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Inhibition of Nitrate Uptake by Ammonium in Barley. Analysis of Component Fluxes[1]

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NO_3^- uptake by plant roots is rapidly inhibited by exposure to NH_4^+. The rapidity of the effect has led to the presumption that the inhibition results from the direct effects of NH_4^+ at the plasma membrane. The mechanism of this inhibition, however, has been in contention. In the present study we used the radiotracer ^{13}N to determine the relative effects of short-term exposures to NH_4^+ on the $^{13}NO_3^-$ influx, efflux, and partitioning of absorbed ^{13}N in barley (*Hordeum vulgare*) roots. Plants were grown without NO_3^- or NO_2^- (uninduced for NO_3^- uptake), or with 0.1, 1.0, 10 mM NO_3^-, or 0.1 mM NO_2^- (to generate plant roots induced for NO_3^- uptake). Exposure to 1 mM NH_4^+ strongly reduced influx; the effect was most pronounced in plants induced for NO_3^- uptake when NO_3^- absorption was measured at low external NO_3^-. At higher $[NO_3^-]$ and in uninduced plants the inhibitory effect was much diminished, indicating that NH_4^+ inhibition of influx was mediated via effects on the inducible high-affinity transport system rather than on the constitutive high-affinity transport system or the low-affinity transport system. Exposure to NH_4^+ also caused increased NO_3^- efflux; the largest effect was at low external $[NO_3^-]$ in uninduced plants. In absolute terms, the reduction of influx made the dominant contribution to the observed reduction of net uptake of NO_3^-. Differences in response between plants induced with NO_3^- and those induced with NO_2^- indicate that NO_2^- may not be an appropriate analog for NO_3^- under all conditions.

The inhibitory effects exerted by the NH_4^+ ion upon NO_3^- uptake by the roots of higher plants have been studied extensively (Weissman, 1950; Lycklama, 1963; Fried et al., 1965; Minotti et al., 1969; Jackson et al., 1976; Rao and Rains, 1976; Doddema and Telkamp, 1979; MacKown et al., 1982a; Deane-Drummond and Glass, 1983; Rufty et al., 1983; Breteler and Siegerist, 1984; Glass et al., 1985; Ingemarsson et al., 1987; Oscarson et al., 1987; Lee and Drew, 1989; Warner and Huffaker, 1989; de la Haba et al., 1990; Ayling, 1993; Aslam et al., 1994, 1997; Chaillou et al., 1994). It is evident that there are short-term effects of NH_4^+ on NO_3^- uptake that are presumed to result from the direct effects of NH_4^+ on the plasma membrane; these short-term effects are apparent within minutes of exposure to NH_4^+. Moreover, longer-term effects due to NH_4^+ and/or assimilation products of NH_4^+ are thought to operate at the transcriptional level (Glass and Siddiqi, 1995; Krapp et al., 1998; Zhuo et al., 1999).

Despite the efforts of many investigators, a lack of consensus persists concerning the mechanism(s) responsible for the short-term inhibition of NO_3^- uptake by NH_4^+; specifically, whether the NH_4^+ effect is achieved by the direct inhibition of influx or by stimulating efflux. Although early reports suggested that NH_4^+ enhanced NO_3^- efflux (Jackson et al., 1976; Doddema and Telkamp, 1979; MacKown et al., 1982a; Deane-Drummond and Glass, 1983; Deane-Drummond, 1985, 1986), later studies using $^{13}NO_3^-$ clearly documented an inhibition of influx (Glass et al., 1985; Lee and Clarkson, 1986; Ingemarsson et al., 1987; Oscarson et al., 1987; Lee and Drew, 1989; Ayling, 1993; King et al., 1993).

The debate has recently been revived by Aslam and coworkers (1994, 1997), who concluded that the main effect of NH_4^+ on net NO_3^- uptake was through stimulation of NO_3^- efflux; they discounted the significance of influx inhibition. A resolution of this controversy has proved difficult because the experiments have used different species or cultivars; more importantly, different techniques were used to determine NO_3^- fluxes. Furthermore, in none of the above studies were both influx and efflux of NO_3^- measured directly and simultaneously; rather, conclusions were based upon measurements of net flux or influx or, where efflux was determined, upon the use of NO_3^- analogs such as ClO_3^- (Deane-Drummond and Glass, 1983; Deane-Drummond, 1985, 1986) and NO_2^- (Aslam et al., 1994). Glass et al. (1985) and Siddiqi et al. (1992) have questioned whether analogs of this sort are appropriate. In addition, Glass et al. (1985) and Ingemarsson et al. (1987) have argued that design features of experiments may have caused perturbations from a steady state, which might explain the sometimes large increases in NO_3^- efflux that were observed in some of the above studies.

We have designed the present study to address such issues. To eliminate problems associated with the choice of plant material, we used the same barley cultivar (CM-72)

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Abbreviations: CAE, compartmental analysis by efflux; CHATS, constitutive high-affinity transport system; IHATS, inducible high-affinity transport system; LATS, low-affinity transport system; $[NO_3^-]_o$, external $[NO_3^-]$ Φ, ionic (N) flux (component fluxes denoted by subscripts, as indicated in text).

used by Aslam and coworkers (1994, 1997), whose studies led to the conclusion that the short-term inhibition of NO_3^- uptake by NH_4^+ resulted exclusively from the stimulation of efflux. In this study we have determined the influx and efflux of NO_3^- by using $^{13}NO_3^-$ under both steady-state and perturbation protocols, by directly measuring influx and efflux, and by estimating influx calculated from CAE. To investigate possible differential effects of NH_4^+ on the CHATS, IHATS, and LATS for NO_3^- transport, we measured NO_3^- uptake at $[NO_3^-]$ that characterize these transport systems and we used plants induced or uninduced for NO_3^- transport. In addition, the experiments were designed to compare the effect of NH_4^+ on NO_3^- influx and efflux in plants induced for NO_3^- uptake by prior exposure to either NO_3^- or NO_2^-.

MATERIALS AND METHODS

Plant Growth Conditions

Barley (*Hordeum vulgare* L. cv CM-72) seeds were surface-sterilized in 1% NaOCl for 10 min, rinsed with deionized water, and germinated in sterilized moist sand in the dark as described by Siddiqi et al. (1989). Seeds were placed on plastic mesh fitted into Plexiglas (Atohaas Americas, Philadelphia, PA) disks, with 40 to 50 seeds per disk for influx experiments and 15 to 20 seeds per disk for efflux experiments (Siddiqi et al., 1989, 1991). After 3 d of germination in the dark, seedlings were transferred to 8-L hydroponic Plexiglas tanks located in walk-in controlled-environment growth chambers. The seedlings grew in hydroponic tanks for 4 d, after which we performed labeling experiments as described below. Growth chambers were maintained at 20°C ± 2°C, 70% RH, and set to a 16-h light/8-h dark photoperiod. Fluorescent lamps (model 1500, F96T12/CW/VHO, 215 W, Philips, Eindhoven, The Netherlands) provided a photon flux of approximately 300 μmol m^{-2} s^{-1}, measured at plant level (LI-189 light meter and LI-190SA quantum sensor, LI-COR).

Nutrient Solutions

After the 3-d germination treatment in sand, seedlings were cultivated for 4 d in hydroponic media in 8-L Plexiglas tanks. We used deionized distilled water and reagent-grade chemicals in the preparation of all nutrient solutions. Modified, one-quarter-strength Johnson's nutrient solution (2 mM KH_2PO_4, 2 mM K_2SO_4, 1 mM $MgSO_4$, 4 mM Ca^{2+} provided as $CaSO_4$ and/or $Ca[NO_3]_2$, and the micronutrients 50 μM Cl, 25 μM B, 20 μM Fe as Fe-EDTA, 2 μM Mn, 2 μM Zn, and 0.5 μM Cu) was used in all experiments (Siddiqi et al., 1989). NO_3^- was provided (in the form of $CA[NO_3]_2$) at 0.1, 1.0, or 10 mM starting 24 h before initiating the experiments. When experiments used NO_2^- to induce NO_3^- transport, it was provided as $NaNO_2$ (at 0.1 mM). During labeling experiments NH_4^+ was added as $(NH_4)_2SO_4$ at 1 mM. Electric circulating pumps (model IC-2, Brinkmann) continuously mixed the nutrient solutions in tanks. Continuous infusion of nutrient stock solution via peristaltic pumps (Technicon Proportioning Pump II, Technicon Instrument Co., Tarrytown, NY) allowed steady-state control of nutrient concentrations in the tanks. We checked the solutions daily for $[K^+]$ using a spectrophotometer (model 443, Instrumentation Laboratory, Lexington, MA). Powdered $CaCO_3$ maintained the solution pH at 6.5 ± 0.3. We monitored the pH daily with a microprocessor-based pocket-size pH meter (pH Testr2 model 59000-20, Cole Parmer, Chicago, IL). The $[NO_3^-]_o$ was measured spectrophotometrically by the method of Cawse (1967).

Influx Analysis

The radiotracer ^{13}N (with a half-life of 9.98 min) was produced by the Tri-University Meson Facility cyclotron at the University of British Columbia (Vancouver, Canada) by proton irradiation of water, producing mostly $^{13}NO_3^-$ with high radiochemical purity (Kronzucker et al., 1995b). The irradiated solutions were supplied in sealed 20-mL glass vials with a starting activity of 700 to 740 MBq. At this activity level, sufficient counts were present in eluates and plant samples even after loading periods of up to 60 min and a total elution period of 22 min in efflux experiments (see below). Procedures for the removal of radiocontaminants were carried out as described in detail elsewhere (Kronzucker et al., 1995a, 1995b). A volume of 100 mL of purified $^{13}NO_3^-$-containing "stock" solution was prepared in a fume hood and transferred into the controlled-environment chambers where the experiments were performed. All uptake solutions were premixed and contained in individual 500-mL plastic vessels behind lead shielding.

The chemical composition of the uptake, prewash, and desorption solutions was identical to the growth solution in the hydroponic tanks (see above) and contained 0.1, 1.0 or 10 mM NO_3^-. When NH_4^+ was present in uptake solutions it was provided at a concentration of 1 mM. In experiments where NO_2^- was used to induce NO_3^- transport (King et al., 1992; Aslam et al., 1997), NO_2^- was not present during loading with $^{13}NO_3^-$, but only during the induction period (24 h); it was replaced by NO_3^- during $^{13}NO_3^-$ loading and flux measurement. Uninduced plants received no N during growth but were exposed to 0.1 mM NO_3^- for flux determinations.

To minimize plant perturbation during experiments, a syringe was used to add tracer to the individual uptake vessels. At the start of the influx experiments, barley seedlings were transferred from the hydroponic growth tanks to prewash solutions in 1-L vessels for 5 min prior to addition of radioisotope to the uptake solutions. This protocol minimized plant perturbation and allowed the roots to equilibrate to the exact solution temperature and composition used during flux analysis. The roots were then exposed to tracer for 5 min. Immediately after loading with isotope, roots were dipped into nonlabeled solutions for 5 s to minimize the carryover of label by the root surface to the desorption solution. Roots were then desorbed for 2 min in unlabeled solution, which was otherwise chemically identical to the influx solution, to remove the $^{13}NO_3^-$ contained in the cell-wall free space. The duration of these steps was based on the half-lives of exchange of NO_3^- for the root

surface, the free space, and the cytoplasm as determined by efflux analysis (see below; Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b, 1995e).

We chose exposure times of 5 min for $^{13}NO_3^-$ influx, because we expected the contribution of tracer efflux from the cytoplasm to be negligible during this time (Lee and Clarkson, 1986; Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b, 1995d, and 1995e). After desorption, seedling roots were excised from the shoots; the roots were spun in a low-speed centrifuge for 30 s to remove any surface liquid; and the fresh weights of the roots and shoots were measured. The plant organs were then introduced into 20-mL scintillation vials, and a γ-counter (Minaxi δ, Auto-γ 5000, Packard Instruments, Meriden, CT) determined the radioactivities of the roots and shoots, measuring the 511-keV positron-electron annihilation radiation generated by the recombination of ambient electrons and β^+ particles emitted from ^{13}N. Using the specific activity ($^{13}N/[^{13}N + {}^{14}N]$ cpm μmol^{-1}) of the loading solution and the total root fresh weight of each seedling, we calculated the NO_3^- fluxes and expressed them in micromoles per gram root fresh weight per hour.

In addition to direct influx determinations by ^{13}N count accumulation over the 5-min loading periods (designated as Φ_{oc}*), influx was also determined by CAE (Φ_{oc}) and net flux was determined by ^{14}N depletion over a period of up to 2 h (Φ_{net}*). We repeated all experiments at least three times. Each experimental treatment consisted of three to four replicates ($n \geq 9$).

CAE

The protocol for CAE was essentially as described elsewhere (Lee and Clarkson, 1986; Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b, 1995e). Roots of intact barley seedlings were immersed for 60 min in 120-mL darkened plastic beakers containing the $^{13}NO_3^-$-labeled solution. NO_3^- concentrations were 0.1, 1.0, or 10 mM. NH_4^+ was added at a 1 mM concentration unless otherwise indicated for the duration of loading and elution; or it was added only at a specified time during the elution of tracer from the cytoplasm (Figs. 2 and 3) to study the immediate effect of NH_4^+ upon NO_3^- efflux. Pretreatment of uninduced and NO_2^--induced plants took place as described above. Conditions closely approximating a steady state with respect to all other nutrients were maintained throughout growth by completely replacing solutions in the 8-L tanks every day.

We maintained steady-state conditions during loading and elution. We chose the duration of the loading period on the basis of the half-lives of exchange for the cytoplasmic compartment for NO_3^- in barley (compare below with Siddiqi et al., 1991). Therefore, 60 min of exposure to tracer ensured that cytoplasmic specific activity was ≥95% of that in the loading solution (Kronzucker et al., 1995e). After loading with ^{13}N, seedlings were transferred to efflux funnels (Siddiqi et al., 1991; Kronzucker et al., 1995b) and the roots were eluted with 20-mL aliquots of nonradioactive solution after varying time intervals. Using an elution protocol lasting 22 min, these time intervals ranged from 5 s to 2 min, as described by Kronzucker et al. (1995b), except when we monitored the response of the NO_3^- efflux to the NH_4^+ addition during elution (Figs. 2 and 3); we used 1-min intervals in those cases to ensure appropriate time resolution.

Eluates from a total of 25 time intervals were collected, and the γ-counter (see above) determined the radioactivities of 20-mL subsamples from each eluate. After each final elution, we excised the seedling roots from the shoots, spun the roots in a low-speed centrifuge for 30 s to remove surface liquid, and determined the fresh weights of the roots and shoots. We then introduced the plant organs into 20-mL scintillation vials and determined the radioactivities of the roots and shoots as described previously for the influx experiments. We repeated the experiments three times with two replicates per experiment. We pooled the data from several experiments ($n \geq 6$) to calculate means and SE. Symbols and calculation of fluxes in CAE were as follows: Φ_{co}, efflux from the cytoplasmic compartment at time 0 divided by the specific activity of ^{13}N in the loading solution; Φ_{net}, net flux, obtained from the accumulation of ^{13}N in the plants at the end of the loading period (60 min); Φ_{oc}, unidirectional influx, calculated from $\Phi_{net} + \Phi_{co}$; Φ_{xylem}, flux of ^{13}N to the shoot at the end of the elution period; and $\Phi_{red/vac}$, combined flux to reduced N and the vacuole, resulting in $\Phi_{net} - \Phi_{xylem}$. Calculations of half-lives of exchange and cytosolic concentrations were done as described in detail elsewhere (Lee and Clarkson, 1986; Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b, 1995c, 1995e).

RESULTS AND DISCUSSION

Effect of NH_4^+ on Half-Lives of Cellular NO_3^- Exchange

As in our previous studies with barley, rice, and spruce (Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b, 1997; H.J. Kronzucker, A.D.M. Glass, and M.Y. Siddiqi, unpublished results), CAE revealed NO_3^- exchange with three subcellular compartments (Fig. 1). These corresponded to a surface film, a binding component in the cell wall, and the cytoplasm, an interpretation substantiated by previously reported compartment identity tests for the CAE technique with ^{13}N (Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b, 1995e). Half-lives of exchange for the surface film and the cell wall free space were very similar (approximately 2 and 30 s, respectively) to those reported in our previous studies (Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b) and did not change significantly as a function of $[NO_3^-]_o$. The half-lives for cytoplasmic NO_3^- exchange without NH_4^+ additions are shown in Table I. Cytoplasmic half-life values of NO_3^- exchange ranged from 7.75 to 12.94 min, with slightly shorter half-lives at higher $[NO_3^-]_o$ and longer half-lives in NO_2^--induced plants (Lee and Clarkson, 1986; Siddiqi et al., 1991; Devienne et al., 1994; Kronzucker et al., 1995a, 1995b, 1995c, 1995e, 1997).

In general, half-life values for the cytoplasm were longer in the cv CM-72 used in the present study compared with the Klondike variety of barley used in previous studies (Siddiqi et al., 1991; Devienne et al., 1994; Kronzucker et al.,

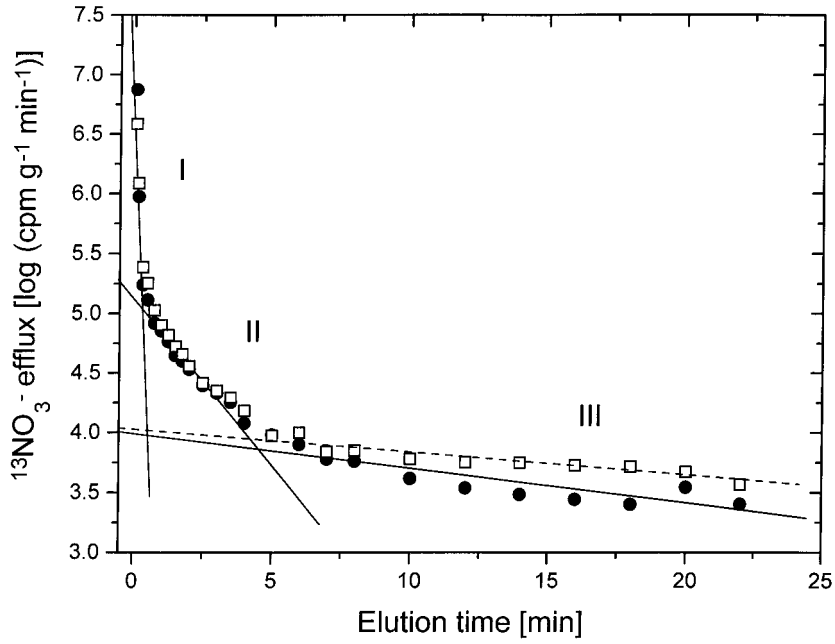

Figure 1. Representative semilogarithmic plots for the rate of release of $^{13}NO_3^-$ versus time of elution for roots of intact cv CM-72 seedlings maintained under steady-state conditions of 0.1 mM $[NO_3^-]_o$ without NH_4^+ (●) and following the addition of 1 mM NH_4^+ (□). Plots include linear regression lines for the three phases of efflux: I, surface film; II, cell wall; and III, cytoplasm. Regression lines are dashed for the plus-NH_4^+ treatment and solid for the control (phases I and II overlapped). For derivation of compartmental parameters, see text.

1995a, 1995b), indicating a larger relative accumulation capacity for NO_3^- (at comparable fluxes) in the former variety. The addition of NH_4^+ significantly affected cytoplasmic exchange kinetics in NO_2^--induced plants as well as in plants grown at 0.1 and at 1 mM $[NO_3^-]_o$: Half-lives increased in all of these cases (Fig. 1), whereas they remained unaffected in uninduced plants and at 10 mM $[NO_3^-]_o$ (Table I). These differences in half-life values are direct reflections of differences in flux partitioning, as discussed below.

Effect of NH_4^+ on NO_3^- Uptake and Subcellular N-Flux Partitioning

The addition of NH_4^+ led to a reduction of net NO_3^- uptake, as estimated by compartmental analysis (Table II) or as ascertained by direct methods (Table III). Moreover, there was a close correspondence between the values for the percentage of inhibition determined by CAE (Table II) and those determined independently by ^{14}N depletion (Table III), although the absolute flux values tended to be higher in CAE determinations. The inhibitory effect of externally applied NH_4^+ is in agreement with other studies (see the introduction), although genetic variability and even a stimulation of NO_3^- uptake by NH_4^+ in isolated cases (Bloom and Finazzo, 1986) have been reported in the responses, which ranged from strong to low levels of inhibition (Schrader et al., 1972; Pan et al., 1985). In the present study the inhibition of net NO_3^- uptake by 1.0 mM NH_4^+ depended upon $[NO_3^-]$ and the N species used to induce NO_3^- uptake prior to exposure to NH_4^+. In plants that were uninduced for NO_3^- uptake, NH_4^+ reduced net NO_3^- uptake by approximately 25% when measured in solutions containing 0.1 mM $[NO_3^-]_o$. In plants induced for NO_3^- uptake, the corresponding values for inhibition were 35% and 25% when net uptake was measured in solutions containing 0.1 and 1.0 mM $[NO_3^-]_o$, respectively, whereas at 10 mM $[NO_3^-]_o$, 1 mM external NH_4^+ had no significant

Table I. *Half-lives of NO_3^- exchange for the cytoplasmic compartment and cytoplasmic $[NO_3^-]$ ($[NO_3^-]_{cyt}$) in roots of intact cv CM-72 plants, determined by compartmental analysis*

Plants were exposed to and labeled at the indicated concentrations of NO_3^- (steady state). Uninduced plants were grown in N-free solution but were exposed to 0.1 mM NO_3^- during labeling and elution. NO_2^--induced plants were exposed to 0.1 mM NO_2^- for 24 h prior to labeling and elution at 0.1 mM NO_3^-. NH_4^+ was present at 1.0 mM during labeling and elution in + treatments. Data are ±SE ($n \geq 6$).

Treatment	$t_{1/2}$, Cytoplasm		$[NO_3^-]_{cyt}$	
	−	+	−	+
	min		*mM*	
0.1 mM NO_3^-	10.94 ± 0.57	14.78 ± 1.04	41 ± 2.89	38.75 ± 3.06
1.0 mM NO_3^-	8.52 ± 0.4	13.32 ± 0.99	51.69 ± 1.49	59.4 ± 2.8
10 mM NO_3^-	7.75 ± 0.37	6.75 ± 0.24	76.18 ± 1.03	67.27 ± 9.34
Uninduced	9.17 ± 0.98	8.55 ± 0.49	2.05 ± 0.45	2.19 ± 0.31
NO_2^- induced	12.94 ± 0.53	17.59 ± 0.98	48.8 ± 3.1	50.6 ± 2.71

Table II. *Component fluxes of NO_3^- as determined by compartmental analysis*

Barley plants were exposed to and labeled at the indicated concentrations of NO_3^- for 24 h prior to experiments. Uninduced plants were grown in N-free solution but exposed to 0.1 mM NO_3^- during labeling and elution. "NO_2^--induced" plants were exposed to 0.1 mM NO_2^- for 24 h prior to labeling and elution at 0.1 mM NO_3^-. NH_4^+ was present at 1.0 mM during labeling and elution in + treatments. Data are ±SE ($n \geq 6$).

Treatment	Φ_{oc}		Φ_{co}		Φ_{net}		$\Phi_{red/vac}$		Φ^*_{xylem}	
	−	+	−	+	−	+	−	+	−	+
	μmol g^{-1} h^{-1}									
0.1 mM NO_3^-	8.61 ± 0.77	5.77 ± 0.59	0.57 ± 0.02	0.84 ± 0.13	7.57 ± 0.76	4.93 ± 0.7	5.3 ± 0.6	3.49 ± 0.62	2.27 ± 0.25	1.44 ± 0.1
1.0 mM NO_3^-	12.93 ± 0.88	10.02 ± 0.13	1.6 ± 0.56	1.46 ± 0.36	11.33 ± 1.44	8.55 ± 1.06	6.85 ± 1.22	5.86 ± 1.08	4.47 ± 0.22	2.69 ± 0.02
10 mM NO_3^-	20.94 ± 0.73	21.58 ± 0.57	10.56 ± 3.97	12.14 ± 0.98	10.38 ± 4.7	9.44 ± 1.54	6.68 ± 2.08	5.95 ± 1.76	3.7 ± 2.62	3.49 ± 2.17
Uninduced	0.53 ± 0.12	0.48 ± 0.04	0.07 ± 0.01	0.13 ± 0.01	0.46 ± 0.1	0.34 ± 0.02	0.36 ± 0.07	0.28 ± 0.03	0.096 ± 0.03	0.055 ± 0.005
NO_2^- induced	8.1 ± 0.69	7.16 ± 0.74	0.29 ± 0.02	0.47 ± 0.09	7.8 ± 0.75	6.69 ± 0.49	6.67 ± 0.62	5.46 ± 0.49	1.13 ± 0.22	1.23 ± 0.09

effect. The inhibition of net NO_3^- uptake was significantly less (approximately 14%–17%) when plants were induced with NO_2^- and NO_3^- uptake was measured using 0.1 mM $[NO_3^-]_o$. The shortcomings of measuring net fluxes by depletion have been discussed previously (Kronzucker et al., 1995d, 1996). In particular, unless short-term (5–10 min) estimates are used, it is possible that plant acclimation will occur in response to declining $[NO_3^-]_o$ during the measurement.

NH_4^+ had a distinct and more potent effect on NO_3^- uptake in the high-affinity transport range (i.e. on the IHATS), which is evident below 1 mM $[NO_3^-]_o$, than in the range of the LATS, which operates at $[NO_3^-]_o \geq 1$ mM (Siddiqi et al., 1989; King et al., 1993; Kronzucker et al., 1995d). This provides additional support for the argument that high- and low-affinity transport systems are biochemically distinct modes of transport (for review, see Glass and Siddiqi, 1995). NO_2^--induced plants were substantially less sensitive to NH_4^+ inhibition of NO_3^- uptake than NO_3^--induced plants. Aslam and coworkers (1994, 1997) used plants induced by and "labeled" with NO_2^- as model systems for NO_3^--induced plants. Although NO_2^- and NO_3^- have been shown to act competitively at the level of uptake (Aslam et al., 1992; Siddiqi et al., 1992), the present findings suggest that NO_2^- may not serve as a satisfactory quantitative analog for NO_3^-. This is consistent with the finding by Siddiqi et al. (1992) that NO_2^- was not capable of inducing NO_3^- reductase activity in barley. Aslam et al. (1987, 1993, 1997), however, reached the opposite conclusion.

The effect of 1 mM NH_4^+ on $^{13}NO_3^-$ influx followed the same pattern (with one exception) as that observed for net uptake, i.e. the extent of inhibition declined with increasing $[NO_3^-]_o$, with 33% inhibition at 0.1 mM, 23% at 1.0 mM, and no effect at 10 mM. Inhibition was much smaller in NO_2^--induced plants (11.6%). This was true when influx was determined by CAE and count accumulation after a 5-min exposure to tracer (Tables II and III). The one exception was that influx in uninduced plants (determined by CAE) was unaffected by NH_4^+, in contrast to the situation for net uptake by uninduced plants. We interpret this result to indicate that, like LATS, the NO_3^- influx via CHATS (King et al., 1992; Kronzucker et al., 1995d) is unaffected by NH_4^+. That exposure to NH_4^+ diminished net uptake in uninduced plants indicates an effect on efflux of NO_3^- in uninduced plants.

We measured CAE on uninduced plants by exposing roots to 0.1 mM $[NO_3^-]_o$ for the duration of the efflux analyses (the labeling and elution procedures lasted 82 min); by this time, the physiological induction of the IHATS and NO_3^- reductase would be relatively small (Friemann et al., 1992; Glass and Siddiqi, 1995; Kronzucker et al., 1995a, 1995b). In contrast to its lack of effect on constitutive influx, NH_4^+ stimulated NO_3^- efflux by as much as 86% (Table II) in uninduced plants. Although not measured directly, efflux was probably even larger during the shorter 5-min exposures to NH_4^+ (see discussion of short-term efflux enhancements below), which may explain the apparent depression of influx that we saw in the short-term

Table III. *Estimates of NO_3^- influx and net flux into roots of intact cv CM-72 plants by methods independent of compartmental analysis*

Plants were exposed to, and fluxes were measured at, the indicated concentrations of NO_3^- (steady state). Uninduced plants were grown in N-free solution prior to flux measurement at 0.1 mM NO_3^-. NO_2^--induced plants were exposed to 0.1 mM NO_2^- for 24 h prior to flux measurement at 0.1 mM NO_3^-. NH_4^+ was present at 1.0 mM during uptake in + treatments. Data are ±SE ($n \geq 9$).

Treatment	Φ^*_{oc}		Φ^*_{net}	
	−	+	−	+
	μmol g^{-1} h^{-1}			
0.1 mM NO_3^-	5.16 ± 0.45	3.26 ± 0.47	3.97 ± 0.7	2.56 ± 0.61
1 mM NO_3^-	6.91 ± 0.34	5.37 ± 0.38	8.57 ± 0.16	6.34 ± 0.27
10 mM NO_3^-	10.52 ± 0.4	10.04 ± 0.93	N/D	N/D
Uninduced	0.42 ± 0.02	0.24 ± 0.02	N/D	N/D
NO_2^- induced	4.49 ± 0.16	3.87 ± 0.08	5.55 ± 0.13	4.59 ± 0.16

determinations after the addition of NH_4^+ (Table III), but not in the CAE determinations (Table II).

Efflux stimulation was also substantial in NO_2^--induced plants (approximately 62%), whereas in plants grown at 0.1 mM $[NO_3^-]_o$, efflux was enhanced by less than 50% and no efflux stimulation at all was seen in plants grown at 1.0 and 10 mM $[NO_3^-]_o$ (Table II; Fig. 1). NO_3^- efflux (expressed as a percentage of influx) also increased as $[NO_3^-]_o$ was raised from 0.1 to 10 mM even in the absence of NH_4^+ (Table II), as previously reported (Siddiqi et al., 1991; Kronzucker et al., 1995b; Volk, 1997). Our results confirm a distinct difference between inducible high-affinity transport and low-affinity transport; they also confirm the fact that NO_2^- induction of NO_3^- transport cannot be used as a quantitative model for NO_3^- induction and provision under steady-state conditions. The latter point is particularly important, because experiments on plants pretreated in this way have led to the conclusion that the NH_4^+ inhibition of net NO_3^- uptake results exclusively from the effects on NO_3^- efflux and that influx is unaffected (Aslam et al., 1994). Furthermore, the relative effect of NH_4^+ was high in the present study (>50% stimulation) because efflux is typically low under control conditions, and the absolute contribution to reduced net uptake was still small in comparison with the contribution arising from reduced influx.

Our experiments show that in NO_2^--induced plants, the combined flux of $^{13}NO_3^-$ and ^{13}N-assimilation products to the shoot (Φ^*_{xylem}) was unaffected by the addition of NH_4^+, whereas it was reduced in all other treatments (Table II). Also, Φ^*_{xylem} in NO_2^--induced plants was substantially lower than in NO_3^--induced plants, and approximated that of plants induced at 0.1 mM $[NO_3^-]_o$ after the application of 1 mM NH_4^+. Because significant suppression of NO_3^- reductase activity by NH_4^+ is well documented (MacKown et al., 1982a, 1982b; Breteler and Siegerist, 1984; Pan et al., 1985; de la Haba et al., 1990; Aslam et al., 1997; however, see Oaks et al., 1979), Φ^*_{xylem} under these conditions will be mostly in the form of $^{13}NO_3^-$ after NH_4^+ addition. Therefore, the lack of an effect of NH_4^+ on

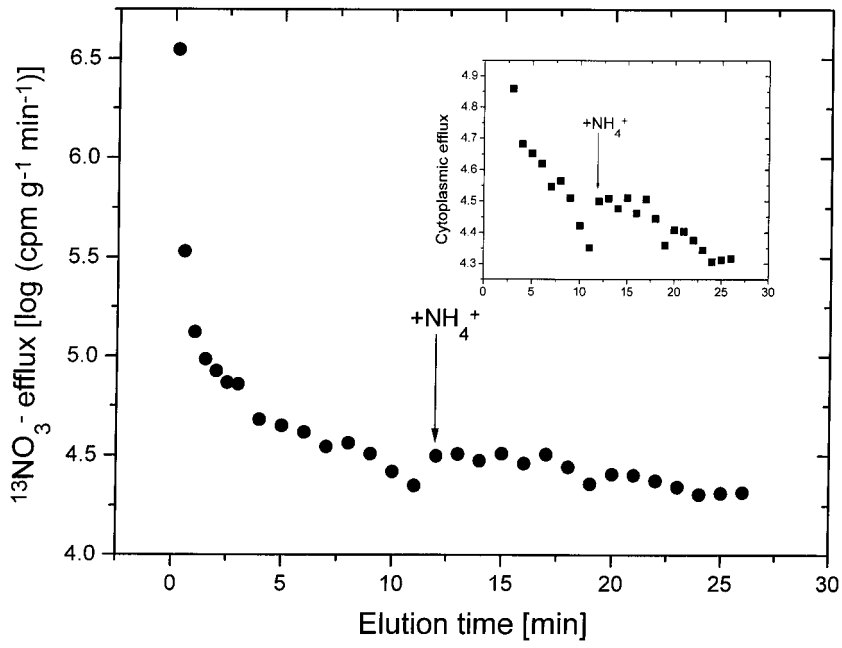

Figure 2. $^{13}NO_3^-$-efflux plot for intact seedlings of cv CM-72 maintained at 0.1 mM $[NO_3^-]_o$ with a one-time addition (and continued presence) of 1.0 mM NH_4^+ during cytoplasmic efflux (at 12 min during tracer elution). Inset shows magnified cytoplasmic exchange.

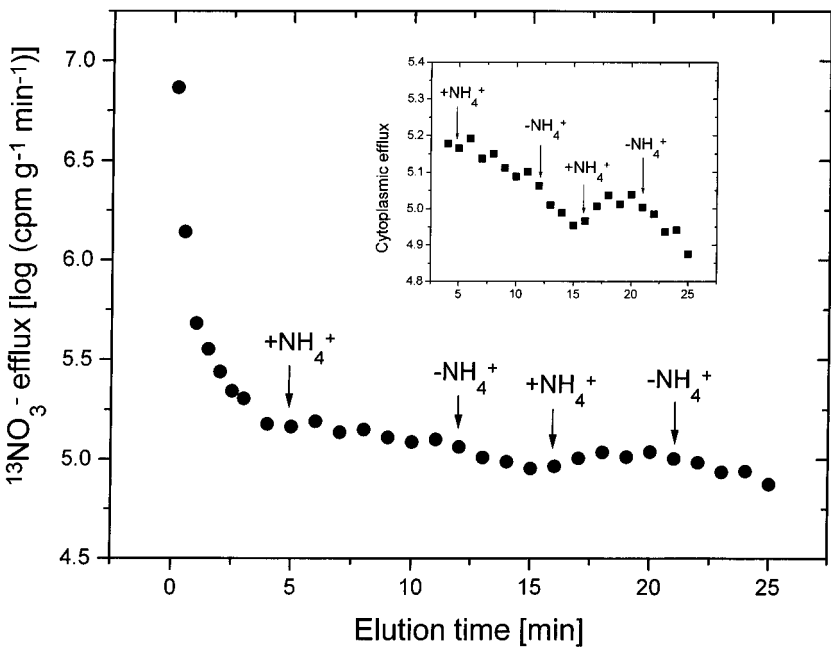

Figure 3. $^{13}NO_3^-$-efflux plot for intact seedlings of cv CM-72 maintained at 0.1 mM $[NO_3^-]_o$ with two-time addition and subsequent withdrawal of 1.0 mM NH_4^+ during cytoplasmic efflux (additions at 5 and 16 min; withdrawals at 12 and 21 min during tracer elution). Inset shows magnified cytoplasmic exchange.

Φ^*_{xylem} in NO_2^--induced plants (measured at 0.1 mM $[NO_3^-]_o$) appears to support the biochemically based conclusion arrived at by Siddiqi et al. (1992) and King et al. (1993) that NO_3^- reductase activity is not induced to any significant extent by NO_2^- in barley roots, in contrast to its full induction by NO_3^-. Claims to the contrary have been made by Aslam et al. (1987, 1993; see Glass and Siddiqi, 1995).

Our CAE analyses do not allow a separation of the reduced and unreduced components of the xylary ^{13}N-translocation term, nor do they permit separation of the components of $\Phi_{red/vac}$, namely the biochemical N flux to reduced N, and the N flux to the vacuole (Lee and Clarkson, 1986; Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b). However, given that the cytoplasmic $[NO_3^-]$ values in NO_2^-- and NO_3^--induced plants were indistinguishable after ^{13}N loading (Table I), and thus similar values of Φ^*_{xylem} for NO_3^- are to be expected, we must conclude that NO_2^- induction leads to a relatively greater stimulation of the NO_3^- flux to the vacuole (in both the control and NH_4^+-treated plants). The reduction in $\Phi_{red/vac}$ after NH_4^+ addition to NO_2^--induced plants (Table II) appears to be a direct result of the inhibition of influx and the stimulation of efflux rather than the effect upon NO_3^- reductase activity.

The clear differences in flux partitioning between NO_3^-- and NO_2^--induced plants revealed by CAE lead us to caution against the use of NO_2^- as an "analog" for NO_3^-. Similar concerns regarding the use of $^{36}ClO_3^-$ as an analog for NO_3^- were expressed in earlier work (Deane-Drummond and Glass, 1983; Dean-Drummond, 1985, 1986), although the basis of the failure to faithfully trace NO_3^- in the case of ClO_3^- was more straightforward (Glass et al., 1985; Lee and Drew, 1989; Siddiqi et al., 1992; Aslam et al., 1994; Glass and Siddiqi, 1995). Doddema and Telkamp (1979) also observed a significant rise in NO_3^- efflux upon the addition of NH_4^+; however, this response was transient and restricted to the perturbational condition (see below). Our present analyses demonstrate that an enhancement of the efflux component makes only a small contribution to the reduction of net uptake, whereas the principal effect of NH_4^+ on NO_3^- uptake comes through the inhibition of influx (except in uninduced plants and in plants pretreated with NO_2^-, where the efflux contribution is magnified).

Kinetics of the Response

It has been shown previously that the inhibition of NO_3^- influx by NH_4^+ is an immediate phenomenon, detectable even within 15 s (Glass et al., 1985; Ingemarsson et al., 1987; Lee and Drew, 1989; Ayling, 1993; Aslam et al., 1994), and that it is reversible with relaxation times of only 2 to 3 min (Lee and Drew, 1989). In the present study we tested the immediacy and reversibility of the stimulation of NO_3^- efflux by NH_4^+ (in plants grown with 0.1 mM $[NO_3^-]_o$); we added NH_4^+ under perturbational conditions only during the elution of the cytoplasmic compartment in $^{13}NO_3^-$ efflux experiments. Figures 2 and 3 show that the effect of NH_4^+ on NO_3^- efflux was evident immediately after its addition to the elution solutions (Fig. 2). Upon withdrawal of NH_4^+ from these solutions, NO_3^- efflux rapidly returned to normal (Fig. 3); repeated additions/withdrawals confirmed that the process was fully reversible. Notwithstanding this clear response, the absolute contribution to the inhibition of NO_3^- net uptake was small compared with the large reduction of influx. Moreover, in a separate study on rice (H.J. Kronzucker, A.D.M. Glass, and M.Y. Siddiqi, unpublished results), we found that in long-term studies under steady-state conditions in which both NO_3^- and NH_4^+ are provided, NO_3^- efflux was reduced rather than enhanced. Under these conditions efflux as a percent-

age of influx was very similar to that seen when only NO_3^- was provided; thus the efflux enhancement appears to be temporary.

The rapidity of the response to NH_4^+ on both the influx and efflux components of NO_3^- uptake provides a compelling argument that the NH_4^+ effect occurs directly at the plasma membrane. Lee and Drew (1989) demonstrated a logarithmic relationship between the inhibition of NO_3^- influx by NH_4^+ and external [NH_4^+], which led to the suggestion that membrane depolarization by NH_4^+ may inhibit the $NO_3^-/2H^+$ cotransport system due to effects on the proton motive force (Ullrich et al., 1984; Ayling, 1993). However, the provision of K^+, which also depolarizes the plasma membrane to an extent similar to that of NH_4^+, fails to inhibit NO_3^- uptake (Glass and Siddiqi, 1995; Wang et al., 1996), arguing for a more specific effect of NH_4^+. Given the rapidity of the response, the inhibition probably occurs allosterically, rather than by involving the products of NO_3^- reduction and N assimilation, or possibly the effects of transcription or translation.

Similar conclusions have been reached by others (Deane-Drummond and Glass, 1983; Ingemarsson et al., 1987; Lee and Drew, 1989; Warner and Huffaker, 1989; Aslam et al., 1994). In agreement with de la Haba et al. (1990) and Aslam et al. (1994), we found that pretreatment of barley and rice plants with the Gln synthetase inhibitor Met sulfoximine for 6 h at 1 mM did not alleviate the inhibitory effect exerted by externally added NH_4^+ (data not shown). Thus it seems unlikely that N assimilates downstream of NH_4^+ are involved in the inhibition of NO_3^- uptake. We must stress that these conclusions apply only to the rapid effects of NH_4^+ on NO_3^- uptake; some (Krapp et al., 1998; Zhuo et al., 1999) have suggested that there may be long-term effects of Gln and other amino acids at the transcription level.

SUMMARY

Our analyses provide evidence that the inhibitory effect of NH_4^+ upon NO_3^- uptake is mediated primarily by inhibiting NO_3^- influx, with only a small contribution from the enhancement of NO_3^- efflux, which: (a) is both transient and reversible, (b) is associated with a large efflux only in uninduced plants and plants induced by NO_2^- (i.e. under conditions where influx is very low), (c) is dependent on $[NO_3^-]_o$, (d) is strong for IHATS but small for CHATS and LATS, (e) occurs directly at the plasma membrane (i.e. it does not involve NO_3^- reduction or N-assimilation products in the short term, although it may in the long-term); and (f) cannot be modeled quantitatively by the use of NO_2^- as an analog of NO_3^-.

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