Plant Physiol. (1994) 106: 1293–1301

# Stimulation of Nitrate and Nitrite Efflux by Ammonium in Barley (*Hordeum vulgare* L.) Seedlings[1]

**Muhammad Aslam, Robert L. Travis, and Ray C. Huffaker***

Department of Agronomy and Range Science, University of California, Davis, California 95616

**The inhibitory effect of $NH_4^+$ on net $NO_3^-$ uptake has been attributed to an enhancement of efflux and, recently, to an inhibition of influx. To study this controversy, we devised treatments to distinguish the effects of $NH_4^+$ on these two processes. Roots of intact barley (*Hordeum vulgare* L.) seedlings, uninduced or induced with $NO_3^-$ or $NO_2^-$, were used. Net uptake and efflux, respectively, were determined by following the depletion and accumulation in the external solutions. In roots of both uninduced and $NO_2^-$-induced seedlings, $NO_3^-$ efflux was negligible; hence, the initial uptake rates were equivalent to influx. Under these conditions, $NH_4^+$ had little effect on $NO_3^-$ uptake (influx) rates by either the low- or high-$K_m$ uptake systems. In contrast, in plants preloaded with $NO_3^-$, $NH_4^+$ and its analog $CH_3NH_3^+$ decreased net uptake, presumably by enhancing $NO_3^-$ efflux. The stimulatory effect of $NH_4^+$ on $NO_3^-$ efflux was a function of external $NH_4^+$ and internal $NO_3^-$ concentration. These results were corroborated by the absence of any effect of $NH_4^+$ on $NO_2^-$ uptake unless the roots were preloaded with $NO_2^-$. In this case $NH_4^+$ increased efflux and decreased net uptake. Hence, the main effect of $NH_4^+$ on net $NO_3^-$ and $NO_2^-$ uptake appears to be due to enhancement of efflux and not to inhibition of influx.**

The influence of $NH_4^+$ on net $NO_3^-$ uptake (defined as the difference between influx and efflux) in higher plants has been widely studied. Results of those studies have varied considerably, with reports ranging from little or no effect (Smith and Thompson, 1971; Schrader et al., 1972; Oaks et al., 1979) to strong inhibition (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; Chaillou et al., 1994). Since net uptake is a balance between influx and efflux, an inhibitory effect of $NH_4^+$ could be due to either inhibition of influx or enhancement of efflux. In the reports cited above, the question of whether $NH_4^+$ affects $NO_3^-$ influx and/or efflux is also controversial.

Early investigators reported that external $NH_4^+$ increased $NO_3^-$ efflux in *Arabidopsis* (Doddema and Telkamp, 1979), barley (*Hordeum vulgare*; Deane-Drummond and Glass, 1983), pea (*Pisum sativum*) seedlings, and *Chara corallina* (Deane-Drummond, 1985, 1986). In one recent report of longer-term studies with soybeans, Chaillou et al. (1994) showed increased periods of net $NO_3^-$ efflux in the presence of $NH_4^+$. In accordance with the findings that $NH_4^+$ increased $NO_3^-$ efflux, several reports also showed that $NH_4^+$ had no effect on $^{36}ClO_3^-$ influx when the latter was used as an analog for $NO_3^-$ (Deane-Drummond and Glass, 1983; Deane-Drummond, 1985, 1986).

On the other hand, based on the use of $^{13}NO_3^-$, the recent consensus is that $NH_4^+$ inhibits net $NO_3^-$ uptake by inhibiting its influx per se rather than by enhancing efflux (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). Decreased influx of $^{13}NO_3^-$ was correlated with depolarization of membrane potentials of barley and tomato (*Lycopersicon esculentum*) roots (Ayling, 1993) and of *Lemna* (Ullrich et al., 1984). Investigators using $^{15}NO_3^-$ and longer term studies also reported that $NH_4^+$ inhibited $NO_3^-$ influx and had no effect on efflux in wheat (*Triticum aestivum*; Jackson et al., 1976) and corn (*Zea mays*; MacKown et al., 1982a).

The results of the studies that showed no effect of $NH_4^+$ on $^{36}ClO_3^-$ influx have been attributed to anomalies that occurred when $^{36}ClO_3^-$ was used as an $NO_3^-$ analog (Glass et al., 1985). In addition, the finding of increased efflux by $NH_4^+$ has been attributed to using plants that have been perturbed from steady state, i.e. removed from a high concentration of $NO_3^-$ and placed in a low concentration (Glass et al., 1985; Ingemarsson et al., 1987).

If the effect of $NH_4^+$ were on $NO_3^-$ influx, then one would expect that an inhibitory effect on net $NO_3^-$ uptake would always be found when $NH_4^+$ is present. Evidently, this has not been the case. To study this problem, we have used plant systems in which the effect of $NH_4^+$ on $NO_3^-$ influx can be measured initially with little efflux occurring. In addition, we have compared the effect of $NH_4^+$ on $NO_3^-$ efflux in roots of seedlings, both loaded and not loaded with $NO_3^-$. Included also are comparable uptake experiments with $NO_2^-$, which has similar transport systems. $NO_2^-$ is a competitive inhibitor of $NO_3^-$ uptake (Aslam et al., 1992), it induces the uptake systems for both ions (Aslam et al., 1993), and it is also metabolized after uptake. We have included short-term experiments that (a) minimize changes in properties of transporters and possible secondary regulation of their activity by metabolites and (b) compare roots that were either perturbed or not perturbed from steady state during the measurement of efflux.

[1] This work was supported in part by a grant to R.C.H. from the U.S. National Aeronautics and Space Administration (NASA NCC 2–9).

* Corresponding author; fax 1–916–752–4361.

In this study, we show that $NH_4^+$ had little effect on either $NO_3^-$ or $NO_2^-$ influx. $NH_4^+$ increased $NO_3^-$ and $NO_2^-$ efflux as a function of their internal concentrations. Hence, when $NH_4^+$ decreased net uptake of $NO_3^-$ and $NO_2^-$, it did so by presumably facilitating their efflux.

## MATERIALS AND METHODS

### Plant Material

Barley (*Hordeum vulgare* L. var CM-72) seedlings were grown hydroponically as described before (Aslam et al., 1979). Seedlings were grown in 0.2 mM $CaSO_4$ in the dark for 6 d. The seedlings were then transferred to aerated one-quarter-strength Hoagland solution lacking N (Hoagland and Arnon, 1950) and placed in the growth chamber under continuous light at 25°C and 60 to 65% RH. The PPFD (400–700 nm) at the seedling canopy was 400 $\mu$mol $m^{-2}$ $s^{-1}$ and was provided by incandescent and cool-white fluorescent lamps. The seedlings either were grown in N-free solution for 48 h in light (uninduced seedlings) or were transferred after 24 h to large volumes (5–10 L) of the induction solutions containing 0.002, 0.1, or 1.0 mM $NaNO_3$ or 0.1 mM $NaNO_2$ for an additional 8 h (0.002 mM $NO_3^-$ treatment only) or 24 h to induce the $NO_3^-$ and $NO_2^-$ uptake systems.

In some experiments, seedlings induced with 0.1 mM $NO_2^-$ were loaded with $NO_3^-$ or $NO_2^-$ by placing them into 2.5 L of the appropriate 2.0 mM solution for 1 h. Cycloheximide (2 $\mu$g/mL) was added to the loading solution containing $NO_3^-$ to inhibit further induction (if any) of the $NO_3^-$ uptake system.

### Measurement of $NO_3^-$ and $NO_2^-$ Uptake

Intact seedlings were used in all experiments. Net uptake was determined by following the depletion of $NO_3^-$ or $NO_2^-$ from the uptake solutions. All experiments were done in a mini-growth chamber set at 25°C, 700 $\mu$mol $m^{-2}$ $s^{-1}$ light intensity (400–700 nm), and 60 to 65% RH. The growth chamber was part of a fully automatic system described by Goyal and Huffaker (1986). Uptake was started by placing 15 seedlings in a Pyrex beaker (80 × 50 mm) containing 50 mL of the appropriate solution. The first sample was obtained about 2 min after the transfer of the seedlings into the uptake solutions. The solutions contained 1.0 mM Mes (pH 6.0), 0.2 mM $CaSO_4$, $NO_3^-$ or $NO_2^-$, and $NH_4^+$ or $CH_3NH_3^+$ as indicated in the figure and table legends. Ammonium was supplied as ammonium sulfate. The solutions were aerated vigorously to ensure thorough mixing. The beaker was fitted with a stainless steel screen about 10 mm above the bottom, and a magnetic bar was placed below the screen. The roots were held above the screen. The beaker was placed on the magnetic stirrer for thorough and rapid mixing of the solutions. Aliquots (0.4 mL) for $NO_3^-$ and $NO_2^-$ determination were removed automatically at 1.0-, 1.5-, or 3.0-min intervals by the HPLC system. In one study samples were withdrawn manually every 15 s. The cumulative uptake was computed from the concentration and volume data (Goyal and Huffaker, 1986). The experiments were repeated three to five times, and the results from representative experiments are shown. The initial uptake rates were calculated by linear regression analysis of the cumulative data during a 10- to 12-min period. The $r^2$ values of the regression analysis were significant at $P = 0.001$.

### Measurement of $NO_3^-$ and $NO_2^-$ Efflux

$NO_3^-$ efflux was determined where specified by following the accumulation of the ion in the uptake solutions after the addition of 1 or 2 mM $NO_2^-$ or by transferring the seedlings into specific efflux solutions containing 1.0 mM Mes (pH 6.0), 0.2 mM $CaSO_4$, 1.0 mM $NO_2^-$ with or without 1.0 mM $NH_4^+$ or $CH_3NH_3^+$. The solution for the measurement of $NO_2^-$ efflux contained 1.0 mM $NO_3^-$ instead of $NO_2^-$. Since $NO_3^-$ and $NO_2^-$ are competitive inhibitors of each other's uptake (Aslam et al., 1992; Siddiqi et al., 1992), the presence of the counterion at 1.0 or 2.0 mM in the efflux solutions markedly inhibited the reabsorption of the effluxed ion (Table I). For the measurement of $NO_3^-$ and $NO_2^-$ efflux, the roots were rinsed for 5 s in an N-free solution and transferred to 50 mL of efflux solution contained in a Pyrex beaker fitted with the stainless steel screen as described above. Efflux was determined by removing aliquots (0.4 mL) at 1-min intervals for 12 to 15 min by the automatic HPLC system. Cumulative efflux was calculated from the concentration and solution volume data as previously described (Goyal and Huffaker, 1986).

### $NO_3^-$, $NO_2^-$, and $NH_4^+$ Determination

The concentrations of $NO_3^-$ and $NO_2^-$ were determined spectrophotometrically by measuring their $A_{210}$ after separation by HPLC on a Partisal-10 SAX anion-exchange column (Thayer and Huffaker, 1980). For analysis of internal $NO_3^-$, $NO_2^-$, and $NH_4^+$ concentrations, roots (1.0–1.5 g) were washed with distilled deionized water and homogenized in 4.0 mL/g fresh weight of cold, distilled, deionized water in a chilled mortar and pestle in the presence of acid-washed sand. The extracts were centrifuged at 30,000*g* for 10 min, and the supernatants were used for determination of the ions. $NO_3^-$ was determined by HPLC as described above; $NO_2^-$ was determined at $A_{540}$ after color development for 15 min with a 1:1 mixture of 1% (w/v) sulfanilamide in 1.5 N HCl and 0.02% (w/v) *n*-naphthylethylenediamine dihydrochloride. $NH_4^+$ was determined by measuring the electrical conductivity of ammonia gas evolved after reacting the sample with KOH using the continuous flow system of Carlson (1978). All of the results are presented on the basis of fresh weight of the roots.

## RESULTS AND DISCUSSION

We devised two sets of experimental conditions to distinguish between the effect of $NH_4^+$ on $NO_3^-$ influx and efflux. Influx can be measured when efflux is prevented or minimized. Similarly, efflux can be measured when influx is inhibited. These experiments are described in sequence below.

**Table I.** *Effect of $NO_2^-$ on $NO_3^-$ uptake (influx) by roots of intact seedlings induced with 0.1 mM $NO_2^-$ for 24 h*

The seedlings were grown and induced with 0.1 mM $NO_2^-$ as described in "Materials and Methods." Cumulative uptake of $NO_3^-$ was then determined during a 12-min period by following its depletion from the uptake solutions initially containing 0.025, 0.05, and 0.1 mM substrate and 0, 1.0, or 2.0 mM $NO_2^-$. Uptake rates were calculated by linear regression analysis of the cumulative uptake data. The depletion of $NO_3^-$ from the uptake solution containing 0 $NO_2^-$ was 20 to 25%. The values are means ± SD of two replicate measurements.

| $[NO_2^-]$ | $NO_3^-$ Uptake Rates at | | |
|---|---|---|---|
| | 0.025 mM | 0.05 mM | 0.1 mM |
| mM | | $\mu mol\ g^{-1}\ h^{-1}$ | |
| 0 | 3.63 ± 0.15 | 4.57 ± 0.26 | 6.07 ± 0.27 |
| 1 | 0.40 ± 0.02 | 0.77 ± 0.02 | 1.47 ± 0.11 |
| 2 | 0.23 ± 0.01 | 0.43 ± 0.04 | 0.66 ± 0.09 |

### Effect of $NH_4^+$ on $NO_3^-$ and $NO_2^-$ Uptake (Influx) by Uninduced Seedlings

Seedlings that have not been previously exposed to $NO_3^-$ or $NO_2^-$ (uninduced seedlings) have constitutive low-$K_m$ uptake systems for both ions and yet contain little if any $NO_3^-$ or $NO_2^-$ (Aslam et al., 1993). Thus, when $NO_3^-$ or $NO_2^-$ is added to the substrate solution, efflux of either ion would not be expected or would be minimal during the initial phase of uptake. Under these conditions, any effect of $NH_4^+$ should be primarily on influx. $NH_4^+$ at 1 mM had little effect on uptake of either ion from a 0.05 mM substrate solution during the first 6 to 9 min (Fig. 1).

### Effect of $NH_4^+$ on $NO_3^-$ and $NO_2^-$ Uptake (Influx) and Efflux by $NO_2^-$-Induced Seedlings

We recently reported that $NO_2^-$ effectively induces the high-$K_m$ uptake systems of both $NO_3^-$ and $NO_2^-$ with little accumulation of either ion (Aslam et al., 1993). Thus, when the $NO_3^-$ uptake system is induced with $NO_2^-$, efflux would be minimized during the initial phase of uptake. This was substantiated by linear cumulative uptake (constant rate) during the initial 20 min (Fig. 2A). Therefore, any effect of $NH_4^+$ should be primarily on influx. An uptake rate decreasing with time would be expected if efflux began. In this experiment, seedlings were induced with 0.1 mM $NO_2^-$ for 24 h and then placed in 0.1 mM $NO_3^-$ for measurement of uptake (Fig. 2A). $NH_4^+$ had little effect on $NO_3^-$ uptake whether it was added at the beginning or 15 or 22 min after the initiation of uptake (Fig. 2A). Similarly, $NH_4^+$ had little effect on $NO_2^-$ uptake by either $NO_3^-$-induced (data not shown) or $NO_2^-$-induced roots not loaded with $NO_2^-$ (Table II). de la Haba et al. (1990) also found no effect of $NH_4^+$ on $NO_2^-$ uptake in $NO_3^-$-induced sunflower seedlings.

To substantiate the observation that little $NO_3^-$ efflux was occurring, uptake at 20 min was interrupted by the addition of 1 mM $NO_2^-$ (Fig. 2B). At this time, the $NO_3^-$ concentration in the uptake solution was depleted to about 0.025 mM. At such $NO_3^-$ concentrations, $NO_2^-$ at 1 or 2 mM inhibits $NO_3^-$ uptake (influx) 90% or more (Table I). Under these conditions, net uptake ceased, no efflux was detected, and there was no effect of $NH_4^+$ on efflux observed (Fig. 2B). Since efflux would be manifested by the appearance of $NO_3^-$ in the external solution, it would be expressed as a decrease in cumulative uptake.

$NO_2^-$ is a strong competitive inhibitor of $NO_3^-$ uptake (Aslam et al., 1992; Siddiqi et al., 1992). The effect of $NO_2^-$ is likely on $NO_3^-$ influx, since inhibition occurs immediately in roots of uninduced seedlings or in seedlings induced in $NO_2^-$, both of which contain little $NO_3^-$ (Aslam et al., 1993).

### Effect of $NH_4^+$ on $NO_3^-$ Uptake and Efflux in $NO_3^-$-Induced Seedlings

In contrast to seedlings containing little $NO_3^-$, when seedlings were preloaded by incubation in 0.1 mM $NO_3^-$ for 24 h, $NH_4^+$ rapidly decreased net uptake whether added at the beginning or 12 or 20 min after the initiation of uptake (Fig. 3). This effect was a function of both the external $NH_4^+$

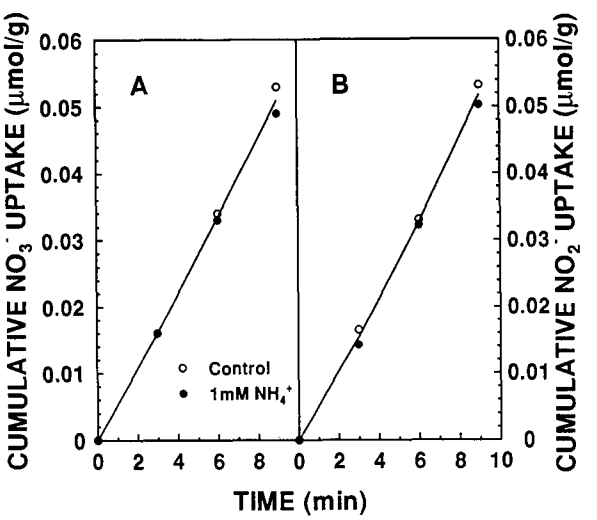


**Figure 1.** Effect of $NH_4^+$ on $NO_3^-$ (A) and $NO_2^-$ (B) uptake by roots of uninduced, intact seedlings. The seedlings were grown in N-free Hoagland solution for 6 d in the dark followed by 2 d in the light. The seedlings were then transferred to uptake solutions containing 0.05 mM $NO_3^-$ or $NO_2^-$ and 0 or 1 mM $NH_4^+$. Cumulative uptake of the ions was determined by following their depletion as described in "Materials and Methods."

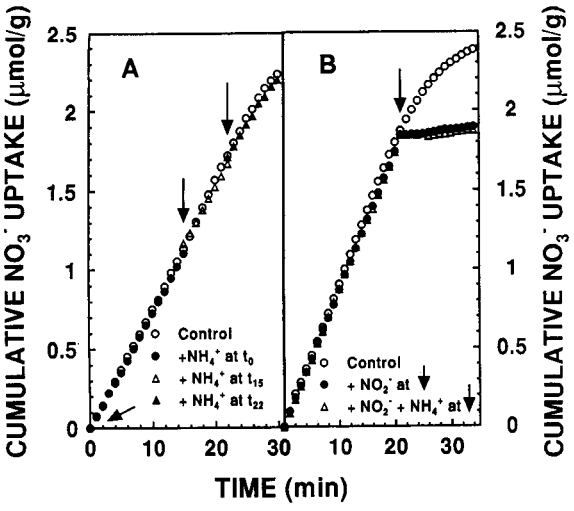


**Figure 2.** Effect of $NH_4^+$, supplied at different intervals either alone (A) or with $NO_2^-$ (B) on net $NO_3^-$ uptake by roots induced with 0.1 mM $NO_2^-$ for 24 h. The seedlings were grown in N-free Hoagland solution as described in Figure 1. After 24 h of light, they were transferred to solutions containing 0.1 mM $NO_2^-$ for 24 h in continuous light. Uptake was then determined from solutions containing 0.1 mM $NO_3^-$. A, $NH_4^+$ (1 mM) was added to the uptake solutions at different intervals. B, $NO_2^-$ (1 mM) ±1 mM $NH_4^+$ was added to the uptake solutions at the time indicated by the arrow. At this time, the $NO_3^-$ concentration in the uptake solution was depleted to about 0.025 mM. Cumulative uptake was determined by sampling at 1-min intervals as described in "Materials and Methods." $NO_3^-$ concentration of the roots was 0.40 ± 0.13 μmol/g fresh weight.

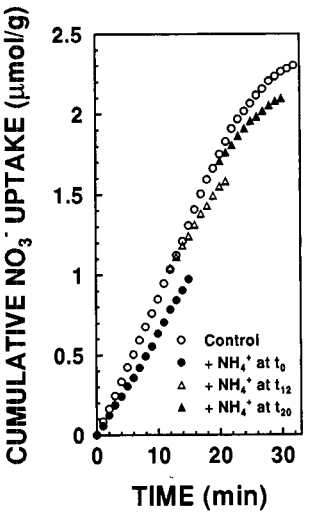


**Figure 3.** Effect of $NH_4^+$, supplied at different intervals, on $NO_3^-$ uptake by roots induced with 0.1 mM $NO_3^-$ for 24 h. Experimental details are the same as described in Figure 2A, except that the seedlings were induced with 0.1 mM $NO_3^-$. $NO_3^-$ concentration of the roots was 27.6 ± 1.0 μmol/g fresh weight.

concentration and the internal concentration of $NO_3^-$ in the tissue (Table III).

The results shown in Tables II and III establish that $NH_4^+$ had little effect on net $NO_3^-$ uptake by roots low in $NO_3^-$ whether the uptake system was induced with either $NO_3^-$ or $NO_2^-$. To further compare the effect of $NH_4^+$ on $NO_2^-$- and $NO_3^-$-induced uptake systems, roots of intact seedlings were induced in 0.1 mM $NO_2^-$ and then loaded with $NO_3^-$ for a brief period (Table II). Cycloheximide was added to the solution to inhibit any further induction by $NO_3^-$ during loading. $NH_4^+$ appreciably inhibited net $NO_3^-$ uptake only when the seedlings, induced in either $NO_3^-$ or $NO_2^-$, had been loaded with $NO_3^-$.

To determine the effect of $NH_4^+$ on efflux from roots containing differing internal concentrations of $NO_3^-$, seedlings were induced for 24 h in either 0.1 or 1 mM $NO_3^-$ (Fig. 4). After the 24-h induction, linear cumulative net uptake at 0.1 mM $NO_3^-$ during a 15-min period showed a steady-state rate in both sets of seedlings. At this time, the $NO_3^-$ concentration was about 0.04 mM, and the addition of 2 mM $NO_2^-$ restricted net uptake (Table I). Net $NO_3^-$ efflux (indicated by the negative slope for cumulative uptake) was detected within 1 min from the roots of both sets of seedlings and was greater from those induced in 1 mM $NO_3^-$. The presence of $NH_4^+$ increased the efflux of $NO_3^-$ in both sets of seedlings, with the effect being greater in the seedlings induced in 1 mM $NO_3^-$.

Other investigators have attributed the enhancement of $NO_3^-$ efflux by $NH_4^+$ to the perturbation of plant roots from

**Table II.** *Effect of $NH_4^+$ on $NO_3^-$ and $NO_2^-$ uptake rates in intact roots of seedlings induced with 0.1 mM $NO_2^-$ for 24 h and then some were loaded with $NO_3^-$ or $NO_2^-$*

For loading, the seedlings were placed in 2.0 mM $NO_3^-$ or $NO_2^-$ solutions for 1 h. The loading solution containing $NO_3^-$ also contained cycloheximide (2 μg/mL) to inhibit further induction (if any) of $NO_3^-$ uptake system. The cumulative uptake of the ions was then determined during a 12-min period. The uptake solutions contained 0.1 mM $NO_3^-$ or $NO_2^-$ and 0 or 1.0 mM $NH_4^+$. The uptake rates were determined by linear regression analysis of the cumulative uptake data. The values are means ± SD of two replicate measurements.

| $[NH_4^+]$ | $NO_3^-$ Uptake Rates | | $NO_2^-$ Uptake Rates | |
|---|---|---|---|---|
| | Not loaded | Loaded | Not loaded | Loaded |
| mM | μmol $g^{-1}$ $h^{-1}$ | | | |
| 0 | 4.95 ± 0.12 | 4.24 ± 0.29 | 4.35 ± 0.45 | 3.14 ± 0.22 |
| 1.0 | 4.51 ± 0.35 | 3.26 ± 0.32 | 4.12 ± 0.33 | 2.20 ± 0.27 |

**Table III.** *Effect of different concentrations of $NH_4^+$ on $NO_3^-$ uptake by seedlings varying in root $NO_3^-$ content*

The seedlings were induced with 0.1 mM $NO_2^-$ for 24 h or 0.002 mM $NO_3^-$ for 8 h to obtain low, 0.05 mM $NO_3^-$ for 24 h to obtain medium, or 1.0 mM $NO_3^-$ for 24 h to obtain high $NO_3^-$ concentrations in the roots. The uptake solutions contained 0.1 mM $NO_3^-$ and different concentrations of $NH_4^+$. Rates of $NO_3^-$ uptake were determined by linear regression anaysis of the cumulative uptake curves during a 12-min period. The $NO_3^-$ concentrations of the roots (μmol/g) were 0.65 to 0.72 (low), 8.5 (medium), and 29.7 (high).

| $[NH_4^+]$ | $NO_3^-$ Uptake Rates[a] | | | |
|---|---|---|---|---|
| | $NO_2^-$ induced | $NO_3^-$ induced | | |
| | Low $NO_3^-$ | Low $NO_3^-$ | Medium $NO_3^-$ | High $NO_3^-$ |
| mM | | μmol $g^{-1}$ $h^{-1}$ | | |
| 0 | 4.18a | 3.53a | 4.36a | 5.44a |
| 0.1 | 4.03a | 3.35a | 3.24b | 3.57b |
| 1.0 | 3.95a | 3.22a | 3.00b | 2.99c |
| 10.0 | 3.88a | 3.16a | 2.89b | 2.25d |

[a] Uptake rates within each column followed by different letters are significantly different at $P \leq 0.05$.

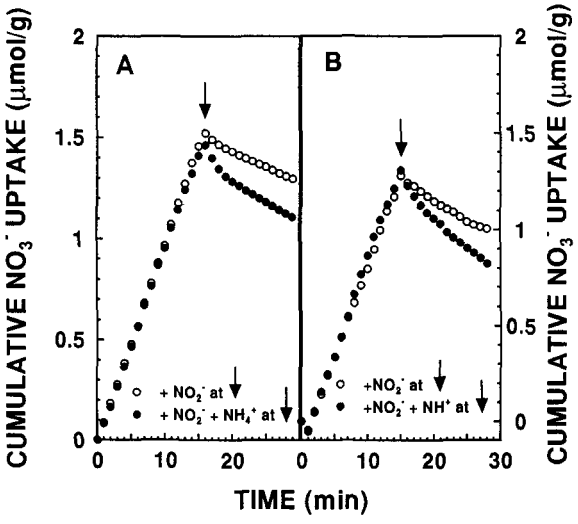


**Figure 4.** Effect of $NH_4^+$ on $NO_3^-$ efflux by roots of intact seedlings induced with 0.1 mM (A) or 1 mM (B) $NO_3^-$ for 24 h. The induced seedlings were rinsed in $NO_3^-$-free solution for 5 s and transferred to the uptake solutions containing 0.1 mM $NO_3^-$. Net uptake was measured over 15 min by following $NO_3^-$ depletion. At the time indicated by the arrow, $NO_3^-$ was depleted from the uptake solution to about 0.04 mM. At this point, 2 mM $NO_2^-$ ± 1 mM $NH_4^+$ were added to the uptake solutions, and the appearance of $NO_3^-$ from the roots into the external solution was followed to determine cumulative efflux. Under these conditions, the rate of net efflux is indicated by the negative slope of cumulative net uptake. $NO_3^-$ concentrations of the roots were 25.9 ± 0.7 and 41.3 ± 1.0 μmol/g fresh weight, respectively, for roots fed 0.1 mM and 1.0 mM $NO_3^-$.

steady state, i.e. removed from a high concentration of $NO_3^-$ and placed in a low or zero concentration (Glass et al., 1985; Ingemarsson et al., 1987). This was not a problem here, since the $NO_2^-$ and $NH_4^+$ were added when cumulative uptake was linear (uptake rate was at steady state) and the concentration of $NO_3^-$ was not changed to determine efflux.

To determine whether the $NO_2^-$ treatment had changed the transport characteristics of the plasma membrane, $NO_3^-$ uptake by the roots was compared before and after treatment with $NO_2^-$. The rate of net $NO_3^-$ uptake by the roots treated with 2.0 mM $NO_2^-$ was within 10% of the control rate determined before treatment with $NO_2^-$ (Fig. 5). We have found no evidence for any toxic effects from the short-term treatments (15 min) with 1.0 or 2.0 mM $NO_2^-$ or from inducing the barley seedlings at 0.1 mM for 24 h.

### Effect of Methylamine

Methylamine, an analog of $NH_4^+$, increased $NO_3^-$ efflux (Fig. 6) and affected $NO_3^-$ and $NO_2^-$ uptake in the same manner as $NH_4^+$ (Table IV). Inhibition of net uptake occurred only when there was sufficient $NO_3^-$ inside the root tissues. Other cations such as Na, K, and Ca did not replace $NH_4^+$ in this effect (data not shown).

### Effect of Brief Loading with $NO_2^-$ and $NO_3^-$ on Efflux and Uptake

Figure 7A shows the effect of $NH_4^+$ on $NO_2^-$ efflux by roots of seedlings induced in 0.1 mM $NO_2^-$ for 24 h. A low level of $NO_2^-$ accumulated, a small amount of efflux occurred, and little effect of $NH_4^+$ was detected. However, when the roots were preloaded for a brief period with $NO_2^-$ by placing the seedlings into a solution of 2 mM $NO_2^-$ for 1 h, considerable efflux occurred and $NH_4^+$ increased the amount of $NO_2^-$ effluxed. $NH_4^+$ decreased net uptake only

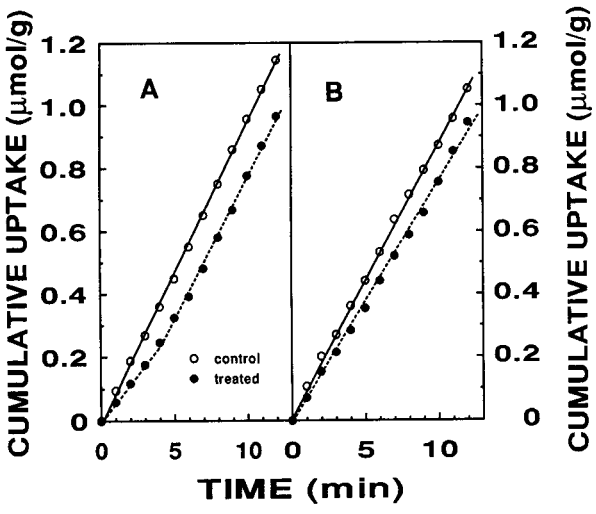


**Figure 5.** Effect of pretreatment with $NO_2^-$ on subsequent $NO_3^-$ uptake by roots of intact seedlings induced with 0.1 mM $NO_3^-$ (A) or $NO_2^-$ (B) for 24 h. The seedlings were induced as described in Figure 2. After induction, cumulative uptake of $NO_3^-$ at 0.05 mM $NO_3^-$ was determined during a 12-min period (control). The seedlings were then placed into solutions containing 0.05 mM $NO_3^-$ and 2.0 mM $NO_2^-$ (treated). After 15 min, the seedlings were rinsed with distilled water for 30 s, and cumulative uptake was determined from uptake solutions containing 0.05 mM $NO_3^-$ as described in "Materials and Methods."

in seedlings preloaded with a high concentration of $NO_2^-$ (Table II).

As described above (Fig. 2B), $NO_3^-$ efflux was not detected from roots of seedlings induced in $NO_2^-$ for 24 h; however, when these seedlings were briefly loaded (1 h) in 2 mM $NO_3^-$, efflux was readily detected and was increased by $NH_4^+$ (Fig. 7B). Interestingly, $NO_2^-$ efflux from loaded roots was greater than that of $NO_3^-$, although the internal $NO_2^-$ concentration was about 4 times less than that of $NO_3^-$ (1.58 versus 6.13 μmol/g fresh weight). Even though these seedlings were perturbed from steady state by complete removal of $NO_2^-$ or $NO_3^-$ from the external solutions, and perhaps showed greater efflux as a result, their efflux was still increased by the presence of $NH_4^+$. Table II shows the effect of the same treatments on net uptake of $NO_2^-$ and $NO_3^-$. $NH_4^+$ inhibited net uptake only when the plants were preloaded with either $NO_2^-$ or $NO_3^-$.

### Rapidity of $NH_4^+$ Response

As noted above, increased efflux of $NO_3^-$ from $NO_3^-$-loaded roots was observed within 1 min after the addition of $NH_4^+$ (Fig. 4). This is in agreement with the observed decrease in cumulative net $NO_3^-$ uptake within 15 s after $NH_4^+$ was supplied to the roots of $NO_3^-$-loaded seedlings (Fig. 8). Other investigators have found similar rapid effects of $NH_4^+$ on $NO_3^-$ uptake. Lee and Drew (1989) and Ayling (1993), using $^{13}NO_3^-$, found inhibition by $NH_4^+$ within 2 min. Ingemarsson et al. (1987) reported immediate inhibition by $NH_4^+$ following the disappearance of $NO_3^-$.

### Requirement for $NH_4^+$ in External Solution

Several investigators reported that $NH_4^+$ must be present in the external solution to affect $NO_3^-$ uptake (Deane-Drummond and Glass 1983; Ingemarsson et al., 1987; Lee and Drew, 1989). Inhibition by $NH_4^+$ was rapidly reversed in barley roots after it was removed from the external solution (Lee and Drew, 1989; Warner and Huffaker, 1989). Conversely, King et al. (1993) pretreated barley seedlings for a longer time (24 h) with high concentrations of $NH_4^+$ and found that the pretreatment greatly decreased $NO_3^-$ influx. The decreased influx was attributed to possible $NH_4^+$ feedback inhibition.

When barley seedlings were preloaded with a high concentration of $NH_4^+$ (10 mM; with or without Met sulfoximine, an inhibitor of $NH_4^+$ assimilation) for 1 h, followed by the removal of both compounds, there was little effect on ensuing uptake of either $NO_3^-$ or $NO_2^-$, even though the treated roots had accumulated 17 to 24 times more $NH_4^+$ than the control roots (Table V).

The results presented here suggest that the enhancement effect of $NH_4^+$ on $NO_3^-$ efflux occurs externally to the plasma membrane. However, it is also possible that the stimulation of $NO_3^-$ efflux could occur by the $NH_4^+$ in the cytoplasm. Roberts and Pang (1992) and Wang et al. (1993) reported that $NH_4^+$ is rapidly sequestered in the vacuole. Thus, when $NH_4^+$ is removed from the external medium, its concentration in the cytoplasm may quickly decrease. If this does in fact occur, and if the regulation by $NH_4^+$ is at the level of the cytoplasm, then this might account for the lack of an effect

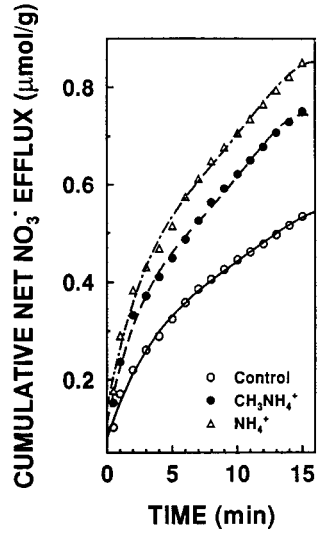


**Figure 6.** Comparative effect of $NH_4^+$ and $CH_3NH_3^+$ on $NO_3^-$ efflux from intact roots induced with 1.0 mM $NO_3^-$ for 24 h. The seedlings were grown and induced with 1 mM $NO_3^-$ as described in the legend for Figure 2. For the measurement of $NO_3^-$ efflux, the roots were rinsed for 5 s in a $NO_3^-$-free solution and transferred immediately to efflux solutions containing 1 mM Mes (pH 6.0), 1 mM $NO_2^-$, and 0 (control) or 1 mM $NH_4^+$ or 1 mM $CH_3NH_3^+$. Cumulative efflux was determined during a 15-min period as described in "Materials and Methods." The $NO_3^-$ concentration of the roots was 39.0 ± 1.2 μmol/g fresh weight.

**Table IV.** *Effect of $CH_3NH_3^+$ on $NO_3^-$ and $NO_2^-$ uptake in roots of intact seedlings induced with 1.0 mM $NO_3^-$ or 0.1 mM $NO_2^-$ for 24 h*

Uptake rates were determined by following the depletion of the ions from the uptake solutions initially containing 0.1 mM substrate and different concentrations of $CH_3NH_3^+$ as described in Table I.

| $[CH_3NH_3^+]$ | Uptake Rate[a] | | | |
|---|---|---|---|---|
| | $NO_3^-$ induced | | $NO_2^-$ induced | |
| | $NO_3^-$ | $NO_2^-$ | $NO_3^-$ | $NO_2^-$ |
| mM | $\mu mol\ g^{-1}\ h^{-1}$ | | | |
| 0 | 4.22a | 4.29a | 4.13a | 4.32a |
| 1 | 2.93b | 4.05a | 3.89a | 4.11a |
| 10 | N.D.[b] | 3.85a | N.D. | 4.11a |

[a] Uptake rates within each column followed by different letters are significantly different at $P \leq 0.05$. [b] N.D., Not determined.

of $NH_4^+$ on efflux following its removal from the external medium.

### $NH_4^+$ on $NO_3^-$ Kinetics

Attempts to show the effect of $NH_4^+$ on the kinetics of $NO_3^-$ influx have led to variable results. Inhibition of $NO_3^-$ uptake by $NH_4^+$ has been described as noncompetitive in *Neurospora* (Schloemer and Garrett, 1974); however, Lee and Drew (1989) reported that simple noncompetitive inhibition did not describe their results. In their work (Lee and Drew, 1989), the slopes of the double-reciprocal plots were not directly proportional to the $NH_4^+$ concentration but were approximately proportional to the logarithm of the $NH_4^+$ concentration. If the effect of $NH_4^+$ is mainly on $NO_3^-$ efflux and not on influx, then the above results are not surprising.

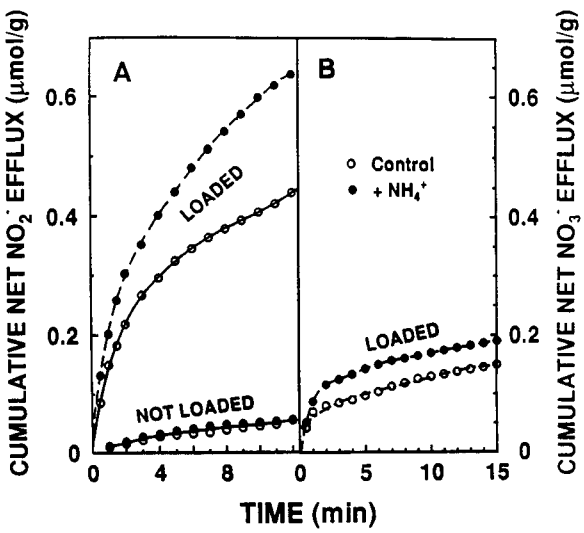


**Figure 7.** Effect of $NH_4^+$ on $NO_2^-$ (A) and $NO_3^-$ (B) efflux from roots of intact seedlings induced with 0.1 mM $NO_2^-$ for 24 h and then loaded with 2 mM $NO_2^-$ or $NO_3^-$ for 1 h. $NO_2^-$ and $NO_3^-$ efflux were then determined as described in "Materials and Methods." The $NO_2^-$ and $NO_3^-$ concentrations of the roots loaded with $NO_2^-$ and $NO_3^-$ were, respectively, 1.6 ± 0.1 and 6.1 ± 0.1 μmol/g fresh weight.

### Relation to Other Work

It appears that the contrary views regarding which component of net $NO_3^-$ uptake is affected by $NH_4^+$ may be related to the methods used by the different investigators. For example, the use of $^{36}ClO_3^-$, $^{13}NO_3^-$, and $^{15}NO_3^-$ isotopes to determine the effect of $NH_4^+$ on $NO_3^-$ uptake has led to opposite interpretations. Deane-Drummond and Glass (1983) and later work by Deane-Drummond (1985, 1986), using $ClO_3^-$ as an analog for $NO_3^-$ to determine $NO_3^-$ influx, showed no effect of $NH_4^+$ on influx and attributed decreased net uptake to efflux by *C. corallina* cells. However, the use of $^{36}ClO_3^-$ as an analog of $NO_3^-$ seems suspicious. Glass et al. (1985) pointed out that contamination of $^{36}ClO_3^-$ with $^{36}Cl$ may introduce significant error when it is used as a tracer for

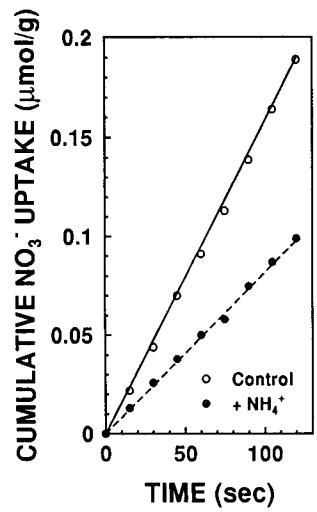


**Figure 8.** Effect of $NH_4^+$ on cumulative uptake of $NO_3^-$ by intact roots induced with 0.1 mM $NO_3^-$ for 24 h. The experimental details are the same as described in Figure 2A, except that uptake was determined by sampling at 15-s intervals for 2 min only.

**Table V.** *Effect of pretreatment with $NH_4^+$ on subsequent $NO_3^-$ and $NO_2^-$ uptake by roots of intact seedlings induced with 1.0 mM $NO_3^-$ for 24 h*

For pretreatment with $NH_4^+$, the seedlings were placed in 500 mL of solutions containing 10 mM $NH_4^+$, 1.0 mM $NO_3^-$, and 0 or 1.0 mM methionine sulfoximine (MSO) for 1 h. After pretreatment, the roots were rinsed in a $NH_4^+$-free solution for 30 s. The uptake solutions contained 0.1 mM $NO_3^-$ or $NO_2^-$ and 0 (pretreated) or 10 mM (not pretreated) $NH_4^+$. [$NH_4^+$] in the roots was determined prior to measurement of uptake. Uptake rates were computed by linear regression of the cumulative uptake data.

| Treatments | Uptake Rates[a] | | [$NH_4^+$] in Roots |
|---|---|---|---|
| | $NO_3^-$ | $NO_2^-$ | |
| | $\mu mol\ g^{-1}\ h^{-1}$ | | $\mu mol\ g^{-1}$ |
| No pretreatment | | | |
| Control | 4.75a | 4.33a | 0.90 ± 0.03 |
| +10 mM $NH_4^+$ | 2.32b | 3.92a | 0.90 ± 0.03 |
| Pretreatment | | | |
| $NH_4^+$ − MSO | 4.48a | 3.89a | 15.49 ± 0.98 |
| $NH_4^+$ + MSO | 4.31a | 4.06a | 21.71 ± 2.14 |

[a] Uptake rates within each column followed by different letters are significantly different at $P \leq 0.05$.

$NO_3^-$. Kosola (1991) and Siddiqi et al. (1992) found that $ClO_3^-$ may be a poor analog for $NO_3^-$ in some organisms. We also found $ClO_3^-$ to be a poor analog for $NO_3^-$ (data not shown).

To our knowledge, all studies in which $^{13}NO_3^-$ has been used to determine the effect of $NH_4^+$ on $NO_3^-$ uptake have concluded that $NO_3^-$ influx is inhibited by the presence of $NH_4^+$ (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). Lee and Drew (1986) suggested, however, that the influx of $NO_3^-$, as measured by $^{13}NO_3^-$, may be underestimated by as much as 25 to 30% at concentrations of 0.15 mM because of concurrent efflux of the tracer during the uptake period. Siddiqi et al. (1989) pointed out that this depends on the rate of increase of cytoplasmic specific activity and the rate of efflux. Perhaps the presence of $NH_4^+$ can facilitate such efflux. This would be detected as decreased $NO_3^-$ influx in the presence of $NH_4^+$. In $^{15}NO_3^-$ experiments, decreased $NO_3^-$ influx ascribed to $NH_4^+$ (MacKown et al., 1982a) required longer uptake times (2 h) relative to the half-life of cytoplasmic exchange, e.g. 4 min (Lee and Clarkson, 1986) and 7 min (Siddiqi et al., 1991); therefore, these studies may actually reflect net uptake (Fried et al., 1965).

Other factors may also contribute to the reported variability. Genotypic variation occurs in barley (Bloom and Finazzo, 1986) and corn (Pan et al., 1985) roots relative to the effect of $NH_4^+$ on $NO_3^-$ uptake. Decreased in vivo reduction of root $NO_3^-$ in the presence of $NH_4^+$ has been proposed as a possible secondary effector causing decreased $NO_3^-$ uptake (MacKown et al., 1982b; Pan et al., 1985). Although this may occur in long-term studies, evidence indicates that it likely does not affect shorter-term studies. The double barley mutant, which lacks both the NADH and NAD(P)H nitrate reductase isozymes and, therefore, can reduce very little $NO_3^-$, had net $NO_3^-$ uptake rates similar to those of the wild type (Warner and Huffaker, 1989).

## SUMMARY

Our results show that $NH_4^+$ had little effect on $NO_3^-$ influx in roots of plants that contained negligible concentrations of $NO_3^-$. As expected, no $NO_3^-$ efflux was detected from such seedlings. In contrast, $NH_4^+$ inhibited net $NO_3^-$ uptake as a function of the internal concentration of $NO_3^-$. This was the direct result of increased $NO_3^-$ efflux in the presence of $NH_4^+$.

The results were substantiated by determining the effects of $NH_4^+$ on $NO_2^-$ uptake. There was little if any effect of $NH_4^+$ on $NO_2^-$ uptake unless the roots were loaded with $NO_2^-$ just prior to determination of uptake. Under those conditions, net $NO_2^-$ uptake was decreased as a result of increased efflux in the presence of $NH_4^+$.

Received March 21, 1994; accepted July 20, 1994.


## LITERATURE CITED

**Aslam M, Huffaker RC, Rains DW, Rao KP** (1979) Influence of light and ambient carbon dioxide concentration on nitrate assimilation by intact barley seedlings. Plant Physiol **63:** 1205–1209

**Aslam M, Travis RL, Huffaker RC** (1992) Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (*Hordeum vulgare* L.) seedlings. Plant Physiol **99:** 1124–1133

**Aslam M, Travis RL, Huffaker RC** (1993) Comparative induction of nitrate and nitrite uptake and reduction systems by ambient nitrate and nitrite in intact roots of barley (*Hordeum vulgare* L.) seedlings. Plant Physiol **102:** 811–819

**Ayling SM** (1993) The effect of ammonium ion on membrane potential and anion flux in roots of barley and tomato. Plant Cell Environ **16:** 297–303

**Bloom AJ, Finazzo J** (1986) The influence of ammonium and chloride on potassium and nitrate absorption by barley roots depends on time of exposure and cultivar. Plant Physiol **81:** 67–69

**Breteler H, Siegerist M** (1984) Effect of ammonium on nitrate utilization by roots of dwarf bean. Plant Physiol **75:** 1099–1103

**Carlson RM** (1978) Automated separation and conductimetric determination of ammonia and dissolved carbon dioxide. Anal Chem **50:** 1528–1531

**Chaillou S, Rideout JW, Raper CD, Morot-Gaudry J-F** (1994) Responses of soybean to ammonium and nitrate supplied in combination to the whole root system or separately in a split-root system. Physiol Plant **90:** 259–268

**de la Haba P, Aguera E, Maldonado JM** (1990) Differential effects of ammonium and tungsten on nitrate and nitrite uptake and reduction by sunflower plants. Plant Sci **70:** 21–26

**Deane-Drummond CE** (1985) Regulation of nitrate uptake into *Chara corallina* cells via $NH_4^+$ stimulation of $NO_3^-$ efflux. Plant Cell Environ **8:** 105–111

**Deane-Drummond CE** (1986) Nitrate uptake into *Pisum sativum* L cv. Feltham First seedlings: commonality with nitrate uptake into *Chara corallina* and *Hordeum vulgare* through a substrate cycling model. Plant Cell Environ **9:** 41–48

**Deane-Drummond CE, Glass ADM** (1983) Short term studies of nitrate uptake into barley plants using ion-specific electrodes and $^{36}ClO_3^-$. II. Regulation of $NO_3^-$ efflux by $NH_4^+$. Plant Physiol **73:** 105–110

**Doddema H, Telkamp GP** (1979) Uptake of nitrate by mutants of *Arabidopsis thaliana* disturbed in uptake or reduction of nitrate. II. Kinetics. Physiol Plant **45:** 332–338

**Fried M, Zsoldos F, Vose PB, Shatokhin IL** (1965) Characterizing

the $NO_3^-$ and $NH_4^+$ uptake process of rice roots by use of $^{15}N$ labelled $NH_4NO_3$. Physiol Plant **18:** 313–320

**Glass ADM, Thompson RG, Bordeleau L** (1985) Regulation of $NO_3^-$ influx in barley. Studies using $^{13}NO_3^-$. Plant Physiol **77:** 379–381

**Goyal SS, Huffaker RC** (1986) A novel approach and a fully automated microcomputer-based system to study kinetics of $NO_3^-$, $NO_2^-$, and $NH_4^+$ transport simultaneously by intact wheat seedlings. Plant Cell Environ **9:** 209–215

**Hoagland DR, Arnon DI** (1950) The water culture method for growing plants without soil. Calif Agric Exp Stn Bull **347:** 32

**Ingemarsson B, Oscarson P, af Ugglas M, Larsson C-M** (1987) Nitrogen utilization in *Lemna*. III. Short term effects of ammonium on nitrate uptake and nitrate reduction. Plant Physiol **85:** 865–867

**Jackson WA, Kwik KD, Volk RJ, Butz RG** (1976) Nitrate influx and efflux by intact wheat seedlings: effects of prior nitrate nutrition. Planta **132:** 149–156

**King BJ, Siddiqi MY, Ruth TJ, Warner RL, Glass ADM** (1993) Feedback regulation of nitrate influx in barley roots by nitrate, nitrite, and ammonium. Plant Physiol **102:** 1279–1286

**Kosola KR** (1991) Transport of ammonium, nitrate, methylammonium, and chlorate in tomato (*Lycopersicon esculentum*, Mill, cv T5), and characterization of a nitrate reductase deficient tomato mutant. PhD thesis, University of California, Davis

**Lee RB, Clarkson DT** (1986) Nitrogen-13 studies of nitrate fluxes in barley roots. I. Compartmental analysis from measurements of $^{13}N$ efflux. J Exp Bot **37:** 1753–1767

**Lee RB, Drew MC** (1986) Nitrogen-13 studies of nitrate fluxes in barley roots. II. Effect of plant N status on the kinetic parameters of nitrate influx. J Exp Bot **37:** 1768–1779

**Lee RB, Drew MC** (1989) Rapid, reversible inhibition of nitrate influx in barley by ammonium. J Exp Bot **40:** 741–752

**MacKown CT, Jackson WA, Volk RJ** (1982a) Restricted nitrate influx and reduction in corn seedlings exposed to ammonium. Plant Physiol **69:** 353–359

**MacKown CT, Volk RJ, Jackson WA** (1982b) Nitrate assimilation by decapitated corn root systems: effects of ammonium during induction. Plant Sci Lett **24:** 295–302

**Oaks A, Stulen I, Boesel IL** (1979) Influence of amino acids and ammonium on nitrate reduction in corn seedlings. Can J Bot **57:** 1824–1829

**Oscarson P, Ingmarsson B, af Ugglas M, Larsson C-M** (1987) Short term studies of $NO_3^-$ uptake in *Pisum* using $^{13}NO_3^-$. Planta **170:** 550–555

**Pan WL, Jackson WA, Moll RH** (1985) Nitrate uptake and partitioning by corn root systems. Differential effects of ammonium among genotypes and stages of root development. Plant Physiol **77:** 560–566

**Rao KP, Rains DW** (1976) Nitrate absorption by barley. I. Kinetics and energetics. Plant Physiol **57:** 55–58

**Roberts JKM, Pang MKL** (1992) Estimation of ammonium ion distribution between cytoplasm and vacuole using nuclear magnetic resonance spectroscopy. Plant Physiol **100:** 1571–1574

**Rufty TW, Raper CD, Jackson WA** (1983) Growth and nitrogen assimilation of soybeans in response to ammonium and nitrate nutrition. Bot Gaz **144:** 466–470

**Schloemer RH, Garrett RH** (1974) Nitrate transport system in *Neurospora crassa*. J Bacteriol **118:** 259–269

**Schrader LE, Domska D, Jung PE, Peterson LA** (1972) Uptake and assimilation of ammonium-N and nitrate-N and their influence on the growth of corn (*Zea mays* L.). Agron J **64:** 690–695

**Siddiqi MY, Glass ADM, Ruth TJ** (1991) Studies of the uptake of nitrate in barley. III. Compartmentation of $NO_3^-$. J Exp Bot **42:** 1455–1463

**Siddiqi MY, Glass ADM, Ruth TJ, Fernando M** (1989) Studies on the regulation of nitrate influx by barley seedlings using $^{13}NO_3^-$. Plant Physiol **90:** 806–813

**Siddiqi MY, King BJ, Glass ADM** (1992) Effects of nitrite, chlorate, and chlorite on nitrate uptake and nitrate reductase activity. Plant Physiol **100:** 644–650

**Smith FW, Thompson JF** (1971) Regulation of nitrate reductase in excised barley roots. Plant Physiol **48:** 219–223

**Thayer JR, Huffaker RC** (1980) Determination of nitrate and nitrite by high-pressure liquid chromatography: comparison with other methods for nitrate determination. Anal Biochem **102:** 110–119

**Ullrich WR, Larsson M, Larsson C-M, Lesch S, Novacky A** (1984) Ammonium uptake in *Lemna gibba* Gl, related membrane potential changes, and inhibition of anion uptake. Physiol Plant **61:** 369–376

**Wang MY, Siddiqi MY, Ruth TJ, Glass ADM** (1993) Ammonium uptake by rice roots. I. Fluxes and subcellular distribution of $^{13}NH_4^+$. Plant Physiol **103:** 1249–1258

**Warner RL, Huffaker RC** (1989) Nitrate transport is independent of NADH and NAD(P)H nitrate reductases in barley seedlings. Plant Physiol **91:** 947–953