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Effect of Exogenous and Endogenous Nitrate Concentration on Nitrate Utilization by Dwarf Bean[1]

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ABSTRACT

The effect of the exogenous and endogenous NO_3^- concentration on net uptake, influx, and efflux of NO_3^- and on nitrate reductase activity (NRA) in roots was studied in *Phaseolus vulgaris* L. cv. Witte Krombek. After exposure to NO_3^-, an apparent induction period of about 6 hours occurred regardless of the exogenous NO_3^- level. A double reciprocal plot of the net uptake rate of induced plants *versus* exogenous NO_3^- concentration yielded four distinct phases, each with simple Michaelis-Menten kinetics, and separated by sharp breaks at about 45, 80, and 480 micromoles per cubic decimeter.

Influx was estimated as the accumulation of ^{15}N after 1 hour exposure to $^{15}NO_3^-$. The isotherms for influx and net uptake were similar and corresponded to those for alkali cations and Cl^-. Efflux of NO_3^- was a constant proportion of net uptake during initial NO_3^- supply and increased with exogenous NO_3^- concentration. No efflux occurred to a NO_3^--free medium.

The net uptake rate was negatively correlated with the NO_3^- content of roots. Nitrate efflux, but not influx, was influenced by endogenous NO_3^-. Variations between experiments, *e.g.* in NO_3^- status, affected the values of K_m and V_{max} in the various concentration phases. The concentrations at which phase transitions occurred, however, were constant both for influx and net uptake. The findings corroborate the contention that separate sites are responsible for uptake and transitions between phases.

Beyond 100 micromoles per cubic decimeter, root NRA was not affected by exogenous NO_3^- indicating that NO_3^- uptake was not coupled to root NRA, at least not at high concentrations.

Nitrate utilization in higher plants is essentially the integration of three processes: acquisition, conversion, and translocation of nitrate-nitrogen. The amount of NO_3^- taken up by the roots represents the upper limit of a plant's NO_3^- utilization capacity. Despite its great biological and agricultural significance, the uptake of NO_3^- has been studied much less extensively than the uptake of other important ions. It is noteworthy that recent reviews on the concentration kinetics of ion transport do not give data on NO_3^- (14, 26). Although kinetic parameters of NO_3^- uptake have been reviewed (7, 18), no uniform pattern emerges. It is unclear whether the relationship between uptake rate and NO_3^- concentration is best described by single, dual, or multiphasic isotherms. Furthermore, the kinetic parameters in the various concentration ranges remain unknown. The published values depend largely on the experimental and analytical techniques employed, and are often questionable due to lack of steady-state conditions and sufficiently rigorous analytical procedures.

In this study, several techniques were combined, *e.g.* measurement of net uptake, influx, and efflux of NO_3^- and the assay of nitrate reductase activity, to construct a kinetic picture of the uptake and reduction of NO_3^- by bean roots. Additionally, a comparison of characteristics of net NO_3^- uptake with those of unidirectional fluxes was made and the kinetic behavior of plants during environmental depletion of NO_3^- was studied. Short reports of our findings have appeared (4, 5).

MATERIALS AND METHODS

Plant Cultivation. *Phaseolus vulgaris* L. cv. Witte Krombek was germinated in Perlite and transferred to N-free basal medium (3), the pH of which was kept between 4.2 and 6.5 with dilute KOH or at 5.0 ± 0.1 with a pH stat. Plants were grown for 10 d in the former and 7 d in the latter treatment. After these periods, the roots were unnodulated, the primary leaves had expanded, and the contribution of trifoliate leaves to plant weight or plant NRA[2] was negligible (3). No major differences in plant weight, development, rate of NO_3^- uptake, and NRA occurred between plants grown with manual or automatic pH control. Barley (*Hordeum vulgare* L. cv. Herta) was grown for 10 d with manual pH control.

Nitrate utilization was initiated by adding $Ca(NO_3)_2$ to plants grown in basal medium. Germination, cultivation, and experiments were performed at 20 ± 1°C, 65 ± 10% RH, and a 16-h photoperiod at 30 w m^{-2}. Dwarf bean was grown for 10 d on $CaCl_2$ solutions (0.5 mmol dm^{-3}) to produce low-salt roots. We refer to previous papers for detailed conditions of cultivation and experimentation (2, 3).

Experiments. Net uptake of NO_3^- and $H_2PO_4^-$ was assayed by following nutrient depletion in the ambient solution for several hours. In experiments with various starting concentrations, NO_3^- was regularly replenished to the initial levels in long-term experiments, while in short-term experiments the depletion in a certain interval was plotted against the average concentration in that interval. In experiments on the time course of NO_3^- uptake, the uptake rate was considered constant when depletion proceeded either at a constant rate or when a constant time was required to cover a specified concentration range. In experiments on $^{15}NO_3^-$ influx, medium depletion was kept within 20% by adoption of suitable volume to plant ratios.

Nitrate influx was measured by analysis of ^{15}N in plants after a 1-h absorption period with $^{15}NO_3^-$. Nitrate efflux was calculated as the difference between influx and net uptake, or measured as the amount of ^{15}N recovered from a $^{14}NO_3^-$ solution in which $^{15}NO_3^-$-preloaded plants had grown for 1 h. To remove adhering $^{15}NO_3^-$, $^{15}NO_3^-$-treated roots were rinsed for 1 min in a 10-fold

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[2] Abbreviations: NRA, nitrate reductase activity; NUR, nitrate uptake rate.

excess of $^{14}NO_3^-$ in basal medium. Nitrogen-15 in the efflux medium was exclusively in the NO_3^- fraction; no NO_2^-, NH_4^+, amino acids, amides, peptides, or proteins were detected. The accumulated limits of detection of these compounds represented a contribution of at most 0.7% to NO_3^--N in the solution. Nitrate efflux to basal medium was estimated by measuring the light absorption at 202 nm (see below).

Analytical Procedures. Phosphate was determined by measuring the radioactivity of a ^{32}P-labeled NaH_2PO_4 solution in a liquid scintillation counter equipped with a flowcell. Counts were recorded at 2-min intervals, corrected for background, and processed as described for NO_3^- depletion experiments. No differences in counting efficiency for Cerenkov radiation were found between solutions sampled before and after a depletion experiment.

Nitrate in solution was determined intermittently or continuously by either measuring samples, or by circulating the experimental solution through a UV monitor (LKB Uvicord III, 206 nm filter) or a Beckman 26 spectrophotometer. A wide concentration range (10 μmol–10 mmol dm^{-3}) could be studied by changing the cell path width in the former and the wavelength in the latter instrument. This method was checked regularly with conventional NO_3^- procedures as described before (3).

Nitrate in plant material was extracted with water and analyzed by an automated hydrazine reduction procedure (2).

Nitrogen-15 was determined in digests of plants and in nutrient solutions after concentration *in vacuo*. The assay for ^{15}N was by emission spectrophotometry (2) or MS. In the latter case, N_2 was evolved from the plant samples in a Carlo Erba ANA 1300 automatic nitrogen analyzer and analyzed in a Varian MAT 250 mass spectrometer. Results of experiments with ^{15}N are given as the amount of all N tagged with the isotope, thus correcting for the isotope abundance in the applied NO_3^-. Nitrate reductase activity in roots was measured by an *in vivo* procedure (3) which yielded results in good agreement with the actual rate of NO_3^- reduction (2). Proteins and peptides were analyzed with Coomassie Brillant G dye (Bio-Rad) with egg albumin as reference (34). Amino compounds and NH_4^+ were assayed with ninhydrin (31), using α-alanine as reference. Nitrite was measured as described for the incubation medium in the NRA assay (3). All results are expressed per unit of dry root mass.

RESULTS

Time Course of Nitrate Uptake. The rate of NO_3^- uptake increased continuously after exposure of bean roots to NO_3^- and became constant after about 6 h. The ambient NO_3^- concentration of the medium had no significant effect either on the length of the apparent induction period (Table I) or on the uptake pattern.

Steady-State Kinetics of Net Nitrate Uptake. The concentration dependence of NO_3^- uptake was examined in plants which had established a constant uptake rate (*cf.* 3). When plants were allowed to deplete their medium, a complex kinetic pattern emerged (Fig. 1). There were at least four separate phases, each obeying simple Michaelis-Menten kinetics, with phase transitions at about 45, 80, and 480 μmol dm^{-3} (Table II). The net uptake rate increased with ambient NO_3^- level except in an intermediate concentration range. The data points from discontinuous measurements of net NO_3^- uptake were plotted in a log-log fashion (Fig. 2) and, speculatively, interconnected according to the phase pattern discerned in double-reciprocal plots from continuous measurements.

Table I. *Time Required to Attain a Constant Rate of Net Nitrate Uptake by Dwarf Bean at Various Nitrate Concentrations*

Data are given ±SD with the number of independent experiments for each concentration in parentheses.

NO_3^-	Lag Period
mmol dm^{-3}	*h*
0.01	6.4 ± 0.4 (n = 5)
0.03	6.4 ± 0.3 (n = 4)
0.05	6.4 ± 0.2 (n = 4)
0.1	6.2 ± 0.6 (n = 3)
0.3	6.1 ± 0.9 (n = 5)
1	6.5 ± 0.9 (n = 3)
3	7.2 ± 1.0 (n = 3)
10	6.3 ± 1.0 (n = 5)

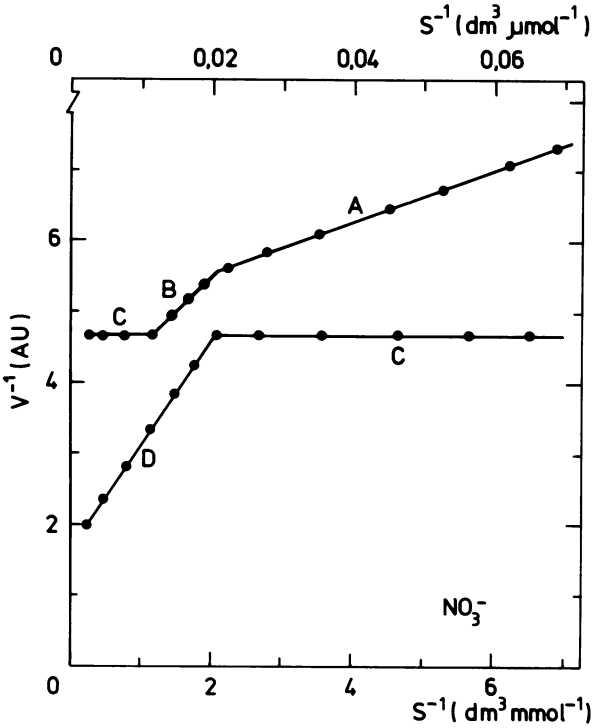

FIG. 1. Lineweaver-Burk plot of net NO_3^- uptake by dwarf bean, calculated from depletion experiments starting at 0.4 mmol NO_3^- dm^{-3} (upper abscissa, phases A, B, and C) and 5 mmol NO_3^- dm^{-3} (lower abscissa, phases C and D). The continuous monitoring technique provided many more data points than plotted in the figure. Kinetic constants are summarized in Table II. Plants were induced for at least 7 h at 0.5 mmol NO_3^- dm^{-3}, and depletion experiments lasted less than 6 h. The mean uptake rate (ordinate) is given in arbitrary units (AU) and approximated 40 μmol h^{-1} g^{-1} in phase C. Phase C values were the same in depletion experiments started either in phase C or phase D and were therefore equalized in the lower and upper graphs.

The concentration-independent nature of net nitrate uptake in phase C (80–480 μmol dm^{-3}; Figs. 2 and 3) was checked by transfer of plants from one concentration (130) to another (130, 200, and 400) within this phase or by transfer from a phase C (130) to a phase B concentration (60 μmol dm^{-3}). The uptake rate was unaffected in the former, but 40% lower in the latter case. Net uptake was constant for at least 7 h, provided the NO_3^- concentration was kept within the range of phase C.

To see whether the observed kinetic phenomena are of wider importance, we determined the low-concentration kinetics (<0.4 mmol dm^{-3}) of another species (barley) under similar conditions, and of another anion ($H_2PO_4^-$) by excised low-salt bean roots. The kinetic properties of NO_3^- uptake were similar for dwarf bean and barley (Table II), and a phase transition, predicted from discontinuous ^{32}P accumulation experiments (24) appeared in continuous $H_2PO_4^-$ depletion experiments at about 50 μmol dm^{-3} (not shown).

Unidirectional Fluxes. Nitrate influx into $^{14}NO_3^-$-induced roots

Table II. *Kinetic Characteristics of Net Nitrate Uptake by Dwarf Bean and Barley.*

The data represent the ranges observed in at least five (beans) or three (barley) independent depletion experiments, started at 0.4 (phases A, B, and C) or 5 mmol NO_3^- dm^{-3} (phases C and D, beans). Phases as indicated in Figures 1 and 2. Plants were induced for at least 7 h at 0.5 mmol NO_3^- dm^{-3}, and depletion experiments lasted less than 6 h.

	Transitions			Michaelis Constants				V_{max}		
	A–B	B–C	C–D	A	B	C	D	A:B	B:C	C:D
	μmol dm⁻³			*μmol dm⁻³*				*ratio*		
Beans	41–48	75–92	458–496	5–15	16–120	<0.1	900–1,170	0.3–0.7	1.2–2.4	0.3–0.6
Barley	54–57	67–93		1–25	17–118	<0.1		0.3–0.8	1.3–2.3	

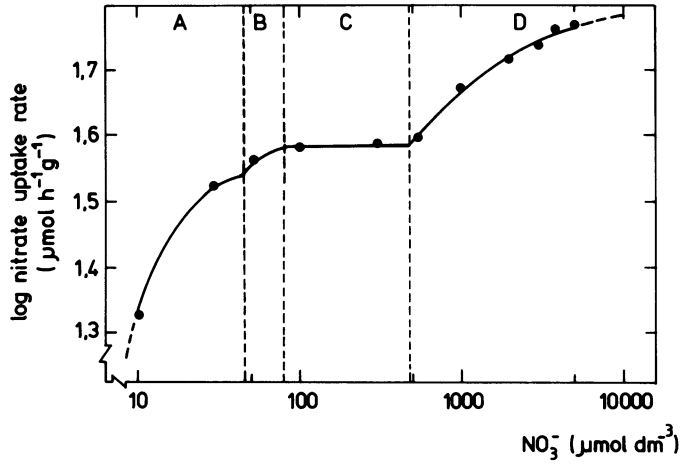

FIG. 2. Log-log plot of the rate of NO_3^- depletion by dwarf bean *versus* NO_3^- concentration. Plants were grown for 7 h at 0.5 mmol NO_3^- dm^{-3} prior to depletion experiments. The data points are interconnected to fit the four phases observed in Figure 1.

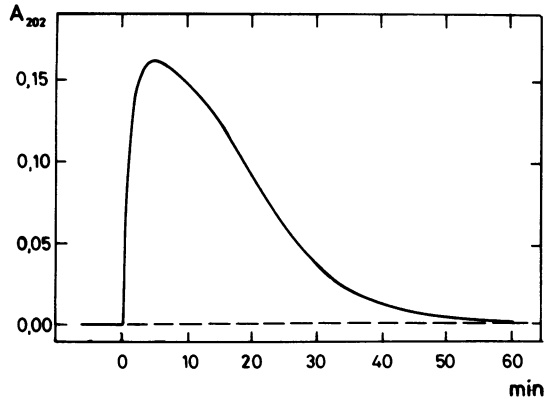

FIG. 3. Nitrate efflux by dwarf bean to a NO_3^--free medium. Plants were grown for 16 h on a NO_3^- medium (1 mmol NO_3^- dm^{-3}), and transferred for 1 h to a NO_3^--free medium. The maximal contribution of NO_3^- to the light absorption at 202 nm can be calculated with the millimolar extinction coefficient of 9.41.

was estimated by measuring the accumulation of ^{15}N in plants treated for 1 h with $^{15}NO_3^-$. The concentration-kinetics of NO_3^- influx matched the pattern that was computed from more detailed continuous data (*cf.* Fig. 1). At higher concentrations (>5 mmol dm^{-3}) only limited data are available, and further kinetic complexity cannot be precluded, neither for net uptake nor for influx.

Nitrate efflux was indicated by an excess of ^{15}N accumulation over net uptake. On the first day of NO_3^- nutrition, efflux was a constant proportion of net influx (Table III), and occurred in roots of induced plants as well as during the apparent induction of

Table III. *Nitrate Fluxes through Roots of Dwarf Bean in the Course of Nitrate Nutrition*

Nitrogen-depleted plants were supplied with $^{15}NO_3^-$ (0.5 mmol dm^{-3}) and transferred for 1 h to equimolar $^{14}NO_3^-$ media at indicated intervals. Disappearance of ambient nitrate and $^{15}NO_3^-$ recovered from the $^{14}NO_3^-$ solution were considered as net uptake and efflux, respectively. Influx was calculated as the sum of efflux and net uptake.

Measurement After	Influx	Efflux	Net Uptake	Efflux / Net Uptake
h	*μmol NO_3^- g^{-1} h^{-1}*			
2 .5	43.9	7.1	36.8	0.19
6	59.5	8.9	50.6	0.18
7 .5	62.8	10.2	52.6	0.19
11 .5	62.4	8.4	54.0	0.16
24	59.0	9.4	49.6	0.19

Table IV. *Nitrate Fluxes through Roots of Dwarf Bean at Various Nitrate Concentrations*

After 16 h of $^{15}NO_3^-$ supply (1 mmol dm^{-3}), plants were transferred for 1 h to $^{14}NO_3^-$ solutions of various NO_3^- content. Disappearance of ambient nitrate and $^{15}NO_3^-$ recovered from the $^{14}NO_3^-$ solution were considered as net uptake and efflux, respectively. Influx was calculated as the sum of efflux and net uptake.

Nitrate Concentration	Influx	Efflux	Net Uptake	Efflux / Net Uptake
μmol dm^{-3}	*μmol NO_3^- g^{-1} h^{-1}*			
0	0	0	0	0.09
50	19.9	1.7	18.2	0.17
100	34.3	5.0	29.3	0.27
500	43.8	9.2	34.6	0.47
1,000	66.9	21.5	45.4	

NO_3^- uptake. At increasing NO_3^- concentration, the efflux rate and also the proportion of efflux relative to the net uptake increased (Table IV). A transient efflux occurred to media deprived of NO_3^- (Fig. 3). After 5 min of efflux, the released NO_3^- started to become reabsorbed, resulting in practically zero (<1 μmol dm^{-3}) NO_3^- levels after 1 h. In contrast, efflux to NO_3^- media was essentially constant with time for at least 1.5 h.

Inhibition of Net Uptake by Endogenous Nitrate. After 6 h of NO_3^- nutrition, tissue NO_3^- in roots and shoots was proportional to the NO_3^- concentration in the medium and net uptake of NO_3^- appeared to be progressively inhibited by NO_3^- in roots (Fig. 4). After 6 h of NO_3^- supply, the influx of NO_3^- was unaffected by tissue NO_3^- level (Fig. 5).

Nitrate Reductase Activity. Six h after the onset of NO_3^- nutrition, NO_3^- is mainly reduced in the roots of dwarf bean (2) and the bulk of the nitrate reductase activity is also in the root system at low (0.1) or high (5 mmol dm^{-3}) NO_3^- concentration.

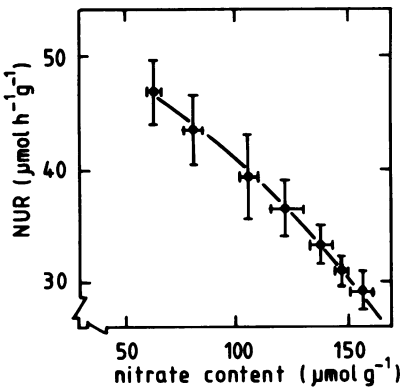

FIG. 4. Relationship between endogenous nitrate concentration in roots of dwarf bean and net NO_3^- uptake rate (NUR). Plants were grown for 6 h at various NO_3^- concentrations, and NO_3^- contents refer to roots collected prior to the determination of the NO_3^- uptake rate at 0.15 mmol NO_3^- dm^{-3}.

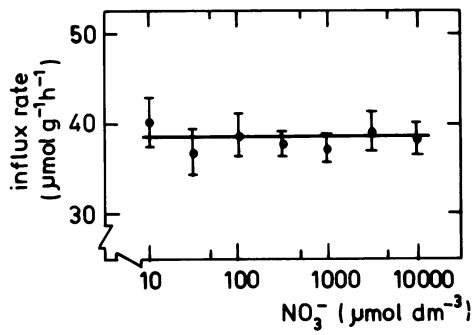

FIG. 5. Effect of exogenous NO_3^- concentration on NO_3^- influx rate into roots of dwarf bean. Plants were grown for 6 h at various $^{14}NO_3^-$ concentrations prior to 1 h exposure to $^{15}NO_3^-$ (0.15 mmol dm^{-3}). Nitrate influx was calculated as the total amount of N tagged with the isotope and recovered in the plants. Vertical bars represent ±SD (n = 3).

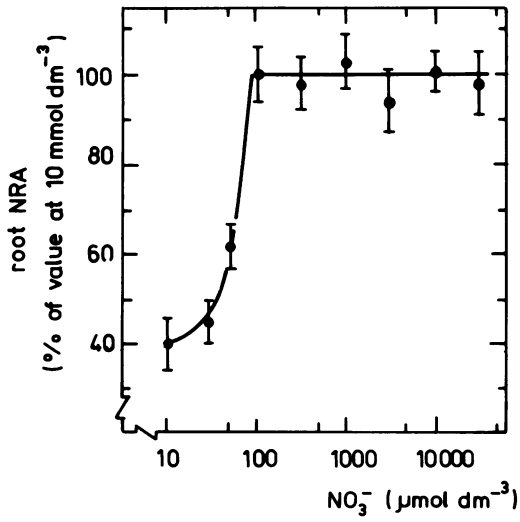

FIG. 6. Effect of NO_3^- concentration on nitrate reductase activity (NRA) of roots of dwarf bean after 6 h of NO_3^- nutrition. Vertical bars represent ±SD (n = 5). The value for root NRA without NO_3^- was 0.06 ± 0.02, and the 100% value was 5.8 ± 0.3 μmol NO_2^- h^{-1} g^{-1}.

Over a wide range of ambient NO_3^- concentrations, root NRA after 6 h was independent of the level of NO_3^- supply (Fig. 6). At the time of NRA assay, the NO_3^- content in roots was 1.5 times higher at 10 than at 0.1 mmol dm^{-3} (158 and 105 μmol g^{-1}, respectively). Concentration dependence of NRA occurred only at NO_3^- levels below 0.1 mmol dm^{-3}. No indications of a complex kinetic pattern were found.

DISCUSSION

The constant length of the lag period at various NO_3^- concentrations suggests that only traces of NO_3^- are required for the apparent induction of NO_3^- uptake in dwarf bean (Table I). In contrast, the apparent induction has been reported to be faster or slower with increasing NO_3^- concentrations in maize (22) and *Arabidopsis* (13), respectively.

The concentration dependence of both net uptake and influx is in accord with the concept of multiphasic uptake mechanisms (Figs. 1 and 2). Short-term influx and long-term accumulation of NH_4^+-N, $H_2PO_4^-$, K^+, Ca^{2+}, Mg^{2+}, and Zn^{2+} have been shown to behave multiphasically over long growing periods in a number of species (28). Our finding of a phase with an extremely high affinity for NO_3^- (Table II, phase C), and K_m and V_{max} values lower than those of the adjacent lower concentration phase (phase B) cannot be reconciled with models other than the multiphasic one. The sharp breaks evident in NO_3^- (Fig. 1) and $H_2PO_4^-$ depletion experiments are furthermore only consistent with discontinuous kinetics.

We envisage the multiphasic pattern as resulting from changes in kinetic properties caused, in an all-or-none fashion, by the exogenous ion concentration (27). Evidence for the multiphasic concept comes, so far, from experiments performed at a number of discrete ion concentrations. The present experiments were either carried out in this way (Fig. 2) or roots were exposed to a continuously changing NO_3^- concentration, due to progressive depletion of the uptake solution (Fig. 1). The existence of concentration-determined phases was also indicated by the rapid change of the uptake rate upon transfer of plants from a concentration of phase C to one of phase B. The estimation of uptake by depletion as performed for $H_2PO_4^-$ (23), NO_3^- (37), and K^+ (10) reduces the biological variability and is better equated to natural conditions than is exposure to various salt conditions.

An uptake rate which is independent of the NO_3^- concentration as in phase C, is an attractive feature from an experimental point of view. The physiological significance of phase C could be the maintenance of a constant provision with NO_3^- in a concentration range occurring in natural and agricultural ecosystems (40). The phase pattern for net uptake and influx of NO_3^- is essentially similar to that for alkali cations and Cl^- (25). For many nutrients, the kinetic pattern seems to be fairly uniform among higher plant species (24, 25). At least for the three low-concentration phases, the kinetic pattern of NO_3^- uptake did not differ between dwarf bean and barley (Table II).

Previously published K_m values for net nitrate uptake may pertain to only one or two of the four uptake phases established for dwarf bean. Affinities as in phase A (average $K_m \leq 10$ μmol dm^{-3}) have been found in a bacterium (36), a fungus (16), algae (8, 17), and higher plants (11, 21). A typical phase B K_m value of 50 to 250 μmol dm^{-3} occurs in a fungus (32), algae (42, 43), and higher plants (9, 30). A K_m below 1 μmol dm^{-3}, or a phase (phase C) in which uptake proceeds almost independently of NO_3^- concentration, also occurs in algae (8, 17, 38) and higher plants (11, 21, 37). Last, K_m values in the mmol dm^{-3} range (phase D) came from experiments with a bacterium (36) and higher plants (6, 22).

The NO_3^- uptake isotherm deviates also from a single Michaelis-Menten curve in algae (42, 43), a microorganism (36), and higher plants (22, 30). We believe therefore, that the various K_m values found for NO_3^- uptake can be ascribed to various kinetic phases, rather than to differences in the molybdenum status of roots as proposed by Butz and Jackson (7).

Efflux of NO_3^- during net uptake of NO_3^- has been reported for several species (18, 29). In the present experiments, NO_3^- efflux varied between 10% and 50% of the net incoming NO_3^-. During and after the apparent induction of NO_3^- uptake, the proportion of efflux to influx was fixed (Table IV) at a ratio which depended on the exogenous NO_3^- level (Table IV). Exogenous NO_3^- or NO_3^- influx was required for efflux, and circulation of NO_3^- through the root periphery was proportional to influx (Table IV; Fig. 3).

A 'NO_3^- compensation concentration' as sometimes reported (21, 37) was not observed with dwarf bean. Complete exhaustion of nutrient NO_3^- indicates that the effect of an unstirred layer or medium heterogeneity was negligible. Efflux occurred during and after the recovery from N-deficiency (Table III) and was thus not caused by an excessive NO_3^- status, at least not during the first day of NO_3^- supply. In contrast to our finding, NO_3^- leakage to N-free media occurred in wheat (19). These plants, however, were grown considerably longer with NO_3^- than our bean plants.

Net NO_3^- uptake appeared to be inhibited by unmetabolized NO_3^- in the roots (Fig. 4). This control is merely exerted on the efflux, not on the influx component (Fig. 5). For K^+, in contrast, the influx rate decreased at increasing endogenous K^+ levels (15). It is uncertain whether endogenous NO_3^- inhibits NO_3^- uptake specifically. Cram (12), for example, reported that the KCl status of carrot roots was negatively correlated with the net uptake of NO_3^- and the influx of a number of other compounds.

Induction and experimental period were not rigorously standardized in the present work. This presumably resulted in variation of the plant NO_3^- status between experiments. Nevertheless, induced plants took up NO_3^- at a constant rate for many hours. Variation in NO_3^- status may have affected the efflux rate and the values for V_{max} and K_m in the various concentration phases (Table II). The concentrations at which phase transitions occurred, however, were hardly affected by NO_3^- status (Table II) and were similar for influx and net uptake (Table II; Figs. 1 and 2) and for dwarf bean and barley. These results strongly indicate that K_m and V_{max} for NO_3^- uptake are governed by both endogenous and exogenous NO_3^-, but that phase transitions solely reflect an interaction between ambient NO_3^- and entities in the plasmalemma of absorbing root cells. For K^+, V_{max}, and K_m for uptake into barley roots depend upon the K^+ status of the roots, while phase transitions were also indifferent to the cellular K^+ concentration (27). By different means, the existence of separate sites involved in uptake and phase transitions was also demonstrated for SO_4^{2-} uptake (39).

The distribution of NO_3^- reduction between roots and leaves has been suggested to change with ambient NO_3^- level (1, 20). In dwarf bean (3), neither root NRA, nor the proportion of root NRA to total plant NRA was affected by the NO_3^- concentration in the range of 0.1 to 5 mmol dm^{-3}. At least for the former finding, this range can now be extended to 30 mmol dm^{-3} (Fig. 6). The concentration of exogenous NO_3^- required for maximal NRA varies widely between organs and species (35). For roots, saturating concentrations may vary between 10 μmol dm^{-3} in barley (33) and 100 mmol dm^{-3} in maize (41). In this respect, dwarf bean roots appear relatively efficient in their use of exogenous NO_3^-.

In phases A and B, root NRA was proportional to NO_3^- uptake. Increased NO_3^- uptake in phase D, however, is neither followed by, nor the consequence of, increased root NRA. Nitrate accumulation in roots was proportional to exogenous NO_3^- as a consequence of increased uptake and constant NRA. At least at higher concentrations, NO_3^- uptake and root NRA thus seem to be independently regulated.

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