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Light and Dark Controls of Nitrate Reduction in Wheat (*Triticum aestivum* L.) Protoplasts[1]

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ABSTRACT

Protoplasts were isolated from the leaves of nitrate-cultured wheat (*Triticum aestivum* L. var. Frederick) seedlings. When incubated in the dark, protoplasts accumulated nitrite under anaerobic, but not under aerobic, conditions. The assimilation of [^{15}N]nitrite by protoplasts was strictly light-dependent, and no loss of nitrite from the assay medium was observed under dark aerobic conditions. Therefore, the absence of nitrite accumulation under dark aerobic conditions was the result of an O_2 inhibition of nitrate reduction and not a stimulation of nitrite reduction. In the presence of antimycin A, protoplasts accumulated nitrite under dark aerobic conditions. The oxygen inhibition of nitrate reduction was apparently due to a competition between nitrate reduction and dark respiration for cytoplasmic-reducing equivalents.

Oxygen control of nitrate reduction was not observed in the light, since protoplasts assimilated [^{15}N]nitrate to amino-N under light aerobic conditions. It has been proposed that the increase of the ATP-to-ADP ratio in the light inhibits dark respiration and allows nitrate reduction to occur under aerobic conditions. To test this hypothesis, protoplast N and C assimilation was assayed in the presence of nigericin, an uncoupler of photophosphorylation. The dark to light increase of the protoplast energy charge was not observed in the presence of nigericin, and CO_2 fixation was completely inhibited by the uncoupler. In contrast, rates of *in vivo* nitrate reduction (N_2 and air) and nitrite reduction were relatively unaffected by nigericin, and light-driven nitrate assimilation was inhibited by only 20%. Nigericin had no effect on the dark-to-light increase of protoplast NADH and NADPH levels. It is proposed that the light-induced increase of cytoplasmic-reducing equivalents suppresses the competition between nitrate reduction and dark respiration and allows nitrate reduction to occur under aerobic conditions. Dark-to-light changes of the ATP-to-ADP ratio apparently are not critical to the regulation of nitrate reduction.

The leaf *in vivo* NR[3] assay is routinely run under dark anaerobic conditions by following nitrite accumulation in the leaf tissue and/or assay medium (9). Aerobic conditions have been found to inhibit the accumulation of NO_2^- in the dark (2, 5, 18, 22, 25). Because NO_2^- assimilation by leaf tissue was not observed under dark aerobic conditions (4), the inhibitory effect of oxygen on the *in vivo* NR assay was concluded to be a direct effect on nitrate reduction (2, 4). O_2 inhibition of NO_2^- accumulation could be prevented by the addition of respiratory electron transport inhibitors to the assay medium (5, 18, 22, 25). This implied a competition between NO_3^- reduction and dark respiration for reducing equivalents, with the latter the more successful competitor under dark aerobic conditions (5). Although these studies support this interpretation, the effect of O_2 on NO_2^- accumulation in the dark is a controversial issue (3) and is reviewed in this study.

In the light, NO_3^- is assimilated to amino-N in the leaf under either anaerobic or aerobic conditions (4). This suggests that light negates the O_2 inhibition of NO_3^- reduction. In the presence of DCMU (an inhibitor of light-driven nitrite reduction, [2]), leaf tissue accumulated NO_2^- in the light under anaerobic, but not under aerobic, conditions (2, 27). Therefore, the effect of light on the O_2 regulation of NO_3^- reduction is mediated through a photosynthetic function. It has been proposed that the light-induced increase of the cellular ATP-to-ADP ratio is sufficient to inhibit the mitochondrial electron transport chain, thereby releasing reducing equivalents for NO_3^- reduction (24, 25). Alternatively, the increase of reducing equivalents exported from the chloroplast to the cytoplasm in the light may saturate the requirements for both NO_3^- reduction and dark respiration (15).

The purposes of this study were to clarify the regulatory role of O_2 on NO_2^- accumulation in the dark and to examine the effect of light on the O_2 control of NO_3^- reduction in terms of the two hypotheses outlined above. By assaying NO_3^- assimilation in the light with photophosphorylation uncoupled, we reasoned that the relative significance of the theories could be assessed. For this study, leaf protoplasts were preferred to a whole leaf assay system to minimize permeability restrictions to exogenous molecules and to have a chlorophyllous system relatively free of nonchlorophyllous tissue.

MATERIALS AND METHODS

Plant Material and Protoplast Preparation. Wheat (*Triticum aestivum* L. var. Frederick) was grown in vermiculite in plastic trays (24 × 16 × 8 cm) which were perforated on the bottoms to allow for subirrigation. For the first 4 d, the seedlings were subirrigated daily with H_2O and, subsequently, with a modified half-strength Hoagland solution (12) containing 10 mM KNO_3. The seedlings were grown in a growth chamber under a 24-h regime of 10-h light (450 μE $m^{-2}s^{-1}$), 14-h dark, at 25°C and 18°C, respectively.

The first leaf of 7-d-old seedlings was harvested 4 h after onset of the light period and washed in deionized H_2O. The leaves (10 g) were cut into 1-mm^2 sections under a 0.35 M sorbitol solution. The leaf sections were vacuum-infiltrated in 40 ml of medium A (medium A contained 0.5 M sorbitol, 25 mM Mes (pH 5.5), 5 mM $MgCl_2$, 10 mM K_2SO_4, 0.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5% (w/v) BSA, 2% [w/v] Cellulase [Cellulase 'Onozuka' R-10], 0.3% pectinase [Macerozyme R-10; Kinki Yacult Manufacturing Co, Nishinomiya, Japan], and 1% PVP) and incubated, without shaking, in a water bath illuminated from the bottom (400 μE $m^{-2}s^{-1}$ at the

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[3] Abbreviations: NR, nitrate reductase; NRA, nitrate reductase activity; CCCP, carbonyl-cyanide-*m*-chlorophenylhydrazone; OAA, oxaloacetate; PGA, 3-phosphoglyceric acid; DHAP, dihydroxyacetone phosphate.

surface of the leaf sections) at 28°C for 2.5 h. The enzyme medium was removed, and the leaf sections were gently washed, first in 100 ml medium B (medium B contained 0.5 M sorbitol, 50 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 5 mM K_2SO_4, 0.5 mM KH_2PO_4, and 1 mM $CaCl_2$) for 30 min and then in 60 ml medium B for 5 min to release the protoplasts. Both washings were done at 4°C. After each washing, the suspension was filtered through a 200-μm nylon mesh. The filtrates were pooled and centrifuged repeatedly (3×) at 100*g* for 5 min until the supernatant was clear. The protoplast pellet was resuspended in medium B after each centrifugation, and the final resuspension was normally in 30 ml. The protoplast preparation was maintained at 4°C at all times during the purification procedures and subsequent storage before assays.

Assays containing protoplasts at 250 μg Chl were carried out in 50-ml Erlenmeyer flasks, and assays containing 60 μg Chl were carried out in scintillation vials. All assays were run in a shaking water bath at 27°C. Light was provided by banks of 50-w reflector lamps (350 μE $m^{-2}s^{-1}$ at the surface of the flasks) beneath the water bath, and, for any dark reactions, the flasks were wrapped in aluminum foil. Each assay routinely contained, in a total volume of 2.22 ml medium B: protoplast suspension (250 μg or 60 μg Chl); 50 mM KNO_3; 10 mM $NaHCO_3$; and 1% (v/v) ethanol. All assays were run in duplicate and repeated at least twice.

NO_3^- Reduction *in Vivo*. Assays were conducted in the dark. The flasks were capped with serum stoppers which were linked in series by tubing and 18-gauge hypodermic needles to allow continuous gassing with N_2 or air (130 ml min^{-1}). At specific time intervals, assay flasks were removed, and a 1-ml aliquot of the reaction mix was added to 0.2 ml 1 M zinc acetate in a 12-ml conical tube which was kept on ice. The volume was made up to 2.2 ml with cold medium B, and, after vortexing and centrifugation, the supernatant was assayed for NO_2^- (9). The assay was linear over an h, and the rate of NO_3^- reduction routinely was calculated from the difference in NO_2^- accumulation between 15 and 45 min.

NO_2^- Reduction *in Vivo*. Assays were run in the light and the dark, under aerobic conditions. All assays contained 0.32 mM $NaNO_2$, 10 mM $NaHCO_3$, 1% ethanol, and protoplasts in 2.22 ml medium B. At 15, 30, and 45 min after the initiation of the assays, a 0.2-ml aliquot was added to 0.2 ml 1 M zinc acetate, and NO_2^- was determined as described previously. The rate of NO_2^- reduction was calculated from the slope of the line through these points.

NO_3^- and NO_2^- Assimilation.

Amino-N Accumulation. The assay components and assay conditions were the same as those described for *in vivo* NO_3^- and NO_2^- reduction. All assays were run under aerobic conditions. At 15, 30, and 45 min after initiation of the assays, a 0.1-ml aliquot of the reaction mix was added to 0.2 ml 10% (w/v) TCA in a 12-ml conical tube. The volume was made up to 1 ml with H_2O, and, after vortexing and centrifugation, the supernatant was assayed for NH_4^+ and free amino groups (28). The rates of amino-N accumulation were calculated from the slope of the line through the time points.

^{15}N Studies. As a result of technical peculiarities associated with the ^{15}N assay system, the following modifications were made in the preparation of the protoplasts. First, to minimize dilution of $^{15}NO_3^-$ by $^{14}NO_3^-$ in the protoplasts, the wheat seedlings were watered with 5 mM, rather than 10 mM, NO_3^-. NRA was lowered by less than 10% as a result of this change. Wheat seedlings were watered with 10 mM NO_3^- for $^{15}NO_2^-$ assimilation studies. Second, 25 mM Hepes buffer was used in the protoplast suspension medium (medium B) to lower inorganic salt contamination of the amino acid fraction used for ^{15}N analysis. All other assay conditions were as described previously (amino-N accumulation), except that $K^{15}NO_3$ (95% ^{15}N) and $Na\ ^{15}NO_2$ (95% ^{15}N) were used as the appropriate replacements. At specific times, reaction flasks were removed, and a 2-ml aliquot from each assay was added to 5 ml 95% (v/v) ethanol (acidified with HCl) and 50 mg celite in a 12-ml conical tube. The samples were vortexed and put in a water bath at 80°C for 5 min. After centrifugation of the mixture, sequential extractions of the pellet were made at 80°C with 5 ml 40% (v/v ethanol), 5 ml H_2O, and 5 ml 80% (v/v) ethanol, in that order. All extracts were combined, and further purifications of the NH_4^+ and amino acid fraction were as described by Canvin and Atkins (4). An additional purification of the amino acid sample with ethanol containing 10% (v/v) 1 N HCl was included to remove inorganic salts. Salt contamination of the amino acid sample was found to interfere with the combustion of the sample. Preparation of the ^{15}N samples and analysis of enrichment by optical emission spectroscopy was as described previously (4).

Photosynthetic $^{14}CO_2$ Fixation. All components of the protoplast assay mixture, except $NaHCO_3$, were preincubated in the light at 27°C for 25 min, and the assay was initiated by the addition of 100 μl 0.2 M $NaH^{14}CO_3$ (32 nCi/μmol). At 5 and 15 min after the addition of $NaH^{14}CO_3$, 50-μl aliquots were removed to scintillation vials containing 500 μl 2 N acetic acid. The samples were dried overnight at 50°C, and the fixed ^{14}C was determined by liquid scintillation counting. The rate of $^{14}CO_2$ fixation was calculated over the 10-min time interval. $^{14}CO_2$ fixation was found to be linear up to 25 min after the addition of $NaH^{14}CO_3$.

Determination of Nicotinamide Nucleotides. The extraction and assay of nicotinamide nucleotides were a modification of previous methods (6, 18). The protoplast assay mixture (containing 50 mM KNO_3, 10 mM $NaHCO_3$, and 1% v/v ethanol) was incubated under aerobic conditions in the light or dark, as described previously. After 30 min, the reactions were terminated by the addition of 0.1 ml 2.5 M $HClO_4$ (acid extraction) or 10 μl 10 N KOH (alkaline extraction). The acid-treated samples were kept on ice for 30 min, with periodic shaking, and centrifuged at 30,000*g* for 15 min. The supernatant was neutralized with 10 N KOH, and the $KClO_4$ precipitate was removed by further centrifugation. All extraction steps were conducted at 4°C. The final supernatant was frozen prior to analysis for NAD and NADP. The alkaline-treated samples were similarly kept on ice for 30 min before boiling for 90 s. The samples were returned to the ice bath, neutralized (pH 7.6 with 0.1 ml 1 M KH_2PO_4), and centrifuged at 30,000*g* for 15 min. The supernatant from each sample was assayed for NADH and NADPH.

Assays for NAD(H) and NADP(H) were as described by Mann *et al.* (18). In total assay volumes of 1 ml, 12.5 units of alcohol dehydrogenase and 1.5 units of glucose-6-P dehydrogenase were used, respectively, and both assays contained 1 μmol EDTA.

Determination of Adenine Nucleotides. The protoplast assay mixture was incubated in the light or dark for 30 min, and each reaction was terminated by the addition of 0.1 ml 2.5 M $HClO_4$. The extraction procedures for ATP, ADP, and AMP were the same as those described for NAD and NADP. ATP, ADP, and AMP were assayed enzymically, as described by Chaparro *et al.* (6), using 0.5 ml of the acid extract for each assay. The ATP assay contained 0.5 units hexokinase and 3.6 units glucose-6-P dehydrogenase in a total assay volume of 1 ml. The ADP and AMP assay contained 5 units pyruvate kinase, 5 units lactate dehydrogenase, and 3 units myokinase, similarly in a total assay volume of 1 ml. Calibration curves for the three adenine phosphates (standards were acid-treated) were linear from 0 to 20 nmol.

RESULTS AND DISCUSSION

In agreement with previous studies with leaf tissue (5, 18, 22), wheat protoplasts accumulated NO_2^- in the *in vivo* NR assay under dark anaerobic, but not under dark aerobic, conditions (Table I). NO_2^- accumulation under aerobic conditions was observed only when 40 μM antimycin A was included in the assay. Salicylhydroxamic acid was ineffective in restoring NO_2^- accumulation under aerobic assay conditions.

Table I. *Effects of Antimycin A and Salicylhydroxamic Acid on Protoplast in Vivo NRA, Assayed under Dark Aerobic and Anaerobic Conditions*

Each assay contained protoplasts at approximately 60 μg Chl.

Treatment	NO_2^- Formed		
	N_2 (A)	Air (B)	B/A × 100
	$\mu mol \cdot mg^{-1}\ Chl \cdot h^{-1}$		
Control (2% acetone)	1.04	0.05	5
Antimycin A, 4.10^{-5} M	1.06	0.66	62
Salicylhydroxamic acid, 10^{-3} M	1.04	0.06	6

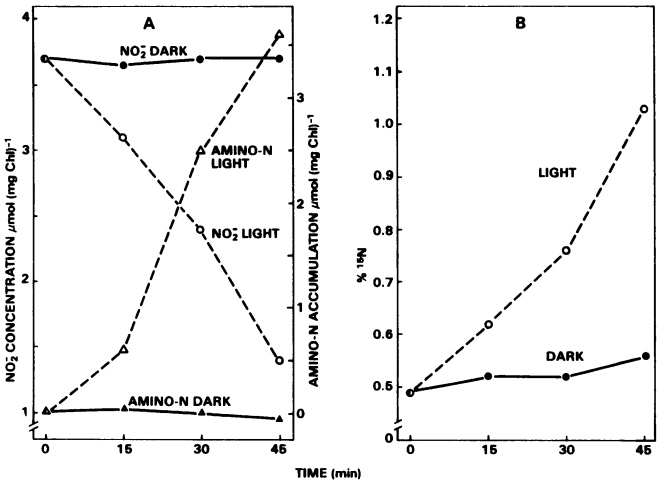

FIG. 1. NO_2^- assimilation by protoplasts incubated in the light and dark under aerobic conditions. A, $NaNO_2$ (0.32 mM) was added to the protoplast assay mixture, and assimilation was monitored simultaneously as NO_2^- disappearance and amino-N accumulation. The initial amino-N concentration in each assay was 18.8 μmol (mg Chl)$^{-1}$. B, $Na^{15}NO_2$ (0.32 mM) (95% ^{15}N) was added to the protoplast assay mixture, and the ^{15}N enrichment of the NH_4^+ and amino-N fraction was determined. All assays contained approximately 250 μg Chl.

To characterize the aerobic inhibition of NO_2^- accumulation, conditions for NO_2^- reduction and assimilation by protoplasts were investigated. When protoplasts were incubated with $NaNO_2$, there was a linear loss of NO_2^- from the assay mixture in the light but no NO_2^- disappearance in the dark (Fig. 1A). Similarly, over the same time period, there was a corresponding increase of amino-N in the assay mixture in the light but no increase in the dark. The absence of 1:1 stoichiometry between NO_2^- loss and amino-N accumulation was probably due to assimilation of endogenous NO_3^-, but this alone did not account for the total amino-N accumulation observed (Fig. 2A). When protoplasts were incubated with $^{15}NO_2^-$, there was a linear increase in the ^{15}N content of the NH_4^+ and amino-N fraction in the light but no incorporation of ^{15}N into the same reduced-N fraction in the dark (Fig. 1B). These results show that NO_2^- assimilation by leaf protoplasts is strictly light dependent, and no loss or assimilation of NO_2^- occurs under dark aerobic conditions. In addition, when protoplasts were allowed to accumulate NO_2^- in the *in vivo* NR assay for 30 min and then transferred to either light or dark aerobic conditions for a further 30 min, loss of NO_2^- was only observed in the light (A. J. Reed and D. T. Canvin, unpublished data). The absence of NO_2^- assimilation in the dark was apparently not due to permeability limitations of the protoplasts to NO_2^-.

Collectively, these data (Table I and Fig. 1) support the concept that O_2 prevents NO_2^- accumulation by leaf protoplasts in the dark *in vivo* NR assay by an inhibition of NO_3^- reduction and not by a stimulation of NO_2^- assimilation. Relief of the O_2 inhibition

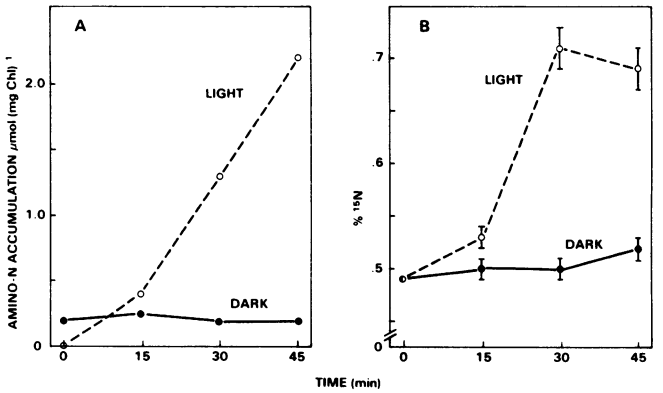

FIG. 2. NO_3^- assimilation by protoplasts incubated in the light and dark under aerobic conditions. A, Protoplasts were incubated with 50 mM KNO_3, and assimilation was assayed as amino-N accumulation. The amino-N concentrations at zero time were 18.1 and 18.3 μmol (mg Chl)$^{-1}$ for the light and dark assays, respectively. B, Protoplasts were incubated with 50 mM $K^{15}NO_3$ (95% ^{15}N), and the ^{15}N enrichment of the NH_4^+ and amino-N fraction was determined. All assays contained approximately 250 μg Chl. Bars around points indicate 1 SD.

by antimycin A suggested a competition between the CN^--sensitive mitochondrial electron transport chain and NR for the oxidation of reducing equivalents. The CN^--insensitive oxidative pathway was apparently not involved in this competitive system.

NO_3^- assimilation by protoplasts was measured in the light and dark under aerobic conditions by accumulation of NH_4^+ and amino-N and by incorporation of $^{15}NO_3^-$ into the reduced-N of the NH_4^+ and amino fraction. In the light, there was a linear increase of amino-N over the 45-min assay period, whereas, in the dark, there was no change in the amino-N content of the assay mixture over the same time period (Fig. 2A). Similarly, $^{15}NO_3^-$ assimilation proceeded in the light but not the dark (Fig. 2B). The absence of NO_3^- assimilation in the dark was not surprising in view of the lack of both NO_3^- (Table I) and NO_2^- (Fig. 1) reduction under dark aerobic conditions. However, NO_3^- assimilation in the light showed that the aerobic inhibition of NO_3^- reduction is negated in the light.

Two possible mechanisms have been presented to explain the light effect on the O_2 regulation of NO_3^- reduction: the dark-to-light increase of the cellular ATP-to-ADP ratio inhibits dark respiration (24, 25); or the dark-to-light increase of cytoplasmic-reducing equivalents saturates the oxidative requirements of both NR and the mitochondria (15). It was reasoned that the validity of these hypotheses could be assessed by assaying NO_3^- assimilation when photophosphorylation was uncoupled.

Compounds previously identified as inhibitors of photophosphorylation in chloroplasts (14) were screened for their uncoupling capacity in protoplasts (Table II). The fixation of $^{14}CO_2$ was measured to monitor uncoupling activity, and *in vivo* NO_2^- reduction was measured in parallel to eliminate compounds that inhibited $^{14}CO_2$ fixation by blocking photosynthetic electron transport (2). At the upper limits of solubility or physiological feasibility, of the uncouplers tested, only CCCP and nigericin were effective inhibitors of $^{14}CO_2$ fixation. However, as CCCP inhibited NO_2^- assimilation, nigericin was considered the more suitable uncoupling agent, since, at 0.1 μM, $^{14}CO_2$ fixation was inhibited 98%, and NO_2^- assimilation was slightly stimulated (20%).

The effect of nigericin on the adenine nucleotide content of protoplasts in the light is shown in Table III. The relatively high protoplast concentration used in this and all subsequent assays necessitated the use of 0.2 μM nigericin for complete inhibition of CO_2 fixation (Fig. 3). The protoplast ATP level increased 50% from dark to light, but no increase was observed in the presence

Table II. *Effect of Inhibitors of ATP Synthesis on CO_2 Fixation and in Vivo NO_2^- Reduction by Wheat Protoplasts*

NO_2^- reduction was assayed as NO_2^- disappearance from the assay mixture in the light. The assays contained approximately 60 μg Chl, and the control rates of CO_2 fixation and NO_2^- reduction were 109 and 2.7 μmol·mg^{-1} Chl·h^{-1}, respectively.

Treatment	CO_2 Fixation	NO_2^- Reduction
	% of control	
Control	100	100
Arsenate, $5 \cdot 10^{-2}$ M	96	
Gramicidin-D, $4 \cdot 10^{-6}$ M	95	134
Dinitrophenol, 10^{-5} M	105	98
Valinomycin, 10^{-5} M	59	
CCCP, 10^{-6} M	3	40
Nigericin, 10^{-7} M	1.8	120

Table III. *Effect of Nigericin on the Adenine Nucleotide Content of Wheat Protoplasts*

Protoplasts were incubated for 30 min in the light or dark under aerobic conditions before determinations of the adenine nucleotides were made. Each assay contained 50 mM KNO_3, 10 mM $NaHCO_3$, and approximately 250 μg Chl.

Treatment	ATP	ADP	AMP	ATP/ADP	ATP + One-Half ADP / ATP + ADP + AMP
	nmol·mg^{-1} Chl				*ratio*
Dark, control	45	62	99	0.73	0.37
Light, control	67	76	72	0.88	0.49
Light, 0.2 μM nigericin	33	92	93	0.36	0.37

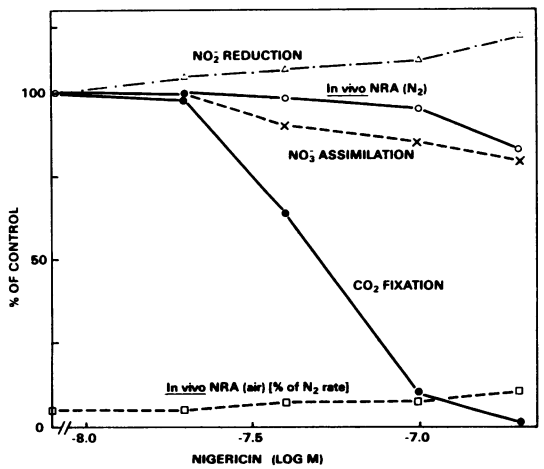

FIG. 3. Effect of nigericin on CO_2 fixation, NO_3^- assimilation, and *in vivo* NO_3^- and NO_2^- reduction by protoplasts. NO_3^- assimilation was assayed as amino-N accumulation in the light (control rate, 2.8 μmol h^{-1} [mg Chl]$^{-1}$). *In vivo* NRA was measured as NO_2^- accumulation under dark anaerobic and aerobic conditions (control N_2 rate, 1.1 μmol NO_2^- h^{-1} [mg Chl]$^{-1}$). *In vivo* NO_2^- reduction was assayed as NO_2^- disappearance from the assay mixture in the light (control rate, 3.2 μmol NO_2^- h^{-1} [mg Chl]$^{-1}$). The control rate of CO_2 fixation was 109 μmol h^{-1} (mg Chl)$^{-1}$. All assays contained approximately 250 μg Chl.

of nigericin. In a separate experiment, protoplast ATP levels in the dark (D) and light (L) with and without 0.2 μM nigericin (N) were (nmol [mgChl]$^{-1}$): D, 43; D + N, 35; L, 73; L + N, 36. Collectively, these data suggest that nigericin fully uncouples photophosphorylation and, either fully or partially, uncouples oxidative phosphorylation. Consideration of the dark-to-light changes of the other adenine nucleotides reinforces this conclusion, but metabolic complexities were apparent. Both the ATP-to-ADP ratio and the energy charge increased in the light, but, in the presence of nigericin, these ratios were either less or equal, respectively, to those observed in the dark. The absence of a dark-to-light reciprocal relationship between ATP and ADP was most probably due to high adenylate kinase activity (23), as was evident from the dark-to-light changes of AMP. For this reason, the energy charge equation (which incorporates all three adenine nucleotides) was a better indicator of the energy status of the protoplasts than was the ATP-to-ADP ratio.

The complexities of the dark-to-light changes of the adenine nucleotides stemmed partially from an increase of the total adenine nucleotides in the light and from the high base levels of AMP in the protoplasts. The adenine nucleotide content of chloroplasts isolated from leaves that had been subjected to short-term light/dark transitions has been shown to remain constant over the transitions (23). However, over longer time periods (15 min), the adenine nucleotide content of wheat leaves has been found to increase in the light (19). This may have resulted from a light-activated breakdown of NAD to AMP (8). The high AMP content of the protoplasts remains an enigma and may have resulted from the metabolic stress imposed during protoplast preparation or from the breakdown of NAD or other adenine nucleotides during the 30-min incubation period before the assays.

The effect of nigericin on the dark to light redox changes of the pyridine nucleotides is shown in Table IV. Both NADH and NADPH increased in the light, and nigericin had no effect on the absolute levels of the reduced pyridine nucleotides in the light. Similarly, in the presence or absence of nigericin, both the NADH-to-NAD and NADPH-to-NADP ratios increased from dark to light, but changes in these ratios were also influenced by dark-to-light changes in the total NAD(H) and NADP(H) content of the protoplasts.

These results were not unusual for leaf cells. The small dark-to-light increases in the steady-state levels of the reduced pyridine nucleotides have also been observed in leaves from a number of plant species (10). In addition, Ogren and Krogmann (20) have shown that NADP and NADPH increase in the light at the expense of NAD. The relatively high levels of NADP(H) compared to NAD(H) in the dark suggest that this process may have occurred appreciably during protoplast preparation.

Overall, the data of Tables III and IV show that nigericin is a potent inhibitor of photophosphorylation, but the molecule has little effect on the light-driven increase of pyridine-nucleotide-reducing equivalents and apparently acts purely in an uncoupling capacity in the protoplasts.

The effects of nigericin on protoplast CO_2 fixation, *in vivo* NO_3^- and NO_2^- reduction, and light-dependent NO_3^- assimilation are shown in Figure 3. At 0.2 μM nigericin, CO_2 fixation was inhibited 99% relative to the control, consistent with the uncoupling action of the molecule. At the same nigericin concentration, *in vivo* NRA (dark, N_2) was inhibited 15%, and light-dependent NO_2^- reduction was stimulated 17% relative to controls. In addition, the dark aerobic *in vivo* NRA was slightly stimulated (5%). These results show that, while nigericin acts as a potent uncoupler in protoplasts, it has relatively little effect on the individual components and regulatory characteristics of the NO_3^- assimilation pathway (NO_3^- to NH_4^+). NO_3^- assimilation, measured as amino-N accumulation in the light, was inhibited 20% relative to the control by 0.2 μM nigericin, and, as a function of nigericin concentration, NO_3^-

Table IV. *Effect of Nigericin on the Pyridine Nucleotide Content of Wheat Protoplasts*

Incubation and assay conditions were as described in the legend of Table III.

Treatment	NAD	NADH	NADP	NADPH	NADH/NAD	NADPH/NADP
	nmol·mg⁻¹ Chl				*ratio*	
Dark, control	19.4	1.7	24.5	6.1	0.09	0.25
Light, control	16.5	2.5	24.5	8.2	0.15	0.33
Light, 0.2 μM nigericin	18.7	2.4	19.2	8.1	0.13	0.42

assimilation and *in vivo* NRA (N_2) followed similar profiles.

The effects of nigericin on the rates of both NO_3^- and NO_2^- assimilation in the light were confirmed using the ^{15}N assay system (Figs. 4 and 5). The results are shown in comparison to the ^{14}N assays, and, for both $^{15}NO_3^-$ and $^{15}NO_2^-$ assimilation, 0.2 μM nigericin had no effect on the rates of ^{15}N enrichment of the NH_4^+ and amino-N fraction. Since NH_4^+ was included in the amino acid fraction for ^{15}N analysis, effects of nigericin on NH_4^+ assimilation were not considered in this study.

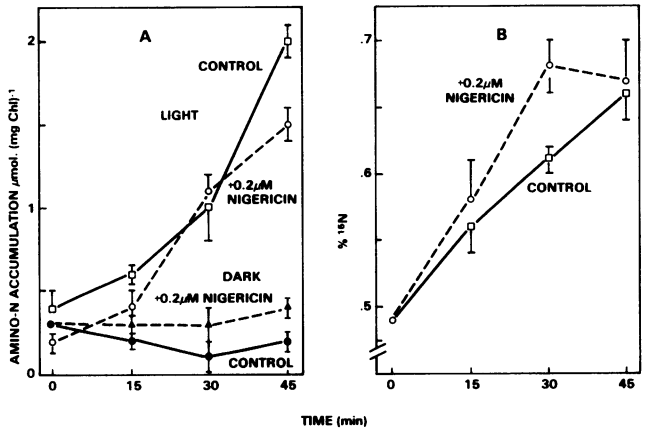

FIG. 4. Effect of nigericin on NO_3^- assimilation by wheat protoplasts. A, Protoplasts were incubated with 50 mM KNO_3, and assimilation was assayed as amino-N accumulation. The initial amino-N concentration in the light control assay was 21.2 μmol (mg Chl)$^{-1}$. B, Protoplasts were incubated with 50 mM $K^{15}NO_3$ (95%), and ^{15}N enrichment of the NH_4^+ and amino-N fraction was determined. All assays contained approximately 250 μg Chl. Bars around points indicate 1 SD.

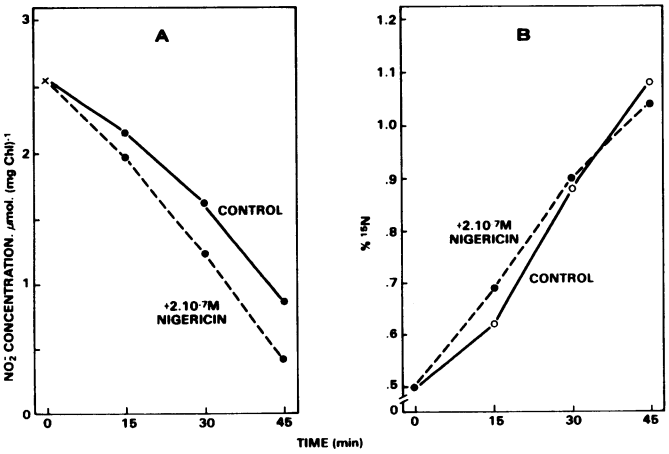

FIG. 5. Effect of nigericin on NO_2^- assimilation by wheat protoplasts. A, Protoplasts were incubated with 0.32 mM $NaNO_2$, and assimilation was measured as NO_2^- disappearance from the assay mixture. B, Protoplasts were incubated with 0.32 mM $Na^{15}NO_2$ (95%), and the ^{15}N content of the NH_4^+ and amino-N fraction was determined. All assays contained approximately 250 μg Chl.

Collectively, these data (Figs. 3–5) show that NO_3^- reduction and assimilation in the light continue when photophosphorylation is completely inhibited. Although this data does not rule out the possibility that cellular changes of the ATP-to-ADP ratio regulate dark respiration in the light, it is apparent that the dark/light control of NO_3^- reduction is not expressed through changes in the ratio of these nucleotides. NO_3^- reduction occurs in the light but not the dark, under aerobic conditions, most probably because of the dark-to-light increase of cytoplasmic-reducing equivalents, sufficient to saturate the oxidative requirements of both NR and the mitochondria.

GENERAL DISCUSSION

Although O_2 inhibition of the leaf *in vivo* NR assay has been observed in numerous studies (5, 18, 22), interpretation of this phenomenon has remained controversial. Loss of NO_2^- from spinach (18), wheat (16), and barley (1) leaves has been shown under dark aerobic conditions. In contrast, no $^{15}NO_2^-$ assimilation to amino-N was found in the dark for wheat (26), barley, corn, and bean (4) leaves. NO_2^- can be lost as gaseous oxides of nitrogen from leaves of certain plant species (3). This phenomenon may explain the apparent discrepancy in the results of the above studies. Alternatively, since NO_2^- is reduced in the dark in non-photosynthetic tissue (7), differences in the nonchlorophyllous components of the leaves used in the above studies may also provide a resolution to the controversy. In the present study, wheat protoplasts provided an assay system free of nonchlorophyllous vascular tissue. No loss or assimilation of NO_2^- was found under dark aerobic conditions. For this reason, it is concluded that O_2 prevents NO_2^- accumulation in the dark *in vivo* NR assay by an inhibition of NO_3^- reduction. Canvin and Woo (5) concluded that NR and mitochondria compete for the oxidation of cytosolic NADH, and NO_3^- reduction can only occur in the dark when the mitochondrial electron transport chain is inhibited. In support of this conclusion, wheat protoplasts accumulated NO_2^- under dark aerobic conditions in the presence of antimycin A.

Competition for reducing equivalents between the mitochondria and NR is apparently negated in the light. Light may inhibit mitochondrial electron transport through an increase of the cellular ATP-to-ADP ratio (25). In the light, a rapid consumption of ADP occurs in the chloroplasts. Through ATP/ADP translocation systems (11), the demand for ADP in the chloroplasts is transduced to the rest of the cell (8). Under these conditions, mitochondrial electron transport would be limited by the availability of ADP. However, this explanation for the absence of O_2 inhibition of NO_3^- reduction in the light is unsatisfactory for the following reasons. First, as a result of adenylate kinase activity, protoplast ADP levels were higher in the light than in the dark. Effectively, adenylate kinase buffers the ADP demand of the chloroplasts. Even low levels of ADP in the mitochondria would allow appreciable rates of electron transport. Second, light-dependent NO_3^- assimilation continued when photophosphorylation was uncou-

pled. Under these conditions, mitochondrial electron transport may be uncoupled also or no longer be limited by the availability of ADP. The specific uncoupling action of nigericin (14) rules out the possibility that mitochondrial electron transport could be limited by ADP translocation. Overall, if mitochondrial electron transport is inhibited by the lack of ADP in the light, it should be restored when photophosphorylation is uncoupled.

Mitochondrial electron transport may be inhibited in the light by another mechanism. However, it is more likely that the export of reducing equivalents from the chloroplast to the cytoplasm in the light saturates the oxidative demands of both NR and the mitochondria. Transfer of reducing equivalents from the chloroplast to cytoplasm can be achieved through malate/OAA and PGA/DHAP shuttle systems (11). Coupling of these redox shuttles to NO_3^- reduction has been demonstrated (13, 21). Even though nigericin would inhibit the PGA/DHAP shuttle, the malate/OAA shuttle would be unaffected by the uncoupler and may compensate for the PGA/DHAP shuttle. In agreement with the work of Canvin and Atkins (4), NO_3^- assimilation can proceed in the absence of concurrent CO_2 fixation. This concept of regulation of NO_3^- reduction is appealing in its simplicity, because NADH acts as both substrate and regulator. The scheme is supported by the fact that leaf tissue accumulates NO_2^- under dark aerobic conditions when high concentrations of glycolytic intermediates (15, 25) or malate (27) are added to the *in vivo* NR assay medium.

Based on stimulation of the dark anaerobic *in vivo* NR assay by Krebs cycle intermediates, a scheme has been proposed whereby reducing equivalents for NO_3^- reduction in the light are derived from the mitochondria (3, 24, 27). According to this metabolic scheme, NO_3^- acts as an alternate electron acceptor to O_2, and it would allow carbon flow through the Krebs cycle to succinate in the light. Although the results of the present study do not dispute this possibility, they do suggest that reducing equivalents for NO_3^- reduction in the light are primarily derived directly from the chloroplast. In addition, for bean leaves in the light, carbon flow through succinate has been shown (17). This would suggest that O_2, rather than NO_3^-, is the terminal electron acceptor for mitochondrial reducing equivalents in the light. The NADH generating systems for dark anaerobic NO_3^- reduction are probably different from those for NO_3^- reduction in the light.

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