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Nitrate Uptake into Barley (*Hordeum vulgare*) Plants[1]

A NEW APPROACH USING $^{36}ClO_3^-$ AS AN ANALOG FOR NO_3^-

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ABSTRACT

Evidence is presented that chlorate is an extremely good analog for nitrate during nitrate uptake by intact barley (*Hordeum vulgare* cv. Fergus) roots. The depletion of ClO_3^- or NO_3^- from uptake media over 2 to 6 hours by seedlings was found to be dependent on combined NO_3^- plus ClO_3^- concentrations, and total anion uptake was equivalent at different NO_3^-/ClO_3^- ratios. After loading barley seedlings with $^{36}ClO_3^-$ for 6 hours, kinetic parameters were derived from the analysis of efflux of [^{36}Cl] chlorate into unlabeled solution. On the basis of this analysis, the half times for exchange for the cytoplasmic and vacuolar phases were 17 minutes and 20 hours, respectively.

Data pooled from a number of different experiments were used to calculate kinetic constants (K_m and V_{max}) for $^{36}ClO_3^-$ influx into barley roots at different external ClO_3^-/NO_3^- ratios, using short (10 minutes) influx times. There appeared to be no discrimination by the root cells between ClO_3^- and NO_3^-. Lineweaver-Burk analysis of the interaction between nitrate and chlorate were characteristic of competitive inhibition at low nitrate concentrations (0–0.5 mM). At higher concentrations, in the range of >1 mM, similar interactions between these ions were evident.

There is relatively little information available on the detailed kinetics of nitrate transport into barley plants. Even the more recent investigations (2, 7, 21) have described net nitrate flux into barley plants over periods of greater than 2 h duration. The use of the heavy isotope $^{15}NO_3^-$ as a tracer is not without difficulties, and again for whole plant studies uptake periods of 9 h or greater are no exception (1, 14). In part, this is due to the large internal pools of nitrogen (6). As far as the authors are aware $^{13}NO_3^-$ has so far only been used for the study of nitrate assimilation in tobacco culture cells (24) and soybean roots and nodules (23). Moreover, this isotope is not very convenient for routine experiments because of its extremely short half-life (10 min).

Doddema and Telkamp (9) have shown that nitrate competes with chlorate for uptake into *Arabidopsis thaliana*. Other workers have obtained similar K_m and V_{max} values for nitrate reductase activity (NR) using chlorate or nitrate as substrates for the enzymes from *Chlorella* (25) or tomato (13). In higher plants, the chlorite which results from the action of NR is not reduced by nitrite reductase and apparently accounts for many of the toxic symptoms caused by chlorate treatment (15). Indeed, chlorate resistance has been used to screen for nitrate reductase mutants in *Aspergillus* (3) and *Pisum* (12).

Tromballa and Broda (27) have studied the uptake and reduction of $^{36}ClO_3^-$ and $^{36}ClO_4^-$ in *Chlorella fusca*. They found that increasing external nitrate concentrations depressed accumulation of tracer in the cells, and the authors assumed that competition between ClO_3^- and NO_3^- took place at both the uptake and reduction steps. Cram (4) and Smith (26) have suggested that accumulated nitrate and chloride exert a common negative feedback on the subsequent uptake of these ions. The demonstration, therefore, that Cl^- accumulation by carrot discs led to a reduction of $^{36}ClO_3^-$ uptake (5) is consistent with the belief that this ion may serve as a tracer for nitrate. To date these are the only examples in the literature which give some indication that $^{36}ClO_3^-$ could be used as a tracer for NO_3^-.

There are other known examples where two ions are so similar in chemical properties that the cellular transport mechanisms fail to discriminate between them. Such examples are sulfate-selenate, chloride-bromide, calcium-strontium, and rubidium-potassium (10, 11, 18). The convenience associated with the considerably longer half-life of $^{86}Rb^+$ (18.7 d) than $^{42}K^+$ (12.4 h) has led to the widespread use (11) of the former as a tracer for K^+. Provided that certain limitations of this method are taken into account (22), the technique has greatly enhanced current understanding of K^+ transport in higher plants.

From the foregoing discussion, the available evidence in the literature suggests that the use of $^{36}ClO_3^-$ (half-life 3.07×10^5 years) as a tracer for NO_3^- is a reasonable proposition. The aim of the present inquiry was to explore this possibility during NO_3^- uptake by barley plants.

MATERIALS AND METHODS

Growth of Plants. Barley plants (*Hordeum vulgare* cv. Fergus) were grown as described previously (7) in modified Johnson's solution at one-tenth concentration with calcium sulfate and potassium sulfate replacing nitrates. Nitrate was provided at 10 μM. Previous work has shown that at this concentration there is no net nitrate flux into the plants, but that the nitrate uptake system is fully 'induced' (7). In all experiments, barley plants were 7 to 9 d old.

Net Flux Measurements. These were obtained by measuring depletion of the uptake solution containing 0.5 mM $CaSO_4$ + 0.25 mM K_2SO_4 + (0–0.5 mM) nitrate and/or chlorate. Disappearance of chlorate and nitrate was monitored using an HNU (HNU Systems Inc., MA) nitrate electrode. We have found that nitrate and chlorate are virtually indistinguishable by this method of analysis (Fig. 1, insert).

$^{36}ClO_3^-$ Synthesis. $^{36}ClO_3^-$ was prepared by the electrolysis of $^{36}Cl^-$ and the products were separated using TLC. $^{36}ClO_3^-$ was counted in a Triton X-based scintillation fluid by means of a Searle Isocap scintillation counter (8).

Efflux Analysis. Barley plants were loaded for 6 h in 0.1 mM ClO_3^- + 0.25 mM K_2SO_4 + 0.5 mM $CaSO_4$ labeled with $^{36}ClO_3^-$ (final specific activity, 0.013 μCi/μmol). The unlabeled medium contained 0.1 mM KNO_3 + 0.25 mM K_2SO_4 + 0.5 mM $CaSO_4$. After loading, the roots were briefly rinsed in distilled H_2O to prevent carry-over of label from $^{36}ClO_3^-$ loading solution and then

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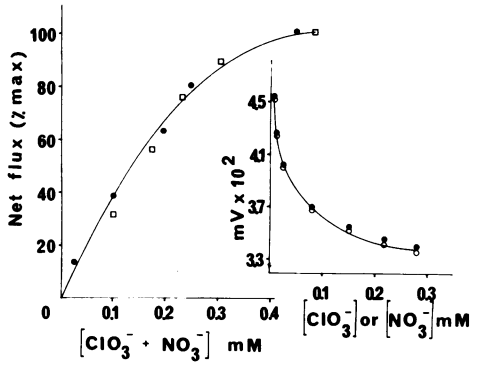

FIG. 1. Net nitrate and (nitrate plus chlorate) flux into barley roots. (●), net nitrate flux at 0.05, 0.1, 0.2, 0.25, and 0.4 mM NO_3^-. (□), net anion flux at 0.1 mM NO_3^- and 0.175 mM NO_3^- + 0, 0.05, 0.13, 0.28 mM ClO_3^-. The maximum net flux was 16 μmol g^{-1} fresh weight h^{-1}. Insert shows response of nitrate electrode to different chlorate (○, □) and nitrate (●, ■) concentrations.

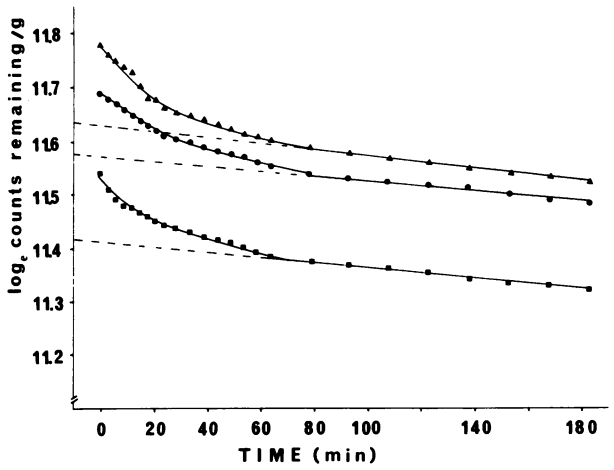

FIG. 2. Time course of $^{36}ClO_3^-$ efflux from barley roots loaded with 0.1 mM [^{36}Cl]chlorate for 6 h. Regression lines were drawn for the slow phases for three experiments with r^2 values = 0.99 (▲), 0.98 (●), 0.98 (■), respectively, and $t_{1/2}$ = 17.3 (▲), 20.2 (●), and 20.2 (■) h, respectively.

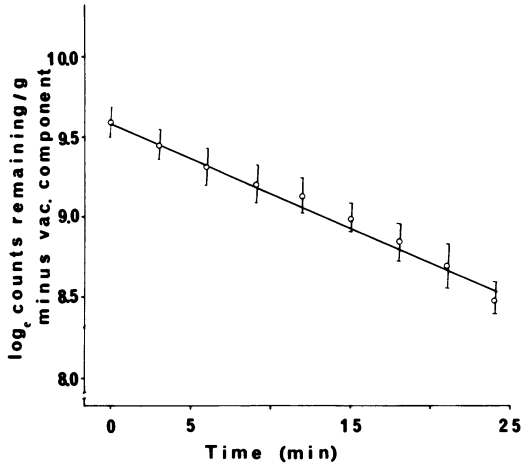

FIG. 3. Time course of $^{36}ClO_3^-$ efflux from barley after subtraction of slow phase. (○), data pooled from the three experiments shown in Figure 2. SE indicated by bar lines. The regression line with r^2 = 0.97 gave a calculated value for $t_{1/2}$ = 17.3 min.

subjected to repeated washes with 10 ml unlabeled medium. ^{36}Cl remaining in the roots was calculated after subtraction of the total counts which appeared in the 'washout' solution from that present in the tissue initially. The assumption made, in the present context, is that there was a negligible contribution by [^{36}Cl]chlorite (resulting from chlorate reduction) to the observed efflux of label. This assumption is based on the argument, in the particular experimental regime used here, that nitrate reduction is less than 10% of nitrate uptake (7). The assumption was checked by TLC of the activity remaining in the tissue at the end of the experiment, which revealed that the major activity cochromatographed with authentic $^{36}ClO_3^-$. The following equations were used to calculate various flux components:

$$\phi_{oc} = \frac{I_{v/t} + I_c \cdot k_c}{S_o}$$

$$\phi_{co} = \frac{I_c \cdot k_c}{S_o} + k_v \cdot Q_v$$

$$\phi_{cv} = \phi_{co} \frac{(I_{v/t})}{I_c \cdot k_c}$$

$$\phi_{vc} = \phi_{cv} - (\phi_{oc} - \phi_{co})$$

$$Q_c = \frac{(\phi_{co} + \phi_{cv})}{k_c}$$

where ϕ_{oc} = flux from external solution to cytoplasm, ϕ_{co} = flux from cytoplasm to external solution, ϕ_{cv} = flux from cytoplasm to vacuole, ϕ_{vc} = flux from vacuole to cytoplasm, Q_c = content of cytoplasmic compartment, Q_v = content of vacuole (about 90% tissue content at the end of the experiment), k_c = rate constant for exchange of cytoplasmic compartment (Fig. 3), k_v = rate constant for exchange of vacuole compartment (Fig. 2), I_c = apparent isotope content of cytoplasm, I_v = isotope content of vacuole after exchange of isotope in cytoplasm, S_o = specific activity of external solution during isotope uptake, t = duration of exposure of tissue to isotope solution. Derivations of these equations have been described in detail elsewhere (4, 19, 20).

Desorption Analysis. Efflux of label from the barley roots was measured at 2°C after a 30-min loading period at 27°C in 0.5 mM KNO_3 labeled with $^{36}ClO_3^-$ at a specific activity of 0.013 μCi/μmol.

$^{36}ClO_3^-$ Influx. The radioactivity taken up by the roots of intact plants was measured after a 10-min influx period at 27°C followed by a 5-min desorption at 2°C. The uptake medium contained 0.5 mM $CaSO_4$, 0.25 mM K_2SO_4 plus various nitrate or chlorate concentrations labeled with $^{36}ClO_3^-$ to give final specific activity of 0.6 μCi/mmol. After desorption, excess solution was removed from roots by a short spin in a basket centrifuge, and then root samples were weighed into scintillation vials. The external channels ratio method was used to derive a quench curve, *i.e.*, ^{36}Cl counting efficiency as a function of quenching associated with different root mass (0.2–1.0 g). All activity measurements were subsequently corrected for quenching.

FCCP[2] Inhibition of $^{36}ClO_3^-$ Influx. FCCP was dissolved in 10% (v/v) acetone/water solution and added to uptake and desorption solutions at a concentration of FCCP ranging between 0 and 0.5 μM. The concentration of acetone was only 0.005% (v/v) even at the highest FCCP concentration used, but to allow for any possible side effects of acetone it was included at this concentration in control experiments.

$K^+/^{86}Rb^+$ Uptake. The uptake medium contained 0.5 mM $CaSO_4$ + various concentrations of KNO_3 or $KClO_3$ (0 to 0.5 mM).

[2] Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

$^{86}Rb^+$ was added, and a 10-min influx period at 27°C was followed by a 5-min wash in unlabeled solution at 2°C. The roots were excised, and excess solution was removed by a short spin using a basket centrifuge and weighed into glass vials. After washing for 16 h at 500°C, 10 ml of H_2O were added to each sample, and $^{86}Rb^+$ activity was measured by Čerekov counting in a Searle Isocap/300 scintillation counter.

RESULTS AND DISCUSSION

As already stated in the introduction, there is evidence in the literature to suggest that plant cells fail to discriminate between nitrate and chlorate at both the uptake and reduction steps (5, 9, 12, 15, 25, 27). It was of interest that the nitrate electrode also failed to distinguish between nitrate and chlorate (Fig. 1, insert). Moreover, the potential difference (*i.e.* mv reading) depended on the total anion concentration and did not vary with different ClO_3^-/NO_3^- ratios (C. E. Deane-Drummond, unpublished results). Barley roots also appeared not to be able to discriminate between these ions since the total net anion flux (chlorate plus nitrate) was dependent on the sum of the anion concentration and independent of their ratio. The kinetics for this total anion uptake were identical with those obtained when NO_3^- alone was used. At the highest chlorate concentrations, there was a decrease in uptake after about 2 to 3 h, which suggests that some toxic effects become important after this time (C. E. Deane-Drummond and A. D. M. Glass, unpublished results). The apparent lack of discrimination between NO_3^- and ClO_3^- both in the case of the NO_3^- electrode and the barley plants may indicate that the hydrated molecular

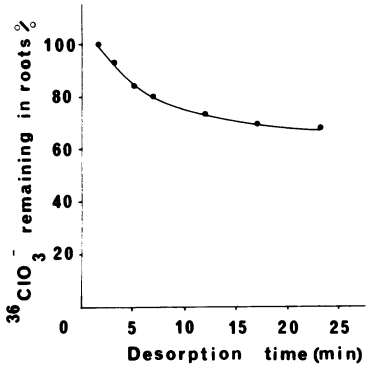

FIG. 4. Time course of desorption of barley roots at 2°C after 0.5 h loading at 27°C in 0.5 mM KNO_3 + 0.25 mM K_2SO_4 + 0.5 mM $CaSO_4$ + [^{36}Cl]chlorate. The desorption medium contained 0.5 mM $CaSO_4$. Points are means of three replicates; SE < dimensions of points.

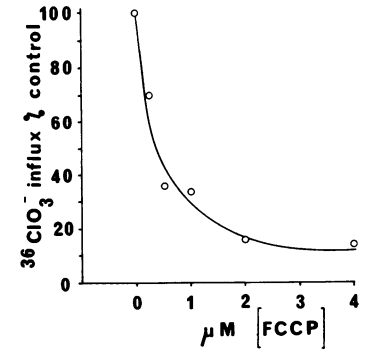

FIG. 5. The effect of FCCP on [^{36}Cl]chlorate labeled nitrate uptake into barley seedlings. The uptake medium contained 0.5 mM KNO_3 + 0.25 mM K_2SO_4 + 0.5 mM $CaSO_4$ + [^{36}Cl]chlorate + FCCP. All points are means of three replicate; SE <10% mean.

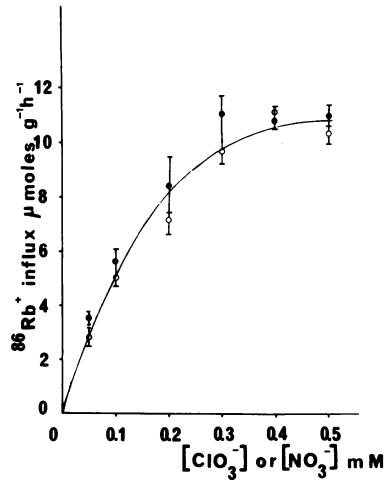

FIG. 6. The effect of chlorate on $^{86}Rb^+/K^+$ uptake into barley seedlings. The uptake solution contained 0.5 mM $CaSO_4$ + KNO_2 (●) or $KClO_3$ (○) at 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM. Points are means of three replicates; SE shown by bar lines.

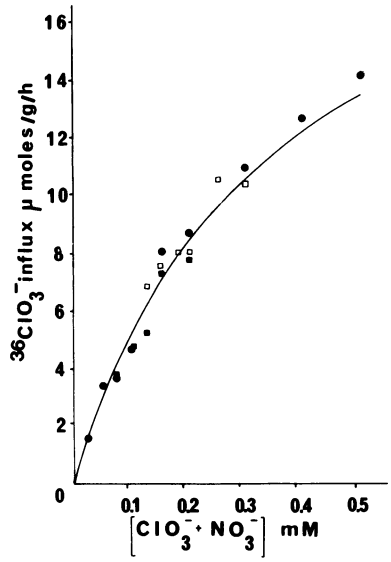

FIG. 7. Influx of $^{36}ClO_3^-$ chlorate as a function of total anion concentration with different proportions of NO_3 + ClO_3^-. The line of best fit was drawn through all the points, using the kinetic constants (K_m = 0.308 ± 0.07 mM, V_{max} = 21.5 ± 2.4 μmol g^{-1} h^{-1}) which were calculated from a Hofstee plot of the data (r^2 = 0.70). (●), NO_3^- + $^{36}ClO_3^-$ (<5 μM); (■), NO_3^- + 0.05 mM ClO_3^- + $^{36}ClO_3^-$; (□), NO_3^- + 0.1 mM ClO_3^- + $^{36}ClO_3^-$. All points are means of three replicates; SE <10% of the mean.

dimensions of these ions are very similar. ClO_3^- is usually considered to be pyramidal in configuration, while NO_3^- is planar; however, there is some evidence that in oxyacids Cl—O bonding also shows some planar character (17). Efflux of labeled chlorate from barley seedlings showed classical 'washout' kinetics thought to represent the emptying of the cytoplasmic and vacuolar phases (18–20). We are well aware that the results of such an analysis need to be treated with caution (18) in view of the assumptions made in the calculations of the various flux components (18, 20). In the present work, roots were left intact during efflux so that possible artifacts caused by cutting (18) were eliminated. Bearing in mind the limitations of this technique, the fluxes derived from such an analysis seem to be reasonably close to expectation. The following parameters were derived from the equations given in

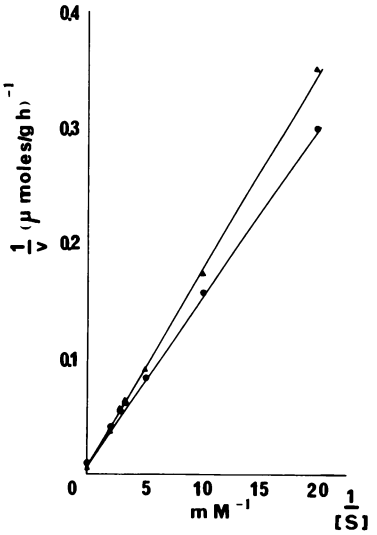

FIG. 8. Lineweaver-Burk plot demonstrating competition between ClO_3^- and NO_3^- for $^{36}ClO_3^-$ labeled nitrate uptake into barley seedlings. r^2 values for regression were 0.954 and 0.988, respectively, for NO_3^- alone (●) and ClO_3^- (0.15 mM) + NO_3^- (▲). Data points were means of three replicate determinations.

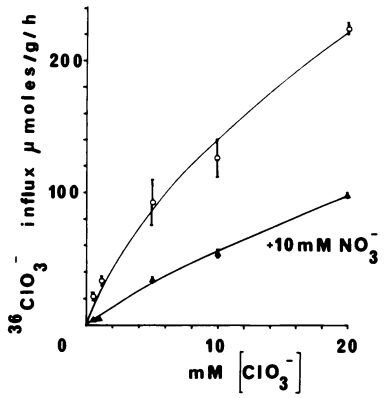

FIG. 9. Competition between NO_3^- and $^{36}ClO_3^-$ labeled chlorate uptake into barley seedlings at 0.5, 1.0, 5.0, 10.0, and 20.0 mM chlorate. (○), chlorate alone; (▲), chlorate + 10 mM NO_3^-. All points are means of three replicates; SE indicated by bar lines.

"Materials and Methods" and Figures 2 and 3:

Time for half exchange of vacuolar component:

$$t_{1/2v} = \log e\ 2/k_v = 19.9 \text{ h}$$

Time for half exchange of cytoplasmic component:

$$t_{1/2c} = \log e\ 2/k_c = 17.3 \text{ min}$$

Cytoplasmic pool size

$$Q_c = 8.19\ \mu\text{mol g}^{-1} \text{ fresh weight root}$$

On the basis of these estimates and the kinetics of $^{36}ClO_3^-$ efflux during desorption at 2°C (Fig. 4), a 10-min influx and 5-min cold wash was selected for further experiments. This short-term influx should give a reasonable estimate of plasmalemma flux (4).

The following were also calculated from the data.

$$\phi_{oc} = 16.59\ \mu\text{mol g}^{-1} \text{ fresh weight h}^{-1}$$

$$\phi_{co} = 12.19\ \mu\text{mol g}^{-1} \text{ fresh weight h}^{-1}$$

$$\phi_{vc} = 3.48\ \mu\text{mol g}^{-1} \text{ fresh weight h}^{-1}$$

$$\phi_{cv} = 7.88\ \mu\text{mol g}^{-1} \text{ fresh weight h}^{-1}$$

The estimate for net flux (4.4 μmol g^{-1} fresh weight h^{-1}) is in good agreement with that obtained at this nitrate concentration (0.1 mM) using the nitrate depletion method (4.8 μmol g^{-1} fresh weight h^{-1} [Fig. 1]).

To check that the tracer flux was dependent on metabolic energy rather than a passive isotopic entry, we added FCCP at various concentrations to the influx medium. This compound has been widely used as an uncoupler of ion transport. Lin (16), *e.g.* has reported K_i values for inhibition of H^+ efflux, K^+, and Pi influx to be 0.5, 1.0, and 1.5 μM, respectively, in corn roots. Figure 5 demonstrates that $^{36}ClO_3^-$ influx is strongly inhibited by FCCP. The value for K_i (0.69 μM), calculated by linear regression of a plot of relative velocity against inhibitor concentration, is in the same order of magnitude as that obtained for ion fluxes in corn roots (16).

It is unlikely that toxic levels of ClO_2^- (arising from reduction of ClO_2^-) could accumulate during a 10-min influx period. However, to check for any side effects of ClO_3^- on ion transport, $^{86}Rb^+$ influx into barley seedlings was measured at different chlorate and nitrate concentrations (Fig. 6). There was clearly no significant depression of $^{86}Rb^+$ uptake even at an external concentration of 0.5 mM ClO_3^-. Thus, at least in the case of short-term experiments, any toxic effects of chlorate or chlorite on ion transport are unlikely to be of any significance. Furthermore, when $^{36}ClO_3^-$ is used as a tracer for NO_3^-, the absolute concentration of chlorate in the uptake medium is <0.5 μM.

A series of experiments was carried out using different combinations of chlorate and nitrate in the uptake medium labeled with $^{36}ClO_3^-$ (see "Materials and Methods" for details). $^{36}ClO_3^-$ influx into barley roots was calculated treating NO_3^- and ClO_3^- as equivalent in the determination of $^{36}ClO_3^-$ specific activity. Influx values at various nitrate or nitrate plus chlorate concentrations were essentially identical. The data obtained in this way were pooled to obtain a single Hoffstee plot from which the kinetic constants K_m and V_{max} were derived by linear regression. These constants were used to draw a single influx isotherm (Fig. 7). It is evident that all data fit this curve. These results imply that NO_3^- and ClO_3^- are taken up by the same transporter. Competition between these ions was further emphasized by treating chlorate, in the presence of nitrate, as a competitive inhibitor, *i.e.* specific activity of $^{36}ClO_3^-$ was calculated using nitrate concentration alone. Results of one experiment, drawn as a Lineweaver-Burk plot (Fig. 8) show that there was a large increase in slope/intercept ratio in the presence of the competing ion (chlorate). A '*t*' test demonstrated significant differences of slopes at the 0.001 level of probability. The increase in slope with no increase in intercept is characteristic of competitive inhibition. Moreover, the K_i value, calculated assuming that there was competition between NO_3^- and ClO_3^-, was of the same order of magnitude as K_m (0.79 and 0.86 mM, respectively). It is worth noting that the K_m and V_{max} varied from one batch of plants to the next, and the value of these parameters is unlikely to be constant but rather to be dependent on the particular growth conditions and stage of development of the plants. It is therefore of obvious importance to include experimental controls at each stage. The most important point in the present context is the similarity between K_i and K_m. Epstein and Hagen (10) have considered that allowing for experimental error even a 2-fold difference in these parameters for $^{86}Rb^+$ was within an acceptable range for validation of the hypothesis that there was no discrimination between these ions by K^+ transport carriers in barley plants.

The competition between nitrate and chlorate in the high concentration range (0.5 to 20 mM) is shown in Figure 9. The effect of NO_3^- on $^{36}ClO_3^-$ influx was selected since any reduction in NO_3^- influx by high ClO_3^- concentrations might be attributed to toxic effects of chlorate. There was negligible uptake of $^{36}ClO_3^-$ at 0.5 mM ClO_3^- + 10 mM NO_3^-, but at 10 mM ClO_3^- + 10 mM

NO_3^- uptake of $^{36}ClO_3^-$ was near to 50% of that obtained using 10 mM ClO_3^- alone. These results are consistent with competition between NO_3^- and ClO_3^-. More detailed analysis of these results was not appropriate because of the complex nature of the influx isotherm over this concentration range. The uptake of chlorate at an external concentration of 20 mM is higher than might be expected in comparison with that found in the presence of 10 mM NO_3^-, but this may be due to some side effects of ClO_3^- or ClO_2^- at this high concentration.

Doddema and Telkamp (9) have studied the competition between ClO_3^- and NO_3^- for uptake into *Arabidopsis thaliana*. They found that in the high concentration range (2.5 mM) there was a marked reduction in chlorate uptake in the presence of 2.5 mM NO_3^-. These results are consistent with competitive effects. The effect of chlorate on nitrate uptake was more difficult to interpret, since the rate of nitrate uptake appeared to be increased by low chlorate (a decrease in K_m) and at higher concentrations ClO_3^- also stimulated the release of a substance absorbing at 208 nm (presumed to be nitrate). In spite of these anomalies, the authors concluded that chlorate and nitrate were taken up by the same 'carrier system.' Their results may have been complicated by stimulation of nitrate efflux by chlorate. Since nitrate-starved plants were used for the present investigation, there was no possible interference by changes in NO_3^- efflux. Influx times for estimates of plasmalemma flux were sufficiently short that recycling of label to the external solution was unlikely.

In conclusion, the results described here give clear evidence to support the hypothesis that there is little discrimination between NO_3^- and ClO_3^- influx into barley roots in short-term experiments. Thus, $^{36}ClO_3^-$ can be used as a tracer for NO_3^- and provides a sensitive and rapid method for the exploration of nitrate uptake in higher plants.

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