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Studies of the Uptake of Nitrate in Barley[1]

V. Estimation of Root Cytoplasmic Nitrate Concentration Using Nitrate Reductase Activity—Implications for Nitrate Influx

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ABSTRACT

The cytoplasmic NO_3^- concentration ($[NO_3^-]_c$) was estimated for roots of barley (*Hordeum vulgare* L. cv Klondike) using a technique based on measurement of *in vivo* nitrate reductase activity. At zero external NO_3^- concentration ($[NO_3^-]_o$), $[NO_3^-]_c$ was estimated to be 0.66 mM for plants previously grown in 100 μM NO_3^-. It increased linearly with $[NO_3^-]_o$ between 2 and 20 mM, up to 3.9 mM at 20 mM $[NO_3^-]_o$. The values obtained are much lower than previous estimates from compartmental analysis of barley roots. These observations support the suggestion (MY Siddiqi, ADM Glass, TJ Ruth [1991] J Exp Bot 42: 1455–1463) that the nitrate reductase-based technique and compartmental analysis determine $[NO_3^-]_c$ for two separate pools; an active, nitrate reductase-containing pool (possibly located in the epidermal cells) and a larger, slowly metabolized storage pool (possibly in the cortical cells), respectively. Given the values obtained for $[NO_3^-]_c$ and cell membrane potentials of −200 to −300 mV (ADM Glass, JE Schaff, LV Kochian [1992] Plant Physiol 99: 456–463), it is very unlikely that passive influx of NO_3^- is possible via the high-concentration, low-affinity transport system for NO_3^-. This conclusion is consistent with the suggestion by Glass *et al.* that this system is thermodynamically active and capable of transporting NO_3^- against its electrochemical potential gradient.

The uptake of NO_3^-, like that of other ions, appears to be mediated by at least two distinct systems in barley (*Hordeum vulgare* L.) and other higher plants (10, 11, 29, 31 and references therein). The first is an inducible HATS[2], which has a low K_m for NO_3^-, shows Michaelis-Menten saturation kinetics, is sensitive to metabolic inhibitors, is regulated according to the plant nitrogen status, and appears to be an active transport system (see review by Clarkson [6]).

The second system is a constitutive LATS, which operates at higher external NO_3^- concentrations, shows linear kinetics, and is considerably less sensitive to metabolic inhibitors. The LATS, therefore, has some of the characteristics that would be expected of an energetically passive, channel-mediated transport system (see review of plant ion channels by Hedrich and Schroeder [15]) that facilitates the movement of NO_3^- down its electrochemical potential gradient.

The direction of NO_3^- transport through such a passive system depends upon the electrochemical potential difference for nitrate. This is determined by three factors: $[NO_3^-]_o$, $[NO_3^-]_c$, and the cell membrane electrical potential difference, $\Delta\Psi$. $[NO_3^-]_c$ has been estimated in several studies, using various methods. The technique of compartmental analysis has been applied to roots of several plant species using the tracer ^{13}N or ^{15}N, subsequent to the early work of Deane-Drummond and Glass (8) using $^{36}ClO_3^-$. Presland and McNaughton (24) and Lee and Clarkson (18), using ^{13}N, obtained values of 50 to 100 and 26 mM for maize and barley roots, respectively. Macklon *et al.* (20), using ^{15}N, obtained values of 40 to 50 mM in onion roots. Siddiqi *et al.* (29), using ^{13}N, found that $[NO_3^-]_c$ in barley roots increased from 12 to 37 mM with increased $[NO_3^-]_o$ from 0.01 to 1 mM.

Miller and Zhen (23), using NO_3^--specific microelectrodes, estimated $[NO_3^-]_c$ in *Chara* cells to be 1.6 mM. Recently, Zhen *et al.* (33) applied a similar technique to barley roots, obtaining values of 5.4 and 3.2 mM for $[NO_3^-]_c$ in epidermal and cortical cells, respectively.

In contrast, Robin *et al.* (25) estimated $[NO_3^-]_c$ in leaves of pea, soybean, alfalfa, barley, maize, and rice using the *in vivo* rates of NRA under anaerobic conditions and obtained estimates ranging from approximately 10 to 100 μM. Belton *et al.* (3) used this method to analyze the data of Brunetti and Hageman (4) for whole wheat plants and that of Jordan and Fletcher (16) for cultured rose cells. They obtained values of 65 and 140 μM, respectively. Using ^{14}N NMR, the same authors estimated $[NO_3^-]_c$ to be quite low compared with the vacuolar $[NO_3^-]$ in barley, maize, and pea roots, although the precise value could not be determined by this method.

It is evident that the estimates of $[NO_3^-]_c$ from compartmental analysis and NO_3^--specific microelectrodes are 2 to 3 orders of magnitude higher than those obtained with the NR-based technique of Robin *et al.* (25). Siddiqi *et al.* (31) proposed that $[NO_3^-]_c$ is a critical factor in determining whether passive NO_3^- uptake via the LATS is possible. If

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[2] Abbreviations: HATS, high-affinity NO_3^- transport system; E^N, Nernst membrane electrical potential difference; FAD, flavin adenine dinucleotide; LATS, low-affinity NO_3^- transport system; $[NO_3^-]_c$, cytoplasmic NO_3^- concentration; $[NO_3^-]_o$, external nitrate concentration; NR, nitrate reductase; NRA, nitrate reductase activity; $\Delta\Psi$, cell membrane electrical potential difference.

$[NO_3^-]_c$ is in the millimolar range as suggested by compartmental analysis, passive influx of NO_3^- via the LATS would be essentially impossible, requiring a positive value for $\Delta\Psi$ (31). However, if it is in the micromolar range as suggested by the NR-based method, passive transport might be possible given $\Delta\Psi$ values from approximately −50 to −100 mV (31).

Siddiqi *et al.* (29) proposed a model, based on studies of barley roots, to reconcile the results of compartmental analysis with those of the NR-based method and with the apparently passive characteristics of the LATS. They suggested that compartmental analysis measures $[NO_3^-]_c$ primarily in the root cortical cells, whereas the method based on NRA measures $[NO_3^-]_c$ of epidermal cells. The cytoplasm of the cortical cells may serve as a pool for unmetabolized NO_3^- (29), which is eventually either effluxed to the external medium or translocated to the shoot (21), and $[NO_3^-]_c$ in this pool might be in the millimolar range (29). Recently supplied NO_3^-, however, may be reduced in a separate epidermal pool (29) or translocated to the shoot (21), and, indeed, in corn roots the majority of NR appears to occur in the epidermis (27). The presence of NR in the epidermal cells and a rapid rate of NO_3^- turnover might maintain $[NO_3^-]_c$ at a micromolar level, sufficiently low for passive influx of NO_3^- to occur via the LATS (31).

Recently, however, Glass *et al.* (10), using microelectrodes, measured $\Delta\Psi$ values ranging from −200 to −300 mV in epidermal and cortical root cells of barley. For the LATS to allow passive NO_3^- uptake into the cells under such conditions at typical $[NO_3^-]_o$ values, they calculated that $[NO_3^-]_c$ would have to be in the nanomolar range, much lower than the estimates of any previous studies. Furthermore, they observed that transport via the LATS caused depolarization of $\Delta\Psi$, which is not consistent with a passive channel-mediated mechanism. They proposed, instead, that the LATS is an active, electrogenic system, possibly involving cotransport with H^+.

In light of the discrepancy between estimates of $[NO_3^-]_c$ obtained with the NR-based method and those obtained using compartmental analysis, we decided to estimate $[NO_3^-]_c$ of barley roots using the NR-based method (25). In earlier papers in this series, we described the kinetics, energetics, and electrophysiology of the LATS and HATS in barley roots (10, 11, 31) and compartmental analysis of NO_3^- in the root cells (29). In the present study, we have compared the results from the NR-based method with those obtained for barley by Siddiqi *et al.* (29) using compartmental analysis. Our goal was to determine whether $[NO_3^-]_c$ is sufficiently low in NO_3^--reducing cells to allow passive NO_3^- transport into these cells via the LATS, given the $\Delta\Psi$ values obtained for barley roots by Glass *et al.* (10). The study constitutes an evaluation of the contrasting hypotheses of Siddiqi *et al.* (29) and Glass *et al.* (10) referred to above, using the same barley variety grown under similar conditions. The study provides further evidence regarding the nature of the LATS for NO_3^- in barley roots.

MATERIALS AND METHODS

Seed Germination and Plant Growth

Seeds of barley (*Hordeum vulgare* L. cv Klondike) were germinated in the dark in moist sand on plastic mesh fitted into Plexiglas discs (approximately 40 seeds per disc) as described by Siddiqi and Glass (28). After 3 d, the discs were transferred to 25-L Plexiglas hydroponic tanks containing 1/80-strength modified Johnson's nutrient solution (30) with 100 μM NO_3^- supplied as $Ca(NO_3)_2$ and grown for 4 d. NO_3^- and K^+ concentrations were monitored daily, and the nutrient supply was maintained by means of peristaltic pumps, with the delivery rate adjusted according to the rate of NO_3^- depletion. The plants were maintained in a controlled environment room at 20 ± 2°C with a 16-h light/8-h dark cycle and 70% RH. The light was provided by fluorescent tubes having a spectral composition similar to that of sunlight, at an intensity of 300 $\mu E\ m^{-2}\ s^{-1}$ at plant level.

Spectrophotometric Assays

NO_2^- was assayed essentially as described by Long and Oaks (19) except that the reagents were added at higher concentrations to reduce volume and improve the sensitivity. *N*-1-Naphthylene-diamine-dihydrochloride (0.25 mL of 0.08% [w/v]) and sulfanilamide (0.5 mL of 2% [w/v] dissolved in 5 N HCl) were added to 1.5 mL of sample in a 10-mL test tube and mixed. The sample was allowed to stand at least 30 min, and then A_{540} was measured spectrophotometrically. High NO_3^- concentrations were found to give a slight color reaction in this assay, so this interference was corrected for when necessary by subtracting a blank containing the appropriate NO_3^- concentration.

NO_3^- was assayed essentially according to the procedure of Cataldo *et al.* (5), involving nitration of salicylic acid under acidic conditions. The sample (0.1 mL) was added to 0.4 mL salicylic acid (w/v, dissolved in concentrated H_2SO_4), mixed, and allowed to stand for 20 min at room temperature; then 9.5 mL of 2 N NaOH was added slowly with mixing. The samples were allowed to cool, and then A_{410} was determined spectrophotometrically.

Protein was determined using Coomassie blue G-250 (Protein Assay kit, Bio-Rad, Mississauga, Ontario). The assay reagent (0.2 mL) was added to 0.1 mL sample and 0.7 mL water, and mixed, and the A_{595} was determined spectrophotometrically.

In Vivo NR Assay

In vivo NRA was measured by anaerobic incubation of excised roots using a method similar to that of Robin *et al.* (26) (see review of Beevers and Hageman [2] for general discussion). Four milliliters of 100 mM KPO_4 buffer (pH 7.7) containing the desired concentration of KNO_3 was added to each of several 10-mL Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) and equilibrated in a water bath at 27 ± 1°C. Approximately 0.3 to 0.5 g fresh weight of roots were excised, rinsed with 50 μM $CaSO_4$, and placed in each tube. The tubes were capped and purged for 5 min with helium, which was introduced through each cap septum by 20-gauge 3.5-inch spinal needles (Becton-Dickinson) connected by a manifold to a compressed gas cylinder. The tubes were returned to the water bath and incubated for the desired period. They were then uncapped and placed in a boiling water bath for 10 min to extract tissue NO_2^-, and a 1.5-mL sample was withdrawn and assayed for NO_2^- as described above. The root fresh

weight was determined, and NRA was expressed as μmol NO_2^- g^{-1} fresh weight h^{-1}.

In Vitro NR Assay

This procedure was carried out essentially according to the method of Long and Oaks (19). For the extraction procedure, barley roots were placed in a mortar kept on ice, ground in liquid nitrogen, transferred to precooled vials, and stored at −80°C for later use (within 1–2 d). Approximately 5 g of frozen powder was ground with 5 mL of chilled extraction buffer in a mortar on ice and filtered through nylon mesh into a small beaker. The extraction buffer (17) contained Tris-HCl (25 mM, pH 8.5), EDTA (1 mM), FAD (20 μM), BSA (1%, w/v), DTT (1 mM), and cysteine (10 mM). Chymostatin (dissolved in DMSO; final concentration 10 μM) was added as a protease inhibitor, which is known to stabilize NRA extracted from maize roots (19). After extraction, the filtrate volume was determined, and the filtrate was centrifuged at 45,000*g* for 45 min at 4°C. The sample supernatant was kept on ice before subsequent steps.

To determine the NO_3^- concentration dependence of NRA, it was necessary to remove endogenous NO_3^- from the extracted samples by gel filtration, because a large amount of NO_3^- (amounting to 3–4 mM in the final extract) was released from the vacuole during grinding of the tissue. A 16- × 2-cm column of coarse mesh Sephadex G-25 (Pharmacia) was equilibrated with approximately 200 mL of 25 mM Tris HCl (pH 8.5) and then with 50 mL of the extraction buffer as described above but modified by the omission of BSA and FAD. Approximately 10 mL of the sample supernatant was applied to the column, and elution was carried out with an additional 50 mL of modified extraction buffer to elute soluble proteins, followed by approximately 100 mL of 25 mM Tris HCl. Fractions of 1.5 mL were collected in microcentrifuge tubes and assayed for protein, NO_3^-, and NRA.

All of the NRA (amounting to approximately 80% of the activity of the original extract) was eluted with the soluble protein within a volume of 40 mL, and NO_3^- was eluted much later, well separated from the protein peak. Therefore, in subsequent experiments, the protein-containing fractions were pooled and stored on ice before NRA was assayed at various NO_3^- concentrations.

NRA was assayed essentially as described by Long and Oaks (19). For each assay, 0.2 mL of Hepes buffer (0.65 M, pH 7.0), 0.14 mL FAD (0.2 μM), and 0.2 mL of KNO_3 solution (from concentrated stocks according to the desired final concentration) were added to 10-mL test tubes. A predetermined volume of water was added to give a final volume of 1.4 mL for incubation after the sample and other reagents were added, and the mixture was equilibrated in a water bath at 28 ± 1°C. An aliquot of sample, typically 0.4 to 0.5 mL, was added, and the reaction was started by the addition of 0.1 mL of NADH (3.6 mg mL^{-1}) with mixing. The tubes were incubated at 28°C for 30 min, and then the reaction was stopped by boiling for 10 min. The tubes were cooled to 28°C, and 0.1 mL of pyruvic acid (5.3 g L^{-1}) and 2 μL of lactate dehydrogenase suspension (Boehringer-Mannheim, Montreal, PQ) were added with mixing. An additional 10-min incubation was carried out to oxidize residual NADH, which interferes with color development in the NO_2^- assay (19). The samples were then assayed for NO_2^- as described above.

In one experiment, NRA was assayed at various NO_3^- concentrations using NADPH as the electron donor. In this case, 0.1 mL of NADPH (2.64 mg mL^{-1}) was added to start the reaction instead of NADH, and lactate dehydrogenase and pyruvate were included during the incubation with NADPH to eliminate any NADH produced by phosphatase action on NADPH (32). The slight interference of NADPH with color development in the NO_2^- assay was corrected for by including the same concentration of NADPH when running NO_2^- standards.

Terminology and Calculations

The *in vitro* NRA determined with NADH as the sole electron donor is referred to in the text and figures as "total activity," because this includes activity due to an NADH-specific NR and an NAD(P)H-bispecific NR (19, 32, and references therein). The activity obtained with NADPH alone can be attributed to the NAD(P)H-bispecific enzyme and is referred to here as "NADPH activity." Activity due to the NADH-dependent form ("NADH activity") is obtained by subtraction of the NADPH activity from the total activity.

The K_m and V_{max} for NO_3^- for the NADH and NAD(P)H enzymes were determined by Eadie-Hofstee analysis of the NADH and NADPH activities. The rate of *in vivo* NRA due to the NADH-specific enzyme (V) at various external NO_3^- concentrations ($[NO_3^-]_o$) was estimated by subtraction of the NADPH activity, which was considered to be saturated at all values of $[NO_3^-]_o$ (see "Discussion"). The $[NO_3^-]_c$ was then determined for various values of $[NO_3^-]_o$ by rearranging the Michaelis-Menten equation:

$$[NO_3^-]_c = \frac{V \cdot K_m}{V_{max} - V}$$

This value was then compared to that obtained theoretically from the Nernst equation at 20°C for various values of $[NO_3^-]_o$ and $\Delta\Psi$, assuming passive equilibration of NO_3^- (in which case $E^N = \Delta\Psi$):

$$[NO_3^-]_c = [NO_3^-1]_o \cdot 10^{(E^N/(RT/zF))}$$

where R represents the gas constant; T, temperature (K); z, the charge of the ion; and F, the Faraday constant.

RESULTS

Figure 1 shows the *in vivo* production of NO_2^- due to NRA of excised barley roots incubated anaerobically during a 6-h period in the presence and absence of exogenous NO_3^- (100 mM $[NO_3^-]_o$). For both treatments, NO_2^- accumulation was most rapid in the first 20 to 30 min (Fig. 1, inset). The rates of NRA, calculated by regression of the mean values during the first 20-min period, were 0.77 ± 0.08 and 1.04 ± 0.17 μmol g^{-1} fresh weight h^{-1} ($n = 4$) for the $-NO_3^-$ and $+NO_3^-$

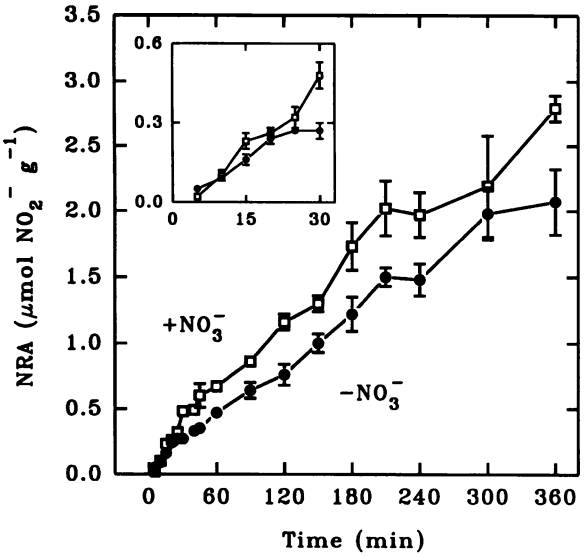

Figure 1. Time course of *in vivo* NRA of excised barley roots incubated under anaerobic conditions in the presence (□) and absence (●) of exogenously supplied NO_3^- (100 mM). Each point is the mean ± SE of four to ten replicates. The inset shows the pattern of NRA during the first 30 min period, with symbols as above.

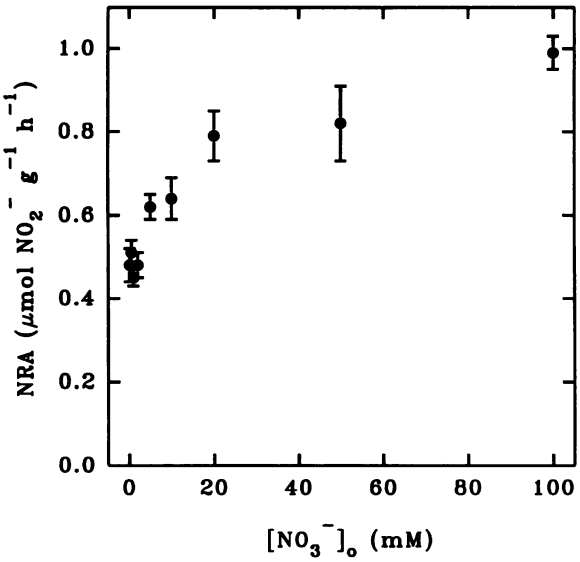

Figure 2. The effect of exogenous NO_3^- on *in vivo* NRA of excised barley roots incubated under anaerobic conditions for 20 min. Each point is the mean ± SE of 18 replicates from three separate experiments of six replicates each.

treatments, respectively. Subsequently, the rates were lower, with NO_2^- production continuing in a linear pattern until 3.5 h, at an average rate of 0.39 ± 0.01 and 0.50 ± 0.02 μmol g^{-1} fresh weight h^{-1} (n = 9) for the two treatments, respectively. Between 3.5 and 6 h, the pattern was more variable. For both treatments, some samples continued to produce NO_2^- at a considerable rate, whereas in others, NO_2^- production declined to near zero. Furthermore, between 1.5 and 6 h, NO_3^- was lost from the tissue of the $-NO_3^-$ samples to the surrounding medium at a rate of 0.85 ± 0.03 μmol g^{-1} fresh weight h^{-1} (n = 4). Because of the high NO_3^- background in the medium, it was not possible to determine whether there was also NO_3^- efflux from the $+NO_3^-$ samples.

At all times except the earliest (5 min), NRA was considerably higher in the presence of 100 mM $[NO_3^-]_o$ than in its absence (Fig. 1), although the magnitude of the differences was variable and failed to show a clear trend with time. Averaged over the period from 10 min to 6 h, NRA for the $+NO_3^-$ treatment was 25.8% ± 2.7% (n = 14) higher than the $-NO_3^-$ rate.

Figure 2 illustrates the $[NO_3^-]_o$ dependence of NRA *in vivo*, based on measurement of NO_2^- production of excised roots incubated anaerobically for 20 min. The four lowest $[NO_3^-]_o$ values used (0.1, 0.5, 1, and 2 mM) were not significantly different in NRA relative to that at zero $[NO_3^-]_o$ (0.57 ± 0.03 μmol g^{-1} fresh weight h^{-1}; n = 18). However, higher $[NO_3^-]_o$ (5–100 mM) resulted in higher rates of NRA, with the maximum rate (0.99 ± 0.04 μmol g^{-1} fresh weight h^{-1}; n = 18) obtained at 100 mM $[NO_3^-]_o$. Concentrations higher than 100 mM were strongly inhibitory, with a 70% ± 16% (n = 18) reduction of NRA occurring at 500 mM $[NO_3^-]_o$ relative to that at 100 mM.

The *in vitro* NO_3^- concentration dependencies of total NRA

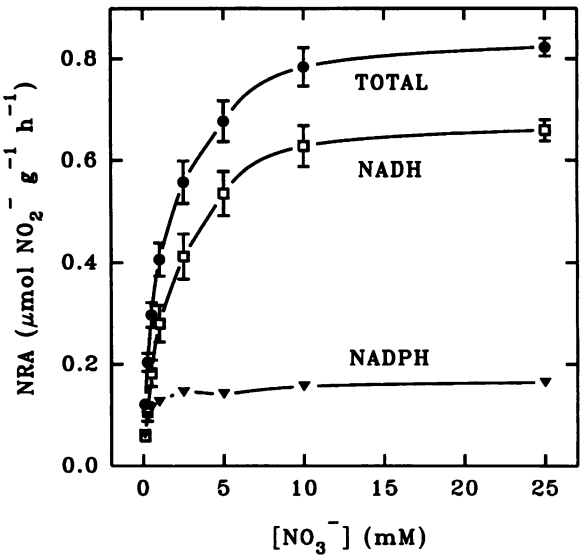

Figure 3. The NO_3^- concentration dependence of *in vitro* NRA. ●, Total NRA (nine replicates ± SE derived from three separate experiments of three replicates each); □ and ▼, the estimated NADH- and NADPH-dependent NRA, respectively, as explained in "Materials and Methods." The NADPH curve represents the mean of three replicates; SE is smaller than the dimensions of the symbols. The NADH curve was obtained by subtraction of NADPH-dependent activity from the total activity at each NO_3^- concentration.

and the NADH- and NADPH-dependent activities are shown in Figure 3. All three curves showed saturation kinetics, with the maximum rates obtained at 25 mM NO_3^-. Concentrations higher than 25 mM were somewhat inhibitory (data not shown), with 100 mM resulting in a 25% ± 5% (n = 9) reduction of the total NRA relative to that at 25 mM, and in a corresponding reduction of the NADH- and NADPH-dependent activities.

Eadie-Hofstee analysis of the data for total NRA from Figure 3 revealed biphasic kinetics (data not shown), suggesting the presence of more than one NR component. Therefore, kinetic parameters were determined separately for the NADH- and NADPH-dependent activities rather than for the total activity. Figure 4 is an Eadie-Hofstee plot of the NADH- and NADPH-dependent activities from Figure 3, both of which showed a much better straight line fit than the total activity. The K_m values for NO_3^- were 1.41 and 0.16 mM and the V_{max} values, 0.69 and 0.15 μmol g^{-1} fresh weight h^{-1}, for the NADH- and NADPH-dependent activities, respectively (see Fig. 4 legend for SE and r^2 values).

Figure 5 shows the relationship between $[NO_3^-]_o$ and the predicted values for $[NO_3^-]_c$, calculated for nonsaturating values of $[NO_3^-]_o$, as described in "Materials and Methods." At zero $[NO_3^-]_o$ (the y intercept), $[NO_3^-]_c$ was 0.66 mM, and it increased to 3.9 mM at 20 mM $[NO_3^-]_o$, as determined from the regression line.

Finally, Figure 6 illustrates the logarithmic relationship between $[NO_3^-]_c$ and E^N for four values of $[NO_3^-]_o$ from 0.01

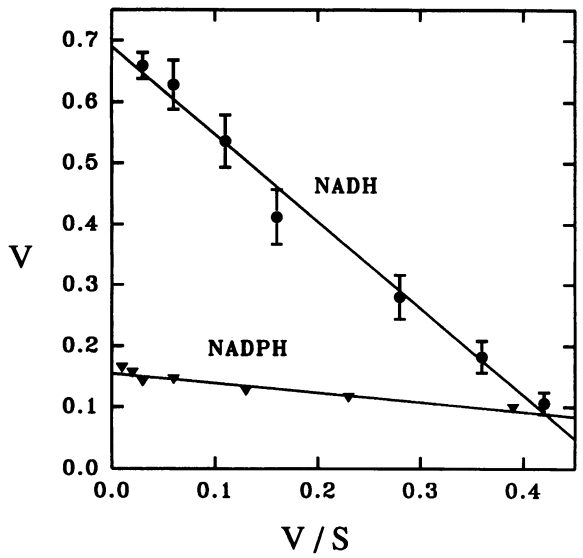

Figure 4. Eadie-Hofstee analysis of the *in vitro* NO_3^- dependence of NRA based on the data shown in Figure 3. V represents NRA (μmol g^{-1} fresh weight h^{-1}), and S represents NO_3^- concentration (mM). The K_m values for NO_3^- for the NADH-dependent (●) and NADPH-dependent (▼) activities, as determined from the slopes, were 1.41 ± 0.07 and 0.16 ± 0.02 mM, respectively, and the V_{max} values were 0.69 ± 0.03 and 0.15 ± 0.01 μmol g^{-1} fresh weight h^{-1}, respectively. The r^2 values were 0.99 and 0.91, respectively. The data points for the lowest NO_3^- concentration (0.1 mM; not shown) deviated considerably from the rest of the data and were not included in the regression.

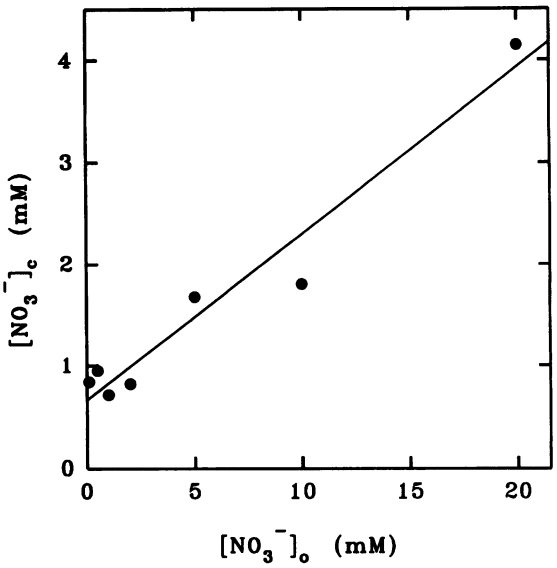

Figure 5. The relationship between $[NO_3^-]_o$ and $[NO_3^-]_c$, based on the data for NO_3^- dependence of *in vivo* NADH-dependent NRA, derived from the data shown in Figure 2; a K_m value for the NADH-dependent activity of 1.41 mM was used, as determined from Figure 4. The slope of the regression line was 0.16 ± 0.02, and the y intercept was 0.66. The SE of the y estimates from the regression was 0.30, and the r^2 value was 0.95. The SE values of the data used in the calculations are given in Figure 2 and in the legend to Figure 4.

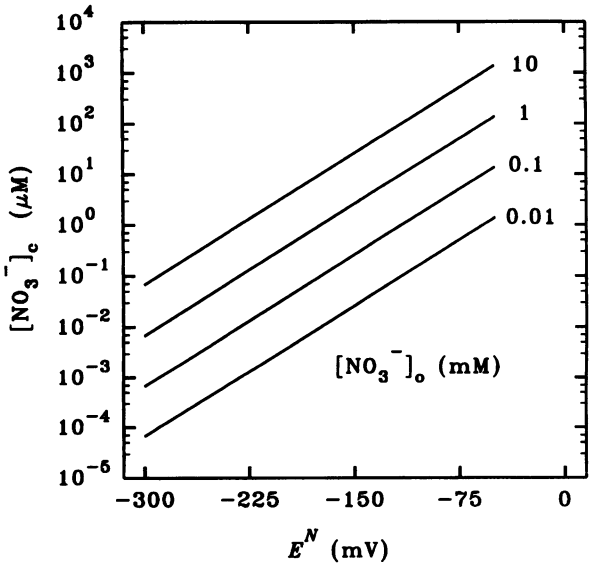

Figure 6. The theoretical equilibrium relationship between E^N and $[NO_3^-]_c$ for four values of $[NO_3^-]_o$ as calculated from the Nernst equation assuming passive entry of NO_3^-.

to 10 mM, as predicted theoretically by the Nernst equation, assuming passive equilibration of $[NO_3^-]_o$ and $[NO_3^-]_c$ according to E^N.

DISCUSSION

In Vivo Nitrate Reduction

Figure 1 demonstrates that excised barley roots incubated under anaerobic conditions are capable of producing NO_2^- at substantial rates for at least 6 h in the absence of exogenous NO_3^-. Because approximately 2 μmol g^{-1} fresh weight of NO_2^- were produced during this time, a NO_3^- reserve of at least that magnitude must be present within the roots and available to support NRA. This result agrees with those of Aslam (1) who showed that NO_2^- continues to be produced over an 8-h period by leaves of soybean and barley incubated anaerobically, although, as in our system, the rates gradually declined over time. Likewise, Robin *et al.* (25, 26) observed that pea leaves incubated anaerobically produced NO_2^- in a linear fashion over a 3-h period, with reductions in rate occurring subsequently.

We observed that, on average, the addition of exogenous NO_3^- at 100 mM enhanced NO_2^- production by 26% (Fig. 1), in close agreement with the results of Robin *et al.* (25). This result clearly indicates that NRA is limited by the availability of NO_3^- under these conditions rather than by reductant supply or other factors. This observation is critical to the interpretation of the data presented here, because to use the NR-based method to estimate $[NO_3^-]_c$, NRA *in vivo* must be NO_3^- limited.

Ferrari *et al.* (9), using cultured tobacco cells, found that NO_2^- production essentially ceased after 1 h of anaerobic incubation, despite the fact that the total tissue NO_3^- was not significantly depleted. The cessation was not due to depletion of reductant supply or other factors, because addition of exogenous NO_3^- caused resumption of NO_2^- production. Ferrari *et al.* (9) therefore inferred the presence of two NO_3^- pools, a metabolic pool (presumably cytoplasmic), which became depleted of NO_3^- within a 1-h period but could be replenished by exogenous NO_3^-, and a storage pool, presumably vacuolar, which was much larger but inaccessible to the metabolic pool over the time period studied.

Our results and those of Robin *et al.* (25, 26) differ from those of Ferrari *et al.* (9) in that NO_2^- production, although limited by NO_3^- supply, continued over an extended time period. The successive declines in rates that were observed after 30 min and after 3.5 h occurred in both the presence and absence of exogenous NO_3^- and were, therefore, clearly not due to NO_3^- limitation.

We believe our results, and those of Robin *et al.* (25), indicate that the vacuolar NO_3^- storage pool is readily available to the cytoplasmic pool and is capable of supplying NO_3^- so as to maintain NRA at a relatively constant rate. For example, between 30 min and 3.5 h, the accumulation of NO_2^- was essentially linear. Under conditions where NRA is limited by NO_3^-, this must indicate that NO_3^- is being delivered to the metabolic pool at a steady rate; otherwise, changes in NRA should be apparent with time. This idea is supported by previous observations with the same barley cultivar, showing that the pool of cytoplasmic NO_3^- is relatively small and unable by itself to maintain NRA for prolonged periods (29), and that total root $[NO_3^-]$ (mainly vacuolar) declines rapidly when external NO_3^- is removed (30). It seems quite reasonable that the vacuole should, under conditions of low $[NO_3^-]_o$, be able to deliver NO_3^- to maintain the cytoplasmic pool and support NRA.

Three concerns might arise regarding the accuracy of the NRA values obtained using the *in vivo* method. First, the anaerobic treatment might be expected to cause depolarization of the plasma membrane, interfering with the uptake of exogenous NO_3^-. This could account for the fact that there was no increase in NRA between 0 and 2 mM $[NO_3^-]_o$ (Fig. 2). However, concentrations between 2 and 100 mM clearly caused a significant increase in NRA, indicating that the exogenous NO_3^- was able to enter the tissue and allowing an estimate of the maximum *in vivo* NRA to be obtained.

Second, anaerobiosis could have an adverse effect on NRA (for example, by decreasing the reductant supply for NRA). However, the fact that NRA continued for several hours and was NO_3^- limited clearly indicates that the reductant supply was not the limiting factor for NRA during the experiments.

Finally, anaerobiosis might not cause complete inhibition of NO_2^- reductase activity, resulting in further metabolism of NO_2^-, again resulting in an underestimation of NRA (13). To test this possibility, we incubated roots anaerobically with $^{13}NO_3^-$ over a 10 min period (see refs. 11 and 29–31 for methodology), boiled the roots to extract the label that was taken up, and separated the cationic fraction from the anionic/neutral fraction by cation exchange under acidic conditions. Essentially all of the label was present in the anionic/neutral fraction, which would comprise mainly NO_3^- and NO_2^-, and none was in the cationic fraction, which would include NH_4^+ and amino acids. This indicated that further metabolism of NO_2^- was blocked under the conditions we used.

For the above reasons, we believe our study has given reasonable estimates of the true *in vivo* NRA. In support of this statement, the *in vivo* rates of NRA during the first 30 min agree quite well with those obtained by the *in vitro* assay (see below). In any case, even a quite substantial error in our estimates would not significantly alter the conclusions of this study (see final section of "Discussion").

We chose a 20-min incubation period to examine the *in vivo* response of NRA to varying $[NO_3^-]_o$ (Fig. 2). NRA showed saturation kinetics, increasing nearly twofold between 0.1 and 100 mM, where the maximum rate was obtained. The curve did not resemble a typical rectangular hyperbola, mostly because substantial rates of NRA (approximately 0.5 μmol g^{-1} fresh weight h^{-1}) were obtained even at low $[NO_3^-]_o$ values. This is because, even at zero $[NO_3^-]_o$, $[NO_3^-]_c$ can be maintained at a level that supports NRA for extended periods due to the vacuolar flux, as explained above.

The results are similar to those obtained by Robin *et al.* (25) except that we found that $[NO_3^-]_o$ values higher than 100 mM were inhibitory, whereas in their study full NRA was obtained up to nearly 1 M. Our results clearly show that, with a 20-min anaerobic incubation, $[NO_3^-]_c$ is limiting for NRA.

In Vitro Nitrate Reduction

Figure 3 shows the *in vitro* dependence of NRA on NO_3^- concentration for the total activity and the NADH- and NADPH-dependent activities, which were determined as described in "Materials and Methods." The total activity approached saturation at 25 mM NO_3^-, with a rate of approximately 0.8 μmol g^{-1} fresh weight h^{-1}. This compares favorably with the *in vivo* rate obtained at 100 mM $[NO_3^-]_o$ for a 20-min incubation, which was approximately 1 μmol g^{-1} fresh weight h^{-1}.

The NADH-dependent activity was much higher than the NADPH-dependent activity, accounting for approximately 80% of the total at high NO_3^- concentrations. Its K_m value was 1.41 mM (Fig. 4). The NADPH-dependent activity showed a considerably higher NO_3^- affinity, with a K_m for NO_3^- of 0.16 mM (Fig. 4).

These values differ considerably from those previously reported. For leaves of barley cv Steptoe, Dailey *et al.* (7) obtained values of 0.13 and 1.2 mM for the NADH and NAD(P)H enzymes, respectively, and Harker *et al.* (14) obtained a value of 0.61 mM for the NAD(P)H enzyme. Typically, K_m values of approximately 0.2 mM have been obtained for the NADH enzyme from several plant species (13). The reason for the difference between our results and those of previous studies is not clear. However, considerable interspecific variation appears to occur among K_m values for both enzyme forms (12), and the previous investigators have used leaves rather than roots. It seems reasonable that differences might occur between roots and leaves and among cultivars of the same species.

Estimation of $[NO_3^-]_c$

If it can be assumed that the *in vivo* kinetic parameters and relative magnitudes of the NADH- and NADPH-dependent activities are similar to those determined *in vitro*, then two conclusions can be drawn. First, given the low K_m of the NADPH-dependent activity, it is likely that this activity *in vivo* is always NO_3^- saturated (compare Figs. 2 and 3). At low $[NO_3^-]_o$ (0–2 mM), it would account for approximately 50% of the *in vivo* activity shown in Figure 2; whereas at high $[NO_3^-]_o$ (20–50 mM), it would account for approximately 20%. Second, due to its lower NO_3^- affinity and higher relative activity, the NADH-dependent activity likely accounts for most, if not all, of the response to increased $[NO_3^-]_o$ shown in Figure 2. Therefore, we based our calculation of $[NO_3^-]_c$ on the NADH-dependent activity, after correcting the *in vivo* data shown in Figure 2 for the estimated NADPH-dependent activity as seen in Figure 3. Figure 5 shows the estimates for $[NO_3^-]_c$ with increasing $[NO_3^-]_o$. At zero $[NO_3^-]_o$, $[NO_3^-]_c$ was 0.66 mM, and it increased in an approximately linear fashion with $[NO_3^-]_o$, reaching 3.9 mM at 20 mM $[NO_3^-]_o$. At higher $[NO_3^-]_o$ values, $[NO_3^-]_c$ could not be calculated directly from the data shown in Figure 2, due to saturation of the NADH-dependent activity. However, if the linear relationship holds for higher values of $[NO_3^-]_o$, then at 100 mM (which gave the maximum *in vivo* NRA), $[NO_3^-]_c$ would be approximately 17 mM.

Recently, Zhen *et al.* (33), using NO_3^--specific microelectrodes, estimated $[NO_3^-]_c$ to be 5.4 and 3.2 mM, respectively, for epidermal and cortical root cells of barley plants grown in 10 mM NO_3^-. The two values were judged not to be significantly different. This result would appear to contradict the suggestion of Siddiqi *et al.* (29) that $[NO_3^-]_c$ is higher in the cortical cells than in the epidermal cells (see the introduction). However, growth in 10 mM NO_3^- (in contrast with 100 μM as used in our studies) may have elevated $[NO_3^-]_c$ in the epidermal cells to a level that saturates NR, eliminating any difference between the two cell types. In any case, the results of Zhen *et al.* (33) are consistent with our observation that $[NO_3^-]_c$ is in the low millimolar range for values of $[NO_3^-]_o$ from 5 to 20 mM (Fig. 5).

Our results support those of Siddiqi *et al.* (29) using compartmental analysis, which show that $[NO_3^-]_c$ increases with increasing $[NO_3^-]_o$, although compartmental analysis yields much higher values (see the introduction). Siddiqi *et al.* (29) suggested that $[NO_3^-]_c$ is not as rigorously controlled as cytoplasmic concentrations of other major ions such as K^+ and Pi. This seems reasonable because NO_3^-, unlike these ions, does not directly regulate cellular metabolism (except for the induction of NO_3^- transport and NR). Although varying $[NO_3^-]_c$ would have effects on charge and osmotic balance, presumably these could be compensated for by other solute fluxes.

Implications for NO_3^- Influx

Figure 6 shows the relationship between $[NO_3^-]_c$ and E^N for four different values of $[NO_3^-]_o$, calculated according to the Nernst equation, which assumes passive equilibration of NO_3^- across the plasma membrane, in which case $\Delta\Psi$ is equivalent to E^N. The E^N values span the range of measured $\Delta\Psi$ values for barley as determined by Glass *et al.* (10) (−200 to −300 mV) as well as including less negative values. Given our estimates of $[NO_3^-]_c$ (between 0.6 and 1 mM for $[NO_3^-]_o$ values between 0.1 and 1 mM; Fig. 5), it is clear that passive NO_3^- influx via the LATS could not occur at $\Delta\Psi$ values between −200 and −300 mV. At such values, $[NO_3^-]_c$ would have to be in the nanomolar range (10).

The values that have been previously obtained for $\Delta\Psi$ of barley root cells are rather variable. For example, Mertz and Higinbotham (22) obtained values between −100 and −200 mV, and Zhen *et al.* (33) recently obtained a mean value of approximately −70 mV, considerably less negative than the values obtained by Glass *et al.* (10). The reason for the difference between the results of these studies is not clear. However, given a $\Delta\Psi$ value of −70 mV and our estimates of $[NO_3^-]_c$, passive net entry of NO_3^- via the LATS is still not possible except at very high $[NO_3^-]_o$ (approximately 10 mM).

The use of a K_m of 0.13 mM for the NADH enzyme, as obtained by Dailey *et al.* (7), rather than our value of 1.4 mM, yields lower values for $[NO_3^-]_c$, *e.g.* 60 μM at zero $[NO_3^-]_o$. With such values, at a $\Delta\Psi$ of approximately −70 mV as obtained by Zhen *et al.* (33), influx of NO_3^- via the LATS could be passive (Fig. 6). However, we consider such an extreme scenario unlikely for several reasons. First, the agreement between our *in vivo* and *in vitro* data for NO_3^- concentration dependence of NRA suggests that the K_m value we obtained, although different from those of other studies, is

correct for the barley cultivar we used. Second, in our analysis, we used kinetic parameters and $\Delta\Psi$ values from the same cultivar grown to the same age under similar conditions. Finally, consistent with our observations, the depolarization of $\Delta\Psi$ observed by Glass *et al.* (10) strongly suggests that the LATS is an active system.

CONCLUSIONS

Determination of $[NO_3^-]_c$ in barley roots using the NR-based method of Robin *et al.* (25) yields values of approximately 0.6 to 1 mM for 0.1 to 1 mM $[NO_3^-]_o$, in contrast to the values of 12 to 37 mM obtained by compartmental analysis (29). The present findings support the proposal of Siddiqi *et al.* (29) that the two techniques measure $[NO_3^-]_c$ in two different pools; respectively, a large, slowly metabolized pool (possibly the cytoplasm of the cortical cells) and a smaller, NR-containing, actively metabolized pool (possibly the cytoplasm of the epidermal cells).

$[NO_3^-]_c$ increased with increasing $[NO_3^-]_o$, up to 3.9 mM at 20 mM $[NO_3^-]_o$, confirming the results of Siddiqi *et al.* (29) from compartmental analysis and supporting their suggestion that $[NO_3^-]_c$ is not as closely regulated as the cytoplasmic pools of other major ions.

At the values we obtained for $[NO_3^-]_c$, passive transport via the LATS is essentially impossible given the $\Delta\Psi$ values measured by Glass *et al.* (10). Our results support their suggestion that the LATS is energetically active. Nevertheless, the LATS shows characteristics that are very puzzling for an active system, including linear kinetics, lower sensitivity to metabolic inhibitors than the HATS, and a lower temperature coefficient (11, 31). The paradoxical characteristics of this transport system clearly warrant further investigation.

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