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Chloride Accumulation by Mung Bean Root Tips

A LOW AFFINITY ACTIVE TRANSPORT SYSTEM AT THE PLASMALEMMA[1]

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DONALD F. GERSON[2] AND RONALD J. POOLE
Department of Biology, McGill University, P. O. Box 6070, Montreal 101, Quebec, Canada

ABSTRACT

Net uptake of Cl^- into root tips of mung bean (*Phaseolus aureus*) increases steadily with increasing external concentrations from 1 to 60 mM. Membrane potentials were measured to determine the equilibrium concentration of Cl^- in the tissue which could be due to diffusion. This concentration was readily exceeded in both the relatively nonvacuolate tips (0 to 1 mm) and the vacuolate, mature upper sectons (1 to 11 mm) of the roots. The activity coefficient of both cytoplasmic and vacuolar Cl^-, measured with Cl^- sensitive microelectrodes, was approximately the same as that of a pure KCl solution of the same concentration. It is concluded that the "second mechanism" of ion uptake involves a large increase in the rate of active transport at the plasmalemma as the external concentration is increased above 1 mM.

The kinetics of ion influx into plant cells suggest the involvement of more than one transport mechanism for a given ionic species. For many anions and cations, the curve of influx *versus* concentration characteristically approaches saturation at external concentrations less than 1.0 mM. Influx in this concentration range is clearly against an activity gradient (9, 16, 28) and follows simple Michaelis-Menten kinetics (15). At higher external concentrations (1–100 mM), the influx rate is greatly increased over the saturation level of the first mechanism.

The additional influx has been ascribed to a second mechanism involving carrier sites at the membrane with very different properties from those of mechanism 1. This interpretation is supported by many detailed studies of the kinetics of isotope influx (12–14, 21, 29, 30, 34, 35). However, in the high concentration range it becomes difficult to apply the usual criteria for active transport, since the ion activities may approach thermodynamic equilibrium across the cell membrane (28), and since the influx rate is usually not described by simple Michaelis-Menten kinetics (15, 23). Indeed the influx often shows no sign of saturation at high concentrations and may be more suggestive of diffusion across the cell membrane (24, 29, 31). When saturation does appear, it may in some cases reflect a limitation of influx at the tonoplast rather than at the cell membrane (11, 31). Thus it is still a matter of controversy whether or not the second mechanism involves active transport at the plasmalemma.

This investigation examines Cl^- uptake by root tips of mung bean (*Phaseolus aureus*) from solutions in the concentration range of the second mechanism. It is shown that the second mechanism involves a large increase in the accumulation of Cl^- as the external concentration is increased above 1 mM, and that this accumulation takes place against an electrochemical gradient of Cl^- at the plasmalemma.

MATERIALS AND METHODS

Mung bean seeds (*Phaseolus aureus*) were sterilized in 0.5% sodium hypochlorite (v/v) for 15 min, washed for 10 min in running tap water, and then rinsed several times with distilled water. The seeds were planted on eight mesh stainless steel screens and covered with a single layer of cheesecloth. The screen was suspended over aerated 0.5 mM $CaSO_4$, which was changed after the first 24 hr, and again several hours before an experiment. The seeds were constantly illuminated by a bank of four 40-w Sylvania GRO-LUX fluorescent bulbs located 30 cm above the plants. It should be noted that Ca^{2+} is necessary for healthy root growth: plants grown in distilled water produced roots with extremely flaccid tips. Experiments were performed on the tips (apical 1 mm) and upper sections (1–11 mm) of 3- to 5-day-old roots.

All experimental solutions contained 0.5 mM $CaSO_4$ with the required concentration of KCl. Experiments were performed at 30 C; uptake solutions were aerated with filtered, water-saturated air at the same temperature. Net Cl^- uptake was determined for the roots of intact plants. Transport of chloride to the rest of the plant did not affect our conclusions, since similar results were obtained with excised segments. To measure net uptake, the plants were transferred to stainless steel screens over 1-liter beakers containing the appropriate solutions; at the end of the uptake period segments were cut, washed in 0.5 mM $CaSO_4$ for 15 min at 4 C, and weighed. Chloride was extracted from the tissue at room temperature with 0.1 M HNO_3, 10% acetic acid (v/v) for 2 to 5 hr. The tissue was then macerated with a stirring rod, and the slurry was titrated with a Buchler-Cotlove chloridometer. The gelatin additive often used in this technique was omitted since it introduces a nonlinearity at low concentrations.

Membrane potentials were measured with glass microelectrodes which were drawn with a gravity-operated electrode puller from Kimax-51, 1 mm internal diameter capillaries. Tip diameters were less than or equal to 1 μ, and resistances were between 15 and 30 megohms. The electrodes were filled with 3 M KCl by boiling. Improved reliability and decreased tip potentials resulted from filtering the 3 M KCl through 0.8 μ pore diameter Millipore filters to remove insoluble impurities

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[2] Present address: Department of Biophysics, University of Western Ontario, London 72, Ontario, Canada.

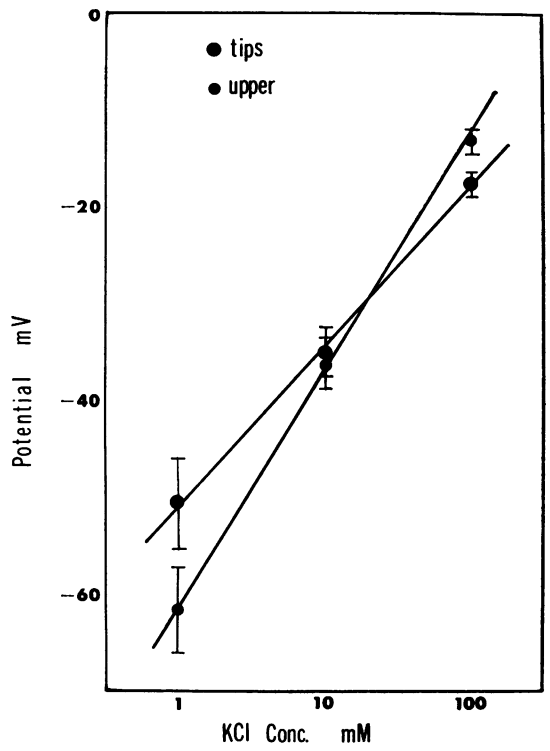

FIG. 1. Membrane potentials of mung bean root cells in KCl solutions containing 0.5 mM $CaSO_4$. Standard errors of the mean of at least 30 measurements are given for each point. Potentials were measured in newly excised roots. Potentials in roots of intact plants were similar. Tips = apical 1.0 mm; upper = 1 to 11 mm from tip.

The electrodes were connected through a 3 M KCl salt or salt-agar (3%) bridge and a Radiometer K-401 calomel reference electrode to a Vibron model 33B-2 electrometer. Tip potentials in the experimental solutions were less than ±10 mv (usually positive) and were subtracted from measurements.

Chloride-sensitive ion-selective microelectrodes were made by a method modified from Brown *et al.* (2). Electrodes were drawn as before, and the tips were dipped for as short a time as possible into 0.5% (v/v) Siliclad (Clay-Adams, Inc., Parsippany, N.J.) in 1-chloronaphthalene. Following this they were placed tip up in holes in an aluminum block and baked at 280 C for 1 to 3 hr. The electrode tips often clogged with Siliclad, as Walker noted (32); we found that clogging was a very sensitive function of tip diameter, and with suitable adjustment it could be virtually eliminated. When cool, electrodes were filled with 0.5 M KCl by boiling. To fill the tip of the KCl-filled electrodes with chloride liquid ion exchange resin (either that made by Orion, Cambridge, Mass. or Corning, Corning, N.Y.) a small rubber tube was attached to the microelectrode, and the tip was dipped into the resin. Gentle suction pulled a column of resin about 0.2 mm long into the tip. Electrodes were stored on glass microscope slides in staining dishes filled with 0.5 M KCl. Each electrode was calibrated individually over a suitable range of KCl concentrations and the corresponding activities were calculated. The specificity of the microelectrodes was not tested, since it was assumed to be dependent only on the resin; the manufacturers report no interfering ions which are likely to be present in sufficient concentrations to cause concern. (Chloride sensitivity of similar microelectrodes has been measured by Walker [32].) Each electrode was used only once because the high internal pressure of the cells forced the resin a short distance up the tip, filling the extreme tip with cellular contents, and thus permanently contaminating the electrode.

Preparation of the roots for microscopy to measure the relative vacuolar volumes followed the methods of Mesquita (25), except that the tissue was embedded in Araldite resin. Sections 1 μ thick were stained with 1% toluidine blue for 20 min at 50 C.

RESULTS

Membrane potentials (Fig. 1) of both tip and mature cells of roots grown in 0.5 mM $CaSO_4$ were measured to allow calculation of the maximum internal chloride concentrations which could be due to diffusion at a given external concentration. Essentially identical values were obtained with roots which had been allowed to accumulate salt for 2 days from 10 mM KCl, 0.5 mM $CaSO_4$. Equilibrium internal concentrations of Cl^- were calculated by the Nernst equation from these potentials and are plotted in Figures 2 and 3 along with internal concentrations observed after an uptake period. The differences between potentials in the tips and the upper segments produce statistically indistinguishable differences in equilibrium chloride concentrations.

In external KCl concentrations up to 10 mM, the calculated equilibrium concentration (Fig. 2) was less than the initial level of Cl^- in the tissue (3.4 mM). Thus, in this concentration range, diffusion across the plasmalemma could make no positive contribution to the net uptake. Nevertheless, the tissue concentrations (Fig. 2) increased during the 21-hr experiment to approximately 10 times the calculated equilibrium levels. Moreover, the amount of Cl^- taken up over the uptake period shows a steep increase with increasing external concentrations above 1 mM, as one would expect for the second mechanism. Although the internal concentration after 21 hr shows only the average rate of uptake during that period, experiments over shorter time periods (down to 2.25 hr) confirmed that the general pattern of net uptake seen in Figure 2 does reflect the relationship between uptake rate and external concentration.

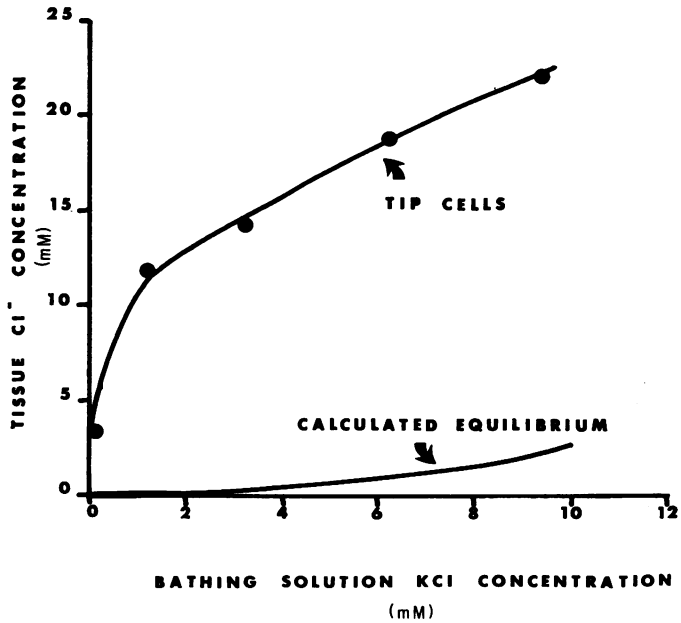

FIG. 2. Chloride concentrations in mung bean root tips after 21 hr of uptake from KCl (+0.5 mM $CaSO_4$) compared with the maximum concentration which could be due to diffusion. The plants remained intact during the uptake period. Approximately 50 plants were used at each concentration.

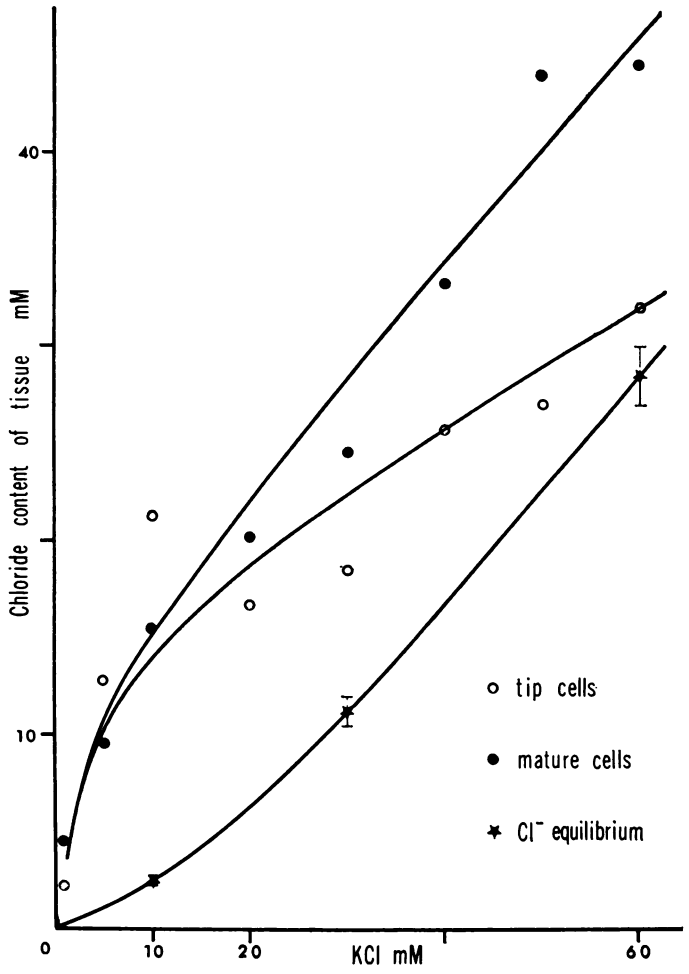

FIG. 3. Chloride concentration in mung bean root tissue after 19 hr of uptake compared with the maximum internal concentration which could be due to diffusion. The range indicated for the latter is due to the variability of the membrane potential (see Fig. 1). The plants remained intact during the uptake period. Approximately 50 plants were used for each determination.

The data of Figure 2 show therefore that chloride is accumulated against an electrochemical gradient in root tips of mung bean. In addition, there is a large increase in the rate of active transport as the concentration is increased from 1 to 10 mM. This increase in active transport is in the concentration range in which "mechanism 2" is commonly thought to operate. (Mung beans, like many other species, show a typical "mechanism 1" at much lower concentrations [20].)

In a similar experiment (Fig. 3) net uptake was measured in both tips and mature sections for external concentrations up to 60 mM. Over this concentration range, the net uptake of chloride continues to increase with concentration, but the theoretical equilibrium value increases exponentially, so that at the highest concentrations there is no significant diffusion gradient across the plasmalemma in root tips. Presumably the active transport observed at 10 mM external concentration also operates at higher concentrations, but it is not possible to say, from these data, whether diffusion makes a significant contribution to net uptake from 60 mM KCl.

Since the most significant barrier to transport in root tips is presumably the plasmalemma, it seems reasonable to conclude that the active transport system responsible for mechanism 2 is located at this membrane. There are, however, two possibilities which could cause the rejection of this conclusion: (*a*) if the activity coefficient, γ, of chloride in the tissue is approximately equal to 0.1, due to binding or to sequestration in an organelle, then the activity of chloride in the tissue will roughly equal that predicted for diffusion, or (*b*), if vacuolation in the first millimeter of the root tips is sufficiently advanced, then the measured potentials or ion gradients could apply to the tonoplast. Both of these possibilities have been examined; neither objection appears to be justified.

Intracellular chloride activities measured with chloride-sensitive microelectrodes are compared in Table I with chloride concentrations measured by extraction and titration. The roots used for these measurements had accumulated salt from 10 mM KCl for 24 hr. The ratio between the chloride activity in the cell and the activity in a free KCl solution at the same concentration for both the tips (presumably cytoplasm) and the upper sections (presumably vacuolar sap) is essentially unity. This precludes the possibility that chloride is bound or that a significant portion is concentrated in a small organelle which could not be penetrated by the electrodes.

Vacuolar volume as a percentage of tissue volume was obtained from photographs of longitudinal root tip sections with standard stereological techniques (6). Volumes were determined for every alternate 0.2 mm band across the root beginning at the tip and ending at 2.0 mm. The net precentage of vacuolation (vacuole volume/tissue volume) for the first millimeter was 21%. If we assume, as did Pitman (27), that cell walls and intercellular spaces account for a total of 10% of the tissue volume, then the cytoplasm must account for 69% of the tissue. Of the intracellular volume alone, however, cytoplasmic volume is around 77%: this represents the likelihood that the tip of a microelectrode is in the cytoplasm when it is in a cell. Since the data obtained with the microelectrodes were quite uniform, either the electrodes failed to penetrate the vacuoles as often as expected, possibly because of the small size of vacuoles in the tip, or activities in the cytoplasm and vacuolar sap were nearly the same. Vacuolar volumes of mature roots have been measured by others (3, 4) and are roughly 85% of the total volume of the tissue. Using the same argument, vacuoles comprise 94% of the intracellular volume in mature tissue.

DISCUSSION

Anion Transport by the Second Mechanism. The object of this investigation has been to characterize the component of Cl^- uptake which is operationally defined as mechanism 2, *i.e.*, the component which increases as the external concentration is increased above 1 mM. It is shown above (Figs. 2, 3) that the rate of net uptake of Cl^- by mung bean root tips increases steadily with increasing external concentration, although the initial diffusion gradient of Cl^- is in the outward direction at external concentrations up to at least 10 mM. These results pro-

Table I. *Activity Coefficients for Cl^- in Mung Bean Root Cells*

Root Segment	Concn	Activity	γCell[1]	γKCl[2]	$\frac{\gamma Cell}{\gamma KCl}$
	mM				
Tip (0–1 mm)	27.1 ± 1.4	21.8 ± 2.0[3]	0.80	0.85	0.94
Mature (1–11 mm)	24.7 ± 2.1	20.1 ± 3.2[3]	0.82	0.85	0.96

[1] Observed activity coefficient (activity/concentration).

[2] Calculated activity coefficient for pure KCl solution of the observed concentration.

[3] Standard error of the mean of at least 30 measurements.

vide direct electrochemical evidence that the second (low affinity) uptake mechanism in plant cells accomplished active transport at the plasmalemma over and above that brought about by mechanism 1. The possibility of an additional contribution to net uptake by diffusion across the plasmalemma at high external concentrations (10 to 60 mM) cannot be ruled out. This possibility, of course, in no way negates the evidence for low affinity active transport across the plasmalemma.

The present data on cytoplasmic Cl^- activities also aid in the interpretation of earlier transport studies. For instance, the work of Torii and Laties (31), which purports to show that Cl^- diffuses across the plasmalemma into corn root tips from solutions more concentrated than 1 mM, may be given an opposite interpretation. Torii and Laties showed that influx of Cl^- into corn root tips may reach a rate of more than 10 μmoles/gram·hr from 40 mM NaCl. It was established that uptake was linear for 8 hr, in which time the influx would amount to 80 μmoles/g. Since it is unlikely that much Cl^- was present initially to exchange with the external isotope, it appears that net accumulation of Cl^- in this experiment raised the internal concentration (and activity) of Cl^- well above that of the external solution. These data, taken with any likely value of membrane potential, suggest that mechanism 2 is indeed an active transport process at the plasmalemma of corn root tip cells.

A number of previous studies have been concerned with anion fluxes in vacuolated cells. The data of Pitman (27) indicate an increase in net active transport of Br^- at the plasmalemma of beet cells with increasing concentrations in the range of the second mechanism. Pitman was reluctant to draw this conclusion because the cytoplasmic Br^- (estimated at up to 25 mM) may have been sequestered, for instance, in the mitochondria. However, mitochondria are believed to be impermeable to halides (5), and the data of the present study indicate that cytoplasmic Cl^- has a high activity coefficient. (The same may not hold for photosynthetic material. Coster and Hope [8] summarize work on the *Characeae* showing that most of the cytoplasmic Cl^- is in the chloroplasts.) In oat coleoptiles, according to Pierce and Higinbotham (26), Cl^- is very far from equilibrium across the plasmalemma: at an external concentration of 10 mM, cytoplasmic Cl^- was estimated at 83 mM.

An analysis of chloride compartmentation by Cram (9) differs from the present results in showing little additional net uptake as concentration is increased into the range of the second mechanism, but concludes that "the main change in tracer fluxes at the plasmalemma above 8 mM is in a 1-for-1 exchange component." Weigl (33) also showed that Cl^- efflux in corn roots is dependent on the external concentration of Cl^-. Finally, it was suggested by Cram and Laties (11) that 90% of the plasmalemma influx of Cl^- in barley roots is attributable to one for one exchange rather than to diffusion. Thus, although the relative importance of active transport *versus* exchange diffusion varies from one investigation to another, the data are consistent in minimizing the role of free diffusion in Cl^- uptake.

Interpretation of Kinetic Data. An important basis for the view that the second mechanism involves diffusion across the plasmalemma has been the observation that in corn root tips (31) as well as in potato tissue (24, 29) the chloride influx increases proportionately, or even exponentially, with increasing external concentration, rather than showing the saturation kinetics often characteristic of carrier transport. The influx rates in these studies could, however, be correlated with the predicted effects of the membrane potential on Cl^- diffusion.

It was observed in the present work that root tips of mung bean, like those of corn, show no saturation either of Cl^- net uptake (Fig. 2) or isotope influx (unpublished data). Nevertheless, it appears that Cl^- is transported against a diffusion gradient at the plasmalemma in root tips of both species. This system may be similar to the nonsaturable carrier-mediated component of amino acid transport in Ehrlich cells described by Christensen and Liang (7). It must be concluded that the shape of the influx curve is not a reliable indicator of the predominant mode of transport. This conclusion is reinforced by the finding (11) that the shape of the influx curve is a function of experimental technique.

The characterization of the second mechanism as an active transport process at the plasmalemma has no direct bearing on the question of whether this membrane is rate-limiting for isotope influx. Thus, Torii and Laties (31) may be correct in ascribing the kinetic differences between influx into root tips and vacuolated cells to the influence of transport at the tonoplast. Arguments against such an influence of the tonoplast (34, 35) have been answered by Cram (10). The work of Cram and Laties (11) shows that the magnitude of the influx, especially at high concentrations, may be greatly affected by the procedure used for washing the tissue. For this reason, the detailed characterization of influx kinetics by Epstein and others (12, 13, 14, 15, 21, 29, 30, 34, 35) may not apply directly to the plasmalemma transport system described here.

In conclusion, it may be noted that the evidence we have presented for low affinity active transport of Cl^- across the plasmalemma is in line with the data of Fisher and Hodges (18) and Fisher *et al.* (19) showing a requirement for high concentrations to activate a plant membrane ATPase.

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