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# Studies of the Regulation of Nitrate Influx by Barley Seedlings Using $^{13}NO_3^{-}$[1]

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## ABSTRACT

**Using $^{13}NO_3^-$, effects of various $NO_3^-$ pretreatments upon $NO_3^-$ influx were studied in intact roots of barley (*Hordeum vulgare* L. cv Klondike). Prior exposure of roots to $NO_3^-$ increased $NO_3^-$ influx and net $NO_3^-$ uptake. This 'induction' of $NO_3^-$ uptake was dependent both on time and external $NO_3^-$ concentration ($[NO_3^-]$). During induction influx was positively correlated with root $[NO_3^-]$. In the postinduction period, however, $NO_3^-$ influx declined as root $[NO_3^-]$ increased. It is suggested that induction and negative feedback regulation are independent processes: Induction appears to depend upon some critical cytoplasmic $[NO_3^-]$; removal of external $NO_3^-$ caused a reduction of $^{13}NO_3^-$ influx even though mean root $[NO_3^-]$ remained high. It is proposed that cytoplasmic $[NO_3^-]$ is depleted rapidly under these conditions resulting in 'deinduction' of the $NO_3^-$ transport system. Beyond 50 micromoles per gram $[NO_3^-]$, $^{13}NO_3^-$ influx was negatively correlated with root $[NO_3^-]$. However, it is unclear whether root $[NO_3^-]$ *per se* or some product(s) of $NO_3^-$ assimilation are responsible for the negative feedback effects.**

In higher plants, $NO_3^-$ uptake is unique in that it is subject to both positive and negative feedback regulation. Plants which have not been pretreated with $NO_3^-$ show low levels of tissue $NO_3^-$ and low rates of net $NO_3^-$ uptake (sometimes referred to as 'constitutive' uptake [4]). Following exposure to $NO_3^-$, root $[NO_3^-]$ and $NO_3^-$ uptake increase several fold with time (4, 10, 13). This increase in $NO_3^-$ uptake ('induced' by $NO_3^-$) appears to be a specific response to $NO_3^-$ provision, since other sources of N (*e.g.* $NH_4^+$) appear to be ineffective. Thus it is probable that $NO_3^-$ *per se* is the inducing agent. Consistent with this observation is the finding that $NO_3^-$ uptake is independent of the activity of the enzyme nitrate reductase (12, for review see 4).

Numerous studies have shown that, during post induction periods, *net* $NO_3^-$ uptake rates are under negative feedback control: they increase following N-starvation and decrease following $NO_3^-$ pretreatment (15, 18 and references therein). However, in other species (24, 29) the existence of negative feedback may be obscured because removal of $NO_3^-$ from the external medium causes an immediate decrease of $NO_3^-$ uptake.

The mechanism(s) responsible for exerting negative feedback control upon net $NO_3^-$ uptake have been in dispute. According to Deane-Drummond and Glass (6, 7) and Glass (10), who used $^{36}ClO_3^-$ and $^{13}NO_3^-$, respectively, to examine $NO_3^-$ influx after various $NO_3^-$ pretreatments, influx was independent of prior exposure to $NO_3^-$. By contrast, Lee and Drew (17) showed that withholding $NO_3^-$ for 3 d increased $V_{max}$ for $NO_3^-$ influx. It has been suggested that both influx and efflux may be regulated independently and that the contribution of efflux in the control of net flux may be a feature of perturbation rather than steady state (4).

The apparently opposing effect of $NO_3^-$, with respect to induction and negative feedback, presents a much more complicated scenario for understanding the regulation of $NO_3^-$ uptake than is the case for most other ions. Furthermore, the metabolism of $NO_3^-$ to generate a host of reduced nitrogen derivatives further complicates prospects for identifying feedback signals. It is evident that to clarify these processes it is critical to examine $NO_3^-$ fluxes over as short a time scale as is feasible and to undertake time course studies so that the component processes (induction and negative feedback) can be identified and isolated. The experiments reported here, using $^{13}NO_3^-$, were designed to meet these goals.

## MATERIALS AND METHODS

### Seed Germination and Plant Growth

Seeds of barley (*Hordeum vulgare* L. cv Klondike) were germinated in sterilized moist sand on plastic mesh fitted into plexiglass discs (40–50 seeds per disc) as described by Siddiqi and Glass (26). After 3 d of germination in the dark, seedlings were transferred to Plexiglas hydroponic tanks (approximately 40 L capacity) containing modified Johnson's nutrient solution at appropriate strength (1/10 or 1/80 as indicated) with or without appropriate amounts of $Ca(NO_3)_2$. The composition of modified Johnson's solution was as follows: $KH_2PO_4$ 2 mM. $K_2SO_4$ 2 mM, $MgSO_4$ 1 mM, $Ca^{2+}$ [as $CaSO_4$ and/or $Ca(NO_3)_2$] 4 mM; micronutrients and Fe (as Fe-EDTA) $\mu$M: Cl 50, B 25, Mn 2, Zn 2, Cu 0.5, Fe 20.

In $NO_3^-$ treatments, an appropriate amount of $Ca(NO_3^-)_2$ was added to provide the required concentration of $NO_3^-$. In all experiments, solution $NO_3^-$ and $K^+$ concentrations were maintained by means of peristaltic pumps. Concentrations of these ions were measured at least twice a day, and pump speed and/or concentration of stock solution was adjusted accordingly. Other nutrients were supplied by reference to $K^+$ (in the same proportions as in the original solution).

The plants were maintained in a controlled environment

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room at 20 ± 2°C, 16 h light-8 h dark cycle, and 70% RH. The light was provided at 300 $\mu E\ m^{-2}\ S^{-1}$ (plant level) by fluorescent tubes having spectral composition similar to sunlight. In all the experiments, induction was carried out in light; for final 30 h, all plants remained in continuous light.

### Induction of Nitrate Uptake

After 3 d of germination in sand, seven sets of seedlings (40 seedlings per set) were transferred to modified 1/10 Johnson's solution without N (−N tank) and one set of seedlings to 1/10 Johnson's solution containing 1.5 mol $m^{-3}$ $NO_3^-$ (+N tank). Seedlings were transferred from −N to +N tank at intervals to give 0, 12, 24, 48, 72, 96, 120, and 144 h of $NO_3^-$ pretreatment (experiment 1). In another experiment exactly the same procedure was followed except that the solution concentration was 1/80 Johnson with or without 0.1 mol $m^{-3}$ $NO_3^-$ (experiment 2). Effects of shorter exposures to $NO_3^-$ were investigated in 7 d old seedlings by pretreating seedlings with 0.1 mol $m^{-3}$ $NO_3^-$ for 0, 2, 4, 6, 12 and 24 h (experiment 3).

The effect of external nitrate ($[NO_3^-]_0$) and duration of pretreatment at these $[NO_3^-]_0$ on the induction of $NO_3^-$ uptake were investigated by pretreating the seedlings after germination for 0, 6, 12, 24, or 96 h with 0.01, 0.1, 1.0, or 10.0 mol $m^{-3}$ $NO_3^-$ (experiment 4).

### Decay of Induction (Deinduction) of $NO_3^-$ Uptake and Reinduction

It was important in these comparative experiments to ensure that (a) all pretreatments terminated at the anticipated time of $^{13}NO_3^-$ arrival, (b) there be no treatment differences arising from diurnal or age differences in plants, and (c) differences arising from variations between separate experiments were eliminated. Hence, in the first deinduction/reinduction experiment (experiment 5), treatments were carefully staggered so that all plants received 4 d $NO_3^-$ (1.5 mol $m^{-3}$) exposure followed by variable periods of N-deprivation ((0, 12, 24, 48, and 72 h). A second set of all treatments was staggered by a further 12 h, so that they could be reinduced by a final 12 h exposure to 1.5 mol $m^{-3}$ $NO_3^-$. One set of plants remained in $+NO_3^-$ for the entire period (7 d) (experiment 5).

In a separate experiment, deinduction was followed over shorter periods by removing exogenous $NO_3^-$ for 0, 2, 4, 5, 7, and 14 h (experiment 6). This experiment differed from experiment 5 in two other respects: (a) treatments were not staggered; thus 0 deinduction treatment had 96 h exposure to $NO_3^-$ whereas the period of deinduction (2, 4, 5, 7, and 14 h) for other treatments resulted in a variable exposure to $NO_3^-$; for example, the 14 h deinduction treatment was exposed to $NO_3^-$ for 82 h followed by 14 h in the absence of $NO_3^-$. (b) Nutrient concentration during growth was 1/80 Johnson's solution with or without 0.1 mol $m^{-3}$ $NO_3^-$.

All experiments were repeated two or three times. Each treatment was replicated four or five times and each replicate consisted of approximately 10 seedlings.

### Measurement of Influx

In all experiments influx was measured from 0.13 mol $m^{-3}$ $NO_3^-$ labeled with $^{13}NO_3^-$; all other nutrients were provided as in the respective growth medium (1/80 or 1/10 Johnson's solution). The pH of the uptake solution was adjusted to 6 with KOH. The volume of influx solution ($cm^3$): root weight (g) ratio was approximately 80:1 (40 seedlings in 400 $cm^3$ solution) so that the depletion of $NO_3^-$ during the 10 min influx period was approximately 10% or less.

Influx was measured into intact roots that were prewashed for 5 min in fresh nonradioactive solution (identical to influx solution in all other respects). In the case of 'step-down' experiments (*i.e.* plants grown in 1.5 mol $m^{-3}$ $NO_3^-$ and influx measured from 0.13 mol $m^{-3}$ $NO_3^-$) roots were prewashed in 0.13 mol $m^{-3}$ $NO_3^-$ for 20 min. A preliminary experiment demonstrated that in this situation, there was an initial rapid efflux of $NO_3^-$: however, by 15 to 20 min this transient effect of perturbation had ceased. Therefore, in all such experiments, roots were prewashed for 20 min in 0.13 mol $m^{-3}$ $NO_3^-$ before transfer to $^{13}NO_3^-$ labeled solutions. The roots were transferred to influx solution labeled with $^{13}NO_3^-$ for 10 min. The uptake was terminated by transferring roots to 40 $dm^3$ of an identical but nonradioactive solution for 2 min to desorb $^{13}NO_3^-$ from the free space. The desorption time of 2 min was selected on the basis of efflux experiments to maximize removal of cell wall $^{13}NO_3^-$ while minimizing loss from a cytoplasmic pool (our unpublished data). Roots were placed in scintillation vials and counted immediately in a Searle Isocap scintillation spectrometer without scintillation cocktail. Root samples were then weighed and their $NO_3^-$ content determined.

After correction for decay, fluxes were corrected for quenching by the root tissue. A quench curve was established by comparative counting of $\gamma$-emissions in a $\gamma$-counter and positron emission in the scintillation counter. Unfortunately, the $\gamma$-counter was remote from our laboratory and its use involved loss of approximately 1 half-life in transporting the samples across campus. Hence, for all of the experiments described, counting of the positron emission was obtained by scintillation counting.

### Net $NO_3^-$ Uptake

Rates of net uptake of $NO_3^-$ were determined by measuring the disappearance of $NO_3^-$ from the external medium.

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

The $^{13}N$ species were produced by the proton irradiation of $H_2O$ on the TRIUMF-ACEL CP42 cyclotron using 20 MeV protons. The 3 mL target volume was loaded remotely and an overpressure of 3 atm was used during irradiation. Typically, the irradiations were performed for 10 min with a 10 $\mu$A beam. These conditions provided $^{13}N$ primarily as $NO_3^-$ (>90%) (28).

The samples were transported from the TRIUMF facility to the University of British Columbia campus via an underground pipeline with transit times of 2 to 3 min.

However, $^{13}NH_4^+$, $^{13}NO_2^-$ and some $^{18}F$ were present in the

sample as contaminants. These were removed by the following procedures. $^{18}F$ was removed by passing the sample through a SEP-PAK Alumina N Cartridge (Waters Associates) twice. Then 2 mL of 1 N $H_2SO_4$ and 1 mL of 20% $H_2O_2$ were added and the sample was boiled for 2.5 min to remove $NO_2^-$ (16). The sample was cooled in an ice bath and passed through a cation exchange column (AG 50W-X8, 100–200 mesh, hydrogen form, Bio-Rad) which effectively removed $^{13}NH_4^+$ from the sample. We routinely determined the $t_{1/2}$ of the purified samples which were always found to be extremely close to the reported literature value (9.97 min).

### $NO_3^-$ Analysis in Plant and Solution Samples

$NO_3^-$ from roots was extracted by boiling the tissue in deionized distilled water. $NO_3^-$ concentration in the extract was determined by two procedures: (a) UV absorption: to 1 $cm^3$ sample, 4 $cm^3$ of 5% $HClO_4$ were added and absorbance was measured at 210 nm (3). (b) Cadmium-copper reduction method using a Technicon Autoanalyzer (30). Selected samples were analyzed using this procedure. In solution samples the two procedures concurred closely. In root samples, the UV procedure consistently overestimated $[NO_3^-]$ by ~10 $\mu$mol $g^{-1}$. Thus, in samples which were analyzed by the UV procedure, appropriate correction was made for this overestimate.

## RESULTS

### Induction of Influx of $NO_3^-$

Figures 1 to 4 show that $^{13}NO_3^-$ influx is a highly inducible process. In plants pretreated in various concentrations of $NO_3^-$ (0.1 to 10 mol $m^{-3}$) influxes increased for the first 12 to 24 h of exposure and then declined to a steady value, reached after 48 to 72 h. These $NO_3^-$ pretreatments caused $^{13}NO_3^-$ influx to increase by factors of four- to fivefold compared to uninduced plants (plants never exposed to $NO_3^-$ except for a 5 min prewash prior to influx measurement). The time taken to attain maximum induction varied with $[NO_3^-]_0$, increasing with decreasing $[NO_3^-]_0$ (Fig. 4). During the induction period (up to the time when influx peaked), influx was positively correlated with root $[NO_3^-]$ (Figs. 1–5). Subsequently, influxes declined while root $[NO_3^-]$ either continued to increase or showed little change. At 0.01 mol $m^{-3}$, however, both influxes and root $[NO_3^-]$ continued to increase throughout the experimental period (96 h) (Figs. 4 and 5).

A plot of $^{13}NO_3^-$ influx *versus* root $[NO_3^-]$ yielded a parabola. In the range from 0 to ~50 $\mu$mol $g^{-1}$, influxes appear to be positively related to root $[NO_3^-]$, whereas beyond 50 $\mu$mol $g^{-1}$ they are negatively related to root $[NO_3^-]$ (Fig. 6).

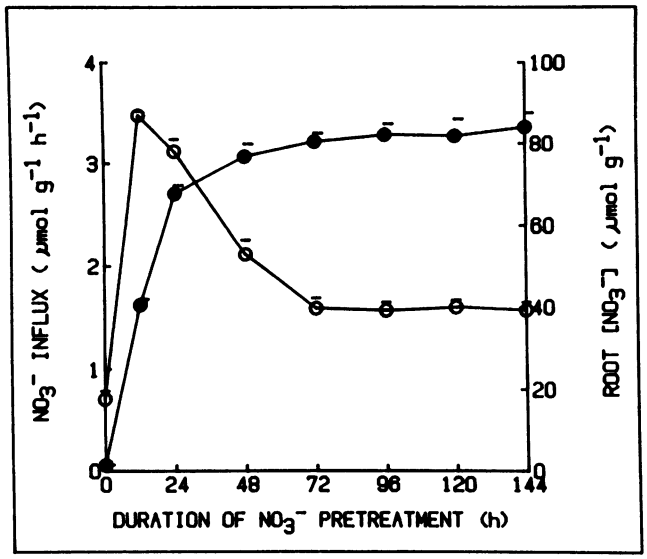


**Figure 1.** $NO_3^-$ influx (○) (± SE) and root $[NO_3^-]$ (●) (± SE) after pretreatment with 1.5 mol $m^{-3}$ $NO_3^-$ for 144 h (see text).

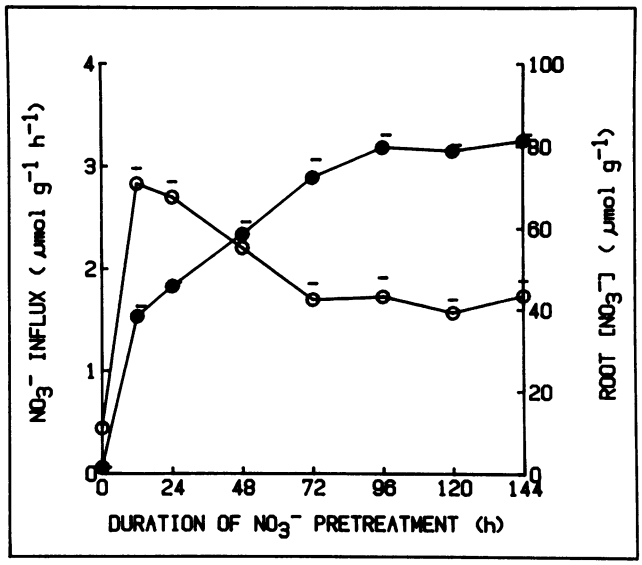


**Figure 2.** $NO_3^-$ influx (○) (± SE) and root $[NO_3^-]$ (●) after pretreatment with 0.1 mol $m^{-3}$ $NO_3^-$ for 0 to 144 hours (see text).

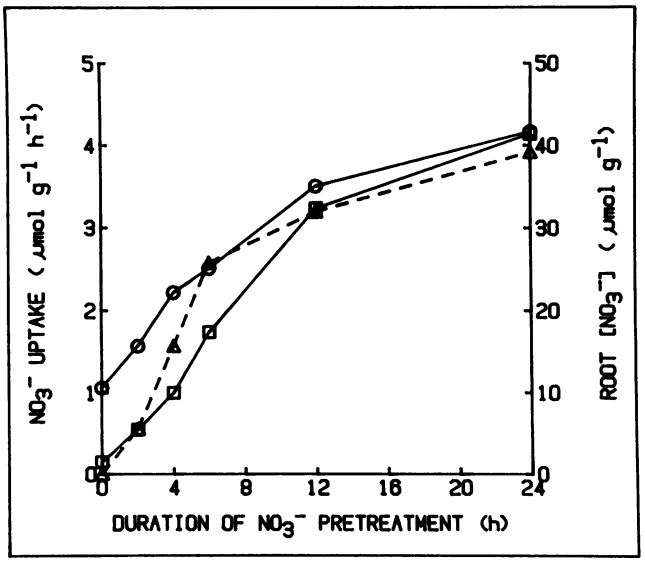


**Figure 3.** $NO_3^-$ influx (○), rate of net $NO_3^-$ uptake (△) and root $[NO_3^-]$ (□) after pretreatment with 0.1 mol $m^{-3}$ $NO_3^-$ for 0 to 24 h (see text). Standard errors (not shown) were within 10% of the respective means.

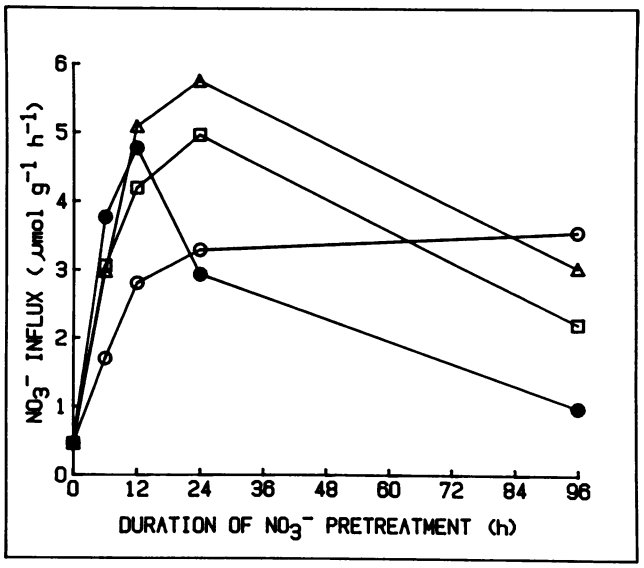


**Figure 4.** $NO_3^-$ influx after pretreatment with 0.01 (○), 0.1 (△), 1.0 (□) or 10.0 (●) mol $m^{-3}$ $NO_3^-$ for 0 to 96 h (see text). Standard errors (not shown) were within 10% of the respective means.

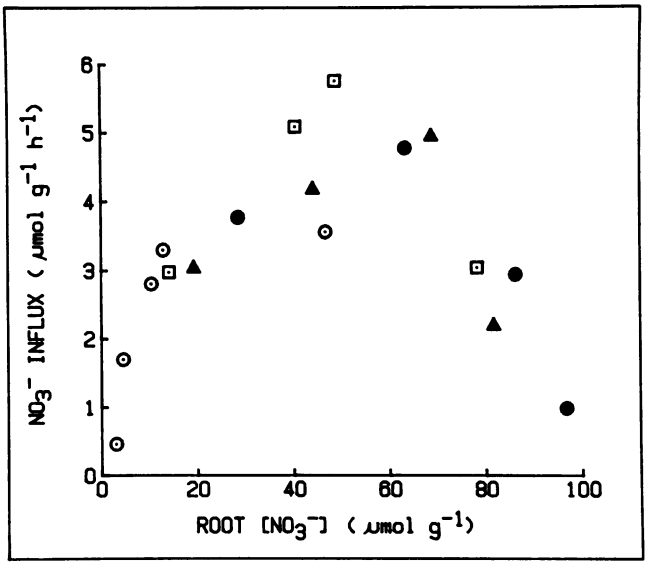


**Figure 6.** $NO_3^-$ influx plotted against root [$NO_3^-$] from Figures 4 and 5. Plants were exposed to 0.01 (○), 0.1 (□), 1.0 (△) or 10.0 (●) mol $m^{-3}$ $NO_3^-$ for 0 to 96 h.

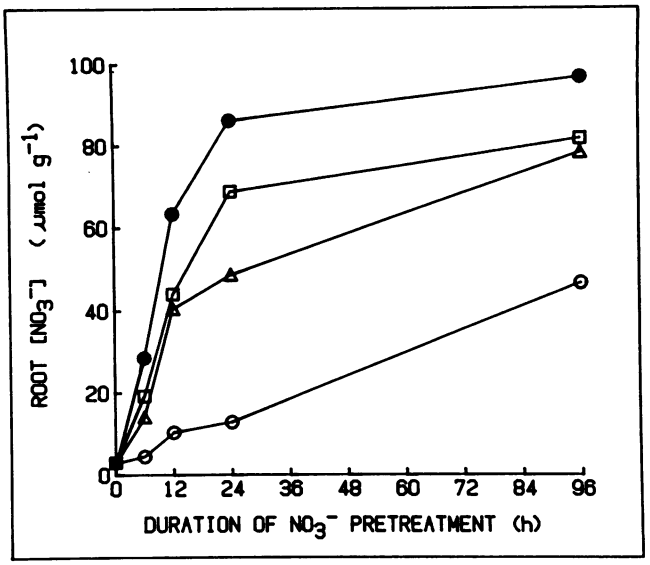


**Figure 5.** Root [$NO_3^-$] after pretreatment with 0.01 (○), 0.1 (△), 1.0 (□) or 10.0 (●) mol $m^{-3}$ $NO_3^-$ for 0 to 96 h. Standard errors (not shown) were within 10% of the respective means.

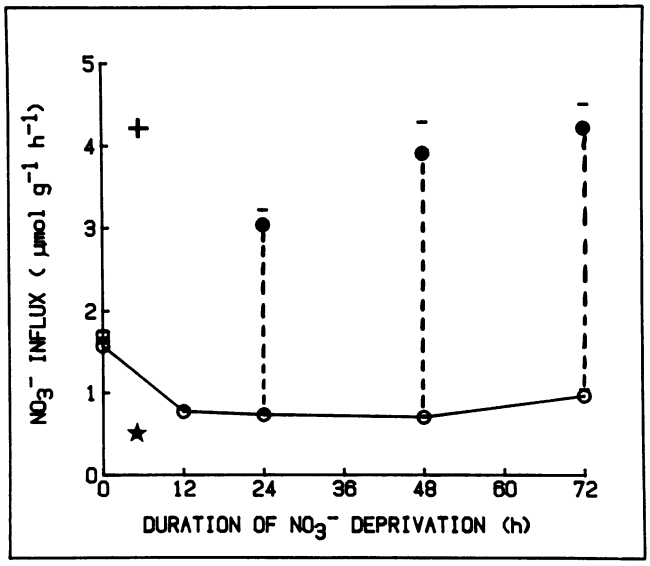


**Figure 7.** $NO_3^-$ influx (± SE) following $NO_3^-$ deprivation for 0 to 72 h of plants that were pretreated with 1.5 mol $m^{-3}$ $NO_3^-$ for 4 days. (○) Plants not reinduced after $NO_3^-$ deprivation; (●) plant reinduced by resupplying 1.5 mol $m^{-3}$ $NO_3^-$ for 12 h after the respective $NO_3^-$ deprivation treatments. Vertical broken lines indicate the extent of induction. Also shown are influx of plants grown in 1.5 mol $m^{-3}$ $NO_3^-$ for 7 days (□), always without $NO_3^-$ (★) and exposed to 1.5 mol $m^{-3}$ $NO_3^-$ only for the last 12 h (+). See text.

### Deinduction/Reinduction of $NO_3^-$ Influx

When plants previously fed with $NO_3^-$ were transferred to N-free medium, there was a substantial decline in influx within the first few hours of N-deprivation (Figs. 7 and 9). However, $^{13}NO_3^-$ fluxes of these 'deinduced' plants were ~1.5 times higher than those of plants which had never been exposed to $NO_3^-$. Two hours after removal of $NO_3^-$, $^{13}NO_3^-$ influx had declined significantly, although there was no detectable change in root [$NO_3^-$] (Fig. 9). Subsequently, N deprivation over a period of 3 days caused a gradual decrease in root [$NO_3^-$] with little change of influxes (Figs. 7 and 8). In the same experiment, one set of plants each from the 24, 48, and 72 h N-deprivation treatments was resupplied with 1.5 mol $m^{-3}$ $NO_3$ for 12 h (reinduction). This treatment resulted in a four- to fivefold increase in $^{13}NO_3^-$ influx compared to uninduced plants (Figs. 7 and 8), concomitant with increases in root [$NO_3^-$]. These reinduced plants (24–72 h of N-deprivation followed by 12 h of $NO_3^-$ exposure) showed an apparent negative relationship between $^{13}NO_3^-$ influx and root [$NO_3^-$]. With increasing duration of N deprivation, fluxes

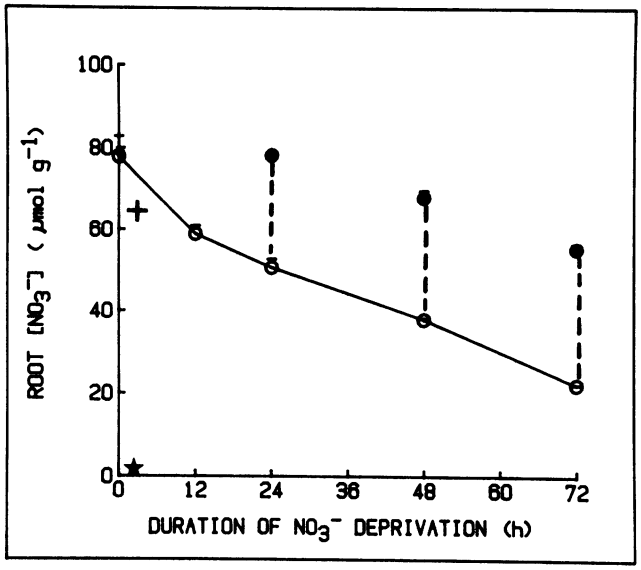


**Figure 8.** Root $[NO_3^-]$ (± SE) following various $NO_3^-$ deprivation treatments whose influxes are shown in Figure 6. For details and symbols, see Figure 6.

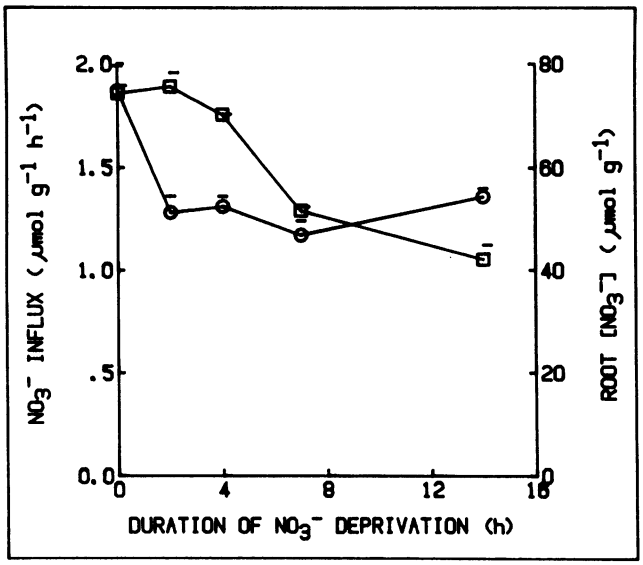


**Figure 9.** $NO_3^-$ influx (± SE) (○) and root $[NO_3^-]$ (± SE) (□) following $NO_3^-$ deprivation for 0 to 14 h of plants that were pretreated with 0.1 mol $m^{-3}$ $NO_3^-$ for 3 d (see text).

were higher and root $[NO_3^-]$ was lower (Figs. 7 and 8) following reinduction. However, root $[NO_3^-]$ of plants which had never been deprived of $NO_3^-$ (Fig. 8) was similar to that of the 24 h reinduced plants although $^{13}NO_3^-$ influx of the latter was twofold higher.

## DISCUSSION

In the measurement of unidirectional influx of ions, using radioactive tracers, there is an inherent error (underestimation) involved due to a concurrent efflux of the tracer during the experimental period. The magnitude of this error depends on the rate of increase of cytoplasmic specific activity and the rate of efflux. Lee and Drew (17) have considered this aspect in detail regarding $^{13}NO_3^-$ influx in barley. They estimated that influx measured over a period of 15 min from 0.15 mol $m^{-3}$ $NO_3^-$ would be underestimated by 26 to 29%. Ideally, then, the influx period should be short relative to the half-life of exchange. However, influx period <5 min may result in underestimation due to failure to equilibrate the apparent free space. In the case of $^{13}NO_3^-$ ($t_{1/2}$ = 9.97 min) studies, there is an additional requirement that the influx be sufficiently long to accumulate measurable counts. Using influx periods of 1, 2.5, 5, 7.5, and 10 min we observed, like Lee and Drew (17), that a 10 min influx period did underestimate influx (Table I). Taking the 1 min influx period as equivalent to 100%, 10 min influx values were 74% of the former. However, since determination of specific uptake rates (μmol $g^{-1}$ $h^{-1}$) involved multiplying by 60, 24, 11, 8, 6, respectively, any error associated with remaining apparent free space $^{13}NO_3^-$ would overestimate influx based upon a 1 min influx period. Given the need to consider the factors discussed above as well as the technical problem of handling large numbers of samples, the 10 min influx period, 2 min desorption was considered an acceptable compromise, particularly since the investigation sought to examine comparative effects of induction and negative feedback.

**Table I.** *$^{13}NO_3^-$ Influx as Determined by Exposing Roots to 0.13 mol $m^{-3}$ $NO_3^-$ for Varying Periods (Duration of Influx)*

These plants were pretreated with 0.13 mol $m^{-3}$ $NO_3^-$ for 24 h.

| Duration of Influx | Influx |
|---|---|
| *min* | *μmol $g^{-1}$ $h^{-1}$ ± SE* |
| 1.0 | 9.47 ± 0.08 |
| 2.5 | 8.64 ± 0.21 |
| 5.0 | 7.28 ± 0.09 |
| 7.5 | 6.90 ± 0.17 |
| 10.0 | 6.98 ± 0.10 |

### Induction and Deinduction of $NO_3^-$ Uptake

It is well established that net $NO_3^-$ uptake is subject to induction by the presence of external $NO_3^-$ (4, 10 for review). In agreement with those of Lee and Drew (17), our results showed that plasmalemma $^{13}NO_3^-$ influx was increased by $NO_3^-$ pretreatment; maximum induction caused a four- to fivefold increase of influx compared to the uninduced plants (Figs. 1–4). Kinetic studies have suggested that constitutive and inducible $NO_3^-$ uptake are mediated by two distinct 'carrier' systems and that the latter requires *de novo* protein synthesis (13, 17).

There are conflicting reports in the literature regarding the concentration dependence of the induction of $NO_3^-$ uptake. For example Breteler and Nissen (1) observed that induction was independent of $[NO_3^-]_0$ in beans. By contrast, Neyra and Hageman (22) and Maeck and Tischner (19) reported that induction *was* concentration dependent. In our study, the induction of $^{13}NO_3^-$ influx was dependent upon $[NO_3^-]_0$ (Fig. 4). This is contrary to the results of Maeck and Tischner (19) who found that in sugarbeet, induction was more rapid at lower $[NO_3^-]_0$ in the range 0.1 to 5 mol $m^{-3}$. It is clear that in the barley cultivar we have used, 0.01 mol $m^{-3}$ $[NO_3^-]_0$

was not adequate to produce maximum induction even after 96 h of pretreatment (*cf.* ref. 1).

There is general agreement that induction of the $NO_3^-$ uptake system specifically requires the presence of external $NO_3^-$ (12, 13, 23). Neither $NH_4^+$ (12) and by implication, no other product of $NO_3^-$ reduction, is capable of inducing $NO_3^-$ uptake. Although the induction of $NO_3^-$ uptake and $NO_3^-$ reduction appear to be synchronized, there is now strong evidence that the former is independent of the latter (21, for review see 4, 10). However, Deane-Drummond (5) suggested two components of induction of $NO_3^-$ uptake in *Chara*: one independent of nitrate reductase activity and the other related to (perhaps dependent upon) the reduction of $NO_3^-$ or subsequent steps.

An increase of influx and net flux with increasing root $[NO_3^-]$ during induction (Figs. 1–5) suggests that maximum induction required a critical value of root $[NO_3^-]$. The deinduction experiments (Figs. 7–9), however, clearly show that the induction of $NO_3^-$ uptake is not a function of *mean* root $[NO_3^-]$; influx (Fig. 9) and net uptake (data not shown) had decreased within 2 h of removal of the external $NO_3^-$ source. Yet there was no detectable change in root $[NO_3^-]$ (24, 29). Further starvation decreased root $[NO_3^-]$ by ~30% with little change in influx (Figs. 7–9) Resupplying $NO_3^-$ to these starved plants increased their root $[NO_3^-]$ to almost the same level as unstarved plants (Fig. 8) yet influx of the former was two- to threefold higher than the latter (Fig. 7). It appears safe to assume that induction is dependent upon the cytoplasmic $[NO_3^-]$ (23, 29). Considering the reported short half life for $NO_3^-$ exchange and small pool size of cytoplasmic $NO_3^-$ (6, 16) (RJ Ritchie, personal communication), it is likely that in our deinduction experiments cytoplasmic $[NO_3^-]$ was significantly reduced within 2 h although mean root $[NO_3^-]$ (principally vacuolar $[NO_3^-]$) showed no appreciable change.

In some plants, *e.g.* barley cv Midas (17, 18), two corn cultivars (27) and *Arabidopsis thaliana* (8), $NO_3^-$ starvation for a few hours to days actually increased $NO_3^-$ uptake; further deprivation, however, caused reduction in $NO_3^-$ uptake, eventually to the constitutive levels. These genotypes may be more efficient in the retrieval of vacuolar $NO_3^-$ to maintain cytoplasmic $[NO_3^-]$ at some value which is critical for the maintenance of the induced condition. The corn cultivars which were the subject of the study by Theyker *et al.* (27) differed substantially in the duration of elevated fluxes following $NO_3^-$ deprivation. There is also evidence that there are substantial genotypic differences among barley cutlivars in the retrieval of vacuolar $K^+$ (20). Note that in Midas (the barley cultivar used by Lee and Rudge [18], and Lee and Drew [17]), root $[NO_3^-]$ of plants deprived of $NO_3^-$ for 1, 2, or 3 d were approximately 20, 10, and 2 $\mu$mol $g^{-1}$, respectively, compared to ~80 $\mu$mol $g^{-1}$ in unstarved plants (Table 3 in Lee and Rudge [18]). By contrast, in the cultivar Klondike (employed in the present study), starvation for 1, 2, or 3 d decreased root $[NO_3^-]$ to ~50, 40, and 20 $\mu$mol $g^{-1}$ compared to ~80 $\mu$mol $g^{-1}$ in unstarved plants (Fig. 8). These dramatic differences in the rates of depletion of vacuolar $[NO_3^-]$ between cultivars must reflect the differences in their capacities to mobilize vacuolar $NO_3^-$.

Clarkson (4) has questioned the existence of a constitutive (uninduced) $NO_3^-$ uptake system on the basis of a failure to measure net $NO_3^-$ uptake in uninduced plants. We have also observed that net uptake was not detectable until 3 to 4 h after exposure to $NO_3^-$. However, the same plants showed a $^{13}NO_3^-$ influx of ~0.7 $\mu$mol $g^{-1}h^{-1}$ upon first exposure to $NO_3^-$ (5 min prewash and 10 min uptake in $NO_3^-$ solution) (Fig. 3). This indicates that the constitutive $NO_3^-$ uptake system was present but that during the first few hours of induction efflux nearly matched the influx. After about 6 h of exposure to $NO_3^-$, efflux, relative to influx, decreased (Fig. 3) to a steady value.

### Negative Feedback Regulation of $NO_3^-$ Uptake

In contrast to the situation for induction, the signals responsible for negative feedback effects on $NO_3^-$ uptake are not known with certainty. Potential candidates might include $NO_3^-$ and products of $NO_3^-$ reduction such as $NO_2^-$, $NH_4^+$, or amino acids. In earlier reports from this laboratory (6, 7, 11), it was suggested that $NO_3^-$ influx is insensitive to prior $NO_3^-$ treatment and that the major source of regulation of $NO_3^-$ uptake was through effects on efflux (7, 8, 13). Lee and Drew (17), by contrast, have demonstrated that withholding exogenous $NO_3^-$ for 3 days caused a significant increase in the $V_{max}$ for $^{13}NO_3^-$ influx.

Our results suggest that the situation is much more complex than hitherto realized. We observed that during the induction period (until the peak induction was achieved), influxes were positively correlated with root $[NO_3^-]$ (Figs. 1–5). Subsequently influxes declined, and root $[NO_3^-]$ showed typical negative feedback effects on influx (Figs. 1, 2, 4, and 5). A plot of $[NO_3^-]$ influx against root $[NO_3^-]$ from Figures 4 and 5 yielded a parabola which nicely summarizes these 'positive' and negative feedback effects during induction and postinduction periods, respectively (Fig. 6). It is clear that similar fluxes can be attained at very different $[NO_3^-]_0$ pretreatments (and root $[NO_3^-]$) simply because they are in two different phases. This may explain why Glass *et al.* (11) observed no negative feedback effects of various $NO_3^-$ pretreatments on $^{13}NO_3^-$ influx in barley.

Although influx was negatively correlated with root $[NO_3^-]$ during the postinduction period (Fig. 6), the arguments advanced by Lee and Rudge (18) suggest that neither root $[NO_3^-]$ *per se* nor $[NH_4^+]$ was responsible for the negative feedback regulation of $NO_3^-$ influx but, rather, some products of $NH_4^+$ assimilation (18). Their argument is based, in part, upon the work of Breteler and Siegerist (2). The latter observed that methionine sulfoximine and azaserine, inhibitors of glutamine synthetase and glutamate synthase, respectively, increased tissue $[NH_4^+]$ but relieved, rather than increased, the negative feedback effects (referred to as 'repression' by Breteler and Siegerist) of $NH_4^+$ supply. In addition, it has long been known that net $NO_3^-$ uptake is inhibited by provision of certain amino acids (4, 10 for review). However, there appear to be differences among species in sensitivity to particular amino acids (8, for review 4). In addition, the study by Lee and Rudge (18) demonstrated that prior accumulation of $NO_3^-$ or $NH_4^+$ reduced net $NH_4^+$ uptake to similar extents, indicating a common negative feedback regulation for $NO_3^-$ and $NH_4^+$ uptake which they interpreted as being from some

product of $NH_4^+$ assimilation. We have also observed that preloading barley for 24 h with 10 mol $m^{-3}$ $NH_4^+$ (in the presence of 0.2 mol $m^{-3}$ $NO_3^-$) reduced influx to the same extent as preloading with 10 mol $m^{-3}$ $NO_3^-$ although root $[NO_3^-]$ in the latter was much greater than the former (our unpublished data, see also ref. 14). Though the above arguments are suggestive, we feel that they are not sufficiently convincing to warrant eliminating $NO_3^-$ and $NH_4^+$ from consideration as sources of negative feedback.

For example, vacuolar $[NO_3^-]$ may still influence $NO_3^-$ influx across the plasmalemma through indirect effects. By controlling $NO_3^-$ fluxes across the tonoplast, cytoplasmic $[NO_3^-]$ and flux of N through the glutamine synthetase-GOGAT pathway may be determined. This, in turn, according to the above arguments would exert negative feedback inhibition upon plasmalemma $NO_3^-$ influx. Such a model is consistent with the observed negative correlation between influx and root $[NO_3^-]$ when $NO_3^-$ was the sole source of N (Fig. 6). It would also explain the fact that this negative relationship no longer existed when plants were fed with $NH_4^+$ (our unpublished data).

It has been argued that growth rates and/or root:shoot ratios are the prime regulators of the uptake of $NO_3^-$ and other nutrient ions, *e.g.* $K^+$, $PO_4^-$, $Cl^-$ (9, 25). We determined growth parameters (data not shown) and, consistent with the results of Lee and Rudge (18), found no correlations between $NO_3^-$ influx and growth rates or root:shoot ratios. Recently, we have demonstrated (26) that these growth factors were *not* directly involved in the regulation of $K^+$ influx.

### Induction and Negative Feedback Regulation Are Independent Processes

It has been shown that pretreatment with $NH_4^+$ or certain amino acids failed to inhibit the induction of $NO_3^-$ uptake by external $NO_3^-$; the same treatments were, however, effective in inhibiting $NO_3^-$ uptake after induction (4). Our experiments further demonstrate the independence of induction and negative feedback inhibition processes; when $NO_3^-$ was resupplied to plants which were deprived of external $NO_3^-$ for 1 to 3 d, they all showed elevated fluxes (compared to unstarved plants) despite the fact that their root $[NO_3^-]$ were vastly different (Figs. 7 and 8). In these experiments, although fluxes were substantially increased in all $NO_3^-$ deprivation treatments (1–3 d of deprivation), there was some indication of a negative relationship between influx and root $NO_3^-$ within 12 h of reexposure to $NO_3^-$ (Fig. 7). It appears that root $[NO_3^-]$ (vacuolar $[NO_3^-]$) did not affect the process of induction but had accelerated the appearance of negative feedback effects. These observations are consistent with the model described above.

## CONCLUSIONS

1. Induction of $^{13}NO_3^-$ influx required the presence of external $NO_3^-$ even when mean root $[NO_3^-]$ was relatively high. We suggest that the maintenance of an induced state depends upon some critical cytoplasmic $[NO_3^-]$. Upon removal of nitrate from the medium, plants depend on retrieval of $NO_3$ from the vacuole for the maintenance of cytoplasmic $[NO_3^-]$ and the state of induction. Interspecific and intraspecific genetic differences in the efficiency of retrieval of vacuolar $NO_3^-$ may account for the reported differences in influx patterns upon removal of $NO_3^-$ from the medium.

2. Induction is not an all or none phenomenon; it increases with time depending on $[NO_3^-]_0$ until peak induction has been attained.

3. The inducible 'carrier' system responsible for $NO_3^-$ uptake, appears to be very labile (4) and decays within a few hours of removal of external $[NO_3^-]$ since the cytoplasmic $NO_3^-$ pool is small with a short half life of exchange.

4. $NO_3^-$ influx is subject to negative feedback inhibition. However, we consider that it may be premature to focus exclusively upon products of ammonium assimilation as the source(s) of negative feedback inhibition of $NO_3^-$ influx.

5. Induction and negative feedback inhibition of $NO_3^-$ influx appear to be independent processes. Although vacuolar $NO_3^-$ may not affect induction directly it may, nevertheless, exert an indirect effect upon induction through its effect upon cytoplasmic $[NO_3^-]$. Slow release of $NO_3^-$ from the vacuole may result in 'deinduction.' Likewise (through effects upon cytoplasmic $[NO_3^-]$ and, hence, the flux of N through the GS-GOGAT pathway) vacuolar $NO_3^-$ may exert indirect effects upon negative feedback via reduced N derivatives.

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