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Effects of Nitrite, Chlorate, and Chlorite on Nitrate Uptake and Nitrate Reductase Activity[1]

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ABSTRACT

Effects of NO_2^-, ClO_3^-, and ClO_2^- on the induction of nitrate transport and nitrate reductase activity (NRA) as well as their effects on NO_3^- influx into roots of intact barley (*Hordeum vulgare* cv Klondike) seedlings were investigated. A 24-h pretreatment with 0.1 mol m^{-3} NO_2^- fully induced NO_3^- transport but failed to induce NRA. Similar pretreatments with ClO_3^- and ClO_2^- induced neither NO_3^- transport nor NRA. Net ClO_3^- uptake was induced by NO_3^- but not by ClO_3^- itself, indicating that NO_3^- and ClO_3^- transport occur via the NO_3^- carrier. At the uptake step, NO_2^- and ClO_2^- strongly inhibited NO_3^- influx; the former exhibited classical competitive kinetics, whereas the latter exhibited complex mixed-type kinetics. ClO_3^- proved to be a weak inhibitor of NO_3^- influx (K_i = 16 mol m^{-3}) in a noncompetitive manner. The implications of these findings are discussed in the context of the suitability of these NO_3^- analogs as screening agents for the isolation of mutants defective in NO_3^- transport.

Most high-affinity solute transport systems in plants appear to be derepressible; when their solutes are withheld, transport rates increase severalfold (14, 22). Ion transport systems involved in the absorption of Cl^-, SO_4^-, $H_2PO_4^-$, K^+, Na^+ (see ref. 14 for review), and NH_4^+ (34) conform to this this pattern.

The HATS[2] for NO_3^- absorption is, therefore, exceptional in being substrate inducible as well as subject to negative feedback (6, 24, 31). Enzymes involved in nitrate assimilation, NR and NiR, are also substrate inducible.

The absence of a readily available radiotracer for NO_3^- has led to the widespread use of ClO_3^- as a NO_3^- analog in studies of NO_3^- uptake and assimilation in both plants and microorganisms (10). $^{36}ClO_3^-$, which can be generated by electrolysis of $^{36}Cl^-$, has provided a useful tracer for NO_3^-. However, at high concentrations (0.5 mol m^{-3} and above) and during prolonged exposures, ClO_3^- has proven to be toxic to most plants. Indeed, ClO_3^- was previously used extensively as a herbicide (16). This toxicity has commonly been ascribed to the greater toxicity of chlorite (ClO_2^-) produced by reduction of absorbed ClO_3^- by the enzyme NR (21 and references therein).

Hence, the toxic effects of ClO_3^- have been exploited by many researchers to screen for two classes of mutants: those defective in NO_3^- transport and those defective in NRA. Both of these mutants should survive exposure to ClO_3^-. It is interesting that, although mutations of the second category have commonly been obtained, virtually none of the first category has been isolated. As part of a study directed to the isolation of NO_3^- transport mutants, we have examined the capacity of various transport analogs (NO_2^-, ClO_3^-, and ClO_2^-) to induce transport of NO_3^- and to induce NR. In addition, we have examined the interactions between these ions and NO_3^- at the transport step through classical competitive kinetics.

The results of these experiments demonstrate that ClO_3^- is an exceedingly poor analog of NO_3^-. The affinity of the nitrate HATS for ClO_3^- is so low that extremely high concentrations of ClO_3^- must be used to bring about toxic effects, particularly in tissue culture systems in which ambient NO_3^- concentrations are already high. As a consequence, ClO_3^- will enter tissues largely via the low-affinity constitutive nitrate transporter (31). Thus, lesions in the HATS rarely render the genotype immune to the toxic effects of ClO_3^-, and such mutants are not isolated. Rather, genotypes defective in NRA are isolated, supporting the contention that ClO_2^- rather than ClO_3^- is the effective toxin. Notwithstanding these observations, Cove (8) demonstrated that in *Aspergillus* the toxic effects of ClO_3^- were not necessarily related to the extent of induction of NR.

The results of the present study are discussed in the context of the regulation of nitrate uptake and nitrate reduction by nitrate and its analogs.

MATERIALS AND METHODS

Plant Growth

Seeds of barley (*Hordeum vulgare* L. cv Klondike) were germinated in moist sand in the dark for 3 d. Seedlings were then transferred to Plexiglas tanks (25- to 40-L capacity) containing appropriate nutrient solutions (1/80 modified Johnson's solution) (30). The concentrations of nutrients were maintained by continuous infusion of appropriate stock solutions as described by Siddiqi et al. (30). The plants were maintained and the experiments were performed in a controlled environment room at 20 ± 2°C, 16-h light/8-h dark cycle, and 70% RH. The light was provided by fluorescent

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[2] Abbreviations: HATS, high-affinity transport system; NR, nitrate reductase; NiR, nitrite reductase; NRA, nitrate reductase activity; NiRA, nitrite reductase activity; LATS, low-affinity transport system; $[NO_2^-]_i$ and $[NO_3^-]_i$ ($[NO_2^-]_o$ and $[NO_3^-]_o$), internal (external) concentrations of NO_2^- and NO_3^-, respectively; $[NO_2^-]_c$, cytoplasmic NO_2^- concentration.

tubes (300 $\mu E\ m^{-2}\ s^{-1}$) with a spectral composition similar to sunlight. All experiments were repeated.

Induction of Uptake and Reduction of NO_3^-

Plants were grown in 1/80-strength modified Johnson's nutrient solution (pH 6) without nitrogen for 3 d. They were then transferred for 1 d to 1/80-strength modified Johnson's solution containing 0.1 mol m^{-3} NO_3^-, NO_2^-, ClO_3^-, or ClO_2^- or none of these. In plants so pretreated, nitrate ($^{13}NO_3^-$) influx was measured from 1/80-strength modified Johnson's solution containing 0.1 mol m^{-3} NO_3^-. In these plants, in vivo and in vitro NRAs were also measured (see below for the procedures).

Interactions at the Uptake Step

Plants were grown in 1/80-strength modified Johnson's solution (pH 6) without nitrogen for 3 d. They were then transferred to 1/80-strength modified Johnson's solution containing 0.1 mol m^{-3} NO_3^- for 1 d. In these plants, nitrate ($^{13}NO_3^-$) influx was measured from 1/80-strength modified Johnson's solution containing various concentrations of NO_3^- ($[NO_3^-]_o$) with or without NO_2^-, ClO_3^-, or ClO_2^- at a fixed concentration, as indicated.

Measurement of Influx and Net Flux

Influx of NO_3^- was measured by exposing intact roots to the appropriate solutions (pH 6.0), labeled with ^{13}N, for 10 min. Before the exposure to radioactive solutions, roots were prewashed for 5 min in an identical but nonradioactive solution. Influx was followed by a 2-min wash of intact roots in an identical but nonradioactive solution to remove $^{13}NO_3^-$ from the free space (30). Roots were then excised and counted in a Packard γ-counter (Minaxi, Auto-γ 500 series). Influx values given in 'Results' are means of three to four replicates, each replicate consisting of about 10 seedlings.

Net uptake was measured by the disappearance of the given ion from the external solution during times lasting from minutes to hours.

In Vivo NRA

In vivo NRA in roots was assayed by measuring NO_2^- production under anaerobic conditions (12). Briefly, excised roots were incubated in potassium phosphate buffer (pH 7.7) containing 100 mol m^{-3} NO_3^-, maintained at 25°C for 20 min. Before the transfer of roots to anaerobic conditions, the buffer was purged with He for 5 min. At the end of the incubation period, the tubes were transferred to a boiling water bath for 10 min to extract NO_2^- from the roots. NO_2^- content was measured spectrophotometrically: the color reaction was produced by adding 1 mL of 1% (w/v) sulfanilamide (in 1 N HCl) and 1 mL of 0.02% (w/v) *N*-1-napthylenediamine dihydrochloride (in water) and incubating for 30 min. Absorbance was then measured at 540 nm. In the case of NO_2^--pretreated plants, the NO_2^- concentrations of plants, before anaerobic incubation, were determined and subtracted from the values of test plants (i.e. after anaerobic incubation). Details of the procedure are described by King et al. (18).

In Vitro NRA

The procedure of Long and Oaks (25) was followed. Root samples were ground in liquid nitrogen and then in Tris-HCl buffer (25 mol m^{-3}, pH 8.5) containing EDTA (1 mol m^{-3}), flavin adenine dinucleotide (20 mmol m^{-3}), BSA (1%, w/v), DTT (1 mol m^{-3}), cysteine (10 mol m^{-3}), and chymostatin (stock solution dissolved in DMSO, final concentration 10 mmol m^{-3}). The extract was filtered through a nylon mesh and then centrifuged at 10,000*g* for 20 min at 4°C. In the supernatant, NRA was measured in an assay mixture that contained 0.2 mL of Hepes buffer (0.65 M, pH 7.0), 0.2 mL of KNO_3 (0.1 M), 0.6 mL of water, and 0.4 mL of enzyme extract. The reaction was started by adding 0.1 mL of NADH (3.6 mg mL^{-1}) with mixing. The tubes were incubated at 28°C for 30 min, after which the reaction was stopped by transferring the tubes to a boiling water bath for 10 min. After the tubes were cooled to 28°C, 0.1 mL of pyruvic acid (5.3 mg mL^{-1}) and 2 μL of lactate dehydrogenase suspension (Boehringer-Mannheim, Montreal, PQ, Canada) were added to each sample, which was incubated for 10 min to oxidize residual NADH. NO_2^- concentrations were measured spectrophotometrically as described above.

Measurement of Ion Concentrations

The concentrations of NO_3^-, NO_2^-, ClO_3^-, and ClO_2^- in the solutions were measured by ion chromatography (Dionex Corp., Sunnyvale, CA). In the case of plant tissues, NO_3^- and NO_2^- were extracted in deionized distilled water at 90°C for 30 min. NO_3^- and NO_2^- concentrations of the extract were measured spectrophotometrically: NO_3^- by the procedure of Cataldo et al. (5) and NO_2^- as described in the preceding sections.

Production and Purification of $^{13}NO_3^-$

$^{13}NO_3^-$ was produced by proton irradiation of H_2O on the TRIUMF-ACEL CP42 cyclotron using 20 MeV protons (30). The contaminants ^{18}F, $^{13}NO_2^-$, and $^{13}NH_4^+$ were removed as described by Siddiqi et al. (30) and Glass et al. (15).

RESULTS

Induction of $^{13}NO_3^-$ Influx and NR

$^{13}NO_3^-$ influx was effectively induced by pretreatment with either NO_3^- or NO_2^- (Table I). However, pretreatments with NO_2^- failed to induce NRA (Table I). Table II shows the tissue NO_3^- and NO_2^- contents of NO_3^-- or NO_2^--pretreated plants. By contrast, ClO_3^- and ClO_2^- pretreatments failed to induce either NO_3^- uptake or NRA (Table I). However, although ClO_3^- uptake was induced by pretreatment with NO_3^-, ClO_3^- pretreatment failed to induce ClO_3^- uptake: plants pretreated with NO_3^- for 1 d took up ClO_3^- from 0.1 mol m^{-3} $[ClO_3^-]_o$ at the rate of 1.37 $\mu mol\ g^{-1}\ h^{-1}$ (Fig. 1), whereas in plants pretreated with ClO_3^- for 1 d, net ClO_3^- uptake (measured by ClO_3^- depletion of the media) was not detectable even after 4 h had elapsed.

Table I. *Induction of $^{13}NO_3^-$ Influx and NRA by Pretreatment with 0.1 mol m^{-3} NO_3^-, NO_2^-, ClO_3^-, or ClO_2^- for 1 d*

$^{13}NO_3^-$ influx was measured from uptake solutions containing 0.1 mol m^{-3} NO_3^- (see text).

Pretreatment	$^{13}NO_3^-$ Influx	NRA	
		In vivo	In vitro
	μmol g⁻¹ fresh wt h⁻¹	*μmol NO₂⁻ g⁻¹ fresh wt h⁻¹*	
NO_3^-	9.55 ± 0.06	1.43 ± 0.11	1.78 ± 0.06
NO_2^-	10.86 ± 0.48	0.003 ± 0.02	0.30 ± 0.17
ClO_3^-	0.29 ± 0.01	0.34 ± 0.02	0.11 ± 0.05
ClO_2^-	0.33 ± 0.02	0.33 ± 0.01	0.19 ± 0.04
None	0.22 ± 0.01	0.65 ± 0.03	0.24 ± 0.07

Interactions at the Uptake Step

Inhibition of $^{13}NO_3^-$ Influx by NO_2^-

The presence of NO_2^- in the influx medium inhibited NO_3^- influx in a competitive manner: increasing $[NO_2^-]_o$ increased K_m for NO_3^- influx with little effect on V_{max} (Fig. 2, Table III). Inhibition constants (K_i), calculated according to the kinetics of competitive inhibition, were found to range from 115 to 134 mmol m^{-3} NO_2^-.

Inhibition of $^{13}NO_3^-$ Influx by ClO_3^-

The presence of up to 1 mol m^{-3} ClO_3^- had little effect on $^{13}NO_3^-$ influx: neither V_{max} nor K_m showed any significant change. A higher ClO_3^- concentration (5 mol m^{-3}), however, reduced the V_{max} for $^{13}NO_3^-$ influx by approximately 25% without altering K_m values (Fig. 3, Table IV). The K_i value, calculated according to the kinetics of noncompetitive inhibition, was approximately 16 mol m^{-3}.

In a separate experiment, rates of net uptake of ClO_3^- and NO_3^- were compared (Table V). Net ClO_3^- uptake appeared to saturate the transport system at approximately 0.25 mol m^{-3} $[ClO_3^-]_o$; at 0.5 mol m^{-3} $[ClO_3^-]_o$, the rate was substantially higher and did not fall on the saturation curve (Fig. 1). This corresponds to the expression of the HATS for ClO_3^- uptake at low external ClO_3^- and the LATS at $[ClO_3^-]$ >0.25 mol m^{-3}. V_{max} for net ClO_3^- uptake was approximately 18% of the V_{max} for NO_3^- uptake, and the K_m for ClO_3^- uptake was about twice that for NO_3^- (Table V).

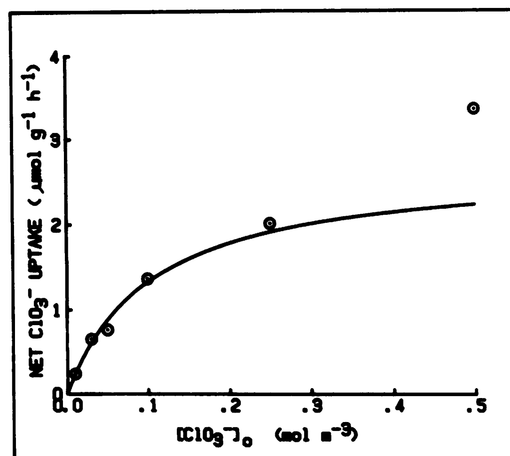

Figure 1. Rates of net ClO_3^- uptake into plants pretreated with 0.1 mol m^{-3} NO_3^- for 1 d (see text) at varying $[ClO_3^-]_o$ in the range of 0 to 0.5 mol m^{-3}. The kinetic parameters V_{max} and K_m, determined from Eadie-Hofstee plots, are given in Table V.

Inhibition of $^{13}NO_3^-$ Influx by ClO_2^-

The presence of ClO_2^- in the medium substantially decreased V_{max} and increased K_m for $^{13}NO_3^-$ influx (Fig. 4, Table VI).

DISCUSSION

NO_3^-/NO_2^- Interactions

Induction

It is generally held that the induction of the HATS for NO_3^- and the enzyme responsible for the reduction of NO_3^- (NR) depend in an obligate fashion on the flux of NO_3^- to the particular tissue. In some instances, the extent of induction of the HATS and NR were strongly correlated with the level of NO_3^- accumulation in the tissue (3, 30). In the study by Aslam et al. (3), NO_2^- pretreatment at relatively high concentrations (0.5 mol m^{-3}) induced NR in detached barley leaves, but according to the authors, only as a result of the formation and accumulation of NO_3^- in the tissue. They showed further that the quantitative relationship between $[NO_3^-]_i$ and the induction of NR was maintained irrespective

Table II. *NO_3^- and NO_2^- Concentrations of Roots and Shoots (μmol g^{-1} fresh weight) of Plants Pretreated with Nutrient Solutions Containing 0.1 or 0.5 mol m^{-3} NO_2^- or 0.1 mol m^{-3} NO_3^- for 1 d*

Control plants remained in nutrient solution without N (see text).

Pretreatment Solutions	NO_2^-		NO_3^-	
	Roots	Shoot	Roots	Shoot
	μmol g⁻¹ fresh wt			
Control	ND[a]	ND	2.30 ± 0.90	2.43 ± 0.96
0.1 mol m^{-3} NO_2^-	0.140 ± 0.009	0.008 ± 0.001	1.36 ± 0.12	2.70 ± 0.07
0.5 mol m^{-3} NO_2^-	0.733 ± 0.112	0.009 ± 0.001	1.21 ± 0.10	3.83 ± 0.13
0.1 mol m^{-3} NO_3^-	0.030 ± 0.001	0.010 ± 0.001	46.9 ± 2.1	39.13 ± 6.50

[a] ND, Not done.

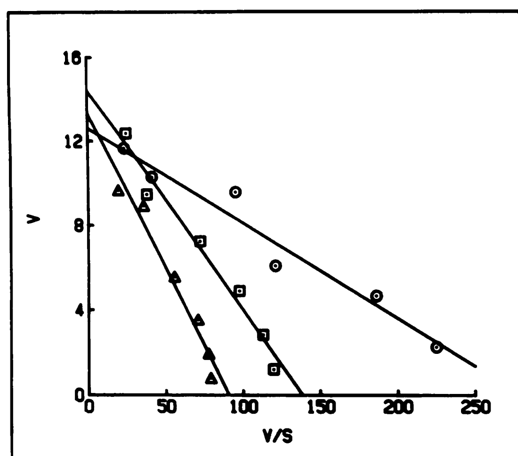

Figure 2. Eadie-Hofstee plots of $^{13}NO_3^-$ influx isotherms in the presence of 0 (⊙), 0.1 (⊡), or 0.3 (△) mol m^{-3} NO_2^- in the uptake solution (see Table III). Plants were pretreated with 0.1 mol m^{-3} NO_3^- for 1 d (see text).

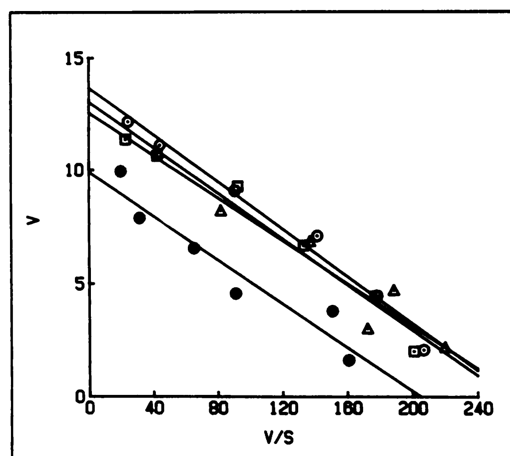

Figure 3. Eadie-Hofstee plots of $^{13}NO_3^-$ influx isotherms in the presence of 0 (⊙), 0.1 (△), 1 (⊡), or 5 (●) mol m^{-3} ClO_3^- in the uptake solution (see Table IV). Plants were pretreated with 0.1 mol m^{-3} NO_3^- for 1 d (see text).

of whether N was supplied as NO_3^- or NO_2^-. In our study, by contrast, NO_2^- pretreatment fully induced NO_3^- uptake (but not NR) (Table I) without any appreciable accumulation of either NO_2^- or NO_3^- (Table II). In fact, in these plants, $[NO_3^-]_i$ was similar to that of uninduced plants. Moreover, although NO_3^-- and NO_2^--pretreated plants exhibited a similar level of induction of NO_3^- uptake (Table I), their $[NO_3^-]_i$ values were very different: 46.9 and 1.36 μmol g^{-1} fresh weight, respectively. A potential role for some product of NO_2^- reduction in the induction of NO_3^- uptake can immediately be discounted because pretreatment with NH_4^+ failed to induce NO_3^- uptake (data not shown).

Warner and Huffaker (35) demonstrated that NR double mutants (defective in both NADH- and NADPH-dependent NR) of barley were virtually indistinguishable from the wild type in the rapidity and extent of induction of net NO_3^- uptake. Hence, as a minimum, it is apparent that NO_3^- itself is capable of inducing NO_3^- uptake.

The present experiments indicate that NO_2^-, too, may be an effective inducer. In NO_2^--pretreated roots, $[NO_2^-]_i$ increased from undetectable levels to 0.14 μmol g^{-1} fresh weight. If all of this NO_2^- were present in the cytoplasm, this would represent a $[NO_2^-]_c$ of 2.8 mol m^{-3}, on the basis of the assumption that the cytoplasm occupies approximately 5% of the cell (23). It is, therefore, possible that $[NO_2^-]_c$ was increased to a level sufficient to induce NO_3^- uptake. Unkles et al. (33) reported that in *Aspergillus nidulans* the *crnA* gene product (responsible for encoding the NO_3^- transporter) is subject to induction by either NO_3^- or NO_2^-.

It might be argued that $[NO_3^-]_c$ increased because of the formation of NO_3^- from NO_2^- (2, 3); a small increase sufficient to increase $[NO_3^-]_c$ substantially (e.g. 0.1–0.2 μmol g^{-1} root fresh weight) would be hardly detectable in the whole tissue analysis against a background $[NO_3^-]_i$ of approximately 1 μmol g^{-1} in uninduced plants. However, we are unable to accept this interpretation because NR was not induced under these conditions.

The fact that NO_2^- pretreatment selectively induced NO_3^- uptake but not NR provides further evidence for the now prevalent view that NO_3^- uptake and NO_3^- reduction are independent processes (6, 17, 35). The earlier evidence was based on the observations that NO_3^- induced NO_3^- uptake even when NRA was absent, as in NR mutants (35) or

Table III. *Effect of NO_2^- in the Influx Media on V_{max} and K_m for $^{13}NO_3^-$ Influx in the Concentration Range from 0 to 0.5 mol m^{-3} NO_3^-*

V_{max} and K_m were estimated from Eadie-Hofstee plots (r^2 values are for linear regressions). Also shown are inhibition constants (see text). Plants were pretreated with 0.1 mol m^{-3} NO_3^- for 1 d (see text) to induce $^{13}NO_3^-$ influx.

$[NO_2^-]_o$	V_{max}	K_m	r^2	K_i
mol m^{-3}	*µmol g^{-1} fresh wt h^{-1}*	*mmol m^{-3}*		*mmol m^{-3}*
0	12.60	45.0	0.95	
0.1	14.40	84.0	0.97	115
0.3	13.30	146.0	0.96	134

Table IV. *Effect of the Presence of ClO_3^- in the Uptake Medium on V_{max} and K_m for the Influx of Nitrate in the Range of 0 to 0.5 mol m^{-3} NO_3^-*

V_{max} and K_m were estimated from Eadie-Hofstee plots (r^2 for linear regressions are given). Inhibition constants (K_i), where appropriate, are also given (see text). Plants were pretreated with 0.1 mol m^{-3} NO_3^- for 1 d (see text).

$[ClO_3^-]_o$	V_{max}	K_m	r^2	K_i
mol m^{-3}	*µmol g^{-1} fresh wt h^{-1}*	*mmol m^{-3}*		*mol m^{-3}*
0	13.01	50	0.97	
0.1	13.65	52	0.98	
1	12.54	47	0.93	
5	9.91	48	0.91	15.98

Table V. *V_{max} and K_m for the Rates of Net Uptake of NO_3^- (in the range of 0 to 0.5 mol m^{-3}) and ClO_3^- (in the range of 0 to 0.25 mol m^{-3}) by Plants Pretreated with 0.1 mol m^{-3} NO_3^- for 1 d (see text)*

V_{max} and K_m were estimated by Eadie-Hofstee plots (r^2 for linear regressions are given).

Ion	V_{max}	K_m	r^2
	μmol g⁻¹ fresh wt h⁻¹	*mmol m⁻³*	
NO_3^-	14.63	52	0.97
ClO_3^-	2.69	102	0.90

Table VI. *Effect of the Presence of ClO_2^- in the Uptake Medium on V_{max} and K_m for the Influx of NO_3^- in the Range of 0 to 0.5 mol m^{-3}*

V_{max} and K_m were estimated from Eadie-Hofstee plots (r^2 for linear regressions are given).

$[ClO_2^-]_o$	V_{max}	K_m	r^2
mol m⁻³	*μmol g⁻¹ fresh wt h⁻¹*	*mmol m⁻³*	
0	11.46	42	0.94
0.04	9.33	45	0.99
0.4	7.17	77	0.98
0.65	4.53	225	0.89

tungstate-treated plants (17). These observations demonstrate the independence of NO_3^- uptake and NRA at the functional level. Likewise, the differential effects of NO_2^- on the induction of NO_3^- uptake and NRA suggest that these processes are independent at the transcriptional and/or translational level.

Transport

At the uptake step, NO_2^- inhibited NO_3^- influx in a classically competitive manner: the K_m for NO_3^- influx was increased with increasing $[NO_2^-]_o$ without a significant effect on the V_{max} (Fig. 2, Table III). The inescapable conclusion that NO_3^- and NO_2^- share the same transporter and the same binding site accords with a number of other reports (13, 32). Nevertheless, some algae appear to transport NO_3^- and NO_2^- by separate transporters (7, 32).

The toxic effect of NO_2^- accumulation within cells is well known (19). Given that NO_2^- uses the same carrier, NO_2^- pretreatments could potentially be used to isolate NO_3^- transport mutants. However, the level of NiRA is typically much higher (up to 30 times) than the rate of NO_2^- uptake (1). We observed that barley shoots exhibited no signs of toxicity at $[NO_2^-]_o$ up to 5 mol m^{-3}. Nevertheless, it may still be possible to use NO_2^- toxicity as a screening technique by selectively blocking the NiRA, e.g. by anoxia or using NiR mutants.

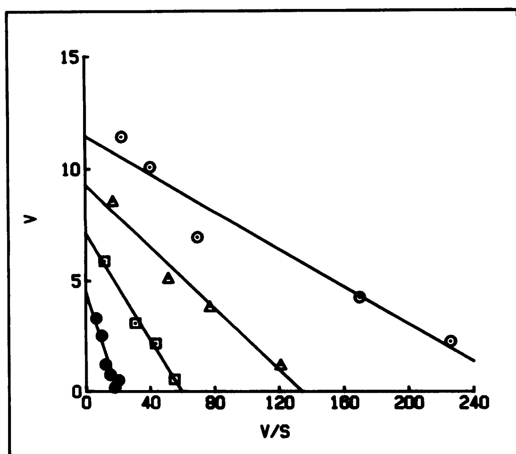

Figure 4. Eadie-Hofstee plots of $^{13}NO_3^-$ influx isotherms in the presence of 0 (⊙), 0.04 (△), 0.4 (⊡), or 0.65 (●) mol m^{-3} ClO_2^- (see Table VI). Plants were pretreated with 0.1 mol m^{-3} NO_3^- for 1 d (see text).

NO_3^-/ClO_3^- Interactions

Induction

It is evident that ClO_3^-, which apparently utilizes the same transporter as NO_3^- (10) and serves as a substrate for NR (4), cannot substitute for NO_3^- as an inducer of the NO_3^- transporter and NR at the transcriptional and/or translational level: neither NO_3^- uptake nor NRA were induced by ClO_3^- (Table I). Furthermore, when plants induced for 24 h with NO_3^- were transferred to ClO_3^- for another 24 h, the state of induction for NO_3^- uptake as well as for NO_3^- reduction was lost (data not shown). Our study, in agreement with that of McClure et al. (27), showed that ClO_3^- uptake was induced by NO_3^- but not by ClO_3^- itself (see "Results"). It may be noted, however, that in some cases ClO_3^- induced NO_3^- uptake (e.g. in *Chara corallina* [9]) and ClO_3^- uptake (in *Arabidopsis thaliana* [11]). Recently LaBrie et al. (21) reported that in *A. thaliana* ClO_3^- pretreatment increased the level of NR mRNA but not of NR protein. They have speculated that NR protein is synthesized but is inactivated by ClO_2^- (produced from ClO_3^- by NR) and that this inactivated NR is rapidly degraded. It is not clear, however, how ClO_2^- production and accumulation can occur to effect toxicity if NR is so rapidly inactivated as to be undetectable immunologically.

Transport

Competition between NO_3^- and ClO_3^- at the uptake step (Fig. 3, Table IV) revealed that at $[ClO_3^-]_o$ up to 1 mol m^{-3} there was little effect on NO_3^- influx. However, 5 mol m^{-3} $[ClO_3^-]_o$ substantially reduced the V_{max} for NO_3^- influx without altering the K_m. This type of inhibition, termed "pure noncompetitive inhibition," has been interpreted in terms of the inhibitor binding to the enzyme at a site different from the binding site for the substrate to form the enzyme-inhibitor complex or to the enzyme-substrate complex to form an inhibitor-enzyme-substrate complex (26). However, in a complex system such as transport into intact roots, the inhibitor may also have some indirect effects, e.g. by altering the membrane potential or affecting the characteristics of the plasma membrane in some way.

The K_i value (approximately 16 mol m^{-3}, Table IV) indicates

that ClO_3^- competes weakly with NO_3^-. This conclusion is further corroborated by the fact that the V_{max} for net ClO_3^- uptake by the HATS was <20% that for net NO_3^- uptake (Table V). The K_m for ClO_3^- uptake was twice that for NO_3^- uptake.

In several systems ranging from microbes to higher plants, ClO_3^- has been used to isolate mutants deficient in NO_3^- reduction and, in a limited number of cases, deficient in NO_3^- uptake (8, 20, 28, 29). It is noteworthy that, in all such studies, the $[ClO_3^-]_o$ used ranged from 10 to > 50 mol m^{-3}.

When screening for NR mutants, the aim is to get ClO_3^- into the cells, and elevated $[ClO_3^-]_o$ can be used without difficulty. However, the same is not true when screening for NO_3^- transport mutants. The transport of ClO_3^- (like NO_3^-) has been reported to be mediated by two distinct transport systems: a HATS, which saturates at <1 mol m^{-3} $[ClO_3^-]_o$, and a linear LATS, which operates at high $[ClO_3^-]_o$ (>1 mol m^{-3}) (16). It is apparent that, in the studies cited above, for the screening of ClO_3^--resistant mutants, the $[ClO_3^-]_o$ used (>10 mol m^{-3}) was well into the range of LATS. At these concentrations, the contribution of LATS to the total ClO_3^- uptake would be much greater than the V_{max} for HATS (16). It follows, therefore, that any ClO_3^- (NO_3^-) transport mutants obtained by the application of such high $[ClO_3^-]_o$ would have to be defective in LATS or in both of the transport systems. The only report in which it is claimed that mutants defective in HATS for NO_3^- were obtained is that of Ruiz et al. (29), who used 10 mol m^{-3} ClO_3^- as a screening method.

NO_3^-/ClO_2^- Interactions

Like ClO_3^-, ClO_2^- failed to induce either NO_3^- transport or NR (Table I). However, at the uptake step, in contrast to the situation for ClO_3^-, ClO_2^- appeared to be a potent inhibitor of NO_3^- influx (Fig. 4, Table VI). The pattern of inhibition by ClO_2^-, i.e. increasing $[ClO_2^-]_o$ decreased the V_{max} and increased the K_m for NO_3^- influx, is characteristic of "mixed-type noncompetitive" inhibition. However, as we stated earlier, in the case of transport into roots (as compared to isolated enzymes), alternate interpretations may apply, particularly when such complex inhibition patterns are exhibited. It may well be that the increase in K_m resulted from a direct competitive inhibition of NO_3^- influx but that the effect on V_{max} was an indirect one, e.g. some indirect effects on the characteristics of the plasma membrane. We have observed that prolonged (24 h) pretreatment with ClO_2^- (in contrast to the effects of ClO_3^-) abolished net uptake of K^+ as well as of NO_3^- (from ClO_2^--free solution). K^+ uptake resumed after a lag of 1 h and took 3 to 4 h to attain full recovery (data not shown).

CONCLUSIONS

We have reached the following conclusions concerning the effects of NO_2^-, ClO_3^-, and ClO_2^- on nitrate transport and NRA:

It appears that NO_2^- per se is capable of inducing the synthesis of NO_3^- transporters but not the synthesis of NR. It is evident that not only are NO_3^- uptake and NO_3^- reduction physiologically independent but their induction at the transcriptional and/or translational level differs with respect to the inducer molecules.

NO_2^- is a competitive inhibitor of NO_3^- uptake. It apparently shares the same transporter and the same binding site as NO_3^-. Given that NO_2^- appears to be toxic, it is plausible that NO_2^- might be used for screening NO_3^- transport mutants, provided that NiRA is blocked.

Although ClO_3^- may be a substrate for NR, it cannot act as an inducer for the induction of NR or NO_3^- transporters.

At the uptake step, ClO_3^- was only a weak, noncompetitive inhibitor of NO_3^- uptake. Apparently ClO_3^- enters via the same transporter as NO_3^-, but the inhibitory effect of ClO_3^- is due to binding at sites other than the NO_3^--binding site or to the transporter-NO_3^- (enzyme-substrate) complex. In any event, ClO_3^- transport by HATS was very inefficient relative to that of NO_3^- (V_{max} for net ClO_3^- uptake was approximately 18% and K_m was twice that for net NO_3^- uptake).

The above may explain why relatively high $[ClO_3^-]_o$ (>10 mol m^{-3}) are required to induce toxicity. Moreover, although $[ClO_3^-]_o$ may be inconsequential when screening for NR mutants, it is not appropriate to use such high $[ClO_3^-]$ to screen for transport mutants deficient in HATS.

ClO_2^- did not induce either NR or NO_3^- uptake but, at the uptake step, proved to be a potent inhibitor of NO_3^- uptake.

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