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Dependency of Nitrate Reduction on Soluble Carbohydrates in Primary Leaves of Barley under Aerobic Conditions[1]

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ABSTRACT

Nitrate reduction was studied as a function of carbohydrate concentration in detached primary leaves of barley (*Hordeum vulgare* L. cv Numar) seedlings under aerobic conditions in light and darkness. Seedlings were grown either in continuous light for 8 days or under a regimen of 16-hour light and 8-hour dark for 8 to 15 days. Leaves of 8-day-old seedlings grown in continuous light accumulated 4 times more carbohydrates than leaves of plants grown under a light and dark regimen. When detached leaves from these seedlings were supplied with NO_3^- in darkness, those with the higher levels of carbohydrates reduced a greater proportion of the NO_3^- that was taken up. In darkness, added glucose increased the percentage of NO_3^- reduced up to 2.6-fold depending on the endogenous carbohydrate status of the leaves. Both NO_3^- reduction and carbohydrate content of the leaves increased with age. Fructose and sucrose also increased NO_3^- reduction in darkness to the same extent as glucose. Krebs cycle intermediates, citrate and succinate, did not increase NO_3^- reduction, whereas malate slightly stimulated it in darkness.

In light, 73 to 90% of the NO_3^- taken up was reduced by the detached leaves; therefore, an exogenous supply of glucose had little additional effect on NO_3^- reduction. The results indicate that in darkness the rate of NO_3^- reduction in primary leaves of barley depends upon the availability of carbohydrates.

It is well established that under dark anaerobic conditions, soluble carbohydrates furnish the metabolic energy required for the reduction of NO_3^- in chlorophyllous tissue (18, 22). However, reports are contradictory regarding the role of carbohydrates in NO_3^- reduction, especially in darkness, under aerobic conditions. Some researchers report that light is required for the reduction of NO_3^- in green leaves under aerobic conditions (7, 9, 10, 23, 26, 27). Others observed NO_3^- reduction in green leaves under dark aerobic conditions (1, 3, 14, 17, 24, 33), suggesting that carbohydrates may supply the metabolic energy for the reduction of NO_3^- even under dark aerobic conditions. The apparent contradiction in the literature may be partly due to differences in carbohydrate status of the leaves used in various studies.

The purpose of the present study was to determine whether carbohydrates limit the NO_3^- reduction in primary leaves of barley.

MATERIALS AND METHODS

Plant Material. The primary leaves of barley (*Hordeum vulgare* L. cv Numar) were used. The seedlings were grown in plastic pots filled with vermiculite. The pots were subirrigated with a modified half-strength Hoagland solution that either lacked N (13) or contained 2 mM KNO_3. Micronutrients were supplied as reported earlier (12). The seedlings were grown at 70% RH in a controlled environment growth chamber, under continuous light (400 $\mu E\ m^{-2}\ s^{-1}$) at 25°C for 8 d or under a regimen of 16-h light at 25°C and 8-h darkness at 15°C for 8 to 15 d.

The leaves from the seedlings grown under a regimen of light and dark were harvested after 5 h of illumination. In some experiments, the seedlings grown under continuous light were placed in darkness for 24 h to deplete photosynthate (1).

Nitrate Uptake. The apical 10 cm of primary leaves were detached from the seedlings and 10 leaves/treatment were placed base down in small glass vials (20-ml total capacity) filled with 10 ml of the uptake solution. The basal 2 cm of the leaves were in the solutions, and the uptake of NO_3^- was facilitated via transpiration in light (400 $\mu E\ m^{-2}\ s^{-1}$) or darkness at 25°C. The uptake solutions contained 5 to 50 mM KNO_3 and various concentrations of glucose and other respiratory metabolites (see legends of each table and figure for details). Nitrate uptake was measured by following depletion of NO_3^- from the uptake solutions at 4-h intervals over a 24-h time course as previously described (3, 11). Rates of NO_3^- uptake were calculated from a linear regression of these curves. Nitrate was not lost from uptake solutions which did not contain leaves. Each experiment was reported at least twice and each treatment was replicated 3 times. All results are reported on the basis of the fresh weight of the

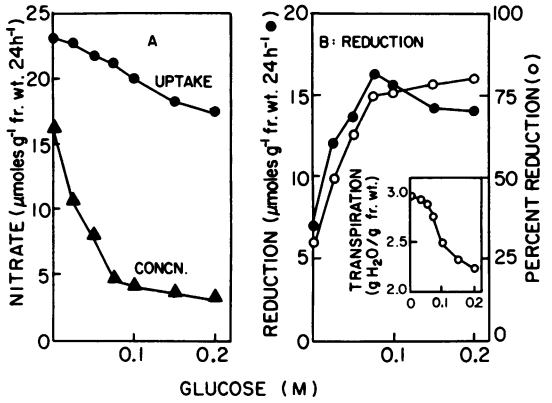

FIG. 1. Effect of exogenous glucose levels on NO_3^- uptake (A), tissue concentration (A), and reduction (B) in detached leaves in darkness. The seedlings were grown under a regimen of 16-h light and 8-h dark in N-free nutrient solution for 8 d. Detached leaves were placed in uptake solutions containing 10 mM KNO_3 and 0 to 0.2 M glucose. NO_3^- uptake and reduction were determined after a period of 24 h of darkness as described in "Materials and Methods." The inset in B shows the transpiration of water by the leaves at various levels of glucose.

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Table I. *Effect of Light (L), Dark (D), and Glucose on NO_3^- Assimilation Rates in Detached Leaves of Barley Seedlings Grown under Continuous Light*

The seedlings were grown in continuous light for 8 d (8-d L) or thereafter placed in darkness for 24 h (8-d L + 24-h D) in N-free nutrient solutions. Detached leaves were placed in uptake solutions containing 5 mM KNO_3 (control) or 10 mM KNO_3 and 0.1 M glucose. The rates of NO_3^- uptake and reduction were determined as described in "Materials and Methods." Correlation coefficients were significant at 0.05 (*) and 0.001 (**) probability.

Growth Conditions	Uptake Conditions	Control			Glucose			Increase of R/U by Glucose
		Uptake (U)	Reduction (R)	R/U	Uptake (U)	Reduction (R)	R/U	
		μmol/g·h			μmol/g·h			%
8-d L	L	1.66**	1.50**	0.90	1.70**	1.74**	1.02	13
8-d L	D	1.31**	0.68**	0.52	1.53**	1.34**	0.88	69
8-d L + 24-h D	L	1.94**	1.41**	0.73	2.12**	1.92**	0.91	24
8-d L + 24-h D	D	0.91**	0.21*	0.23	0.91**	0.55**	0.60	160

Table II. *Comparative Carbohydrate Concentration in Leaves of Seedlings Grown for 8 Days in Continuous Light (8-d L), Continuous Light + 24-Hour Darkness (8-d L + 24-h D), or under a Regimen of 16-Hour Light and 8-Hour Dark (8-d, 16-h L, 8-h D)*

The apical 10 cm of leaves were excised and analyzed for sugars as described in "Materials and Methods." The values are means of three replicate samples.

Growth Conditions	Sugar Content[a]		
	Reducing	Nonreducing	Total Soluble
	mg glucose eq/g		
8-d L	10.2 ± 0.7	11.5 ± 0.8	21.7 ± 1.9
8-d L + 24-h D	2.8 ± 0.4	1.7 ± 0.3	4.5 ± 0.6
8-d, 16-h L, 8-h D	3.9 ± 0.4	1.5 ± 0.2	5.4 ± 0.5

[a] Means ± SD.

leaves.

***In Vivo* Reduction.** The leaves were removed from the uptake solutions at the same intervals that uptake was determined. Any NO_3^- on the leaf surface was transferred quantitatively into the respective vials with deionized H_2O. The leaves were blotted dry, weighed, and ground with a cold mortar and pestle in 10 volumes of cold, deionized H_2O. The extracts were centrifuged at 30,000*g* for 15 min and the NO_3^- and NO_2^- concentrations in the supernatant solutions were determined. Reduction of NO_3^- was determined by subtracting the total amount of NO_3^- accumulated in the tissue from the total taken up at each assay period (3, 11). Reduction rates were calculated from a linear regression of the 24-h time course curves after linearity was attained.

The NO_2^- concentration in the leaves was negligible. Using $^{15}NO_2^-$ as substrates, it was shown previously that detached barley leaves reduced NO_2^- quantitatively to the level of amino N (2). Use of $^{13}NO_3^-$ in short-term experiments also verified the above result (J. R. Thayer and R. C. Huffaker, unpublished results).

Nitrate and Nitrite Analysis. Nitrate was determined spectrophotometrically at 210 nm following separation by HPLC on a Whatman Partisil-10-SAX anion exchange column (31). Nitrite was determined colorimetrically by adding a 1:1 mixture of color reagents (25).

Nitrate Reductase Assay. Leaves (about 1.0 g) were ground with sand in 5 volumes of the extraction medium, which contained 100 mM K-phosphate (pH 7.5), 1 mM EDTA, 1 mM cysteine, and 3% (w/v) casein. The extracts were centrifuged at 30,000*g* for 10 min, and the supernatants were assayed according to the method described by Scholl *et al.* (28) for corn leaves, using phenazine methosulfate as a postassay treatment for the oxidation of excess NADH. Nitrite from the assay mixtures was determined colorimetrically.

Sugar Analysis. Soluble sugars were extracted by grinding 1 g of leaves (apical 10 cm) in a pestle and mortar with 80% (v/v) ethanol. The extract was filtered through Whatman filter paper No. 1. The filtrate was heated to evaporate the ethanol, filtered through Whatman filter paper No. 1, and made to a 100-ml volume. Reducing sugars and the total soluble sugars after acid hydrolysis (30) were assayed using the Somogyi method (21).

Transpiration Measurements. The amount of water transpired by the leaves was measured gravimetrically and is expressed on the basis of fresh weights.

RESULTS

Effect of Glucose Concentration. Nitrate reduction increased with increasing external glucose concentration up to 0.1 M and then leveled off (Fig. 1B); hence, 0.1 M glucose was used in other treatments as indicated. The accumulation of NO_3^- in the leaves

Table III. *Effect of Light (L), Dark (D), and Glucose on NO_3^- Assimilation Rates in Detached Leaves of Barley Seedlings Grown under 16-Hour Light and 8-Hour Dark Regimen for 8 Days*

The seedlings were grown in N-free or NO_3^- (2 mM) containing nutrient solutions. Other experimental details are the same as in Table I except that all uptake solutions contained 10 mM KNO_3. Correlation coefficients were significant at 0.1 (*), 0.05 (**), and 0.001 (***) probability.

Growth NO_3^-	Uptake Conditions	Control			Glucose			Increase of R/U by Glucose
		Uptake (U)	Reduction (R)	R/U	Uptake (U)	Reduction (R)	R/U	
mM		μmol/g·h			μmol/g·h			%
0	L	2.20***	2.00***	0.91	2.25***	2.73**	0.99	8
0	D	1.00***	0.23*	0.23	0.89***	0.66***	0.74	222
2.0	L	2.50***	2.24***	0.90	2.79***	2.56***	0.92	2
2.0	D	0.81***	0.20*	0.24	0.76***	0.65***	0.86	258

Table IV. *Nitrate Reductase Activities (In Vitro) in Detached Leaves of Seedlings Grown with and without 2 mM KNO_3 under 16-Hour Light and 8-Hour Dark*

For experimental details, see Table III.

Darkness Time	NRA[a]			
	Noninduced		Induced	
	Control	+Glucose	Control	+Glucose
h	*μmol NO_2^-/g·h*			
0	0	0	4.0 ± 0.3	4.0 ± 0.3
6	1.8 ± 0.4	2.0 ± 0.4	6.2 ± 0.2	6.6 ± 0.2
12	2.6 ± 0.3	3.0 ± 0.3	8.3 ± 0.4	9.3 ± 0.3
24	4.2 ± 0.4	5.9 ± 0.5	10.4 ± 0.5	12.0 ± 0.6

[a] Means ± SD.

decreased sharply with the increase of glucose to about 0.1 M, reflecting the increased NO_3^- reduction.

Nitrate uptake in darkness by detached leaves from plants grown in a light-and-dark regimen decreased slowly as glucose in the uptake solutions increased (Fig. 1A). In detached leaves, the uptake of solutes occurs via the transpiration stream; hence, the small decrease in NO_3^- uptake with increasing glucose was due to a decrease in transpiration rate of the leaves (inset, Fig. 1B). Glucose at 0.1 M decreased transpiration about 50% in leaves from plants grown in continuous light (data not shown). Since induction of NR[2] (29) and the rate of NO_3^- reduction (11) depend on NO_3^-, the concentration of NO_3^- in the uptake solutions containing glucose was doubled where specified to decrease the effect of NO_3^- flux as a variable. To further remove uptake of NO_3^- as a variable in the presence of glucose, the results are also compared as a ratio of reduction to uptake.

Nitrate Assimilation in Light and Dark. The leaves detached from plants grown in continuous light took up NO_3^- at similar rates in light from uptake solutions; however, the rate of NO_3^- reduction was 16% greater in the presence of added glucose (Table I). The percentage of NO_3^- reduced was high (90%) and increased only 13% in the presence of glucose. All of the NO_3^- taken up was reduced in the presence of glucose. When the experiment was conducted in darkness, 52% of the NO_3^- taken up was reduced. When glucose was supplied, 88% of available NO_3^- was reduced. There was a 69% increase in reduction in the presence of added glucose (Table I).

The leaves detached from plants grown in continuous light and then placed in darkness for 24 h had high rates of NO_3^- reduction in light, and glucose increased the NO_3^- assimilation by 24% (Table I). When the uptake was conducted in darkness, the NO_3^- reduction rate was low; however, glucose greatly increased the rate of reduction and caused a 260% stimulation in the percentage of NO_3^- assimilated. The concentration of carbohydrates in leaves of these plants was about 5-fold lower than in those grown in continuous light (Table II).

Leaves detached from plants grown under a regimen of 16-h light and 8-h dark also had high rates of NO_3^- reduction in subsequent light and glucose increased the percentage of NO_3^- reduced by only 8% (Table III). In darkness, the rate of NO_3^- reduction was very low, and the percentage of NO_3^- assimilated in the presence of glucose increased 222%. The low rate of reduction without glucose was not due to loss of NR since its activity (determined *in vitro*) did not decrease between 12 and 24 h (Table IV). The carbohydrate concentration in leaves of these plants was about 4-fold lower than in leaves of plants grown in constant light (Table II).

In the above studies, seedlings were grown in NO_3^--free solutions and were initially lacking NR. The amount of NR induced was always more than that required to account for the rate of *in vivo* reduction (Table IV). Leaves detached from plants grown in the presence of NO_3^- (to induce NR before initiation of the experiments) under a light-and-dark regimen had high rates of reduction in light (Table III). Added glucose had no effect on the percentage of NO_3^- reduced. In darkness, low rates of reduction and low percentage reduction resulted. Again, the low reduction rate was not due to loss of NR, since its activity (*in vitro*) was still increasing between 6 and 24 h (Table IV). The reduction rate was markedly increased in the presence of glucose resulting in a 258% increase in the percentage of NO_3^- assimilated (Table III). When the leaves were fed cycloheximide along with NO_3^-, significantly more NO_3^- was reduced as compared to the control (Table V). The increased reduction occurred even though cycloheximide stopped the further induction of NR. Although WO_4^{2-} prevented a further increase in NR activity, it had no effect on NO_3^- reduction.

The ability of leaves of seedlings grown under a light-and-dark regimen to reduce NO_3^- in darkness increased considerably with increasing light duration (Table VI) as well as with leaf age (Table VII). The ability to reduce NO_3^- was related to the carbohydrate status. The accumulation of soluble sugars in leaves of 8-day-old seedlings grown under a light-and-dark regimen also increased with light duration (Table VI). As these seedlings aged, the concentration of total soluble sugars in the primary leaves increased dramatically (Table VII) and approached the same level as that in leaves of 8-day-old seedlings grown in continuous light (Table II).

Effect of NO_3^- Supply. Increasing the availability of NO_3^- to the leaves increased NO_3^- reduction (Fig. 2); the increase was

[2] Abbreviations: NR, nitrate reductase; NRA, nitrate reductase activity.

Table V. *Effect of Cycloheximide (CHI) and WO_4^{2-} on Nitrate Reduction in Darkness in Detached Leaves of Barley Seedlings*

The seedlings were grown under a regimen of 16-h light and 8-h dark in nutrient solutions containing 2 mM KNO_3 for 8 d. Detached leaves were placed in darkness in the uptake solutions containing 20 or 15 mM (CHI treatment only) KNO_3 with or without 70 μM CHI or 2 mM Na_2WO_4. NO_3^- uptake, reduction and NRA were determined after a period of 24 h of darkness as described in "Materials and Methods."

Treatments	NRA		NO_3^-[a]			
	Initial	Final	Uptake	Concentration		Reduction
				Initial	Final	
	μmol NO_2^-/g·h		*μmol/g·24 h*			
None (control)	4.5	10.2	43.3 ± 3.3	5.2 ± 1.0	40.7 ± 2.5	7.8
CHI (70 μM)	4.5	4.2	45.9 ± 2.4	5.2 ± 1.0	32.4 ± 2.5	18.7
WO_4^{2-} (2 mM)	4.5	3.1	39.3 ± 1.6	5.2 ± 1.0	35.3 ± 0.7	9.2

[a] Means ± SD.

Table VI. *Effect of a Light Pretreatment on Subsequent NO_3^- Reduction in Darkness in Detached Leaves*

Seedlings were grown under a regimen of 16-h light and 8-h darkness in N-free nutrient solutions. On the 8th day after 5 and 15 h of illumination, the leaves were detached and placed in darkness in uptake solutions containing 20 mM KNO_3 and 0 or 0.1 M glucose. NO_3^- uptake and reduction were determined after 24 h of darkness as described in "Materials and Methods."

Light Pretreatment	Soluble Carbohydrate	NO_3^-[a]			Reduction/ Uptake
		Uptake	Concn.	Reduction	
h	*mg glucose eq/q*	*μmol/g·24 h*			
−Glucose					
5	4.8	38.0 ± 3.7	29.4 ± 2.4	8.6	0.23
15	10.6	43.0 ± 2.7	24.2 ± 1.9	18.8	0.44
+Glucose					
5	4.8	32.5 ± 1.8	11.7 ± 1.5	20.8	0.64
15	10.6	35.8 ± 0.4	8.5 ± 0.9	27.3	0.76

[a] Means ± SD.

Table VII. *Effect of Seedling Age on the Accumulation of Carbohydrates and NO_3^- Reduction in Leaves in Darkness*

The seedlings were grown under a regimen of 16-h light and 8-h dark in N-free nutrient solutions for 8 to 15 d. On the 8th, 11th, 13th, and 15th d after planting, the primary leaves were detached and placed in 10 ml of uptake solutions containing 20 mM KNO_3. NO_3^- uptake and reduction were determined following a period of 24 h of darkness as described in "Materials and Methods."

Seedling Age	Soluble Carbohydrate Level	NO_3^-[a]		Reduction/ Uptake
		Uptake	Reduction	
d	*mg glucose eq/g*	*μmol/g·24 h*		%
8	4.8	35.4 ± 1.9	5.5 ± 1.1	16
11	7.5	35.8 ± 0.4	11.3 ± 1.4	32
13	12.8	35.1 ± 1.3	17.8 ± 0.3	51
15	16.9	33.1 ± 2.0	18.5 ± 0.5	56

[a] Means ± SD.

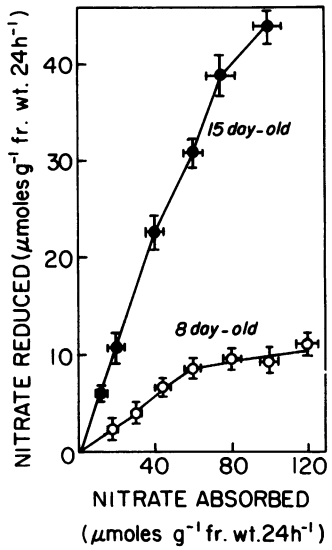

FIG. 2. Effect of NO_3^- supply on NO_3^- reduction in darkness in detached leaves of 8- and 15-d-old seedlings. Seedlings were grown under a regimen of 16-h light and 8-h dark in N-free nutrient solutions for 8 and 15 d. Detached leaves were placed in uptake solutions containing 5 to 50 mM KNO_3. NO_3^- uptake and reduction were determined after a period of 24-h darkness as described in "Materials and Methods." The bars represent SD.

much steeper in leaves of 15-d-old seedlings than in leaves of 8-d-old seedlings. In leaves of 8-d-old seedlings, NO_3^- reduction plateaued at a NO_3^- supply of about 60 μmol/g and only 9 μmol of NO_3^-/g were reduced over a period of 24 h. In leaves of 15-d-old seedlings, NO_3^- reduction did not reach a plateau even at 90 μmol of NO_3^-/g, and the total amount of NO_3^- reduced was 5 times as much as that in leaves of 8-d-old seedlings. The increased reduction in the older leaves was also related to their carbohydrate status (Table VII).

Effect of Sucrose, Fructose, and Intermediary Metabolites. The effectiveness of citrate, succinate, malate, sucrose, and fructose in stimulating NO_3^- reduction was compared with that of glucose (Table VIII). Sucrose, at half the concentration, and fructose, at an equimolar concentration, were as effective in stimulating NO_3^- reduction as glucose, whereas malate was only mildly effective. Citrate and succinate did not stimulate NO_3^- reduction.

DISCUSSION

In darkness, the rate of reduction and the proportion of the absorbed NO_3^- that was reduced (Tables I, III, VI, and VII) depended on the carbohydrate status of the leaves (Tables II, VI, and VII). The carbohydrate status, in turn, was regulated by the length of the light or dark treatment during growth (Tables II and VI) and by the age of the leaves (Table VII). Leaves grown in continuous light for 8 d reduced NO_3^- at a rate equal to 52% of that taken up over a subsequent dark period (Table I). Leaves of similar age grown under a light-and-dark regimen reduced NO_3^- in darkness at a rate equal to only 23% of the NO_3^- taken up (Table III). The carbohydrate concentration in leaves from plants grown in continuous light was 4-fold greater than in leaves from the light-and-dark regimen (Table II), allowing the former leaves to reduce over two times more of the NO_3^- that was taken up in darkness than the latter (Tables I and III). In light, current photosynthesis furnished additional reducing power above that supplied by the basal dark reactions to reduce 73 to 90% of the NO_3^- taken up (Tables I and III). No NO_2^- was detected in the leaves under any experimental conditions, indicating further reduction of NO_2^- to the reduced N level (2, 3).

In darkness, exogenously supplied carbohydrates, glucose, fructose, and sucrose increased the reduction of NO_3^- in carbohydrate-deficient leaves to the same level as in high carbohydrate leaves previously grown in continuous light (Tables I, III, and VIII). Exogenous glucose had a minor effect on NO_3^- reduction in light (Tables I and III).

The NRA level in the leaves was depressed 2.5- to 3-fold by inhibition with cycloheximide or WO_4^{2-} without decreasing *in*

Table VIII. *Comparative Effect of Sugars and Intermediary Metabolites on NO_3^- Reduction in Detached Leaves in Darkness*

Seedlings were grown under a regimen of 16-h light and 8-h dark in N-free nutrient solution for 8 d. Detached leaves were placed in uptake solutions containing 10 mM KNO_3 and metabolites. NO_3^- uptake and reduction were determined after a period of 24 h of darkness as described in "Materials and Methods."

Treatment		NO_3^-[a]		
Metabolite	Concn.	Uptake	Reduction	Reduction/ Uptake
	mM	μmol/g·24 h		%
None		25.7 ± 0.6	3.9 ± 0.1	15
Glucose	50	24.3 ± 1.3	14.3 ± 1.4	59
	100	22.6 ± 1.9	15.5 ± 1.2	69
Fructose	100	22.4 ± 1.5	15.2 ± 0.5	68
Sucrose	25	25.4 ± 1.8	16.6 ± 0.7	65
	50	22.2 ± 0.6	16.0 ± 0.7	72
Succinate	100	18.5 ± 0.5	3.8 ± 0.9	21
Citrate	100	19.5 ± 0.3	3.3 ± 0.6	17
Malate	100	20.1 ± 0.8	6.8 ± 0.6	34

[a] Means ± SD.

vivo NO_3^- reduction (Table V). Although NRA was about 2.5-fold less in the cycloheximide treatment than in the control, over twice as much NO_3^- was reduced in the presence of cycloheximide. Extractable NRA was always greater than the *in vivo* rate of NO_3^- reduction under the conditions of our experiments (compare Tables III and IV). Preinduced or uninduced leaves reduced the same proportion of the NO_3^- taken up, indicating that induction of NR in darkness and under aerobic conditions was always ahead of its *in vivo* activity. These observations indicate that NR may have been in excess at the NO_3^- concentrations supplied and that the rate of NO_3^- reduction may have been regulated more by the carbohydrate status of the leaves.

Other investigators have concluded that NO_3^- reduction does not occur in darkness under aerobic conditions (7, 9, 10, 23, 26, 27). The apparent lack of NO_3^- reduction may have been due to a low carbohydrate status of the leaf tissue used, since the seedlings used were grown under a light-and-dark regimen. As the present studies show, young barley leaves grown under a light-and-dark regimen contained fewer carbohydrates (Tables II, VI, and VII) (12) and reduced less NO_3^- in subsequent darkness (Tables III, VI, and VII). Leaf age can also affect NO_3^- reduction: barley leaves increased in carbohydrate concentration with age and this was related to increased NO_3^- reduction (Table VII). Recently, Reed *et al.* (24) observed differential but significant rates of NO_3^- reduction in darkness in five plant species. The variation in rates among the species might be due to a variable carbohydrate status of their leaves, which in turn may be due to both growth conditions and leaf age.

Based on the assumption that NO_2^- is not reduced in darkness, some earlier studies (10, 26, 27) concluded that an absence of NO_2^- accumulation in leaves in darkness shows a lack of NO_3^- reduction. Recent evidence, however, shows that both NO_3^- (1–3, 8, 24) and NO_2^- (2, 3, 16, 24, 33) are reduced in darkness in leaves and in chloroplast particles supplied with appropriate carbon sources to supply electrons (19). It seems likely that NO_2^- accumulation did not occur in leaves in the earlier studies because the NO_2^- formed was further reduced to ammonium in darkness.

Under aerobic conditions, Krebs cycle intermediates succinate and citrate did not stimulate *in vivo* NO_3^- reduction in darkness, whereas malate stimulated NO_3^- reduction slightly (Table VIII). In contrast, all three intermediates stimulated NO_3^- reduction in wheat (27) and soybean (22) leaves under anaerobic conditions. Glucose is still more effective in stimulating NO_3^- reduction under anaerobic conditions than Krebs cycle intermediates (22). Since NO_3^- reduction occurs in the cytoplasm (10, 23), under aerobic conditions the reducing equivalents generated by the Krebs cycle intermediates may not be readily available for NO_3^- reduction but may preferentially be oxidized by the mitochondrial electron transport chain. However, under anaerobic conditions, the electron transport chain is inhibited and the NADH generated in the mitochondria is not oxidized. Electrons from the intramitochondrial NADH may be transported to the cytosol by mitochondrial membrane transport systems, *e.g.* malate aspartate shuttle (20), and thus become available for NO_3^- reduction. Under aerobic conditions, however, malate may supply reducing equivalents for NO_3^- reduction in the cytosol via the cytoplasmic malate dehydrogenase (10).

Some proponents of the light requirement for NO_3^- reduction (9, 10, 23, 26, 27) argue that under aerobic conditions, the competition between NO_3^- reduction and the mitochondrial electron transport chain is eliminated only in light, not in darkness. According to one group (26, 27), light eliminates the competition for reducing equivalents between mitochondria and NO_3^- reduction by inhibiting the mitochondrial electron transport chain through rapid consumption of ADP in the chloroplast. The decrease in the availability of ADP would limit mitochondrial electron transport. However, NO_3^- reduction in light continued even when consumption of ADP in the chloroplast was inhibited by uncoupling photophosphorylation (8, 23). Reed and Canvin (23) and Jones and Sheard (15) proposed that the export of reducing equivalents from the chloroplast to the cytoplasm in light saturates the oxidative demands of both NO_3^- reduction and mitochondria. The present results suggest that even in darkness under aerobic conditions, reducing equivalents generated by the metabolism of stored photosynthate or exogenously supplied carbohydrates may also satisfy oxidative demands of both NO_3^- reduction and the mitochondrial electron transport chain.

In addition to supplying reducing equivalents for NO_3^- reduction, carbohydrates may affect NO_3^- reduction by regulating the synthesis/induction of NR in darkness (4, 32) as well as the availability of NO_3^- in the metabolic pool (5, 6). The lesser reduction of NO_3^- in carbohydrate-deficient leaves may be due to simultaneous utilization of available energy for the induction/synthesis of NR, nitrite reductase, and other proteins. When protein synthesis was inhibited by cycloheximide, significantly more NO_3^- was reduced in darkness, even in the absence of glucose (Table V).

In summary, the results indicate that NO_3^- reduction in darkness in primary leaves of barley is regulated by the carbohydrate level of the leaves. As long as these leaves have a sufficient supply of photosynthate, NO_3^- can be reduced in darkness under aerobic conditions, and the presence of light *per se* is not obligatory. Light, however, greatly increased the proportion of NO_3^- reduced, especially in carbohydrate-deficient leaves.

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