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# Comparative Induction of Nitrate and Nitrite Uptake and Reduction Systems by Ambient Nitrate and Nitrite in Intact Roots of Barley (*Hordeum vulgare* L.) Seedlings[1]

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**The induction by ambient $NO_3^-$ and $NO_2^-$ of the $NO_3^-$ and $NO_2^-$ uptake and reduction systems in roots of 8-d-old intact barley (*Hordeum vulgare* L.) seedlings was studied. Seedlings were induced with concentrations of $NaNO_3$ or $NaNO_2$ ranging from 0.25 to 1000 μM. Uptake was determined by measuring the depletion of either $NO_3^-$ or $NO_2^-$ from uptake solutions. Enzyme activities were assayed in vitro using cell-free extracts. Uptake and reduction systems for both $NO_3^-$ and $NO_2^-$ were induced by either ion. The $K_m$ values for $NO_3^-$ and $NO_2^-$ uptake induced by $NO_2^-$ were similar to those for uptake induced by $NO_3^-$. Induction of both the uptake and reduction systems was detected well before any $NO_3^-$ or $NO_2^-$ was found in the roots. At lower substrate concentrations of both $NO_3^-$ and $NO_2^-$ (5–10 μM), the durations of the lag periods preceding induction were similar. Induction of uptake, as a function of concentration, proceeded linearly and similarly for both ions up to about 10 μM. Then, while induction by $NO_3^-$ continued to increase more slowly, induction by $NO_2^-$ sharply decreased between 10 and 1000 μM, apparently due to $NO_2^-$ toxicity. In contrast, induction of $NO_3^-$ reductase (NR) and $NO_2^-$ reductase (NiR) by $NO_2^-$ did not decrease above 10 μM but rather continued to increase up to a substrate concentration of 1000 μM. $NO_3^-$ was a more effective inducer of NR than was $NO_2^-$; however, both ions equally induced NiR. Cycloheximide inhibited the induction of both uptake systems as well as NR and NiR activities whether induced by $NO_3^-$ or $NO_2^-$. The results indicate that in situ $NO_3^-$ and $NO_2^-$ induce both uptake and reduction systems, and the accumulation of the substrates per se is not obligatory.**

The literature pertaining to the physiological bases of the induction of $NO_3^-$ and $NO_2^-$ uptake and their respective reductases contains several conflicting reports. At issue is whether those ions induce only their own uptake and reduction systems or whether they are each capable of inducing either system. The controversy likely stems from at least two inherent complications. First, because NR is constitutively present in the roots (Aslam et al., 1990), they are exposed to both ions when $NO_3^-$ is the sole N source, making it difficult to determine which is the inducer. Second, the situation is further complicated by the fact that $NO_3^-$ appears in tissues of various plant species via oxidation of $NO_2^-$ when the latter is the N source (Lips et al., 1973; Kaplan et al., 1974; Sahulka and Lisa, 1978; Aslam et al., 1987; Aslam and Huffaker, 1989). Muhammad and Kumazawa (1974) found $NO_3^-$ in rice seedlings supplied with $NH_4^+$. These observations must be accounted for in studies of induction of uptake and reduction systems by $NO_2^-$.

Because $NO_3^-$ and $NO_2^-$ are competitive inhibitors of their respective uptake and apparently share the same transporters and binding sites (Aslam et al., 1992a), the induction of their uptake and reduction systems is especially interesting. The induction of the $NO_3^-$ and $NO_2^-$ uptake systems in the presence of their respective substrates is well established (Jackson et al., 1973, 1974a, 1974b; Goyal and Huffaker, 1986b; Aguera et al., 1990; Siddiqi et al., 1990). In addition, both uptake systems clearly appear to be induced by $NO_3^-$ (Aslam et al., 1992a). In plant species in which the formation of $NO_2^-$ is inhibited, e.g. double-mutant barley (*Hordeum vulgare* L.) seedlings containing neither the NADH nor NAD(P)H NRA, $NO_3^-$ still induced $NO_3^-$ uptake (Warner and Huffaker, 1989). In $WO_4^{2-}$-treated tissues, in which the formation of an active NR and, hence, of $NO_2^-$ is inhibited, $NO_3^-$ application induced $NO_2^-$ uptake (de la Haba et al., 1990).

The situation regarding the induction of the $NO_3^-$ uptake system by ambient $NO_2^-$ is less clear. Hole et al. (1990) found no induction of $NO_3^-$ uptake by $NO_2^-$ in corn roots. When $NO_2^-$ and $NO_3^-$ were both present in the incubation solution, the induction of the $NO_3^-$ uptake system was delayed in wheat seedlings (Jackson et al., 1974b; Tompkins et al., 1978). When corn seedlings were pretreated with $NO_2^-$ and then transferred to a solution containing only $NO_3^-$, induction of the $NO_3^-$ uptake system was delayed (Jackson et al., 1973). In contrast, in dwarf bean seedlings, pretreatment with $NO_2^-$ shortened the lag period for the subsequent induction of the $NO_3^-$ uptake system by $NO_3^-$ (Breteler and Luczak, 1982). The initial rates of $NO_3^-$ uptake were higher in $NO_2^-$-pretreated bean seedlings (Breteler and Luczak, 1982) and tobacco cells (Heimer, 1975) than in those not pretreated with $NO_2^-$, indicating the development of the $NO_3^-$ uptake system by ambient $NO_2^-$. Recently, Siddiqi et al. (1992) reported that a 24-h pretreatment with 0.1 mM $NO_2^-$ fully induced the $NO_3^-$ uptake system in barley roots. However, it was not clearly determined in their study whether $NO_2^-$ induced the $NO_3^-$ uptake system per se or whether induction was the

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Abbreviations: CHI, cycloheximide; NiR(A), $NO_2^-$ reductase (activity); NR(A), $NO_3^-$ reductase (activity).

result of $NO_3^-$ produced by the oxidation of $NO_2^-$. They did not find net accumulation of $NO_3^-$ in $NO_2^-$-fed barley roots, but the endogenous $NO_3^-$ concentration in their control roots that received no $NO_3^-$ was relatively high. Thus, as they stated, they would not have detected a small increase in $NO_3^-$ concentration from an $NO_2^-$ application.

We previously reported that NRA was induced in barley leaves by either $NO_3^-$ or $NO_2^-$ (Aslam et al., 1987). However, a much higher concentration of $NO_2^-$ than $NO_3^-$ was required for induction. In fact, no induction was found with $NO_2^-$ until $NO_3^-$ appeared, presumably from the internal oxidation of $NO_2^-$ in the leaves.

Studies with roots are less clear. In a preliminary report, we showed that NR was induced in barley roots by both $NO_3^-$ and $NO_2^-$ and also that $NO_3^-$ appeared in roots after feeding with $NO_2^-$ (Aslam et al., 1992b). In contrast, Siddiqi et al. (1992) recently reported that 0.1 mM $NO_2^-$ did not induce NR in barley roots during a 24-h induction period.

Studies of induction of NiR are also controversial. $WO_4^{2-}$, which inhibits the formation of active NR, did not inhibit induction of NiR by $NO_3^-$ in wheat embryos (Gupta et al., 1983), tobacco cells (Kelker and Filner, 1971), and sunflower roots (de la Haba et al., 1990). This suggests that $NO_3^-$ can induce NiR independently of $NO_2^-$. On the other hand, because $NO_3^-$ may result from the internal oxidation of $NO_2^-$, the possibility exists that $NO_3^-$ is the real inducer of NiR even in roots supplied with $NO_2^-$. Our earlier studies also showed that induction of NiR in barley leaves did not occur until $NO_3^-$ was detected in the tissues (Aslam and Huffaker, 1989). Yet, in contrast to the results obtained with leaves, we found that induction of NiR, like induction of NR, in roots occurred with either $NO_3^-$ or $NO_2^-$ in the nutrient solution and before either ion was detected in the root tissue (Aslam et al., 1992b).

In this paper we report that $NO_2^-$ and $NO_3^-$ were equally effective in inducing the uptake systems of either ion, and we demonstrate that $NO_2^-$ will induce NRA and NiRA. We further show that the induction of both uptake and reduction systems occurred several hours before either ion was detected in the roots.

## MATERIALS AND METHODS

### Plant Culture

Barley (*Hordeum vulgare* L. var CM-72) seedlings were grown hydroponically as described by 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 25% full-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 plant canopy was 400 $\mu$mol $m^{-2}$ $s^{-1}$ and was supplied with incandescent and cool-white fluorescent lamps.

### Induction of Uptake and Reduction Systems

After 24 h in the light, the seedlings were transferred to large volumes (5–10 L) of the induction solution containing 0 to 1000 $\mu$M $NaNO_3$ or $NaNO_2$ in 25% full-strength Hoagland solution (Hoagland and Arnon, 1950). The seedlings were then placed in a controlled environment growth chamber set for continuous light, 25°C, and 60 to 65% RH. The induction solutions were analyzed periodically for $NO_3^-$ and $NO_2^-$, and their concentrations were maintained by adding appropriate volumes of the stock solutions. The depletion of the substrates was never more than 20%. In one experiment, the seedlings were pulsed with 250 $\mu$M $NO_3^-$ or $NO_2^-$ for 1 h and then transferred to the N-free solutions. The uptake rates of $NO_3^-$ and $NO_2^-$ and NRA and NiRA were determined at various intervals. In some induction studies, $WO_4^{2-}$ (data not shown) and CHI were also supplied at varying concentrations, as indicated in the respective tables. Sodium salts were used because even the reagent grade $KNO_2$ contained measurable amounts of $NO_3^-$, whereas $NaNO_2$ was free of any $NO_3^-$.

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

Intact seedlings were used in all experiments. Uptake was determined in a minigrowth 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 (1986a). Uptake was started by placing 15 seedlings in a Pyrex culture tube (25 × 150 mm) containing 50 mL of the appropriate solution. Uptake solutions contained 1.0 mM Mes (pH 6.0), 0.2 mM $CaSO_4$, and $NO_3^-$ or $NO_2^-$ as indicated in the figures and tables. All solutions were aerated vigorously to ensure thorough mixing. Aliquots (0.4 mL) for $NO_3^-$ and $NO_2^-$ determination were removed automatically at 1.0- to 3.0-min intervals by the HPLC system. Uptake rates were determined by measuring the disappearance of $NO_3^-$ and $NO_2^-$ from the uptake solutions per unit of time. The rates were computed from determinations of concentration and volume at consecutive sampling times (Goyal and Huffaker, 1986a).

All experiments were repeated at least two times, and the results from representative experiments are shown. In kinetic studies (Fig. 4), the double-reciprocal plots of the uptake rates versus concentration were subjected to linear regression analysis, and the regression equations are shown in the figure legends. All $r^2$ values were significant at $P = 0.001$. The kinetic constants ($K_m$, $V_{max}$) were calculated from these regression equations.

### NR and NiR Assays

Roots (about 2 g per treatment) were washed with distilled, deionized water, detached from the shoots at the scutellar node, and homogenized with 4 mL of buffer $g^{-1}$ of root in a chilled mortar and pestle in the presence of acid-washed sand. The extraction buffer contained 0.05 M Tris-HCl (pH 8.5), 1 mM DTT, 10 $\mu$M flavin adenine dinucleotide, 1 $\mu$M $Na_2MoO_4$, 1 mM EDTA, and 10 mM leupeptin (Kuo et al., 1982). The homogenates were centrifuged at 30,000*g* for 15 min, and the supernatants were used for the assay of NRA, NiRA, $NO_3^-$, and $NO_2^-$.

The enzyme activities were assayed by in vitro methods as described by Aslam and Huffaker (1989). NRA was assayed using NADH as the electron donor, and NiRA was assayed using methyl viologen that was reduced by $Na_2S_2O_4$.

### $NO_3^-$ and $NO_2^-$ Determination

Both $NO_3^-$ and $NO_2^-$ from the uptake solutions and $NO_3^-$ from the root extracts were determined spectrophotometrically by measuring their $A_{210}$ after separation by HPLC on a partisil-10 SAX anion-exchange column (Thayer and Huffaker, 1980). $NO_2^-$ from the root extracts was determined colorometrically 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. The $A_{540}$ was read.

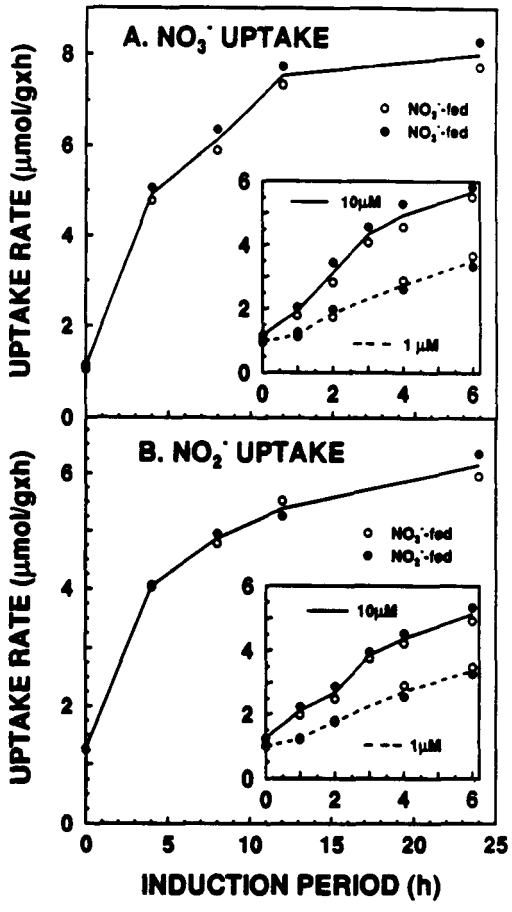


**Figure 1.** Time course of the induction of $NO_3^-$ and $NO_2^-$ uptake systems in roots of intact barley seedlings induced with 1 and 10 μM ambient $NO_3^-$ or $NO_2^-$. Seedlings were grown hydroponically in N-free solutions for 6 d in continuous darkness followed by 24 h of continuous light. The seedlings were then transferred to the induction solutions (10 L) containing 1 or 10 μM $NO_3^-$ or $NO_2^-$ in 25% full-strength Hoagland solution and placed in continuous light. The induction solutions were analyzed periodically and were brought to the original concentration by adding appropriate volumes of stock solutions. The depletion of the substrate from the induction solutions was not more than 20%. Uptake rates of $NO_3^-$ and $NO_2^-$ were determined by analyzing the depletion of the ions from 200 μM solutions for 12 min (sampling every 1.5 min) at each time interval. The insets show the lag periods in the induction of $NO_3^-$ (A) and $NO_2^-$ (B) uptake systems at 1 μM (dotted lines) and 10 μM (solid lines) $NO_3^-$ and $NO_2^-$ concentrations.

**Table I.** *Time course of the accumulation of $NO_3^-$ in roots of intact barley seedlings supplied with 1 and 10 μM $NO_3^-$ or $NO_2^-$*

For experimental details see legend for Figure 1.

| Induction Period | 1 μM Substrate[a] | | 10 μM Substrate[a] | |
|---|---|---|---|---|
| | $NO_3^-$ fed | $NO_2^-$ fed | $NO_3^-$ fed | $NO_2^-$ fed |
| *h* | *nmol g⁻¹* | | | |
| 0 | 9[b] | 9 | 10 | 10 |
| 1 | 11 | ND[c] | 82 | 12 |
| 2 | 39 | 10 | 348 | 10 |
| 3 | 106 | 12 | 865 | 34 |
| 4 | 199 | 17 | 2112 | 69 |
| 6 | 364 | 34 | 3216 | 111 |
| 12 | 564 | 65 | 5832 | 187 |
| 24 | | | 8928 | 369 |

[a] No accumulation of $NO_2^-$ was detected at any time interval. [b] Average coefficient of variation for the data set was 13%. [c] ND, Not determined.

The results are reported on the basis of fresh weights of the roots.

## RESULTS

### Induction of $NO_3^-$ and $NO_2^-$ Uptake Systems

#### *Time Course of Induction at Low Substrate Concentration*

Both the $NO_3^-$ and $NO_2^-$ uptake systems were induced by either ambient $NO_3^-$ or $NO_2^-$. At a substrate concentration of 10 μM, the induction patterns for $NO_3^-$ and $NO_2^-$ uptake were similar (Fig. 1). Induction proceeded at a relatively rapid rate during the initial 4 h and then continued at a reduced rate up to 12 h. Maximal activity was approached between 12 and 24 h. At 1 μM concentration, $NO_3^-$ and $NO_2^-$ were also equally effective inducers of both uptake systems (Fig. 1, insets). After a brief lag period, activity increased linearly throughout the 6-h induction period. At 6 h, total activity was about two-thirds of that obtained with 10 μM substrate. Induction levels with 5 μM substrate were similar to those obtained with 10 μM for both systems (results not shown).

$NO_3^-$ accumulation increased with time and in proportion to substrate concentration in roots supplied with 1 or 10 μM $NO_3^-$ (Table I). Conversely, no accumulation of $NO_2^-$ occurred in roots supplied with either 1 or 10 μM $NO_2^-$ during the 24-h incubation period (results not shown). When seedlings were induced with 1 μM $NO_2^-$, no net accumulation of $NO_3^-$ occurred during the first 3 h, and in the presence of 10 μM $NO_2^-$, only trace amounts of $NO_3^-$ were detected during the same period. After 3 h, $NO_3^-$ accumulation was low, but appreciable, and was apparently dependent on the concentration of $NO_2^-$ in the induction medium. No accumulation of $NO_2^-$ occurred in $NO_3^-$-supplied roots during the 24-h induction period (results not shown)

#### *Time Course of Induction at High Substrate Concentration*

At 1000 μM substrate, the time course and the level of induction of both systems by $NO_3^-$ were different from those obtained with $NO_2^-$ (Fig. 2). In the presence of $NO_3^-$, both

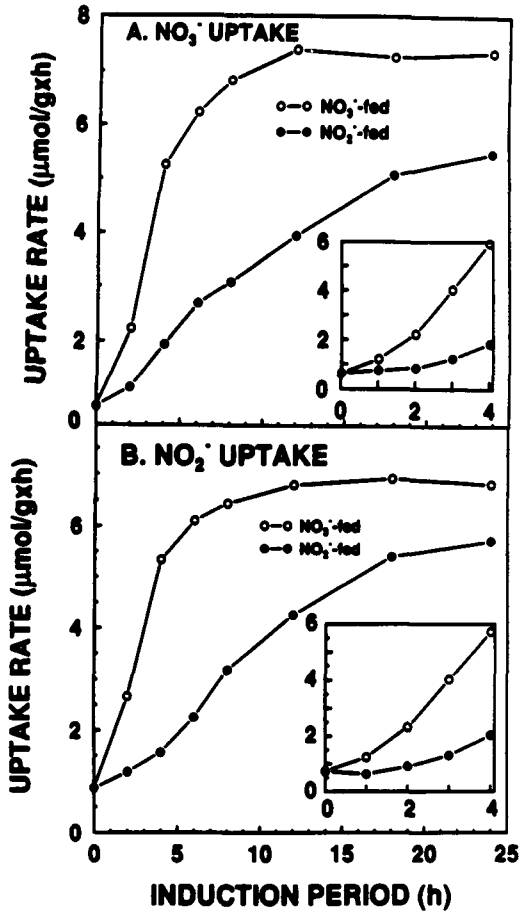


**Figure 2.** Time course of the induction of $NO_3^-$ and $NO_2^-$ uptake systems in intact roots of barley seedlings induced with 1000 μM $NO_3^-$ or $NO_2^-$. The experimental details are the same as in Figure 1. The insets show the lag periods in the induction of $NO_3^-$ (A) and $NO_2^-$ (B) uptake systems as a function of $NO_3^-$ and $NO_2^-$ concentrations.

uptake systems increased rapidly, with little lag, during the initial 12 h. Thereafter, little increase in activity occurred. In contrast, with $NO_2^-$, induction was slower but, following a lag period, continued to increase for 18 h before approaching saturation. Induction with $NO_3^-$ reached saturation after 12 h. At that point, uptake induced by $NO_2^-$ was only 50 to 60% of that induced by $NO_3^-$.

Roots supplied with 1000 μM $NO_2^-$ accumulated increasing amounts of $NO_3^-$ during the 24-h induction period (Table II); however, the levels were much less than those in $NO_3^-$-fed roots. $NO_2^-$ also accumulated in both $NO_2^-$- and $NO_3^-$-fed roots, but the accumulation in $NO_3^-$-fed roots was only 10 to 15% of that in $NO_2^-$-fed roots. In both cases, the concentration of $NO_2^-$ decreased with time.

### *Concentration Dependence of Induction*

To elucidate further the concentration dependence of induction on $NO_3^-$ and $NO_2^-$ accumulation, seedlings were induced for 4 h in a range of substrate concentrations up to 1000 μM (Fig. 3). The induction of both uptake systems was

**Table II.** *Time course of the accumulation of $NO_3^-$ and $NO_2^-$ in roots of intact barley seedlings supplied with 1000 μM $NO_3^-$ or $NO_2^-$*

For experimental details and $NO_3^-$ and $NO_2^-$ uptake rates see Figure 2.

| Induction Period | [$NO_3^-$] | | [$NO_2^-$] | |
|---|---|---|---|---|
| | $NO_3^-$ fed | $NO_2^-$ fed | $NO_3^-$ fed | $NO_2^-$ fed |
| *h* | *μmol g⁻¹* | | | |
| 0 | 0.01[a] | 0.01 | 0.0 | 0.0 |
| 1 | 1.05 | 0.05 | 0.03 | 1.1 |
| 2 | 3.47 | 0.10 | 0.03 | 2.13 |
| 3 | 7.25 | 0.16 | 0.06 | 3.25 |
| 4 | 10.71 | 0.21 | 0.08 | 2.81 |
| 6 | 15.31 | 0.36 | 0.13 | 1.82 |
| 12 | 28.60 | 1.05 | 0.09 | 1.48 |
| 24 | 42.44 | 1.52 | 0.07 | 0.76 |

[a] Average coefficient of variation for this data set was 9%.

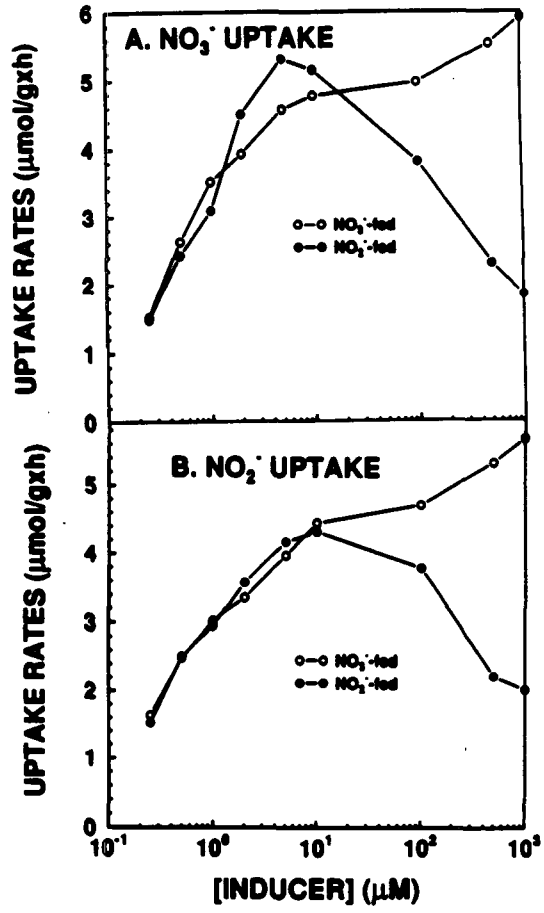


**Figure 3.** Induction of $NO_3^-$ and $NO_2^-$ uptake systems in roots of intact seedlings supplied with varying concentrations of $NO_3^-$ or $NO_2^-$ for 4 h. For experimental details see Figures 1 and 2. Uptake rates were determined by analyzing the depletion of the ions from 200 μM solutions for 12 min (sampling every 1.5 min) at each time interval.

linear up to 5 μM, and then $NO_3^-$ transport gradually increased with increasing concentrations up to 1000 μM $NO_3^-$. In contrast, the induction of both transport systems significantly decreased at concentrations of $NO_2^-$ greater than 10 μM.

The accumulation of $NO_3^-$ increased with increasing concentration of $NO_3^-$ in the induction solution. On the other hand, only a slight accumulation of $NO_2^-$ occurred at $NO_3^-$ concentrations greater than 100 μM (Table III). In contrast, no accumulation of $NO_3^-$ occurred until roots were supplied with 5 μM and greater $NO_2^-$ for 4 h; no accumulation of $NO_2^-$ occurred up to 10 μM $NO_2^-$. However, at higher concentrations of $NO_2^-$ (100 μM and more), both ions accumulated, with the accumulation of $NO_2^-$ being much greater than that of $NO_3^-$. After 24 h, a significant level of $NO_3^-$ had accumulated in roots supplied with 5 μM $NO_2^-$, and the accumulation increased with increasing $NO_2^-$ concentrations (data not shown).

*Kinetics of the Inducible Uptake Systems*

The uptake kinetics for both systems induced by ambient $NO_2^-$ (Fig. 4) were similar to the kinetics of uptake induced by $NO_3^-$. The apparent $K_m$ values for the $NO_3^-$ and $NO_2^-$ uptake systems induced by $NO_2^-$ were, respectively, 30 and 64 μM (Fig. 4B). The $V_{max}$ of $NO_3^-$ and $NO_2^-$ uptake systems were, respectively, 6.6 and 8.0 μmol $g^{-1}$ $h^{-1}$. Corresponding $K_m$ and $V_{max}$ values for $NO_3^-$-induced systems were about 35 and 45 μM and 8.4 and 6.6 μmol $g^{-1}$ $h^{-1}$, respectively, for $NO_3^-$ and $NO_2^-$ (Aslam et al., 1992a).

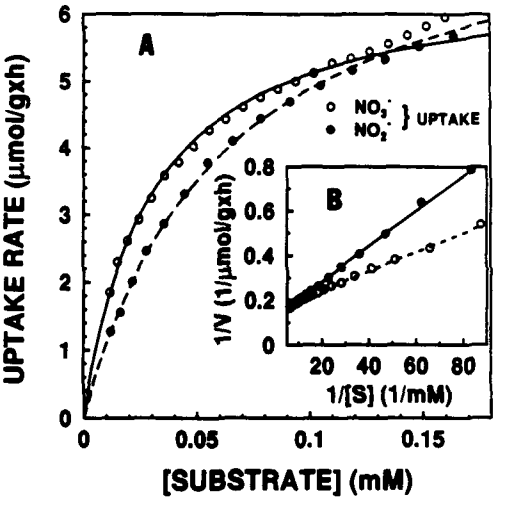


**Figure 4.** Kinetics of inducible $NO_3^-$ and $NO_2^-$ uptake systems in roots of intact seedlings induced with $NO_2^-$. Seedlings were induced with 1000 μM $NO_2^-$ for 24 h as described in Figure 2. Uptake rates were then determined at 3-min intervals by analyzing the depletion of the ions from uptake solutions initially containing 175 μM $NO_3^-$ or $NO_2^-$. A, Rates of uptake as a function of substrate concentration. The Michaelis-Menten equations are, for $NO_3^-$, $y = 6.64\ [S]\ (0.030)^{-1} + [S]^{-1}$ and, for $NO_2^-$, $y = 7.99\ [S]\ (0.064)^{-1} + [S]^{-1}$, where $S$ is the substrate. B, Lineweaver-Burk plot of the data in A. The regression equations of the double-reciprocal plots are, for $NO_3^-$, $y = 0.15056 + 0.004477x$ ($r^2 = 0.9984$) and, for $NO_2^-$, $y = 0.12799 + 0.007834x$ ($r^2 = 0.9978$). The apparent $K_m$ values for $NO_3^-$ and $NO_2^-$, respectively, were 30 and 64 μM, and $V_{max}$ values were 6.6 and 8.0 μmol $g^{-1}$ of root $h^{-1}$.

## Time Course and Concentration Dependence of Induction of NRA and NiRA

NRA and NiRA were also induced by both ions (Fig. 5). However, unlike induction of the uptake systems, induction of the enzyme systems did not show a lag at 1000 μM $NO_2^-$ (compare Figs. 2 and 5). The levels of the enzyme activities induced at 10 μM substrate were about two-thirds of those induced at 1000 μM substrate. Also, whereas the levels of NRA induced by ambient $NO_2^-$ were about 60 to 75% of that induced by $NO_3^-$ (Fig. 5A), similar levels of NiRA were induced by both $NO_3^-$ and $NO_2^-$ at each substrate concentration (Fig. 5B). At 1 μM substrate, some induction of NRA occurred (Fig. 5A); however, no NiRA was detected. This was probably due to the lower sensitivity of the NiRA assay.

Increasing concentrations of $NO_3^-$ or $NO_2^-$ in the induction solutions led to increased levels of both enzyme activities in the roots (Fig. 6). The increase in enzyme activity in response to substrate was more rapid at concentrations of less than 100 μM. At greater than 100 μM, little increase in NRA occurred; however, NiRA continued to increase (Fig. 6). Similar levels of NiRA were induced by both $NO_3^-$ and $NO_2^-$ (Fig. 6B); however, the level of NRA induced by $NO_2^-$ was only 60 to 75% of that induced by $NO_3^-$ (Fig. 6A).

**Table III.** *$NO_3^-$ and $NO_2^-$ accumulation in roots of intact barley seedlings supplied with different concentrations of $NO_3^-$ or $NO_2^-$ for 4 h*

For experimental details and $NO_3^-$ and $NO_2^-$ uptake rates see Figure 3.

| [Substrate] | [$NO_3^-$] | | [$NO_2^-$] | |
|---|---|---|---|---|
| | $NO_3^-$ fed | $NO_2^-$ fed | $NO_3^-$ fed | $NO_2^-$ fed |
| μM | *nmol $g^{-1}$* | | | |
| 0 | 10[a] | 10 | 0 | 0 |
| 0.25 | 10 | 8 | 0 | 0 |
| 0.50 | 41 | 11 | 0 | 0 |
| 1 | 267 | 13 | 0 | 0 |
| 2 | 572 | 12 | 0 | 0 |
| 5 | 1224 | 27 | 0 | 0 |
| 10 | 2040 | 64 | 0 | 0 |
| 100 | 5394 | 122 | 38 | 622 |
| 500 | 7465 | 182 | 53 | 1851 |
| 1000 | 9250 | 224 | 65 | 2918 |

[a] Average coefficient of variation for this data set was 13%.

## Induction of Uptake and Reduction Systems after a 1-h Pulse with Substrates

The induction of both the $NO_3^-$ and $NO_2^-$ uptake systems increased gradually during a 6-h period following a 1-h pulse with 250 μM $NO_3^-$ or $NO_2^-$ (Fig. 7). In contrast, NRA and NiRA increased only during the initial 2 h (Table IV). Although the levels of NRA were similar in both $NO_3^-$- and $NO_2^-$-pulsed roots, the levels of NiRA were about 2-fold

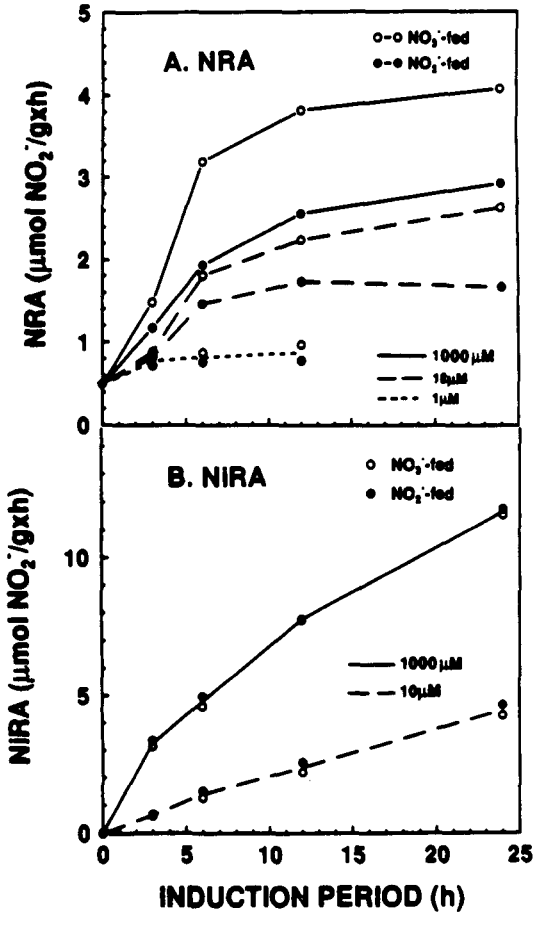


**Figure 5.** Time course of the induction of NRA and NiRA in roots of intact seedlings supplied with 1 µM (broken lines), 10 µM (dotted lines), and 1000 µM (solid lines) $NO_3^-$ or $NO_2^-$. Seedlings were grown and induced as described in Figures 1 and 2. The enzyme activities were determined at various intervals during the induction.

higher in roots pulsed with $NO_2^-$ as compared to $NO_3^-$-pulsed roots. The concentration of $NO_3^-$ in roots pulsed with $NO_3^-$ decreased gradually, whereas $NO_2^-$ taken up by roots pulsed with $NO_2^-$ disappeared during the initial 2 h (Table V).

### Effect of CHI on the Induction of Uptake and Reduction Systems

The induction of both $NO_3^-$ and $NO_2^-$ uptake systems as well as NRA and NiRA was inhibited by CHI (Table VI). This indicates that the induction of both uptake and reduction systems by $NO_3^-$ and $NO_2^-$ requires the synthesis of new protein(s).

## DISCUSSION

### Induction of Uptake Systems

The results presented here support the hypothesis that either ion will induce $NO_3^-$ or $NO_2^-$ uptake. Furthermore, these results, together with the observation that both ions are competitive inhibitors of the uptake of either ion, suggest that they share the same transporter(s) and binding sites. The alternative to this is that $NO_3^-$, resulting from the internal oxidation of $NO_2^-$, is responsible for induction. In fact, we did observe an increase in internal $NO_3^-$ in roots incubated in $NO_2^-$ (Tables I and II). The oxidation of $NO_2^-$ to $NO_3^-$ in several plant species has been reported: barley leaves (Kaplan et al., 1974; Aslam et al., 1987; Aslam and Huffaker, 1989), bean cotyledons (Lips et al., 1973), and pea roots (Sahulka and Lisa, 1978). Muhammad and Kumazawa (1974) reported the appearance of $NO_3^-$ in rice seedlings when $NH_4^+$ was the substrate. However, equal induction in roots supplied with 0.25 to 2.0 µM substrates (Figs. 1 and 3), when no net accumulation of either ion occurred during the first 2 to 3 h (Tables I and III), indicates that accumulation of either $NO_3^-$ or $NO_2^-$ is not obligatory for the induction of the uptake systems. Siddiqi et al. (1992) also concluded that $NO_2^-$ will

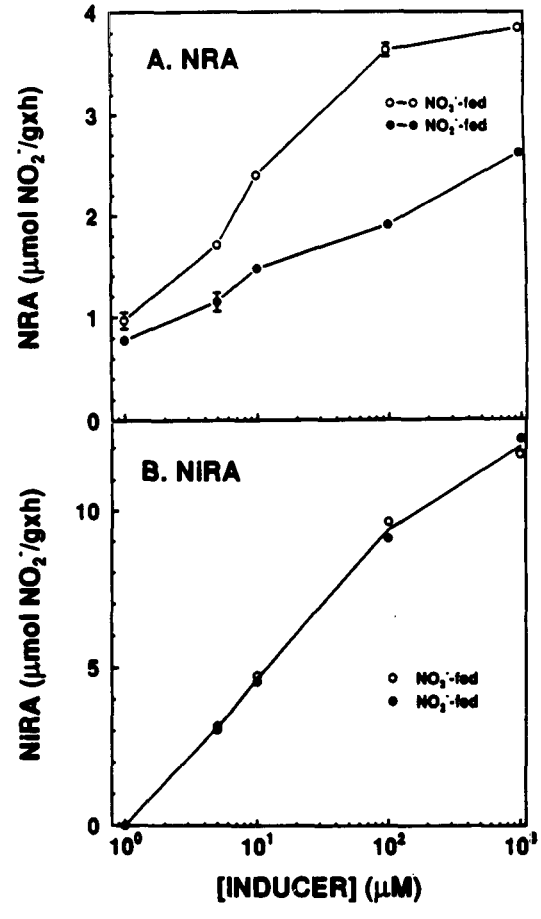


**Figure 6.** Effect of different concentrations of $NO_3^-$ and $NO_2^-$ on the induction of NRA (A) and NiRA (B) in roots of intact seedlings. Seedlings were induced as described in Figures 1 and 2, except that the induction solutions contained different concentrations of $NO_3^-$ and $NO_2^-$. The induction solutions were changed after 12 h. Enzyme activities were assayed after 24 h of induction. The depletion of either ion from its respective induction solutions did not exceed 20%. NRA and NiRA in roots of uninduced seedlings were, respectively, 0.32 ± 0.04 and 0 µmol of $NO_2^-$ $g^{-1}$ $h^{-1}$.

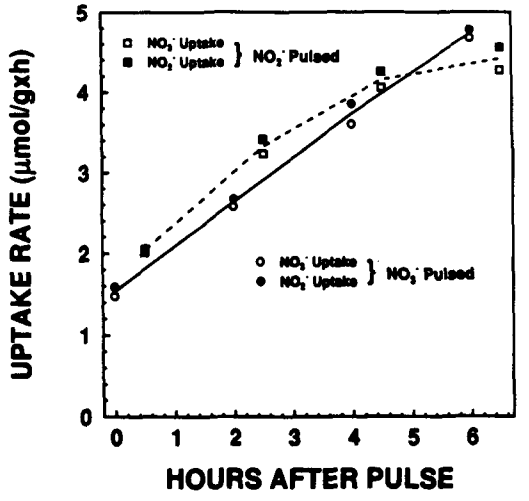


**Figure 7.** Induction of the $NO_3^-$ and $NO_2^-$ uptake systems in roots of intact seedlings pulsed with 250 μM $NO_3^-$ or $NO_2^-$ for 1 h. Uninduced seedlings were supplied with 250 μM $NO_3^-$ or $NO_2^-$ for 1 h. The seedlings were then rinsed with distilled water and transferred to the N-free solutions. Rates of $NO_3^-$ and $NO_2^-$ uptake were determined at different intervals as described in Figure 1. See Tables IV and V for corresponding enzyme activities and $NO_3^-$ and $NO_2^-$ concentrations, respectively.

induce $NO_3^-$ uptake, but their results did not rule out the possibility that $NO_3^-$, resulting from $NO_2^-$ oxidation, could have caused the induction.

The $NO_3^-$- and $NO_2^-$-induced systems showed similar activity patterns at low substrate concentrations (Figs. 1 and 3); however, they responded differently to increasing substrate concentrations. At higher substrate levels, there was a longer lag in the induction of both systems by $NO_2^-$ (Fig. 2), and induction by 1000 μM $NO_2^-$ was slower than that by 10 μM $NO_2^-$ (Figs. 1–3). This difference is likely due to a toxic effect of $NO_2^-$. At 1000 μM $NO_2^-$, there was an initial buildup of $NO_2^-$ in the roots (Table II). As the $NO_2^-$ concentration in $NO_2^-$-fed roots decreased with time, a concomitant increase in uptake activity occurred (Fig. 2).

Kinetic data reported here (see Aslam et al., 1992b, for a preliminary report) suggest that the uptake systems are similar whether induction is by $NO_3^-$ or $NO_2^-$. For example, the $K_m$ values of both systems, when induction was by $NO_2^-$ (Fig. 4), were similar to those obtained with induction by $NO_3^-$ (Aslam et al., 1992a). Further evidence in support of this hypothesis is the similar competitive, reciprocal inhibition of both $NO_2^-$-induced (our unpublished data) and $NO_3^-$-induced systems (Aslam et al., 1992a). In *Aspergillus nidulans*, the *crnA* gene product, which is responsible for encoding the $NO_3^-$ transporter, is induced either by $NO_3^-$ or $NO_2^-$ (Unkles et al., 1991). Thus, not only do the two ions apparently share the same transport system, it appears that either will induce that system.

It has been proposed that induction of the uptake systems by $NO_2^-$ was a "consequence of $NO_2^-$ metabolism rather than $NO_2^-$ per se" (Breteler and Luczak, 1982). These investigators reported a decrease in the lag period of $NO_3^-$ uptake in roots of dwarf beans pretreated with $NO_2^-$. They also suggested that a similar "consequence" causes induction of $NO_3^-$ uptake in $NO_3^-$-fed roots. Induction of either system by possible changes in N status of the seedlings was ruled out in our study by noting that $NH_4^+$ did not promote induction (results not shown).

**Table IV.** *Induction of NRA and NiRA in roots of intact barley seedlings pulsed with 250 μM $NO_3^-$ or $NO_2^-$ for 1 h*

For experimental details and corresponding $NO_3^-$ and $NO_2^-$ uptake rates see Figure 7.

| Period after Pulse | NRA | | NiRA | |
|---|---|---|---|---|
| | Pulsed with $NO_3^-$ | Pulsed with $NO_2^-$ | Pulsed with $NO_3^-$ | Pulsed with $NO_2^-$ |
| *h* | *μmol $NO_2^-$ $g^{-1}$ $h^{-1}$* | | | |
| 0 | 0.80[a] | 0.82 | 0.85 | 0.99 |
| 2 | 1.02 | 1.26 | 1.28 | 2.56 |
| 4 | 0.96 | 1.10 | 1.49 | 2.61 |
| 6 | 0.97 | 1.09 | 1.30 | 2.52 |

[a] Average coefficient of variation for this data set was 5%.

**Table V.** *$NO_3^-$ and $NO_2^-$ concentrations in roots of intact seedlings pulsed with 250 μM $NO_3^-$ or $NO_2^-$ for 1 h*

See Figure 7 for experimental details and corresponding uptake rates. See Table IV for corresponding enzyme activities.

| Period after Pulse | $[NO_3^-]$ | | $[NO_2^-]$ | |
|---|---|---|---|---|
| | Pulsed with $NO_3^-$ | Pulsed with $NO_2^-$ | Pulsed with $NO_3^-$ | Pulsed with $NO_2^-$ |
| *h* | *nmol $g^{-1}$* | | | |
| 0 | 772[a] | 20 | 0 | 880 |
| 2 | 689 | 12 | 0 | 0 |
| 4 | 614 | 10 | 0 | 0 |
| 6 | 472 | 4 | 0 | 0 |

[a] Average coefficient of variation for this data set was 8%.

**Table VI.** *Effect of CHI on the induction of $NO_3^-$ and $NO_2^-$ uptake systems and reductases in roots of intact barley seedlings supplied with 100 μM substrates*

Seedlings were induced with 100 μM $NO_3^-$ or $NO_2^-$ solutions containing 0 or 8 μM CHI. $NO_3^-$ uptake rates and NRA and NiRA were determined after 6 h of induction. Uptake rates were determined as described in Figure 1.

| [CHI] | Uptake Rate | | Enzyme Activity | |
|---|---|---|---|---|
| | $NO_3^-$ | $NO_2^-$ | NRA | NiRA |
| μM | *μmol $g^{-1}$ $h^{-1}$* | | *μmol $NO_2^-$ $g^{-1}$ $h^{-1}$* | |
| | *$NO_3^-$-fed seedlings* | | | |
| 0 | 6.02[a] | 5.41 | 2.52 | 3.62 |
| 8 | 0.81 | 0.57 | 0.51 | 1.25 |
| | *$NO_2^-$-fed seedlings* | | | |
| 0 | 6.41 | 5.67 | 1.84 | 3.39 |
| 8 | 0.75 | 0.65 | 0.35 | 1.06 |

[a] Average coefficient of variation for the data set was 14%.

The mechanism by which $NO_3^-$ and $NO_2^-$ stimulate the induction process in the absence of cellular accumulation of the substrate is an open question. It is plausible that, as $NO_3^-$ or $NO_2^-$ is transported across the plasma membrane by constitutive uptake systems (Behl et al., 1988; Aslam et al., 1992a), either anion may trigger the induction of uptake. A 1-h pulse with either 250 $\mu$M $NO_3^-$ or 250 $\mu$M $NO_2^-$ was equally effective in inducing both the $NO_3^-$ and $NO_2^-$ uptake systems (Fig. 7), although both ions totally disappeared 2 h after the pulse with $NO_2^-$ (Table V). These results indicate that induction of the uptake systems does not require a continuous supply of $NO_3^-$ or $NO_2^-$. In contrast, Minotti et al. (1968) and Rao and Rains (1976) suggested that the synthesis of the protein that accelerates $NO_3^-$ uptake depended on the level of $NO_3^-$ entering the roots. In corn roots, a 1-h pulse with 10 to 250 $\mu$M $NO_3^-$ accelerated the development of the $NO_3^-$ uptake system similarly during the subsequent 3 h (MacKown and McClure, 1988). MacKown and McClure concluded that the development of the $NO_3^-$-induced $NO_3^-$ uptake system can be achieved by a small accumulation of endogenous $NO_3^-$.

Induction of both uptake systems by either $NO_3^-$ or $NO_2^-$ was inhibited by CHI (Table VI). This suggests that induced transport was not the result of activation of preexisting proteins but required the synthesis of new proteins.

### Induction of NR

NR was induced by $NO_2^-$, albeit at levels only 60 to 75% of those induced by $NO_3^-$, at all substrate concentrations (Fig. 5A). This is in conflict with the results of Siddiqi et al. (1992), who found that $NO_2^-$ did not induce NR. In our study, the accumulation of $NO_3^-$ in the roots supplied with higher concentrations of $NO_2^-$ suggests that enzyme induction may have resulted from $NO_3^-$ produced by the oxidation of absorbed $NO_2^-$. However, there was no relationship between NR and $NO_3^-$ concentration. In roots supplied with $NO_2^-$, the accumulation of $NO_3^-$ was much less than in $NO_3^-$-supplied roots (Table II); yet, NRA was only about 25% lower (Fig. 5A). The induction of NR at 1 $\mu$M $NO_2^-$ (Fig. 5A) without significant accumulation of $NO_3^-$ or $NO_2^-$ (Table I) indicates that, similar to induction of the uptake systems, the accumulation of the substrate is not required for the induction of the enzyme activities. Thus, at 1 $\mu$M $NO_3^-$, NRA did not increase after 3 h (Fig. 5A), although $NO_3^-$ accumulation continued to increase (Table I). This is in contrast to the situation in leaves in which a positive correlation was observed between NR induction and $NO_3^-$ concentration and no NRA was detected in the $NO_2^-$-fed leaves unless $NO_3^-$ accumulated in those leaves (Aslam et al., 1987).

The induction of NRA by both ions was inhibited by CHI (Table VI). This indicates that increased activity was a result of new protein synthesis rather than activation of a preexisting protein. In contrast, Kaplan et al. (1978) reported in vitro activation of NRA by $NO_2^-$. We were unable to detect any in vitro activation of NRA by $NO_2^-$ (data not shown). Kaplan et al. (1978) reported that the $NO_3^-$-induced and $NO_2^-$-activated NRs are different in that the mol wt of $NO_2^-$-activated NR is lower compared with that of the $NO_3^-$-induced protein. They postulated that the $NO_2^-$-activated component of NR is a constitutive subunit that becomes incorporated into the NR protein during the synthesis of the Cyt *c* component (Kaplan et al., 1979).

### Induction of NiR

Unlike NR, both the activity and the time course for NiR induction by $NO_2^-$ were similar to those obtained with $NO_3^-$ (Figs. 5B and 6B). The induction of NiR was not inhibited by $WO_4^{2-}$ in either $NO_3^-$- or $NO_2^-$-fed roots (data not shown), indicating that in $NO_3^-$-fed roots an inhibition of the reduction of $NO_3^-$ to $NO_2^-$ did not affect the induction of NiR. de la Haba et al. (1990) reported that in sunflower roots $WO_4^{2-}$ completely inhibited the induction of NR without affecting the induction of NiR. $WO_4^{2-}$ also did not inhibit the induction of NiR by $NO_3^-$ in barley leaves (Aslam et al., 1987), wheat embryos (Gupta et al., 1983), or tobacco cells (Kelker and Filner, 1971). Like the induction of NRA, the induction of NiR by both $NO_3^-$ and $NO_2^-$ was inhibited by CHI (Table VI), indicating the requirement for new protein synthesis.

The mechanism by which $NO_2^-$ induces NiR is open to speculation. It is possible that $NO_3^-$ produced by the oxidation of $NO_2^-$ in the roots is also the inducer of NiR. However, unlike the induction of NR, the absence of any lag period in the induction of NiR by $NO_2^-$ (Fig. 5B) indicates that $NO_2^-$ induces NiR directly. The greater amounts of NiR in roots pulsed with $NO_2^-$ support this conjecture (Table IV). Although the roots pulsed with $NO_3^-$ had much higher concentrations of $NO_3^-$, the activity of NiR was lower than in roots pulsed with $NO_2^-$ (Table IV). This is in contrast to leaves in which a positive correlation occurred between $NO_3^-$ concentration and NiR even when the leaves were supplied with $NO_2^-$ (Aslam and Huffaker, 1989).

In summary, our results show that both the $NO_3^-$ and $NO_2^-$ uptake systems and the corresponding reductases are induced by either $NO_3^-$ or $NO_2^-$. Induction of both uptake and reduction systems occurred well before any $NO_3^-$ or $NO_2^-$ accumulated in the tissue. This eliminates the possibility that $NO_3^-$, resulting from the oxidation of $NO_2^-$, is responsible for induction. Inhibition of $NO_2^-$ induction of NR and NiR by CHI indicates that the inductions require synthesis of new protein(s). The results indicate that, in barley roots, the induction of the uptake and reduction systems by $NO_3^-$ and $NO_2^-$ may occur by similar mechanism(s).

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