Can unidirectional influx be measured in higher plants? A mathematical approach using parameters from efflux analysis

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Summary

• A comprehensive and pragmatic approach to the design of unidirectional ion transport experiments in plants is presented here, revising and simplifying classical models.

• The kinetic constant for cytosolic ion exchange (k_c) is critical to the understanding of the interrelated flux processes occurring simultaneously at the cellular level. This constant is most effectively estimated using the compartmental analysis by efflux method, which, by providing values for additional kinetic parameters (i.e. unidirectional influx and efflux) can be used to determine the extent of distortion inherent in assessments of influx at, or close to, the steady state.

• Focusing on the kinetics of nitrogen exchange, with tracer efflux experiments in barley (*Hordeum vulgare*) using $^{13}NH_4^+$, conducted under perturbation conditions, it was demonstrated that a transitional state was rapidly established following a concentration shift, characterized by a restoration of the preperturbational (steady-state) k_c value. It is concluded that a reasonably accurate estimate of unidirectional influx can be made when influx measurements are conducted subsequent to the establishment of this transitional state.

• A mathematical treatment of unidirectional flux processes allows the exact determination of errors caused by ionic counterfluxes under steady-state conditions.

Key words: ammonium, barley, compartmental analysis, efflux, influx, *k* constant, N-13, steady state.

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 & 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 (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 & Ayling, 1993). To date, this claim remains unrefuted. In the present paper we use kinetic parameters obtained from efflux analysis to show why it is incorrect, presenting, in a simplified yet comprehensive form, a mathematical treatment of unidirectional flux processes which allows the exact determination of errors caused by ionic counterfluxes under steady-state conditions. We supplement this with original experiments for ammonium (NH_4^+) acquisition under steady-state and perturbational conditions in the model system barley. From these theoretical and experimental considerations, procedural recommendations emerge, the careful consideration of which is essential to the execution and interpretation of steady-state and perturbational influx measurements.

Materials and Methods

Plant growth conditions and isotope preparation

Barley seedlings (*Hordeum vulgare* L., cv. CM-72 and cv. Midas) were cultured hydroponically for 7 d in controlled-environment chambers as described elsewhere (Siddiqi *et al.*, 1990, 1991; Kronzucker *et al.*, 1999). The hydroponic tanks contained aerated 1/4-strength modified N-free Johnson's solution (Kronzucker *et al.*, 1999). NH_4^+ was supplied as $(NH_4)_2SO_4$. N concentration during growth was 0.1 mM or 10 mM. During ^{13}N-labelling N was also provided at the growth concentration. The radiotracer ^{13}N was provided by the Tri-University Meson Facility (TRIUMF) at the University of British Columbia, and $^{13}NH_4^+$ was prepared according to previously described procedures (Wang *et al.*, 1993; Kronzucker *et al.*, 1995b,c).

Efflux experiments

Roots of intact barley seedlings (grown at steady-state provision of 0.1 or 10 mM NH_4^+) were equilibrated in nonlabelled preloading solution for 5 min before transfer to the ^{13}N-labelling solution. Roots were then immersed in ^{13}N-labelled solution for 60 min to maximize specific ^{13}N activity in the cytosolic phase (Lee & Clarkson, 1986; Siddiqi *et al.*, 1991; Kronzucker *et al.*, 1995a,b,c). Seedlings were transferred to efflux funnels (Siddiqi *et al.*, 1991; Wang *et al.*, 1993) and the roots eluted successively with 20-ml aliquots of nonlabelled solution for varying time periods. NH_4^+ concentration in the eluant was identical to that of the growth and labelling solutions, except in concentration shift experiments, where seedlings grown and labelled at 0.1 mM NH_4^+ were subjected to either an upward (to 10 mM) or downward (to 0.01 mM) concentration shift during elution (see Fig. 4). Serial elution proceeded for up to 26 min in steady-state trials and up to 32 min in concentration shift experiments with sampling intervals as shown in Figs 1 and 4. The eluates were counted in a Packard γ-counter (Minaxi δ, Auto-γ 5000 Series (Canberra-Packard Canada, Mississauga, ON, Canada)). Roots were excised from the shoots after the final elution and spun for 30 s, and plant organs were weighed and also counted. Treatment of data was as described elsewhere (Lee & Clarkson, 1986; Siddiqi *et al.*, 1991). Experiments were repeated eight to nine times. Standard errors were within 10% of the means. Representative experiments were chosen for semilogarithmic plots of the rate of ^{13}N-release vs elution time (Figs 1 and 4). By convention, the y-axes of efflux plots are on a decadal logarithmic scale, while *k*-values given are based upon natural logarithms.

Results and Discussion

Steady-state conditions

Before we can discuss quantitatively the errors associated with influx determinations, some key concepts underlying tracer studies need to be reiterated. If chemical fluxes and pool sizes remain constant in the plant over the duration of flux measurement, a condition referred to throughout this paper 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 & Dainty, 1958; Pitman, 1963; Pallaghy & Scott, 1969; MacRobbie, 1971; Pitman, 1971; Poole, 1971; Cram, 1973; Walker & Pitman, 1976; Jeschke & Jambor, 1981; MacRobbie, 1981; Behl & Jeschke, 1982). The exponentiality of the compartmental labelling 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 & Clarkson, 1986). As such, when a solute pool within the cytosol of a plant cell is labelled directly from an essentially nonchanging 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, $t_{1/2} = 0.693/k$, when natural logarithms are used) describing the time dimension of pool turnover. As described in Walker & Pitman, 1976), the rise in s_c during a labelling time t can therefore be modelled using this parameter in the following exponential equation:

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

However, this simple equation does not consider delivery fluxes of unlabelled 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 addition, backflow from such pools inside the plant which gradually become labelled in longer-term tracer experiments, must be considered. In such cases, tracer content of the compartment will increase in a stepwise, compoundly exponential, manner, as these fluxes become labelled. When labelled tissue is exposed to nonlabelled external solution, and in the absence of other compartments sufficiently labelled such that they back-deliver tracer to the cytosol, the decline in tracer content of the cytosol over time can be described in the equation:

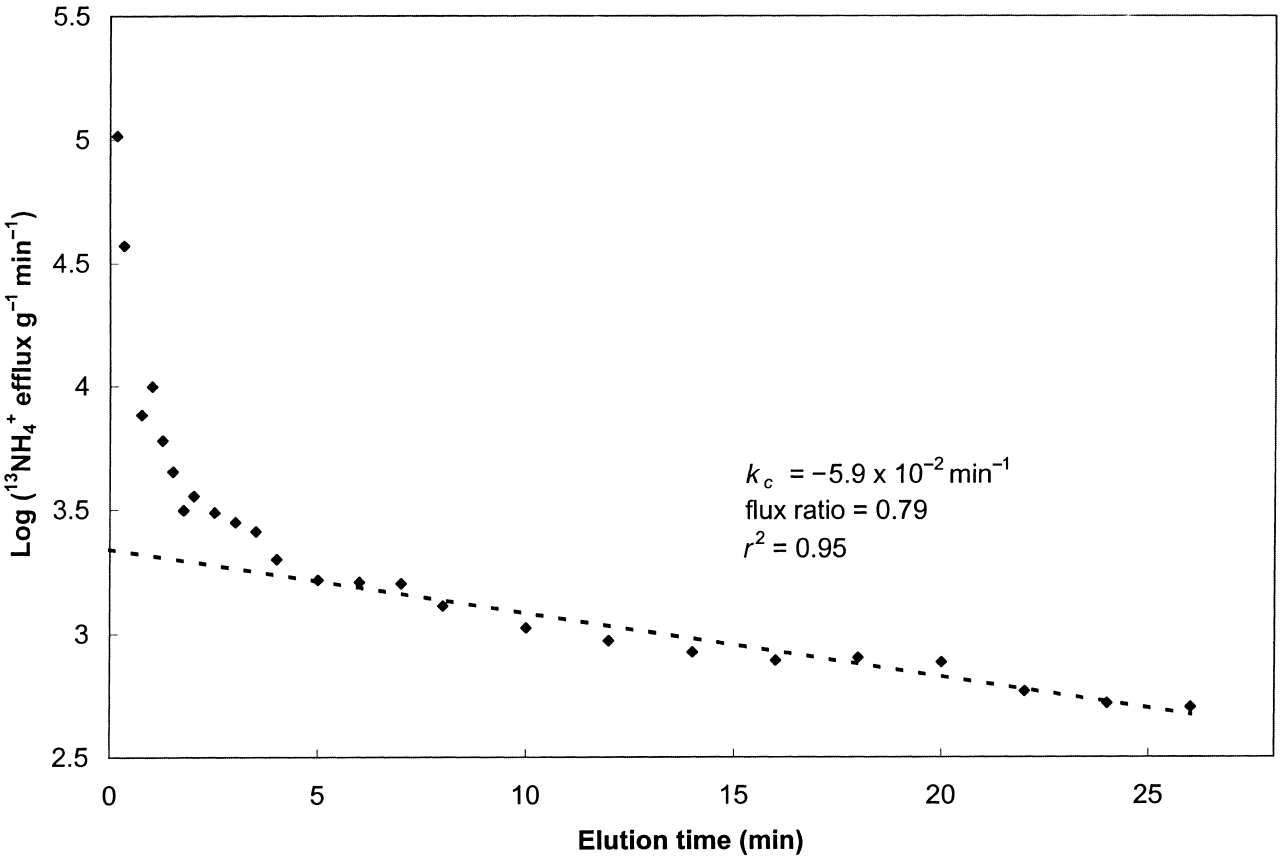

Fig. 1 Representative plot of $^{13}NH_4^+$ efflux from roots of intact barley (*Hordeum vulgare* L., cv. CM-72) seedlings at 10 mM $[NH_4^+]_o$. Seedlings were labelled in ^{13}N-containing solution for 60 min and then subjected to a 26-min elution protocol as described in Materials and Methods. The logarithm of the rate of release of radioactivity from root tissue was plotted vs time of tracer elution, and linear regression on the semilogarithmic plots was then used to resolve separate phases. The dashed line represents tracer elution from the root cytosol (Kronzucker *et al.*, 1995c). The kinetic exchange constant (k_c), flux ratio and the coefficient of determination (r^2) for this phase are as indicated.

$$s_c = s_{initial}e^{-kt}$$

($s_{initial}$, the cytosolic specific activity at the start of elution). If tracer is delivered from additional pools within the plant, the elution process is no longer governed by a single exponential, 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 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), 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. For instance, in the case of a cytosolic pool labelled by an external tracer source, variations in the kinetics of specific activity buildup in the cytosol as a result of fluxes from initially unlabelled, predominantly vacuolar, pools inside the plant are entirely accounted for in the cytosolic *k* term (k_c). However, as emphasized above 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 Fig. 3 below). In other words, while initially the processes of cytosolic labelling and elution are governed by the same *k* constant (cf. Lee & Ayling, 1993), the cytosolic pool cannot in the short term reach a specific activity identical to that of the external solution, contrary to what is often presumed (Siddiqi *et al.*, 1991).

Because tracer efflux is directly proportional to tracer activity within a source compartment, it must follow the same kinetics of rise or decline. The exponential nature of this decline is verified by the observation (Fig. 1) that the serial elution of tracer from a labelled root system follows a compoundly exponential decay pattern (Lee & Clarkson, 1986; Siddiqi *et al.*, 1991; Wang *et al.*, 1993). When transformed semilogarithmically, this pattern may be dissected to resolve individual phases of tracer release, which must in turn 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 the slope of the appropriate segment of the tracer-efflux plot[1], as long as k values 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 measurement of influx across the plasmalemma 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 labelling, to remove tracer adsorbed in the apparent free space (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 release during desorption form the very basis of efflux analysis.

Since k_c and efflux values often vary considerably between plant species, ion species, and ion supply conditions, any modelling of these processes needs to be case-specific. However, the following model provides a generalized means of estimating steady-state unidirectional influx by taking into account the effect of efflux during labelling and desorption. It is important to note that this simplified equation applies only to a plant system in which fluxes delivering unlabelled quanta of the measured ion to the cytosol (e.g. from the vacuole) are negligable 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 labelling and desorption periods.

B = quantity of tracer lost from cytosol during the labelling 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 labelling 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

$$\phi^*_{oc} = Q^*/t_L$$

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

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

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

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

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

Therefore

$$\phi^*_{oc} = \left\{A + \phi^*_{co}\left[t_L - \frac{1}{k_c}(e^{-k_c t_D} - e^{-k_c(t_L+t_D)})\right]\right\}/t_L .$$

This expression is a novel summary of the essential terms that contribute to tracer influx (cf. Cram, 1969), and circumvents the unnecessarily complicated mathematical treatments repeatedly presented in the literature (MacRobbie, 1971; Walker & 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. 2(a), for example, shows how true influx can be underestimated in a labelling experiment due to efflux removing tracer from the cytosol during labelling. Fig. 2(b) shows how the extent of this deflection depends on k_c and the magnitude of efflux relative to influx (i.e. the flux ratio). In this figure, three flux 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} ($t_{1/2}$ of 7 min) and a flux ratio of 1, true influx is underestimated by 37% after a labelling 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. 2c). 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 labelling period, and, especially, when the flux 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 a flux 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 minimized 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

[1]In the case of nonmetabolised 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).

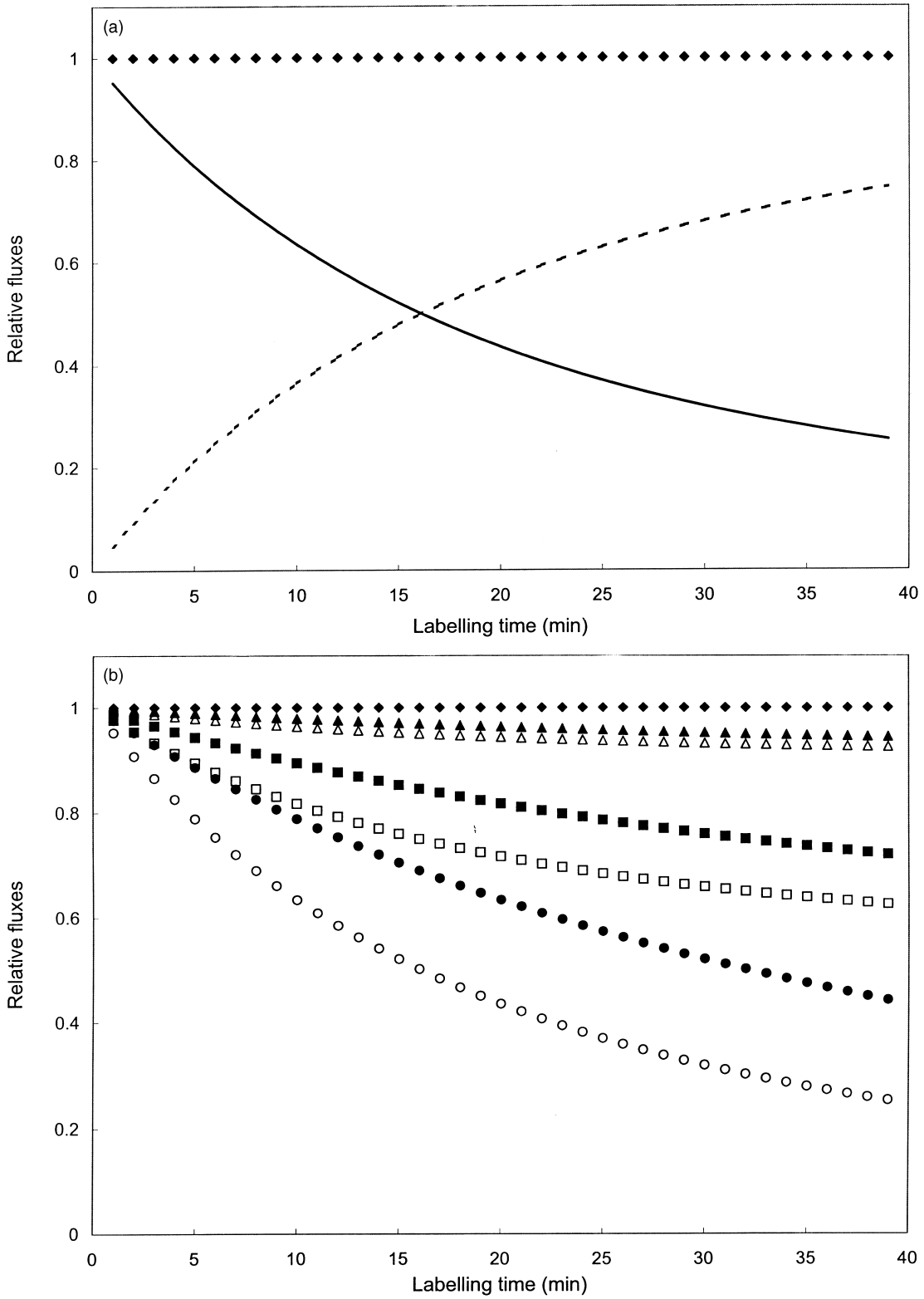

Fig. 2 (a) Graphical depiction of the effect of efflux on measurement of influx as a function of labelling time. In this example, a root system with a cytosolic exchange half-life of 7 min and a flux ratio of 1 is shown. For clarity, no desorption period is considered here. Solid line, net (measured) tracer influx; dashed line, tracer efflux. (b) The model as applied to systems with half-lives of 7 min (open symbols) and 14 min (closed symbols), at flux ratios of 0.1 (triangles), 0.5 (squares), and 1 (circles). Again, desorption was not considered. (c) The effect of varying periods of desorption on the system shown in (a). No desorption (solid diamond); 3 min desorption (solid triangle); 5 min desorption (solid square); 10 min desorption (solid circle).

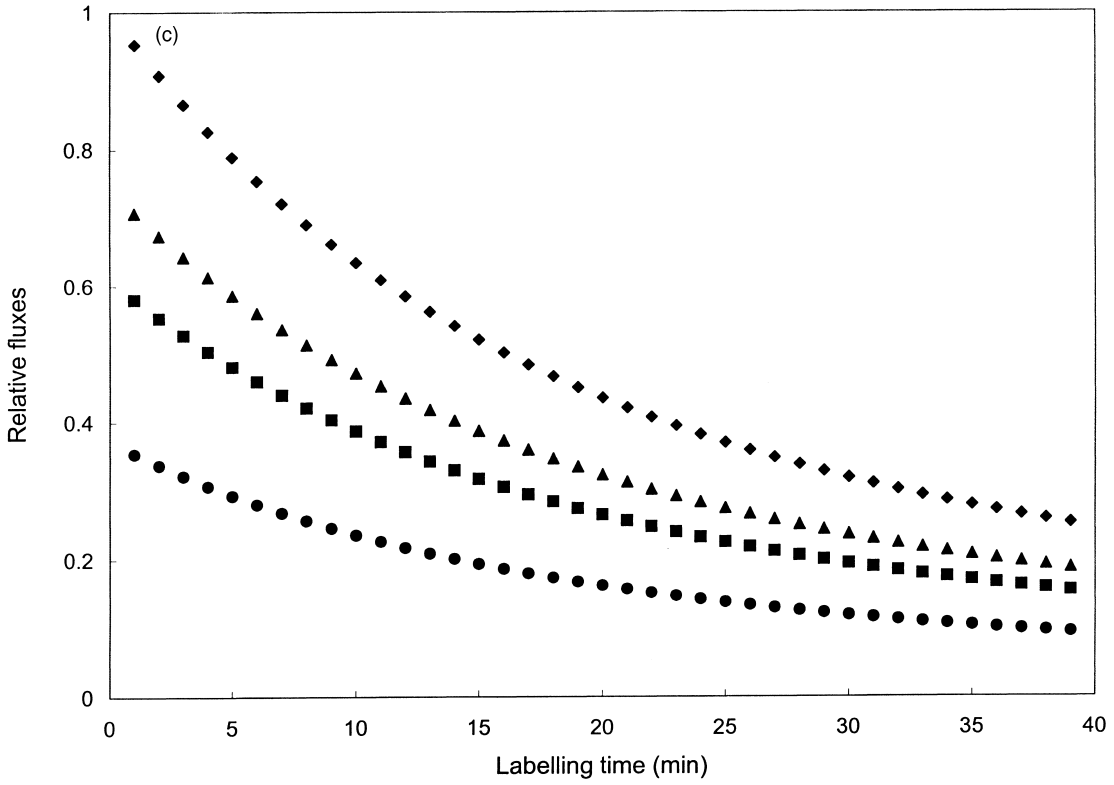

Fig. 2 *continued.*

over in this space can be modelled by considering that it, too, will diminish exponentially during the desorption step according to an equation analogous to that describing cytosolic tracer loss:

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

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

As suggested earlier, the analysis of influx underestimates is further complicated if delivery fluxes into the cytosol from sources other than traced delivery from the external medium are present. Potentially sizeable internal fluxes, from the vacuole (ϕ_{vc}) or from other endogenous pools (Feng *et al.*, 1998), will lower the effective specific activity of the cytosolic pool, and therefore will attenuate the problem of tracer efflux during influx measurement procedures. Here we use experimentally determined $t_{1/2}$ values for vacuolar NH_4^+ exchange from Macklon *et al.*, 1990 (after compartment reassignment following rationale presented in Kronzucker *et al.* (1995c); this $t_{1/2}$ value of approximately 100 min has been confirmed by our unpublished results using ^{15}N), and vacuolar pool sizes estimated from total tissue analysis (Wang *et al.*, 1993; our unpublished results), to calculate tonoplast fluxes, thereby permitting a modelling of tracer rise in both cytosol and vacuole (Fig. 3). Under the assumption that tonoplast fluxes are equal in both directions (i.e. $\phi_{cv} = \phi_{vc}$), we used the equation

$$\phi_{cv} = \frac{[NH_4^+]_v}{\Omega t_{1/2}}$$

(ϕ_{cv}, expressed in μmol g^{-1} h^{-1}, $[NH_4^+]_v$ (the vacuolar ammonium concentration) in mM; and $t_{1/2}$, 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 $t_{1/2}$ 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. 3 (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, the value of which is determined by the ratio of ϕ_{oc} to ($\phi_{oc} + \phi_{vc}$) (Lee & 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),

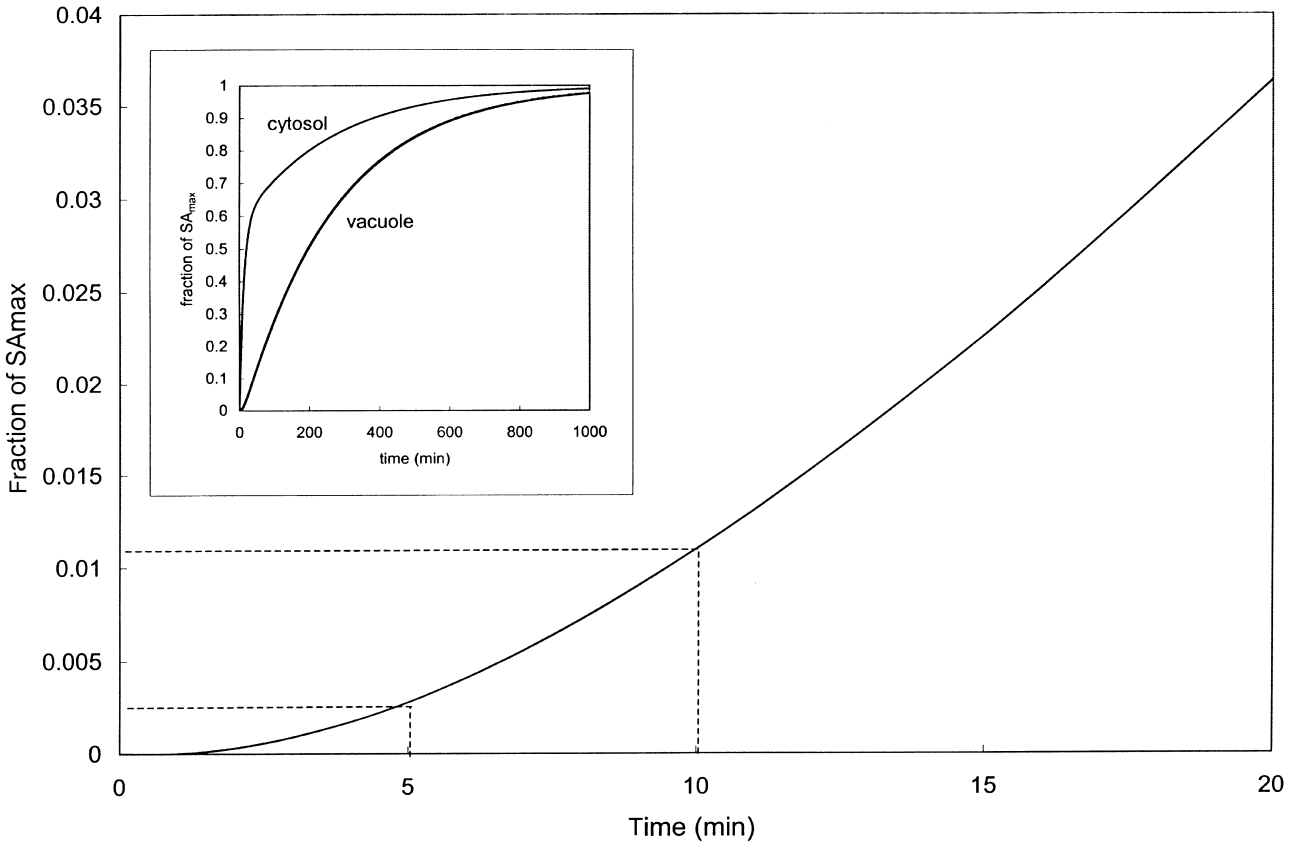

Fig. 3 Demonstration of the sigmoidal nature of tracer activity rise in the vacuole, resulting from the compound exponentiality of tracer increase in the cytosolic pool which labels the vacuole (inset). Maximal specific activity in either compartment is designated SA_{max} (= 1). The fraction of SA_{max} attained after labelling times of 5 and 10 min are as indicated by dashed lines. Data used for model are discussed in the text.

accordingly reducing the impact of ϕ^*_{co} on influx measurements, and reflecting more realistic experimental conditions:

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

Even when tonoplast fluxes are large compared to influx across the plasmalemma (but cf. MacRobbie, 1971), back-delivery of tracer from the vacuole into the cytosol (and hence available for efflux during labelling) will be negligible, as specific activity in the vacuole will have reached only 1.1% of its maximal value over a standard 10-min labelling period (Fig. 3). As illustrated in Fig. 3, the specific activity in the vacuole rises sigmoidally due to the requirement that the pool which labels it, that is the cytosol, is itself only gradually labelled. Interestingly, this entails a discrepancy in the kinetics of vacuolar labelling 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 & Pitman, 1976), during the early phases of elution, some, finite, amounts of tracer will still be delivered to the vacuole, effectively elevating the intercept, albeit marginally, of the vacuolar regression line, and causing a marginal deviation from first-order elution kinetics (E. A. C. MacRobbie, pers. comm.). The extent of these deviations will be diminished with longer loading times and with increased differences between cytosolic and vacuolar half-lives. In the present example, $t_{1/2}$ for the vacuole was determined in washout experiments to be 100 min, while the apparent half-time of filling this compartment with tracer 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 5–10 min influx measurement.

In summary, efflux analysis for a given steady-state condition must precede any kinetic evaluation of influx processes, in order to minimize, or at least quantify, errors associated with tracer efflux during labelling and desorption. Similar reasoning by Cram (1969, 1973) led to the formulation of a guideline which stated that the time period of labelling 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. 2 show that this rule pertains only to the specific condition of a system having a flux ratio of 1 and having

ϕ_{oc} much greater than all other fluxes into the cytosol, and hence may be overly generic. In fact, partly due to the indiscriminate application of this guideline, Lee & 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, $t_{1/2}$ for cytosolic NH_4^+ exchange was calculated only indirectly from flux data and NMR-derived estimates of pool size (Lee & Ratcliffe, 1991), rather than from direct measurements such as more recently made using efflux analysis (Wang *et al.*, 1993; Kronzucker *et al.*, 1995c; Min *et al.*, 1999; our unpublished results); the latter show that the cytosolic $t_{1/2}$ 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 earlier. 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 flux ratio in the abscence of MSO (Wang *et al.*, 1993; Kronzucker *et al.*, 1995c; our unpublished results). While the flux discrepancies between short- and long-term measurements reported in their work, then, may be attributable to an increasing significance of an efflux term, we reiterate that evaluations of this sort must include *direct* measurements of efflux and $t_{1/2}$ terms[3]. In contrast to the conclusions drawn by Lee & 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 flux ratios.

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 isotherms imply that the measured flux at each concentration represents the condition of the influx transporter at the moment a new state of ion provision 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 analysed is at steady state with respect to only one concentration, that is 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 undermined by the fact that our 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, is severely limited. We do know that, upon transitions in external ion concentrations, plant cells may undergo substantial and rapid changes in essential parameters such as membrane electrical potential (Ullrich *et al.*, 1984; Ayling, 1993; Wang *et al.*, 1994; Crawford & Glass, 1998) and cytosolic pH (Kosegarten *et al.*, 1997). These changes occur over a time scale of seconds to minutes and must affect the thermodynamic conditions influencing fluxes across the plasma membrane. Moreover, the spatial configuration, and thus substrate affinity, 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 from the variation of a single parameter (i.e. external concentration), but from a suite of response characteristics 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. 4, which shows that $^{13}NH_4^+$ efflux from prelabelled 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 semiquantitative interpretations are possible. It is well established that, in the long term, the capacity of plants to take up and process 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 & Rudge, 1986; Morgan & Jackson, 1988a,b; Kronzucker *et al.*, 1998). Compartment sizes of nitrate and ammonium are also known to increase or decrease over the long term with increasing or decreasing external nutrient provision, respectively (Siddiqi *et al.*, 1991; Wang *et al.*, 1993; Kronzucker *et al.*, 1995a,b). A relatively large cytosolic compartment size, in combination with a relatively downregulated influx, will dilate the period

[2]The authors observed substantial decreases in ammonium fluxes when methionine sulphoximine (MSO)-treated barley plants were labelled 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).

[3]Interestingly, in the case of inorganic nitrogen exchange, the apparent constancy of $t_{1/2}$ vis-à-vis changing conditions of N-suppy (Kronzucker *et al.*, 1995a,b) renders this task somewhat simpler, but still necessitates measurements of efflux for the determination of flux ratio.

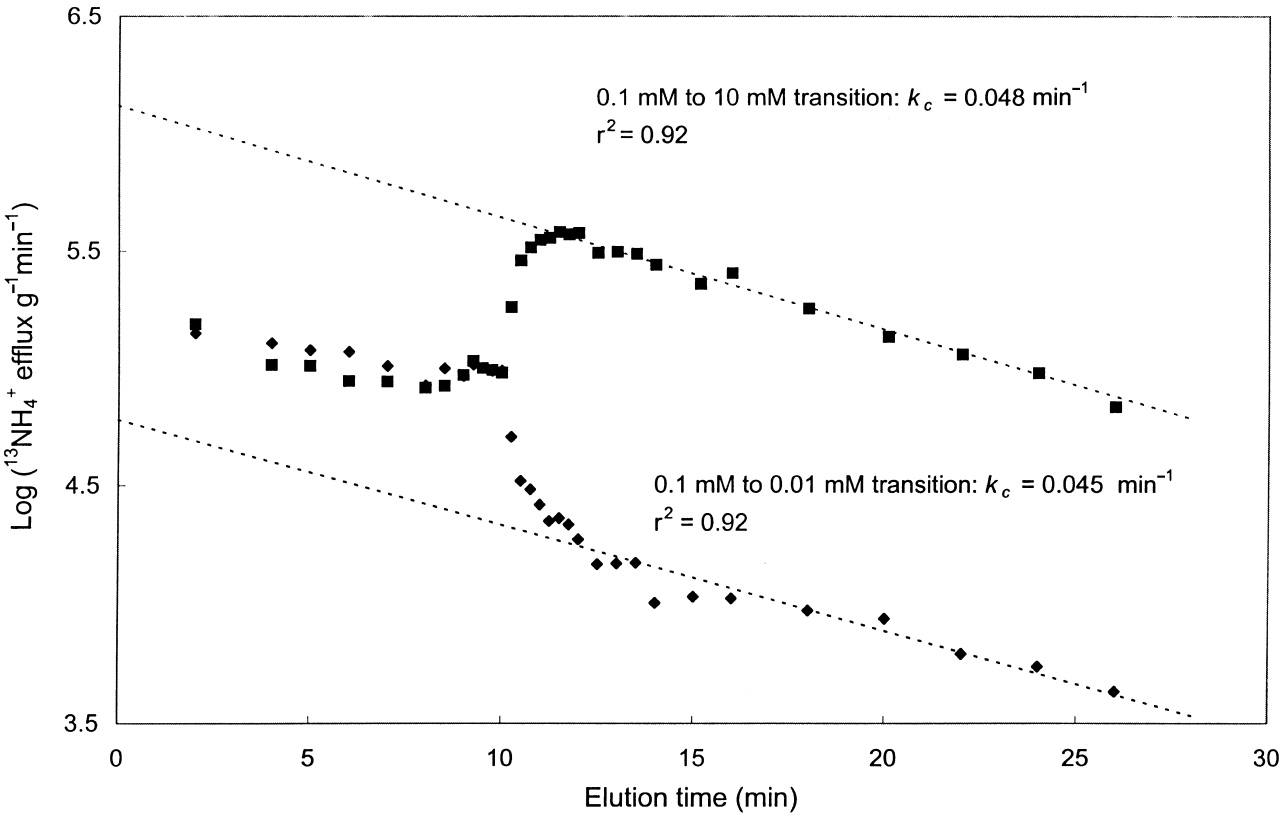

Fig. 4 Patterns of $^{13}NH_4^+$ efflux from roots of barley (cv. Klondike) seedlings with 0.1 mM NH_4^+ provided continuously (middle line), or with imposition of a concentration shift from 0.1 to 10 mM (upper line) and from 0.1 to 0.01 mM (lower line). Note the return to first-order exchange kinetics after 5 min following perturbation.

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 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 exceeds that of the steady-state growing condition.

Most interestingly, however, the short-term concentration-dependent changes evident in modulated tracer efflux (Fig. 4) only appear to show for a few minutes the chaotic behaviour expected of a perturbed system. After this initial phase of disturbance, the rate of tracer elution returns to similar first-order exponential decline characteristics 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 minimized by introducing a standard equilibration step (prewash), lasting for the duration of the chaotic adjustment period, for each concentration, with an unlabelled solution at that concentration (see, e.g. Siddiqi *et al.*, 1990; Kronzucker *et al.*, 1995c, 1996). Knowledge of the *k* value and the time-course of its resumption would then permit the application of Cram's guideline (see the section titled 'Steady-state conditions') 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 flux 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

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.

The conclusion drawn by Lee & Ayling (1993), that influx (ϕ_{oc}) under steady-state conditions is not measurable, is shown to be incorrect.

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.

By contrast, the timecourse 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 unlabel it.

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 labelled. However, the initial plateau to which tracer activity in the cytosol tends can be estimated.

Tracer activity carried over in the cell wall after a labelling 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 labelling can reduce this tracer contribution, but tracer efflux from the cytosol during this time must be accounted for, as presented in our model.

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

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 we show, in the case of NH_4^+, to be complete within approx. 5 min following the onset of the concentration shift. Procedurally, we recommend the inclusion of an equilibration step preceding the actual flux assay, the duration of which should at least equal the adjustment period.

Our analyses emphasize the necessity for caution in making inferences about the mechanistic properties of transport proteins from measurements of ϕ_{oc}, and equally in attributing transport functions to gene sequences. 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.

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References

Ayling SM. 1993. The effect of ammonium ions on membrane potential and anion flux in roots of barley and tomato. *Plant, Cell & Environment* **16**: 297–303.

Behl R, Jeschke WD. 1982. Potassium fluxes in excised barley roots. *Journal of Experimental Botany* **33**: 584–600.

Cheeseman JM. 1986. Compartmental efflux analysis: an evaluation of the technique and its limitations. *Plant Physiology* **80**: 1006–1011.

Cram WJ. 1969. Short term influx as a measure of influx across the plasmalemma. *Plant Physiology* **44**: 1013–1015.

Cram WJ. 1973. Chloride fluxes in cells of the isolated root cortex of *Zea mays*. *Australian Journal of Biological Sciences* **26**: 757–779.

Cram WJ. 1974. Influx isotherms – their interpretation and use. In: Zimmermann U, Dainty J, eds. *Membrane transport in plants*. New York, USA: Springer-Verlag, 334–337.

Crawford NM, Glass ADM. 1998. Molecular and physiological aspects of nitrate uptake in plants. *Trends in Plant Science* **3**: 389–395.

Epstein E. 1966. Dual patterns of ion absorption by plant cells and by plants. *Nature* **212**: 1324–1327.

Feng J, Volk RJ, Jackson WA. 1994. Inward and outward transport of ammonium in roots of maize and sorghum: contrasting effects of methionine sulphoximine. *Journal of Experimental Botany* **45**: 429–439.

Feng J, Volk RJ, Jackson WA. 1998. Source and magnitude of ammonium generation in maize roots. *Plant Physiology* **118**: 835–841.

Jeschke WD, Jambor W. 1981. Determination of unidirectional sodium fluxes in roots of intact sunflower seedlings. *Journal of Experimental Botany* **32**: 1257–1272.

Kosegarten H, Grolig F, Wieneke J, Wilson G, Hoffmann B. 1997. Differential ammonia-elicited changes of cytosolic pH in root hair cells of rice and maize as monitored by 2′,7′-bis-(2-carboxyethyl) -5 (and -6) -carboxyfluorescein-flourescence ratio. *Plant Physiology* **113**: 451–461.

Krapp A, Fraisier V, Scheible W, Quesada A, Gojon A, Stitt M, Caboche M, Daniel-Vedele F. 1998. Expression studies of *Nrt2: 1Np*, a putative high-affinity nitrate transporter: Evidence for its role in nitrate uptake. *Plant Journal* **14**: 723–731.

Kronzucker HJ, Glass ADM, Siddiqi MY. 1999. Inhibition of nitrate uptake by ammonium in barley. Analysis of component fluxes. *Plant Physiology* **120**: 283–292.

Kronzucker HJ, Schjoerring JK, Erner Y, Kirk GJD, Siddiqi MY, Glass ADM. 1998. Dynamic interactions between root NH_4^+ influx and long-distance N translocation in rice: Insights into feedback processes. *Plant and Cell Physiology* **39**: 1287–1293.

Kronzucker HJ, Siddiqi MY, Glass ADM. 1995a. Compartmentation and flux characteristics of nitrate in spruce. *Planta* **196**: 674–682.

Kronzucker HJ, Siddiqi MY, Glass ADM. 1995b. Compartmentation and flux characteristics of ammonium in spruce. *Planta* **196**: 691–698.

Kronzucker HJ, Siddiqi MY, Glass ADM. 1995c. Analysis of $^{13}NH4^{+}$ efflux in spruce roots. A test case for phase identification in compartmental analysis. *Plant Physiology* **109**: 481–490.

Kronzucker HJ, Siddiqi MY, Glass ADM. 1996. Kinetics of NH_4^+ influx in spruce. *Plant Physiology* **110**: 773–779.

Lee RB, Ayling SM. 1993. The effect of methionine sulphoximine on the absorption of ammonium by maize and barley roots over short periods. *Journal of Experimental Botany* **44**: 53–63.

Lee RB, Clarkson DT. 1986. Nitrogen-13 studies of nitrate fluxes in barley roots. I. Compartmental analysis from measurements of ^{13}N efflux. *Journal of Experimental Botany* **37**: 1753–1756.

Lee RB, Ratcliffe RG. 1991. Observations on the subcellular distribution of the ammonium ion in maize root tissue using *in vivo* ^{14}N-nuclear magnetic resonance spectroscopy. *Planta* **183**: 359–367.

Lee RB, Rudge KA. 1986. Effects of nitrogen deficiency on the absorption of nitrate and ammonium by barley plants. *Annals of Botany* **57**: 471–486.

Liu K-H, Huang C-Y, Tsay Y-F. 1999. CHL1 is a dual-affinity nitrate transporter of Arabidopsis involved in multiple phases of nitrate uptake. *Plant Cell* **11**: 865–874.

Macklon AES, Ron MM, Sim A. 1990. Cortical cell fluxes of ammonium and nitrate in excised root segments of *Allium cepa* L., studies using ^{15}N. *Journal of Experimental Botany* **41**: 359–370.

MacRobbie EAC. 1971. Fluxes and compartmentation in plant cells. In: Machlis L, Briggs WR, Park RB, eds. *Annual review of plant physiology*. Palo Alto, CA, USA: Annual Review Inc., 75–96.

MacRobbie EAC. 1981. Ion fluxes in 'isolated' guard cells of *Commelina communis* L. *Journal of Experimental Botany* **32**: 545–562.

MacRobbie EAC, Dainty J. 1958. Ion transport in *Nitella obtusa*. *Journal of General Physiology* **42**: 335–353.

Min XJ, Siddiqi MY, Guy RD, Glass ADM, Kronzucker HJ. 1999. A comparative study of fluxes and compartmentation of nitrate and ammonium in early-successional tree species. *Plant, Cell & Environment* **22**: 821–830.

Morgan MA, Jackson WA. 1988a. Inward and outward movement of ammonium in root systems: transient responses during recovery from nitrogen deprivation in the presence of ammonium. *Journal of Experimental Botany* **39**: 179–191.

Morgan MA, Jackson WA. 1988b. Suppression of ammonium uptake by nitrogen supply and its relief during nitrogen limitation. *Physiologia Plantarum* **73**: 38–45.

Pallaghy CK, Scott BIH. 1969. The electrochemical state of cells of broad bean roots. II. Potassium kinetics in excised root tissue. *Australian Journal of Biological Sciences* **22**: 585–600.

Paulsen IT, Sliwinski MK, Nelissen B, Goffeau A, Saier Jr MH. 1998. Unified inventory of established and putative transporters encoded within the complete genome of *Saccharomyces cerevisieae. FEBS Letters* **430**: 116–125.

Pitman MG. 1963. The determination of salt relations of the cytoplasmic phase in cells of beet root tissue. *Australian Journal of Biological Sciences* **16**: 647–668.

Pitman MG. 1971. Uptake and transport of ions in barley seedlings. I. Estimation of chloride fluxes in cells of excised roots. *Australian Journal of Biological Sciences* **24**: 407–421.

Platt SG, Anthon GE. 1981. Ammonia accumulation and inhibition of photosynthesis in methionine sulfoximine treated spinach. *Plant Physiology* **67**: 509–513.

Poole RJ. 1971. Effect of sodium on potassium fluxes at the cell membrane and vacuole membrane of red beet. *Plant Physiology* **47**: 735–739.

Rescigno A. 1999. Compartmental analysis revisited. *Pharmacological Research* **39**: 471–478.

Siddiqi MY, Glass ADM, Ruth TJ. 1991. Studies of the uptake of nitrate in barley. III. Compartmentation of NO_3^-. *Journal of Experimental Botany* **42**: 1455–1463.

Siddiqi MY, Glass ADM, Ruth TJ, Rufty TW. 1990. Studies of the uptake of nitrate in barley. I. *Kinetics of* $^{13}No_3^-$ *Influx. Plant Physiologm* **93**: 1426–1432.

Thain JF. 1984. The analysis of radioisotopic tracer flux experiments in plant tissues. *Journal of Experimental Botany* **35**: 444–453.

Touraine B, Glass ADM. 1997. Nitrate and chlorate fluxes in the *chl1–5* mutant of *Arabidopsis thaliana.* Does the *CHL1–5* gene encode a low-affinity nitrate transporter? *Plant Physiology* **114**: 137–144.

Tsay Y-F, Schroeder JL, Feldmann KA, Crawford NM. 1993. The herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. *Cell* **72**: 705–713.

Ullrich WR, Larsson M, Larsson C-M, Lesch S, Novacky A. 1984. Ammonium uptake in *Lemna gibba* G 1, related membrane potential changes, and inhibition of anion uptake. *Physiologia Plantarum* **61**: 369–376.

Walker NA, Pitman MG. 1976. Measurement of fluxes across membranes. In: Lüttge U, Pitman MG, eds. *Encyclopedia of Plant Physiology, Vol. 2 Part a.* Berlin, Germany: Springer-Verlag, 93–126.

Wang MY, Glass ADM, Shaff JE, Kochian LV. 1994. Ammonium uptake by rice roots. III. Electrophysiology. *Plant Physiology* **104**: 899–906.

Wang MY, Siddiqi MY, Ruth TJ, Glass ADM. 1993. Ammonium uptake by rice roots. I. Fluxes and subcellular distribution of $^{13}NH_4^+$. *Plant Physiology* **103**: 1249–1258.

Wieneke J, Roeb GW. 1998. Effect of methionine sulphoximine on ^{13}N-ammonium fluxes in the roots of barley and squash seedlings. *Zeitschrift für Pflanzenernährung und Bodenkunde* **161**: 1–7.

Zierler K. 1981. A critique of compartmental analysis. *Annual Reviews of Biophysics and Bioengineering* **10**: 531–562.