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INVITED REVIEW

Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function

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ABSTRACT

The transport of mineral ions into and out of tissues and cells is central to the life of plants. Ion transport and the plasma membrane transporters themselves have been studied using a variety of techniques. In the last 15 years, measurement of specific ion fluxes has contributed to the characterization of transport systems. Progress in molecular genetics is allowing gene identification and controlled expression of transporter molecules. However the molecular expression of transporter gene products must be characterized at the functional level. The ion-selective microelectrode technique to measure specific ion fluxes non-invasively is ideally suited to this purpose. This technique, its theory, its links with others and its application and prospects in plant science, are discussed. Ions studied include hydrogen, potassium, sodium, ammonium, calcium, chloride and nitrate. Applications discussed include: solute ion uptake by roots; gravitropism and other processes in the root cap, meristematic and elongation zones; Nod factor effect on root hairs; osmotic and salt stresses; oscillations; the effects of light and temperature. Studies have included intact roots, leaf mesophyll and other tissues, protoplasts and bacterial biofilms. A multi-ion capability of the technique will greatly assist functional genomics, particularly when coupled with imaging techniques, patch clamping and the use of suitable mutants.

Key-words: diffusion; electric current; electrochemical potential; MIFE; oscillation; plasma membrane; vibrating probe.

Abbreviations: CCCP, carbonyl cyanide m-chlorophenyl-hydrazone; IAA, indole acetic acid; LIX, liquid ion exchanger; NAA, napthalene acetic acid; TEA, tetra ethyl ammonium; VDI, valid data interval.

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INTRODUCTION

The transport of mineral nutrient ions into and out of plant roots has long been studied using various methods of chemical analysis and tracer techniques. More recently, attention has been focused on the transporters themselves, located in the plasma membrane of cells. Membrane potential measurements, patch clamp, ion-and pH-sensitive dyes with advanced imaging techniques, and ion-selective microelectrodes have provided more specific information on ion distribution and movement, and thus on transporter properties. In a number of laboratories in the last 15 years, an additional technique, non-invasive measurement of ion fluxes using ion-selective microelectrodes, has contributed to the functional characterization of the transporter systems. This technique, with its achievements and prospects and its links with others mentioned, is the subject of this review.

How does the technique work? It is first described, and its principles, theory, limitations and implementations are outlined.

How has it been useful in plant science, for roots in particular? In its application in diverse areas of plant physiology, it has come to form a useful link between slow, bulk tissue (chemical and tracer) measurements and fast, localized measurements (using dyes and markers with imaging techniques or using individual transporter patch clamping). Its use in elucidating the transport of the main mineral nutrient ions is discussed, as well as some applications beyond transport physiology.

What are its prospects for the future? How does it team with other techniques to build our understanding of ion transport systems? How can it help in the functional characterization of expressed transporter gene products?

The proposal to use ion concentrations (strictly, electrochemical potentials) measured outside plant tissues to calculate tissue flux of the ion came from Bill Lucas. It was presented at a NATO Advanced Studies Institute in Italy in 1984 (Lucas & Kochian 1986). It was natural to apply the theory to root nutrition and the first study was on the stoichiometry of H^+ and K^+ fluxes in corn roots (Newman *et al*. 1987). Further development of the theory and its application to a range of ion transport issues in roots and other tissues has been carried out in a number of laboratories including those of the biophysics group in Tasmania.

THE ION-SELECTIVE MICROELECTRODE ION FLUX TECHNIQUE

Ions crossing the surface of a tissue in solution are carried to or from that surface by diffusion or by mass flow. In the absence of convection or water uptake, and in steady or only slowly changing conditions, measurements of the net diffusive flux of the ion in solution close to the tissue must give the net flux of the ion across the tissue surface.

Theory

Substances in solution tend to move by diffusion from high concentration to low. For ions, electric forces are also involved, so the movement is from high electrochemical potential to low. If the electrochemical potential gradient can be measured, the net ion movement by diffusion can be calculated from that gradient and the mobility and concentration of the ion in solution. For H^+, for example, the gradient can be measured using a pH meter, by moving the pH electrode between two positions, with the reference electrode fixed some distance away. For another ion, the principle is the same but the electrode must be sensitive specifically to that ion instead. To achieve good spatial resolution requires the use of a microelectrode.

Consider a microelectrode containing a specific ion carrier, or liquid ion exchanger (LIX), in the tip (Fig. 1), which is initially at a distance x (metres) from the tissue. The electrochemical potential of that ion in the solution is μ (joules mol^{-1}) at the distance x. In terms of the ion's free concentration c and the electric potential V_b at that point in the bathing solution, μ is given by the equation,

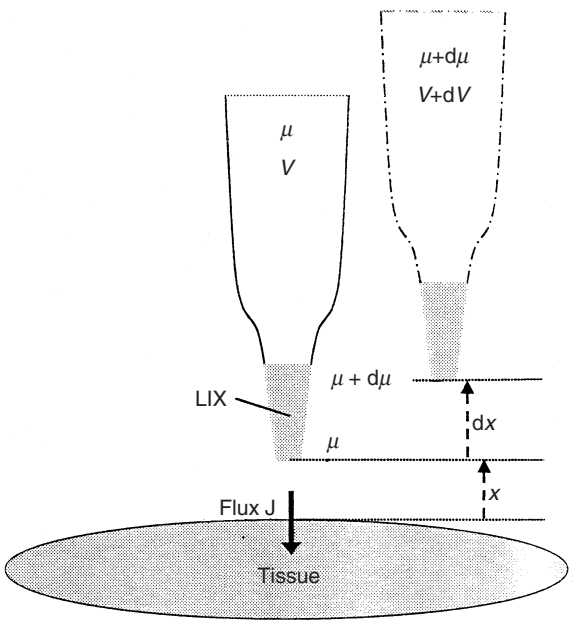

Figure 1. To illustrate the theory, where a microelectrode with an ion selective barrier (LIX) in the tip is moved through a known distance dx and an electrometer measures the associated change in electrode voltage dV.

$$\mu = \mu_0 + RT\ln\gamma c + zFV_b \quad (1)$$

where μ_0 is a reference value of electrochemical potential. The ion's valency is z, γ is the activity coefficient of the ion in the solution, F is the Faraday number (96 500 C mol^{-1}), R is the gas constant (8·3 J mol^{-1} K^{-1}) and T is the temperature (K).

The microelectrode tip is now moved slowly (not to disturb the solution) away through a small distance dx. (The lateral offset shown in Fig. 1 is only to avoid overlapping of labels.) At this new position in solution the electrochemical potential is μ + dμ, where dμ incorporates both the change in c and the change in V_b to the new position, with the same reference μ_0.

Standard electrochemical theory, e.g. Dainty (1962; Eqn 9) or Nobel (1974; Eqn 3.6), shows that the net flux of the ion J (mol m^{-2} s^{-1}) is given in terms of the ion concentration (*not* activity) c (mol m^{-3}), the mobility of the ion u (speed per unit force, m s^{-1} per newton mol^{-1}), and the force per mole which is the electrochemical potential gradient (dμ/dx).

$$\text{Thus } J = cu(\mathrm{d}\mu/\mathrm{d}x) \quad (2)$$

Here J is considered positive if *into* the tissue, opposite to x. When μ is expressed in terms of c and V_b from Eqn 1, Eqn 2 is sometimes (Meyer & Weisenseel 1997) known as the Nernst–Planck equation. Because the LIX allows free passage of the ion in question (but no others), the electrochemical potential of the ion inside the electrode initially is also μ and its change is also dμ (Fig. 1). Inside the electrode the concentration is fixed, so dμ there is expressed only in terms of the change dV in internal voltage V (volts), so ideally: d$\mu = zF$dV. The voltage V is measured by an electrometer connected via suitable half-cells to the electrode solution and to a reference electrode some distance away in the bath solution. The electrometer measures dV as the electrode is moved through the chosen distance dx. For the ion, u is a known constant.

Because the LIX does not usually show ideal Nernstian behaviour, the internal dV in practice underestimates the external dμ by a factor g which is a little greater than unity, typically approximately 1·1. For this reason, we should relate the external dμ to the internal, measured dV by the equation:

$$\mathrm{d}\mu = zFg\mathrm{d}V \quad (3)$$

This factor g is found from the measured *Nernst slope* as described below. Hence Eqn 2 for the flux may be written,

$$J = cuzFg(\mathrm{d}V/\mathrm{d}x) \quad (4)$$

The only quantity still to be determined for Eqn 4 is the average bath solution concentration near the tip. This is adequately calculated in practice from the average value of V during the move, using the results of the electrode calibration in solutions of known concentration. The calibration is done in solutions having no electric field, so an implicit assumption for this calculation of c is that, during the experiment, V_b near the tissue is little different from the V_b near the reference electrode, some distance away in the

bath solution. This is normally the case (Ryan, Shaff & Kochian 1992).

Because the electrode moves in a short time between the two positions, all steady or slowly drifting voltages in the measurement circuit will cancel out in calculating dV. The effect of the electrochemical gradient dμ/dx between the two positions is all that remains. Because of this useful lack of sensitivity to reference or other values, the electrodes in this technique are sometimes called 'self referencing' (Smith *et al*. 1999).

The theory can be expressed alternatively, using Fick's Law of diffusion, in terms of the diffusion coefficient D for the ion instead of the mobility u (to which it is related: $D = uRT$) (Henriksen, Bloom & Spanswick 1990; Smith, Sanger & Jaffe 1994, Eqn 2; Smith *et al*. 1999). However if Fick's law alone is used in the theory, the relevance of the electric potential gradient in solution is not acknowledged, and yet the ion selective electrodes actually measure the local μ with respect to a fixed reference, not a function (logarithm, see Eqn 1) of the local activity γc alone. Workers who have followed Kuhtreiber & Jaffe (1990), Smith *et al*. (1994) and Smith *et al*. (1999) have obtained 'equivalent activities' from their microelectrode measurements, which incorporate the effect of the local electric potential change in the medium. The flux values obtained from Fick's law by using the difference between those 'equivalent activities' thus turn out to be essentially correct. The fact that the difference is not the true chemical activity difference is unimportant for the flux calculation. The average concentration calculated from these activities is also not greatly in error, and indeed is just what is used in practice for c in Eqn 4 above. Smith (1995) showed experimentally that measured dV values agreed reasonably well with a theoretical model of the same voltage gradient.

When membrane transport of an ion changes (from a previously steady state or in an oscillation), the consequent movement of that ion in solution has electrical effects on other ions, thereby causing them to move, even though they were not transported through the membrane. Smith & Eberl (1993; Eqn 7) have analysed this interaction and identified a diffusion length ξ which depends on an average diffusion coefficient D for the ions in solution and on the time course of the change or on the period T of the oscillation [$\xi = (DT/\pi)^{1/2}$]. For KCl ($D \cong 1{\cdot}5 \times 10^{-9}$ m^2 s^{-1}) and supposing $T = 100$ s, this gives $\xi = 200$ μm. For other experimental solutions D would not be greatly different. Thus if flux measurements are made well within 200 μm of the tissue, the interactions discussed by Smith & Eberl (1993) can be neglected for changes or oscillations of period > 100 s. This has been the case for work reviewed here.

Geometrical issues

In a typical use of the technique, the initial distance x may be 2–20 μm and the change dx may be 10–60 μm. It is usually estimated that the flux measured is associated with a region of tissue surface whose diameter is roughly the mean electrode distance from it, 7–50 μm for the ranges in this example. This region would usually include tens of cells at most. Henriksen *et al*. (1992) present a detailed assessment.

In the above theory it is assumed that the measurement is made close enough to the tissue surface that it can be treated as being flat, so that the ionic movement is normal to the surface. This is not valid if there are local features, e.g. root hairs, the root tip or for single cell studies. In these cases, the theory is geometrically adequate if the measurements can be made close enough to the surface so that both x and dx are small compared with the dimensions of the feature.

For a root, away from the features mentioned above, the cylindrical geometry straightforwardly brings a logarithmic term into Eqn 4 (Newman *et al*. 1987; Henriksen *et al*. 1992; Lew 1999). For protoplasts, their spherical geometry brings a 1/x term into Eqn 4. (See Eqns 8 & 9 below.) In all cases, the experimenter must assess the suitability of the geometrical assumptions in relation to the movement of the electrode tip and the tissue geometry.

An interesting issue arises when flux from a tissue in which there are cell layers is compared with flux from a protoplast obtained from that tissue type (Shabala & Newman 2000). The flux that emerges into solution from the flat tissue surface has come not only from the membrane immediately adjacent to the solution but also, through the apoplast, from membranes on the other sides of those outer cells and from deeper cell layers. The relative contributions of these membrane fluxes to the observed tissue surface flux have not been quantified. Qualitatively, the observed flux (which is per unit area) from the tissue surface is greater than the flux through any single cell membrane by an unknown and variable factor. Thus when we measured protoplast fluxes (Shabala & Newman 2000), they were smaller than the fluxes from the tissue from which the protoplasts had been derived. As well as this 'geometrical' factor, the very preparation of the protoplasts must also modify the behaviour of the protoplast membrane, further complicating such comparisons.

Time resolution

The microelectrode tip is kept at each position x or x + dx (Fig. 1) for a time usually in the range 1–10 s. Calculation of the dV, from which the flux value is calculated, requires one cycle of twice that duration to measure the voltage at the two positions. This period sets the time resolution of the technique. Two practical considerations prevent this time being much shortened.

1 The solution must not be disturbed by having too fast a movement. Movement between the positions takes 10–20% of each half cycle. If the flux is expected to be large however, a small dx could be used and traversed in a small time.

2 The LIX must have time to 'settle' to the new μ value (Fig. 2; Kochian *et al*. 1992, Fig. 2; Shabala, Newman & Morris 1997, Fig. 1). Electrodes vary considerably, with age, with conditioning in solution (Cl^- requiring several hours) and for different ions' LIX. For those we have used,

we have found Ca^{2+} to settle the quickest after movement, followed by H^+, K^+, Na^+, NO_3^-, NH_4^+, Cl^- roughly in that order. Smith *et al*. (1999) also discuss these issues, but the time allowed by their system may be insufficient for adequate settling. Could it be the cause of the underestimation of fluxes by their system, requiring the use of a correction factor (Smith *et al*. 1999, Fig. 4 and Table 1)?

The time resolution of 2–20 s is achievable for responses to treatments such as illumination (Shabala & Newman 1999) that do not themselves disturb the solution around the tissue. It is also achievable for comparisons between time courses of responses from different ions measured simultaneously (Shabala *et al*. 1997, Fig. 2a; Felle *et al*. 1998, Fig. 5a). Effects of inhibitors or of different solution chemical composition, when initiated by solution change, have a time resolution of minutes, as discussed in the next section.

Bulk solution flow

Central to the theory is the requirement that there is no convection, mixing or other bulk solution flow, so that ionic movement in the solution adjacent to the tissue (regardless of the membrane transport processes) is solely by diffusion under the influence of electric and chemical forces in solution. After a solution change, and depending on both x and dx, it may take minutes for steady, diffusion-limited conditions to be established in the region between tissue and electrode tips. Data obtained during this interval are not valid and are usually omitted from figures (Ryan, Newman & Arif 1992; Shabala and Newman 1998, for example). The time required can be estimated in advance by applying diffusion theory to the planned distance using the diffusion coefficient for the ion. This time limits the speed with which one can follow effects of application of inhibitors or other chemical treatments. An elegant way around this limitation may be to use micro-injection or chemicals that are 'caged' in dispersed vesicles and then released by a pulse of light.

Plassard *et al*. (1999) defied convention by making measurements in flowing solution (presumably parallel to the root axis) to ensure steady-state conditions for their roots. The measured radial H^+ concentration profile (Plassard *et al*. 1999, Fig. 2) was stable from the root surface to about 0·5 root radius outside it. This would seem to validate their work if the ion flux at the root surface was constant for some distance 'upstream' from the measuring site. Future workers may also wish to consider using (properly validated) flowing solutions when measuring fluxes.

Experimenters have also assumed that the water flow into roots is small enough that bulk streaming of the solution contributes negligibly to the ionic fluxes. Henriksen *et al*. (1992) have carried out an analysis that confirms the general validity of the assumption. Calculations have also been carried out for imbibing seeds (Shabala *et al*. 2000a) which took up water to increase their volume by 10% per hour. Their conclusion was that the contribution to fluxes by bulk solution flow was negligible.

Ion fluxes and currents

Electrodes are sometimes referred to as 'vibrating', but the conditions are very different from those for the long established 'vibrating probe' (Jaffe & Nuccitelli 1974). For ion flux measurements it is necessary that the electrodes move slowly, so that the solution remains still. The net flux or the equivalent electric current (Ryan, Newman & Shields 1990) of one or more individual ions is measured as described above. The total electric current in solution is the sum of the currents carried by the fluxes of *all* ions present in the solution. This total current is only known if the contributions from all ions are measured. It cannot be calculated from a dV using a standard KCl-filled microelectrode because (ionic) current is driven by chemical activity gradients as well as by the electric potential gradient. Smith *et al*. (1994, Eqn 1), Smith (1995) and Smith *et al*. (1999) are in error on this theoretical point, although it has not been incorporated into their theory or data. Lucas & Kochian (1986) have presented a more detailed analysis of this error.

For the vibrating probe, its rapid vibration thoroughly mixes the solution in its vicinity. This mixing collapses all the chemical concentration gradients in the region of the probe, replacing them (for electrochemical continuity) by an electric voltage gradient that the probe measures along its axis of vibration. From this voltage gradient and solution conductivity, the net electric current component in that direction is calculated using Ohm's law (Jaffe & Nuccitelli 1974). Thus the rapidity of the vibrating probe is necessary for the validity of the net current measurement, as well as to allow noise reduction through narrow band filtering of the alternating signal. Ferrier & Lucas (1986) present a *caveat* on the vibrating probe relating to the effect of disparate ionic mobilities.

If a combination ion-selective electrode (having the reference electrode alongside the ion-selective one, instead of fixed at a distance) is used to measure ion fluxes (Meyer & Weisenseel 1997; for example), it is the ion's activity gradient alone that is thereby measured. In that case, the electric potential gradient in the unstirred medium must be measured simultaneously and combined according to the Nernst Planck equation. The attempt by Meyer & Weisenseel (1997) to do this separately using the vibrating probe to obtain dV was incorrect, and their calculated fluxes are in error to the extent of the errors in the dV values.

Electrodes and LIX

The preparation of the electrodes is generally regarded as the most challenging, and at times frustrating, aspect of the entire technique. The standard text on ion selective electrodes is by Ammann (1986). Standard micropipettes for membrane potential or patch measurements may be used for flux studies. The tips are coated by various methods with a hydrophobic material (e.g. tributylchlorosilane) to hold the LIX. The specific LIX are available commercially for a

wide range of nutrient ions and new ones are being developed.

The filling solution is injected into the rear of the pipette to fill the tip. The tip is then usually filled to tens of micrometres length by touching it to a drop of LIX in a capillary. A very informative, detailed description of the process is given by Smith *et al*. (1999) in their aptly named section 'Making Ion Selective Electrodes: The Key to Success'. Special considerations apply to the making and filling of multibarrel microelectrodes (Taylor & Bloom 1998).

The filling solution must contain chloride to provide a stable junction potential at the Ag/AgCl electrode inserted into the back of the micropipette. The filling solution must also contain a reasonable concentration of the ion of interest to stabilize the chemical component of its electrochemical potential. Despite this requirement, some authors report using KCl as the filling solution for some other ions (Lew 1998).

Electrodes are usually made for use during the same day. They are calibrated, before or after use, in solutions with a series of concentrations covering the expected range of the ion in question. This calibration is usually done with a background of the solution to be used for measurements. There are two reasons for this:

1 The activity of the ion depends on the ionic strength of the solution.
2 It takes some account of effects of other ions for which the LIX may have partial sensitivity. Where significant non-selectivity effects are expected, other measures need to be taken (Henriksen *et al*. 1990; Smith *et al*. 1999, 'Selectivity and Response Times' section).

If the fit of the line to the calibration points is poor (e.g. correlation coefficient $r < 0{\cdot}9996$), it may indicate that the calibration is outside the linear range for the LIX (see LIX data sheets from suppliers). A close fit is desirable in order to ensure reliable interpolation to obtain the value for the concentration. Both the slope and intercept of the calibration line are used to calculate the concentration c in Eqn 7 from values of V measured during the experiment.

The slope of the straight line fitted to the calibration data (mV against $\log_{10}$ concentration) is usually expressed in terms of the *Nernst slope*, because ideally the line follows the Nernst relationship (Dainty 1962; Nobel 1974),

$$V - V_{\mathrm{ref}} = (RT/zF)\ln(a/a_{\mathrm{ref}})$$

Concentrations can be used in place of the activities a if the activity coefficient γ is constant over the calibration range. For voltages measured in mV, at 20 °C, using base-10 logarithms and combining the constants V_{ref} and $\ln a_{\mathrm{ref}}$ into V_0 which is the intercept on the mV axis of the calibration graph, this equation becomes,

$$V = V_0 + (58/z)\log_{10}(c)$$

In practice, the mV change is smaller by the factor g, defined above, so the experimental calibration graph has the equation,

$$V = V_0 + (58/gz)\log_{10}(c) \quad (5)$$

The slope of this calibration graph is the *Nernst slope*, which can therefore be written,

$$\textit{Nernst slope} = (58/gz) \quad (6)$$

Measured *Nernst slopes* are usually above 50 (or above 25 for divalent ions). Equation 6 allows substitution for gz in Eqn 4, which becomes,

$$J = cuF(58/\textit{Nernst slope})(\mathrm{d}V/\mathrm{d}x) \quad (7)$$

This is the equation used for planar geometry by the MIFE system software (see below). Equations used with spherical and cylindrical geometry, respectively, using r for the radius of the sphere or cylinder source, are obtained by replacing the dx in Eqn 7 with

$$\mathrm{d}x = r^2[1/(r+x) - 1/(r+x+\mathrm{d}x)], \text{ for the sphere, or} \quad (8)$$

$$\mathrm{d}x = r\ln[(r+x+\mathrm{d}x)/(r+x)] \text{ for the cylinder.} \quad (9)$$

Flux measurement in buffered solution

H^+-selective microelectrodes detect free H^+ in solution. If the solution is buffered, any membrane flux of H^+ is carried in solution partly by protonated buffer molecules. Thus the measured H^+ flux in solution underestimates the membrane flux of H^+ that is of interest. Arif, Newman & Keenlyside (1995) have provided a method that workers can use to correct measured H^+ fluxes for the presence of buffers, including the effect of water itself around pH 7 and above. Demarest & Morgan (1995) also evaluated a correction for buffers in terms of diffusion coefficients for one particular buffer, but they ignored the (sometimes substantial) effect of water. Both those sources noted, without giving details, that Ca^{2+} buffers will have a similar effect on Ca^{2+} fluxes.

Sensitivity

The systems designed to implement this technique have a data sampling rate ≥ 10 Hz and minimize noise by digital averaging over longer time periods (typically 1–10 s). All approaches have the same ultimate limitation on their sensitivity, which is set by the thermal electronic 'Johnson' noise in the high resistance of the ion selective LIX in the micropipette tip. This theoretical limit on sensitivity is discussed by Ryan *et al*. (1990, Eqn 3 and Table 1) who give the minimum detectable fluxes and equivalent current densities for several medium concentrations. Those quantities for other ions and concentrations can be calculated from their results or from Eqn 7. They also draw attention to other limitations on the sensitivity, including electrode drift.

Practical systems are able to get close to those theoretical limits. As discussed by Kochian *et al*. (1992), the digital averaging over a 10 s cycle is equivalent to reducing the bandwidth to approximately 0·1 Hz. This improves the sensitivity 10-fold through reducing 'Johnson' noise, since noise $\propto\sqrt{(\text{bandwidth})}$. If a very short time resolution is not essential, sensitivity is optimized by having the half cycle

time longer – perhaps > 10 s – so that digital averaging reduces the noise of the voltage in each position. The improvement in sensitivity goes as √period. For an electrode resistance R_e, the noise amplitude is $\propto \sqrt{R_e}$, so microelectrodes having R_e much reduced from the usual 1–5 GΩ would improve the sensitivity. Improved sensitivity would permit a smaller dx (to a few micrometres) and hence a tissue localization of that order. It would also permit flux measurements in media having higher concentrations of the ion. This is because the sensitivity threshold (the flux associated with the minimum detectable dV, see Eqn 7) is directly proportional to the concentration of the ion. This is a problem, for example, in measuring Na^+ or Cl^- fluxes in seawater or for salinity studies.

The MIFE system

The MIFE™ system, described below, was developed at the University of Tasmania where it has been used to acquire and manage the data in our ion flux studies since 1996 (Newman 2000). The name was first used as an acronym for 'microelectrode ion flux estimation' (Arif & Newman 1993; Henriksen & Spanswick 1993).

The method of calculating dV values from the recorded data is illustrated in Fig. 2, which shows a small portion of the mV recording for a H^+ flux. It is an improvement on what was used by Shabala *et al.* (1997) and has not been described fully before.

Under computer control, the manipulator moved the electrodes to the position close to the tissue surface at the time 60 s (Fig. 2). The electrochemical potential, measured by the H^+ electrode, quickly decreased by about 1 mV equivalent (with an origin that is dependent on the existing solution pH and the electrometer offset, which are included in the subsequent calculation). At 65 s, the manipulator moved the electrodes to the more distant position, and the H^+ electrochemical potential increased again. The cycle was repeated with the 10 s period. The higher electrochemical potential at the more distant position means a higher concentration (and/or V_b) at that position, for the (positive) H^+ ion in this example. The associated diffusive movement of the ion down its electrochemical potential gradient is towards the tissue, i.e. there is a net influx. To calculate the magnitude of this net flux, the electrometer's dV was found as follows for use in Eqn 7.

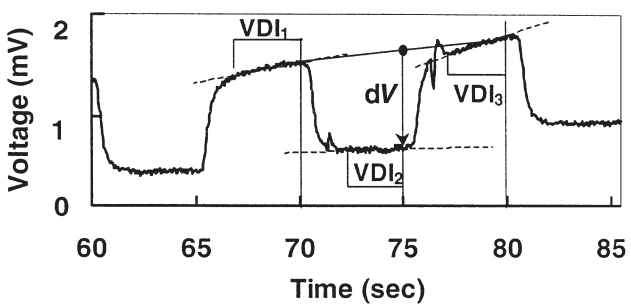

Figure 2. A small subset of voltage data recorded from a H^+ microelectrode that was moved between two positions with a 5 min half period. The move at 60 s was towards the tissue. The data were recorded at 17 samples s^{-1}. The electrode takes about 2 s to settle after a move. There is also a general upward drift of the signal. The dV used in subsequent flux calculations for 75 s is shown related to the end points of the triplet of VDIs.

A valid data interval (VDI) was defined as the time interval (chosen to be 3 s in Fig. 2) for which the measured voltage had become settled, after the electrode movement and up to the start of the following movement. Three consecutive VDIs (VDI_1, VDI_2 and VDI_3 in Fig. 2) were chosen and straight lines (dashed in Fig. 2) fitted to the data in each. The end point values (at 70 and 80 s, respectively) of those lines for VDI_1 and VDI_3 were found and a line was drawn between them. The dV, computed by the software and used in the subsequent flux calculation, was the vertical distance from this line to the end point mV value for VDI_2, as shown in Fig. 2. The dV value was regarded as being at the end time of VDI_2, at 75 s in Fig. 2. For the flux 5 s later, VDI_1 was dropped and the next VDI was added to give the next triplet to be treated similarly. Fluxes were then calculated from dV values obtained by this process, at 5 s intervals, using calibration results and other experimental parameters including buffer concentration (Arif *et al.* 1995) and root geometry (Eqns 7, 8 & 9).

Another system designed for non-invasive ion flux measurement was developed at the National Vibrating Probe Facility at Woods Hole MA in the USA (Kuhtreiber & Jaffe 1990). Its analysis was improved by omitting data points occurring during electrode settling after movement (Kochian *et al.* 1992). It was described and reviewed by Smith (1995), and in detail by Smith *et al.* (1999). From the description, the Woods Hole system appears not normally to store the raw voltage data but only to record the running average flux results obtained from its real-time analysis. That system has been used by visitors at the National Vibrating Probe Facility (Miller 1989; Felle & Hepler 1997; Lew 1998; Lew 1999). Versions are in some other laboratories (Kochian *et al.* 1992; Cardenas *et al.* 1999). Other workers (including J. Dunlop and A. Walker, personal comm.) have developed their own systems, without the automation of the two discussed above (Henriksen & Spanswick 1993; Taylor & Bloom 1998; Plassard *et al.* 1999).

ION FLUXES IN PLANT TISSUES

Plant roots have a wide variety of ion transporters suited to the functions of the different regions of the root (Flowers & Yeo 1992; Marschner 1995). Measurements of electric potential distribution, net electric current paths and pH profiles have been made with the aim of elucidating the function and regulation of the transporters. Localized measurements of specific ion fluxes, with fine time resolution, are now enabling a much tighter functional characterization of the transport systems in different regions than was possible with those less specific techniques.

Most specific ion flux measurements have been made on roots. In assessing the flux studies on ion transport, work done using other plant organs is included here where it is also relevant to mineral nutrient transport by roots. An outline of some non-specific electrical measurements is given first.

Profiles of electric potential, currents and ion concentrations along the root

The electrical activity of plants, as a phenomenon, has attracted interest for over 200 years, with argument over whether electric fields exerted a central control over plant growth and development or were merely an effect of growth. Scott (1967 and references therein) interpreted electric potentials, measurable in the bathing solution around the root, as representing the net electric currents through the root surface, the currents being the electric manifestation of the movement of mineral ions (and possible organic anions).

This idea led to the modern focus on the electrophysiology of ion transport, in which electric fields in membranes and solution affect ion movement, but conversely the movement of ions themselves modifies those electric fields. For example, Watanabe *et al*. (1995 and see references therein) observed negative potential outside *Vigna mungo* L. roots, within 5 mm of the tip. From concurrent microelectrode H^+, K^+ and Cl^- concentration measurements, they interpreted the negative electric potential in terms of diffusion potentials of the ions.

Before ion fluxes could be measured non-invasively, direct measurement of electric currents was a useful approach. Since Jaffe & Nuccitelli (1974) developed the vibrating probe, it has been used in a number of laboratories to measure the normal component of the net electric current at the root surface. Most authors report considerable variability within their current measurements. Ion flux oscillations (Shabala *et al*. 1997; Shabala & Newman 1998) may well be the cause. Variations between authors, on the other hand, even with the same plant material, are largely due to their use of media having different ionic composition.

Miller & Gow (1989) examined 12 diverse species growing at pH 6 and reported that currents consistently entered the growing regions and left from mature regions. They thus supported the general interpretations of the early simple electric potential studies.

In an attempt to identify the ions carrying these electric currents, Weisenseel, Dorn & Jaffe (1979) substituted for one ion at a time in their medium containing K^+, Na^+, Ca^{2+} and Cl^- at approximately pH 5·4. For barley they found current to be inward in the meristematic and elongation regions, outward in the root hair region, and parallel to the surface in between. They assessed that most of the current was carried by H^+. Extending that work using ion substitution and altered pH with corn, Miller (1989) reported strong enhancement of inward currents by low pH. He emphasized the role of H^+ in the currents because a reduction of inward current was caused by 1 μM fusicoccin (a stimulator of the proton extruding ATPase) or by 0·2 mM IAA (also known to acidify the apoplast).

It is difficult to interpret a measured electric current, carried by all the ions in solution, in terms of the membrane transport of one or more of them. With the advent of the ability to measure specific ion fluxes (Lucas & Kochian 1986; Newman *et al*. 1987; Kuhtreiber & Jaffe 1990), and with the growing interest in the functioning and regulation of the transporters themselves, the utility of electric current measurements diminished.

Ryan *et al*. (1990) were the first to report on the general profiles of H^+, K^+ and Ca^{2+} fluxes, from the apex to the mature regions of corn roots in 0·2 mM $CaSO_4$ at approximately pH 6. Fluxes showed great variability in time, and they had poor sensitivity in the absence of digital averaging. In the meristematic and apical elongation regions there was significant net influx of Ca^{2+}, efflux of K^+ (into the low-K^+ solution) and probable H^+ influx. In the basal elongation and mature regions there was significant H^+ efflux, with small, variable K^+ and Ca^{2+} fluxes, thus supporting the general involvement of H^+ in the currents but also a possible role for Ca^{2+} in the growing region. Subsequent flux studies, focussing on the functionality of these and other ion fluxes in very diverse areas of plant physiology, will now be considered. The flux measurements have contributed to knowledge of ion transport and have given information not available from other methods.

Measurements of pH and H^+ fluxes and their interpretation

Hydrogen ions are intimately involved with the biochemistry of the cell. Internal pH is closely regulated by homeostatic mechanisms, including internal buffering and the transport of H^+ through the plasma membrane, to maintain slightly alkaline cytosolic pH. The main H^+ efflux system is the electrogenic proton extruding ATPase, which is the principal determinant of the plasma membrane potential which itself also regulates the ATPase. Many transport systems employ the resulting $\Delta\mu_H$ across the plasma membrane for inward cotransport of other ions or uncharged organic molecules. Net plasma membrane proton flux is the sum of all of these transport processes.

Apoplastic pH is regulated as well, because enzymatic activity there also and wall loosening are pH-dependent. The efflux of respiratory products and metabolically generated organic acids or their anions, depending on their pK and the pH, also contributes to changing the pH of the wall and external medium (Felle 1998; Peters & Felle 1999). Finally H^+ exchanges with other ions in the cell wall's complex Donnan system whose strength is itself pH-dependent (Ryan *et al*. 1992a).

The effect of known buffers in solution can be taken into account in H^+ flux calculations (Arif *et al*. 1995), but not the effect of unknown extrusion of unknown buffers through

the plasma membrane. For all these reasons, an externally measured H^+ flux, particularly over a short time interval, may be different from the plasma membrane H^+ flux. With these complex interactions, it is very difficult to control the relevant variables in investigations. Interpretations are thus fraught with pitfalls. Nevertheless, microelectrode measurements of both H^+ concentrations and fluxes have shone some light onto parts of this complex picture.

Where H^+ fluxes are involved with the transport of other ions, they are discussed in those contexts in subsequent sections.

In unbuffered solution, typically at approximately pH 5·5, roots placed horizontally usually show net H^+ influx, with alkalization of the adjacent medium, in the root cap, meristematic and apical elongation regions (Ryan *et al*. 1990, 1992b; Taylor & Bloom 1998). On average, these and other observers found a net H^+ efflux over the bulk of the elongation zone and the mature and root hair regions. Indeed, Taylor & Bloom (1998) regarded the H^+ efflux in the elongation zone (independent of nitrogen source) as important for elongation growth. In more acid media, at pH 4·4, the net efflux becomes a large and continued influx, whereas in media near neutral pH the efflux region expands (Monshausen, Zieschang & Sievers 1996; Shabala *et al*. 1997; Felle *et al*. 1998). Zieschang, Kohler & Sievers (1993, Fig. 3), however, with measurements spaced at 0·1 mm intervals, give evidence that strong influx close to the meristem may remain at the higher pH.

These fluxes are consistent with the previously found electric current profiles and with the colour change of bromocresol purple (Weisenseel *et al*. 1979, who used it to estimate fluxes; Mulkey & Evans 1981). The pH-dependence of the H^+ fluxes is not surprising. They reflect the different processes taking place in the different regions, some of which have been studied.

In an elegantly designed study of gravitropic mechanisms in vertical *Phleum pratense* roots in unbuffered pH 6 solution, Zieschang *et al*. (1993), showed that H^+ fluxes in the apical 2 mm changed towards efflux, particularly on the top, for some time at least after the root was tilted from vertical. The pH change began at 8 min, which was 9 min before the bending response began. As well as implicating H^+ flux in gravitropism, the study criticises the use of horizontal roots for 'normal' flux studies.

The H^+ pump is affected by osmotic stress in an osmoticum-dependent manner (Reinhold, Seiden & Volokita 1984). Experiments with inhibitors (vanadate, CCCP and TEA) and the presence or absence of K^+ have indicated that the H^+ pump is not directly affected (Shabala, Babourina & Newman 2000). Rather it is modified by membrane potential changes caused by K^+ and Cl^-, whose fluxes were sufficient to provide the osmotic balance.

Although the technique measures the net flux, it was possible to use oscillatory characteristics of H^+ fluxes in the elongation zone of corn to distinguish between operation of the extrusion pump and passive inward transport (Shabala *et al*. 1997). The pump oscillated with about 7 min period, whereas the inward transport systems showed 80 min oscillations. The observed suppression of the 7 min oscillations by 100 mM mannitol, with the H^+ fluxes shifting towards influx, confirmed this assessment (Shabala & Newman 1998). The strong oscillatory behaviour of the pump is characteristic of feedback systems having high gain, as may be expected for homeostasis of actively growing cells.

Measurement of H^+ flux can be used to study other processes that incorporate the ubiquitous H^+ ion. The average efflux, particularly in the elongation zone, is subject to large oscillations that can reach into influx (Shabala *et al*. 1997). Shabala & Newman (1997b) correlated the oscillatory H^+ fluxes with corn root nutations, which took place in decapped roots that did not respond to gravity. A model for an intrinsic oscillator was developed to link H^+ flux, the Ca^{2+} flux oscillation also observed, and auxin-induced growth (Shabala & Newman 1997c). The model predicted the range of nutations they had actually observed, and thus supported Zieschang *et al*. (1993) in arguing that H^+ plays an important role in modulation of hormone action.

The measurement of H^+ fluxes has been applied to several other interesting questions. Because the transporters are located in a membrane, the state of fluidity of the membrane will modulate their functioning. In a study of chilling-tolerant and chilling-sensitive species, Shabala & Newman (1997a) identified critical temperatures and signal transduction times for H^+ fluxes, distinguishing between active efflux and passive influx. The common critical temperature found for both fluxes was close to membrane phase transition temperatures. The species' critical temperatures correlated with the species' chilling tolerance in a way that could be applied in screening for tolerant varieties.

Shabala *et al*. (1998) measured H^+ fluxes simultaneously on opposite sides of 60 μm-diameter protoplasts isolated from corn coleoptiles. They deduced that the plasma membrane H^+ fluxes were a complex mosaic that changed with time, sometimes showing oscillations. This observation gives a warning that stoichiometries, involving H^+ at the transporter level, may be masked when H^+ and other fluxes are viewed at the level of whole tissues.

Potassium fluxes

Potassium is a major mineral nutrient ion and is important for cell turgor. Inward- and outward-rectifying potassium channels are known, and a high affinity active transporter is required for uptake from media with < 0·2 mM K^+. There have been recent reviews (Rodriguez-Navarro 2000; Schachtman 2000).

It is generally found that there is net K^+ efflux from the meristematic and apical elongation regions of the root tip into a low-potassium (< 10 μM) medium (Newman *et al*. 1987; Ryan *et al*. 1990, 1992b). This efflux can raise the K^+ concentration to above 50 μM in the bathing medium. The remainder of the root showed K^+ uptake, which must be active at this concentration.

It was natural that the first published study of ion fluxes in plants should have looked at high affinity potassium uptake (Newman *et al*. 1987). From stoichiometries, a H^+/K^+ symport was thought possible, but the following workers (Kochian, Shaff & Lucas 1989; Ryan *et al*. 1990) could not observe the necessary correlations for that symport. Recent studies (Babourina, Shabala & Newman 2000) have shown the presence of a K^+ uptake mechanism which could not be a K^+/H^+ symport, but was possibly a common Na^+/K^+ transporter of which the known example is HKT1 (Rubio, Gassmann & Schroeder 1995; Schachtman & Liu 1999). With much work at the molecular level and the identification of K^+ transporters (Amtmann, Jelitto & Sanders 1999; Schachtman 2000), K^+ flux studies focused on these transporters are indicated.

The MIFE system has recently been used for K^+ flux measurements simultaneously with pressure probe measurements of cell turgor under hyperosmotic treatment (S. Shabala & R. Lew, personal comm.). Preliminary results indicate that turgor recovery was accompanied by K^+ uptake as had been predicted for osmotic balance (Shabala *et al*. 2000a). A striking distinction has been made by Shabala (2000) between osmotic and ionic components of salt stress perception. He found that mannitol at 150 mM concentration caused K^+ influx (contributing to osmotic balance), which continued during at least 1 h. By contrast, isotonic 90 mM NaCl caused rising K^+ efflux during that time, thereby worsening the important intracellular K^+/Na^+ ratio.

Several studies have interpreted K^+ fluxes as providing electrical balancing (implemented via plasma membrane potential-regulation of K^+ inward- and outward-rectifier channels) when there are large fluxes of Na^+ (Shabala 2000) or Cl^- (Felle *et al*. 1998; Shabala *et al*. 2000a).

There is now a great opportunity for K^+ flux measurements to contribute to characterizing the function of known K^+ transport proteins in different tissues in which they are expressed.

Calcium fluxes

Calcium is perhaps the most interesting of the mineral nutrients. It is required in high concentration in cell walls, where it functions in pH-regulated wall stiffening and participates in other ion exchange in the Donnan system of the wall (Ryan *et al*. 1992a; Arif & Newman 1993).

By contrast, the Ca^{2+} concentration in the cytosol must be kept very low, a factor of 10^4 below its concentration outside the cell. Coupled with the plasma membrane potential difference acting on this divalent ion, this means that there is an enormous inward force on Ca^{2+}, with $\Delta\mu_{Ca} \cong -50$ kJ mol^{-1} (Sanders, Brownlee & Harper 1999). Because of this force, a single Ca^{2+} channel can allow a substantial Ca^{2+} influx as an early part of a localized signal transduction pathway from an external receptor to the cellular response. Thus, plants use Ca^{2+} widely for signalling purposes.

One signalling system studied is the transduction of the Nod factor signal in root hairs. Herrmann & Felle (1995), measuring ion activities rather than fluxes directly, found that Ca^{2+} fluxes occur in the tips only of *Sinapis alba* hairs. From closely comparing the timing of ion flux and membrane potential changes, Felle *et al*. (1998) observed Ca^{2+} influx several seconds before the H^+ pump was affected. They cogently argued that Ca^{2+} uptake played a primary role in Nod signal transduction and triggered an anion channel giving Cl^- release and depolarization. This work was an effective use of a multi-ion capability, even though it simply used concentration changes at a fixed position to infer the presence and timing of ion fluxes without their quantitative measurement.

Cardenas *et al*. (1999) identified a reversible doubling, to 280 nmol m^{-2} s^{-1}, of Ca^{2+} influx at the growing hair apex, and an increase in intracellular free Ca^{2+}, within 5 min of the application of Nod factors to *Phaseolus vulgaris* seedlings in 0·05 mM $CaCl_2$, 2·5 mM MES at pH 6·2. By its timing and location, the Ca^{2+} influx was clearly linked to the subsequent deformation of the hair cell in its response to the Nod factor.

This kind of study has demonstrated nicely that ion-selective microelectrode measurement of Ca^{2+} fluxes outside the cell helps to decide on the source of the rise in cytosolic Ca^{2+}, which was observed through the use of calcium-sensitive internal dye (fura-2-dextran) (Felle & Hepler 1997; Cardenas *et al*. 1999). Thus for Ca^{2+} studies, ion-selective microelectrode flux measurements can be effectively teamed up with the use of dyes and their imaging.

At the root tip, tracer studies had showed Ca^{2+} influx in the apical 0·5 mm of corn roots (Ferguson & Clarkson 1975). Ryan *et al*. (1990) reported large and significant Ca^{2+} influx at 1 and 2 mm from the root tip in 0·2 mM $CaSO_4$, and yet Ryan *et al*. (1992b) found only small and variable Ca^{2+} influx in wheat root tips in 1 mM $CaCl_2$ at pH 5. The endodermis restricts apoplasmic solute movement over much of the root (Gierth, Stelzer & Lehmann 1998; Kuhn, Schroder & Bauch 2000, for example). For calcium, which is required in quite large quantities by the growing plant, its uptake at the root apex means that its transport through most of the root symplast is able to be small, allowing its cytoplasmic concentration to be kept low. Indeed, whole cell patched Ca^{2+} currents for the tip region of *Arabidopsis thaliana* roots were identified by Kiegle *et al*. (2000), who suggested that they related to constitutive uptake of Ca^{2+} for cell division and elongation.

Using microelectrodes to measure Ca^{2+} activity at the surface of maize root tips, Bjorkman & Cleland (1991) found a calcium gradient across the gravistimulated tip. This appeared to have a signalling role as well as being linked to wall loosening. It would have been interesting had Zieschang *et al*. (1993) extended their detailed H^+ flux study to Ca^{2+} as well.

Not surprisingly, calcium fluxes have been implicated in plant responses to various environmental stresses. Halperin, Kochian & Lynch (1997) found that the Ca^{2+} uptake in the tip region of barley (but not elsewhere) was reduced by salinity. Huang *et al*. (1992a) found Al^{3+} inhibited Ca^{2+} uptake in this region for an aluminium-sensitive

wheat cultivar, but not for an aluminium-tolerant one. Related studies have been by Huang *et al*. (1992b) and Ryan & Kochian (1993).

In plant cell walls, Ca^{2+} and H^+ have interacting roles and, with other ions, contribute to the Donnan system of the wall. Because of H^+/Ca^{2+} exchange in the wall, a membrane flux of one ion affects the measured external flux of the other. Ryan *et al*. (1992a) and Arif & Newman (1993) have modelled Ca^{2+}/H^+ exchange in cell walls on the basis of ion flux measurements. To interpret the Ca^{2+} efflux induced by 90 mM NaCl treatment *et al*. (Shabala 2000a; Shabala & Newman 2000), we have applied this model to show that the transient Ca^{2+} flux (and the initial transients in H^+ and K^+ fluxes) was due to initial exchange with the external Na^+ and not to membrane transport of Ca^{2+}.

Other issues, in addition to wall exchange and plasma membrane signal transduction, have been considered through the observation of Ca^{2+} fluxes. Hush, Newman & Overall (1992) found that a Ca^{2+} influx (with a minor H^+ influx, a large K^+ efflux and substantial unknown other fluxes) contributed to wound-induced inward electric currents, suggested to be part of a healing response around pea roots (Hush & Overall 1989), or maize roots (Meyer & Weisenseel 1997).

The time courses of H^+, K^+, Ca^{2+} and Cl^- fluxes at the mesophyll and epidermis of *Vicia faba* leaves were observed after illumination (Shabala & Newman 1999). Influx of Ca^{2+} was associated in time with membrane depolarization whereas H^+, K^+ and Cl^- flux changes occurred later. This clearly identified entry of Ca^{2+} as the main depolarizing agent in the membrane potential responses to light, in distinction from Nod factor-induced depolarization (Felle *et al*. 1998).

Ammonium and nitrate fluxes

Nitrogen nutrition has been the subject of attention for ion flux measurement because both NH_4^+ and NO_3^- have LIX available. The interest is in the plant's choice of nitrogen source and the H^+-stoichiometry of the high affinity transporters.

The general uptake patterns of NH_4^+ or NO_3^- over the apical 50 mm of barley roots were measured by Henriksen *et al*. (1992) in unbuffered low salt medium with added NH_4^+ or NO_3^-. They found time-variability and no particular trend for either ion with position in what was largely the mature region. With better spatial resolution, Colmer & Bloom (1998) found a difference between the mature region and the growing (meristematic and elongation) region of (induced) maize roots for NH_4^+ and NO_3^- fluxes. For rice adventitious roots, they found a decrease in uptake rates of both NH_4^+ and NO_3^- at the basal region of the roots containing sclerenchymatous fibres, which they considered might restrict nutrient uptake compared with the growing regions.

Plassard *et al*. (1999) report H^+ flux profiles along the apical 90 mm of corn roots in 1 mM NH_4^+ or 4 mM NO_3^- at pH 6·5. Despite much variability between roots, H^+ fluxes were outward in NH_4^+ but in NO_3^- they were inward at the growing region and were mixed in the mature region. Videodensitometry showed systematic alkalization in the apical 20 mm with NO_3^- treatment, but nitrogen transport mechanisms were not discussed.

Garnett (personal comm.) observed uniform but time-variable uptake by the mature region of NH_4NO_3-pre-treated *Eucalyptus nitens* roots of both NH_4^+ and NO_3^- from 0·1 mM NH_4NO_3 solution. The $NH_4^+ : NO_3^- : H^+$ stoichiometries were 3 : 1 : 6. These could not readily be interpreted in the absence of measurements of the individual nitrogen ions alone or of other ions.

When the ions are present together for barley roots (Henriksen *et al*. 1990), in the first nitrogen microelectrode flux study, it appears that NO_3^- flux, but not NH_4^+ flux is diminished compared with the controls without the other ion. Where direct comparisons have been made, the consensus of the above studies is that NH_4^+ net influx is greater than the net influx of NO_3^- from equimolar solutions, and the presence of NH_4^+ tends to inhibit the uptake of NO_3^- (Taylor & Bloom 1998).

Although there is a constitutive uptake system (Glass, Shaff & Kochian 1992), improved influx is inducible by pretreatment with NO_3^-. Henriksen & Spanswick (1993), with barley roots, found that only NO_3^- pretreatment could induce NO_3^- uptake from 0·1 mM $Ca(NO_3)_2$.

The influx of the negative NO_3^- requires secondary active transport into the negative cytosol under normal conditions. It was natural to look for H^+ cotransport and H^+ has been well implicated in NO_3^- uptake (McClure *et al*. 1990; Plassard *et al*. 1999 and references therein). Glass *et al*. (1992), for un-induced barley in 0·2 mM $CaSO_4$ with NO_3^-, related H^+ fluxes to the membrane potential and to previously observed (Siddiqi *et al*. 1990) kinetics of $^{13}NO_3^-$ uptake. They suggested that NO_3^- is taken up in both low and high affinity transport via 2 : 1 H^+/NO_3^- symports.

The problems of N uptake are complex and there are a number of transport systems. Perhaps they can be sorted out by flux studies on suitable mutants. All the studies mentioned have reported time variability in the fluxes, as is also observed for K^+ fluxes. Is variability at the cell level an inherent property of ion transport systems?

Chloride fluxes

The major mineral anion Cl^- has a high electrochemical potential inside cells, so its efflux is passive and suitable anion channels are known. Uptake of Cl^- requires an active, most likely secondary active, mechanism and symport with H^+ has been proposed (Felle 1994).

Relatively few studies of Cl^- fluxes using ion-selective microelectrodes have been made, despite its importance, perhaps because a satisfactory Cl^- LIX was developed later than for the other major ions. In the earliest study on Cl^- fluxes (Ryan *et al*. 1992b), significant Cl^- efflux, contribut-

ing to the net inward electric current, was observed from the meristematic region of wheat roots into 0·1 mM $CaCl_2$ at pH 4·5.

In their studies on Nod factor signalling in *Medicago sativa* root hairs, Felle *et al*. (1998) argued that the calcium-stimulated Cl^- release they observed was the direct cause of the observed depolarization of the membrane potential. Because they used fixed electrodes, they could not observe the subsequent time course of the fluxes.

Concerning the Cl^- influx transport system, Felle (1994) observed membrane potential, cytosolic H^+ and Cl^- responses to a rise in external Cl^-, and found that Cl^- transport depended on the plasma membrane pH gradient. He proposed an $nH^+ : 1\ Cl^-$ symporter, with $n > 1$.

Pharmawati *et al*. (1999) suggested a complex role in plant nutrient homeostasis for natriuretic peptides, possibly including an effect on a H^+/Cl^- symport.

Observations of Cl^- influx associated with auxin (NAA) action on oat coleoptiles (Babourina, Shabala & Newman 1998) led to a model of net uptake in terms of combined activity of a pH-sensitive influx system and Cl^- channels for efflux (Babourina, Knowles & Newman 1998).

Shabala *et al*. (2000a) affirmed the function of Cl^- uptake, with the associated membrane hyperpolarization, in osmoregulation of broad bean leaf mesophyll. Average H^+/Cl^- stoichiometry for H^+ and Cl^- flux changes was – 1·7. This is opposite to the positive correlation expected for a symport, but may result from a coupling of other H^+ transporters to the symporter via the membrane potential. On the other hand, could there be a different kind of Cl^- transporter?

The physiological search for the Cl^- influx transporter is proving difficult. Advances may come from characterization of, as yet unidentified, transporter molecules.

Fluxes of other ions, and other applications of the technique

Some work has been done to measure Na^+ fluxes (Babourina *et al*. 2000a; Shabala 2000) in the context of salt stress. Massive but decreasing (half time, 4–10 min) Na^+ influx followed application of 20–120 mM NaCl (Babourina *et al*. 2000a). Significantly, within 20 min a (K^+-dependent) net Na^+ efflux began. The interesting question for the future may be on the nature and regulation of the plasma membrane Na^+ export system – possibly a Na^+/H^+ exchanger.

Ca^{2+}, H^+ and other fluxes in the elongation region of roots show fascinating oscillations, rich in information (Shabala & Newman 1997b; Shabala *et al*. 1997; Shabala & Newman 1998). These should be the subjects of a separate review.

Flux profiles of H^+, K^+ and Ca^{2+} around germinating wheat seeds suggested that plasma membrane transporters are active in seed germination (Shabala *et al*. 2000b).

Ion-selective microelectrodes have also been used to observe time courses of H^+ and Ca^{2+} fluxes from bacterial biofilms (L. Shabala personal comm.). There was a peak in net H^+ extrusion during exponential growth, with little membrane transport of Ca^{2+}.

Phosphate is the remaining major mineral ion for which a commercial LIX is not yet available.

PROSPECTS FOR ION FLUX MEASUREMENTS IN THE FUTURE

The electrically based studies on mineral ion acquisition by plant roots, reviewed here, have been largely phenomenological in nature, and using them alone it is hard to identify specific transport systems. During its 15 years, the ion flux technique has been refined in precision (time and space), in range of ionophores available, in reliability and recognition of its limitations. It can now be used effectively in conjunction with other techniques in cell and molecular biology. It will have a particular utility in characterization of active transporters – pumps and cotransporters – for which patch clamping is less suited than for channels.

Progress in molecular genetics is allowing identification, cloning and controlled expression of transporter molecules. In some cases, the transporter has been characterized at the molecular level by expression in yeast, oocytes or some other system. However this characterization is still at the electrical currents level and is not *in planta*. Application of a combination of other techniques will be necessary to show the function, regulation and other interactions of the transporters in the growing plant. These techniques include patch clamp, membrane potential measurement, the use of ion-selective microelectrodes and the imaging of suitable dyes and other markers.

In this context the functional genomics of transporters can be facilitated by measurements of multiple specific ion fluxes, made with the time and space resolution provided by the microelectrode technique. In particular, the MIFE system's multi-ion capability and versatility may be particularly effective. With the emerging knowledge of the *Arabidopsis* genome, the ion flux characteristics of mutants, with or without a particular transporter, can be compared in order to screen, identify or confirm the transporter's function *in planta*. For studies on the transporters controlling nutrient acquisition by roots, this application may be a particularly useful one.

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