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Endogenous NO_3^- in the Root as a Source of Substrate for Reduction in the Light[1]

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ABSTRACT

An experiment was conducted to investigate the reduction of endogenous NO_3^-, which had been taken up by plants in darkness, during the course of the subsequent light period. Vegetative, nonnodulated soybean plants (*Glycine max* [L]. Merrill, 'Ransom') were exposed to 1.0 millimolar $^{15}NO_3^-$ for 12 hours in darkness and then returned to a solution containing 1.0 millimolar $^{14}NO_3^-$ for the 12 hours 'chase' period in the light. Another set of plants was exposed to $^{15}NO_3^-$ during the light period to allow a direct comparison of contributions of substrate from the endogenous and exogenous sources. At the end of the $^{15}NO_3^-$ exposure in the dark, 70% of the absorbed $^{15}NO_3^-$ remained unreduced, and 83% of this unreduced NO_3^- was retained in roots. The pool of endogenous $^{15}NO_3^-$ in roots was depleted at a steady rate during the initial 9 hours of light and was utilized almost exclusively in the formation of insoluble reduced-N in leaves. Unlabeled endogenous NO_3^-, which had accumulated in the root prior to the previous dark period, also was depleted in the light. When exogenous $^{15}NO_3^-$ was supplied during the light period, the rate of assimilation progressively increased, reflecting an increased rate of uptake and decreased accumulation of NO_3^- in the root tissue. The dark-absorbed endogenous NO_3^- in the root was the primary source of substrate for whole-plant NO_3^- reduction in the first 6 hours of the light period, and exogenous NO_3^- was the primary source of substrate thereafter. It is concluded that retention of NO_3^- in roots in darkness and its release in the following light period is an important whole-plant regulatory mechanism which serves to coordinate delivery of substrate with the maximal potential for NO_3^- assimilation in photosynthetic tissues.

Available experimental evidence indicates that during the light phase of the diurnal cycle the rate of NO_3^- reduction in plant leaves is regulated to a large extent by the flux of NO_3^- from the xylem. When delivery of NO_3^- to leaves was restricted by decreasing transpiration, exposing roots to low temperature, or limiting the exogenous NO_3^- supply, a rapid decline in leaf nitrate reductase activity was observed (30–32). The decline occurred even though substantial amounts of NO_3^- were present in the leaf tissue, indicating that most of the NO_3^- was sequestered in 'storage' pools and relatively unavailable for reduction (30). It is evident, therefore, that provision of NO_3^- by the root system for transport in the xylem is an important determinant of the rate of NO_3^- reduction in illuminated leaves.

In attempting to evaluate processes in the root which influence NO_3^- flux to the xylem in the light, it is necessary to identify the sources from which NO_3^- originates. Nitrate being acquired from the external medium clearly would be important. A close relationship between concurrent absorption and xylem transport has been demonstrated (11, 15, 29). In addition, there is evidence that NO_3^- originating from endogenous pools in the root may contribute significantly to the flux in the xylem. A recent experiment with soybean revealed that a large portion of the NO_3^- taken-up from the external medium in the dark was retained in the root and then assimilated into reduced-N in leaves in the following light period (28). This nitrate accounted for about 45% of the total NO_3^- reduced in the plant during the light period.

The purpose of this experiment was to characterize, in greater detail, assimilation of dark-absorbed NO_3^- during the course of a subsequent light period using $^{15}NO_3^-$. Assimilation of NO_3^- being absorbed in the light also was examined to allow assessment of the relative contributions of substrate from endogenous and exogenous sources to leaf and whole-plant NO_3^- reduction.

MATERIALS AND METHODS

Plant Culture. Seeds of soybean (*Glycine max* [L.] Merrill, 'Ransom') were germinated in moistened paper in a dark germination chamber at 25°C and 98% RH. The germination paper and seeds were kept moist by capillary action from a 0.1 mM $CaSO_4$ solution. On day 4, 48 seedlings with radical lengths between 8 and 12 cm were selected and placed into two 115-L continuous flow, hydroponic culture systems. The culture systems were located in a controlled-environment growth room programmed for 28/22°C during the 12/12 h light/dark cycle. A photosynthetic photon flux density of 1000 ± 50 μE $m^{-2}s^{-1}$ (at canopy height) between wavelengths of 400 and 700 nm was provided during the 12-h light period from a combination of high pressure sodium and metal halide lamps. The environmental conditions used were sufficient to sustain net photosynthetic rates which exceed those of soybean plants grown in the greenhouse or field.

The culture solution temperature was 24 ± 1.0°C, and the solution pH was maintained at 5.8 ± 0.2 by additions of 0.2 N H_2SO_4. Nutrient concentrations in solution were 1.0 mM NO_3^-, 0.1 mM $H_2PO_4^-$, 1.1 mM K^+, 1.0 mM Ca^{2+}, 1.0 mM Mg^{2+}, 1.0 mM SO_4^{2-}, 17 μM B, 3 μM Mn, 0.3 μM Zn, 0.1 μM Cu, 0.04 μM Mo, and 18 μM Fe as ferric diethylenetriamine pentaacetate (Fe-DPTA, CIBA-Geigy Corp., Greensboro, NC).[2] The solutions

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were changed every 2 d to avoid depletion effects.

Experimental Conditions. Two separate groups of plants were exposed to $^{15}NO_3^-$, one during a dark period and the other during the following light period. Plants from both groups were sampled during that light period. The experiment began on d 17 after germination, when the second trifoliolate leaf had just reached full expansion. At the beginning of the dark period, 24 plants were removed from the culture solution in which they were growing and placed into a separate solution containing an identical nutrient composition except with 1.0 mM $^{15}NO_3^-$ (99 atom % ^{15}N) substituted for 1.0 mM $^{14}NO_3^-$. The plants remained in the $^{15}NO_3^-$ solution for the 12-h dark period (in the same growth room as before). At the end of the dark period, shoots of four plants were excised just below the cotyledonary node with a razor blade. Leaves and stems (including petioles) were separated and frozen promptly. The stem remaining with the root system was blotted just after excision, then the exuding sap was collected with capillary pipets for 20 min. At the end of that time, the exudate from the four plants was combined and frozen, and the roots were removed from the solution, rinsed in a cold 2.0 mM $CaSO_4$ solution, and immediately frozen. While the initial four plants were being sampled, the remaining 20 plants were removed from the $^{15}NO_3^-$ solution, roots were dipped into 2.0 mM $CaSO_4$ (24°C), and the plants were transferred into the culture solution containing $^{14}NO_3^-$. During the following 12 h, in light, four plants were sampled and exudate collected at 2 or 3 h intervals as designated in "Results."

At the end of the dark period, also on d 17, the second group of 24 plants, which had been growing with $^{14}NO_3^-$, was placed into an identical solution but with $^{15}NO_3^-$. The plants remained in the $^{15}NO_3^-$ solution throughout the subsequent 12 h sample period which was in the light. Plants were sampled coincident with those from the plant group which had been exposed to $^{15}NO_3^-$ in the dark. The sampling protocol was the same as stated previously.

No nodules were found on plant roots during the experiment; thus, plants acquired nitrogen solely as NO_3^- from the nutrient solution. Since the concentration of NO_3^- in solution was never depleted more than 5% (including the $^{15}NO_3^-$ exposure periods), plants were in steady-state with respect to solution NO_3^- availability throughout.

Exudate and Tissue Analysis. The xylem exudate samples were analyzed for NO_3^- and reduced nitrogen. Nitrate was determined using a manual modification of the method of Lowe and Hamilton (14). The atom % ^{15}N of the NO_3^- fraction was determined by mass spectrometry using a nitric oxide procedure (34). The analysis of reduced N involved an initial removal of NO_3^- (20), followed by Kjeldahl digestion (17) and colorimetric determination of NH_4^+ (2). The NH_4^+ in the remaining digest was recovered by diffusion and the atom % ^{15}N determined mass spectrometrically using a freeze-layer procedure (33).

The tissue samples were analyzed for NO_3^- and soluble and insoluble reduced nitrogen. After being freeze-dried, weighed and ground, the tissue was extracted with methanol:chloroform:water (13:4:3). Following separation of the chloroform from the methanol:water fraction, the chloroform was added back to the tissue residue, with this constituting the insoluble reduced N fraction. Total nitrogen and ^{15}N in the insoluble reduced N fraction were determined as described for exudate reduced N, omitting the NO_3^- removal procedure. The methanol:water fraction was analyzed for NO_3^- and soluble reduced N. After the methanol was evaporated, an aliquot was taken and NO_3^- and its ^{15}N enrichment were determined as in the exudate analysis. Nitrate in the remainder of the sample was removed and the soluble reduced N and atom % ^{15}N determined as in the analysis of exudate reduced N. All tissue ^{15}N data were calculated as atom % × N content and represent means of four replicates ± SE.

For reasons of clarity, data in tables and graphs are presented as μmol N plant^{-1}. The mean dry weights of leaves, stems, and roots of all plants sampled during the 12 h sampling period were 868 ± 22, 263 ± 6, and 398 ± 10 mg plant^{-1}, respectively. The mean area of the leaf canopies was 830 ± 12 cm^2 plant^{-1}.

RESULTS

Total uptake and assimilation of $^{15}NO_3^-$ by plants in the dark and light exposure periods are summarized in Table I. The summaries are presented to emphasize adjustments which occur in darkness. Two specific effects were apparent: (a) total uptake was 38% less in the dark (236 *versus* 380 μmol plant^{-1}), and (b) a smaller percentage of the NO_3^- taken up in darkness was reduced (30% *versus* 75%). As was noted in a previous experiment (28), most of the $^{15}NO_3^-$ present in the plant at the end of the dark period (83%) was located in the root system.

A large portion of the $^{15}NO_3^-$ remaining in the plant at the end of the dark period was reduced during the subsequent light period, with most of the ^{15}N being assimilated into the insoluble reduced N fraction (Fig. 1A). This was associated primarily with depletion of the root $^{15}NO_3^-$ pool (Fig. 1B) and the incorporation of ^{15}N into insoluble reduced N in leaves (Fig. 1D) and, to a lesser extent, in stems (Fig. 1C). Analysis of xylem sap collected at each sample interval (Table II) indicates that, during the period of rapid depletion of $^{15}NO_3^-$ from the root (0–6 h), about 82% of the ^{15}N in xylem exudate was $^{15}NO_3^-$ and 18% was reduced ^{15}N. Little of the endogenous $^{15}NO_3^-$ in the root was reduced and retained there, as the soluble and insoluble reduced ^{15}N fractions were relatively stable throughout the light 'chase' period (Fig. 1B).

Coincident with the assimilation of the dark absorbed endogenous NO_3^-, plants also were taking up and assimilating NO_3^- from the nutrient solution. Exogenously supplied $^{15}NO_3^-$ was taken up at an increasing rate during the 12h sampling period (Fig. 2A). The rate of uptake increased from about 24 to 29 μmol plant^{-1} h^{-1} during h 4 to 9, and then to 37 μmol plant^{-1} h^{-1} during h 9 to 12 (data calculated from Fig. 2A). An alteration also was apparent in the efficiency of $^{15}NO_3^-$ reduction in the plant tissue. During the initial 6 h of light, a significant portion of the absorbed $^{15}NO_3^-$ was accumulated in the root (Fig. 2B); but, thereafter, net accumulation in the root ceased coincident with (a) increased incorporation of ^{15}N into insoluble reduced N in the root (Fig. 2B), and (b) increased translocation of ^{15}N to the shoot, with the ^{15}N being assimilated predominantly into insoluble reduced N in leaves (Fig. 2D). The relative content of $^{15}NO_3^-$ and reduced ^{15}N in the xylem sap indicates that between 14 and 19% of the ^{15}N translocated to the leaves was reduced N (Table II).

The total amount of NO_3^- ($^{14}NO_3^- + {}^{15}NO_3^-$) in roots declined during the 12 h light period. The mean content in roots of plants from both treatment groups is shown in Figure 3. This decline was accompanied by a parallel decrease in root NO_3^- concentration from 601 to 294 μmol g^{-1} dry weight (data not shown). In addition, using data from the two $^{15}NO_3^-$ treatments, it was possible to estimate the amount of NO_3^- in the tissue at each sample time which had been taken up and retained in the root prior to the $^{15}NO_3^-$ exposure periods. This 'older' pool of endogenous NO_3^- was depleted similarly to the NO_3^- accumulated in the previous dark period, although the depletion rate was lower, 8.3 *versus* 12.2 μmol plant^{-1} h^{-1} (*cf.* Fig. 1B) during the first 9 h of light. The origin of the 'old' endogenous NO_3^- cannot be determined with certainty, but much of this NO_3^- pool may have been that remaining in the root at the end of the previous light period (*e.g. cf.* Fig. 2B). Due to the large variability associated with reduced-^{14}N fractions in the plant, it was not possible to quantify utilization of the older endogenous NO_3^- as was done using the ^{15}N label (Figs. 1 and 2).

Table I. *Accumulation of ^{15}N in Nitrogen Fractions of Different Tissues of Plants which Had Been Exposed to $^{15}NO_3^-$ Solutions for 12 h during the Dark or Light Phase of the Photoperiod*

Exposure Period	Tissue Analyzed	Nitrogen Fractions			
		NO_3^-	Soluble reduced	Insoluble reduced	Total
		μmol ^{15}N plant^{-1}			
Dark (12 h)	Leaves	6.9 ± 0.3	4.7 ± 0.3	25.3 ± 3.4	36.9 ± 3.3
	Stems	21.0 ± 1.3	5.9 ± 1.0	8.3 ± 1.1	35.2 ± 3.2
	Shoot total	27.9	10.6	33.6	72.1
	Roots	136.8 ± 13.4	4.7 ± 0.9	22.7 ± 2.3	164.2 ± 14.5
	Plant total	164.7	15.3	56.3	236.0
Light (12 h)	Leaves	16.7 ± 1.4	29.5 ± 3.8	161.6 ± 11.7	207.7 ± 14.5
	Stems	17.6 ± 1.9	16.0 ± 0.8	21.6 ± 0.8	55.2 ± 2.5
	Shoot total	34.3	45.5	183.2	262.9
	Roots	60.3 ± 2.9	12.2 ± 0.9	45.0 ± 2.1	117.5 ± 2.7
	Plant total	94.5	57.6	228.2	380.4

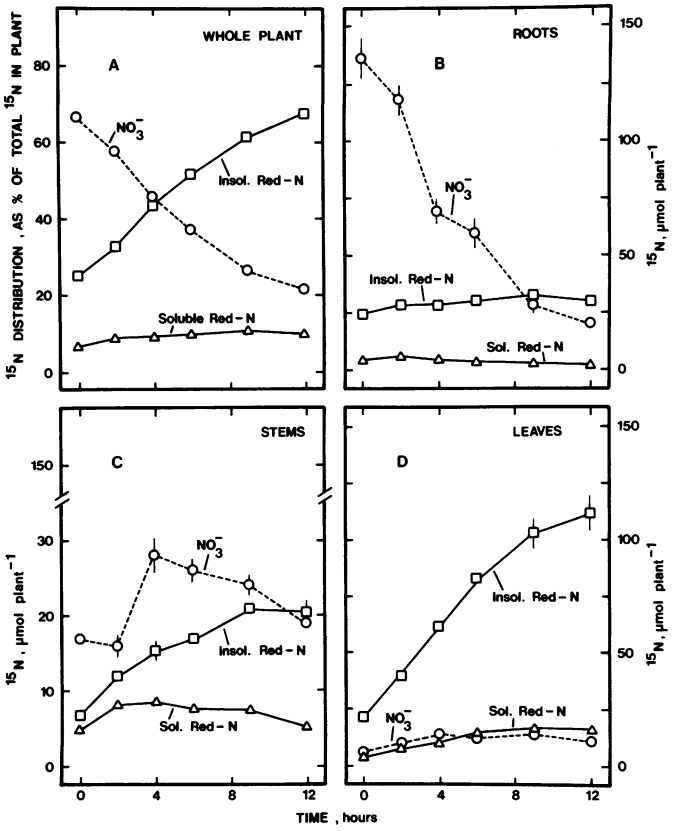

FIG. 1. Distribution of ^{15}N among nitrogen fractions in whole soybean plants and in each plant part following exposure to 1.0 mM $^{15}NO_3^-$ for 12 h in the dark. Plants were exposed to $^{14}NO_3^-$ during the 12 h sample period which was in the light.

Nitrate is reduced and assimilated into soluble reduced N (primarily amino acids) prior to the formation of insoluble reduced N (primarily proteins and nucleic acids). In this experiment, however, ^{15}N in the soluble reduced-N fraction in the two treatment groups always was low (Figs. 1 and 2), indicating that the ^{15}N was readily assimilated into insoluble macromolecules following $^{15}NO_3^-$ reduction. In evaluating the relative contribution of the two NO_3^- sources to insoluble reduced N formation in the whole plant, it is apparent that NO_3^- which had accumulated in the previous dark period was the primary source of N

Table II. *NO_3^- and Reduced N in Xylem Exudate of Plants Exposed to $^{15}NO_3^-$ Solutions during the Previous 12 h Dark Period or during the 12 h Sample Period in the Light*

Data are expressed as μmol plant^{-1} h^{-1}·atom % enrichment of the total N (^{14}N + ^{15}N) present in each fraction is given in parentheses.

Exposure Period	Time of Sampling in the Light	Exudate N Fractions		
		$^{15}NO_3^-$	Reduced-^{15}N	
	h	*μmol ^{15}N*	*μmol ^{15}N*	*% of total ^{15}N*
Dark (12 h)	0	9.9 (78)	2.7 (19)	21
	2	23.1 (49)	3.6 (15)	14
	4	17.1 (32)	3.6 (15)	17
	6	6.3 (19)	1.8 (9)	22
	9	2.7 (10)	0.9 (6)	25
	12	0.9 (5)	1.2 (9)	57
Light (12 h)	0	(0)	(0)	
	2	13.5 (31)	2.1 (11)	14
	4	27.0 (51)	4.5 (17)	14
	6	21.0 (66)	4.8 (24)	19
	9	17.7 (80)	3.9 (30)	18
	12	15.6 (86)	3.6 (25)	19

assimilated during the initial 6 h of the light period (Fig. 4A). The NO_3^- being taken up during the light period was the predominant source thereafter. When considered on a rate basis, total incorporation of N from the two sources increased during the first 6 h of light, reflecting an increasing rate of assimilation of the concurrently absorbed NO_3^- (Fig. 4B). Total incorporation tended to level-off during the last 6 h as the endogenous NO_3^- pool was depleted (*cf.* Fig. 1B), which offset the continuing increase in the assimilation of exogenous NO_3^-.

DISCUSSION

Utilization of Endogenous NO_3^-. The results from this experiment with soybean clearly indicate that NO_3^- taken up and retained by roots during the dark phase of the diurnal cycle can be an important source of substrate for reduction during most of the subsequent light period. The dark absorbed $^{15}NO_3^-$ was utilized almost exclusively in the formation of insoluble reduced-N in shoot tissues (Fig. 1). Contributions to insoluble reduced-N in leaves (the primary end-product) exceeded those from concurrently absorbed NO_3^- during the initial 6 h of light, and contributions from the two were nearly equal after 9 h (*cf.* Figs. 1D and 2D). This occurred even though NO_3^- uptake and the

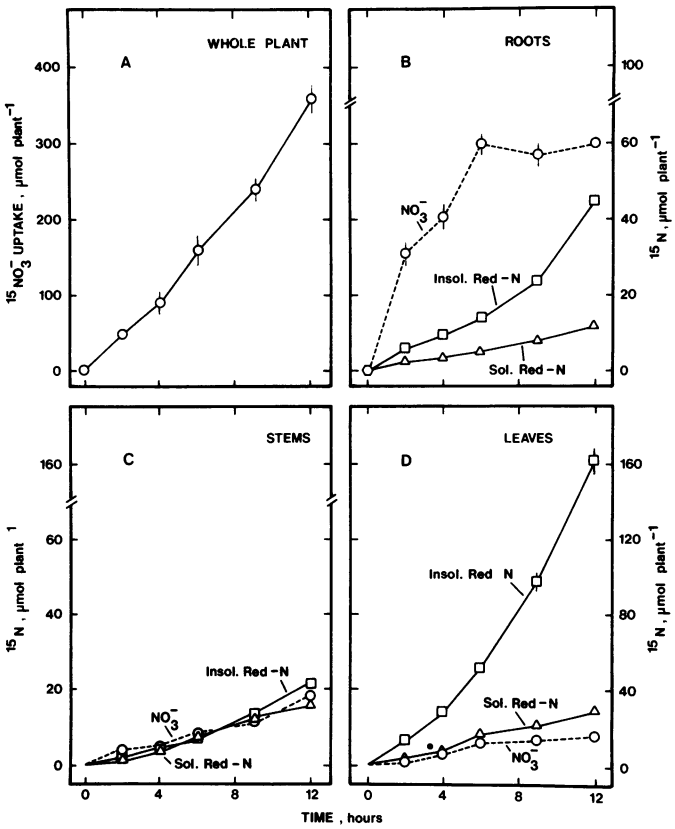

FIG. 2. Plant uptake of $^{15}NO_3^-$ and distribution of ^{15}N among nitrogen fractions in each plant part during exposure to 1.0 mM $^{15}NO_3^-$ for 12 h in the light.

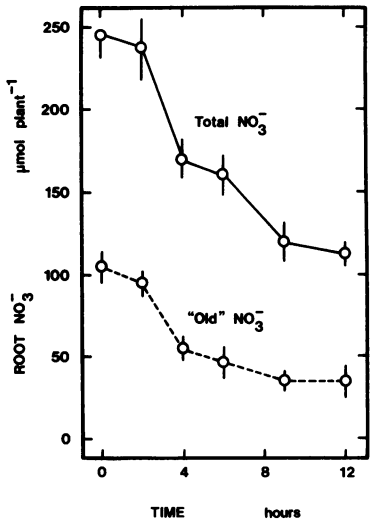

FIG. 3. Alterations in the total NO_3^- ($^{14}NO_3^-$ + $^{15}NO_3^-$) content of the root, and in the content of "old" endogenous NO_3^- accumulated prior to the dark and light $^{15}NO_3^-$ exposure periods. Data for "old" nitrate were calculated from total root NO_3^- ($^{14}NO_3^-$ + $^{15}NO_3^-$) minus dark absorbed $^{15}NO_3^-$ (Fig. 1B) and light absorbed $^{15}NO_3^-$ (Fig. 2B) in roots at each sample time.

associated loading of the endogenous 'pool' in the root were considerably depressed in the dark (Table I). In older, less rapidly growing plants, in which uptake rates were similar in dark and light, the relative contribution of NO_3^- substrate from the endogenous source was even greater (28).

The regulatory mechanism(s) responsible for the restriction of

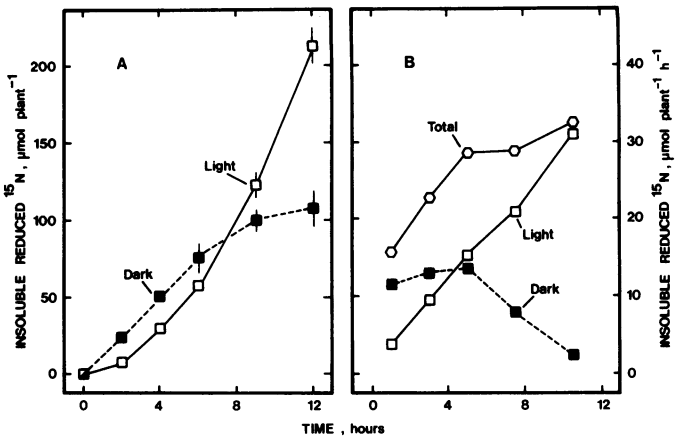

FIG. 4. Incorporation of ^{15}N into the insoluble reduced N fraction in whole soybean plants which were exposed to $^{15}NO_3^-$ during the previous dark period (■) or during the sample period in the light (□). Data are expressed as cumulative totals (A) and as rates (B).

NO_3^- translocation from the root in darkness is somewhat obscure. Similar restrictions have been noted in experiments with other species (19, 23, 24) and with other nutrient ions (7, 8, 35), suggesting nonspecific control. It can be reasonably assumed that stomatal adjustments and the associated changes in water flux through the root and the vascular system would be intimately involved in the regulation of NO_3^- translocation to the shoots. The rapid enhancement of translocation once plants were exposed to light, as indicated by rapid ^{15}N accumulation in the shoot, and the concurrent release of endogenous NO_3^- which had accumulated in the root prior to the previous dark period (Fig. 3), both lend support to a general change occurring in translocation efficiency in response to increased transpiration. In addition, there is evidence indicating that properties of the root directly governing water and nutrient fluxes also are altered in the dark (1, 4, 21, 22). Thus, a complex system of effects is implied, possibly being triggered by changes in turgor which would accompany adjustments in transpiration.

The marked diurnal fluctuation in the root NO_3^- content clearly suggests vacuolar involvement. More than 50% of the endogenous NO_3^- present in the root at the end of the dark period was depleted during the subsequent 12 h of light (Fig. 3), coinciding with a decline in total NO_3^- concentration (endogenous plus currently absorbed), and these changes may well have been paralleled by changes in the content of other inorganic ions. Furthermore, there is an apparent requirement that a relatively stable ionic concentration be maintained in the cytoplasm of root cells (5, 13). It therefore seems likely that retention of NO_3^- by the root in darkness and release in the light would reflect regulation of ion transport across tonoplast membranes as well as transport across xylem parenchyma plasmalemmae, which are directly involved in ion deposition into the vascular system (25). Whether or not these transport systems are responsive to changes in turgor remains to be established.

Xylem sap analyses suggest that about 18% of the endogenous NO_3^- in the root was reduced prior to translocation during the first half of the light period (Table II). This result would seem different from that observed with corn roots, where NO_3^- reduction in the root was closely associated with uptake from the external solution, but endogenous NO_3^- was relatively unavailable for reduction (16). The situation with soybean remains somewhat uncertain, however, as our methodology does not allow assessment of the extent to which (a) endogenous $^{15}NO_3^-$ was reduced after efflux and reabsorption (16) or (b) soluble reduced-^{15}N was cycled rapidly down from the shoot to the root and transferred into the xylem (3, 26).

Utilization of Exogenous NO_3^-. In contrast to the utilization of endogenous NO_3^- (Fig. 1), NO_3^- originating from the external medium was assimilated into insoluble reduced-N in both roots and leaves (Fig. 2). The close association between $^{15}NO_3^-$ uptake and ^{15}N incorporation into insoluble reduced N in roots has been noted previously (18).

Exogenous NO_3^- became the dominant source of substrate for whole-plant reduction as the light period progressed (Fig. 4B), reflecting an increased rate of assimilation and concurrent depletion of the endogenous NO_3^- pool. Increased assimilation was due, in part, to an increase in the rate of NO_3^- uptake (*cf.* Fig. 2A). Uptake of NO_3^- g^{-1} root dry weight also increased during the light period (data not shown), which indicates that a true enhancement of transport activity occurred independently of root growth. The increase in NO_3^- uptake coincided with a decline in NO_3^- concentration in the root tissue (Fig. 3), and thus could have resulted from alleviation of feedback inhibition (12, 15). Alternatively, uptake could have been stimulated by increased availability of energy in the root system (6, 10). Assimilation of ^{15}N into insoluble reduced-N in the root, which also is sensitive to the energy status of the root (10), increased coincident with the increase in uptake.

The progressive increase in assimilation of exogenous NO_3^- during the 12 h light period also was associated with cessation of NO_3^- accumulation in the root (Fig. 2B). During the initial 6 h, 40% of the absorbed $^{15}NO_3^-$ had accumulated in the root, but no net accumulation was observed after that time. Since the concentration of NO_3^- in the root was declining during this period, the capacity for NO_3^- storage was not saturated. The assimilatory pathway for NO_3^- in roots is composed of multiple components, which compete for available NO_3^- (9, 27). The absence of accumulation beyond 6 h thus would suggest that a new equilibrium was attained between transport and reduction functions. From previous arguments, the conclusion that NO_3^- reduction and translocation were increased by an increased supply of carbohydrate at the expense of vacuolar deposition seems tenable.

Compartmentation of Endogenous NO_3^-. This experiment demonstrates that the root can serve an important whole-plant compartmentation function during the dark phase of the diurnal cycle. Increased retention of NO_3^- in the root in darkness and its release during the following light period coordinates delivery of substrate in the xylem with the maximal potential for NO_3^- reduction and reduced-N assimilation into protein in photosynthetic tissues.

Our data suggest there are multiple endogenous pools of NO_3^- in the root, only some of which are involved in the diurnal compartmentation process. The rate of depletion of NO_3^- from the endogenous pools (*cf.* Figs. 1B and 3) diminished noticeably during the latter half of the light period, while considerable amounts of NO_3^- remained in the tissue. There is no obvious reason, physiologically, why release of NO_3^- should have diminished at this time, as stomatal resistance and water flow to the shoot would not have changed appreciably until the end of the light period. The NO_3^- remaining in the root presumably represents a longer-term storage pool. Furthermore, depletion of the dark absorbed NO_3^- occurred at a more rapid rate than depletion of older NO_3^-. The implication is that separate mobilizable endogenous pools exist, each with a differing mobilization efficiency.

As was mentioned previously, the magnitude of the diurnal fluctuation in NO_3^- content in the root suggests the involvement of vacuolar compartmentation. Nitrate would be sequestered in vacuoles in different cellular regions of the root, and it is likely that there is differential sensitivity to the diurnal regulatory stimuli. Tonoplasts at the outer cortex, for example, might be less responsive to turgor adjustments than tonoplasts at the inner cortex and stele. Alternatively, tonoplast transport activity in older, more basal regions may be less responsive than in younger regions nearer root tips. Regardless of the specific factors responsible for the mobilization responses observed here, it is clear that the NO_3^- content of the root and NO_3^- translocation into the xylem are quite dynamic, and at any specific time during a light period will reflect the collective effects of regulatory factors which influence availability of NO_3^- originating from endogenous as well as exogenous sources.

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