Journal of Experimental Botany Advance Access published August 7, 2006

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doi:10.1093/jxb/erl088

Journal of Experimental Botany

**RESEARCH PAPER**

# The face value of ion fluxes: the challenge of determining influx in the low-affinity transport range

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Received 14 March 2006; Accepted 22 June 2006

## Abstract

**The existence of distinct high- and low-affinity transport systems (HATS and LATS) is well established for major nutrient ions. However, influx mediated by these systems is usually estimated using uniformly simple tracer protocols. Two $^{42}$K radiotracer methods to measure potassium influxes in the HATS and LATS ranges in intact barley (*Hordeum vulgare* L.) roots are compared here: a direct influx (DI) method, and an integrated flux analysis (IFA), which is designed to account for tracer efflux from labelled roots and differential tracer accumulation along the plant axis. Methods showed only minor discrepancies for influx values in the HATS range, but large discrepancies in the LATS range, revealing striking distinctions in the cellular exchange properties dominated by the operation of the two transport systems. It is shown that accepted DI protocols are associated with very large errors in the high-conductance LATS range, underestimating influx at least 6-fold due to four characteristics of this transport mode: (i) accelerated cellular $^{42}$K exchange; (ii) a greatly increased ratio of efflux to influx; (iii) increased $^{42}$K loss during the removal of water from roots in preweighing centrifugation or blotting protocols; and (iv) increased $^{42}$K retention at the root–shoot interface, a region of the plant frequently disregarded in DI determinations. The findings warrant a re-evaluation of a large body of literature reporting influx in the LATS range, and are of fundamental importance to ion flux experimentation in plant physiology.**

Key words: Barley, cellular ion exchange, efflux, high-affinity transport, influx, low-affinity transport, potassium.

## Introduction

The unidirectional influx of nutrient ions into the plant cell is the membrane-transport parameter of greatest interest to most researchers in the field of plant ion transport. This emphasis on influx is entirely reasonable, as it is the primary step in plant mineral acquisition from the external environment. The elucidation of influx patterns in plant roots has led to the identification and characterization of the enzymatic basis of ion transport in these tissues, including the determination of velocity and affinity constants ($V_{max}$ and $K_m$, respectively) for distinct transport systems (Epstein *et al*., 1963). In the present day, the application of accurate influx data is essential to the assignment of specific transport functions to gene products.

By comparison, information about ionic efflux from plant cells is available only for a limited number of conditions and systems. Under steady-state conditions, the use of protocols that allow an investigator to quantify both influx and efflux has revealed intriguing aspects of ion exchange in plant cells (Jeschke and Stelter, 1973; Cram and Laties, 1974; Kochian and Lucas, 1982; Lee and Clarkson, 1986; Siddiqi *et al*., 1991; Lasat *et al*., 2000; Britto *et al*., 2001, 2004; Kronzucker *et al*., 1997, 2003*a*, *b*), although only a few molecular mechanisms underlying efflux processes have so far been identified (Gaymard *et al*., 1998; Shi *et al*., 2000; Mäser *et al*., 2001; Zhu, 2001). Information about ionic efflux from plant cells might be viewed as being of peripheral interest to the influx analyst, but, as shown here, rates of efflux can at times be so high, and cellular ion turnover so rapid, that standard measurements of influx by tracers are not possible without substantial supplemental experimentation.

In this paper, an Integrative Flux Analysis (IFA) protocol is used, which draws upon quantitative efflux data and comprehensive accounting of tracer retention in plant tissue, to demonstrate how efflux and other factors can

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Abbreviations: DI, direct influx; IFA, integrated flux analysis; HATS, high-affinity transport system; LATS, low-affinity transport system.

strongly impact direct influx (DI) measurements. By examining $^{42}$K-labelled potassium fluxes in the barley (*Hordeum vulgare* L.) model system, it is shown that the delineated errors have a much greater impact in the low-affinity transport system (LATS) range, compared with the high-affinity (HATS) range, powerfully underscoring the fundamental distinctions between these two modes of transport (Epstein *et al*., 1963; Kochian and Lucas, 1982; Vale *et al*., 1987; Kronzucker *et al*., 2003*a*).

## Materials and methods

### Plant culture

Seeds of barley (*Hordeum vulgare* L. cv. 'Klondike') were germinated on sand for 3 d prior to placement on aerated hydroponic growth medium containing modified ¼-strength Johnson's solution (pH 6.3–6.5) for an additional 4 d. The solution was modified to provide two concentrations of potassium (as $K_2SO_4$): 0.1 mM, which is typical of soil $K^+$ levels and of the operative range of high-affinity $K^+$ transport (HATS); and 40 mM, which is representative of the low-affinity $K^+$ transport (LATS) range, and similar to apparent LATS $K_m$ values reported by others (Epstein *et al*., 1963). Plant fresh and dry weights were identical after 7 d between the two treatments, indicating that the plants were neither $K^+$-deprived at 0.1 external [$K^+$], nor subjected to $K^+$ toxicity at 40 mM. Plants were cultured in walk-in growth chambers equipped with fluorescent lights (Philips Econ-o-watt, F96T12), providing an irradiation of ~200 μmol photons $m^{-2}$ $s^{-1}$ at plant height, for 16 h $d^{-1}$. Daytime temperature was 20 °C, night-time temperature was 15 °C, and relative humidity was approximately 70%.

### Flux experiments

For all flux experiments, each replicate consisted of a bundle of five intact plants (except for experiments comparing blotting and centrifugation, in which eight plants were bundled). Bundles were prepared 1 d prior to the experiment (6 d after the start of germination), by means of a plastic collar made from a 0.5 cm length of tubing. This collar held together the plant stems, just above the interface of seed and stem. A wire clasp was constructed to attach to the collar, and hook the plant bundle to the rim of a beaker filled with 30 ml of experimental solution, sufficient to immerse the roots. This procedure was used to minimize physical disturbance to the plant root system during plant transfer from one vessel to another, and to minimize transfer times.

All experimental solutions (prelabelling, labelling, dip, desorption) were of the same chemical composition as the growth solutions, with the labelling solutions containing the potassium radioisotope $^{42}$K ($t_{½}$=12.36 h), provided by the McMaster Nuclear Reactor, in Hamilton, Ontario, Canada. Solutions were mixed using a fine stream of air bubbles.

The main feature of integrated flux analysis (IFA) experiments was the monitoring of efflux by periodic subsampling of a 30 ml volume of growth solution in which labelled, intact barley roots were immersed. This was performed as described in detail elsewhere (Britto *et al*., 2006). In brief, roots of bundled plants were equilibrated in non-radioactive growth solution for 5 min, followed by labelling in growth solution containing $^{42}$K for 1 h. The roots were then dipped for 5 s in non-radioactive growth solution (for removal of radioactivity in surface water), and sequentially placed into two 30 ml desorption solutions for 5 min and 25–30 min, respectively. 3 ml aliquots were periodically removed from the desorption vessels (and replaced with non-radioactive solution) to sample the tracer released from the labelled roots; samples were taken every 30 s from the first vessel, and every 30 s from the second vessel for the first 5 min, then every minute for the remainder of the experiment.

Radioactivity released by plants over time in the two desorption vessels was quantified by γ-counting and by use of the following formula:

$$cpm_{\text{released}} = cpm_{\text{n}}\left(\frac{v_{\text{vessel}}}{v_{\text{sample}}}\right) + \sum_{i=1}^{n-1} cpm_{\text{i}} \quad (1)$$

This gives the cumulative cpm released after removal of the *n*th sample from the desorption solution. It accounts for the ratio of subsample volume ($v_{\text{sample}}$) to total beaker volume ($v_{\text{vessel}}$), and for the sum of cpm previously removed from the beaker. The tracer released into the external medium was diluted in this process, at a rate sufficiently high to prevent problems resulting from tracer reabsorption (this was confirmed by the lack of effect on flux parameters produced by 4-fold variations in labelling-solution specific activity, and changes in desorption-solution volume ranging from 30 ml to 4.0 l; Britto *et al*., 2006). Released radioactivity was plotted against time, and best-fit exponential equations of the form $A_t=A_0\ (1-e^{-kt})$ were determined using a non-linear, least-squares regression (Microcal Origin software version 6.0; see Fig. 2). In this equation, $k$ is the rate constant of the tracer accrual process, and $A_t$ and $A_0$ are, respectively, cumulative tracer released at time $t$, and maximal cumulative tracer released over the entire time-course. The exponential tracer accrual function represented by the equation is derived by integrating, with respect to time, the exponential decline function $\phi_t^*=\phi_0^* e^{-kt}$ which is expected for tracer efflux from a single compartment (Walker and Pitman, 1976; Lee and Clarkson, 1986), and in which $\phi_t^*$ is tracer released at time $t$, $\phi_o^*$ is initial tracer release, and $k$ is the same rate constant as above. The rationale for fitting a single exponential function to these data sets comes from measurements of exchange kinetics from other compartments in the root, in particular the extracellular spaces (cell wall), and the vacuole (Behl and Jeschke, 1982; Jeschke, 1982; Memon *et al*., 1985; Hajibagheri *et al*., 1988). The 5 min desorption period was long enough to remove 96–99% of tracer from the extracellular regions (with exchange half-times of 0.8–1.1 min), while the labelling times were short enough to prevent labelling of the more slowly-exchanging spaces such as the vacuole (with exchange half-times of many hours), while providing substantial tracer to the cytosol (with exchange half-times of 7 min and 16 min for the LATS and HATS conditions, respectively, in the present study; these important considerations are discussed in detail in Britto *et al*., 2006). These equations were differentiated with respect to time, to determine tracer efflux. The specific activities of the tracer-releasing compartments were determined from the kinetic constants ($k$ values) revealed in the exponential regressions, and used to calculate chemical efflux from tracer efflux (Siddiqi *et al*., 1991; Kronzucker *et al*., 2003*a*). Net flux was determined using tracer accumulated in plant tissue (root, shoot, and root–shoot interface), and in centrifuged liquid from roots. Kinetic constants were used to correct the net flux for additional efflux that would have occurred had the elution continued beyond the end of the experiment (Kronzucker *et al*., 2003*a*). Influx was obtained by summing efflux and net flux terms.

Direct influx experiments (DI) consisted of a 5 min equilibration in non-radioactive growth solution, followed by variable labelling periods in growth solution containing $^{42}$K, and then a 5 s dip followed by a 5 min desorption, both in non-radioactive growth solution. Most experiments (e.g. those in Table 1 and Fig. 4) employed a 5 min labelling, since this is a typical period used by many researchers conducting DI experiments. In experiments comparing centrifugation and blotting, the eight-seedling bundles were separated into two sets of four seedlings following desorption, with each set then subjected to either centrifugation or blotting.

At the end of all flux experiments, roots and shoots were excised from the root–shoot interface (which consisted of a 1 cm segment along the root–shoot axis, where seedlings were bundled together, and which included the basal part of the shoot and the seed coat), prior to weighing. Roots were spun in 16 mm × 100 mm test tubes in a low-speed centrifuge for 30 s (except in blotting experiments), to remove surface water prior to determining fresh weight. The test tubes contained a compacted, absorbent paper tissue (Kimwipe) that was used to capture surface water and any residual $^{42}K$ from the roots during centrifugation. Blotting was carried out by placing the roots in a folded Kimwipe, which was then pressed for 10 s with a 750 g mass. Gamma counts from roots, shoots, subsamples of washing medium, root–shoot interface, and Kimwipes from blotting or centrifugation, were detected using a Canberra-Packard, Quantum Cobra Series II, Model 5003 γ-counter equipped with a NaI crystal/photodiode detector system. All radioactive samples were presented to the counter in 16 mm ×100 mm test tubes for 1 min, with an energy window of 1200–1800 keV.

DI values were initially calculated by adding together the counts accumulated in roots and shoots. Corrections were made to this base value first by adding tracer retained in the root–shoot boundary, and recovered from centrifugation or blotting of roots. This quantity was then corrected for the losses via efflux that occurred during both absorption and desorption according to the following formula:

$$\phi^*_{oc} = \left\{ A + \phi^*_{co} \left[ t_L - \frac{1}{k} \left( e^{-kt_D} - e^{-k(t_L + t_D)} \right) \right] \right\} / t_L \qquad (2)$$

(Britto and Kronzucker, 2001*a*), where $\phi^*_{OC}$ is the corrected tracer influx, $A$ is tracer retained in plant tissue, $t_L$ is labelling time, $t_D$ is desorption time, $\phi^*_{CO}$ is maximal tracer efflux, and $k$ is the rate constant for cytosolic turnover of the ion in question. The parameters $\phi^*_{CO}$ and $k$ were determined using IFA.

## Results and discussion

Table 1 shows the results of DI (direct influx) and IFA (integrated flux analysis) experiments conducted at two external $K^+$ concentrations, one in the HATS (0.1 mM), the other in the LATS (40 mM) range. Values found using DI agree well with prior studies that also used DI to measure potassium fluxes in the steady state (i.e. under growth and assay conditions that are identical to one another; Epstein *et al.*, 1963; Kochian and Lucas, 1982; Wrona and Epstein, 1985; Siddiqi and Glass, 1986). However, at both concentrations, the influx value determined using IFA was significantly higher ($P$ <0.95) than that determined using DI. In the HATS condition, this difference was moderate, but in the LATS condition, it was extremely pronounced, the IFA-derived value being 7-fold higher than the DI-derived value. Given this sizeable discrepancy, and because the vast majority of ion transport data reported in the literature are obtained by DI protocols, it is urgent that the nature of this discrepancy be understood.

Figure 1 shows a short-term time-course of direct influx measurements conducted in the LATS range. The severe drop in influx estimates observed over time is indicative of tracer efflux occurring simultaneously with influx and during post-labelling desorption of the free space (Cram, 1969; Lee and Ayling, 1993; Britto and Kronzucker, 2001*a*), an artifact not seen in the HATS range. Similarly, the method-dependent differences between flux estimates can be partially explained by accounting for the efflux of tracer, a phenomenon that is only directly observed and quantified in the tracer capture enabled by IFA protocols. Tracer lost during DI experiments can be corrected for by the application of flux and turnover parameters according to equation 2 in the Materials and methods (also see Britto and Kronzucker, 2001*a*). The cytosolic turnover rate and the ratio of efflux to influx are the two key physiological parameters that determine the extent of the underestimate of influx occurring in DI experiments, and both must be determined independently using IFA. Figure 2 shows the results of two IFA experiments, one examining the HATS, and the other the LATS, condition. Equation 2 predicts that, when turnover rates and efflux:influx ratios are large, there will be major discrepancies between uncorrected DI and IFA data. When the steady-state external $[K^+]$ was increased to 40 mM, turnover rates and flux ratios both

**Table 1.** *Comparison of $^{42}K$ influx methods in roots of intact barley seedlings, measured in high- and low-affinity transport (HATS and LATS) ranges*

DI, direct influx measured by $^{42}K$ uptake during a short (5 min) labelling period. DI data is uncorrected (see Fig. 4 for correction components), reflecting standard procedures. IFA, integrated flux analysis involving 60 min labelling followed by the monitoring of efflux as a component of influx. Each datum represents the mean ±SEM of 5–33 replicates. Different letters within a row refer to significantly different values ($P$ <0.95).

| $[K^+]_{external}$ (mM) | Influx (μmol $g^{-1}$ root FW $h^{-1}$) | |
|---|---|---|
| | DI | IFA |
| 0.1 (HATS) | 7.31±0.18 a | 10.43±0.55 b |
| 40 (LATS) | 10.30±0.22 a | 74.70±13.78 b |

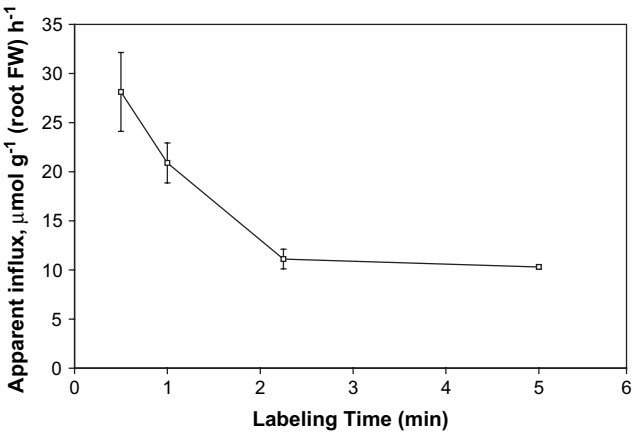


**Fig. 1.** Short-term time-course of uncorrected direct influx (DI) measurements, conducted in the LATS range of $K^+$ transport in intact barley seedlings. Error bars refer to SEM of 3–33 replicates ($n$=33 for the 5 min time point, which was highly replicated due to its use elsewhere in this study, and to its use as a standard labelling period in other studies).

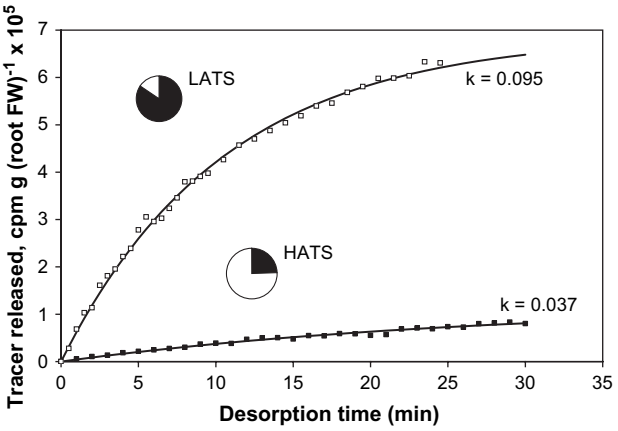


**Fig. 2.** Representative plots of tracer loss from roots of intact barley seedlings grown and assayed at 0.1 mM (HATS range, filled squares) and 40 mM (LATS, open squares) $[K^+]_{external}$. Tracer fluxes were normalized to the same external $^{42}K$ specific activity. Kinetic exchange constants (*k* values) for HATS and LATS conditions are shown for each tracer. Inset: pie-charts demonstrate the relative contribution of efflux (black) to total influx (white plus black) for the two conditions.

become large, relative to those at $K^+$ concentrations in the HATS range (Fig. 2). These increases, as predicted, led to a much greater discrepancy in influx measurements in the LATS range (exchange half-time=7 min; efflux: influx ratio=0.8), relative to the HATS (exchange half-time=16 min; efflux:influx ratio=0.2), range. From equation 2, it follows that the DI values given for HATS and LATS conditions in Table 1 have been underestimated by 7% and 44%, respectively, on account of efflux alone. In the case of $K^+$, the ratio of efflux to influx in the LATS range increases progressively when external $[K^+]$ increases from the HATS range towards the values shown here (MW Szczerba *et al*., 2006), and therefore errors incurred in DI measurement must also increase along this gradient. Similar shortening of exchange half-times and/or increases in efflux:influx ratios have been observed for other ions such as $NO_3^-$, $NH_4^+$, $Cl^-$, and $SO_4^{2-}$ (Kronzucker *et al*., 1999; Min *et al*., 1999; Scheurwater *et al*., 1999; ter Steege *et al*., 1999; Lopez *et al*., 2002; Kronzucker *et al*., 2003*b*; Britto *et al*., 2001, 2004), and thus similar difficulties with influx determinations in the LATS range are to be expected as the external concentrations of these ions increase. In the case of sodium, which is of primary current interest because of salinity research, and is therefore studied at high external concentrations representing the LATS range, short half-times and high efflux:influx ratios appear to be characteristic (Cheeseman, 1980; Essah *et al*., 2003; Davenport *et al*., 2005; Wang *et al*., 2006). This necessitates the application of caution with respect to interpreting direct influx (DI) measurements for these ions, and calls for the complementary execution of IFA experiments.

Even after applying a correction for simultaneous efflux, however, there remained a large discrepancy between LATS influxes measured by DI and by IFA in the present study. It is a common practice in flux analysis for researchers to discard the region of the plant at the root–shoot interface, prior to tracer counting or tissue analysis (Vale *et al*., 1988*a*, *b*; Lazof and Cheeseman, 1988*a*, *b*; Siddiqi *et al*., 1989; Kronzucker *et al*., 1998, 2000). This practice can be partially justified by concerns about tracer contamination of basal shoot portions, or residual seed material, by a radioactive solution intended to label the roots alone. However, as shown in Table 2, the proportion of radioactivity found in this region not only varies with external $K^+$ supply, but also depends strongly on analytical procedure. In short-term DI measurements in the LATS range, the tracer accumulated at the root–shoot interface was 1.7 times greater than that which accumulated in the entire remainder of the plant. By contrast, this factor was only 0.05 in the HATS range, and 0.48 and 0.16 in IFA experiments conducted in the LATS and HATS ranges, respectively. These differences are partly attributable to methodological differences between DI and IFA in the duration of labelling and desorption of roots, but of more physiological importance is the attribution of the differences between HATS and LATS values to a selective-filtration (i.e. salt-restricting) function of this region (Jacoby, 1979; Johanson and Cheeseman, 1983), which accumulates a greater proportion of ions as the external supply increases. In other words, strong tracer accumulation at the root–shoot interface is more characteristic of whole-plant behaviour in the LATS range. Abundant evidence for the often substantial, and highly variable, accumulation of tracer in the basal root and basal shoot regions of the plant can be found in the literature (Jacoby, 1964; Shone *et al*., 1969; Yeo *et al*., 1977; Johanson and Cheeseman, 1983; Johanson *et al*., 1983; Drew and Läuchli, 1985; Lacan and Durand, 1996). The contribution of tracer at the root–shoot interface must be included in the total tracer absorbed by the roots and, therefore, counts retained in this region must be subjected to equation 2 in the same way as the remainder of the tissue.

Even after considering the effects of efflux and tracer accumulation at the root–shoot interface, however, the difference between flux estimates determined by DI and IFA in the LATS range still remained substantial. This observation led to an examination of methods used for the superficial drying of roots following tracer desorption, a process required to obtain accurate fresh weight determination. Such removal of surface water is typically accomplished by blotting (Jacoby, 1964; Cram and Laties, 1971; Jeschke, 1982; Johansen and Cheeseman, 1983; Drew and Läuchli, 1985; Wrona and Epstein, 1985; Lee and Drew, 1986; Cao *et al*., 1993; Elphick *et al*., 2001; Davenport *et al*., 2005) or low-speed centrifugation (Glass and Perley, 1980; Kochian and Lucas, 1982; Kronzucker *et al*., 2003*a*, *b*) of the root tissue. It is generally assumed that surface water removed by either practice will contain negligible quantities of tracer, given a sufficiently long

**Table 2.** *Accumulation of $^{42}K$ in root–shoot interface relative to $^{42}K$ in root and shoot of intact barley seedlings, as measured by two methods in high- and low-affinity transport (HATS and LATS) ranges*

Values are expressed as a fraction of accumulated root and shoot counts in each treatment (=1). DI, direct influx; IFA, integrated flux analysis (see Table 1). Different letters within a column refer to significantly different values ($P$ <0.95).

| $[K^+]_{external}$ (mM) | DI | IFA |
|---|---|---|
| 0.1 (HATS) | 0.05 a | 0.16 a |
| 40 (LATS) | 1.70 b | 0.48 b |

**Table 3.** *Influx estimates corrected for $^{42}K$ captured in centrifugation or blotting of labelled roots of intact barley seedlings in high- and low-affinity transport (HATS and LATS) ranges*

Each datum is the mean of 7–8 replicates (±SEM for fresh weights). The same letters within a given potassium treatment refer to values that are not significantly different ($P$ <0.95).

| $[K^+]_{external}$ (mM) | Method of processing roots | Influx corrected for processing error (μmol $g^{-1}$ root FW $h^{-1}$) | Percentage change after correction | Root fresh weights (g) |
|---|---|---|---|---|
| 0.1 (HATS) | Centrifugation | 9.22±0.31 a | 2.48 | 0.42±0.02 a |
| | Blotting | 8.72±0.19 a | 1.21 | 0.41±0.02 a |
| 40 (LATS) | Centrifugation | 12.18±0.70 a | 24.05 | 0.53±0.10 a |
| | Blotting | 10.30±0.89 a | 17.88 | 0.59±0.10 a |

desorption period prior to the weighing procedure. Surprisingly, however, it was found that there was substantial tracer leaving the plant under all conditions as a result of centrifugation and blotting methods, although with no significant differences between these two methods in terms of resulting influx or fresh weight estimates (Table 3). It was also found that the amount of tracer lost in this manner, relative to tracer retained in the plant tissue, strongly varied with growth condition and labelling time. As depicted in Fig. 3, the radioactivity collected in the centrifugation step equalled as much as 200% of the tracer that accumulated in roots and shoots, in the case of short-term (30 s) labelling in the LATS range. Exponential-decay modelling of tracer clearance from extracellular phases (Britto and Kronzucker, 2001*a*) confirms that a 5 min desorption period will clear the surface-film phase (exchange half-time=10 s) of ≫99% of its tracer content, and will clear the Donnan free space (exchange half-time = 0.79 min) of ~ 99% of its tracer content. This modelling exercise, combined with the observation that the ratio of counts extracted by blotting or centrifugation, to counts remaining in the roots and shoots, is independent of the desorption period (which varied from 5 min to 30 min; data not shown), led to the conclusion that surface water-removal techniques can lead to substantial losses of tracer from *within* the cells of the root. Therefore, a more accurate influx estimate must include any counts collected in this manner, in addition to counts retained within, and effluxed from, plant cells; only procedures that normalize fluxes to measures other than fresh weight (e.g. dry weight, surface area) are essentially free of this problem. It is worth noting two additional aspects of the experiment shown in Fig. 3. First, in the LATS range, the contribution of centrifuged tracer relative to tracer remaining in the roots and shoots declined sharply with labelling time; this represents an increasing development of $^{42}K$-labelled pools in the plant that are resistant to loss by centrifugation (e.g. in the shoot, and in vacuoles of root cells), and indicates that errors incurred by not including these lost counts are much more severe in short-term DI experiments than in longer-term IFA experiments. Second, there was very little contribution of centrifuged tracer in the case of HATS-range studies, and

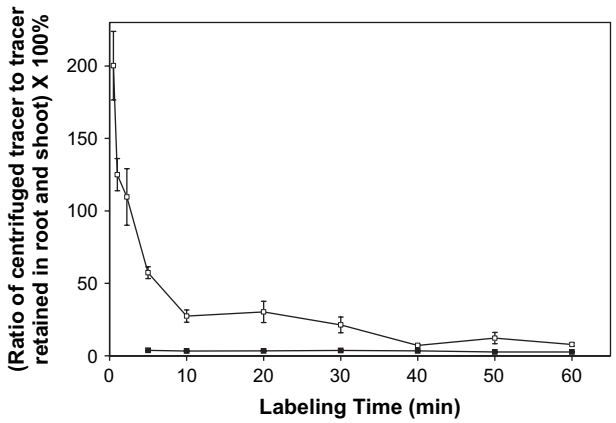


**Fig. 3.** $^{42}K$ counts collected from centrifugation of labelled roots from intact barley seedlings, grown and assayed at 0.1 mM (HATS range, filled squares) and 40 mM (LATS range, open squares) $[K^+]_{external}$, relative to counts retained in root and shoot, and as a function of labelling time. Each datum is the mean ±SEM of 6–30 replicates. Error bars in the HATS range are smaller than the symbols.

the relative contribution did not decline with labelling time. This is attributed to the longer cytosolic exchange half-time, and lower tissue potassium content, of the HATS-range plants. This difference may also be indicative of the activity of mechanosensitive channels (Cosgrove and Hedrich, 1991; Ramahaleo *et al*., 1996; ter Steege and Stulen, 1997; Demidchik *et al*., 2002; Qi *et al*., 2004) operating in LATS ranges, which could cause higher disturbance-enhanced LATS fluxes, a phenomenon that has been observed by Hommels *et al*., (1990).

## Conclusion

This study shows that there are at least four ways in which major errors can routinely be encountered in direct influx analyses. As shown in Fig. 4, the extent of these errors depends, for potassium, critically on the plant's transport mode (Kronzucker *et al*., 2003*a*), and similar considerations are believed to apply to the uptake of other ions in

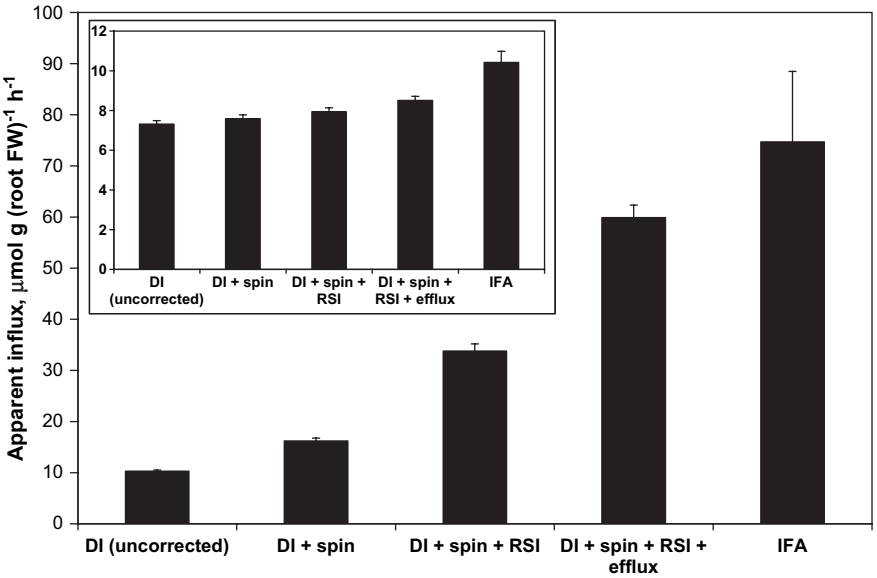


**Fig. 4.** Development of corrections to direct influx (DI) measurements in LATS and HATS (inset) ranges of $K^+$ transport in intact barley seedlings. Corrections are for centrifuged tracer (spin), for contributions from the root–shoot interface (RSI), and efflux occurring during labelling and desorption. Integrated-flux-analysis (IFA) data is shown for comparison. Error bars refer to SEM of 5–15 replicates.

the LATS range (see above). In the HATS mode, correction of these errors makes a modest, though significant, change in the resulting flux estimates, increasing the face-value flux as determined by classic DI procedures by about 16%. This situation is in stark contrast to influx measurements in the LATS range, which increase by an extraordinary 600%, at 40 mM, after all corrections are applied to the initial DI estimate. Interestingly, for both HATS and LATS conditions, full correction of DI data nevertheless still results in slightly lower fluxes than with IFA, although this difference is only significant in the case of the HATS range. It is possible that this residual difference is due to increased relaxation of plants in the IFA study, which undergo less physical handling than plants in the DI study, over the course of the experiment (Britto *et al*., 2006).

The physiological basis of the modal distinction discussed here, and the measurement errors associated with this distinction, are, at present, reasonably clear for potassium. The carrier-mediated transport proteins that catalyse $K^+$ influx in the HATS mode are limited by substrate-binding capacity and require energy to transport $K^+$ across a membrane that is more negatively charged (inside negative) than the Nernst potential, $E_K$. These conditions provide a low membrane conductance that is fundamentally different from the channel-dominated condition found in the LATS mode, in which the plasma membrane is depolarized closer to $E_K$, and high conductances are normal (Cheeseman and Hanson, 1979*a*, *b*; Beilby, 1985). For other ions, mechanistic distinctions between flux modes are less clear; for instance, it is feasible that sodium influx has no high-affinity transport mode under normal physiological conditions (i.e. conditions where $K^+$ is present), but may instead be catalysed by channels, either non-selective or predominantly $K^+$-selective (Demidchik *et al*., 2002; Tester and Davenport, 2003). In such a case, the errors in determining unidirectional influxes associated with the operation of high-conductance pathways may be found at most, or all, levels of substrate provision (see above). The case of anion permeation is also less clear than that of $K^+$ flux. For anions such as $NO_3^-$ and $Cl^-$, influx is likely to be energy-requiring under all circumstances; however, the proportion of incoming ions that are subsequently effluxed has also been shown to increase with increasing provision (see above).

In summary, the fundamental differences in the generation of errors between $K^+$ transport modes are attributable to four distinct characteristics of the LATS range: (i) increased efflux relative to influx; (ii) increased cytosolic turnover; (iii) increased accumulation of tracer at the root–shoot interface; and (iv) increased loss of tracer due to centrifugation or blotting. While this conclusion, therefore, poses no major difficulties to studies, past and present, conducted in the HATS range, it underscores the enormous, and previously unknown, difficulties in measuring fluxes in the LATS range. Moreover, it calls into question virtually all direct influx studies that have been conducted in this range, because, for all ions thus far investigated, the ratio of efflux to influx increases as external ion concentrations increase, often approaching unity (Kronzucker *et al*., 1999, 2003*a*, *b*; Min *et al*., 1999; Scheurwater *et al*., 1999; ter Steege *et al*., 1999; Britto *et al*., 2001, 2004; Lopez *et al*., 2002). In addition, for many ions the cytosolic exchange half-time appears to shorten with increasing external concentrations (a notable exception

is inorganic N; see Britto and Kronzucker, 2001*b*), resulting in even more pronounced errors. Given the current interest in the functioning of plant ion transport systems in the context of salinity toxicity and tolerance (Tyerman and Skerrett, 1999; Blumwald *et al*., 2000; Demidchik *et al*., 2002; Tester and Davenport, 2003), and given that studies in this area usually examine LATS-range transport modes, the findings here must be considered by researchers who seek a realistic understanding of the primary acquisition of potentially toxic ions by plants. More fundamentally, the accuracy of concentration-dependent influx isotherms should be re-examined in the light of these findings, particularly when the range of the isotherm is extended to high external concentrations. Isotherms generated by use of direct influx protocols that do not take into account the factors described here will likely incur ever more serious errors as externally applied concentrations increase.

## Acknowledgements

We thank M Butler at McMaster University in Hamilton, Ontario, Canada, for supplying the $^{42}K$ required to conduct these experiments, and PS Tehrani for assistance with experiments. This work was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC), and the University of Toronto.

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