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Studies of the Uptake of Nitrate in Barley[1]

II. Energetics

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ABSTRACT

Q_{10} values for $^{13}NO_3^-$ influx were determined in 'uninduced' (NO_3^--starved) and 'induced' (NO_3^--pretreated) roots of barley (*Hordeum vulgare* L.) plants at various concentrations of external NO_3^- ($[NO_3^-]_0$). At 0.02 mole per cubic meter $[NO_3^-]_0$, Q_{10} values for influx were from 3 to 4 between 5 and 10°C. As $[NO_3^-]_0$ increased Q_{10} values decreased, reaching values of 1.2 and 2.0, respectively, at 20 moles per cubic meter in uninduced and induced plants. The metabolic dependence of $^{13}NO_3^-$ influx at low and high $[NO_3^-]_0$ (0.1 and 20.0 moles per cubic meter, respectively) in uninduced and induced plants was probed by the use of various inhibitors. These experiments confirmed the findings of the Q_{10} studies, demonstrating that at low $[NO_3^-]_0$ $^{13}NO_3^-$ influx was extremely sensitive to metabolic inhibition. By contrast, at high $[NO_3^-]_0$, influx was relatively insensitive to the presence of inhibitors.

The first definitive analysis of the thermodynamic driving forces for nitrate uptake were undertaken by Higinbotham *et al.* (5) for pea and oat roots. They concluded that the absorption of nitrate by plants grown in inorganic media containing 1 mol m^{-3} NO_3^- was active, *i.e.* against the electrochemical potential difference ($\Delta\tilde{\mu}_{NO_3^-}$) for NO_3^-. This conclusion has consistently been borne out by studies designed to evaluate the effects of metabolic inhibitors, low temperature or anaerobiosis on NO_3^- uptake (2, 4, 9). Without exception, NO_3^- absorption by plant roots is strongly inhibited by such treatments. These studies have generally been undertaken at relatively low external NO_3^- concentration ($[NO_3^-]_0$);[2] typically $[NO_3^-]_0$ values up to and including 1 mol m^{-3} have been used, with plants fully induced for NO_3^- uptake by pretreatment with exogenous NO_3^-.

Recently it has been demonstrated that $^{13}NO_3^-$ influx in barley (10) and net NO_3^- uptake in corn (8) and barley (7) are mediated by a saturable system at low $[NO_3^-]_0$ and a linear (nonsaturating) system at high $[NO_3^-]_0$. Siddiqi *et al.* (10, 12) have proposed that in barley the high-concentration system is constitutive; pretreatment with NO_3^- fails to cause increased $^{13}NO_3^-$ influx in the range from 1 to 50 mol m^{-3} other than that effect due to the 'induction' of the low-concentration system. These authors have proposed that NO_3^- uptake at high $[NO_3^-]_0$ is mediated by NO_3^- channels (12). Fluxes of the order of 10^6 ions s^{-1} occur through channels along the electrochemical potential gradient for the particular ion. For NO_3^-, this would require that at high $[NO_3^-]_0$ $\Delta\tilde{\mu}_{NO_3^-}$ across the plasmamembrane be thermodynamically 'downhill.' As stated at the outset, the dogma, with regard to NO_3^- uptake, is that this process is always active (4).

How, then, can the nonsaturating, constitutive, high-concentration fluxes be channel mediated? Siddiqi *et al.* (10, 12) have suggested that the critical unknown in considerations of the nature of plasmalemma NO_3^- fluxes is cytoplasmic $[NO_3^-]$. Notwithstanding high vacuolar $[NO_3^-]$ (6), low cytoplasmic $[NO_3^-]$ may result from NO_3^- fluxes to the xylem, to the vacuole, to efflux and biochemical fluxes to NO_2^- and NH_4^+. Selecting values for cytoplasmic $[NO_3^-]$ in the range from 10 to 100 mmol m^{-3} and a plasma membrane electrical potential difference ($\Delta\psi$) of −150 mV, it is possible to predict (according to the Nernst equation) that NO_3^- uptake at 10 mol m^{-3} $[NO_3^-]_0$ may be passive (see Siddiqi *et al.* [12] for a discussion of this point).

The experiments described in the present communication were designed to evaluate the metabolic dependence of $^{13}NO_3^-$ influx by means of Q_{10} determinations and through the use of a variety of inhibitors. Effects of pH changes on $^{13}NO_3^-$ influx were also evaluated.

MATERIALS AND METHODS

Plant Growth

Seeds of barley (*Hordeum vulgare* L. cv Klondike) were germinated in washed, sterilized, moist sand as described previously (11). After 3 d of germination in the dark at 20°C, seedlings were transferred to hydroponic tanks containing 26 or 40 L of modified Johnson's nutrient solution at 1/80 strength (11), with or without appropriate amounts of $Ca(NO_3)_2$. Con-

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[2] Abbreviations: $[NO_3^-]_0$, external concentration of nitrate; DIDS, 4,4′-diisothiocyano-2 2′-stilbene disulfonic acid; DES, diethylstilbestrol; PCMBS, P-chloromercuribenzene sulfonate; CCCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; SHAM, sialicylhydroxamic acid; DNP, 2,4-dinitrophenol; $[NO_3^-]$, concentration of nitrate.

centrations of nutrient ions were maintained by continuous infusions of appropriate stock solutions (11). The plants were maintained in a controlled environment room at 20 ± 2°C, on a 16 h light-8 h dark cycle, and 70% RH. The light was provided at 300 μE m^{-2} s^{-1} (plant level) by fluorescent tubes with spectral composition similar to sunlight. Measurements of $^{13}NO_3^-$ influx were undertaken in the same growth rooms under identical ambient conditions (except for root temperatures in Q_{10} experiments) as had prevailed during the growth periods.

Experimental Procedures

$^{13}NO_3^-$ was produced by the proton irradiation of H_2O on the TRIUMF-ACEL CP42 cyclotron as described previously (11). The contaminants, ^{18}F, $^{13}NO_2^-$, and $^{13}NH_4^+$, were removed according to the procedure described by Siddiqi *et al.* (11), with the addition of a final step: excess H_2O_2 was reduced by the addition of 2 mg of catalase to the isotopic solution.

The influx of $^{13}NO_3^-$ into intact roots of barley plants was measured as reported in an earlier communication (11). Effects of various inhibitors, temperature, and pH on $^{13}NO_3^-$ influx were investigated in plants which were either 'uninduced' (not supplied with NO_3^-) or 'induced' (supplied with 0.1 mol m^{-3} NO_3^- for either 1 or 4 d). Before exposure to $^{13}NO_3^-$, plants were typically pretreated for 5 min in 400 mL of unlabeled influx solution. This was identical to the growth medium, consisting of 1/80 strength modified Johnson's inorganic nutrient solution. Plants were then transferred to 400 mL of $^{13}NO_3^-$ labeled influx solution (~40 kBq μmol^{-1}) for a 10-min influx period. Influx was followed by a 2-min desorption period at room temperature (20°C) in nonlabeled influx solution.

Metabolic inhibitors were included in the pretreatment solutions and in the $^{13}NO_3^-$ influx solutions. In the cases of KCN + SHAM, phenylglyoxal and DIDS treatments, pretreatments were extended to 20, 60, and 60 min, respectively, prior to the 10 min influx period in the presence of these inhibitors. During the extended pretreatment periods uninduced plants continued to be deprived of NO_3^- to maintain the uninduced condition; induced plants received NO_3^- during pretreatment periods. By contrast, the solutions used for the 5 min pretreatments contained NO_3^- at the same concentrations as in the influx solution.

RESULTS

Temperature Effects

NO_3^- influx was measured from various $[NO_3^-]_0$ at 5, 10, and 20°C and Q_{10} values were calculated as $(I_2/I_1)^{10/t_2-t_1}$, where I_2 and I_1 represent fluxes at t_2 and t_1, respectively. Q_{10} values between 10 and 20°C followed similar patterns to those between 5 and 10°C but the former values were much lower (data not shown), indicating a tendency towards saturation of $^{13}NO_3^-$ influx at relatively low temperatures. We have therefore presented Q_{10} values obtained only between 5 and 10°C (Table I) which better illustrate the phenomena.

At 0.02 mol m^{-3} $[NO_3^-]_0$, Q_{10} values for induced and uninduced plants were similar. However, at 0.05 mol m^{-3} and higher $[NO_3^-]_0$, induced and uninduced plants showed marked differences: Q_{10} values for uninduced plants declined substantially with increasing $[NO_3^-]_0$ toward unity at 20 mol m^{-3}. By contrast, Q_{10} values of induced plants declined only after $[NO_3^-]_0$ had reached 0.3 mol m^{-3} and at 20 mol m^{-3} they were still relatively high (about 2). Both 1 d and 4 d

Table I. *NO_3^- Influx and Q_{10} Values for NO_3^- Influx (in the range 5–10°C) at Various $[NO_3^-]_0$ for Plants Grown Without NO_3^- (uninduced) or Pretreated with 0.1 mol m^{-3} NO_3^- for 1 d (1 d induced) or 4 d (4 d induced)*

$[NO_3^-]_0$[a]	Uninduced		1 d induced		4 d induced	
	5°C	10°C	5°C	10°C	5°C	10°C
mol m^{-3}						
(a) NO_3^- influx (μmol $g^{-1}h^{-1}$)						
0.02	0.049 ± 0.002	0.097 ± 0.005	1.42 ± 0.01	2.75 ± 0.02	0.87 ± 0.04	1.54 ± 0.01
0.05	0.116 ± 0.004	0.181 ± 0.005	ND[b]	ND	ND	ND
0.10	0.130 ± 0.007	0.172 ± 0.006	3.45 ± 0.14	6.36 ± 0.29	1.37 ± 0.01	2.40 ± 0.06
0.30	0.304 ± 0.004	0.386 ± 0.005	3.42 ± 0.11	5.27 ± 0.05	1.76 ± 0.03	2.86 ± 0.06
0.50	0.429 ± 0.009	0.501 ± 0.003	ND	ND	ND	ND
20.0	10.70 ± 0.57	11.54 ± 0.77	13.87 ± 0.35	20.87 ± 0.52	6.07 ± 0.42	8.02 ± 0.26

$[NO_3^-]_0$[a]	Q_{10}		
	Uninduced	1 d induced	4 d induced
mol m^{-3}			
(b) Q_{10}			
0.02	3.9	3.8	3.1
0.05	2.4	ND	ND
0.10	1.8	3.4	3.1
0.30	1.6	2.4	2.6
0.50	1.4	ND	ND
20.0	1.2	2.3	1.8

[a] $[NO_3^-]$ of the influx solution. [b] Not determined.

Table II. *NO_3^- Influx into Roots of 1 d Induced and Uninduced Plants (Percentage of Control) after Treatment with Various Inhibitors (see "Materials and Methods")*

Inhibitor	Uninduced[a]		Induced	
	0.1	20	0.1	20
	%			
Control	100	100	100	100
DNP (0.1)	31	78	4	32
KCN + SHAM (1.0)	23	88	13	45
PCMBS (1.0)	21	79	10	42
DES (0.05)	34	89	28	57
CCCP (0.01)	19	81	26	48
Phenylglyoxal (1.0)	19	65	6	25
DIDS (1.0)	57	73	54	49

[a] $[NO_3^-]_0$ (mol m^{-3}).

induced plants showed similar Q_{10} values, although the actual influx of 1 d induced plants was much higher than the 4 d induced plants as shown in our previous study (11).

Effects of Various Inhibitors

The inhibitors used included uncouplers (DNP, KCN + SHAM), a nonpenetrating protein modifier (PCMBS), a plasmalemma H^+-ATPase inhibitor (DES), a protonophore (CCCP), and two inhibitors of anion uptake (phenylglyoxal and DIDS). In both induced and uninduced plants, influx was almost always more sensitive to inhibition from 0.1 mol m^{-3} $[NO_3^-]_0$ than from 20 mol m^{-3} $[NO_3^-]_0$. The one exception to this rule was provided by DIDS in induced plants (Table II). At 0.1 mol m^{-3} $[NO_3^-]_0$, inhibition of influx was generally greater in induced plants than in uninduced plants. At 20 mol m^{-3} $[NO_3^-]_0$, uninduced plants showed only a small response (10–20% inhibition) to the inhibitors, except the specific inhibitors of anion uptake, phenylglyoxal and DIDS which inhibited influx by 35 and 27%, respectively; by contrast, in induced plants at this $[NO_3^-]_0$, inhibition of NO_3^- influx was much greater (50–75%).

Effects of pH

In the range from 4.5 to 7.5, pH changes caused only small effects on $^{13}NO_3^-$ influx (Table III). However, the trends evident in the data were consistent in three separate experiments; pH optima were at 4.5 or 6.0, although differences between influx values at these two values of pH were generally small (≤19%). Differences were smaller at high external $[NO_3^-]$ (7 and 6% of the highest value for influx, respectively, in uninduced and induced plants). Likewise, the reduction of influx at pH 7.5 was smaller at high $[NO_3^-]_0$ (15–20%) than at low $[NO_3^-]_0$ (23–34%).

DISCUSSION

At low $[NO_3^-]_0$ the effects of low temperature and metabolic inhibitors were entirely consistent with thermodynamic evidence (4, 5) that NO_3^- transport across the plasmalemma is active. Q_{10} values of 3 to 4 confirm findings of earlier studies for the temperature dependence of NO_3^- uptake (2, 4). However, as $[NO_3^-]_0$ increased from 0.02 to 20.0 mol m^{-3}, Q_{10} values decreased to 1.2 in uninduced plants and about 2.0 in induced plants. Barber (1), investigating temperature effects on ^{32}P-labeled inorganic phosphate uptake, reported similar observations in barley, and argued that the observed Q_{10} of about 1 at [phosphate] above 1 mol m^{-3} was indicative of passive fluxes at high concentration. The decline of Q_{10} values, from 3.9 to 2.4 in uninduced plants (Table I) occurred at a concentration which is characteristic of the saturable low concentration system. This decline may represent a gradual increased contribution of the linear nonsaturating system, even at this low concentration.

In earlier communications dealing with biphasic $^{13}NO_3^-$ influx (10, 12) it was demonstrated that the contribution of the low concentration transport system to measured $^{13}NO_3^-$ fluxes above 1 mol m^{-3} was extremely small in uninduced plants; the V_{max} for $^{13}NO_3^-$ influx in these plants was 0.344 μmol g^{-1} h^{-1}. At 20 mol m^{-3} $[NO_3^-]_0$, measured $^{13}NO_3^-$ influx was about 25 μmol g^{-1} h^{-1}, and hence the low concentration system must have contributed <1% to the measured influx. Under these conditions metabolic effects would act almost exclusively upon the linear (nonsaturating) system. Hence, it is reasonable to interpret the low Q_{10} value (1.2) of uninduced plants at 20 mol m^{-3} $[NO_3^-]_0$ as characteristic of passive $^{13}NO_3^-$ fluxes.

However, fluxes due to the low concentration system in induced plants were increased about 30-fold by NO_3^- pretreat-

Table III. *NO_3^- Influx (μmol g^{-1} h^{-1} ± SE) into Roots of 1 d Induced and Uninduced Plants at Various pH Values and at Low (0.1 mol m^{-3}) and High (20 mol m^{-3}) $[NO_3^-]_0$*

Figures in parentheses are percentage of the highest value in each column.

pH of Uptake Solution	Uninduced		Induced	
	0.1[a]	20	0.1	20
	μmol g^{-1} h^{-1} ± SE			
4.5	0.38 ± 0.01 (100)	10.24 ± 0.38 (93)	7.88 ± 0.12 (81)	28.67 ± 0.53 (100)
6.0	0.32 ± 0.02 (84)	10.98 ± 0.71 (100)	9.67 ± 0.05 (100)	26.94 ± 0.61 (94)
7.5	0.25 ± 0.01 (66)	8.83 ± 0.33 (80)	7.41 ± 0.89 (77)	24.36 ± 1.29 (85)

[a] $[NO_3^-]_0$ (mol m^{-3}).

ment (V_{max} = 9.41 μmol g^{-1} h^{-1}). Hence, the measured $^{13}NO_3^-$ fluxes in induced plants at 20 mol m^{-3} receive a significant contribution (about 40%) from the low-concentration system. Not surprisingly then, Q_{10} values in these plants remained high (1.8 and 2.3, respectively, in plants pretreated with NO_3^- for 1 or 4 d) even at 20 mol m^{-3} $[NO_3^-]_0$, because low temperature would significantly reduce the contribution of the active low concentration system.

Essentially similar conclusions can be drawn from the inhibitor studies (Table II). Typically, the effects of inhibitors were more pronounced on the low concentration system than on the high concentration system, in uninduced or induced plants. For example, DNP reduced $^{13}NO_3^-$ influx in uninduced plants to 31% of control values at 0.1 mol m^{-3} $[NO_3^-]_0$ compared with a value of 78% of controls at 20 mol m^{-3}. This relationship held for all seven inhibitors. Induced plants behaved according to the same pattern with one exception, namely DIDS. However, because DIDS is an anion transport inhibitor rather than a metabolic inhibitor, this exception is understandable. As was the case for the Q_{10} studies, the inhibitors had a greater effect on high concentration fluxes in induced plants than in uninduced plants. We interpret this as due to the stronger effects of inhibitors upon the metabolically dependent low-concentration components of observed high concentration fluxes.

In summary, when the high concentration fluxes were examined in isolation from the low concentration systems (*i.e.* in uninduced plants) seven different inhibitors reduced influx by only 20% on average compared to an average 70% reduction of the low concentration fluxes in induced plants and a 57% reduction of the high concentration fluxes in induced plants.

As proposed earlier (10, 12), we consider that NO_3^- uptake is determined by the additive effects of two transport systems. The low-concentration system, inducible by exogenous nitrate, saturable and thermodynamically active, is thought to be coupled to 'downhill' H^+ fluxes via a 2 H^+:1 NO_3^- symporter (14) or to 'downhill' OH^- efflux via a NO_3^-/OH^- antiport (13) or via a NO_3^-/NO_3^- exchange system (3). We have suggested that high concentration fluxes occur through NO_3^- channels according to the direction and extent of $\Delta\bar{\mu}_{NO_3^-}$ (10, 12).

The Q_{10} and inhibitor studies are consistent with these proposals. However, it should be appreciated that although channel-mediated NO_3^- transport be passive, it should be sensitive to metabolic inhibitors by virtue of the contribution of the electrical potential difference ($\Delta\psi$) to $\Delta\bar{\mu}_{NO_3^-}$. For example, metabolic inhibitors would be anticipated to block active H^+ efflux, thus reducing the electrogenic component of $\Delta\psi$. The end result of this effect would be to facilitate passive NO_3^- entry. However, no increase of influx was observed in the presence of the selected metabolic inhibitors. Rather, there was only a slight reduction (about 20%) of influx in uninduced plants. It is unclear why this should have been the case. However, it is evident that the clear metabolic dependence observed at low $[NO_3^-]_0$ is not present at elevated concentrations of this ion.

We demonstrated in the first paper of this series that the linear high concentration fluxes became apparent at a significantly lower value of $[NO_3^-]_0$ (about 0.3 mol m^{-3}) in uninduced plants than in induced plants (about 1.0 mol m^{-3}). We interpreted this difference as corresponding to the lower value of $[NO_3^-]_0$ at which $\Delta\bar{\mu}_{NO_3^-}$ became sufficient to 'drive' passive NO_3^- uptake in uninduced plants. In view of the proposal that cytoplasmic $[NO_3^-]$ is probably much lower in uninduced plants (12), this interpretation would seem reasonable. It is therefore significant that Q_{10} values begin to decline at lower $[NO_3^-]_0$ values in uninduced plants than in the induced plants. Specifically, Q_{10} values dropped to 1.4 in uninduced plants at $[NO_3^-]_0 > 0.3$ mol m^{-3}, whereas in induced plants Q_{10} was still characteristic of metabolic reactions (2.4 and 2.6, respectively, in 1 d and 4 d-induced plants) at 0.3 mol m^{-3}.

The effects of pH on low- and high-concentration fluxes were investigated with a view to providing further insights into the mechanisms of the two transport systems. It is evident that pH effects on the low concentration transport system were much larger than those on the high concentration system. The pH effects, although in the right direction for a H^+/NO_3^- symport (see particularly the data for 0.1 mol m^{-3} $[NO_3^-]_0$ in uninduced plants: [Table III]), are not large and do not permit clear evaluations of different mechanisms of energy coupling.

The evidence accumulated from the metabolic studies presented here support our initial proposal regarding the driving forces for low and high concentration NO_3^- fluxes. A clear resolution of this problem will require that cytoplasmic $[NO_3^-]$ be determined.

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