PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

# Chloride Transport in Stomatal Guard Cells

E. A. C. MacRobbie

*Phil. Trans. R. Soc. Lond. B* 1982 **299**, 469-481
doi: 10.1098/rstb.1982.0145

*Phil. Trans. R. Soc. Lond.* B **299**, 469–481 (1982)
*Printed in Great Britain*



# Chloride transport in stomatal guard cells

By E. A. C. MacRobbie
*Botany School, Downing Street, Cambridge CB2 3EA, U.K.*

Stomatal guard cells regulate the size of the stomatal pore by the changes in their shape and volume, which are associated with changes in their turgor. Accumulation of potassium salts plays a major role in this process, and frequently chloride, if available, provides the major balancing anion. Measurements have been made of two-way ion fluxes in guard cells, in epidermal strips of *Commelina communis* L., after treatment at low pH to kill all cells except the guard cells. In such material, opening depends on the ion concentration in the bathing solution, and for this purpose the three salts KCl, KBr and RbCl seem to be equivalent. $^{82}Br^-$ and $^{86}Rb^+$ fluxes have been measured in a range of steady states, with different apertures, and in the transitions between one steady state and another. Analysis of the kinetics of tracer efflux in the steady states allows calculation of the cytoplasmic and vacuolar contents, and their changes with aperture, with wider opening produced by increasing concentration in the bathing solution, or by light incubation compared with dark incubation. The results show that the increases with aperture of the cytoplasmic salt concentration are comparable with the osmotic changes required, but the changes in vacuolar concentration are much less than those required osmotically. Opening must therefore be associated with vacuolar accumulation not only of salt, but also of some other solute. The decrease in aperture on addition of $2 \times 10^{-5}$ M abscisic acid to the solution bathing 'isolated' guard cells, or on their transfer from light to dark, is achieved by marked transient increases in ion efflux, with little change in influx. There are also changes in tonoplast fluxes. The aperture is determined by the level of vacuolar solute accumulation, and thus the results show that this responds to environmental signals by control of plasmalemma efflux rather than influx, and by control of tonoplast fluxes. The ability to transfer salt from cytoplasm to vacuole may be critical for the maintenance of turgor and aperture.

## Introduction

The main function of active chloride transport in plant cells is the net salt accumulation responsible for the high cell turgor, involving the creation and maintenance of a large vacuolar volume, distinct from the metabolically active compartments in the cytoplasm. In mature plant cells the vacuole generally occupies the major fraction of the cell volume (90–95%, say), and the formation of a large central vacuole is one of the most striking features of the differentiation of the mature cell from its meristematic precursor, containing only few, small vacuoles. There are, however, a few cells in which the multivacuolate condition is normal in maturity, and in which marked reversible changes in the extent and state of vacuolation are a normal feature of the cells' activities. Such cells include various motor cells and stomatal guard cells, and it may be that a study of ion transport processes in such systems can help to throw light on the processes of salt accumulation, and particularly vacuolar accumulation, in the normal course of development. The most accessible of such systems for experiment is provided by stomatal guard cells.

The ability to limit water loss from the leaves, in spite of the requirement for access of $CO_2$ to the photosynthetic tissue, is essential for terrestrial plants. The conflict of requirements is

resolved by the control of gas exchange through variable pores in the leaf epidermis, whose apertures are sensitive to environmental conditions and are under metabolic control. The regulation of the aperture is provided by the shape changes associated with swelling and shrinking of a pair of stomatal guard cells as a result of osmotic adjustments in these cells or their neighbours, or both. When the turgor of the guard cells is low the pore is closed, but as the turgor increases, as the cells swell, differential thickening of the various guard cell walls produces a curvature in the cell, separating the ventral walls of the pair, opening a pore between them. The key to the control lies in the ability of stomatal guard cells to regulate their solute content in response to environmental signals, provoking osmotic flows of water into or out of the cells, and it is recognized that movements of potassium salts play a major role in this process. From the evidence available, the processes involved in guard cells are likely to be the same as are involved in salt accumulation as a general activity of plant cells, but there are two striking peculiarities of the guard-cell pattern. The first peculiarity is that already mentioned, the ability to switch salt accumulation on and off in response to environmental signals. In the intact leaf in normal conditions the most important of such signals are light and $CO_2$, and in stress conditions the synthesis and redistribution (between cells or within the cell) of abscisic acid (ABA) play an important role. It is important to establish how such control is achieved, and to identify the particular ion fluxes that change, producing the changes in the steady-state levels of salt accumulation, and hence in turgor and aperture.

The second peculiarity of guard cells is the very high level of salt accumulation achieved in open guard cells; the results from the use of different methods, in different plants, suggest that $[K^+]$ increases from about 80 mM in closed guard cells to about 385–880 mM in open guard cells (for detailed figures and references see review by MacRobbie (1981*a*)). Thus, the level of accumulation is much greater than that achieved by more typical cells, whose regulatory mechanisms operate to limit turgor increases well before this level; this is often achieved by inhibition of active influxes as turgor increases.

This paper will first consider processes involved in salt accumulation in plant cells in general, and the evidence for the existence of similar transport processes in guard cells. It will finally present some flux measurement in guard cells aimed at elucidating the nature of the controls on salt accumulation in these cells.

## Ion transport in plant cells

The result of the patterns of active ion transport identified in plant cells is twofold: the maintenance of high osmotic pressure in both cytoplasm and vacuole, allowing the generation of high cell turgor, and the maintenance of cytoplasmic conditions suitable for metabolism and for its control. The essential cytoplasmic features are high K/Na and pH, very low free $Ca^{2+}$, and adequate cytoplasmic osmotic pressure, yet at cytoplasmic ion levels (say 100–200 mM) low enough to avoid inhibition of enzyme reactions, including protein synthesis. (See Wyn-Jones *et al.* (1979) for a discussion of the importance of the cytoplasmic ionic composition in this respect.) In contrast, the osmotic requirements of the vacuolar composition are not complicated by requirements for, or prohibitions on, particular solutes. The typical pattern of active ion transport by which this is achieved involves active excretion of $H^+$, $Na^+$ and $Ca^{2+}$ from the cytoplasm, both to the outside solution and to the vacuole, but active inward transport of chloride at both membranes, from solution to cytoplasm and from cytoplasm to vacuole. (These




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

are processes identified as active by the creation or maintenance of non-equilibrium ion distributions, without separation into primary and secondary active transport.) The cytoplasmic conditions set up are similar to those in animal cells (though not achieved by the same transport processes), and the essential difference lies in the additional generation of a large internal solute compartment in the vacuole.

If chloride is available in the external medium the commonest form of salt accumulation is of chloride, balanced mainly by potassium but with a variable, smaller, proportion of sodium ions. In the absence of external chloride the accumulated potassium is balanced instead by an internally synthesized acid anion, most commonly malate. This represents an expensive diversion of carbon compounds, potential respiratory substrate and synthetic precursor material, to the synthesis of inert vacuolar osmoticum, and is usually suppressed if chloride is available in adequate amounts. It is now generally agreed that both forms of salt accumulation involve primary active transport of protons out of the cytoplasm at the plasmalemma, generating gradients of pH and of electric potential at that membrane, which provide driving forces for processes of secondary active transport. One diagnostic feature of processes secondarily dependent on the primary proton pump is stimulation by the fungal toxin fusiccocin, which produces a marked stimulation of active proton extrusion in a wide range of higher plant cells. (For the effects of fusiccocin see the review by Marré (1979), and for a review of the proton pump and its function in plant cells see Smith & Raven (1979) and Spanswick (1981).) On this basis the view has developed that chloride uptake at the plasmalemma may be achieved by co-transport with protons, with the driving force provided by the activity of the primary proton-translocating ATPase in that membrane.

However, it should be stressed that the overall process of salt accumulation involves two distinct parts: first the sequence of events leading to the entry of $K^+$ and $Cl^-$ into the cytoplasm (or the entry of $K^+$ and the synthesis of malate), and second the transfer of $K^+$ and its balancing anion from cytoplasm to vacuole. There is evidence of feedback inhibition in both forms of salt accumulation, and hence the transfer of ions out of the cytoplasm into the vacuole is an essential prerequisite for continued entry or synthesis or both. It is therefore important to identify also the nature of the transport processes from cytoplasm to vacuole.

### *Chloride transport in characean cells*

The giant algal cells offer an experimental system in which transport processes are accessible for study, and the only really detailed description of the process of chloride transport at the plasmalemma of a plant cell has emerged from the work of Sanders (1980*a*, *b*, *c*; Sanders & Hansen 1981). This comes in part from the study of intact cells, but also from the use of tonoplast-less perfused cells, developing methods originally introduced by Japanese workers (Tazawa *et al.* 1976).

Earlier work on the sensitivity of $Cl^-$ influx to external pH, in intact cells, led to the suggestion that a co-transport with $H^+$ might be involved (Spear *et al.* 1969; Smith 1970). Measurements of the electrochemical gradient for protons showed that, if this were so, a stoichiometry of $2H^+$:1 $Cl^-$ would be required on energetic grounds (Smith & Walker 1976). Evidence for such stoichiometry is provided by observations of the electrical effects of initiating a high rate of chloride uptake, by adding chloride to the solution bathing chloride-starved cells; thus Sanders (1980*b*) found a depolarization by some 10 mV on this change, and Beilby & Walker (1981) found a positive inward current in voltage-clamped cells in response to the addition.




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Strong support for the model of chloride influx by co-transport of $2H^+$ with each $Cl^-$ is given by the results of the detailed kinetic analysis by Sanders. His work shows that chloride influx is sensitive to chloride concentration and pH, both inside and outside the membrane. The influx shows Michaelis–Menten kinetics with respect to $[Cl^-]_o$, the external chloride concentration, but $V_{max}$ is reduced by increasing $[Cl^-]_i$, the internal chloride concentration, or reducing $pH_i$. $K_m$ is not affected. The influx is particularly sensitive to internal pH, with a 10–20-fold inhibition by the reduction of $pH_i$ by 0.75 unit, from 7.75 to 7.0. Sanders & Hansen (1981) have shown that these kinetic properties can be accounted for, quantitatively, by a model of a transport system in which $2H^+$ and 1 $Cl^-$ bind to a recycling carrier; the properties observed demand that chloride is first on, and first off, the carrier, and that the translocation of the loaded carrier, rather than the return of the unloaded carrier, must be the charge-carrying flux.

There is therefore a reasonable model for the process of chloride influx at the plasmalemma, though no isolation or characterization of the protein concerned has been done.

There is very little information on the nature of chloride transport from cytoplasm to vacuole. The process is active, but the nature of the energy supply is unknown. A proton co-transport is not a feasible mechanism, as the proton gradient is wrongly directed for such a system, with the vacuole some 3 pH more acid, and some 10–15 mV positive, with respect to the cytoplasm. The kinetics of the transfer of tracer chloride to the vacuole have some odd features (MacRobbie 1975, 1977*b*). Two components of vacuolar transfer contribute to the measured tracer kinetics. It seems likely that the fast component represents the transfer of tracer to the vacuole associated with action potentials on cutting the cell for analysis, and the slow component represents the 'true' transfer to the vacuole. Two properties of the slow component have been established, but remain to be explained. The first is that there is no discrimination between bromide and chloride in the vacuolar transfer, although the active influx at the plasmalemma can discriminate between the two; such total inability to distinguish between the two ions is unexpected behaviour for an active transport, if single-ion transfer is involved. The second odd feature is the apparent link between the rate of transfer from cytoplasm to vacuole and the rate of entry at the plasmalemma. In view of Sanders's (1980*b*) demonstration that high cytoplasmic chloride is associated with a low rate of uptake at the plasmalemma, this correlation is the more striking. It was suggested that the kinetics observed may be more consistent with the delivery of salt-filled vesicles to the main vacuole, in a process of creation and fusion of new vacuoles, than with a single-ion carrier process at the tonoplast, but the nature of the transport remains unknown. An understanding of tonoplast transport processes must be one of the main aims of future work.

## Ion accumulation in guard cells

The general pattern of salt accumulation in stomatal guard cells seems similar to that in plant cells in general. Potassium uptake is balanced by chloride, or by malate, or by a mixture of the two, depending on the species and the conditions. (See reviews by Raschke (1975) and MacRobbie (1977*a*, 1981*a*).) In maize and onion, chloride accumulation is responsible for opening and closing, rather than malate synthesis (Raschke & Fellows 1971; Schnabl & Zeigler 1977).

Proton extrusion associated with stomatal opening was demonstrated by Raschke & Humble (1973), in epidermal strips of *Vicia faba* floating on solution, and it has been argued (Raschke 1977; Zeiger *et al.* 1978) that proton extrusion is the primary process, with potassium




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

uptake, chloride uptake and/or malate synthesis as secondary consequences of the pH and electrical gradients generated by the proton pump. The stimulation of stomatal opening by fusicoccin (Turner & Graniti 1970; Squire & Mansfield 1972) lends support to this view. It is, however, essential to measure ion fluxes, as distinct from final levels of accumulation, and to identify the nature of the flux changes responsible for changes in net accumulation in different conditions. With this aim, attempts have been made to measure two-way ion fluxes into and out of guard cells in different conditions, by using a preparation of epidermis in which only guard cells are alive (MacRobbie 1981*b*, *c*). The guard cells make up only 2–3 % of the volume of the epidermis, and in the intact leaf the extracellular ion concentrations in the guard cell walls (whether those bordering the neighbouring cells or those bordering the sub-stomatal air cavity) are neither defined nor under experimental control. It is therefore necessary to start with a defined system, such as this 'isolated' guard cell preparation, if some understanding of the transport processes is to be established.

## Flux measurements in guard cells

The plant chosen is *Commelina communis* L., in which a treatment of epidermal strips at low pH (3.9) kills all cells other than guard cells, but leaves the guard cells alive, capable of opening, and of responding appropriately to the most important of the normal controls, namely light, $CO_2$ and ABA. The guard cells in such isolated epidermal strips will open in light, when floated on solutions of KCl, RbCl or KBr, to an extent that depends on the concentration, and these three salts are, for this purpose, equivalent. For this reason the tracer measurements have been made with $^{86}$RbCl or with K$^{82}$Br, on grounds of the cost, and specific activity, of the isotopes; $^{86}Rb^+$ or $^{82}Br^-$ are used as analogues for $K^+$ or $Cl^-$, not as tracers, but it is argued that the conclusions apply to any of these salts. Under these conditions $K^+(Rb^+)$ uptake appears to be balanced by an equivalent amount of $Cl^-(Br^-)$, as might be expected, with no contribution from malate synthesis. The relation between steady-state ion content and steady-state aperture was established by measuring the tracer contents of the tissue after it had been floated overnight on labelled solutions (MacRobbie & Lettau 1980*a*). Essentially the same linear relation between aperture and tracer content was found for $^{82}$Br and for $^{86}$Rb, with slopes in the range 6.6–14.8 pmol mm$^{-2}$ µm$^{-1}$. The values for tracer content were combined with estimates of guard cell volumes, to establish the relation between ion concentration and aperture. The results suggested concentrations of 20–56 mM at 5 µm aperture, rising to 174–167 mM at 15 µm aperture; this also agreed well with the estimate of potassium concentrations in 'isolated' guard cells made with a $K^+$-sensitive electrode, which gave 51 mM rising to 157 mM over the same range. (It should be noted that such 'isolated' guard cells, in the absence of any subsidiary cell turgor opposing the swelling of the guard cell, can open much wider than intact guard cells of the same potassium content (MacRobbie & Lettau 1980*b*).)

These results establish that opening in 'isolated' guard cells is associated with large accumulations of $K^+(Rb^+)$ $Cl^-(Br^-)$, and that various steady-state apertures can be set up in different conditions, allowing flux measurements in these steady states, or in the transitions between one steady state and another. The results do, however, show that the ion changes are much less than the total solute changes that are required osmotically. The osmotic changes required may be estimated from the aperture changes as sucrose is added to the bathing solution (MacRobbie 1980). In the range 7–13 µm the salt changes can account for only about 20 mosmol kg$^{-1}$ of




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

the 74–121 mosmol $kg^{-1}$ $\mu m^{-1}$ required. Thus, some other solute must also be involved; in view of Outlaw's demonstration that sugars, as well as potassium salts, contribute to the osmotic increases as stomata open in *Vicia faba* (Outlaw & Lowry 1977; Outlaw & Kennedy 1978; Outlaw & Manchester 1979), the most likely source is the conversion of starch to sugars, and their vacuolar accumulation.

### *Steady-state fluxes of* $Cl^-(Br^-)$

For a tissue in a steady state, represented by two phases, vacuole ar ı cytoplasm, in series with one another, kinetic analysis of the time-course of tracer efflux fron the tissue can be used to estimate fluxes at both plasmalemma and tonoplast, and cytoplasmic nd vacuolar contents. The tissue is labelled to a steady state, and the rate of tracer washout tı successive portions of inactive solution is then followed. During the washout of tracer, both tracer content ($Q^*$) and rate of loss of tracer ($R$) will be represented by a sum of exponential terms, the number of such terms being equal to the number of kinetically distinguishable compartments in the tissue. After the initial washout of tracer from the free space, tracer exchange can be frequently be fitted to the sum of two exponential terms, and it is then assumed that the tissue can reasonably be represented as cytoplasm and vacuole in series with one another. This method has been used to investigate $^{82}Br^-$ distribution with guard cells opened to varying extents by being floated on $K^{82}Br$. The method is described by MacRobbie (1981 *b*), and has since been extended.

The efflux from such a two-compartment system can be described by the equations for the tracer, $Q^*$, and the rate of loss, $R$:

$$Q = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad \text{and} \quad R = k_1 A_1 e^{-k_1 t} + k_2 A_2 e^{-k_2 t}.$$

An apparent rate constant for exchange, $\bar{k} = R/Q$, may also be calculated; $\bar{k}$ will fall with time until the slow phase is reached, when it will equal $k_2$. The values for best fit of $Q^*$ to two exponentials can be determined by minimizing the squares of deviations from the fitted curve, by using trial values of the parameters. In the guard cells in such an experiment, the internal specific activity at the start of the efflux is equal to that outside, and the quantities $A_1$, $A_2$, $k_1$ and $k_2$ can then be used to estimate the total tissue content $Q_t$, the cytoplasmic and vacuolar ion contents, $Q_c$ and $Q_v$, and the plasmalemma and tonoplast fluxes, $\phi_p$ and $\phi_t$ (equal in each direction, since the analysis is only valid for a tissue in the steady state, a condition that must be justified by measurement). (See MacRobbie (1981 *b*) for details of the analysis.)

The method has been used to look at changes in cytoplasmic and vacuolar content associated with different apertures, produced by floating overnight on different concentrations of KBr (between 20 and 60 mM) in light. The form of the efflux curves can be seen in the control curves in figure 1, in which ln $Q^*$ is plotted against time, and in figures 2 and 3, in which the mean rate constant for exchange, $\bar{k}$, is plotted against time. In strips bathed in 20–60 mM, both $Q_c$ and $Q_v$ increased with aperture. The regressions of $Q_c$ and $Q_v$ on aperture were calculated, for the 12 strips used, and the relations were: $Q_c = -68.5 + 10.3A$ ($r = 0.922$), and $Q_v = 21.5 + 5.9A$ ($r = 0.906$). These relations were used to estimate concentration changes over the aperture range 7–15 μm. If the cytoplasm is about 30% of the total cell volume, and the vacuole 70%, then the cytoplasmic concentration rises by about 46 mM $\mu m^{-1}$, whereas the vacuolar concentration rises by only about 11 mM $\mu m^{-1}$. The osmotic change required, averaged over the range 7–15 μm is about 122 mosmol $kg^{-1}$ $\mu m^{-1}$. The results therefore suggest that the increased ion concentrations ($K^+ + Br^-$) in the cytoplasm can produce an osmotic change of the




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

order required, but the vacuolar salt changes are much too small. This is unexpected, and the opposite of what might have been predicted. It has been argued that when many plant cells increase their salt content at high external osmotic pressure, maintaining their turgor constant, they do so by accumulating salt in their vacuole, but by accumulating a compatible solute such as betaine, or proline, in their cytoplasm, rather than by allowing the cytoplasmic salt content to rise (Wyn-Jones *et al.* 1977); evidence for such regulation is provided by recent measurements of the distribution of salts and betaine in beet tissue from high and low salt environments. (Leigh *et al.* 1981). But the conclusion from the guard-cell experiments is clear; the vacuole must be identified with the slowly exchanging compartment, and this changes relatively little as the stomata open in these conditions.

The results of a further experiment, in which the $^{82}Br^-$ content and distribution was compared in guard cells incubated in 30 mM KBr in light and in dark, lead to a similar conclusion. After overnight incubation in light the aperture was 12–18 μm, whereas in the dark it was only 5–8 μm. The relation between total $^{82}Br^-$ content ($Q_t$ in picomoles per square millimetre) aperture ($A$, in micrometres) was fitted by the regression $Q_t = -23.6 + 9.3A$ ($r = 0.996$, 10 d.f.). The efflux curves were again fitted to two exponentials, and values for $Q_c$ and $Q_v$ calculated; the fitted regressions were $Q_c = -2.6 + 4.4A$ ($r = 0.877$, 10 d.f.) and $Q_v = -21.0 + 4.9A$ ($r = 0.912$, 10 d.f.). For the aperture range between 6 μm in the dark and 13 μm in the light, and a cytoplasmic volume of 30% of the cell, these figures give only about 15 mM $\mu m^{-1}$ for the cytoplasmic increase, and 8 mM $\mu m^{-1}$ for the vacuolar increase. Neither of these figures is as large as the estimate of that required osmotically, but the discrepancy in the vacuole is particularly marked.

The results suggest that in 'isolated' guard cells, opening while floating on KBr, there is accumulation of KBr associated with opening, but that the vacuolar accumulation of some other solute is also involved. The implication is that the transfer from cytoplasm to vacuole of both salt and at least one other solute is stimulated as the cytoplasmic ion concentration increases. This is an important conclusion, even if the nature of the other solute is not yet identified, nor is the mechanism of the stimulation understood.

Measurements with $K^+$-sensitive electrodes in 'intact' guard cells, in epidermal strips in which all cells are still alive, show that in the early stages of opening there is a similar discrepancy between the measured potassium changes and the total osmotic requirement for guard-cell opening (MacRobbie & Lettau 1981*b*). It seems likely that the same range of processes are involved in the experimental system of 'isolated' guard cells as are concerned in the intact leaf. It may well be that higher levels of cytoplasmic halide are achieved in guard cells opened by floating on KCl ($Br^-$) than are normally found in the intact leaf, but the nature of the transport processes is likely to be the same, and the elucidation of the nature of these processes can come only from the simpler experimental system. The results suggest that there are complex controls on the vacuolar transfer of solutes, including salt, which deserve detailed study.

## Nature of the flux changes responsible for closing

Stomata close when the solute accumulation, largely salt, decreases. It is important to establish whether the decreased salt accumulation is the result of reduced influx, or increased efflux, or both, and to identify the primary process responding to the 'closing' condition: whether this condition is high $CO_2$ partial pressure, presence of ABA, transfer from light to dark, etc. It is




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

therefore of interest to measure ion fluxes in the transitional state, after the transfer to 'closing' conditions. Some measurements of this kind have been made with the *Commelina* system of 'isolated' guard cells. Tissue is treated overnight to reach a steady state, in either radioactive solution for subsequent efflux measurements, or in inactive solution for subsequent measurement of tracer influx over short time periods.

*Effects of ABA*

The first condition studied concerned the effects of adding $2 \times 10^{-5}$ M ABA to 'isolated' guard cells (MacRobbie 1981*c*); this produced only a partial closure, as would be expected from the observations of Itai & Meidner (1978) that full closure by ABA was produced only in guard cells surrounded by live, turgid, subsidiary cells. Nevertheless, it seems likely that the same effects on fluxes should be observed in 'isolated' as in 'intact' guard cells, even if subsidiary cell turgor is necessary to translate these flux changes into full rather than partial closure.

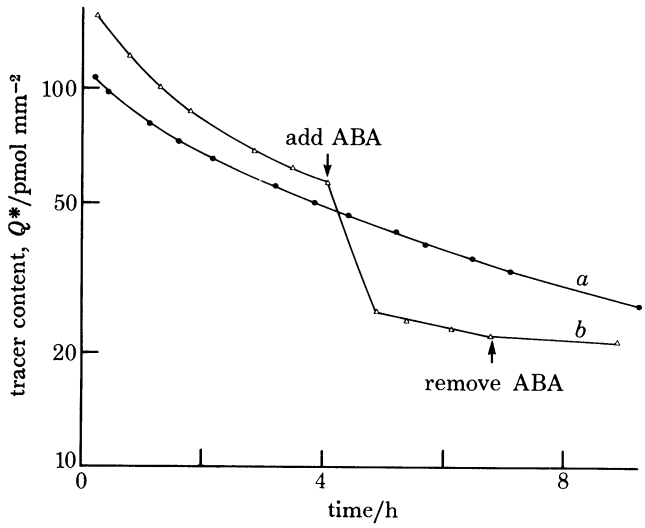


FIGURE 1. Effects of adding $2 \times 10^{-5}$ M ABA during the efflux of $^{82}Br^-$. Semilogarithmic plot of the tissue tracer content ($Q^*$) against time. (*a*) Control; (*b*) ABA added and removed at arrows. Tissue loaded overnight in 60 mM $K^{82}$ Br before the washout.

There were pronounced effect of ABA on efflux of both $^{82}Br$ and $^{86}Rb^+$, but very little effect on influx. The apparent influx of $^{82}Br^-$ was reduced by 15–30% in the period immediately after the addition of ABA, but enhanced efflux must contribute significantly to this reduction. Thus, although it is difficult to measure the influx over a period short enough to avoid underestimation of the true influx by tracer leakage during the uptake period, it is clear that the effects of ABA on influx is small, and may be nil. The same conclusion was reached from measurements of $^{86}Rb^+$ influx.

There were, however, very striking effects of adding ABA during the efflux of $^{82}Br^-$, shown, for example, in figure 1 (the semilogarithmic plot of tracer content against time) and in figure 2 (the plot of the mean rate constant for exchange $\bar{k}$ $(R/Q)$). There is a very large *transient* increase in efflux, but $\bar{k}$ then falls again to near its previous value; the period of enhanced efflux is short, 20 min or less, but the flux is large enough to give significant decreases in the tissue content in this period. Because the fluxes, and contents of cytoplasm and vacuole, are changing, the specific activities become unknown after the addition of ABA, and it is no longer




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

possible to derive accurate flux figures from the tracer efflux. It is, however, possible to deduce which fluxes have changed, and to set rough limits on the extent of the change. When ABA was added to four strips after 3–4 h of efflux (as in figures 1 and 2), the efflux of $^{82}Br^-$ in the first 30–50 min after the addition of ABA was 4–16-fold greater than that calculated for the corresponding period in control conditions. The extra loss during this period was greater than the estimate of the cytoplasmic tracer remaining before the change, and therefore a minimum estimate of the tracer transferred from vacuole to solution in this period can be calculated; combined with the vacuolar specific activity at the time of change, this gives a minimum estimate of the flux $\phi_{vc}$, from vacuole to cytoplasm in that period, for comparison with the control tonoplast flux. In the experiment shown it is clear that the flux $\phi_{vc}$ is increased by more than 2.5–8.9-fold (four strips), in the period immediately after the addition of ABA. The effect of ABA on the plasmalemma efflux of $^{86}Rb^+$ was very similar, but no estimate of tonoplast fluxes of

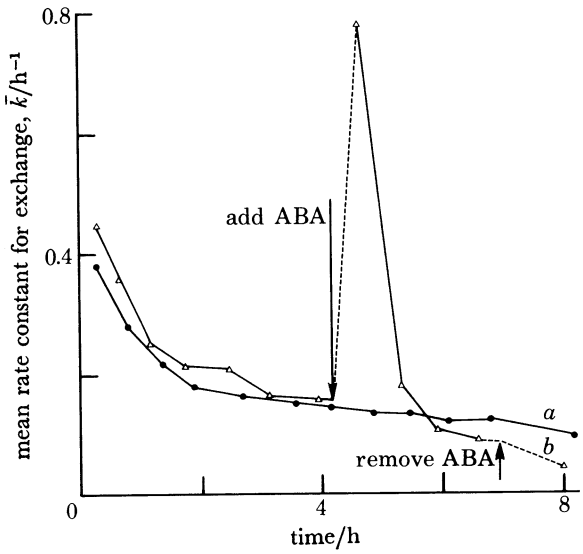


FIGURE 2. Effect of adding $2\times10^{-5}$ M ABA during the efflux of $^{82}Br^-$, shown as the time course of $\bar{k}(R/Q)$. (*a*) Control; (*b*) ABA added and removed at the arrows.

$Rb^+$ can be made because they are large enough, relative to the plasmalemma fluxes, to preclude the appearance of two distinguishable exponentials in the efflux curves.

The results show that the reduction in salt content of 'isolated' guard cells produced by ABA arises, not from inhibition of the influx, but by a transient stimulation of efflux of both anion and cation. Discussing possible mechanisms for ABA action, Raschke (1977, 1979) suggested an effect of ABA on the proton pump at the plasmalemma, or, alternatively, a direct effect of ABA on malate synthesis; he has also suggested that the low cytoplasmic pH or high cytoplasmic malate concentration thereby produced might lead to a leakage of $K^+$, $Cl^-$ and malate from guard cells. However, the flux measurements make it clear that transient changes in fluxes are involved, with stimulated excretion of salt, and that tonoplast fluxes are also markedly affected.

*Effect of light and dark*

Similar experiments have been done, looking at the effect of a transfer from light to dark, on the tracer influx and efflux. This can establish the extent to which light affects the ability of

guard cells to stay open (which may not be identical to the effect of light on the ability to open from a closed state, a study that remains to be done).

In the experiments in which the steady-state apertures in light and dark were compared, after overnight incubation in 30 mM RbCl or KBr, the mean aperture in the light was 14.5 ± 0.2 μm (23 strips, 324 pores), and that in the dark was 7.2 ± 0.2 μm (15 strips, 225 pores) (means ± s.e.m.). On transfer of light strips to the dark, after this opening, the response was sluggish, but the stomatal aperture did begin to fall; in the first 30–50 min in the dark the stomata closed by 1.4–2 μm. Although this response is much less than that observed in the intact leaf, in which increased $CO_2$ levels in the absence of photosynthesis will also contribute to the closure, it seems likely that the flux changes produced in the 'isolated' guard cells by such a transition, are relevant to those in the intact leaf.

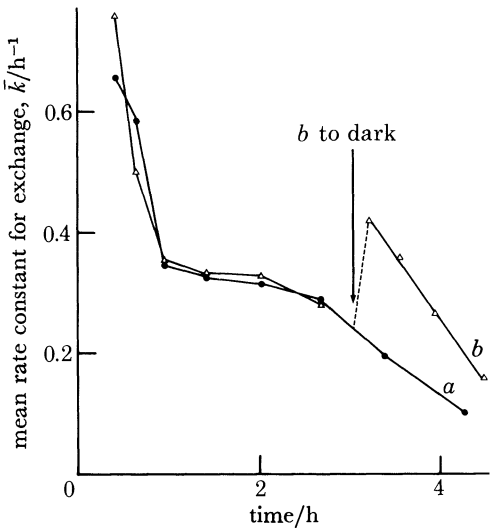


FIGURE 3. Effect of transfer from light to dark during the efflux of $^{82}Br^-$, shown as the time course of $\bar{k}$. Tissue was loaded overnight in 30 mM K$^{82}$Br before the washout. (*a*) Washout in light throughout; (*b*) transfer from light to dark at time shown.

The flux experiments to measure the transients on transfer from light to dark are still incomplete, but the effects so far established are similar to those of the addition of ABA. There is a marked transient increase in efflux, of both $^{82}Br^-$ and $^{86}Rb^+$. Figure 3 shows this, in the plot of the mean rate constant $\bar{k}$ against time, for the washout from tissue labelled in light in 30 mM K$^{82}$Br, and transferred to the dark after 3 h of washout in the light. In three such strips the loss of tracer in the first period in the dark (21 min) was 1.5, 1.5 and 2.8 times higher than that predicted from the efflux curve before the change. In strips transferred from light to dark at the start of the efflux, the rate of loss over the first hour of efflux was much higher than that in light, but the later rates of tracer loss were lower than in the control left in light. The kinetics are compared in table 1. The efflux from tissue transferred to the dark was fitted to two exponentials, for comparison with the control tissue left in light. Although the fluxes and contents cannot be calculated for the non-steady-state tissue, some indication of the effects can be seen. In the dark washout, much more of the total appears in the faster component of efflux, and the rate constants are both reduced, particularly $k_2$, the slower rate constant. This implies that the flux from cytoplasm to vacuole is markedly reduced on transfer to the dark; in the light a fraction of the cytoplasmic tracer is transferred to the vacuole during the first stages of washout,




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 1. LIGHT AND DARK WASHOUT OF $^{82}Br$

| | conditions of washout | |
|---|---|---|
| | light | dark |
| number of strips | 4 | 3 |
| $k_1/h^{-1}$ | $2.2 \pm 0.1$ | $1.73 \pm 0.07$ |
| $A_1$/pmol mm$^{-2}$ | $39 \pm 5$ | $67 \pm 13$ |
| $k_2/h^{-1}$ | $0.27 \pm 0.01$ | $0.096 \pm 0.019$ |
| $A_2$/pmol mm$^{-2}$ | $91 \pm 7$ | $42 \pm 4$ |
| $Q_t$/pmol mm$^{-2}$ | $130 \pm 7$ | $109 \pm 16$ |
| $Q_c$/pmol mm$^{-2}$ | $62 \pm 4$ | |
| $Q_v$/pmol mm$^{-2}$ | $68 \pm 5$ | |

Both sets of tissue were loaded in 30 mM K$^{82}$Br for 15–16 h in light, then washed out in inactive 30 mM Kbr in either light or dark. The tracer content during the washout in each condition was fitted to two exponentials: $Q^* = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$. Tissue remaining in light is in a steady state, and cytoplasmic and vacuolar contents can therefore be calculated from the efflux parameters.

and $A_1$ is therefore significantly less than $Q_c$, whereas after the transfer to the dark the cytoplasmic tracer seems to be lost exclusively to the solution and not to the vacuole.

As yet, the effects of the transfer on the influx of $^{82}Br$ have not been measured. The influx of $^{86}Rb^+$ is not significantly affected by the transfer from light to dark. There are, however, marked effects on the efflux of $^{86}Rb^+$, as with $^{82}Br^-$, with large transient stimulations immediately after the transfer; the ratio of $\bar{k}$ before and after the change was 1.5–3.8, with a mean of $2.27 \pm 0.09$ ($n = 30$).

Thus although the results are incomplete they suggest that, as with ABA, the effects of a transfer to the dark are primarily a transient stimulation of efflux, and not an inhibition of influx, and that there are effects also on the tonoplast fluxes.

## CONCLUSIONS FROM THE FLUX EXPERIMENTS

The results of both the steady-state experiments and those on the transients point to the importance of transfers from cytoplasm to vacuole in the control of salt accumulation. In the experimental conditions the aperture is regulated by the ability to take up chloride from the medium and to retain it, and this ability is sensitive to the appropriate environmental conditions. It is not, however, simply a matter of regulating the chloride influx by external or internal factors. The efflux of ions at the plasmalemma can be stimulated by appropriate signals, to an extent that alters the tissue content. There are also large changes in the tonoplast fluxes, and it may well be that these changes produce changes in cytoplasmic ion levels, which are in turn responsible for changes in ion transport processes in the plasmalemma. Transient responses, rather than simple changes in membrane properties, are involved, and the cells do not simply become more leaky in the presence of ABA, or in the dark. Interaction of the 'closing' factors with the control system, with alteration of the 'set points' of the accumulation, is a possible interpretation of these results.

The importance of tonoplast transport processes might also be suggested by a consideration of the most striking difference between guard cells and more typical plant cells. Closed guard cells seem not to have the ability to maintain the large central vacuole characteristic of open guard cells, or 'normal' mature cells, but instead have many small vacuoles of small total volume. A similar phenomenon is seen in the motor cells of the pulvini of leaves showing sleep movements, which show large volume changes, associated with changes in the vacuolar state. Campbell &




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Garber (1981) showed that the cytoplasmic volume remains essentially the same in the two states, but the number and volume of vacuoles in the cell changes considerably; the total tonoplast area is similar, but there is a transition from a state with many small vacuoles, in the shrunken cell, to a state with a large central vacuole, in the swollen, turgid cell. The changes in guard-cell state are similar, and the same quantitative relation may also hold.

Thus the control of salt accumulation, and hence volume, may well rest with the ability to transfer solutes into vacuoles, and to initiate vacuolar fusions. Guard cells may offer a good experimental system, in which this overall process may be turned on and off under experimental control. The study of chloride accumulation in the 'isolated' guard cell system may therefore throw light on the more general process of salt accumulation, as well as the particular controls in guard cells.

The experimental work described in this paper was done with support from the S.E.R.C., whose help is gratefully acknowledged.

## References

Beilby, M. J. & Walker, N. A. 1981 Chloride transport in *Chara*. *J. exp. Bot.* **32**, 43–54.

Campbell, N. A. & Garber, R. C. 1981 Vacuolar reorganisation in the motor cells of *Albizzia* during leaf movement. *Planta* **148**, 251–255.

Itai, C. & Meidner, H. 1978 Effect of abscisic acid on solute transport in epidermal tissue. *Nature, Lond.* **271**, 653–654.

Leigh, R. A., Ahmad, N. & Wyn-Jones, R. G. 1981 Assessment of glycinebetaine and proline compartmentation by analysis of isolated beet vacuoles. *Planta* **153**, 34–43.

MacRobbie, E. A. C. 1975 Intracellular kinetics of tracer chloride and bromide in *Nitella translucens*. *J. exp. Bot.* **26**, 489–507.

MacRobbie, E. A. C. 1977*a* Functions of ion transport in plant cells and tissues. *Int. Rev. Biochem. Pl. Biochem.* II **13**, 211–247.

MacRobbie, E. A. C. 1977*b* Ion transport processes at the tonoplast. In *Transmembrane ionic exchanges in plants* (ed. M. Thellier, A. Monnier, M. Demarty & J. Dainty), pp. 101–108. Paris: C.N.R.S.

MacRobbie, E. A. C. 1980 Osmotic measurements on stomatal cells of *Commelina communis* L. *J. Membrane Biol.* **53**, 189–198.

MacRobbie, E. A. C. 1981*a* Ionic relations of stomatal guard cells. In *Stomatal physiology* (ed. P. E. Jarvis & T. A. Mansfield), pp. 51-70. Cambridge University Press.

MacRobbie, E. A. C. 1981*b* Ion fluxes in 'isolated' guard cells of *Commelina communis* L. *J. exp. Bot.* **32**, 545–562.

MacRobbie, E. A. C. 1981*c* Effects of ABA in 'isolated' guard cells of *Commelina communis* L. *J. exp. Bot.* **32**, 563–572.

MacRobbie, E. A. C. & Lettau, J. 1980*a* Ion content and aperture in 'isolated' guard cells of *Commelina communis* L. *J. Membrane Biol.* **53**, 199–205.

MacRobbie, E. A. C. & Lettau, J. 1980*b* Potassium content and aperture in 'intact' stomatal and epidermal cells of *Commelina communis* L. *J. Membrane Biol.* **56**, 249–256.

Marré, E. 1979 Fusicoccin: a tool in plant physiology. *A. Rev. Pl. Physiol.* **30**, 273–288.

Outlaw, W. H. & Kennedy, J. 1978 Enzymic and substrate basis for the anaplerotic step in guard cells. *Pl. Physiol.* **62**, 648–652.

Outlaw, W. H. & Lowry, D. H. 1977 Organic acid and potassium accumulation in guard cells during stomatal opening. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4434–4438.

Outlaw, W. H. & Manchester, J. 1979 Guard cell starch concentration quantitatively related to stomatal aperture. *Pl. Physiol.* **64**, 79–82.

Raschke, K. 1975 Stomatal action. *A. Rev. Pl. Physiol.* **26**, 309–340.

Raschke, K. 1977 The stomatal turgor mechanism and its response to $CO_2$ and abscisic acid: observations and a hypothesis. In *Regulation of cell membrane activities in plants* (ed. E. Marré & O. Ciferri), pp. 178–183. Amsterdam: North-Holland.

Raschke, K. 1979 Movements of stomata. In *Encyclopedia of plant physiology* (ed. W. Haupt & M. E. Feinleib), vol. 7, pp. 383–441. Berlin: Springer-Verlag.

Raschke, K. & Fellows, M. P. 1971 Stomatal movement in *Zea mays*. Shuttle of potassium between guard cells and subsidiary cells. *Planta* **101**, 296–316.





PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Raschke, K. & Humble, G. D. 1973 No uptake of anions required by opening stomata of *Vicia faba*: guard cells release hydrogen ions. *Planta* **115**, 47–57.
Sanders, D. 1980*a* Control of plasma membrane $Cl^-$ fluxes in *Chara corallina* by external $Cl^-$ and light. *J. exp. Bot.* **31**, 105–118.
Sanders, D. 1980*b* Control of $Cl^-$ influx in *Chara* by cytoplasmic $Cl^-$ concentration. *J. Membrane Biol.* **52**, 51–60.
Sanders, D. 1980*c* The mechanism of $Cl^-$ transport at the plasma membrane of *Chara corallina*. I. Co-transport with $H^+$. *J. Membrane Biol.* **53**, 129–141.
Sanders, D. & Hansen, U.-P. 1981 Mechanism of $Cl^-$ transport at the plasma membrane of *Chara corallina*. II. Transinhibition and the determination of $H^+/Cl^-$ binding order from a reaction kinetic model. *J. Membrane Biol.* **58**, 139–153.
Schnabl, H. & Ziegler, H. 1977 The mechanism of stomatal movement in *Allium cepa* L. *Planta* **136**, 37–43.
Smith, F. A. 1970 The mechanism of chloride transport in characean cells. *New Phytol.* **69**, 903.
Smith, F. A. & Raven, J. A. 1979 Intracellular pH and its regulation. *A. Rev. Pl. Physiol.* **30**, 289–311.
Smith, F. A. & Walker, N. A. 1976 Chloride transport in *Chara corallina* and the electrochemical potential difference for hydrogen ions. *J. exp. Bot.* **27**, 451–459.
Spanswick, R. M. 1981 Electrogenic ion pumps. *A. Rev. Pl. Physiol.* **32**, 267–289.
Spear, D. G., Barr, J. K. & Barr, C. E. 1969 Localization of hydrogen ion and chloride ion fluxes in *Nitella*. *J. gen. Physiol.* **54**, 397–414.
Squire, G. R. & Mansfield, T. A. 1972 Studies of the mechanism of action of fusicoccin, the fungal toxin that induces wilting, and its interaction with abscisic acid. *Planta* **105**, 71–78.
Tazawa, M., Kikuyama, M. & Shimmen, T. 1976 Electrical characteristics and cytoplasmic streaming of characeae cells lacking tonoplast. *Cell Struct. Funct.* **1**, 165–176.
Turner, N. C. & Graniti, A. 1970 Fusicoccin: a fungal toxin that opens stomata. *Nature, Lond.* **223**, 1070–1071.
Wyn-Jones, R. G., Brady, C. J. & Spiers, J. 1979 Ionic and osmotic relations in plant cells. In *Recent advances in the biochemistry of cereals* (ed. D. L. Laidman & R. G. Wyn-Jones), pp. 63–103. London: Academic Press.
Wyn-Jones, R. G., Storey, R., Leigh, R. A., Ahmad, N. & Pollard, A. 1977 A hypothesis on cytoplasmic osmoregulation. In *Regulation of cell membrane activities in plants* (ed. E. Marré & O. Ciferri), pp. 121–136. Amsterdam: North-Holland.
Zeiger, E., Bloom, A. J. & Hepler, P. K. 1978 Ion transport in stomatal guard cells: a chemiosmotic hypothesis. *What's new Pl. Physiol.* **9**, 29–32.




PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES