion to the cytosol (e.g. from the vacuole) are minor in comparison to traced fluxes. A more general case will be discussed below.

Let Q^* be the total amount of tracer entering the tissue via influx transporters, and consisting of the following three components:

A = quantity of tracer retained in tissue at the end of labeling and desorption periods

B = quantity of tracer lost from cytosol during the labeling period

C = quantity of tracer lost from cytosol during the desorption period

Other essential variables in this analysis are as follows:

t_L = duration of labeling period

t_D = duration of desorption period

ϕ^*_{oc} = tracer influx into cytosol

ϕ^*_{co} = maximal tracer efflux out of cytosol

k_c = rate constant for cytosolic tracer exchange (see slope in Fig. 2)

$$\phi_{oc}^{*} = Q^{*}/t_L$$

$$Q^{*} = A + B + C$$

$$= A + \int_0^{t_L} \phi_{co}^{*}(1 - e^{-k_c t})dt + (1 - e^{-k_c t_L}) \int_0^{t_D} \phi_{co}^{*} e^{-k_c t} dt$$

$$= A + \phi_{co}^{*}\left(\int_0^{t_L} (1 - e^{-k_c t})dt + (1 - e^{-k_c t_L}) \int_0^{t_D} e^{-k_c t} dt \right)$$

$$= A + \phi_{co}^{*}\left(\left[t + \tfrac{1}{k_c} e^{-k_c t} \right]_0^{t_L} + (1 - e^{-k_c t_L}) \left[-\tfrac{1}{k_c} e^{-k_c t} \right]_0^{t_D} \right)$$

$$= A + \phi_{co}^{*}\left[t_L - \tfrac{1}{k_c}\left(e^{-k_c t_D} - e^{-k_c (t_L + t_D)} \right) \right]$$

Therefore

$$\phi_{oc}^{*} = \left\{ A + \phi_{co}^{*}\left[t_L - \tfrac{1}{k_c}\left(e^{-k_c t_D} - e^{-k_c (t_L + t_D)} \right) \right] \right\} / t_L \,. \qquad (3.3)$$

This expression is a novel summary of the terms that contribute to tracer influx (*cf.* Cram 1969), and circumvents the unnecessarily complicated mathematical treatments repeatedly presented in the literature (see, for instance, MacRobbie 1971, Walker and Pitman 1976; see also Thain 1984, for an alternative treatment using unidirectional rate constants in place of compartmental exchange constants; unfortunately, however, the latter approach awaits experimental verification). Applying the above equation to specific cases is relatively straightforward. Fig. 3a, for example, shows how true influx can be

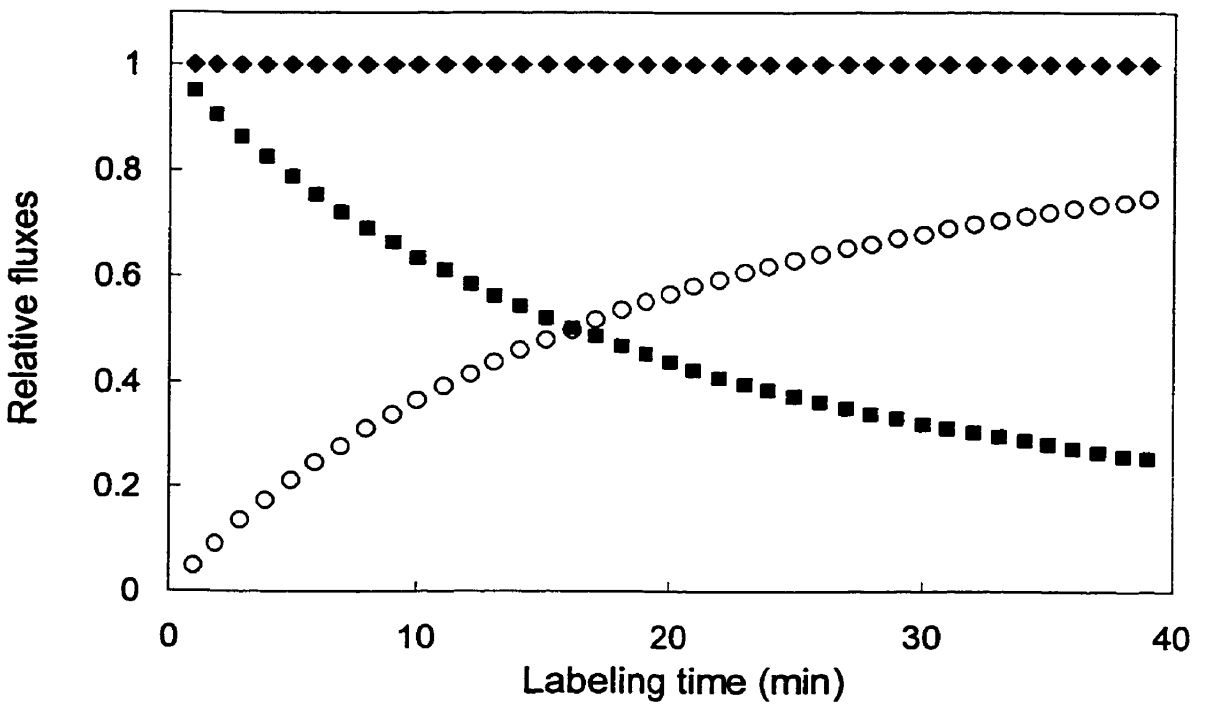

Figure 3a. Graphical depiction of the effect of efflux on measurement of influx as a function of labeling time. In this example, a root system with a cytosolic exchange half-life of 7 min and an efflux:influx ratio of 1 is shown. For clarity, no desorption period is considered here (but see Fig. 3c). ◆, "true" influx; ■, net tracer influx; □, tracer efflux.

underestimated in a labeling experiment due to efflux removing tracer from the cytosol during labeling. Fig. 3b shows how the extent of this deflection depends on k_c and on the magnitude of efflux relative to influx (i.e., the efflux:influx ratio). In this figure, three efflux:influx ratios are modelled against two experimentally determined values of k_c (Siddiqi *et al.* 1991, Kronzucker *et al.* 1995a,b), showing the interaction between these compromising factors. In the most dramatic instance, a transport pattern with a k_c of 0.1 min^{-1} ($t_{½}$ of 7 min) and an efflux:influx ratio of 1.0, true influx is underestimated by 37% after a labeling period of 10 min, even without subsequent desorption. Desorption periods of 3, 5, and 10 min to clear tracer from binding sites in extracellular spaces add 16, 25, and 40% errors, respectively, to the flux measurement (Fig. 3c). Such underestimates are not trivial and are indeed observed (Kronzucker *et al.* 1996, and references therein). It must be noted, however, that when the half-life of a compartment is long relative to the labeling period, and, especially, when the efflux:influx ratio is small, errors due to efflux will also be small; in the case of a 14-min half-life (k_c = 0.05 min^{-1}) with an efflux:influx ratio of 0.1, for instance, the error for a 10-min flux is expected to be only 5%, even with a 10-min desorption period. Moreover, errors due to desorption can be minimised by applying the kinetic exchange constant for the apparent free space of the tissue (k_{AFS}), which, conveniently, is also determinable in efflux analysis. The impact of tracer carried over in this space can be modelled by considering that it too will diminish exponentially according to an equation analogous to that describing cytosolic tracer loss:

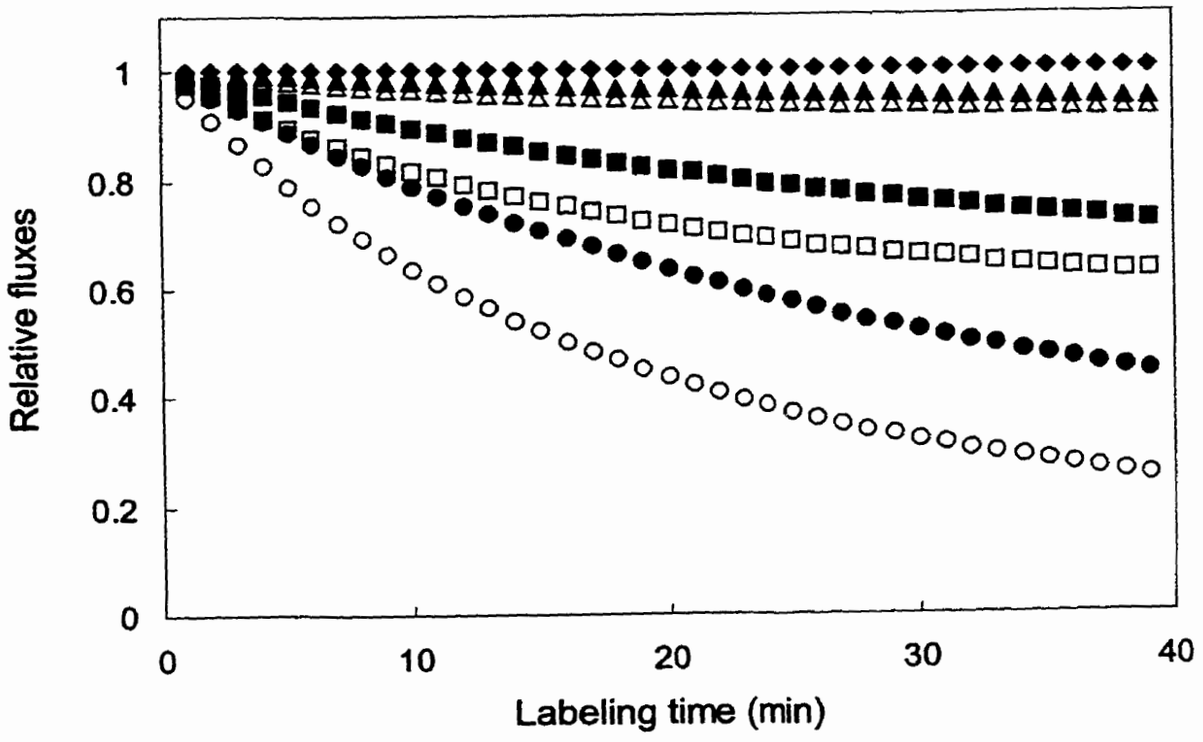

Figure 3b. The model as applied to systems with half-lives of 7 (Δ, □, ○) and 14 min (▲, ■, ●), and at efflux:influx ratios of 0.1 (Δ, ▲), 0.5 (□,■), and 1.0 (○,●). Again, desorption was not considered.

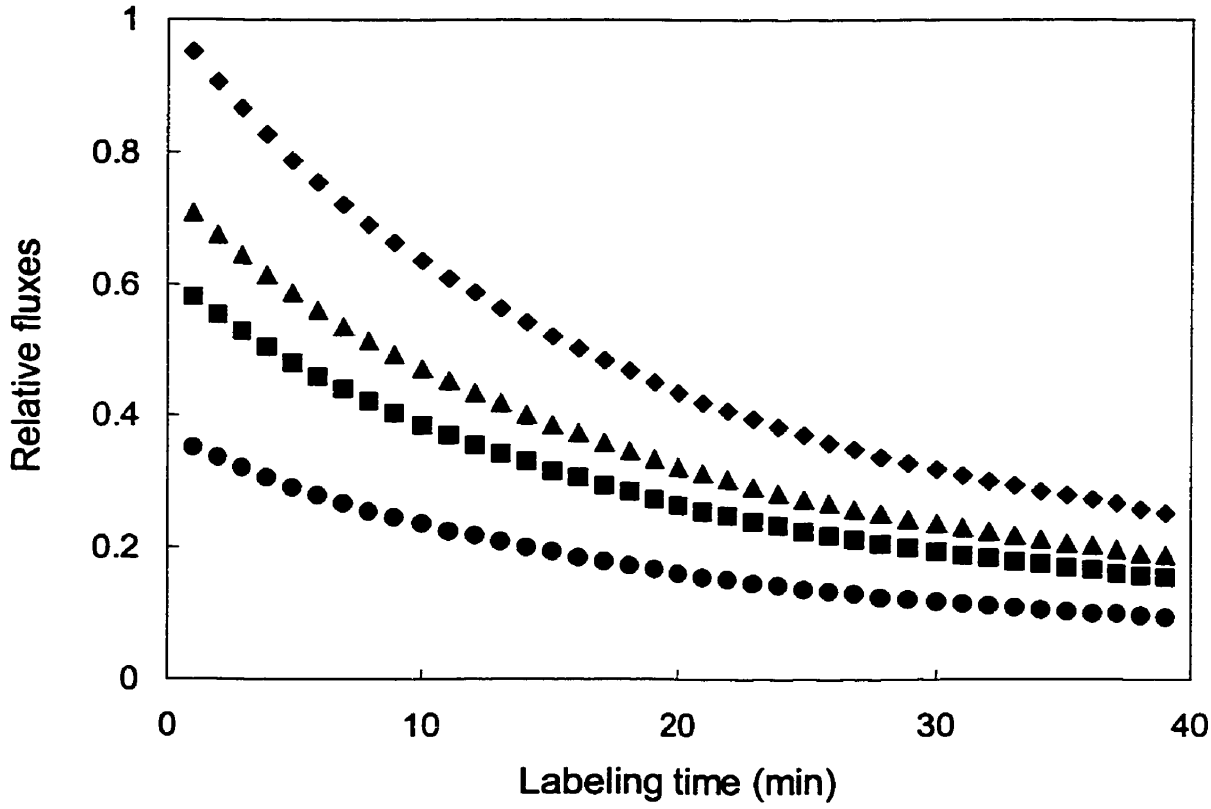

Figure 3c. The effect of varying periods of desorption on the system shown in Figure 3a. ♦, no desorption; ▲, 3 min desorption, ■, 5 min desorption, ●, 10 min desorption.

$$Q^{*}_{AFS} = Q^{*}_{AFS,\max} e^{-k_{AFS} t} \qquad (3.4)$$

where Q^{*}_{AFS} is the tracer quantity in the apparent free space at time t, and $Q^{*}_{AFS,\ max}$ is the maximal tracer content that can be attained in this matrix. Experimental design may thus be optimised to include a desorption period no longer than required for the elimination of excessive extracellular tracer content.

As emphasized above, the analysis of influx underestimates is further complicated if delivery fluxes to the cytosol from internal sources, rather than from the tracer-rich external medium, are present. When unlabeled, internal fluxes such as from the vacuole (ϕ_{vc}) or other endogenous pools (Feng *et al.* 1999), will lower the specific activity of the cytosolic pool, and therefore will lessen the problem of tracer efflux during influx measurement procedures. In the following example, fluxes to the tonoplast experimentally-determined $t_{½}$ values for vacuolar NH_4^+ exchange from Macklon *et al.* 1989 are used (after compartment reassignment following rationale presented in Kronzucker *et al.* 1995c; this vacuolar $t_{½}$ value of approximately 100 min has been tentatively confirmed by results using ^{15}N, not shown), and vacuolar pool sizes estimated from total tissue analysis (Wang *et al.* 1993a, and results not shown), to calculate tonoplast fluxes, thereby permitting a modelling of tracer rise in both cytosol and

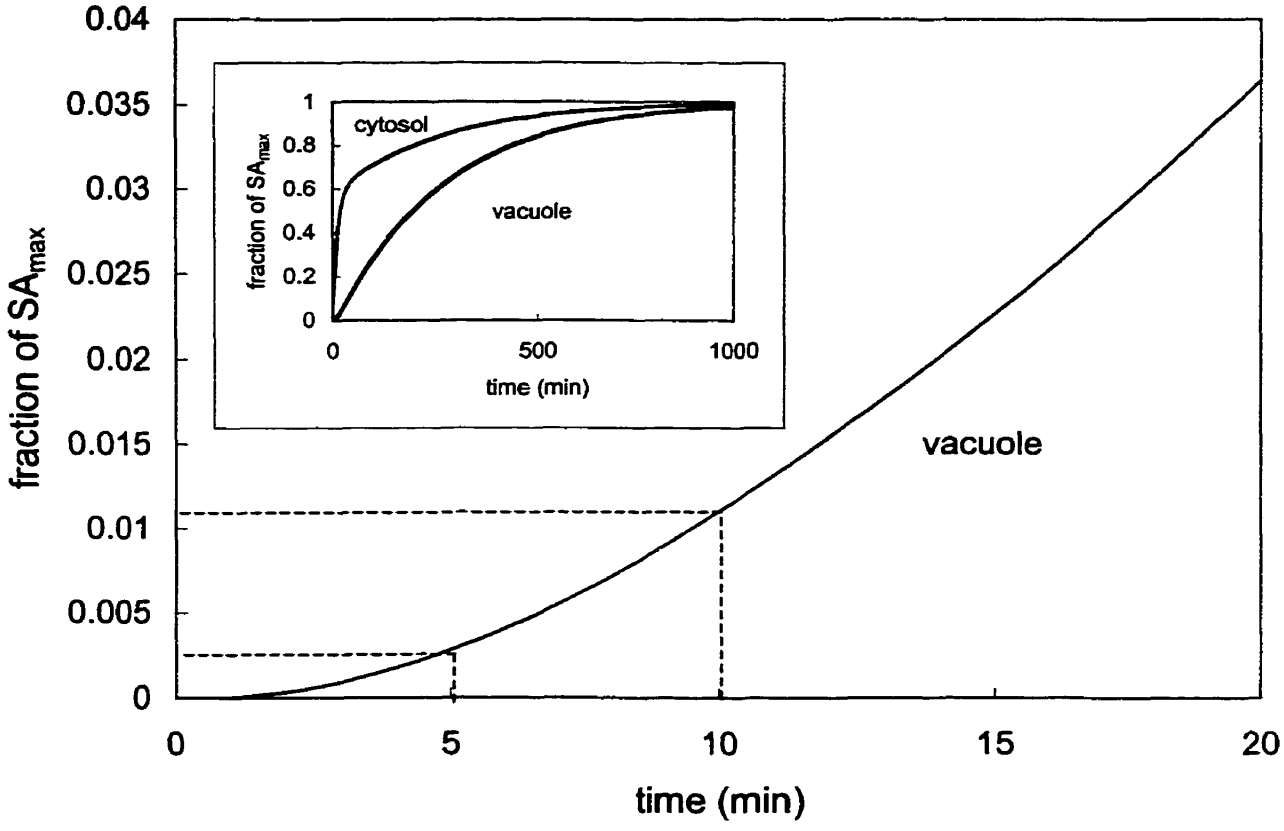

Figure 4. Demonstration of the sigmoidal nature of tracer activity rise in the vacuole, resulting from the compound exponentiality of tracer increase in the cytosolic pool which labels the vacuole (inset). Maximal specific activity in either compartment is designated SA_{max} (=1). The fraction of SA_{max} attained after labeling times of 5 and 10 min are as indicated by broken lines. Data used for model are discussed in the text.

vacuole (Fig. 4). Under the assumption that tonoplast fluxes are equal in both directions (i.e., $\phi_{cv} = \phi_{vc}$), a form of eq. 2.3

$$\phi_{cv} = \frac{[NH_4^+]_v}{\Omega t_{½}} \tag{3.5}$$

was applied, where ϕ_{cv} is expressed in μmol g^{-1} h^{-1}, $[NH_4^+]_v$ (the vacuolar ammonium concentration) in mM, and $t_{½}$ in min. Using these units with an assumption of the vacuolar compartment comprising 90% of the cell volume, Ω comes to 0.0265. Given a $t_{½}$ of 100 min, and $[NH_4^+]_v$ of 9.05 mM, measured for the model system barley grown at an ecologically relevant concentration of 0.1 mM $[NH_4^+]_o$, ϕ_{vc} comes to 3.42 μmol g^{-1} h^{-1}. Fig. 4 (inset), drawing upon these calculations and a measured ϕ_{oc} of 5.46 μmol g^{-1} h^{-1}, indicates that an initial rapid rise in cytosolic activity has superimposed upon it a much slower rise resulting from eventual accumulation and back-delivery of label in and from the vacuole. The initial rise will tend towards a first asymptote (see above), the value of which is determined by the ratio of ϕ_{oc} to (ϕ_{oc} + ϕ_{vc}) (Lee and Ayling 1993), which in this case is 0.61. This ratio should then, in the equation presented above, be included as a multiplier to the collective term (B + C), accordingly reducing the impact of ϕ_{co}^* on influx measurements, and reflecting more realistic experimental conditions:

$$\phi_{oc}^* = \left\{ A + \frac{\phi_{oc}}{\phi_{oc} + \phi_{vc}} \phi_{co}^* \left[t_L - \frac{1}{k_c} \left(e^{-k_c t_D} - e^{-k_c (t_L + t_D)} \right) \right] \right\} \Big/ t_L . \tag{3.6}$$

Even when tonoplast fluxes are large compared to influx across the plasmalemma (as in this example, but *cf.* MacRobbie 1971), back-delivery of tracer from the vacuole into the cytosol (and hence available for efflux during labeling) will be negligible, as specific activity in the vacuole will have reached only 1.1% of its maximal value by the end of a 10-min labeling period (Fig. 4). As illustrated in Fig. 4, the specific activity in the vacuole rises sigmoidally due to the requirement that the pool which labels it, i.e. the cytosol, is itself only gradually labeled. Interestingly, this entails a discrepancy in the kinetics of vacuolar labeling vs. those of specific activity decline in this compartment in washout experiments, where it approximates the form of a simple exponential. However, because the cytosolic and vacuolar compartments are in series (Walker and Pitman 1976), during the early phases of elution, some, finite, amounts of tracer will still be delivered to the vacuole, effectively (albeit marginally) elevating the intercept of the vacuolar regression line, and causing a marginal deviation from first-order elution kinetics (EAC MacRobbie, pers. comm.). The extent of these deviations will be diminished with prolonged loading times and with increased difference between cytosolic and vacuolar half-lives. In the present example, $t_{½}$ for the vacuole was determined in washout experiments to be 100 min, while the apparent half-life of labeling for this compartment is approximately double. From the perspective of influx determinations, the suppressed rise in cytosolic specific activity, and minimal back-delivery from the vacuole, under such conditions, can only enhance the accuracy provided by a standard 5- to 10-min influx measurement.

In summary, efflux analysis for a given steady-state condition must precede any kinetic evaluation of influx processes, in order to minimise, or at least quantify, errors associated with tracer efflux during labeling and desorption. Similar reasoning by Cram (1969, 1973) led to the formulation of a guideline which stated that the time period of labeling for a given solute should not exceed 0.31 times the cytosolic half-life of exchange for that solute, in order to ensure that influx not be underestimated by more than 10%. However, analyses such as that presented in Fig. 3 show that this rule pertains only to the specific condition of a system having an efflux:influx ratio of 1, and having ϕ_{oc} much greater than all other fluxes into the cytosol, and hence is overly generic. In fact, partly due to the indiscriminate application of this guideline, Lee and Ayling (1993) came to the startling conclusion that the half-life of cytosolic NH_4^+ is so short that accurate influx measurements, at least of this ion, are essentially impossible to make. This analysis, while providing some explanation of unusual data[2], is erroneous in several ways, and, due to its wide-ranging implications, must be addressed here. Most problematically, the $t_{½}$ for cytosolic NH_4^+ exchange was calculated only indirectly from flux data and NMR-derived estimates of pool size (Lee and Ratcliffe 1991), rather than from direct measurements such as more recently

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[2] The authors observed substantial decreases in ammonium fluxes when methionine sulphoximine (MSO)-treated barley plants were labeled for increasing lengths of time; however, interpretations of NH_4^+ flux data under MSO treatment remain problematic and should not be used to establish mechanisms underlying transport (Platt & Anthon 1981, Feng et al. 1994, Kronzucker et al. 1995c, Wieneke & Roeb 1998).

made using efflux analysis (Wang *et al.* 1993a, Kronzucker *et al.* 1995c, Min *et al.* 1999, and the present work); the latter show that the cytosolic $t_{½}$ for NH_4^+ exchange in the presence or absence of MSO is much longer than the indirect estimate would suggest, and is not very different from the values used in the model presented above. Moreover, the conditions under which Lee and Ayling's experiments were conducted (1.5 mM external NH_4^+) are not expected to yield an excessively high efflux:influx ratio (Wang *et al.* 1993a, Kronzucker *et al.* 1995c, and the present work). The flux discrepancies between short- and long-term measurements reported in their work, then, cannot be attributed to the increasing significance of an efflux term, and it must be reiterated that evaluations of this sort must include *direct* measurements of efflux and $t_{½}$ terms[3]. In contrast to the conclusions drawn by Lee and Ayling (1993), a consideration of efflux data in this model system indicates that measurement of unidirectional ammonium influx is quite feasible under steady-state conditions bearing modest efflux:influx ratios.

2. Perturbation Conditions

Because the above analysis and the parameters derived from efflux experiments pertain to steady-state conditions, a simple extension of the model to include perturbation conditions is not legitimate. Most interpretations of influx

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[3] Interestingly, in the case of inorganic nitrogen exchange, the apparent constancy of cytosolic $t_{½}$ vis-à-vis changing conditions of N-supply (Kronzucker et al., 1995; also see Chapter 6) renders this task somewhat simpler, but still necessitates measurements of efflux for the determination of flux ratio.

isotherms imply that the measured flux at each concentration represents the condition of the influx transporter at the instant a new ion concentration is imposed. Indeed, since influx isotherms so frequently conform to Michaelis-Menten patterns such as those observed with purified enzyme preparations (Epstein 1966), the assumption that they can yield direct information about the kinetic properties of transport systems is rarely questioned (but see Cram 1974, for an exception). However, because any plant whose concentration-dependence response is analyzed is at steady state with respect to only one concentration, i.e. the concentration it was cultured at, varying degrees of deflection from steady state prevail across the experimental concentration profile and thus results may be confounded by varying degrees of concurrent efflux.

Interpretations of concentration-dependence influx profiles are further undermined by our limited knowledge of the short-term changes in efflux, cytosolic turnover, individual subcellular fluxes, and cellular energetics that occur when a shift in external concentration is imposed. We do know that concentration shifts can cause substantial and rapid changes in essential parameters such as membrane electrical potential (Ullrich *et al.* 1984, Ayling 1993, Wang *et al.* 1994, Crawford and Glass 1988) and cytosolic pH (Kosegarten *et al.* 1997). These changes occur over a time scale of seconds to minutes and must affect the thermodynamic conditions that influence ion fluxes across the plasma membrane. Moreover, the spatial configuration, and hence the substrate affinity (K_m), of transport proteins, cannot be assumed to be unaffected by changes in membrane energization and substrate gradients. We cannot therefore rule out

the possibility that influx isotherms result not purely from the variation of one parameter (i.e., external concentration), but from a suite of responses manifest at the cellular and membrane levels. In turn this may entail that true influx incident upon, and responding to, the imposed concentration shift, is not captured.

The significance of these considerations is substantiated by the data illustrated in Fig. 5, which shows that $^{13}NH_4^+$ efflux from pre-labeled root tissue changes immediately and pronouncedly following both upward and downward shifts in external NH_4^+ concentration. Again, it would be unreasonable to assume that in the presence of such changes, influx will not also undergo transient alterations. While analysis of these flux transients is difficult in the absence of knowledge of changes in solute pool sizes, and hence specific activities, in the cytosol during transition, some semi-quantitative interpretations are possible. It is well established that in the long term the capacity of plants to take up and assimilate nutrients responds powerfully to changes in nutrient status. Flux capacity measured at a given concentration is gradually upregulated in the transition to a lower nutrient provision condition, while it is downregulated when the transition is upward (Lee and Rudge 1986, Wang *et al.* 1993b, Morgan and Jackson 1988a,b, Kronzucker *et al.* 1998). Compartment sizes of nitrate and ammonium have also been observed to increase or decrease over the long term with increasing or decreasing external nutrient provision, respectively (Siddiqi *et al.* 1991, Wang *et al.* 1993a, Kronzucker *et al.* 1995a,b; but cf. Miller *et al.* 1996).

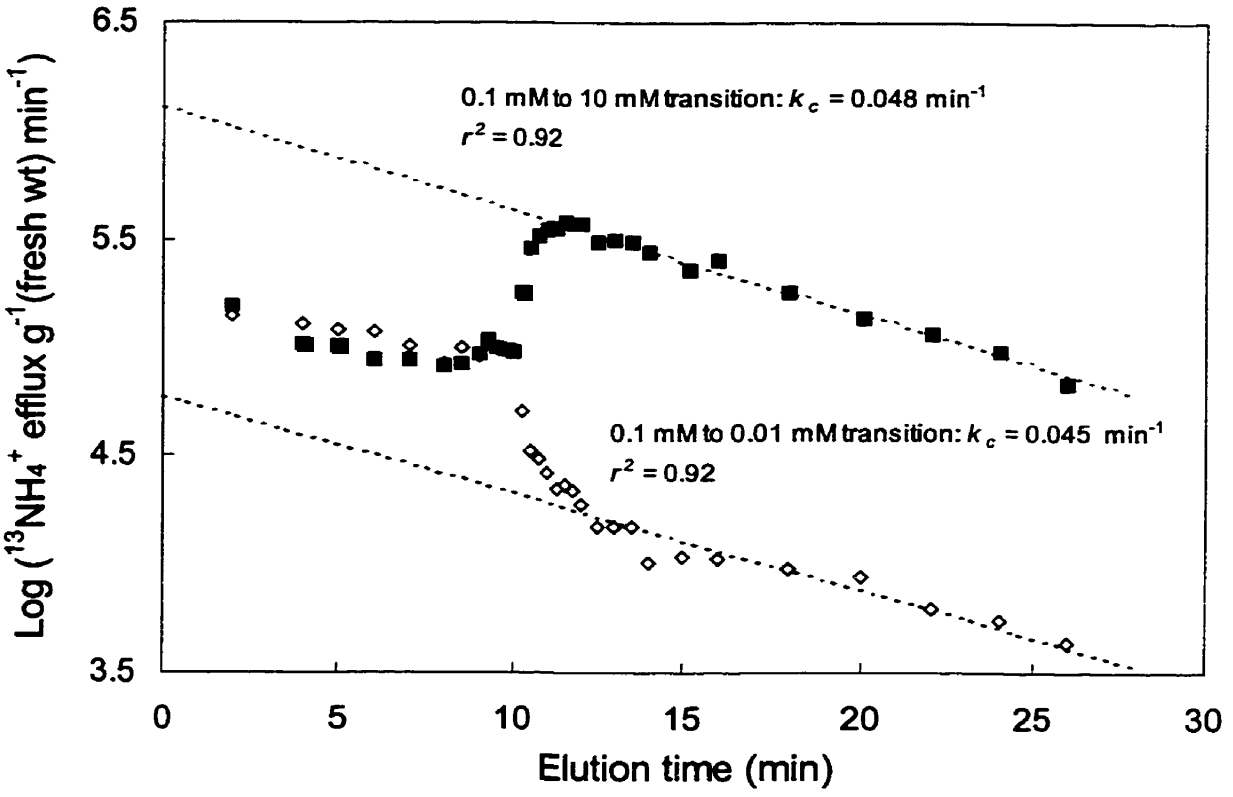

Figure 5. Patterns of $^{13}NH_4^+$ efflux from roots of barley (cv. Klondike) seedlings with 0.1 mM NH_4^+ (the growth-medium concentration) provided initially, followed by the imposition of a concentration shift from 0.1 to 10 mM (upper line) and from 0.1 to 0.01 mM (lower line). Note the return to apparent first-order exchange kinetics after 5 min following perturbation.

A relatively large cytosolic compartment size, in combination with a relatively downregulated influx, will dilate the period of time required to achieve a new steady state when a stepping-down in external concentration is imposed. Conversely, a new steady state will be achieved relatively quickly in an upward transition, due to an upregulated influx which will facilitate a fast rise in the cytosolic pool. Such predictions of differential cellular behavior have been borne out by experimental observations (Kronzucker *et al.* 1998, and references therein), suggesting that perturbational measurements of short-term influx are inherently more distorted as the measuring concentration increases at levels above the steady-state growing condition.

Most interesting, however, are the short-term concentration-dependent changes evident in modulated tracer efflux (Fig. 5). After an initial phase of adjustment, the rate of tracer elution returns to first-order exponential decline characteristics similar to those seen prior to the concentration shift. Because the decline in tracer elution rate subsumes all internal fluxes removing tracer from the cytosol, this surprisingly rapid return to a robust and apparently preset k value strongly suggests that the plant has shifted into what could be termed a *transitional steady state.* Importantly, this is the condition that the plant is usually in when influx is measured with a protocol including an equilibration step of several minutes preceding the influx measurement (see below and, e.g., Siddiqi *et al.* 1990), but clearly this measurement neither represents instantaneous influx into plant cells upon concentration transition, nor reflects the final steady state at the newly imposed concentration.

Since a transitional steady state is achieved by the plant system within minutes of exposure to a new concentration, efflux-derived errors incurred during influx measurement can be minimised by introducing a standard equilibration step (prewash), lasting for the duration of the adjustment period, for each concentration with an unlabeled solution at that concentration (see, e.g., Siddiqi *et al.* 1990, Kronzucker *et al.* 1995, 1996). Knowledge of the k value and the time course of its resumption would then permit the application of Cram's guideline (see Part 1) to ensure that, if such a protocol is followed, measurement error due to simultaneous efflux will in no case exceed 10% (i.e., the error associated with a efflux:influx ratio of 1). In the light of these considerations, commonly used influx procedures involving pretreatment in solutions of chemical composition other than that of the measuring solution (e.g. solutions containing only $CaSO_4$) should be avoided, since in this case an additional perturbation would be imposed upon the system, whose influence upon influx measurements is difficult to evaluate without prior, and substantial, experimental effort.

Conclusions

1) A comprehensive mathematical model is presented to predict errors inherent in measurements of ϕ_{oc} under steady-state conditions, which does not necessitate the complicated mathematical treatments introduced elsewhere. Key to this model is the experimental determination of k values for ion exchange with the cytosolic compartment, and of efflux:influx ratios, by means of efflux analysis.

2) The conclusion drawn by Lee and Ayling (1993), that the influx of NH_4^+ under steady-state conditions is not measurable, is shown to be incorrect.

3) Because the *k* constant subsumes all fluxes contributing to pool turnover, the *k* value describing the rise of tracer activity in the cytosol to an initial plateau is identical to the *k* value determined from the slope of decline in tracer efflux from this compartment as determined in washout experiments.

4) By contrast, the time course of rise in tracer activity in the vacuole is a sigmoidal process and can only be described if both vacuolar and cytosolic *k* values are known. It takes significantly longer to label the vacuole than it takes to de-label it.

5) In the presence of internal delivery fluxes (e.g. fluxes from the vacuole), the specific activity of the cytosol cannot reach the specific activity of the external medium until all such delivery pools have themselves become completely labeled. However, the initial plateau to which tracer activity in the cytosol tends can be estimated.

6) Tracer activity carried over in the cell wall after a labeling period, and hence potentially compromising measurements of ϕ_{oc}, can be accurately determined, if the exchange characteristics of this compartment have also been determined by efflux analysis. In practice, the inclusion of a desorption step following labeling can reduce this tracer contribution, but tracer efflux from the cytosol during this time must be accounted for, as presented in the model.

7) In concentration shifts, as are inherent in influx isotherms, a rapid return to the pre-perturbational value of k_c is observed, which signifies a transitional steady state under which ϕ_{oc} again becomes measurable.

8) However, instantaneous ("true") influx at the time of concentration transition is compromised by rapid changes in other fluxes, notably efflux, rendering ϕ_{oc} not amenable to measurement during this period of adjustment, which is shown, in the case of NH_4^+, to be complete within approximately 5 min following the onset of the concentration shift. Procedurally, the inclusion of an equilibration step preceding the actual flux assay is recommended, the duration of which should at least equal the adjustment period.

9) These analyses emphasise the necessity for caution in making inferences about the mechanistic properties of transport proteins from measurements of ϕ_{oc}. Nevertheless, ϕ_{oc}, measured over a brief interval, and following the establishment of a transitional steady state, is undoubtedly useful, especially for comparative purposes, and particularly in an ecophysiological context.

Chapter 4. Compartmental Analysis by $^{13}NH_4^+$ Efflux in Leaves of Higher Plants

Introduction

In recent years, ^{13}N tracer studies have provided increasing insight into the nature of nitrogenous fluxes and pools in subcellular compartments of plant root systems (e.g. Kronzucker *et al.* 1999, and references therein). However, none to date have explored these phenomena in cells of leaf systems. Little is known about membrane fluxes or subcellular compartmentation of ionic forms of nitrogen in intact leaf cells, although these characteristics have been determined for other ions by use of leaf slices (pioneered by Smith and Epstein 1964, and Rains 1967, 1968; see also Jeschke 1976, for an insightful review), protoplasts (Leonard and Rayder 1985), and cells of *Asparagus* cladophylls (Bown 1982).

Movement and accumulation of ammonium (NH_4^+) in leaf cells are of particular interest because of the numerous processes that can liberate and assimilate this ion within the leaf (Joy 1988, Howitt and Udvardi 2000). NH_4^+ is assimilated in leaf tissue chiefly through the plastidic isoform of glutamine synthetase (GS_2) (Lam *et al.* 1996), while the sources of NH_4^+ in leaves are dominated by endogenous generation via the photorespiratory nitrogen cycle, which can produce up to 10 times as much NH_4^+ as is incorporated by the plant through primary assimilation (Givan *et al.* 1988, Leegood *et al.* 1995). Amino acid and protein catabolism is another local source of leaf NH_4^+ (Joy 1988, Howitt and Udvardi 2000), and lignin biosynthesis may also release NH_4^+ (Razal *et* al. 1996,

Nakashima *et al.* 1997). In addition, there is mounting evidence that NH_4^+ can be delivered *per se* from root to shoot in the translocation stream (Finnemann and Schjoerring 1999). Finally, plant leaves can acquire or emit atmospheric NH_3, depending on internal and environmental factors (Farquhar *et al.* 1980), further emphasizing the need to characterize NH_3/NH_4^+ flux processes in leaves at the cellular level.

Transport systems in the leaf plasma membrane are required to take up NH_4^+ from the xylem stream, as well as for the release and recapture of NH_4^+ lost from the cell after its intracellular production. Raven and Farquhar (1981), using the NH_4^+ analogue methylammonium in leaf slices of *Phaseolus vulgaris*, suggested that NH_4^+ transport in leaf cells may be mediated by an electrogenic uniport mechanism, as has been postulated for low-affinity transport in roots of rice (Wang *et al.* 1994). More recently, Nielsen and Schjoerring (1998) observed very high, concentration-dependent, bi-directional fluxes in leaf discs of *Brassica napus*, one net result of which was the maintenance of apoplastic NH_4^+ levels within a narrow concentration range. Indeed, several genes encoding putative NH_4^+ transporter proteins have now been identified in leaves on the basis of homologies with root transporters (von Wiren *et al.* 2000).

The experiments reported here used the positron-emitting radiotracer ^{13}N and the method of compartmental analysis by efflux (CAE) (Walker and Pitman 1976, Lee and Clarkson 1986, Siddiqi *et al.* 1991) to provide estimates of unidirectional plasma membrane fluxes (influx and efflux), quasi-steady net fluxes (i.e. the difference between traced influx and efflux), cytosolic turnover

constants, and cytosolic pool sizes of NH_4^+ ($[NH_4^+]_c$) in leaf slices of three plant species, wheat, rice, and tomato. To characterize a wider range of plant responses in a single species with this system, wheat leaf slices were tested against concentrations of NH_4^+ varying over four orders of magnitude, and under both light and dark conditions, and also with the glutamine synthetase-inhibitor methionine sulfoximine (MSX) (Kronzucker *et al.* 1995e, Wieneke and Roeb, 1998). Some experiments were performed to verify the methodology, because of the tissue damage that inevitably results from preparing leaf slices, and because of the subsequent imposition of non-steady-state extracellular conditions. The three species were compared because the substantial differences in their abilities to tolerate NH_4^+ as a sole nitrogen source are well documented. Rice performs exceptionally well on NH_4^+ (Wang *et al.* 1993a, Kronzucker *et al.* 1999), wheat less so (Deignan and Lewis 1988), and tomato shows symptoms of toxicity under NH_4^+ nutrition (Wilcox *et al.* 1977, Magalhaes and Wilcox 1983, 1984, Magalhaes and Huber 1989, Gerendas and Sattelmacher 1990).

Materials and Methods

Please refer to Chapter 2.

Results

Fig. 6 is a representative semi-logarithmic plot of the change, with time, in $^{13}NH_4^+$ (tracer) release rate from labeled leaf tissue of 8-d-old wheat seedlings. Two to three kinetically distinct phases of exponentially declining tracer efflux were resolved in every experiment. Treatment of efflux and tracer retention data,

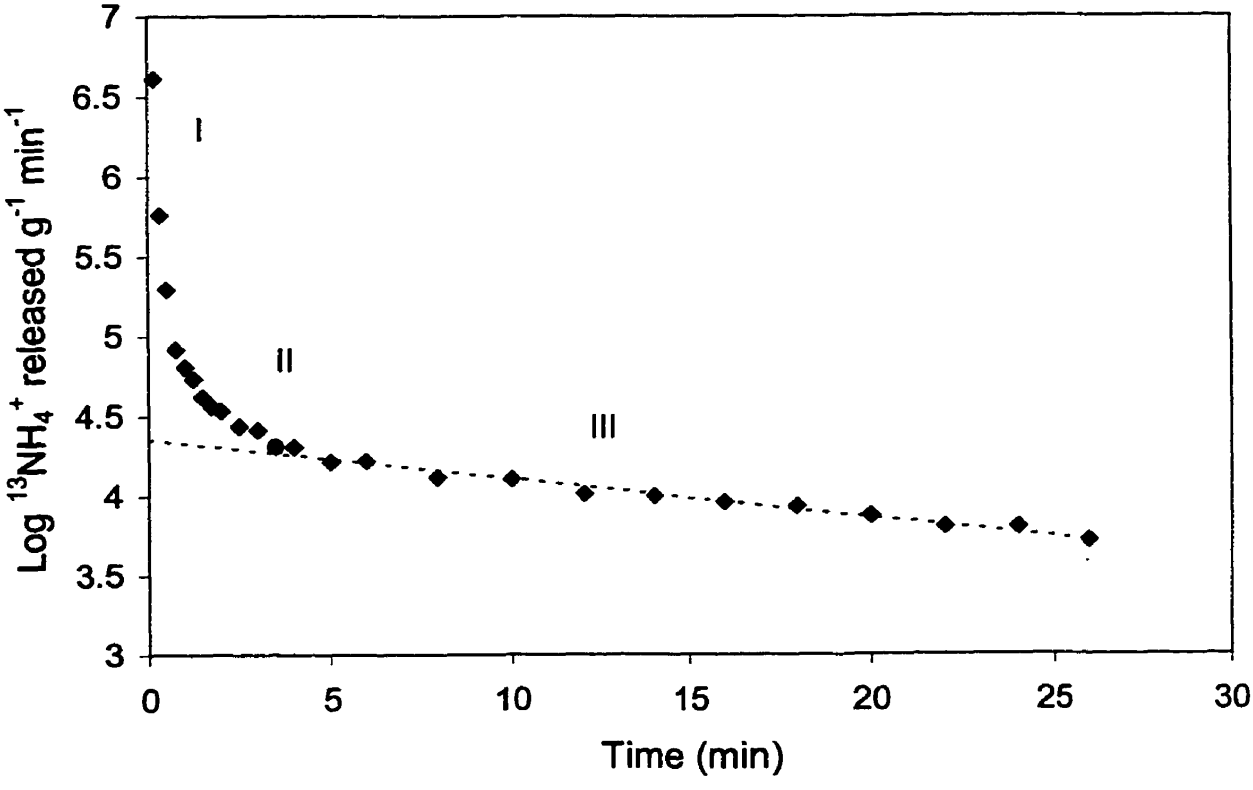

Figure 6. Representative semi-logarithmic plot of $^{13}NH_4^+$ efflux from leaf slices of wheat. Slices had been pretreated for 3 h in non-radioactive uptake medium containing 10 mM NH_4^+ prior to exposure to ^{13}N under the same conditions for a subsequent hour. The broken regression line is assumed to represent tracer released from the cytosolic phase (phase III) of the labeled preparation (see text).

according to the assumption that the compartment releasing phase III tracer corresponds to the leaf cytosol (see Discussion), yielded values for NH_4^+ influx and efflux under a wide range of external NH_4^+ concentrations ($[NH_4^+]_o$). Table 1 shows these values for wheat leaf slices treated with 0.01, 0.1, 1, and 10 mM $[NH_4^+]_o$, and also shows their response to treatments with MSX, or to treatment in the dark. A strong trend of increasing NH_4^+ fluxes was observed in the decadal concentration series from 0.01 to 10 mM $[NH_4^+]_o$. Influx at the highest concentration was 259 times higher than at the lowest, while efflux increased 1700-fold along this gradient. This disparity in the rates of change of efflux and influx underlies the dramatic increase in the efflux:influx ratio, from a low of 0.14 at 0.01 mM to a peak of 0.91 at 10 mM. Treatment of leaf slices at 0.1 mM NH_4^+ with 1 mM MSX increased influx by nearly 50% over controls, and increased efflux by 300%. MSX treatment at 10 mM $[NH_4^+]_o$ had a similar, but less pronounced effect, on the already high values of the controls. MSX increased $[NH_4^+]_c$ by 300% and 13% over controls in 0.1 mM and 10 mM NH_4^+ treatments, respectively (not shown). Dark treatments at 0.1 mM also produced similar results, with a 50% increase in influx and a 240% increase in efflux.

Fig. 7 shows the depletion of unlabeled NH_4^+ observed when wheat leaf slices were exposed for extended periods to an uptake solution containing 0.1 mM NH_4^+. Uptake was tracked over 30- to 50-min periods at various times following excision of tissue. The rate of decrease in $[NH_4^+]_o$ after the first 20 to 60 min (0.92 ± 0.07 μmol gfw^{-1} h^{-1}) following cutting was not significantly different from that seen 30 h later (0.94 ± 0.11 μmol gfw^{-1} h^{-1}). The average rate

Table 1. Influx (ϕ_{oc}), efflux (ϕ_{co}), and efflux:influx ratios (ϕ_{co}:ϕ_{oc}) of ^{13}N-labeled NH_4^+ in leaf slices of wheat. Fluxes are expressed as μmol g $^{-1}$(fresh wt) h^{-1}. Values are means ± 1 SD of 4-8 experiments, except for MSX and dark experiments where n = 2 (or n = 3 for 0.1 mM NH_4^+ + MSX).

	external NH_4^+ concentration (mM)						
	0.01	0.1	1	10	0.1 (+MSX)	10 (+MSX)	0.1 (dark)
ϕ_{oc}	0.37 ± 0.05	1.93 ± 0.75	11.5 ± 1.5	93.1 ± 26.4	2.79 ± 0.42	95.9 ± 4.0	2.73 ± 0.15
ϕ_{co}	0.05 ± 0.01	0.49 ± 0.12	6.9 ± 1.9	85.1 ± 26.8	1.45 ± 0.23	93.5 ± 6.6	1.17 ± 0.08
ϕ_{net}	0.32 ± 0.05	1.44 ± 0.68	4.58 ± 0.83	8.01 ± 3.73	1.23 ± 0.19	2.45 ± 2.61	1.56 ± 0.07
ϕ_{co} :ϕ_{oc}	0.14	0.25	0.60	0.91	0.52	0.97	0.43
$[NH_4^+]_c$	2.58 ± 1.02	13.5 ± 7.8	64.8 ± 5.7	400 ± 33	44.9 ± 35.6	452 ± 13	20.0 ± 1.5

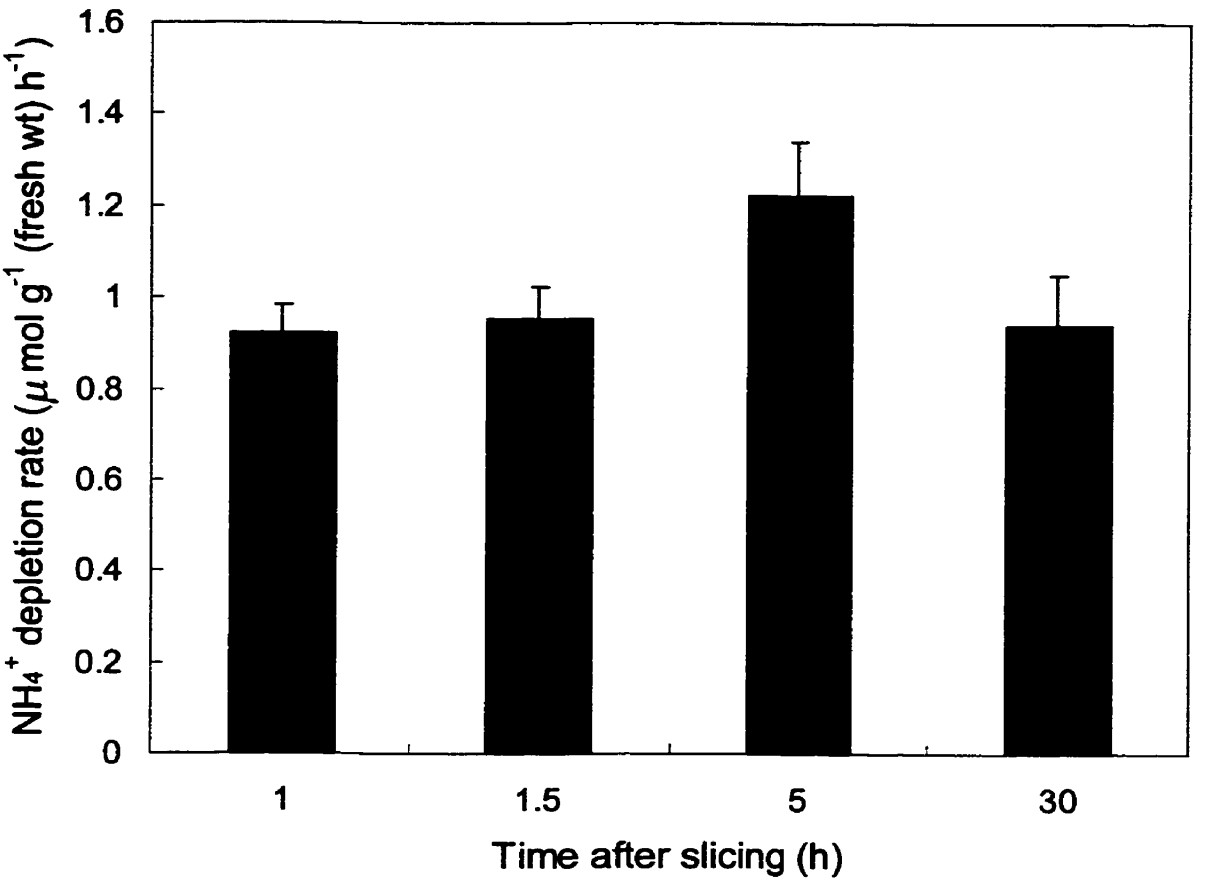

Figure 7. Ammonium depletion (net uptake) rate of wheat leaf slices at 0.1 mM NH_4^+ over 30 h following preparation of slices. The *x*-axis gives the termination of the period over which NH_4^+ depletion was assayed by sub-sampling of the uptake medium. Error bars represent means ± 1 SD (n = 6).

of NH_4^+ acquisition in these trials was 1.00 ± 0.15 μmol gfw^{-1} h^{-1}. For rice leaf slices, the uptake rate was slightly higher than wheat, averaging 1.48 ± 0.11 μmol gfw^{-1} h^{-1} at 0.1 mM $[NH_4^+]_o$, and did not change significantly within the first 6 h following excision (Fig. 8).

The identity of the phase-III tracer source as a membrane-bound compartment was tested by use of the membrane-dissolving detergent SDS. Invariably, treatment of wheat, rice, and barley leaf slices with 1% SDS decreased influx, greatly increased the efflux:influx ratio, decreased the half-life of $^{13}NH_4^+$ exchange, and decreased $[NH_4^+]_c$ relative to controls. Fig. 9 shows the results of an SDS experiment with barley leaves at 0.1 mM $[NH_4^+]_o$. Because these results were so pronounced and consistent among other species and at other concentrations, the experiments were not repeated for all treatments.

Tracer released into efflux aliquots was determined to be 90% positively charged using a cation exchange resin, indicating that only a small amount of ^{13}N released was in the form of glutamine and glutamic acid, the earliest products of NH_4^+ metabolism.

Fig. 10 shows CAE results from experiments conducted to compare NH_4^+ flux and compartmentation characteristics in wheat, rice, and tomato leaf slices. The most pronounced differences among species were found in their NH_4^+ fluxes and efflux:influx ratios at two NH_4^+ concentrations (0.1 mM and 1 mM). At both concentrations, rice was able to retain the largest amount of tracer NH_4^+, and had the lowest efflux:influx ratios of the three species. In all cases, this ratio increased with increasing $[NH_4^+]_o$, but in tomato it changed from a moderate

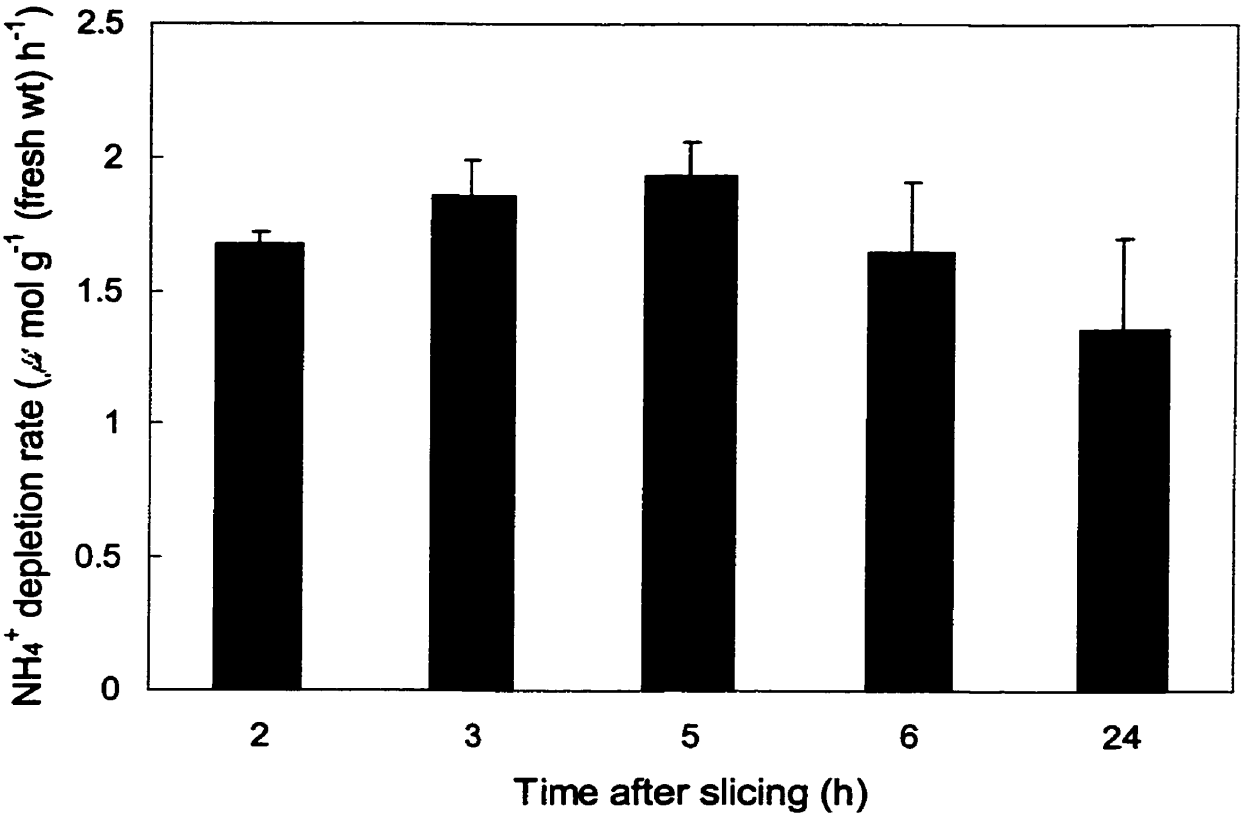

Figure 8. Ammonium depletion rate (net uptake) of rice leaf slices at 0.1 mM NH_4^+ over 24 h following preparation of slices. The *x*-axis gives the termination of the period over which NH_4^+ depletion was assayed by sub-sampling of the uptake medium. Error bars represent means ± 1 SD (n=5).

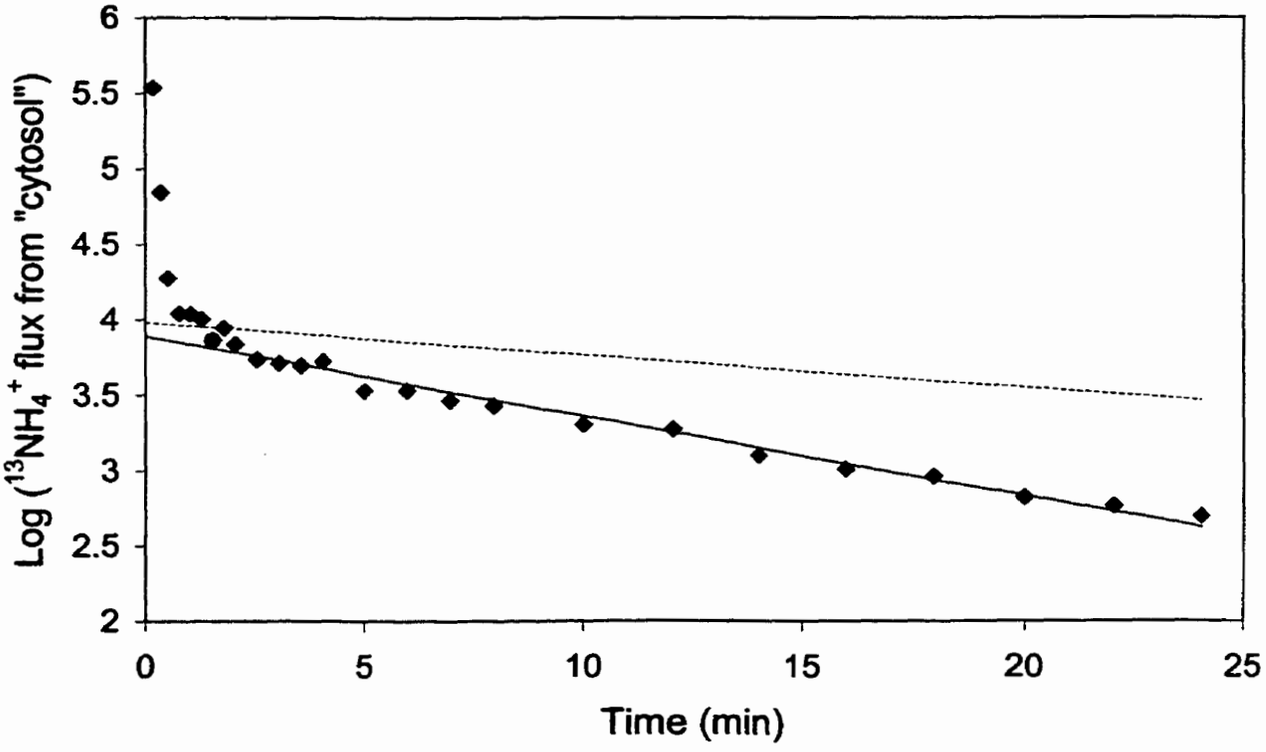

Figure 9. Effect of 1% SDS on tracer flux (0.1 mM NH_4^+) from the compartment hypothesized to be the cytosol of wheat leaf slices. Broken line indicates "phase-III" flux from control material. In this instance, the SDS treatment decreased influx by 20%, increased the efflux:influx ratio to 0.92 (compared to 0.29 in controls), decreased $[NH_4^+]_c$ by 40%, and increased the slope of the phase-III line nearly 3-fold.

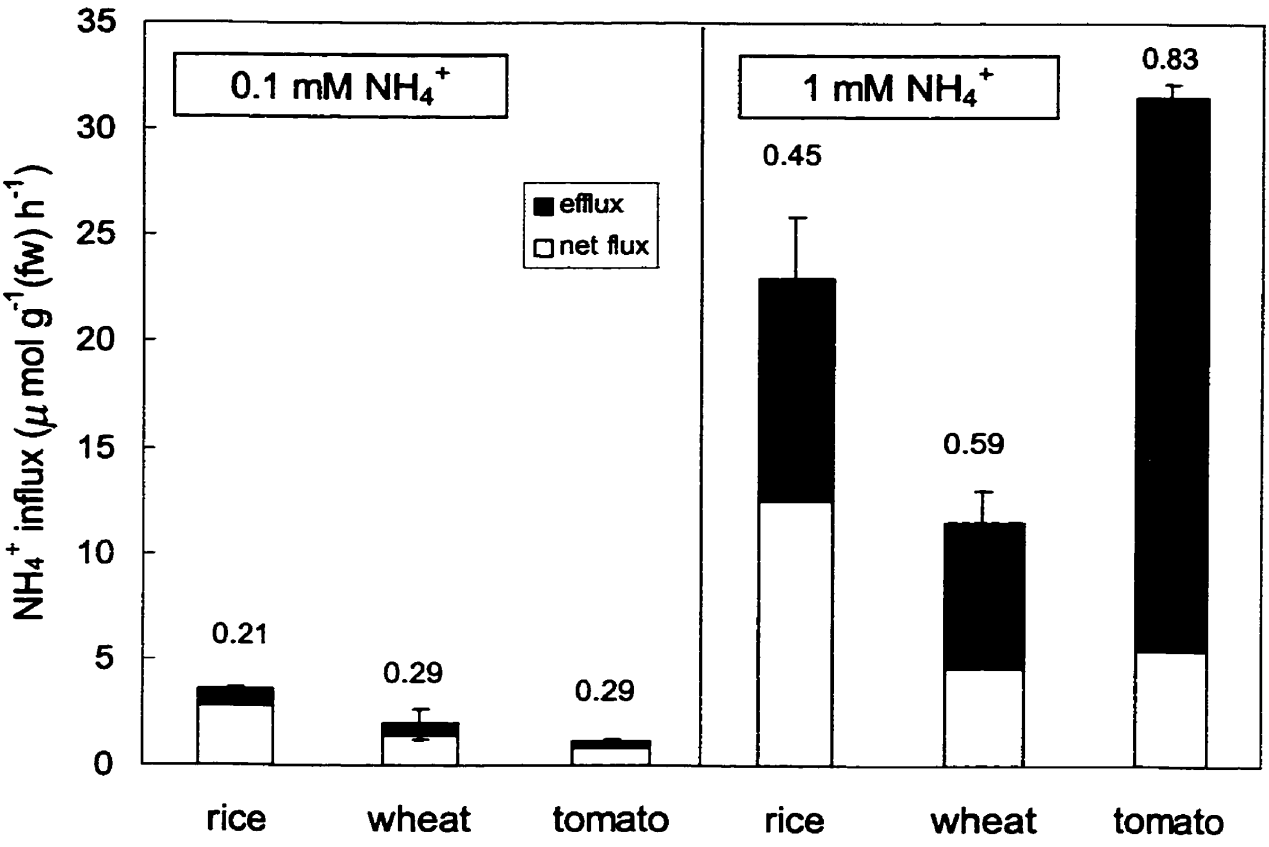

Figure 10. Comparison of fluxes between leaf slices of rice, wheat, and tomato at uptake solution concentrations of (A) 0.1 mM and (B) 1 mM NH_4^+. Bars are broken up into efflux (black segments) and "net flux" (quasi-steady flux to vacuole plus assimilatory fluxes, clear segments), which together comprise the influx term. Error bars represent means of influx values ± 1 SD (for rice and tomato, n = 2; for wheat n = 8 at 0.1 mM and n = 4 at 1 mM. Values above columns indicate ratios of efflux to influx.

value (29%, also seen in wheat) to a very high value (83%), greatly exceeding the ratio in the other species. The absolute magnitudes of both influx and efflux were also highest in tomato under both $[NH_4^+]_o$ conditions.

Table 2 shows half-lives of cytosolic NH_4^+ exchange, which is a measurement of turnover, in the three species, given by the slopes of phase-III regression lines in tracer efflux plots. Values of $t_{½}$ slightly increased in all cases with each ten-fold increase in $[NH_4^+]_o$, except in the case of wheat between 0.01 mM and 0.1 mM $[NH_4^+]_o$, where there was no significant effect. Table 2 also gives $[NH_4^+]_c$, based on $t_{½}$ and influx values, and shows Nernst potentials predicted for the resulting estimates of NH_4^+ concentration differences on either side of the plasma membrane. In all cases, the Nernst potential declined with increasing $[NH_4^+]_o$, except with tomato leaf slices, in which it increased by 18%.

Discussion

Two of the most important requirements to be satisfied in CAE are 1) that the experimental system be at, or near, a steady state with respect to the relevant physiological processes; and 2) that the assignment of compartments releasing tracer are correct. Several lines of evidence suggest that the plant material used in the present work, although subjected to substantial disturbance in preparation for CAE, had nevertheless become adapted to the experimental conditions over the duration of data collection. In most cases, linear regression of phase III in tracer efflux plots (Fig. 6) yielded high coefficients of determination

Table 2. Half-lives ($t_{½}$) of cytosolic NH_4^+ exchange, cytosolic NH_4^+ pool sizes ($[NH_4^+]_c$), and NH_4^+ Nernst potentials in leaf slices from wheat, rice, and tomato. Values are means ± SD of 4-8 experiments for wheat, and 2 experiments for rice and tomato. Nernst potentials were calculated on the basis of cytosolic pool sizes and an ambient temperature of 20°C.

	External NH_4^+ concentration (mM)							
	wheat				rice		tomato	
	0.01	0.1	1	10	0.1	1	0.1	1
$t_{½\,c}$ (min)	14.5 ± 4.0	14.7 ± 4.2	12.2 ± 2.1	9.3 ± 2.3	15.0 ± 1.0	11.4 ± 0.1	11.5 ± 0.7	8.9 ± 0.2
$[NH_4^+]_c$ (mM)	2.6 ± 1.0	13.5 ± 7.8	65 ± 6	400 ± 88	24 ± 1	124 ± 10	6.3 ± 0.1	134 ± 5
Nernst potential (mV)	-141	-120	-107	-94	-140	-123	-106	-125

(typically, $r^2 > 0.9$), suggesting a steadiness of turnover of NH_4^+ in the pool releasing that subset of tracer. The depletion of $^{14}NH_4^+$ by wheat leaf slices, incubated at 0.1 mM $[NH_4^+]_o$, remained substantial and reasonably uniform over a period of time much longer than that required for CAE experiments (Fig. 7), with similar uniformity seen in rice at this concentration (Fig. 8). This indicates that the sum of pertinent rates, i.e. influx and efflux, as well as vacuolar and metabolic fluxes, is reasonably steady over this period. Comparison of net uptake rates in depletion experiments with "net fluxes" observed at these concentrations in CAE experiments (i.e., the differences between influx and efflux in Table 1), however, showed only moderately higher estimates with CAE. This small discrepancy can be explained by the latter ϕ_{net} term representing a traced "quasi-steady" flux which includes assimilation and the "quasi-steady flux to the vacuole" (Cram and Laties 1971), the vacuole being essentially unlabeled over the course of leaf slice incubation with tracer, and hence not accounted for as a source of NH_4^+ efflux.

The broad similarities between the results shown here, and those observed many times with intact roots (see, for example, Wang *et al.* 1993a, 1994, Kronzucker *et al.* 1995a, b, 1999, Min *et al.* 1999), provide additional evidence that leaf NH_4^+ flux systems and compartmentation were near steady state. It is not surprising, despite the very different external conditions to which they are adapted, that similarities exist between the cells of plant roots and shoots, given the common flux and assimilatory processes that have been demonstrated in the two organs (Joy 1988, Lam *et al.* 1996, Howitt and Udvardi

2000, von Wiren *et al.* 2000). Magnitudes of unidirectional fluxes across the plasma membrane, half-lives of cytosolic NH_4^+ exchange and their low variability over a wide concentration range, increasing efflux:influx ratios with increasing $[NH_4^+]_o$, and closeness of $[NH_4^+]_c$ to Nernstian predictions for a plasma-membrane electrical potential of – 90 to – 150 mV (Elzenga *et al.* 1995, and references therein), are all quantitatively similar to values seen with root systems (see references cited above). As these latter organs were cultured at the same $[NH_4^+]_o$ as was used in CAE trials, and therefore more certainly at a physiological steady-state, similarities in essential flux and compartmentation parameters argue in favor of the leaf tissue being under conditions amenable to CAE. Moreover, the observation that, with ^{13}N-labeled barley root systems exposed to physical disturbance and to non-steady-state NH_4^+ concentrations during elution, phase-III efflux lines return to the previously established slope within a few minutes (Chapter 3), provides evidence that plant systems rapidly adjust to short-term changes, further suggesting that leaf slices may be able to achieve new physiological steady states in spite of traumas incurred by slicing and pretreatment.

An exponentially decelerating $^{13}NH_4^+$ efflux is to be expected from a compartment which is turning over NH_4^+ at a constant rate (Lee and Clarkson 1986; also see Chapter 3), and phases of declining tracer flux similar to those observed in the present work were resolved by regression analysis in $^{13}NH_4^+$ - efflux experiments performed on root systems (see references cited above). Agreement between leaf slice data, particularly exchange half-life values, and

those found with roots, therefore, also provides evidence for the identity of the compartments releasing tracer. This assignment, based on such homologies, of phase III as the collective cytosol of the leaf tissue was reinforced by treatment of wheat leaf slices with the membrane-solubilizing detergent SDS (Fig. 9). The substantial decreases in $t_{½}$ and efflux:influx ratio, and decrease in pool size, for this putative compartment following SDS treatment, are as expected of a membrane-bound space (Cram 1968, Siddiqi *et al.* 1991, Kronzucker *et al.* 1995a). Results using the GS-inhibitor MSX in wheat also supported the hypothesis that phase III is cytosolic. MSX increased $[NH_4^+]$ for this phase nearly 3-fold at 0.1 mM $[NH_4^+]_o$, while efflux and the efflux:influx ratio more than doubled (Table 1). These results agree with previous findings that MSX caused ammonium accumulation in leaf discs of spinach (Platt and Anthon 1981), and that MSX increased NH_3 emissions to the atmosphere as well as tissue NH_4^+ concentrations in barley (Mattsson and Schjoerring 1996, Pearson *et al.* 1997) and *Mercurialis perennis* (Pearson *et al.* 1997). The MSX compartmentation results are also consistent with studies that have estimated MSX effects on cytosolic NH_4^+ pools in roots using CAE (Kronzucker *et al.* 1995b) and NMR (Lee and Ratcliffe 1991). Increased influx of NH_4^+ with MSX treatment at 0.1 mM $[NH_4^+]_o$ is also consistent with several studies (Lee and Ayling 1993, Feng *et al.* 1994, Wieneke and Roeb 1998, Rawat *et al.* 1999), and may be due to de-repression of the *AtAMT1* gene by lowered tissue glutamine concentrations (Rawat *et al.* 1999). Increased efflux with MSX treatment has also been previously reported in roots (Feng *et al.* 1994, Kronzucker *et al.* 1995e), and is

likely to be related to the enlarged cytosolic NH_4^+ pool. That MSX did not substantially increase fluxes or pools at 10 mM $[NH_4^+]_o$ in wheat leaf slices (Tables 2 and 3) may be an indication that the leaf system is already at a maximum in terms of these characteristics.

Interestingly, dark pretreatment, labeling, and elution of wheat leaf slices at 0.1 mM $[NH_4^+]_o$, resulted in similar flux and compartmentation characteristics as did MSX treatment at this $[NH_4^+]_o$, possibly reflecting a similar limitation of GS activity and the associated lowering of tissue glutamine, and raising of tissue ammonium. For both treatments, however, the net accumulation of traced NH_4^+ was close to that of controls. This similarity with control material generally agrees with results obtained by Raven and Farquhar (1981) for ^{14}C-labeled methylammonium transport in leaf slices of *P. vulgaris*, in which no differences in tracer accumulation were observed between dark and light, and with an early study by Delwiche (1951) showing no light-dependence of $^{15}NH_4^+$ assimilation in intact leaves of tobacco (*Nicotiana tabacum*). It is also in accord with Lüttge and Ball (1973), in which light, under aerobic conditions, did not greatly affect the uptake of a wide variety of ions (unfortunately, this study did not include measurements of NH_4^+ uptake), although several potassium-uptake studies in leaf segments have shown light-dependence of potassium uptake (Rains 1967, 1968, Nobel 1969). The finding shown here differs from results reported by Canvin and Atkins (1974), who found that light stimulated $^{15}NH_4^+$ incorporation into amino acids 5-fold in leaf segments of barley. However, this had been observed under 20 mM $[NH_4^+]_o$, a 200-fold higher concentration than was used in

the present work. Moreover, in spite of this 5-fold difference, Canvin and Atkins (1974) still found substantial amounts of dark assimilation of $^{15}NH_4^+$, and suggested that the activity of the mitochondrial isoform of glutamate dehydrogenase could account for this observation. In the present work, which was not performed under anaerobic conditions, dark ^{13}N accumulation may have included an assimilatory component, with energy provided by respiration.

Table 1, showing the characteristics of unidirectional influx and efflux for wheat slices across a range of external conditions, indicates that the NH_4^+ flux capacity of leaf tissue is extensive and highly dependent on $[NH_4^+]_o$. The external NH_4^+ concentrations were chosen to include the range of apoplastic concentrations that leaf cells are likely to be exposed to under natural circumstances (Mattsson *et al.* 1997, Finnemann and Schjoerring 1999, but *cf.* Mühling and Sattelmacher 1995). Table 1 shows that the range of influx in response to $[NH_4^+]_o$ is very broad. However, this response is nonlinear in that influx increases 259-fold when $[NH_4^+]_o$ increases by a factor of 1000. Interestingly, efflux increases across this range by a still greater factor (1700), and the different responses of influx and efflux mechanisms to $[NH_4^+]_o$ are suggestive of independent regulatory systems for these opposed processes.

The half-lives of cytosolic NH_4^+ exchange in wheat leaf slices were observed to decline moderately between 0.1 mM and 10 mM $[NH_4^+]_o$ (Table 2), a phenomenon not generally observed with root systems. This decline may be a symptom of ammonium toxicity at high $[NH_4^+]_o$, as it is associated with high cytosolic concentrations of NH_4^+ (Table 2) and low flux efficiency (i.e., high

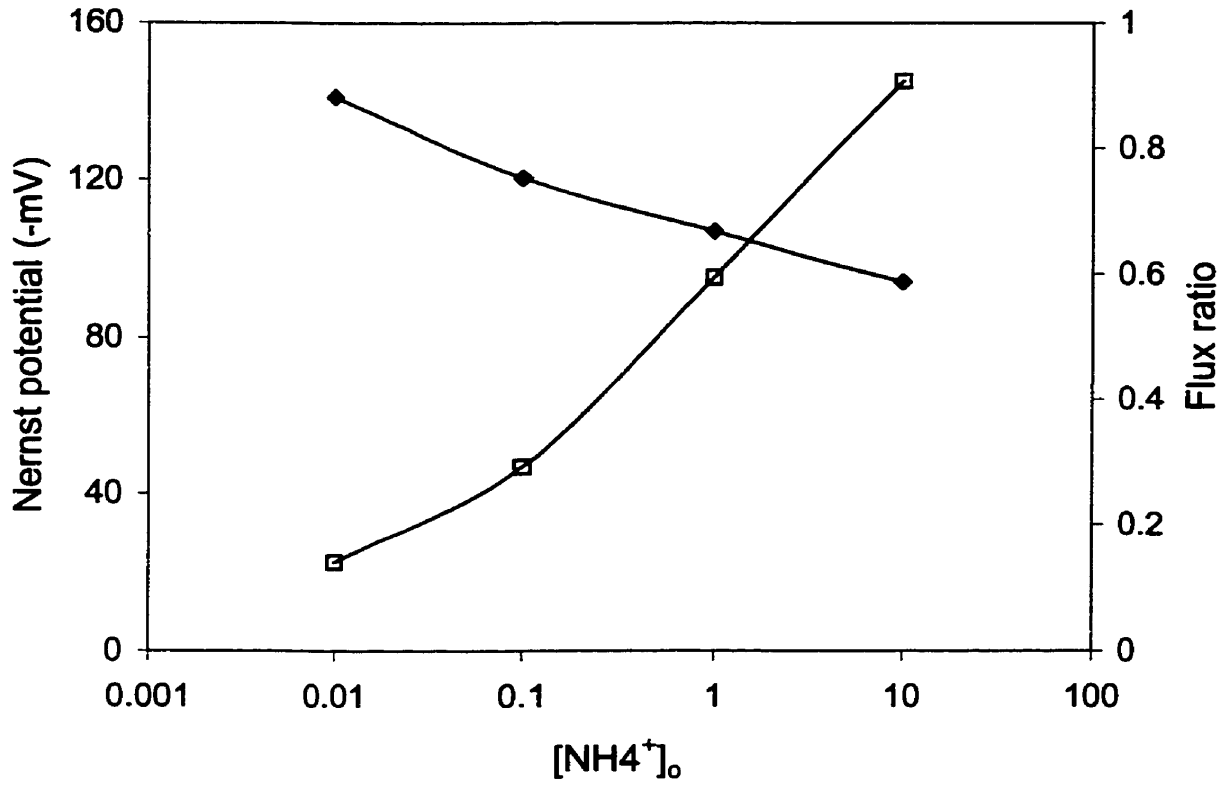

Figure 11. Nernst potentials for NH_4^+ (closed symbols) and efflux:influx ratios (open symbols) as functions of $[NH_4^+]_o$ in wheat leaf slices.

efflux:influx ratio) (Table 1). Declining $t_{½}$ at high $[NH_4^+]_o$ reflects a more rapid turnover in the cytosol, which may work in conjunction with lowered influx and elevated efflux (relative to $[NH_4^+]_o$) to keep internal concentrations at low, perhaps sub-Nernstian levels (see Chapters 5 and 6). Notably, the closeness of estimates of cytosolic NH_4^+ concentrations to Nernstian predictions supports the validity of pool size measurements made using CAE. Fig. 11 illustrates how derived Nernst potentials for NH_4^+ decline with increasing $[NH_4^+]_o$, while the efflux:influx ratio shows the opposite trend.

It was of ecophysiological interest to compare the flux and compartmentation responses to NH_4^+ in rice, wheat, and tomato leaf slices because of what is known about their differing tolerances to NH_4^+ nutrition (see references above). At 0.1 mM $[NH_4^+]_o$, all species showed a moderate ratio of efflux to influx, with rice showing the highest capacity to take up and retain NH_4^+. This capacity is in accordance with the NH_4^+ tolerance commonly observed in rice, and may partly be explained by the high levels of GS found in rice leaves (Magalhaes and Huber 1989). At 1 mM $[NH_4^+]_o$, the differences among species is much more pronounced, with an exceptionally high efflux:influx ratio (0.83) observed in tomato at 1 mM $[NH_4^+]_o$, in comparison with that in wheat (0.59) and rice (0.45). This ratio, under a condition of potential NH_4^+ stress (1 mM), then, is the characteristic that most closely conforms to the hypothesized adaptive gradient. It is noteworthy that even though the quasi-steady net flux in tomato is only half of that seen in rice, influx in tomato at this $[NH_4^+]_o$ level is substantially higher. Lack of apparent coordination between influx systems and NH_4^+ sink

strength of tomato, in contrast to the relatively high coordination in rice, again parallels the greater adaptation to NH_4^+ well-documented in the latter species, and the toxic response to NH_4^+ well-documented in the former (see Introduction).

In summary, the CAE method, which has been applied previously to NH_4^+ fluxes and compartmentation in root systems, has been extended to leaf slices, with interesting results. The concentration dependence of unidirectional fluxes, efflux:influx ratios, cytosolic concentrations and cytosolic half-lives of exchange observed in wheat are indicative of high permeability and accumulation capacity of leaf cells, and possibly of toxicity, to this important metabolite. Comparison of species widely differing in their tolerances to NH_4^+ (i.e. rice, wheat, and tomato) suggests that a characteristic of tolerance to ammonium as sole N source is a high efficiency and more rigorous regulation of plasma membrane flux processes, i.e., a low efflux:influx ratio, as also seen in plant root systems (Min *et al.* 1999, also see Chapter 5). The analysis detailed here opens new possibilities for the study of the ionic relations of a much wider range of plant species than has previously been investigated.

Chapter 5. Futile transmembrane ion cycling: A new cellular hypothesis to explain ammonium toxicity in plants

Introduction

Plants can extract and utilize various forms of nitrogen (N) from soils, most importantly the inorganic ions ammonium (NH_4^+) and nitrate (NO_3^-). While one might expect NH_4^+ to be preferred by plants, as its assimilation requires less energy than that of NO_3^- (Reisenauer 1978), relatively few species perform well when NH_4^+ is the only, or predominant, source of N (Kronzucker *et al.* 1997, and references therein). By contrast, most species develop toxicity symptoms when grown on moderate to high levels of NH_4^+ (see Chapter 1), while normal growth in these species is seen on NO_3^-. Ammonium toxicity is especially problematic in areas with intensive agriculture and cultivation of livestock, where high levels of NH_3 emission, and subsequent NH_4^+ deposition, are observed (Pearson and Stewart 1993, Fangmeier *et al.* 1994). It is estimated that N deposition from atmospheric NH_3 can reach levels as high as 50 kg ha^{-1} yr^{-1} in some parts of Europe, in extreme cases comprising 50 to 80% of total N deposited from all possible sources (Pearson and Stewart 1993). As a result, N saturation in many natural ecosystems is exceeded by as much as 10-fold, and damage to forest and agricultural crops alike has been attributed directly to this phenomenon (Van

Breeman *et al.* 1982, Van Breeman and Van Dijk 1988). This is a problem of serious concern in both Europe and North America.

Several hypotheses have been advanced to explain why NH_4^+ is toxic to plants, but none are considered satisfactory (Gerendas *et al.* 1997, De Graf *et al.* 1998). As NH_4^+ uptake mechanisms are coupled to H^+ extrusion into the rooting medium and H^+ release is also associated with NH_4^+ incorporation into protein (Pearson and Stewart 1993), it has been repeatedly suggested that root medium acidification (Barker *et al.* 1966) and/or intracellular pH disturbance (Bligny *et al.* 1997, Gerendas *et al.* 1997) may explain the observed symptoms. However, in many cases, toxicity is equally observed in pH-buffered media (Blacquière *et al.* 1998), and a recent study on pea (*Pisum sativum*), known to be ammonium-sensitive, has discounted the occurrence of ammonium-induced cytosolic pH disturbance. Others have suggested that carbohydrate limitation may contribute to the toxicity syndrome, based upon the finding that NH_4^+ *per se* is not translocated to the shoot in most plants (Lewis and Chadwick 1983, Wang *et al.* 1993a, Kronzucker *et al.* 1998, and references therein), and all carbon skeletons for nitrogen assimilation must therefore be provided in roots, causing local carbon deprivation (Lewis *et al.* 1982). In some cases, external provision of α-ketoglutarate, a key carbon source for N assimilation, alleviated toxicity symptoms and increased NH_4^+ uptake and assimilation (Magalhaes *et al.*1992, Monselise and Kost 1993), but in other cases it failed to enhance NH_4^+ metabolism (Kronzucker *et al.* 1995e), suggesting that other factors may limit NH_4^+ assimilation. The hypothesis that NH_4^+ toxicity results from the uncoupling

of photophosphorylation in chloroplasts (Pearson and Stewart 1993) has long been shown to be incorrect, as even very large NH_4^+ concentrations do not affect this process in intact chloroplasts (Heber 1984). A diminishment of essential cations such as K^+, Mg^{2+} and Ca^{2+} in tissues of plants grown under prolonged exposure to NH_4^+ has been widely documented (Holldampf and Barker 1993, Cao and Tibbits 1993, Speer and Kaiser 1994), and while this may be a contributor to the toxicity syndrome, it is clearly a longer-term effect.

What these hypotheses have in common is that they are all contingent upon the permeation of NH_4^+ into the cell. Therefore, the study of transmembrane NH_4^+ fluxes is essential to the establishment of a proper context for the discussion of the issue of toxicity. Surprisingly, however, no study of the primary events of NH_4^+ acquisition, including the modes of entry, metabolism, and sequestration, has been undertaken in this context. Here, positron-emission tracing with the short-lived nitrogen isotope ^{13}N was used to characterize these primary events at the cellular level.

Materials and Methods

Please refer to Chapter 2.

Results and Discussion

NH_4^+ transport properties were studied in roots of two major cereal species known to differ markedly in their abilities to tolerate NH_4^+ as the sole N source. $^{13}NH_4^+$ efflux from roots of intact plants of the two species, pre-labeled with this tracer, showed substantial differences in the rates of NH_4^+ extrusion

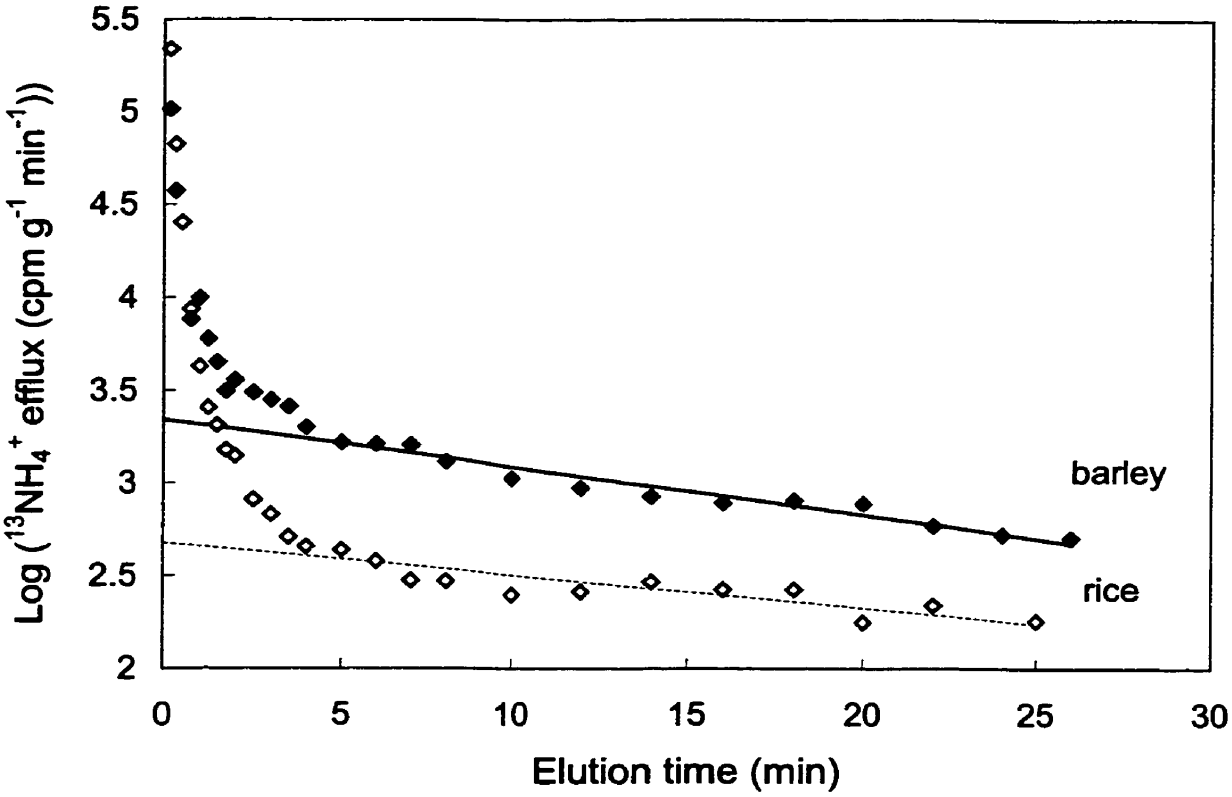

Figure 12. Representative semi-logarithmic plots of $^{13}NH_4^+$ efflux from the cytosolic compartments of barley and rice roots. Plants were pre-labeled under nutritional steady-state conditions at 10 mM $[NH_4^+]_o$. Plots have been corrected for specific activities, allowing a direct comparison of initial efflux rates as represented by the *y*-intercepts of the regression lines.

from root cells into the external medium (Fig. 12). Interestingly, barley (*Hordeum vulgare*), which suffers from NH_4^+ toxicity (Lewis *et al.* 1986), excretes NH_4^+ at a significantly higher rate than rice (*Oryza sativa*), a species whose tolerance to NH_4^+ is considered exceptional (Magalhaes and Huber 1989). Fig. 13, however, depicting bi-directional chemical fluxes of NH_4^+ across the plasma membranes of these two species, documents a large increase in total flux (equivalent to unidirectional influx) as the steady-state external NH_4^+ concentration is stepped up from a moderate level of 0.1 mM to a potentially toxic level of 10 mM (Magalhaes and Huber 1989). Importantly, the increase in barley is much higher (12-fold) than in rice (7-fold). In fact, the magnitudes of the fluxes in barley are the highest ever reported for NH_4^+ in a plant root system, and strongly suggest the operation of channel-type transport systems, whose existence has been repeatedly postulated and supported by recent work employing patch-clamp analysis and channel-specific blocking agents (Tyerman *et al.* 1995, Kaiser *et al.* 1998, Nielsen and Schjoerring 1998). As the steady-state net fluxes are almost identical in the two species (as calculated from the difference between influx and efflux), a substantially larger fraction of incoming nitrogen is subsequently excreted in barley, the ammonium-sensitive species. This pronounced excretion of N is apparently linked to an apparent inability of barley to exclude NH_4^+ at the more primary, intake, step. Preliminary analyses on other species known to suffer NH_4^+ toxicity, including wheat, tomato, and the tree species Douglas-fir (Min *et al.* 1999) and trembling aspen (Min *et al.* 1999), suggest that this phenomenon is not isolated, but rather appears to occur universally among

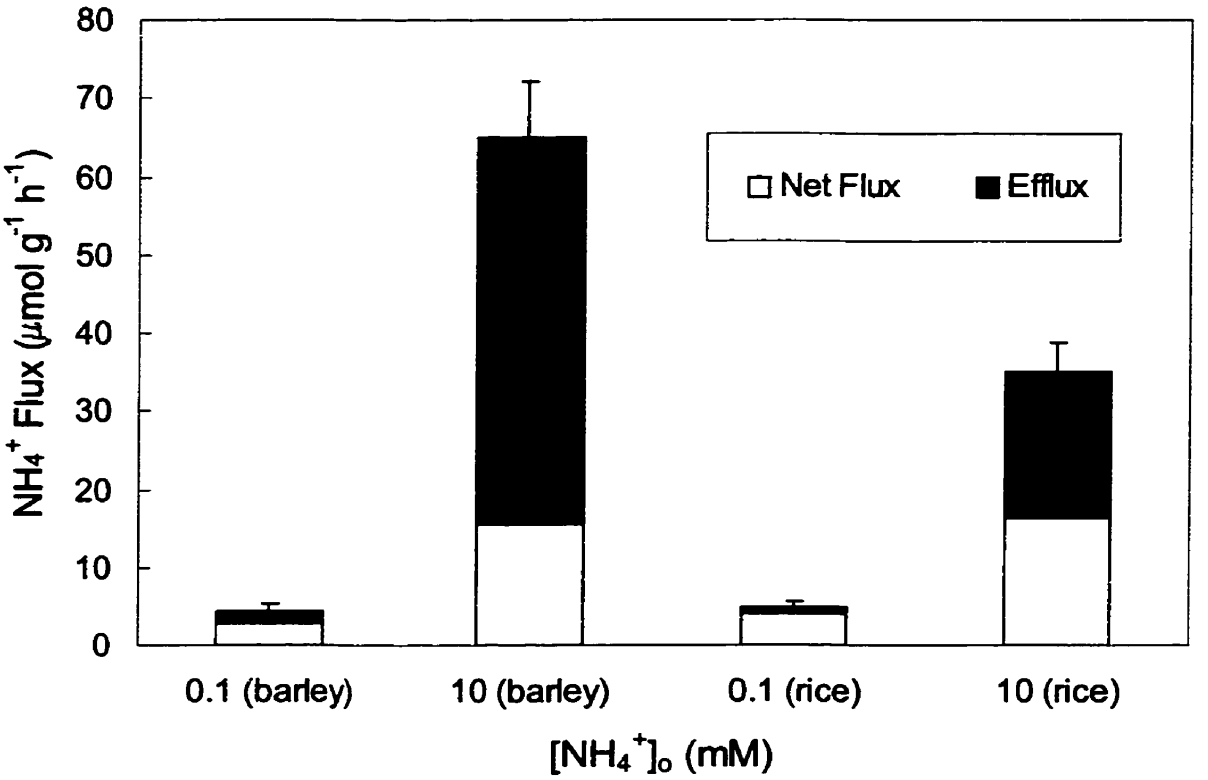

Figure 13. Comparison of steady-state bi-directional plasma-membrane NH_4^+ fluxes in barley and rice roots at 0.1 mM and 10 mM $[NH_4^+]_o$. Column height represents total influx from the external medium, while the filled areas depict the portion of influx returned to the environment by efflux. The net flux is the difference between these two fluxes. Vertical bars indicate standard errors of influx means.

susceptible higher plants. Knowledge of the kinetic constants for NH_4^+ exchange with the cytosolic component in the experimental root system, and of the tracer content in this pool at the onset of the elution protocol, made possible a comparison of NH_4^+ concentrations on either side of the plasma membrane (i.e., $[NH_4^+]_o$ and $[NH_4^+]_c$). Compartmental analysis shows that, at 10 mM $[NH_4^+]_o$, both barley and rice have a higher $[NH_4^+]_c$ than ever previously reported in plant root cells (Table 3; but see Table 2 for still higher estimates), and indeed are irreconcilable with the widely-held dogma that no appreciable quantities of free NH_4^+ can exist in the cytosol (Reisenauer 1978, Pearson and Stewart 1993, Kafkafi and Ganmore-Neumann 1997, De Graaf 1998). However, a thermodynamic analysis, using measurements of electrical potential ($\Delta\Psi$) across the plasma membrane, reveals that, in barley, cytosolic levels of NH_4^+ at 10 mM $[NH_4^+]_o$ are nevertheless substantially lower than predicted by the Nernst equation if NH_4^+ is passively distributed across a highly permeable membrane (Table 3). Rice, by contrast, maintains a lower $[NH_4^+]_c$ than barley under this condition, but due to the relatively depolarised state of its plasma membrane under high $[NH_4^+]_o$ (Wang *et al.* 1994), the transmembrane distribution ratio of NH_4^+ very closely approximates that predicted by Nernstian analysis (Table 3). The stepping-down of the membrane voltage in rice with increasing NH_4^+ provision has the important biophysical consequence of lowering the ceiling for NH_4^+ accumulation in the cytosol, thus eliminating the gradient against which efflux transporters need to work in order to remove excess cytosolic NH_4^+. The

Table 3. Nernstian analysis of cytosolic NH_4^+ pool sizes in barley and rice. The Nernst equation was used to predict equilibrium cytosolic concentrations on the basis of external concentration, membrane potential ($\Delta\Psi$) , and an ambient temperature of 20°C for barley, and 30°C for rice. Cytosolic concentrations were measured using compartmental analysis (see Materials and Methods). Flux experiments were repeated four to five times. Standard errors for fluxes and CAE-estimated pool sizes were < 20% of the means. 67 electrophysiological measurements were made on different barley plants for the 0.1 mM NH_4^+ condition, and 20 measurements for the 10 mM NH_4^+ condition. Standard errors were < 3% of the means.

$[NH_4^+]_o$ (mM)	Influx (μmol gfw^{-1} h^{-1})	Efflux (μmol gfw^{-1} h^{-1})	Flux ratio	$\Delta\Psi$ (mV)	$[NH_4^+]_{cytosol}$ (predicted, mM)	$[NH_4^+]_{cytosol}$ (CAE estimate, mM)
0.1 (barley)	5.46	1.53	0.28	-121	12	28
10 (barley)	65.1	49.4	0.76	-123	1320	358
0.1 (rice)	4.85	0.67	0.14	-110 (ref. 22)	6.8	33.4
10 (rice)	35.0	18.6	0.53	-82 (ref. 22)	233	232

apparent failure of barley to downregulate the activity of its proton ATPase (the primary generator of $\Delta\Psi$ across the plasma membrane), on the other hand, sustains a gradient for very large inward NH_4^+ fluxes, which were in fact observed (Fig. 13). An undiminished $\Delta\Psi$ in barley yields a high potential for NH_4^+ accumulation, and hence a larger demand upon the efflux process (Table 3). The data show that, whereas the net flux of NH_4^+ is nearly identical in both species at 10 mM, the efflux process in barley mediates a 2- to 3- fold higher removal of NH_4^+ from the cytosol, effectively reducing a Nernstian NH_4^+ concentration of 1.32 M by the equivalent of 962 mM (Table 3).

Such a process must carry a substantial energetic burden, and this prediction was consistent with measurements of oxygen consumption in roots of intact barley plants under the same steady-state conditions as with flux measurements. As indicated in Fig. 14, when steady-state $[NH_4^+]_o$ was varied between 0.1 and 10 mM, respiration in barley increased by 41%, while no significant difference was found in rice between growth conditions (Fig. 15). The rapid (1-4 h) development of a high oxygen-consumption state in barley roots was also observed when a transition in growth medium between 0.1 and 10 mM $[NH_4^+]_o$ was imposed (Fig. 16). In order to isolate the flux processes at the barley root plasma membrane from other potentially energy-requiring pathways of NH_4^+, the GS-inhibitor methionine sulfoximine (MSX) was applied. The observation that MSX did not diminish respiratory activity associated with elevated external NH_4^+ (Fig. 14) emphasizes that the respiratory stimulation was attributable to

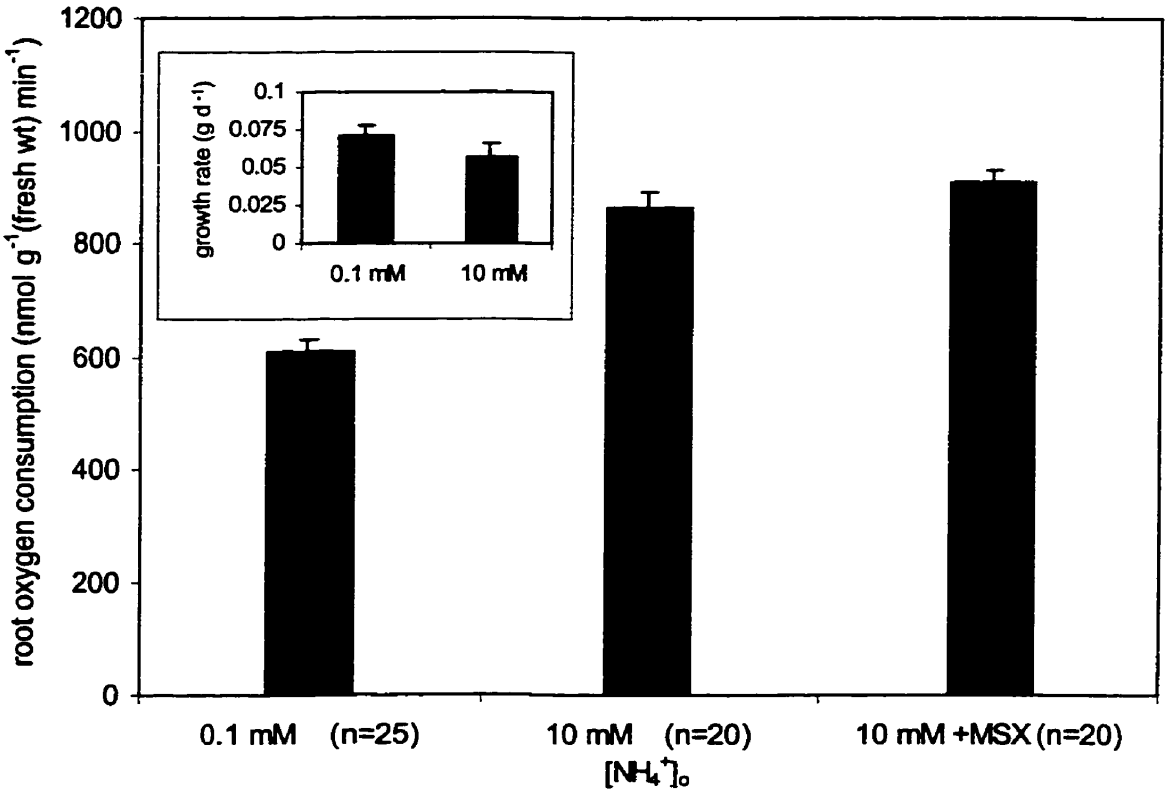

Figure 14. Respiration rates of intact barley roots at 0.1 mM and 10 mM $[NH_4^+]_o$. In one experiment, 1 mM methionine sulfoximine (MSX) was applied (see text). Growth rates (gain in fresh weight averaged over 7 d) under the two conditions are shown in the inset. Error bars indicate S.E. of means.

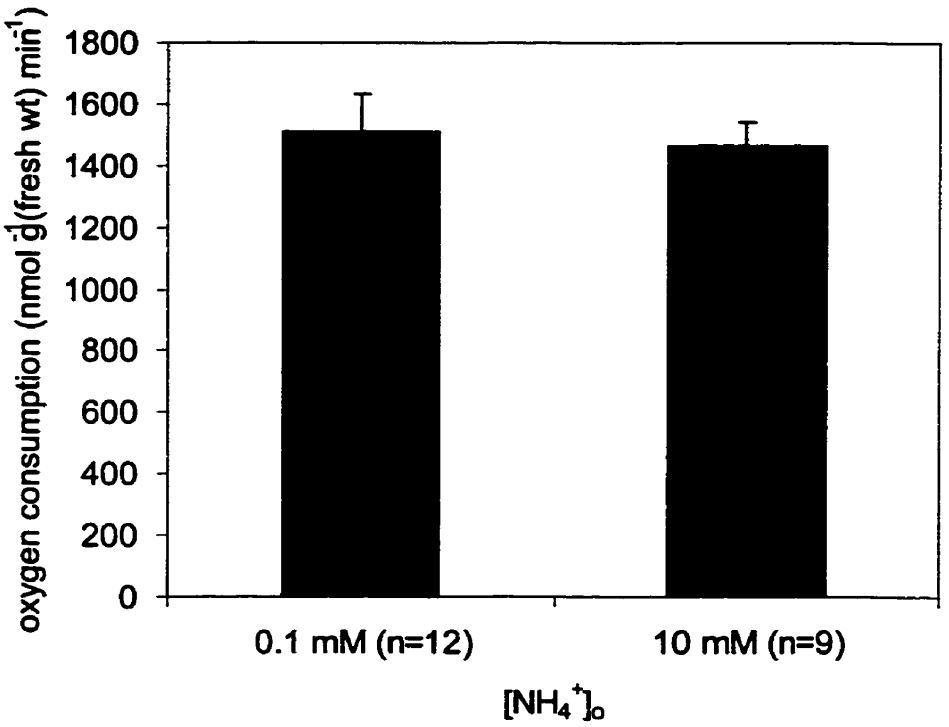

Figure 15. Respiration rates of intact rice roots at 0.1 mM and 10 mM $[NH_4^+]_o$. Error bars indicate S.E. of means.

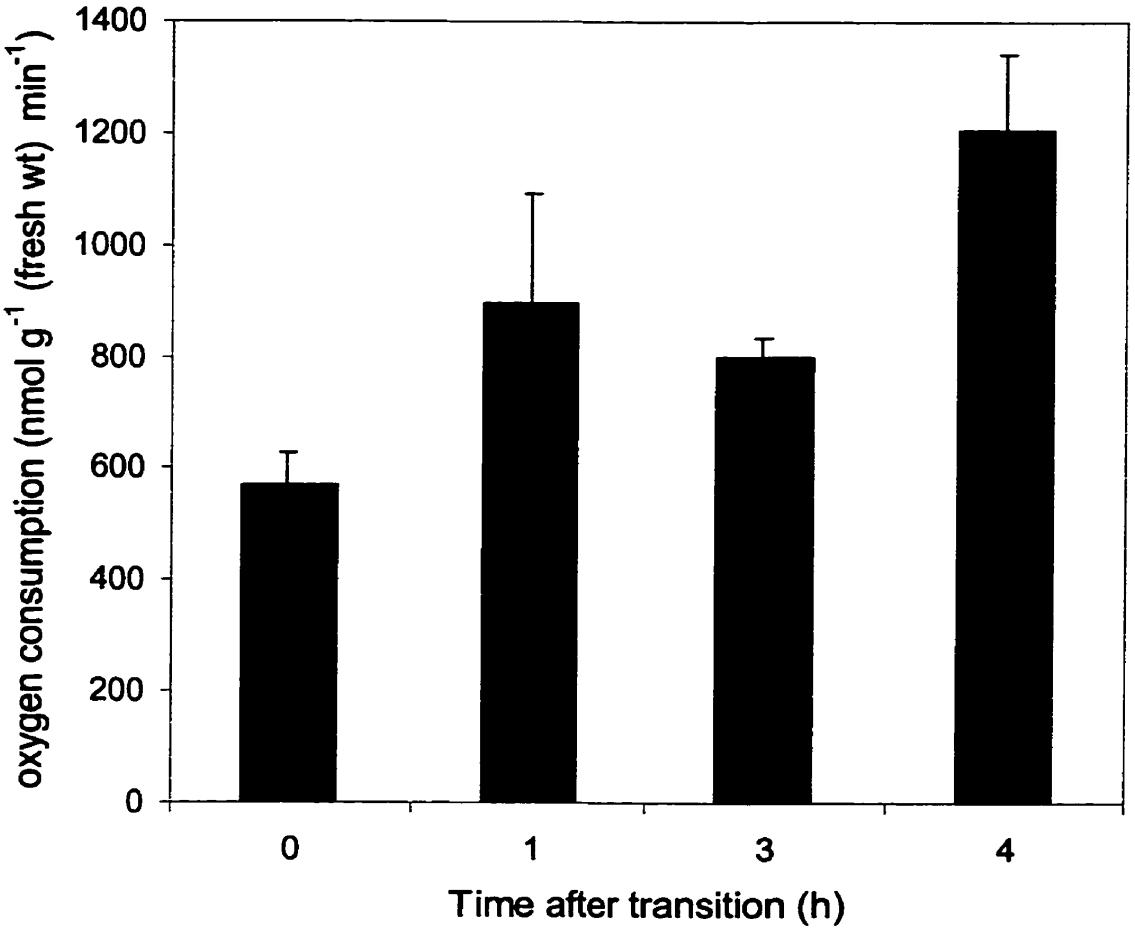

Figure 16. Time course of change in respiration of intact barley roots after transition from 0.1 mM steady-state $[NH_4^+]_o$ to 10 mM $[NH_4^+]_o$. Error bars indicate S.E. of means (n = 3-5).

processes upstream of NH_4^+ metabolism, i.e. residing at the level of membrane transport. Other work has shown that the proportion of total respiration assigned to processes associated with NH_4^+ uptake and assimilation comprises as much as 14% of the root's total respiratory expenditure at 0.1 mM $[NH_4^+]_o$ (Bloom *et al.* 1992). Most of this expenditure appears to be due to plasma membrane transport, hence the observation of a 41% increase in total root respiration under 10 mM $[NH_4^+]_o$ may well be attributable to the approximately 30-fold increase in NH_4^+ efflux from the cytosol under this condition. Previous work with the NH_4^+ analogue methylammonium ($CH_3NH_4^+$) showed that a similar respiratory increase was achieved in the marine alga *Phaedactylum tricormutum* by the addition of this non-metabolised compound (Larson and Rees 1994), indicating that in the absence of metabolic sinks, membrane processes *per se* can impose a substantial energetic burden on the plant root system. Indeed, in barley the increase in respiration at 10 mM $[NH_4^+]_o$ is not correlated with an increase in growth-related energy demands; on the contrary, it accompanies a depression in growth rate at the whole plant level (Fig. 14, inset) not seen in ammonium-tolerant species such as rice (Wang *et al.* 1994, Kronzucker *et al.* 1998, 2000). It is intriguing that in rice, where no respiratory increase was found at elevated NH_4^+ provision (Fig. 15), a thermodynamic equilibrium is achieved across the plasma membrane (Table 3), suggesting that passive inwardly- and outwardly-directed NH_4^+ channel activities may mediate NH_4^+ distribution across this membrane without energetic cost.

The inability of barley, and other ammonium-susceptible species, to exclude NH_4^+ via regulation of plasma-membrane influx systems, is proposed to constitute a fundamental breakdown in plant cell function, which must precede any intracellular toxicity-associated events such as cation displacement or carbohydrate depletion (see Introduction). The operation of the energy-intensive NH_4^+ extrusion mechanism (*cf.* MacRobbie 1971, Mills *et al.* 1985, Nobel 1999) described here appears to be central to the ammonium toxicity syndrome, and is similar in principle to mechanisms evolved by bacteria and carcinomas to actively excrete cytotoxins such as antibiotics and chemotherapeutic agents (McMurry *et al.* 1980, Nikaido 1994). The ecological significance of this syndrome, and of the mechanisms underlying it, is substantial, not only because soil nitrogen profiles profoundly determine the spatial and temporal dynamics of ecosystems (Tilman 1986, Kronzucker *et al.* 1997), but especially in the light of recent interventions by humans in the functioning of the global nitrogen cycle (Pearson and Stewart 1993, Fangmeier *et al.* 1994, Vitousek 1994, De Graaf *et al.* 1998).

Chapter 6. Constancy of Nitrogen Turnover Kinetics in the Plant Cell: Insights into the Integration of Subcellular N Fluxes

Introduction

After the hydrogen and oxygen in water, nitrogen is the element most extensively taken up by higher plants from the soil environment,. Knowledge about its movement, compartmentation, and turnover is critical to plant physiological ecology because N availability limits plant growth and yields more than any other nutritional factor (Vitousek and Howarth 1991, Cassman 1993, Crawford and Glass 1998). Recent genetic analyses and N-tracer studies have shown that the expression and activities of plant plasma-membrane influx systems for nitrate (NO_3^-) and ammonium (NH_4^+), the two major sources of N utilised by plants, are highly variable and are associated with similar variability in metabolic fluxes, fluxes across subcellular membranes, long-distance fluxes between root and shoot, and fluxes from the cytosol to the external medium (Glass and Siddiqi 1995, Kronzucker *et al.* 1995a, Crawford and Glass 1998, Krapp *et al.* 1998, Rawat *et al.* 1999). However, the regulatory processes that integrate these large variations remain unclear. For instance, recent studies involving the up-regulation of nitrate reductase (NR), thought to be the enzyme most limiting to nitrate assimilation in plants, have resulted in no gains in plant

growth or nitrogen content (see references below), a phenomenon which requires explanation.

The present study was designed to quantify changes in subcellular flux partitioning of inorganic N in root tissues of higher plants, and to measure the kinetic constants for N exchange with the cytosolic compartment of cells (k_c), as functions of external NO_3^- or NH_4^+ concentrations. To obtain high-precision and highly time-resolved measurements, the short-lived radiotracer ^{13}N (half-life = 9.98 min) was used. Due to its direct and non-invasive character, the technique of compartmental analysis by efflux was used to obtain estimates of subcellular fluxes and pool sizes, as well as to derive k_c (MacRobbie 1971, Walker and Pitman 1976, Siddiqi *et al.* 1991, Wang *et al.* 1993a). The cytosol was the focus of this study because it exchanges N directly with the external environment via the plasma membrane, and because it is a metabolically critical compartment, containing such key enzymes as NR and glutamine synthetase (cytosolic isoform, GS_1), and providing substrates for inorganic N flux through subcellular and tissue-level transport systems (Campbell 1990, Oaks 1994, Crawford and Glass 1998). The experiments were conducted principally on barley (*Hordeum vulgare*), a cereal species recognised as a model system for the study of NO_3^- and NH_4^+ acquisition (Bloom and Chapin 1981, Siddiqi *et al.* 1991).

Materials and Methods

Please refer to Chapter 2.

Results and Discussion

Figure 17 shows representative semi-logarithmic plots of radiotracer efflux from the roots of intact barley and rice seedlings that had been labeled with $^{13}NO_3^-$ under steady-state nutritional conditions. Phase III in these curves represents $^{13}NO_3^-$ released specifically from the root cytosol (Kronzucker *et al.* 1995b, 1995e) of the two species. Phase-III slopes yield exchange constants k_c (expressed as decadal logarithms, in units of min^{-1}) that describe the decline in the rate of cytosolic $^{13}NO_3^-$ release, which obeys first-order kinetics (MacRobbie and Dainty 1958, MacRobbie 1971, Walker and Pitman 1976), determined by the rapidity of turnover of the cytosolic NO_3^- pool. Hence, k_c reflects the activities of all fluxes introducing and removing NO_3^- to and from this pool, and provides a foundation for direct and inclusive evaluation of cytosolic NO_3^- turnover (Lee and Clarkson 1986, Siddiqi *et al.* 1991).

To obtain information on how plants adjust their cellular exchange kinetics to variations in nutritional conditions, seedlings of barley were cultured under regimes of NO_3^- provision that varied in external concentration ($[NO_3^-]_o$) over four orders of magnitude and, in one case, an uninduced condition was maintained by withholding NO_3^- from the growth medium (see Kronzucker *et al.* 1995a). Table 4 shows that the component fluxes (influx, efflux, flux to assimilation and the vacuole, and flux to the shoot) estimated using compartmental analysis varied as much as 300-fold over the conditions examined, and that the ratios among fluxes also vary substantially from one condition to the next. However, in stark contrast to these flux differences, and despite the operation of entirely distinct transport

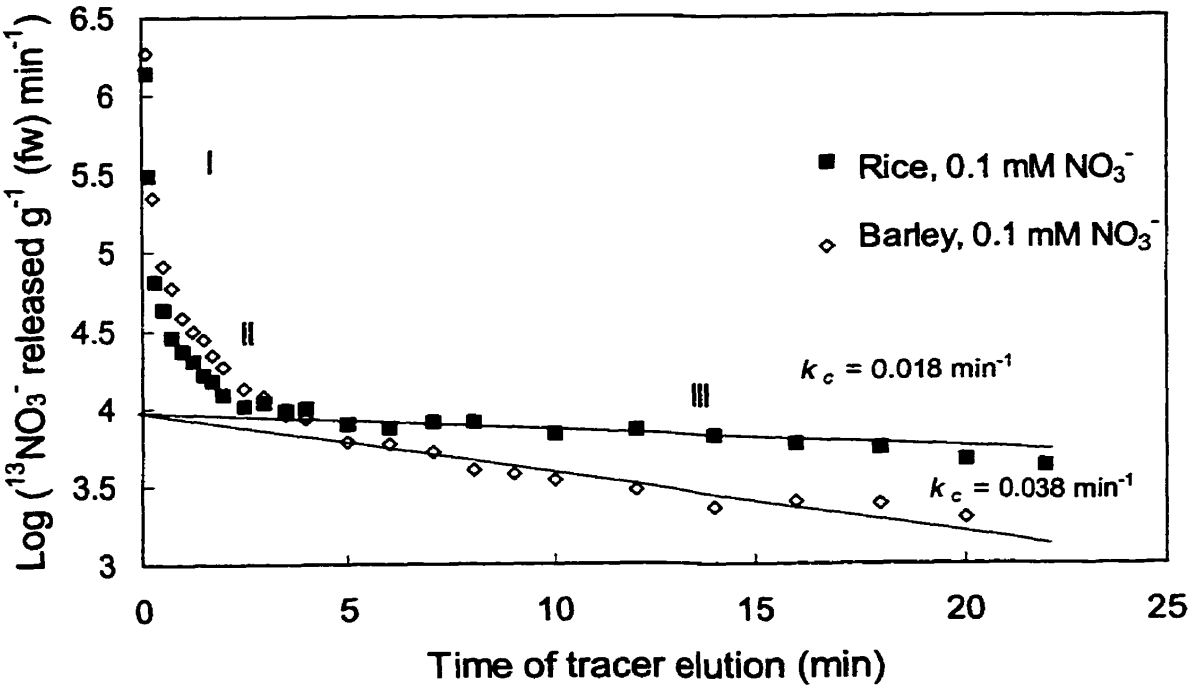

Figure 17. Representative plots of $^{13}NO_3^-$ (tracer) efflux from roots of intact barley (*Hordeum vulgare*, cv. Klondike) and rice (*Oryza sativa* cv. IR-72) seedlings grown, labeled, and eluted in nutrient solution containing 0.1 mM $^{14}NO_3^-$. Linear regression on semi-logarithmic plots was used to resolve phases I, II and III, which represent tracer elution from the surface film, cell wall and cytosol of roots, respectively (Kronzucker et al. 1995e). Kinetic exchange constants for phase III (k_c) are as indicated.

Table 4. Cellular and long-distance fluxes of ^{13}N-labeled NO_3^- in barley roots grown under various nitrogen regimes. Uninduced plants were grown without N and then labeled and measured at 0.01 mM NO_3^-; therefore they were not at a nutritional steady state. In the remaining experiments, plants were grown and measured at 0.01, 0.1, 1, and 10 mM NO_3^-, respectively. ϕ_{oc}, influx; ϕ_{co}, efflux; ϕ_{net}, net flux; $\phi_{met/vac}$, flux to metabolism and vacuole; ϕ_{shoot}, flux to shoot. Fluxes are expressed as μmol g^{-1} (fresh weight) h^{-1} ± S. E. of mean.

	Steady-state NO_3^- condition during growth				
flux	Uninduced (0-0.01 mM)	0.01 mM	0.1 mM	1 mM	10 mM
ϕ_{oc}	0.26 ± 0.01	3.62 ± 0.53	5.69 ± 0.40	11.90 ± 1.02	21.67 ± 1.89
ϕ_{co}	0.03 ± 0.00	0.42 ± 0.05	0.36 ± 0.11	1.89 ± 0.49	6.59 ± 1.97
ϕ_{net}	0.23 ± 0.01	3.20 ± 0.48	5.33 ± 0.30	10.01 ± 0.79	15.07 ± 0.55
$\phi_{met/vac}$	0.21 ± 0.01	2.58 ± 0.36	4.20 ± 0.26	7.30 ± 0.72	8.75 ± 1.09
ϕ_{shoot}	0.02 ± 0.01	0.62 ± 0.11	1.13 ± 0.00	2.71 ± 0.11	6.32 ± 1.12

systems under the different nutrient regimes (Glass and Siddiqi 1995), the essentially uniform slopes of the regression lines in Fig. 18 show that k_c for NO_3^- exchange varied by less than 4% from a mean value of 0.041 min^{-1} across the spectrum of tested conditions. As k_c is essentially equivalent to a frequency term (expressed in min^{-1}), its relative constancy in the presence of substantial changes in individual N fluxes suggests the existence of a co-ordinating mechanism, or system of mechanisms, which set(s) the timing of the entire complex of cytosolic N fluxes involved in NO_3^- acquisition.

The quantity Q of a traced steady-state pool is related (by a proportionality constant Ω, see Chapter 2) to the traced flux ϕ into that pool and to the exchange constant as follows:

$$Q = \Omega \frac{\phi}{k} \tag{6.1}$$

(MacRobbie 1971, Walker and Pitman 1976). In the case of cytosolic NO_3^- pools, a major consequence of the constancy of k_c is therefore a condition in which the size of the pool is linearly related to the steady-state influx of NO_3^- to the cytosol from the external medium. Because many influx values are possible in response to $[NO_3^-]_o$ varying over four orders of magnitude (see above), the size of the cytosolic NO_3^- pool is accordingly adjusted. Hence the "timing mechanism" proposed here not only controls the rate of turnover, but in doing so also determines pool size. Because the frequency of turnover for cytosolic NO_3^- is fixed, while the pool size varies, this condition might be termed one of "amplitude

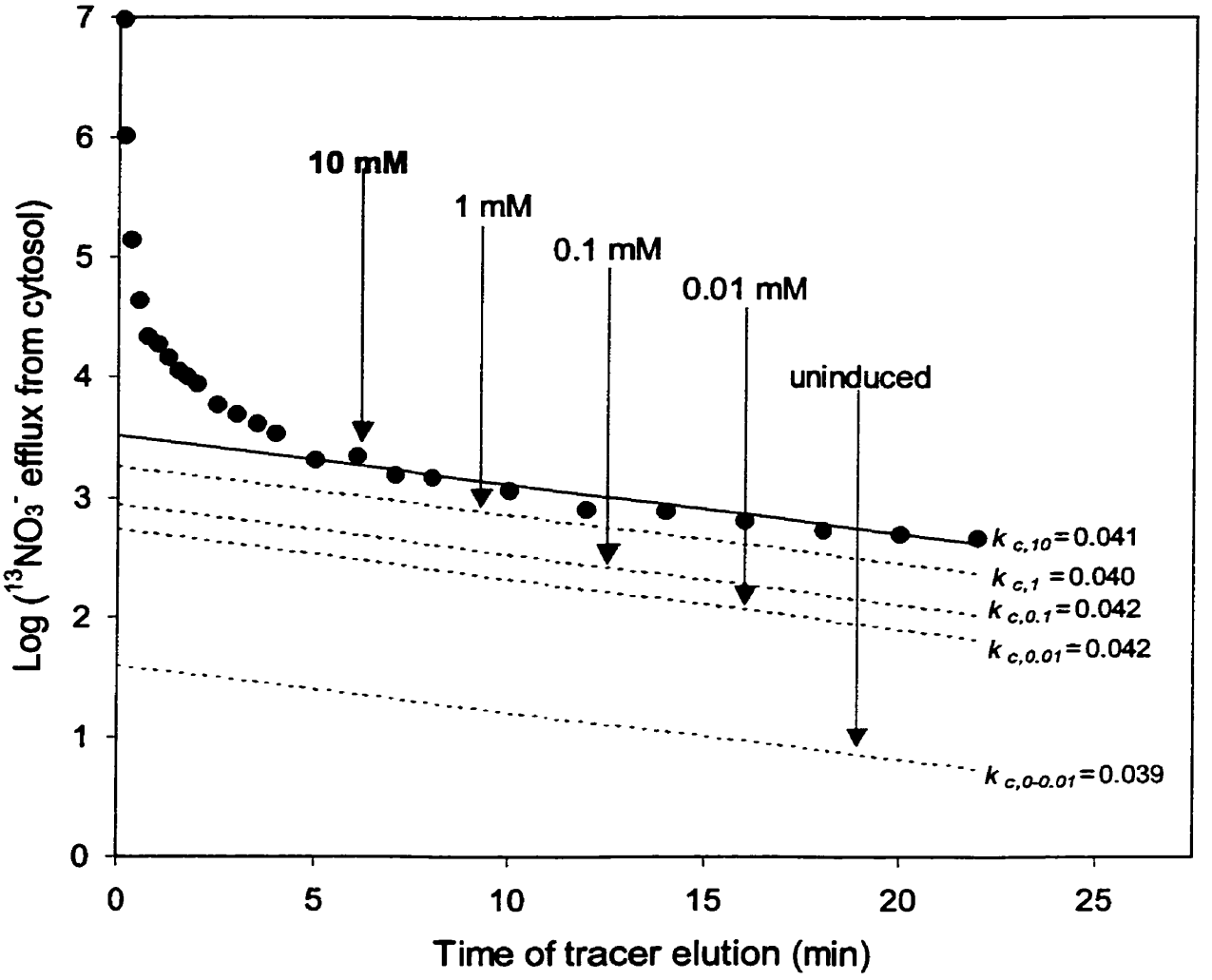

Figure 18. Comparison of cytosolic $^{13}NO_3^-$ efflux patterns in roots of barley seedlings grown and tested under $[NO_3^-]_o$ varying over four orders of magnitude, and under an uninduced condition in which seedlings were deprived of NO_3^- until the time of labeling. Plots have been corrected for differences in root mass, tracer activity, and efflux to influx ratio (see Kronzucker et al. 2000), so that y-intercepts of regression lines directly indicate relative sizes of cytosolic NO_3^- pools. k_c values (min^{-1}), averaged over several experiments (standard errors were within 15% of the mean), are indicated next to respective regression lines).

modulation". However, it should be noted that the terms "amplitude" and "frequency" (see below) here refer to steady-state pool size and pool turnover, respectively, and should be distinguished from the modulated Ca^{++} pulses operating in signal transduction functions (Berridge 1997). It should also be noted that the finding of $[NO_3^-]_c$ modulation described here is incompatible with findings from some studies using nitrate-specific microelectrodes, in which $[NO_3^-]_c$ was estimated to remain constant at around 4 mM as external $[NO_3^-]$ varied 100-fold, from 0.1 to 10 mM (Miller and Smith 1996). Clearly, these methodologies cannot both be correct. However, CAE has advantages over electrode measurement systems, including its high sensitivity (at least with ^{13}N), non-invasiveness, and freedom from calibration uncertainties (see, e.g. Carroll and McGrady 1985, Cuin *et al.* 1999). On the other hand, CAE is beset with the problem of compartment assignment, which appears not to affect protocols using multiple-barrelled electrodes. Nevertheless, this problem has been addressed in several studies (Kronzucker 1995b, 1995e), although more work should be done to resolve this methodological discrepancy.

Table 5, based upon previous compartmental analyses in a large number of plant systems, but performed essentially as described here, shows that the condition of unchanging k_c, and implicitly its association with pool-size variation, applies with equal stringency to other plant species, and to the case of NH_4^+, the second major inorganic N source for plants. As in the case of NO_3^-, the rate of NH_4^+ turnover is held within relatively narrow limits in the presence of large changes in component fluxes resulting from variations in external N (Wang *et al.* 1993a,

Table 5. (Continued on next page.) Kinetic constants (k_c, in min^{-1} ± S. E. of mean) for steady-state cytosolic exchange of NO_3^- and NH_4^+ in selected plant systems.

[a] Steady-state and measurement [N] as indicated.

[b] "0 - 0.01 mM" and "0 - 0.1 mM" treatments refer to growth on zero N and exposure to 0.01 or 0.1 mM N, respectively, during the compartmental-analysis protocol.

[c] Induction of the NO_3^--acquisition pathway was monitored, with plants grown without NO_3^- exposed for the first time to 0.1 mM NO_3^- for 0 – 5 d and assayed at this concentration after the given period.

	NO_3^- [a]						
	0 - 0.01 mM[b]	0.01 mM	0.1 mM	1 mM	1.5 mM	10 mM	Reference
Hordeum vulgare (cv. Klondike)	0.039 ± 0.001	0.042 ± 0.006	0.042 ± 0.004	0.040 ± 0.005	-	0.041 ± 0.003	Siddiqi *et al.* 1991
Hordeum vulgare (cv. CM-72)	-	-	0.028 ± 0.001	0.035 ± 0.002	-	0.039 ± 0.002	Kronzucker *et al.* 1999
Populus tremuloides	-	-	0.030 ± 0.001	-	0.030 ± 0.003	-	Min *et al.* 1999
Picea glauca	0.041 ± 0.013	0.040 ± 0.003	0.0366 ± 0.004	-	0.044 ± 0.005	-	Kronzucker *et al.* 1995b
Pinus contorta	-	-	0.043 ± 0.002	-	0.041 ± 0.002	-	Min *et al.* 1999
Pseudotsuga menziesii	-	-	0.037 ± 0.002	-	0.044 ± 0.002	-	Min *et al.* 1999

Table 5. (Continued from previous page)

	NO_3^-, development of induction[c]						
	0 – 0.1 mM[b]	1d	2d	3d	4d	5d	Reference
Picea glauca	0.049 ± 0.003	0.047 ± 0.006	0.036 ± 0.001	0.040 ± 0.004	0.040 ± 0.004	0.045 ± 0.003	Kron-zucker et al. 1995a

	NH_4^+ [a]						
	0 – 0.01 mM[b]	0.002 mM	0.01 mM	0.1 mM	1 mM	1.5 mM	Reference
Oryza sativa	-	0.043 ± 0.007	-	0.041 ± 0.001	0.036 ± 0.003	-	Wang *et al.* 1993
Hordeum vulgare (cv. Midas)	-	-	-	0.026 ± 0.001	-	0.027 ± 0.001	present work
Triticum aestivum (leaf slices)	-	-	0.021 ± 0.006	0.0216 ± 0.006	0.025 ± 0.004	-	present work
Arabidopsis thaliana	-	-	-	0.039 ± 0.003	0.042 ± 0.005	-	Rawat *et al.* 1999
Populus tremuloides	-	-	-	0.033 ± 0.007	0.032 ± 0.002	-	Min *et al.* 1999
Picea glauca	0.022 ± 0.002	-	0.017 ± 0.001	0.020 ± 0.002	-	0.029 ± 0.001	Kron-zucker *et al.* 1998
Pinus contorta	-	-	-	0.023 ± 0.004	-	0.031 ± 0.004	Min *et al.* 1999
Pseudotsuga menziesii	-	-	-	0.039 ± 0.003	-	0.042 ± 0.005	Min *et al.* 1999

Kronzucker *et al.* 1995c). Most strikingly, as illustrated in Fig. 5 (Chapter 3), sudden deviations in $[NH_4^+]_o$ from the steady state, when imposed midway through the elution period, deflected tracer efflux decline patterns from their initial courses only momentarily. Within minutes, k_c was re-established, even though the whole organism was still hours to days from attaining a steady state in uptake, metabolism, storage or growth (see Kronzucker *et al.* 1998). Such an unexpectedly rapid return to a set flux equilibrium emphasises the fidelity of the control mechanism(s) integrating N fluxes at the cellular level, which is only gradually transduced into a whole-organism response. Transient perturbations in other key cellular parameters, such as membrane electrical potential and cytosolic pH, are also known to rapidly follow changes in extracellular N concentrations of the magnitude imposed here (Wang *et al.* 1994, Kosegarten 1997), suggesting that they also might be involved in the rectification of N turnover kinetics. However, in many cases these processes require significantly longer times to achieve new resting states, when compared to the brief (~5 min) time required for k_c to be rectified (Fig. 5) (Wang *et al.* 1994, Kosegarten *et al.* 1997).

As seen in Table 5, k_c approaches uniformity for a given N form and plant system, from domesticated cereals to wild tree species to the genetic-model-system plant *A. thaliana*. In fact, every organism that has been studied using this form of compartmental analysis demonstrates genotype-specific k_c constancy. Even studies examining $^{13}NH_4^+$ efflux kinetics in leaf slices of wheat (Chapter 4) have yielded k_c values closely similar across a wide range of external N

conditions, indicating that this trait 1) can be detected among distinct cell types; and 2) is highly resilient even under conditions of substantial tissue disturbance (i.e., leaf-slice preparation).

It must be emphasised that k_c constancy could not have been anticipated *a priori*, and is, in fact, not observed for potassium (K^+). Table 6 shows that cytosolic K^+ is exchanged in a manner fundamentally different from that of NH_4^+ and NO_3^- ions. With K^+, k_c is not constant, but increases with external concentration and with associated changes in component fluxes (Memon *et al.* 1985), such that the Q term in the flux-turnover equation (eq. 6.1) is instead held constant. Unlike with inorganic N, then, cytosolic K^+ turnover is modified from state to state, whereas the size of the K^+ pool is maintained at approximately 100 mM, as confirmed by several independent measurement systems (Memon *et al.* 1985, and references therein). To use the previous analogy, cytosolic K^+ is characterized by fixed amplitude and turnover-frequency modulation. Clearly, pools and fluxes of ions in plant cells are subject to high-level integration owing to the regulatory activity of at least two fundamentally differing types of mechanisms within the plant, but questions as to why such divergent strategies may have evolved remain open.

The apparent ubiquity of k_c constancy redefines our view of nitrogen homeostasis at the cellular level, and we suggest that it could profoundly influence the outcome of initiatives to improve plant growth and yield by biotechnological means. Although overexpression of enzymes involved in N

Table 6. Kinetic constants (k_c, in min^{-1} ± S. E. of mean) for steady-state cytosolic exchange of K^+ in roots of three barley cultivars

a. Steady-state K^+ provision as indicated.

	K^+		
	0.01 mM[a]	0.1 mM[a]	Reference
Hordeum vulgare (cv. Fergus)	0.0032 ± 0.0001	0.0089 ± 0.0003	Memon et al. 1985
Hordeum vulgare (cv. Compana)	0.0040 ± 0.0002	0.0092 ± 0.0004	Memon et al. 1985
Hordeum vulgare (cv. Betzes)	0.0054 ± 0.00105	0.0080 ± 0.0005	Memon et al. 1985

metabolism has in special cases been reported to increase growth and yield (Gallardo *et al.* 1999, Migge *et al.* 2000), it has failed to do so in most instances (Foyer *et al.* 1994, Quillère *et al.* 1994, Ferrario *et al.* 1995, Gojon *et al.* 1998, Limami *et al.* 1999). Most pertinently, in the case of NR, the enzyme widely assumed to limit nitrate assimilation (Campbell 1990, Oaks 1994), efforts by leading laboratories to engineer improved plant performance through NR overexpression have, perplexingly, resulted in little or no effect upon either growth or yield (Crawford 1995, and references cited above). This outcome has been difficult to explain. It is unknown whether cytosolic N exchange rates are held constant in the event of over- or under-expression of components, such as NR, of the acquisition pathway, but given the data presented in this paper, one might predict that they are. This inference is supported by the extensively documented observation that plants adapt to changing N conditions, displaying substantial excess capacity of key enzymes and transporters involved in N acquisition. A particularly dramatic example is that of the conifer white spruce (*Picea glauca*), which, although exhibiting an impaired NO_3^--influx capacity (Kronzucker *et al.* 1997), nevertheless exhibits a substantial overshooting of that capacity during the induction of its NO_3^--acquisition pathway (Kronzucker *et al.* 1995a,d). Similar excess capacity has been observed in the timecourse of NR induction itself (Kronzucker *et al.* 1995a, and references therein). Clearly, any increase in plant N demand must be sustained by increased N supply from the external medium, but no enhancement of N uptake has ever been observed in NR overexpressers. Indeed, the reverse appears to be the case (Gojon *et al.*

1998). A consequence of k_c homeostasis might be that other components of the acquisition pathway will counteract the imposed manipulation (for example, through enhanced efflux, diminished influx, altered tonoplast fluxes, or through downregulation of potentially overactive enzymes such as NR (Kronzucker *et al.* 1995a, Gojon *et al.* 1998)), thereby maintaining constancy of cytosolic turnover. Such compensatory shifts in component fluxes are readily observed, perhaps most dramatically in the aforementioned case of *P. glauca* plants undergoing the transition from an uninduced to a steady state of NO_3^- acquisition. Intriguingly, even here, cytosolic k_c appeared not to change over the transitional period (Table 2), indicating the stringency of its regulation. However, as Fig. 17 and Table 5 illustrate, distinct differences in k_c for cytosolic nitrogen exchange do exist between and even within plant species. Thus, it is not impossible for a coordinated complex of flux processes to evolve towards, or to be bred for, different set points, reflecting the plant's growth strategy (Chapin, 1980). In this light, we believe it to be much more likely that nitrogen acquisition in crop species will be improved by means of classical breeding, which, by selecting for a complex of features rather than for isolated components, may circumvent the limitations imposed by k_c constancy.

While individual fluxes, pool sizes and turnover rates involved in plant N acquisition are reliably measurable and must have a genetic basis (see Table 5), the factors integrating them are unknown at the molecular level. Metabolites such as amino acids, as well as NO_3^- and NH_4^+ themselves, are known to regulate activities of transporters and enzymes of nitrogen metabolism (Imsande and

Touraine 1994, Oaks 1994, Crawford and Glass 1998, Rawat *et al.* 1999), but in all likelihood these pools are only single components of a much more complex network of regulating factors, both genetic and environmental. We conclude that flux regulation in plant systems is much more stringent and complex than previously assumed and may generate hitherto unforeseen obstacles for prospects to improve plant nitrogen acquisition.

Chapter 7. Conclusions and Future Directions.

Compartmental analysis by efflux (CAE) of traced N from plant tissues has been shown, here and elsewhere, to be a useful and parameter-rich method by which to understand the movement and subcellular localisation of this important ion. In this work, a number of inferences may be drawn from the model and the data, which can assist in the design of future flux and compartmentation experiments in both roots and leaf slices, shed light on the phenomenon of ammonium toxicity and its ecological implications, and provide insights into the regulation of cellular nitrogen fluxes.

The future directions for the work described here are extensive. The theory underlying compartmental analysis by efflux in higher plant tissues requires further elaboration in order to maximise the explanatory potential of the technique. More complicated investigations, such as those using multiple tracers for the observation of ionic interactions in the cytosol, for instance, could be carried out with greater confidence and insight, allowing for the study of many as yet unexplored variations on the theme of plant-ion relations.

The mechanism of NH_4^+ toxicity discussed in Chapter 5, for instance, could be further studied using CAE, from the perspective of the widely-observed phenomenon that NH_4^+ nutrition tends to suppress the absorption of other cations, such as K^+, Ca^{2+}, and Mg^{2+} by plant roots (Wilcox *et al.* 1973, Claassen and Wilcox 1986, Polizotto *et al.* 1975, Wilcox *et al.* 1977, Magalhaes and Wilcox 1983, Wilcox *et al.* 1985). It is unknown, however, whether the resulting decline in plant mineral cation content entails the lowering of cytosolic concentrations of

any of these ions, or if this decline is limited to vacuolar concentrations. Given the extraordinarily high (though, to remind the reader, sub-Nernstian) NH_4^+ influx values and $[NH_4^+]_c$ reported here under high $[NH_4^+]_o$, one might predict that such ions are displaced from this compartment, possibly to the extent that it leads to stresses on plant survival. CAE using radiotracers such as ^{42}K and ^{28}Mg under varying $[NH_4^+]_o$ could prove to be useful for the testing of this prediction, and its implications for the nutrient balance of the plant. CAE using ^{36}Cl could also be used to test whether the chloride ion accumulates in the cytosol to act as a counter-ion against high $[NH_4^+]_c$, possibly resulting in an additional source of salt stress.

On the other hand, the question as to whether Ca^{2+} is displaced from the cytosol in response to high $[NH_4^+]_c$ is unlikely to be approachable using the methods in the present work. This is because the extremely low reported values for $[Ca^{2+}]_c$, in conjunction with substantial values for Ca^{2+} influx (Pineros and Tester 1997, McLaughlin and Wimmer 1999), would result in a value for k_c so high (i.e., a $t_{½}$ so low) that it would be practically impossible to trace the cytosolic Ca^{2+} phase by observation of ^{45}Ca efflux, even given a much greater time resolution than employed here with $^{13}NH_4^+$. However, this insight leads to the potential re-assignment, as vacuolar, of a ^{45}Ca efflux phase previously observed in onion root segments and in barley roots, and assumed to be cytosolic (Macklon and Sim 1981, Siddiqi and Glass 1984). This re-assignment could be tested in tomato plants, which display substantially lowered tissue Ca^{2+} in response to high $[NH_4^+]_o$ (Wilcox *et al.* 1973, 1977, Magalhaes and Wilcox 1983,

Wilcox *et al.* 1985), a symptom of the susceptibility of this species to NH_4^+ toxicity. Given low expected values for $[Ca^{2+}]_c$, this decrease most likely reflects lower vacuolar $[Ca^{2+}]$, a condition which should be evident in ^{45}Ca tracer efflux plots. This proposed study could be an important test case for compartmental analysis, which at times provides subcellular concentration estimates that vary from those determined by other means (see below).

The use of CAE to observe vacuolar fluxes and pools of NH_4^+ is another potentially productive line of research. Because the turnover of nitrogen in the vacuole is considered to be substantially longer than in the cytosol (Devienne *et al.* 1994a, b), however, a stable isotope such as ^{15}N would be more appropriate for such an investigation than the short-lived tracer ^{13}N. CAE with ^{15}N could answer questions raised by the results presented in Chapters 5 and 6, such as: 1) whether very high fluxes occur in both directions across the tonoplast under high $[NH_4^+]_o$, as they do across the plasmalemma (Chapter 5); and 2) whether vacuolar NH_4^+ turnover (as observed in its *k* value) remains constant over a wide range of $[NH_4^+]_o$, as was observed for cytosolic turnover (Chapter 6).

As mentioned in Chapter 4, the "net flux" term derived from CAE is an overestimate of true net flux, such as that measured in depletion experiments. This is because the vacuolar pool is not labeled to the extent that it will contribute significantly to tracer efflux (see Chapter 3). The term "quasi-steady influx to the vacuole" may therefore be a preferable term for ϕ_{net} (Cram and Laties 1971). However, because in the case of NH_4^+, assimilatory (and, possibly, shoot) fluxes can be substantial, a more general term such as "traced quasi-steady flux" may

be still more appropriate. The exact extent of the ϕ_{net} overestimate could be precisely delineated using longer-term ^{15}N tracing with CAE, which would reveal the efflux contribution from the vacuole.

The NH_4^+ -toxicity question presented in Chapter 5 can also be further explored in terms of the apparent energy-dependence of efflux. Chemical agents which specifically block plasma membrane ATPase activity, for instance, could be used to investigate their effects on the active efflux process. Similarly, channel-blocking agents known for their suppression of low-affinity K^+ fluxes, could be used to see if the very high fluxes seen in NH_4^+-susceptible species are alleviated. Because the energy consumed in the futile cycling of NH_4^+ must ultimately be released as heat, it might be possible to measure thermal dissipation from roots of plants showing NH_4^+-dependent respiration.

The phenomenon of k_c constancy could be further explored in a number of ways, such as through the application of the experimental procedures used in Chapter 6 to plants having alterations in nitrate reductase or glutamine synthetase. The resilience of k_c values following perturbation could be further tested using permutations in external conditions as yet untried. Further mathematical modeling of plant responses to these changes also needs to be undertaken, leading to a greater understanding of the meaning of the "rise and fall" kinetics of tracer efflux.

Finally, workers in this field should seek to further address the doubts sometimes expressed about the veracity of estimates made using CAE protocols (e.g. Epstein 1972, Zierler 1981, Miller and Smith 1996, Howitt and Udvardi

2000). Some of the CAE-derived cytosolic concentrations reported here and elsewhere differ substantially from those derived using other methods (see also Chapter 6, p. 95). For instance, Table 3 (Chapter 5) shows that $[NH_4^+]_c$ at 0.1 mM $[NH_4^+]_o$ is estimated to be 12 mM in barley, while both NMR and microelectrode studies show lower (7-8 mM) $[NH_4^+]_c$ at tenfold higher $[NH_4^+]_o$ (Lee and Ratcliffe 1991, Wells and Miller 2000). Some of this disagreement may lie in differences in plant material (*Zea mays* and *Chara corallina* were used, respectively, in the two latter studies), but in general it is clear that fundamental errors in one or more of the methods must exist, and these need to be investigated. As discussed in Chapter 6, the CAE method is not beset with problems of invasiveness, calibration, or low sensitivity, which may compromise other methods. However, the correct assignment of efflux phases to cellular compartments (the "inverse problem" – see Jacquez 1996) is crucial to the CAE method, and while it has been verified to some extent (Kronzucker *et al.* 1995b, 1995e), further explorations are necessary to unequivocally resolve this question.

To conclude, this thesis presents a number of novel ideas in the field of plant-nitrogen relations, but as is generally the case in scientific work, new questions have been raised with each discovery.

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