

Trans-stimulation of $^{13}\text{NH}_4^+$ efflux provides evidence for the cytosolic origin of tracer in the compartmental analysis of barley roots

Dev T. Britto^A and Herbert J. Kronzucker^{A,B}

^ADepartment of Life Sciences, University of Toronto, 1265 Military Trail, Toronto, ON, Canada M1C 1A4.

^BCorresponding author; email: herbertk@utsc.utoronto.ca

Abstract. The analysis of tracer efflux kinetics is fundamental to membrane transport studies, but requires the rigorous identification of subcellular tracer sources. We present a solution to this problem through the analysis of sharp increases in $^{13}\text{NH}_4^+$ efflux from roots of radiolabelled barley (*Hordeum vulgare* L.) seedlings, in response to a 100-fold increase in external $[\text{NH}_4^+]$. By comparing these trans-stimulation data with a mathematical model incorporating changes in subcellular NH_4^+ fluxes and pool sizes, we show that the cytosol of root cells is the origin of the tracer efflux. Our analysis provides new insight into the rapidly occurring events underlying compensatory flux regulation during transitions from one nutritional steady state to another, and confirms the validity of compartmental analysis by tracer efflux (CATE) in this important model system.

Keywords: compartmental analysis, efflux, inverse problem, ion transport.

Introduction

The compartmental analysis of tracer efflux (CATE) from labelled biological systems offers a powerful means of characterising subcellular compartmentation, exchange, and movement of solutes in these systems (Jacquez 1996). In plants, CATE has been widely used to unravel such fundamental processes as stomatal functioning (MacRobbie 1981), *in vivo* enzyme activities (Kronzucker *et al.* 1995a), and the bioenergetics of ion fluxes across membranes (Macklon and Higinbotham 1970; Siddiqi *et al.* 1991; Britto *et al.* 2001). It has also been used to investigate phenomena of broader practical importance, including plant salinity tolerance and heavy metal tolerance (Hajibagheri *et al.* 1988; Lasat *et al.* 1998) and ecological issues such as agronomic plant performance (Kronzucker *et al.* 1998) and forest succession (Kronzucker *et al.* 1997, 2003a).

A primary appeal of CATE is its non-invasiveness, but this feature entails a rigorous identification of the internal origin of tracer released from labelled systems (Kronzucker 1995c; Jacquez 1996). Previous work towards this end has often involved the use of denaturing chemicals, or relied on the convergence of results with those obtained by other methods (MacRobbie 1981; Siddiqi *et al.* 1991), but the lack of more direct proofs has led to considerable recent scepticism with regard to claims based on CATE methodology (Miller and Smith 1996; Siddiqi and Glass 2002; *cf.* Britto and Kronzucker 2003a). Using a combination of experimental data and mathematical modelling, we present a new,

direct solution to the problem of identifying the tracer source in an important higher plant model system. Specifically, we examine the subcellular fluxes and compartmentation of the ammonium ion (NH_4^+) in roots of barley seedlings (Britto and Kronzucker 2001a, b; Britto *et al.* 2001).

Materials and methods

Barley plants (*Hordeum vulgare* L. cv. 'Klondike') were germinated in sand for 3 d, then grown hydroponically on quarter-strength modified Johnson's solution [pH 6, consisting of macronutrients: 0.05 mM $(\text{NH}_4)_2\text{SO}_4$, 0.5 mM KH_2PO_4 , 0.5 mM K_2SO_4 , 0.25 mM MgSO_4 , 0.2 mM CaSO_4 , 0.02 mM Fe-EDTA; and micronutrients: 50 μM KCl, 25 μM H_3BO_3 , 2 μM MnSO_4 , 2 μM ZnSO_4 , 0.5 μM CuSO_4 , 0.5 μM Na_2MoO_4]. Intact roots of hydroponically cultured, 1-week-old barley seedlings were radiolabelled in a solution that was chemically identical to their growth solution except that it contained the positron-emitting radiotracer $^{13}\text{NH}_4^+$ ($t_{1/2} = 9.98$ min; present in chemically insignificant quantities). After labelling for 40 min, $^{13}\text{NH}_4^+$ -radioactivity was eluted from the plant roots into a timed series of non-radioactive aliquots, which were, initially, identical to the growth solution. After 10 min, and for the remaining 26 min of elution, the $[\text{NH}_4^+]$ in the aliquots was increased, from the growth concentration of 0.1 mM, to 10 mM. Once the final eluate was collected, roots, leaves, and eluted aliquots were separately counted for γ -radioactivity. For more details on growth and experimental protocols, see Britto and Kronzucker (2001a, b), and Britto *et al.* (2001).

Results and discussion

Key observations

The changing rate of $^{13}\text{NH}_4^+$ release from the labelled barley roots is shown in Fig. 1. We base our present analysis on the interpretation of two previously unexplained phenomena

Abbreviations used: CATE, compartmental analysis of tracer efflux; $t_{1/2}$, radioactive half-life.

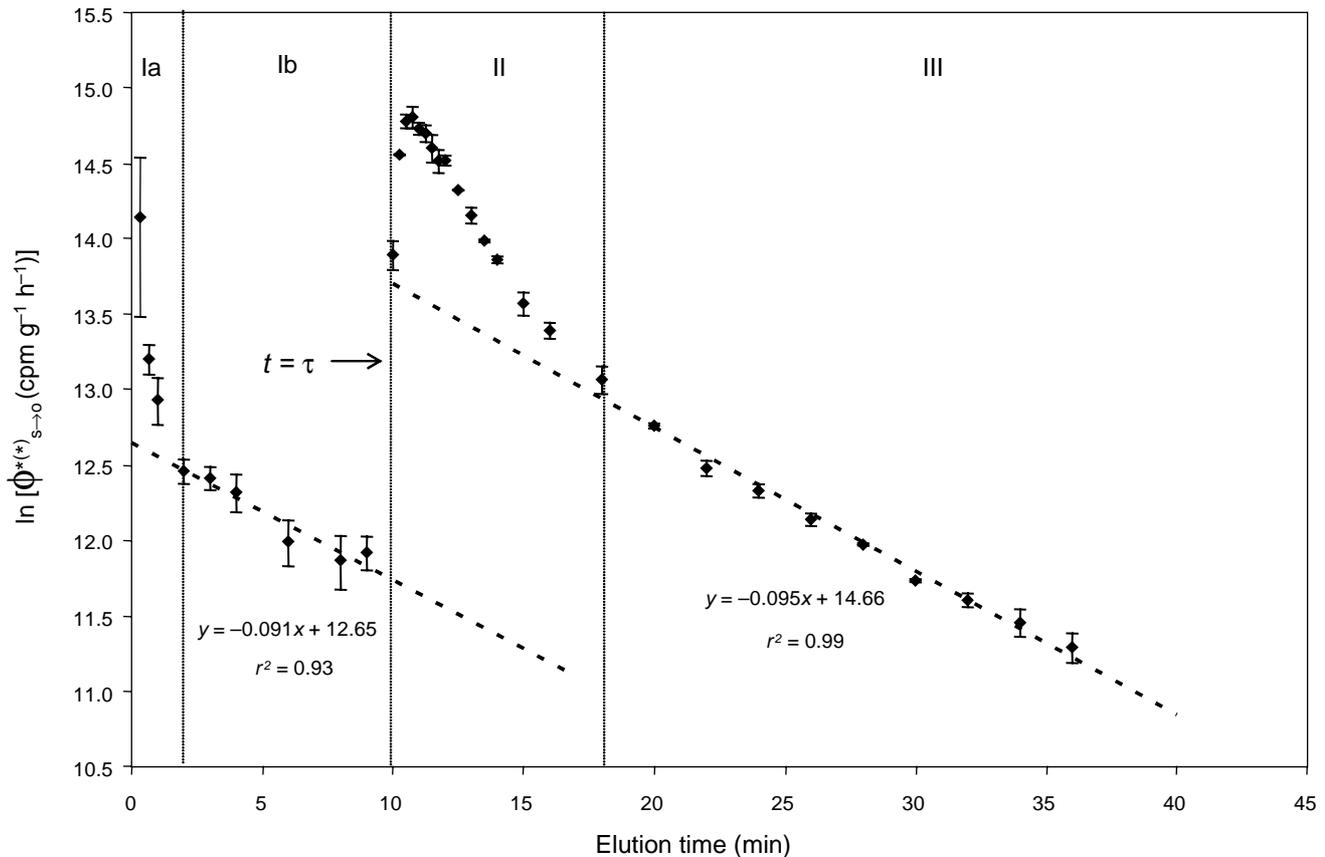


Fig. 1. Semi-logarithmic plot of $^{13}\text{NH}_4^+$ efflux vs elution time from labelled roots of intact barley seedlings, in a concentration-shift experiment (see Britto and Kronzucker 2001a, b). Plants were exposed to a shift in external $[\text{NH}_4^+]$ from 0.1 to 10 mM at $t = 10$ min. The plot shows the means \pm s.d. of two experiments (asymmetrical error bars reflect the logarithmic y-axis). *Phase I* refers to the steady state preceding the shift (see, e.g. Britto *et al.* 2001 for a steady-state trace without a concentration shift), *phase II* is a period of adjustment immediately following it, and *phase III* represents a new steady-state. Linear equations, and coefficients of determination (r^2), pertaining to phases Ib and III, are shown next to regression lines. Phase-Ia data points represent tracer elution from rapidly exchanging spaces other than the compartment presently under examination (MacRobbie 1971; Lee and Clarkson 1986; Kronzucker *et al.* 1995c) and thus, are not regressed.

depicted within this figure (Britto and Kronzucker 2001a, b). First, an upward shift from 0.1 to 10 mM in external concentration of carrier (non-radioactive) ammonium ($^{14}\text{NH}_4^+$) causes an immediate and dramatic increase in the efflux of tracer (radioactive) ammonium ($^{13}\text{NH}_4^+$), from the root system of $^{13}\text{NH}_4^+$ -labelled barley plants, into the external medium (Fig. 1, phases II and III — note logarithmic scale on y-axis). Second, within 10 min following the shift, this trans-stimulated efflux assumes an apparently exponential decline with a rate constant identical to that in the pre-shift 0.1 mM NH_4^+ condition (Fig. 1, compare phases Ib and III). Any model that seeks to identify the origin of the tracer efflux in this system must account for both of these observations.¹

The model

Under steady-state conditions (i.e. conditions under which chemical fluxes and pool sizes remain stable over time, and no environmental changes are introduced over the growth

and experimental procedure), tracer efflux ($\phi_{s \rightarrow o}^*$) at any time (t) from a source compartment (s) in the labelled root system to the outside medium (o) is given by:

$$\phi_{s \rightarrow o}^* = \phi_{s \rightarrow o} \frac{Q^*}{Q} e^{-k_s t}, \quad (1)$$

where $\phi_{s \rightarrow o}$ is the carrier efflux, Q^* is the tracer content of the tracer source pool at $t = 0$, Q is the carrier content in that pool (hence Q^*/Q is the initial specific activity of the pool), and k_s is the first-order rate constant describing its decline in specific activity (Walker and Pitman 1976). The term k_s , a measure of the turnover of Q , can also be expressed as

¹The possibility that the post-shift changes in tracer efflux result from osmotic changes in the external medium can be ruled out by the observation that similar shifts in external $[\text{K}^+]$ do not result in changes in ^{42}K efflux (D. T. Britto and H. J. Kronzucker, unpublished data). However, extremely high increases in external NaCl strength (from 1 mM to 50–100 mM) have been shown to substantially increase efflux of a variety of ions, including NH_4^+ (Shabala *et al.* 2003).

$\phi_{\leftarrow s \rightarrow}^*$ / Q (MacRobbie 1971; Walker and Pitman 1976; Britto and Kronzucker 2001a, b), where $\phi_{\leftarrow s \rightarrow}^*$ denotes the sum of all fluxes removing tracer and carrier in any direction from the source compartment (e.g. to metabolism, across membranes to the external medium, or to other organs or subcellular compartments). An equation modelling the tracer efflux ($\phi_{s \rightarrow o}^{**}$) that follows a concentration shift at time $t = \tau$, such as was imposed in Fig. 1, can be formulated using this substitution:

$$\phi_{s \rightarrow o}^{**} = \frac{\alpha \phi_{s \rightarrow o}^* Q^{**}}{\gamma Q} e^{-\frac{\beta \phi_{\leftarrow s \rightarrow}^*}{\gamma Q} (t - \tau)}, \quad (2)$$

where Q^{**} is the tracer content of the source pool at this time, and the proportionality coefficients α , β , and γ are the factors by which $\phi_{s \rightarrow o}^*$, $\phi_{\leftarrow s \rightarrow}^*$ and Q change, respectively, following the concentration shift. The new apparent steady state that results after this shift (Fig. 1, phase III) can be readily modelled by using the log-linear form of Eqn 2:

$$\ln \phi_{s \rightarrow o}^{**} = \ln \left(\alpha \phi_{s \rightarrow o}^* \frac{Q^{**}}{\gamma Q} \right) - k_s' (t - \tau), \quad (3)$$

where the slope of the new line, k_s' is equal to $\frac{\beta \phi_{\leftarrow s \rightarrow}^*}{\gamma Q}$. This equation can also take the form:

$$\ln \phi_{s \rightarrow o}^{**} = \ln(\delta \phi_{s \rightarrow o}^*, t = \tau) - k_s' (t - \tau), \quad (4)$$

where δ is a coefficient indicating the factor by which the y -intercept of the phase III line in Fig. 1 increases relative to that of the phase Ib line ($\phi_{s \rightarrow o}^*, t = \tau$), as a result of the concentration shift.

Use of the model

The plant root cell is a spatial and functional series of three major compartments (Walker and Pitman 1976) — a central vacuole, the surrounding cytosol, and an extracellular matrix composed of cellulose and other polymers. We used the model to test which of these compartments is the source of the tracer efflux observed in Fig. 1 (given that other subcellular compartments located within the cytoplasm have been shown not to participate in tracer efflux to a significant extent, see Britto and Kronzucker 2003a). We considered the vacuole first, which is enclosed by a single membrane, the tonoplast. Because no NH_4^+ metabolism occurs in this storage compartment, and because the only compartment with which it communicates is the cytosol, there can be only one flux removing NH_4^+ from it, the flux across the tonoplast to the cytosol. The turnover rates of the cytosolic pool and extracellular spaces through which the tracer passes after leaving the vacuole, and before being captured in an eluate, are assumed to be significantly faster than that of the vacuole, largely due to its much greater size relative to other compartments (Walker and Pitman 1976; Kochian and Lucas 1982; Lee and Clarkson 1986; Jacquez 1996). Therefore, for the vacuole, $\phi_{s \rightarrow o}^* = \phi_{\leftarrow s \rightarrow}^*$, assuming that

negligible reabsorption from the cytosol back to the vacuole takes place, and thus the coefficients α and β in Eqn 2 will equal one another. As shown in Fig. 2, we used Eqn 3 to generate the three resulting possibilities for $^{13}\text{NH}_4^+$ efflux from this compartment after the concentration shift, depending on the relationship between α (or β) and γ , i.e. the extent to which efflux from the vacuole ($\phi_{s \rightarrow o}^*$) changes relative to the vacuolar pool size (Q). First, if α (or β) $>$ γ (Fig. 2, case I)², an initial spike in tracer efflux must be followed by a precipitous decline (i.e. k' , the slope of the line following the shift, is greater than k before the shift), because the elevated efflux is not buffered by a commensurate dilution in the specific activity of the pool (note that γ is in the denominator of the slope term in Eqn 3). Thus, the restoration of the apparent rate constant observed in Fig. 1 cannot occur in this case. Second, if α (or β) $=$ γ (Fig. 2, case II), pool size will increase commensurately with efflux, and such a restoration will occur, but an elevated tracer efflux line will not be observed. On the contrary, the post-shift line will be an undeflected continuation of the line previously established (i.e. $k' = k$). Third, if α (or β) $<$ γ (Fig. 2, case III), neither an elevated efflux, nor a restoration of slope, will be observed; instead, the tracer efflux line will initially drop below the previous value, and then flatten out (i.e. $k' < k$). As none of these possibilities fit the data presented in Fig. 1, the vacuole cannot be the source of the tracer efflux seen experimentally.

The model was then applied to the extracellular wall matrix of the plant root system, which behaves like an exchange resin for ions (Sattelmacher 2001), including NH_4^+ (Kronzucker *et al.* 1995b, c). The only flux releasing NH_4^+ from this space is the flux into the aqueous interstitial spaces of the wall matrix ('water free space'), from which nearly 100% of tracer will be captured in the external solution, with only a negligible fraction being symplastically recaptured by the plant, via the plasma membrane (see Kronzucker *et al.* 1995c; Sattelmacher 2001) results in a condition essentially the same as seen with the vacuole. Again, α equals β , indicating that cell-wall behaviour, like that of the vacuole, is limited to the three scenarios depicted in Fig. 2. This limitation eliminates the cell wall as a candidate for the source of tracer efflux, as it did for the vacuole.

From the above instances, it can be seen that the fitting of the model to the data in Fig. 1 demands that several conditions are met. (1) At least two fluxes, responding distinctly to the concentration shift, must simultaneously remove tracer and carrier from the source compartment. (2) The extent to which these fluxes change upon a concentration shift must be anisotropic; specifically, $\alpha > \beta$ (Fig. 3). The cytosol is the only compartment that satisfies these two criteria, exhibiting not

²Note that the inequalities presented in the model are developed explicitly for an upward concentration shift.

only the required duplicity of removal fluxes (i.e. to the outside medium, the vacuole, plastids, xylem stream, and metabolism), but also the required ability to differentially regulate these fluxes (Britto and Kronzucker 2001*b*).

The phenotypic plasticity of plants enables them to grow under a wide variety of nutrient concentration regimes, and to acclimate accordingly from one physiological steady state to another. This acclimation process can require many hours to days (Kronzucker *et al.* 1998). Our solution to the problem of compartment identification provides several fundamental new insights into the rapid-term events that regulate changes in nitrogen fluxes at the cellular level, and initiate longer-term acclimation. The increased intercept, and the conserved slope, of the tracer efflux line (Figs 1, 3, phases I and III) indicate, respectively, that $\alpha > \gamma$, and that $\beta = \gamma$, meaning that efflux to the external medium increases disproportionately, relative to concurrent increases in other fluxes removing carrier and tracer from the cytosol, while changes in the sum of all removal fluxes are directly proportional to pool size changes. The larger increase in efflux, relative to increases in other removal fluxes, prefigures the higher steady-state ratio of NH_4^+ efflux to influx that is well documented under high external $[\text{NH}_4^+]$ regimes (Britto *et al.* 2001, 2002), and the return of the k_s term to its previous value prefigures the conservation of this term over a wide range of steady-state

external $[\text{NH}_4^+]$ regimes (Britto and Kronzucker 2001*b*). The present data show that a high degree of cellular flux co-ordination variably shuttles the nitrogen resource in many directions from a central cytosolic pool (Britto and Kronzucker 2001*b*) and must be responsible for the restoration of the k_s value. This restoration, however, is not immediate. Rather, a brief period of maximal efflux activity (which is at least $90 \mu\text{mol g}^{-1} \text{h}^{-1}$, as can be calculated using instantaneous tracer efflux data, cumulative tracer loss, and external specific activity), which cannot be explained by the $\alpha > \beta = \gamma$ condition, occurs within the first 10 min following the concentration shift (Figs 1, 3, phase II). In this transitional phase, the tracer line is substantially steeper than either of the flanking steady-state lines. Although β and γ ultimately reach the same resting value (see above), the only explanation for the shape of the transitional phase is that the rate at which β attains its resting value is initially higher than that for γ [compare with Fig. 2, case I, in which $\alpha (= \beta) > \gamma$]. In terms of differential calculus, $d\phi_{e \rightarrow s}/dt > dQ/dt$ during this time. The effect of the enhanced tracer efflux (which is the predominant component of the increase in $\phi_{e \rightarrow s}$) on the specific activity of the cytosolic pool, and hence on tracer efflux itself, is initially one of drastic decline, because it is not compensated for by an expanding pool until later in the transition period (phase III).

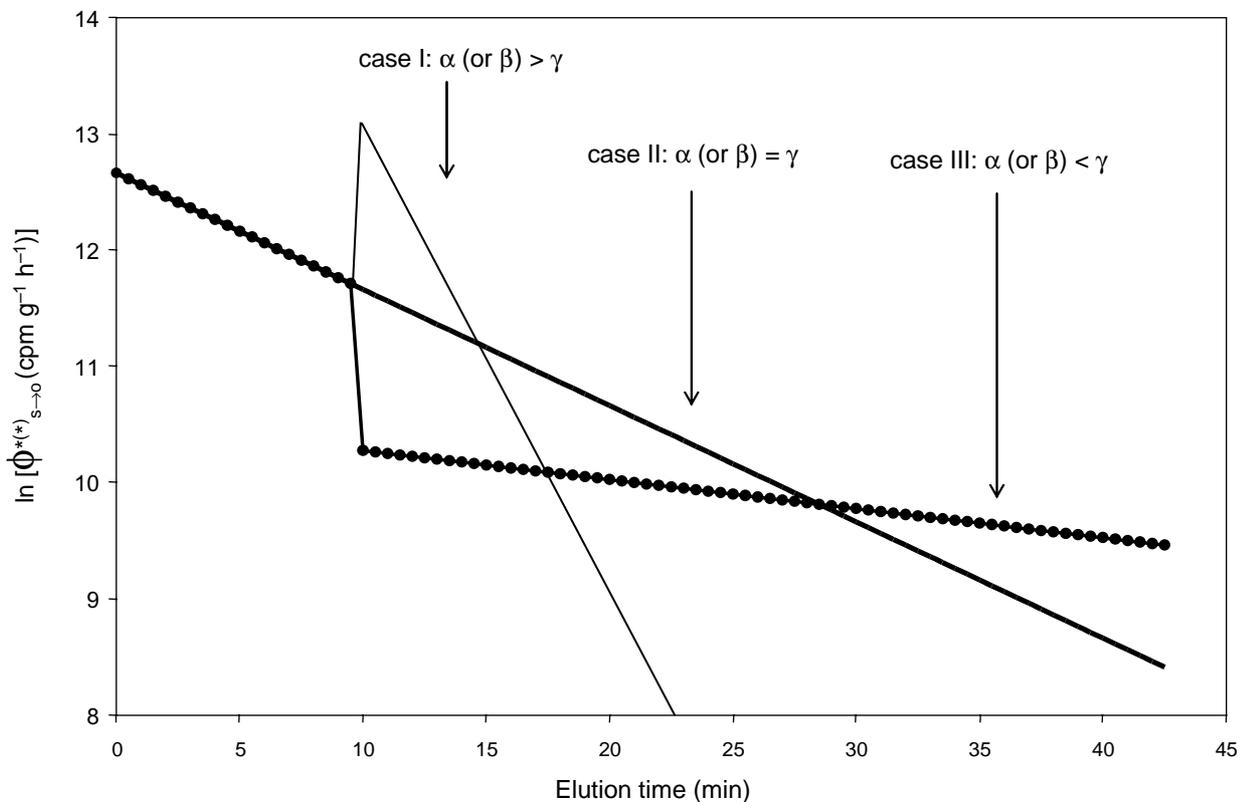


Fig. 2. Three scenarios for tracer efflux following a concentration shift, assuming a tracer source compartment with a single tracer removal pathway (e.g. vacuole or extracellular matrix). Plots were generated using Eqn 3. In case I, the ratio $\alpha/\gamma = 4$, in case II, $\alpha/\gamma = 1$, and in case III, $\alpha/\gamma = 0.25$. For further details, see text.

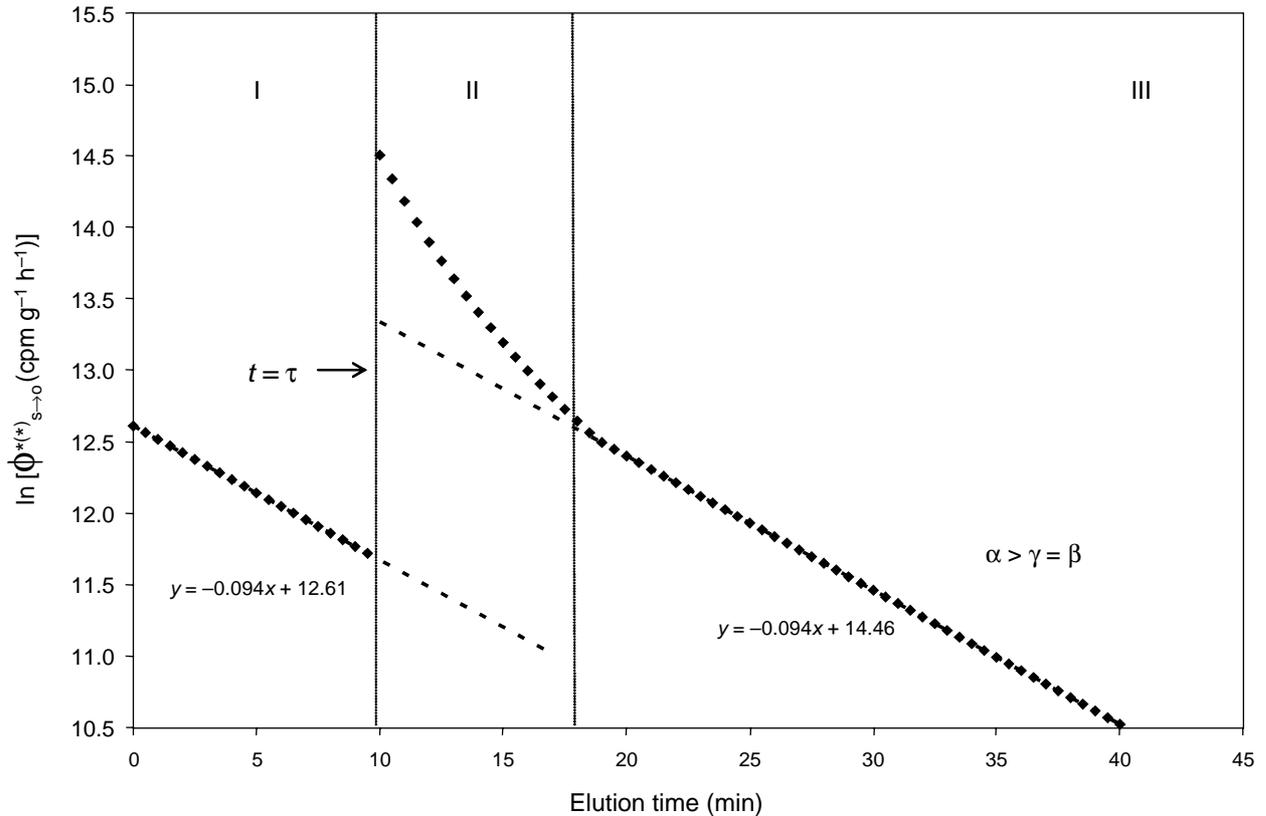


Fig. 3. Model approximation of experimental data. Points in phases I and III were generated by Eqn 3 given in the text. Phase II-points were generated by manipulating values of α , β and γ , i.e. by modelling changes in efflux from the compartment, the sum of all fluxes removing from the compartment, and the pool size of the compartment.

Conclusion

The solution to the problem presented here provides new rigor for the use of tracers to study ion fluxes and compartmentation in living cells. The data dramatically demonstrate the immediacy and precision of subcellular flux regulation in plant cells that follow a sudden change in nutrient supply. The very high efflux rates seen directly after an increase in external substrate concentration have strong implications for the design and interpretation of influx experiments, which are characteristically based upon concentration shifts (Epstein 1966), and involve time intervals of uptake and desorption during which tracer loss from the system is generally unaccounted for (see Britto and Kronzucker 2001a, for specific recommendations in this regard). Equally importantly, tracer-source identification is crucial to the correct use of compartmental analysis in the determination of subcellular pool sizes (Jacquez 1996), a subject of much current controversy (Siddiqi and Glass 2002; Britto and Kronzucker 2003a; and references therein). In turn, correct pool size estimates have important implications for the bioenergetic analysis of thermodynamic gradients across membranes in intact systems (Hirsch *et al.* 1998; Britto *et al.* 2001; Kronzucker *et al.* 2001), for the determination of the kinetics, and concentration

dependence, of outward fluxes from subcellular pools (Cram 1968; MacRobbie 1971, 1981; Jeschke and Jambor 1981; Britto and Kronzucker 2003b; Kronzucker *et al.* 2003b), and for the investigation of cellular ion homeostasis (Britto and Kronzucker 2003a; Kronzucker *et al.* 2003b). This identification also strengthens findings of agronomic and silvicultural importance that have been made using compartmental analysis, including discoveries about nitrogen-source preferences (Kronzucker *et al.* 1995b, 1997), about positive and negative interactions between nitrogen sources and their implications for the growth of cereals (Kronzucker *et al.* 1999a, b), and about the cytosolic hyperaccumulation, and futile cycling at the plasma membrane, of NH_4^+ in ammonium-sensitive plant species (Britto *et al.* 2001; Kronzucker *et al.* 2003a). Possibilities for further investigation are extensive, particularly in the study of cellular responses to environmental change, and to manipulations imposed by techniques in molecular biology.

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