

Regulation of NO_3^- Influx in Barley¹

STUDIES USING $^{13}\text{NO}_3^-$

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ABSTRACT

Short-term (10 minutes) measurements of plasmalemma NO_3^- influx (ϕ_{oc}) into roots of intact barley plants were obtained using $^{13}\text{NO}_3^-$. In plants grown for 4 days at various NO_3^- levels (0.1, 0.2, 0.5 millimolar), ϕ_{oc} was found to be independent of the level of NO_3^- pretreatment. Similarly, pretreatment with Cl^- had no effect upon plasmalemma $^{13}\text{NO}_3^-$ influx. Plants grown in the complete absence of $^{13}\text{NO}_3^-$ (in CaSO_4 solutions) subsequently revealed influx values which were more than 50% lower than for plants grown in NO_3^- . Based upon the documented effects of NO_3^- or Cl^- pretreatments on net uptake of NO_3^- , these observations suggest that negative feedback from vacuolar NO_3^- and/or Cl^- acts at the tonoplast but not at the plasmalemma. When included in the influx medium, 0.5 millimolar Cl^- was without effect upon $^{13}\text{NO}_3^-$ influx, but NH_4^+ caused approximately 50% reduction of influx at this concentration.

was based upon a form of 'pump and leak' system. Previous studies, particularly those of Jackson and his associates (13, 15, 16), have established that net NO_3^- efflux can be considerable under appropriate conditions.

Through the use of $^{13}\text{NO}_3^-$, it is possible to test the above hypothesis directly and to investigate the effect of NO_3^- or Cl^- accumulation upon plasmalemma NO_3^- influx. Using barley plants pretreated for 4 d at various NO_3^- levels and at a single Cl^- level, plasmalemma $^{13}\text{NO}_3^-$ influx values were found to be independent of pretreatment. While preliminary, the results of these experiments confirm the original conclusions regarding the insensitivity of influx to vacuolar $[\text{NO}_3^-]$. Likewise, it is evident that $^{13}\text{NO}_3^-$ influx is insensitive to prior loading with Cl^- , a condition which has been clearly demonstrated to reduce net NO_3^- uptake (3, 7, 19). These results are discussed in the context of current perceptions of the regulation of NO_3^- uptake in roots.

MATERIALS AND METHODS

Growth of Plants. Seeds of barley (*Hordeum vulgare* cv Bonanza) were germinated in sand on plastic gauze glued to Plexiglas discs, as described previously (18). Roots grow down through the gauze, upon which the plants become anchored, making it possible to transfer the discs between solutions with minimal disturbance of the plants. Seedlings were maintained in temperature-controlled greenhouses ($22 \pm 2^\circ\text{C}$) at the Station de Recherches, Agriculture Canada, Ste-Foy, Québec, in February 1983. Natural sunlight was supplemented by banks of fluorescent lamps which extended the short February days to give a standard 16 h day/8 h night. When 3 d old, the seedlings were transferred to hydroponic tanks in the same greenhouses. All solutions contained 0.5 mM CaSO_4 plus various concentrations of KNO_3 or KCl as specified, together with MgSO_4 , micronutrients, and Fe at a concentration equivalent to 1/100 Johnson's modified medium (18). Solutions were replaced daily and, on the evening prior to the influx experiments, the tanks were transported to the control room of the Van de Graaff generator in the Physics department at Université Laval. Room temperature was held at 25°C and at the time corresponding to the beginning of the photoperiod, plants were illuminated from above by a pair of mercury vapor lamps through a heat filter of ice-cold water. At plant level, irradiance was $300 \mu\text{E m}^{-2} \text{s}^{-1}$. Plants were 8 d old at the time of influx measurement.

Preparation of $^{13}\text{NO}_3^-$. $^{13}\text{NO}_3^-$ was prepared by deuteron bombardment of a Li_2CO_3 pellet as outlined by Caldwell *et al.* (1). The pellet was subsequently dissolved in HNO_3 which served to drive off unreacted CO_3^{2-} . The pH of the solution was adjusted to 7 using 1 N KOH and the solution passed through ion exchange columns to remove Li and contaminating ^{18}F . The eluate,

Short-term studies of NO_3^- influx into plant roots have been severely hampered by the lack of a convenient radioactive tracer for NO_3^- . Attempts to use $^{36}\text{ClO}_3^-$ as an analog for NO_3^- have met with some success, but there can be difficulties associated with the use of this tracer (8). Investigations using $^{15}\text{NO}_3^-$ have tended to involve uptake periods which appear to be relatively long by comparison with available estimates of the cytoplasmic half-life for NO_3^- exchange (12). As a consequence, such studies have tended to estimate the ϕ_{ov}^2 rather than ϕ_{oc} . Despite the possibility of using $^{13}\text{NO}_3^-$ as a tracer for NO_3^- in ion transport studies, it is only quite recently (14) that this tracer has been used to measure NO_3^- influx into roots of higher plants.

Previous studies of the net uptake of NO_3^- by barley roots and carrot slices have been interpreted to suggest that negative feedback is exerted upon the uptake process by a common feedback signal from vacuolar $[\text{Cl}^- + \text{NO}_3^-]$ (2, 5, 7, 19). Using $^{36}\text{ClO}_3^-$ as a tracer for NO_3^- ($^{36}\text{ClO}_3^-$), influx was insensitive to levels of NO_3^- pretreatment which caused substantial reductions of net NO_3^- uptake (5). These and more direct observations of NO_3^- efflux as a function of tissue $[\text{NO}_3^-]$ (8) were interpreted to suggest that NO_3^- influx was largely insensitive to vacuolar NO_3^- concentration and that the control of uptake at the plasmalemma

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² Abbreviations: ϕ_{ov} , quasi-steady flux to the vacuole; ϕ_{oc} , plasmalemma influx.

consisting of 2 to 3 ml of a solution of $^{14}\text{NO}_3^-$ labeled with ~ 50 μCi of $^{13}\text{NO}_3^-$, was used to label the uptake solutions. Since complete recovery of carrier NO_3^- (HNO_3) added to the Li_2CO_3 pellet was not always achieved, actual NO_3^- concentrations of the uptake media deviated from planned. However, samples of these solutions were set aside for subsequent NO_3^- analysis by means of an Orion NO_3^- electrode (8). Thus (Table I and elsewhere), the apparent choice of uptake solution containing $[\text{NO}_3^-]$ such as 0.422 mM was unavoidable. In this case, 0.5 mM NO_3^- had been anticipated.

Influx Experiments. Solutions containing 0.5 mM CaSO_4 plus various concentrations of $^{14}\text{NO}_3^-$ labeled with $^{13}\text{NO}_3^-$ and other ions (as specified) were contained in 1-L Plexiglas vessels, designed to accommodate the discs on which seedlings were supported. The solutions were aerated and stirred by means of a 4 place magnetic stirring device. Roots of intact plants were pretreated for 5 min in media which were identical to those to be used for the influx experiment except that they contained no $^{13}\text{NO}_3^-$. Discs were transferred to labeled solutions and exposed to tracer for 10 min. Following this, roots (still intact) were exposed for 5 min to desorption medium (identical to pretreatment solutions) at 2 to 4°C.

Isotope Counting. After desorption, roots were excised and gently blotted dry with paper towels. Each treatment was replicated four times and small (~ 0.5 g) samples were introduced, without weighing, into preweighed glass scintillation vials. One-ml samples of the influx solutions were transferred to scintillation vials in order to estimate the specific activity of these solutions. Radioactivities of the root samples were obtained by scintillation counting (L.K.B. Liquid Scintillation Counter). In the absence of a prescribed method for counting such samples, it was necessary to undertake some brief investigations of counting methods adopting a somewhat *ad hoc* approach. The positron (β^+) emission from $^{13}\text{NO}_3^-$, which is accompanied by gamma radiation, appeared to be counted rather like a Cerenkov radiation, without the need for chemical fluors. Using distilled H_2O additions (10 ml) as we generally employ for Cerenkov counting of ^{86}Rb (9), it was found that the positron emission was efficiently counted in the range employed for tritium counting. However, the counting efficiency appeared to be unaffected by these 10 ml additions of water, so they were subsequently omitted. To check that there was no contamination by other radioisotope product, samples of the uptake solution were counted at each run and then recounted at intervals of 2.5 min. Using the initial cpm and the appropriate half-life for ^{13}N (9.96 min), the cpm values were predicted at 2.5, 5, and 7.5 min (Table I). To correct for quenching due to variation in root mass, a uniform sample of radioactively labeled root material was weighed into 0.2, 0.4, 0.6, 0.8, and 1.0 g portions which were dispensed into scintillation vials. The resulting figures for cpm g^{-1} were used to obtain an exponential regression of cpm g^{-1} against root fresh weight. The regression was employed to estimate the efficiency of counting. Clearly, although there may have been minor inaccuracies with respect to the calculation of absolute $^{13}\text{NO}_3^-$ fluxes into roots, the relative values for various treatments are quite reliable. Using γ -ray detectors and correcting for root free space, McNaughton and Presland (14) obtained values for $^{13}\text{NO}_3^-$ influx in corn roots which are quite close to the values reported here for barley. From completion of the deuteron bombardment of the Li_2CO_3 pellet to arrival at the L.K.B. scintillation counter took approximately 35 min. Each sample was counted for 60 s and a careful note of elapsed time enabled corrections to be made for isotopic decay. All values of cpm g^{-1} fresh weight of roots were corrected for the isotopic decay using a microcomputer program written for the purpose.

RESULTS AND DISCUSSION

Validation of the Identity of the Isotope. In the first experiment the $^{13}\text{NO}_3^-$ was found to be contaminated by a longer-lived isotope which, based upon the half-life for decay, may have been ^{18}F . However, in all subsequent experiments the predicted values for cpm, based upon $t_{1/2} = 9.96$ min, corresponded to within a few per cent of actual values. Table I shows a representative sample of such data.

Influence of Prior Nitrate Provision on $^{13}\text{NO}_3^-$ Influx. Based upon previous studies, it is known that provision of NO_3^- in the range from 0.01 to 0.25 mM causes substantial reduction of net NO_3^- uptake (5). For example, Deane-Drummond (5) showed that barley plants grown in 0.25 mM NO_3^- gave net NO_3^- uptake values which were about one-fifth of the values obtained with plants grown in 0.01 mM NO_3^- . Nitrate was therefore provided at four levels during the 4 d prior to $^{13}\text{NO}_3^-$ influx, namely, 0 (CaSO_4 -grown plants), 0.1 mM, 0.2 mM, and 0.5 mM (as the K salt). It was intended that the influx medium contain 0.5 mM NO_3^- but, due to the failure to recover all of the carrier $^{14}\text{NO}_3^-$ which had been applied to the column, the actual concentration of NO_3^- was found to be 0.422 mM. Table II shows the outcome of this experiment. It is immediately apparent that $^{13}\text{NO}_3^-$ influx is significantly lower in CaSO_4 -grown plants which have received no prior NO_3^- exposure (other than during the 5 min pretreatment). This observation is consistent with the well documented requirement for 'induction' of net nitrate absorption in CaSO_4 -grown plants by exposure, on a time scale of 3 to 4 h, to NO_3^- (11). However, more interestingly, prior exposure to NO_3^- in the range from 0.1 to 0.5 mM apparently caused no reduction of $^{13}\text{NO}_3^-$ influx. This confirms the observations reported using $^{36}\text{ClO}_3^-$ as a tracer for NO_3^- (7, 8). Absolute values of NO_3^- influxes based upon the use of $^{13}\text{NO}_3^-$ or $^{36}\text{ClO}_3^-$ as tracers compare reasonably well considering the uncontrollable variations in growth and influx conditions between our laboratory in Vancouver and facilities at Quebec. In a previous communication, which comes closest to the conditions used for the $^{13}\text{NO}_3^-$ studies, we quoted an influx value of 12.5 $\mu\text{mol g}^{-1} \text{h}^{-1}$ based upon $^{36}\text{ClO}_3^-$. In the present study (Table II), values ranging from 8 to 10 $\mu\text{mol g}^{-1} \text{h}^{-1}$ were obtained using $^{13}\text{NO}_3^-$. The

Table I. Repeated Counts of 1 ml of $^{13}\text{NO}_3^-$ Labeled Uptake Medium at 2.5 Minute Intervals, Together with Predicted Radioactivity Based upon the 9.96 Minute Half-Life for Decay of ^{13}N

Time <i>min</i>	Radioactivity	
	Actual ^a	Predicted ^b
0-1	71,152 \pm 39	71,152
2.5-3.5	59,120 \pm 37	59,726
5.0-6.0	48,803 \pm 35	49,653
7.5-8.5	40,054 \pm 22	40,959

^a Mean of two samples \pm SE.

^b Predicted on the basis of the mean value at 0 to 1 min.

Table II. Influence of Prior NO_3^- Provision upon $^{13}\text{NO}_3^-$ Influx

All plants received 0.5 mM CaSO_4 for 5 d. Where NO_3^- was provided, it was added for the 3 d prior to the influx determination. Uptake medium contained 0.5 mM CaSO_4 plus 0.422 mM KNO_3 . Values for influx are the means \pm SE of four replicates.

Pretreatment Condition	$^{13}\text{NO}_3^-$ Influx $\mu\text{mol g}^{-1} \text{fresh wt h}^{-1}$
CaSO_4	3.41 \pm 0.75
0.1 mM KNO_3	7.88 \pm 0.86
0.2 mM KNO_3	7.19 \pm 0.57
0.5 mM KNO_3	9.98 \pm 0.95

reduction of net NO_3^- uptake associated with NO_3^- or Cl^- preloading must therefore act at the tonoplast, leading to increased cytoplasmic NO_3^- levels and to increased net efflux of NO_3^- across the plasmalemma.

In many respects, this situation appears to be similar to that proposed, on the basis of compartmental analysis, for SO_4^{2-} accumulation (3). This similarity has been noted previously (10).

Influence of Cl^- on $^{13}\text{NO}_3^-$ Influx. Previous studies by Cram (2) and Smith (19) have established that the accumulation of Cl^- results in a substantial reduction of both plasmalemma Cl^- influx and net NO_3^- uptake. Based upon these findings, it has been suggested (2) that Cl^- and NO_3^- uptakes respond to a common feedback signal from the combined $[\text{Cl}^- + \text{NO}_3^-]$ of the vacuole. The availability of $^{13}\text{NO}_3^-$ has enabled us to investigate the locus(i) of this feedback. Table III demonstrates that prior exposure to 0.5 mM Cl^- failed to reduce subsequent $^{13}\text{NO}_3^-$ influx, by comparison with plants grown without Cl^- . Thus, a consistent pattern emerges with respect to the common feedback effect of vacuolar Cl^- or NO_3^- on NO_3^- uptake. It is clear that the reduction of net NO_3^- uptake does not result from reduction of plasmalemma influx. By contrast, in the case of Cl^- , Cram (2) has demonstrated that both the ϕ_{oc} and the ϕ_{ov} are reduced by prior Cl^- or NO_3^- accumulation.

Table III also demonstrates that $^{13}\text{NO}_3^-$ influx is insensitive to the presence of Cl^- in the external medium. This observation is consistent with the report by Smith (19) that net NO_3^- uptake is insensitive to external Cl^- . However, in a previous study of $\text{NO}_3^-/\text{Cl}^-$ interactions, which made use of $^{36}\text{ClO}_3^-$ as a tracer for NO_3^- (7), it was observed that NO_3^- ($^{36}\text{ClO}_3^-$) influx was extremely sensitive to external Cl^- . Furthermore, pretreatment with Cl^- caused inhibition of NO_3^- ($^{36}\text{ClO}_3^-$) influx.

In view of the present observations with $^{13}\text{NO}_3^-$, the anomalous behavior of $^{36}\text{ClO}_3^-$ in the above instance could readily be accounted for by contamination of the $^{36}\text{ClO}_3^-$ employed in the above two experiments with $^{36}\text{Cl}^-$. This is entirely feasible since $^{36}\text{ClO}_3^-$ was generated by electrolysis of $^{36}\text{Cl}^-$, followed by chromatographic separation of the products (4). Alternatively, although ClO_3^- may be a legitimate analog for NO_3^- under a range of circumstances, there may be a failure to faithfully trace NO_3^- under these particular circumstances. We have previously cautioned that $^{36}\text{ClO}_3^-$ may not serve as a satisfactory tracer for NO_3^- in some organisms (8).

Table III. Influence of Cl^- and NH_4^+ upon $^{13}\text{NO}_3^-$ Influx

Plants were grown for 5 d in 'standard' media containing 0.01 mM KNO_3 , 0.1 mM K_2SO_4 , 0.5 mM CaSO_4 , and 0.01 mM MgSO_4 together with the micronutrient components of 1/100 strength modified Johnson's medium (18). In treatment no. 2, 0.5 mM Cl^- (Ca salt) was added to the growth medium. Standard influx medium contained 0.5 mM CaSO_4 plus 0.75 mM KNO_3 , labeled with $^{13}\text{NO}_3^-$. In treatment nos. 3 and 4, 0.5 mM Cl^- (Ca salt) or 0.5 mM NH_4^+ (SO_4^{2-} salt), respectively, were added to the influx media. Values for influx are means of four replicates \pm SE.

Pretreatment	Influx Medium	$^{13}\text{NO}_3^-$ Influx
		$\mu\text{mol g}^{-1}$ <i>fresh wt h</i> ⁻¹
1. Standard*	Standard*	8.95 \pm 0.47
2. Standard + 0.5 mM Cl^-	Standard	9.88 \pm 0.81
3. Standard	Standard + 0.5 mM Cl^-	10.12 \pm 0.98
4. Standard	Standard + 0.5 mM NH_4^+	4.80 \pm 0.41

* As described in legend for table.

Influence of NH_4^+ on $^{13}\text{NO}_3^-$ Influx. Plants previously exposed to low-N conditions (0.01 mM KNO_3) were exposed to 0.25 mM $(\text{NH}_4)_2\text{SO}_4$ during the influx period in 0.75 mM NO_3^- . The results of this experiment clearly demonstrate that $^{13}\text{NO}_3^-$ influx was strongly inhibited by NH_4^+ . This confirms what has been apparent from numerous studies of net NO_3^- absorption (13, 17).

In summary, it is apparent that plasmalemma $^{13}\text{NO}_3^-$ influx is insensitive to the NO_3^- or Cl^- status of the tissue and that the site of the negative feedback upon net NO_3^- absorption is therefore probably at the tonoplast. Inhibition of tonoplast NO_3^- fluxes by NO_3^- or Cl^- accumulation would inevitably lead to increased cytoplasmic $[\text{NO}_3^-]$ and/or increased transport of NO_3^- to the shoot, together with increased efflux of NO_3^- . In these respects, the situation bears close resemblance to the picture for SO_4^{2-} absorption proposed by Cram (3) on the basis of compartmental analysis.

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