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Influence of Light and Ambient Carbon Dioxide Concentration on Nitrate Assimilation by Intact Barley Seedlings[1]

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ABSTRACT

The influence of light, dark, and ambient CO_2 on nitrate assimilation in 8- to 9-day-old barley seedlings was studied. To develop the photosynthetic apparatus fully, the seedlings were grown in nitrogen-free Hoagland solution for 5 days in darkness followed by 3 days in continuous light.

The seedlings reduced nitrate and nitrite in both light and dark, although more slowly in darkness. The slower nitrate reduction in darkness was not due to decreased uptake, since the steady-state internal concentration of nitrate was doubled. The faster nitrate reduction in light was attributed to recent products of photosynthetic CO_2 fixation supplying reducing energy, possibly by shuttle reactions between chloroplasts and cytoplasm. In carbohydrate-deficient tissue, it appeared that recently fixed photosynthate could supply all of the energy required for nitrate reduction. When sufficient metabolites were present in the green tissue, light was not obligatory for the reduction of nitrate and nitrite.

It is well established that light energy is intimately involved in the assimilation of nitrate (1, 4–6, 24). The influence of light is not well understood, however, and reports are contradictory. An obligatory requirement of light for nitrate assimilation has been reported for several species (6, 18, 19), whereas others report nitrate reduction in darkness in tissue slices or detached leaves of many species under both anaerobic (3, 13, 17–19) and aerobic (12, 13) conditions. Sawhney *et al.* (18) recently proposed that reduction of nitrate in light only avoids the accumulation of toxic levels of nitrite in the dark. In contrast, Jones and Sheard (12) observed that both nitrate and nitrite are reduced in leaf slices in darkness. Some have proposed that under aerobic conditions, nitrite is either low or not found because O_2 inhibits nitrate reduction (3, 19), whereas others (12) have shown evidence that O_2 is required for nitrite reduction in dark.

Different effects of CO_2 on nitrate assimilation have also been reported. Some found that ambient CO_2 increased nitrate reduction (22), whereas others reported that decreased nitrate in corn leaves was due to decreased movement of nitrate from other plant parts into the leaves (16). Resolving those questions will involve following each of the processes involved in nitrate assimilation: uptake, tissue concentration, and reduction as a function of the different treatments.

We present evidence that nitrate and nitrite reduction occur in darkness in intact barley plants, although more slowly than in light. Also shown is that ambient CO_2 affected nitrate reduction almost exclusively, rather than uptake.

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MATERIALS AND METHODS

Preparation of Seedlings. Seeds of barley (*Hordeum vulgare* L. var. Numar) were soaked in 1% (v/v) Clorox solution for 15 min, rinsed with distilled H_2O, and germinated at room temperature in aerated-deionized distilled H_2O. After 24 h, the germinating seeds were again rinsed with distilled H_2O, spread on a layer of cheesecloth supported on a stainless steel screen suspended about 1 cm above the surface of 5 liters of aerated 0.2 mM $CaSO_4$ solution, and placed in the dark at room temperature. On the 6th day, the seedlings were transferred to aerated one-fourth-strength Hoagland solution (9) lacking nitrogen and placed in continous light of 500 $\mu E\ m^{-2}\ s^{-1}$ for 3 more days at 25 C and 70 to 75% RH to develop the photosynthetic apparatus (10). When carbohydrate-deficient seedlings were used, they were given 3 days of light treatment and then placed in darkness at 25 C and 70 to 75% RH for the desired period (24–48 h).

Nitrate and Nitrite Uptake. Uptake of nitrate and nitrite was measured as the amounts disappearing from the substrate solution with time. Ten seedlings per treatment (each treatment replicated twice and each experiment repeated two times) were placed in 140 ml of one-fourth-strength Hoagland solution containing 1.0 mM KNO_3 or 1.0 mM $NaNO_2$ and 5.0 mM $CaSO_4$. The initial pH of the solutions was 5.8. The solutions were renewed once after a 12-h absorption period. By this time, nitrate concentration from the uptake medium was reduced to about 0.5 mM. Previous studies in this laboratory have shown that uptake rates between 0.5 and 1.0 mM nitrate were constant (7). When excised roots were used, they were excised at the scutellar node and submerged in the uptake medium for the desired periods. Two g of excised roots per treatment were used. All solutions were aerated during uptake. The addition of 50 μg/ml chloramphenicol to uptake solutions had no effect on the results showing that bacterial contamination was not a problem.

To study the effect of CO_2 on nitrate uptake, the seedlings were placed in a 15-liter Plexiglas chamber. The light intensity inside the chamber at the top of the seedling canopy was 450 $\mu E\ m^{-2}\ s^{-1}$. Normal or CO_2-free air was passed through the chamber at 2 liters/min, giving about eight exchanges/h, with a positive pressure inside the chamber. The treatment solutions were aerated with the same gas. The air from the Plexiglas chamber was passed through a cold bath and recirculated back into the chamber at 6 liters/min. This maintained temperature and RH at about the same level as in the growth chamber.

For studies with excised leaves (see Figs. 6 and 7), the seedlings were grown in Vermiculite for 7 days in continuous light of 500 $\mu E\ m^{-2}\ s^{-1}$ and irrigated with nitrogen-free Hoagland solution. The tip 10 cm of 10 leaves were then excised and placed base down in small glass vials containing 10 ml of uptake solutions. In this way only the basal 2 cm of the leaves were dipping in the solutions and uptake was facilitated via transpiration.

After the desired uptake period, the seedlings were removed

from the solutions, separated into roots and shoots at the scutellar node, weighed, and frozen immediately. The tissue was then ground with mortar and pestle in 4 volumes of 0.1 M K-phosphate (pH 7.4) and centrifuged at 30,000*g* for 15 min. The supernatant was used for nitrate and nitrite analysis.

***In Vivo* Nitrate and Nitrite Reduction.** *In vivo* reduction of nitrate or nitrite was determined by subtracting the total amount of the ion in both roots and shoots from the total uptake of the ion at each harvest period (7).

Nitrate and Nitrite Analysis. Nitrate from both the uptake medium and the tissue extract was determined by enzymic reduction to nitrite with dissimilatory nitrate reductase obtained from *Klebsiella pneumoniae* membrane. The assay medium was essentially the same as used by Schrader *et al.* (20), in which reduced riboflavin-5-P was the source of reductant. The nitrite formed was determined by adding 1.5 ml of a solution containing 0.75 N HCl, 0.5% (w/v) sulfanilamide, and 0.01% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride. After 15 min, *A* was read at 540 nm. Comparison of standard curves of nitrite and of nitrate reduced to nitrite by the disassimilatory nitrate reductase showed that in 30 min all of the nitrate from the medium was reduced to nitrite. Nitrate was always determined from a standard curve newly prepared by enzymic reduction of nitrate to nitrite and calculated on the basis of the fresh weight of the whole seedling.

Transpiration Measurements. The amount of water transpired by the seedlings was measured gravimetrically and is expressed on the basis of fresh weight of the shoot.

RESULTS

Nitrate Assimilation in Light and Dark. After 8 h, nitrate uptake and reduction showed linear rates for an extended period in both light and dark (Fig. 1). Uptake was 20% faster in light, while reduction was 100% faster. After 24 h, about 80% of the nitrate absorbed by the seedlings in light was reduced, compared with 45% reduced in darkness. Nitrate accumulation was 2.3-fold greater in the whole plant in darkness (Fig. 1B), with roots and leaves showing, respectively, increases to 1.5- and 2.8-fold (Fig. 2, B and C). Seedling transpiration in darkness was about half that in light (Fig. 2A), showing little effect of transpiration on nitrate uptake.

Effect of CO_2-free Air. As expected, the absence of photosynthetic CO_2 fixation (Fig. 3) affected nitrate assimilation similarly

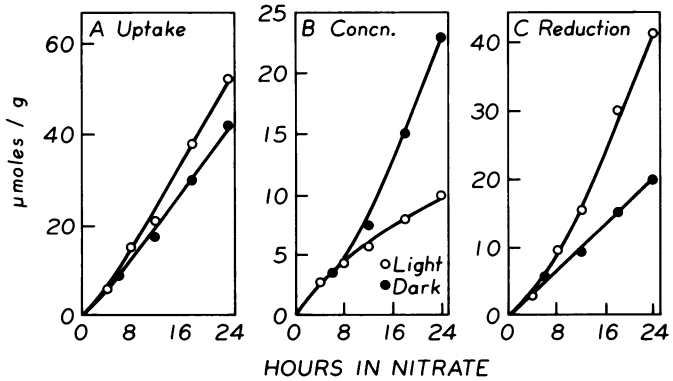

FIG. 1. Time course of nitrate uptake (A), accumulation (B), and *in vivo* reduction (C) in light (○) and darkness (●) in light-grown seedlings. Seedlings were grown 5 days in darkness and 3 days in light, then placed in an uptake medium containing 1.0 mM KNO_3 and 5 mM $CaSO_4$ in one-fourth-strength Hoagland solution. Uptake was determined as the nitrate disappearing from the solution and reported on a fresh weight basis. Solutions were changed every 12 h. *In vivo* reduction was determined by subtracting the total amount of nitrate in both roots and shoots from the total uptake of nitrate at each assay period. Rates were calculated from the slopes after linearity occurred.

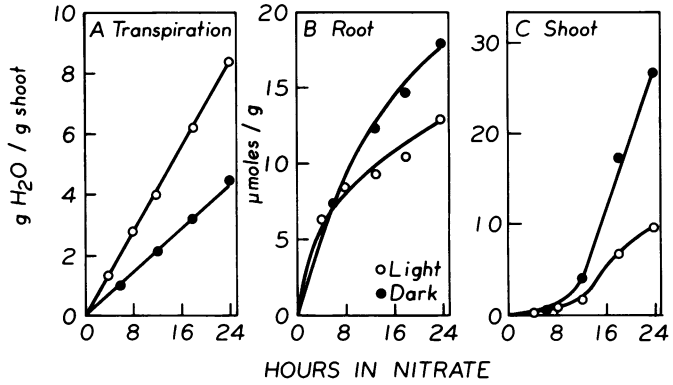

FIG. 2. Time course of transpiration (A) and of nitrate accumulation in roots (B), and shoots (C) in light (○) and darkness (●). For details, see Figure 1.

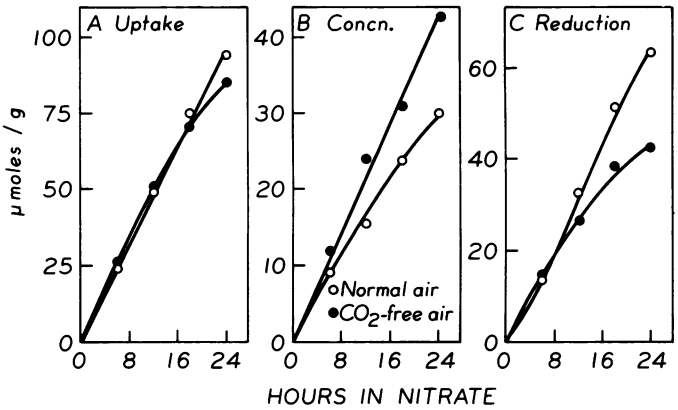

FIG. 3. Effect of CO_2-free (●) and normal (○) air on time course of nitrate uptake (A), accumulation (B), and reduction (C) in carbohydrate-sufficient seedlings in light. Seedlings were grown as in Figure 1. Ten seedlings per treatment were placed in the uptake medium, which contained 1 mM KNO_3 and 5 mM $CaSO_4$ in one-fourth-strength Hoagland solution. Seedlings were then transferred to a Plexiglas chamber through which CO_2-free or normal air was passed at 2 liters/min. Uptake solutions were aerated with the same air.

to darkness (Fig. 1). Nitrate uptake was about the same in both CO_2-free and normal air (Fig. 3). Nitrate reduction after 12 h was markedly less in CO_2-free air than in normal air. After 24 h, the total amount of nitrate reduced was 35% less in CO_2-free air than in normal air. Accordingly, 35% more nitrate accumulated in the seedlings in CO_2-free atmosphere than in normal air (Fig. 3B) and the excessive accumulation was, again, in the shoot (data not presented). Seedling transpiration was not changed at ambient CO_2 concentrations of zero and 300 µl/l (data not shown).

Carbohydrate Deficiency. Nitrate uptake and reduction in darkness or in CO_2-free air and light were apparently driven by reserve materials such as carbohydrates. For further exploration of the contribution of reserve materials, seedlings were made carbohydrate-deficient. Uptake and reduction of nitrate in carbohydrate-deficient seedlings during the first 12 h differed only slightly between light and darkness; thereafter, both continued to be greater in light (Fig. 4). In contrast, further reduction of nitrate essentially ceased in darkness while uptake of nitrate continued, although more slowly. Accumulation of nitrate in whole seedlings was about the same in both light and darkness.

To test further the dependency of nitrate reduction on photosynthetic CO_2 fixation, seedlings were made carbohydrate-deficient and then placed in light in either CO_2-free or normal air. After 6 h in CO_2-free air, nitrate uptake decreased about 40% while reduction nearly ceased (Fig. 5). Nitrate accumulation was the same under both conditions. In carbohydrate-deficient seed-

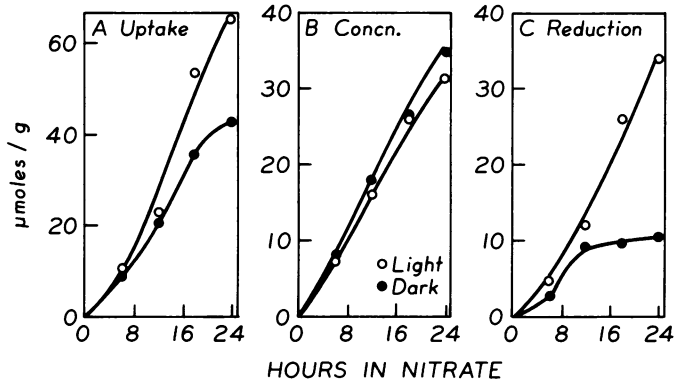

FIG. 4. Time course of nitrate uptake (A), accumulation (B), and reduction (C) in carbohydrate-depleted seedlings in light (○) and darkness (●). Seedlings were grown as in Figure 1. After 3 days in light, they were placed in darkness for 24 h to deplete stored carbohydrates. Other experimental conditions were the same as in Figure 1.

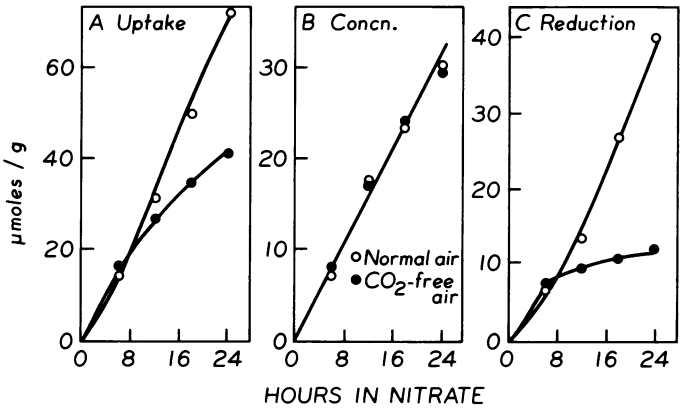

FIG. 5. Effects of normal (○) and CO_2-free (●) air on time course of nitrate uptake (A), accumulation (B), and reduction (C) in carbohydrate-deficient seedlings in light. Seedlings were grown as in Figure 1. After light treatment, seedlings were placed in darkness for 24 h to deplete carbohydrate. Other conditions were the same as in Figure 3.

lings, CO_2-free air in light affected nitrate reduction (Fig. 5C) about the same as did dark incubation (Fig. 4C).

The small amount of nitrate reduction in seedlings with carbohydrate-depleted leaves that occurred in darkness and in light minus CO_2 (Figs. 4 and 5) apparently took place in the roots. Detached carbohydrate-deficient leaves reduced very little nitrate (Fig. 6A), whereas roots (Fig. 6B) reduced nitrate at an amount sufficient to account for the nitrate reduced by the intact carbohydrate-deficient plants (Figs. 4C and 5C). Nitrate reduction in leaves and roots of the carbohydrate-deficient seedlings was respectively one-tenth and two-thirds that of normal leaves and roots.

Uptake and Reduction of Nitrite. In all of the studies, nitrite did not accumulate in the leaves in either light or darkness, indicating that nitrate was reduced to ammonia even in darkness. This suggests that green tissue has the ability to reduce nitrite in darkness. That is in agreement with the recent report by Jones and Sheard (12) that nitrite accumulating in leaf slices in darkness under anaerobic conditions disappeared when the environment was made aerobic. To test that in the barley system, uptake and reduction of nitrite by intact barley seedlings were studied in light and darkness. Although uptake of nitrite over a 24-h period was about 40% less in darkness than in light (27.8 µmol/g fresh weight in darkness, *versus* 49.1 µmol in light), there was no accumulation of nitrite in the roots or leaves of the seedlings under either condition, suggesting that all of the nitrite absorbed by the seedlings was reduced.

It may be argued that the only site of nitrite reduction in darkness is in the roots, so that no nitrite would accumulate in leaves. Figure 7 shows that leaves do reduce nitrite in darkness. As expected, both uptake and reduction of nitrite were doubled in light. Nitrite accumulated in the leaves in a slight amount during the early hours in both light and darkness but disappeared with time.

To determine the fate of nitrite in light and dark, $^{15}NO_2^-$-N was traced into reduced N using detached leaves exactly as in Figure 7. All of the $^{15}NO_2^-$-N absorbed over a 24-h period was recovered as reduced N (unpublished results), thus eliminating the possibility that nitrite was lost as oxides of N.

To determine whether nitrite might be lost nonenzymically during extraction, a known amount of nitrite was added to the extraction buffer along with the plant tissue. All of the nitrite was recovered, showing no loss in this part of the procedure.

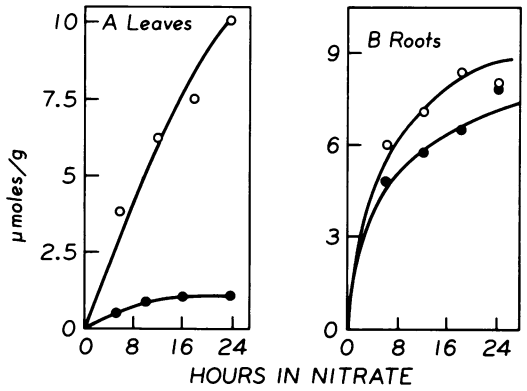

FIG. 6. Nitrate reduction in carbohydrate-rich (○) and carbohydrate-deficient (●) excised leaves (A) and roots (B) in darkness. For excised leaves, seedlings were grown for 7 days in continuous light or transferred to darkness after 6 days to obtain carbohydrate-deficient leaves. For roots, seedlings were grown as in Figure 1. After 3 days of light, one set of seedlings was placed in darkness for 48 h to deplete carbohydrates. Tip 10-cm-long leaves were placed base down in small glass vials containing 10 ml of 5 mM KNO_3 solution and allowed to absorb nitrate. Two g of roots were placed in 140 ml aerated uptake medium containing 1 mM KNO_3 (see Fig. 1). *In vivo* reduction of nitrate was determined by subtracting the total amount of nitrate in the tissue from the total uptake at each time interval.

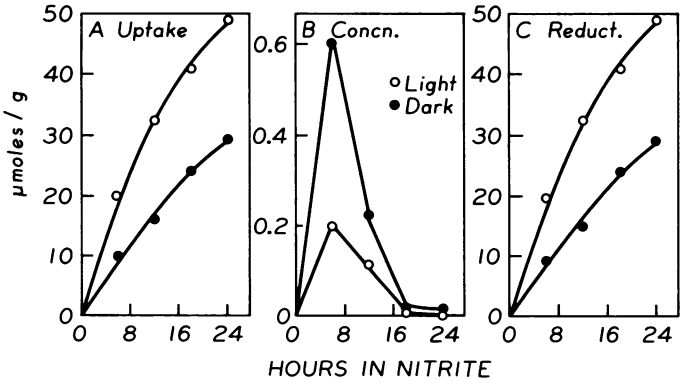

FIG. 7. Nitrite uptake (A), accumulation (B), and reduction (C) by excised leaves in light (○) and darkness (●). Seedlings were grown in Vermiculite at continuous light. After 7 days, tip 10-cm-long leaves were excised, placed base down in glass vials containing 10 ml 5 mM $NaNO_2$, and incubated in light or darkness. *In vivo* reduction of nitrite was determined by subtracting the total amount of nitrite in the tissue (if any) for the total uptake at each time interval.

DISCUSSION

Nitrate Reduction in Light and Dark. Although the role of light on nitrate assimilation has been studied extensively, controversy continues as to whether nitrate reduction normally occurs in darkness. Nitrate reduction has been reported in tissue slices in darkness under anaerobic conditions, detected as nitrite accumulation in the medium (13, 17–19). Under aerobic conditions in the dark, however, nitrite does not accumulate (this report, 3, 13, 17, 19). That observation has been ascribed to two contradictory processes: (*a*) that O_2 inhibited nitrate reduction (3, 19); or (*b*) that both nitrate and nitrite reduction occurred in the presence of O_2 (12, 13, 17). That problem can be resolved only by following each of the processes involved: nitrate uptake, accumulation, and reduction or at least the disappearance of substrate (nitrate), and the appearance and/or disappearance of the product (nitrite). Sawhney *et al.* (18, 19) looked for only the appearance of nitrite in their experiments under aerobic conditions, so it was not determined whether nitrate reduction was inhibited by O_2 or whether nitrite was further reduced. Either case would account for not finding nitrite in their assay. Canvin and Atkins (6) found no conversion of $^{15}NO_3^-$ into amino acids in darkness in barley leaves in short term experiments. They did not determine whether $^{15}NO_3^-$ was converted to any of the intermediate products (NO_2^- or NH_4^+) in darkness.

Our results clearly show that nitrate reduction in intact barley seedlings under aerobic conditions occurs at linear rates in both light and darkness (Fig. 1). In darkness, storage reserves apparently drive nitrate reduction, whereas in light, energy for nitrate reduction might be derived from both stored reserves and recently fixed photosynthate. Nitrate reduction in darkness occurred in both roots and leaves (Fig. 6).

Since uptake supplies the nitrate flux through the metabolic pool (2, 7, 21), decreases in uptake could be reflected in corresponding decreases in nitrate reduction. In the present studies, however, the slightly decreased uptake in darkness probably did not account for the decreased reduction rate, since the steady-state concentration of internal nitrate was doubled.

The slower nitrate reduction in darkness might be partially due to the activation of inhibitors in darkness, as recently proposed by Jolly and Tolbert (11). Our recent results (unpublished) show that when excised barley leaves in darkness are supplied with glucose, they reduce more than 90% of the absorbed nitrate. Hence, reserve substrate supply seems more important than inhibitors in barley leaves.

Nitrite Reduction in Light and Dark. During dark reduction of nitrate (Fig. 1) no nitrite was detected in either roots or leaves of the intact plants, indicating further reduction of nitrite. Nitrite reduction in detached leaves occurred at linear rates in both light and dark (Fig. 7). Since Stutte and Weiland (23) have shown that nitrite can be further converted to gaseous oxides of N, we utilized $^{15}NO_2^-$ to follow the reduction of nitrite into products in a detached barley leaf system done exactly as shown in Figure 7. All of the $^{15}NO_2^-$ absorbed in light or darkness over a 24-h time course was recovered in the reduced N products.

Jones and Sheard (12) reported that nitrite, accumulating in detached wheat leaves in darkness and anaerobic conditions, disappeared when the system was made aerobic while still in darkness. The above results contrast with those of Canvin and Atkins (6), who reported no reduction of nitrite in darkness in barley leaves.

It appears that O_2, instead of inhibiting nitrate reduction, may be a requirement for nitrite reduction in darkness. Fitting that hypothesis is the observation of Sawhney *et al.* (19) that CO treatments caused an accumulation of nitrite in detached wheat leaves under aerobic conditions in darkness. The reduction of nitrite in green tissue is considered to be closely linked to photosynthetic electron transport via reduced ferredoxin (14, 15). How nitrite is reduced in darkness in green leaves is a matter of speculation. Nevertheless, because of the demonstrated great similarities of nitrite reductase from green and nongreen tissue, and given the lack of ferredoxin in nongreen tissue, Beevers and Hageman (5) have questioned the role of ferredoxin in *in vivo* nitrite reduction in green tissue. Since respiration supplies the reducing power for both nitrate and nitrite reduction in nongreen tissue (8, 25), the same may be true for green tissue in darkness.

CO_2 on Nitrate Assimilation. Part of the faster reduction in light is likely a function of recent products of CO_2 fixation. In the intact barley plants, the effect of CO_2 was almost exclusively on nitrate reduction, not uptake (Fig. 3). A concomitant increase in tissue nitrate resulted, with less reduction in the absence of CO_2. The necessity for photosynthetic CO_2 fixation was shown in carbohydrate-deficient seedlings. Light did not increase nitrate reduction unless CO_2 was present (Fig. 5). It appears that recently fixed photosynthate could, if necessary, supply all of the energy required for nitrate reduction. In the presence of CO_2 and light, rates of nitrate reduction were similar for both carbohydrate-sufficient and -deficient plants (compare Figs. 1, 3–5). Recently fixed photosynthate could supply energy for nitrate reduction by chloroplast-cytoplasm shuttle systems. CO_2 and light increased nitrate reduction in corn also (22).

Neyra and Hageman (16) reported that the main effect of CO_2 on nitrate reduction in corn seedlings was not on reduction, but that CO_2 limited uptake or movement of nitrate from the roots or stalk into the leaves. Canvin and Atkins (6) found no effect of CO_2 on conversion of nitrate to amino acids in short term studies using barley leaves.

In summary, nitrate and nitrite assimilation occurs in both light and darkness in barley seedlings, the light rate being two times that in the dark. Both stored carbohydrate and recent products of photosynthetic CO_2 fixation can apparently supply the energy requirement for nitrate and nitrite reduction.

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