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Nitrite Reduction in Reconstituted and Whole Spinach Chloroplasts during Carbon Dioxide Reduction[1]

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ABSTRACT

Nitrite reduction in either whole, isolated spinach chloroplasts (*Spinacia oleracea* L.) or in reconstituted spinach chloroplasts is stimulated by a short period of photosynthetic CO_2 fixation in the light prior to nitrite addition. With reconstituted chloroplasts, a similar stimulation can be obtained in nitrite reduction without CO_2 fixation by the addition of dihydroxyacetone phosphate or fructose 6-phosphate. Specific intermediate metabolites of the photosynthetic carbon reduction cycle may have a regulatory role in nitrite reduction in chloroplasts in the light.

Nitrate can be reduced by illuminated chloroplast grana (4, 14) especially if the chloroplast preparations are provided with ferredoxin and cell-free extracts containing nitrite reductase activity (7). Examination of the location of the nitrite reductase activity in green plant cells by employing nonaqueous chloroplast isolation (14) or isopycnic sucrose density gradients (3) provided evidence for the location of the enzyme in the chloroplasts. It was recently shown that intact chloroplasts, capable of high rates of photosynthetic CO_2 fixation, can reduce nitrite in the light without supplemental cofactors or enzymes (8, 10).

Nitrite reduction with chloroplasts is stimulated by additions of ferredoxin (2, 7), indicating that the photoelectron transport system of chloroplasts provides electrons for nitrite reduction. It has been shown that nitrite reduction in intact chloroplasts is functionally associated with photosystem I and ferredoxin (12).

Since both photosynthetic reduction of products of CO_2 fixation and nitrite reduction apparently require electrons from the photoelectron transport system, and these electrons must be transferred via ferredoxin in both cases, some regulatory or competitive interaction between the two processes might be expected, particularly under circumstances where the regeneration of reduced ferredoxin might be rate-limiting. Under physiological conditions, the rate of CO_2 reduction is 10 to 20 times greater than the rate of nitrite reduction. Therefore, an effect of CO_2 reduction on nitrite reduction might be more easily observed than the converse.

The reconstituted spinach chloroplast system appeared to be a good choice for study of this system, since it is capable of high rates of CO_2 fixation and reduction to normal photosynthetic intermediate compounds (1), yet contains all of the soluble components originally present in the chloroplasts, including presumably those required for nitrite reduction. The absence of an external membrane in such a system is convenient for systematic study involving additions of nitrite and kinetic analysis of its disappearance in the presence of the complete photosynthetic CO_2 fixation and reduction system. We compared such nitrite reduction with that in whole chloroplasts, and examined the interaction, if any, with CO_2 fixation and reduction.

MATERIALS AND METHODS

Spinach plants (*Spinacia oleracea* L) were grown outdoors in 15 cm of vermiculite in flats and were fertilized weekly with Hoagland solution. Much of the problem previously encountered with inactive chloroplasts due to long day lengths in the summer has been overcome by the surprisingly simple expedient of placing the plants where they will be shaded during about half of the daylight hours in the summer. Young spinach leaves of nearly the same age were harvested and used at once. Whole chloroplasts were isolated and used in the experiments of photosynthesis with intact chloroplasts according to the method of Jensen and Bassham (5).

In experiments with the reconstituted chloroplasts, the previously described method was used (1), but with modifications dictated by the nitrite reduction assay. In both the chloroplast suspension medium (solution Y) and the chloroplast-lysing medium (solution Z), glutathione was replaced by 20 mM HEPES, and pH of both buffers was adjusted to 7.8.

Incubations for the kinetic experiments for nitrite reduction and for CO_2 fixation and reduction at the same time were carried out in 15-ml round bottom flasks which were sealed with serum stoppers. All flasks were connected to a gas-flushing system which provided a constant stream of N_2 saturated with water vapor. Each flask initially contained in a final volume of 1 ml: ADP (0.1 mM), $NADP^+$ (0.1 mM), spinach ferredoxin (100 μg), sodium isoascorbate (4 mM), PGA[5] (1 mM), as primer, and soluble chloroplast solution (720 μl). The flasks were attached to a shaker, with immersion of the flask bottoms in a water bath at 25 C, and illumination from below with a light intensity of about 25,000 lux by white fluorescent lights (5).

Experiments were initiated by injection of 50 μl of chloroplast lamellae to give 40 to 70 μg Chl/flask. After 6 min of illumination with shaking, a solution of $NaH^{14}CO_3$ (17 Ci/mol) was added by hypodermic needle through the serum cap to give a final HCO_3^- concentration of 7 mM. The stream of N_2 was closed off to avoid subsequent loss of $^{14}CO_2$ from the gas phase above the solution.

Periodically, 50-μl samples were removed from the flasks and were immediately injected into 450 μl methanol. Total fixation

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[5] Abbreviations: 3-PGA: 3-phosphoglycerate; ribulose-1,5-diP: ribulose 1,5-diphosphate.

of ^{14}C into acid-stable products was determined by acidifying aliquot portions of these samples with glacial acetic acid, drying, and then counting with liquid scintillation. Other aliquot portions of the samples were analyzed for labeled products by two-dimensional paper chromatography and radioautography, followed by ^{14}C measurement of individual compounds by the automatic Geiger counter (6).

For assay of nitrite reduction in both whole chloroplasts and reconstituted chloroplasts, KNO_2 solutions were injected into the flasks at times indicated under "Results" to give a final concentration of 1 mM. Samples (50 μl) were removed from the flasks at times indicated and were immediately transferred into 1 ml of 0.04 M $ZnSO_4$ and 0.05 M NaOH in H_2O. Nitrite was measured as described elsewhere (12) by consecutive additions of 1 ml of 1% (w/v) sulfanilamide in 3 N HCl and 0.95 ml of 0.02% (w/v) *N*-(l-naphthyl)ethylenediamino dihydrochloride. The samples were then centrifuged, and the A_{540} of the supernatant solution was determined after 1 to 3 hr. The earliest sample was always removed from the reaction mixture within 5 sec after the addition of nitrite, and was considered as containing the initial concentration of nitrite in the reaction mixture. In some experiments, the possible effects of NH_4^+, or methyl amine, on nitrite reduction were tested by adding 1 mM NH_4^+ or methyl amine to the reaction flasks containing the reconstituted chloroplast system at the same time as nitrite addition. For other experiments with reconstituted chloroplasts, various metabolites and cofactors were added.

The rate of nitrite reduction by the reconstituted chlorplast system was remarkably higher in the winter than in the summer. The response, however, of the reduction to CO_2 fixation and to cofactors was unchanged.

RESULTS

CO_2 Fixation and Nitrite Reduction in Whole Chloroplasts. With whole spinach chloroplasts, the reduction of nitrite is stimulated by the presence of bicarbonate in the light (Fig. 1). Nitrite addition was made at time zero following 7 min of photosynthesis with bicarbonate and light. The initial rate of the nitrite reduction in the light without bicarbonate was similar to that obtained with bicarbonate, but dropped after about 4 min following the addition of nitrite. The rate of CO_2 fixation was comparable (on a per mg chloroplast basis) with these whole chloroplasts to that found with the reconstituted system, and was not affected by the presence of nitrite.

CO_2 Fixation and Nitrite Reduction in the Reconstituted System. The replacement of glutathione by HEPES buffer was required because of interference with nitrite reduction measurements when glutathione was present. Unfortunately, the fixation of CO_2 is not so well sustained in the reconstituted system (Fig. 2) after this substitution. The initial fixation rate is high (about 120 μg atoms ^{14}C/mg Chl·hr), and comparable to the rate with whole chloroplasts (above) but declines rapidly after 5 min. When nitrite is added 7 min after introduction of bicarbonate, there is no effect on the (diminished) rate of CO_2 fixation. There was also no effect of nitrite on CO_2 fixation when both nitrite and bicarbonate were added simultaneously after preillumination of chloroplast lamellae (experiments not presented).

Upon nitrite addition, there is a very rapid rate of nitrite reduction (with HCO_3^- present) followed by a slower but sustained rate. There is a small background reduction of nitrite initially, but then no further reduction. This constant rate and the similar rate of whole chloroplasts were somewhat higher than the rates obtained by Neyra and Hageman with whole chloroplasts (12). The rate of nitrite reduction is much higher in the presence of bicarbonate than in its absence, in both the initial rapid reduction phase and in the slower sustained reduction phase. The fixation and reduction of CO_2 in this system, which competes with nitrite reduction for electrons from the photoelectron transport system, nevertheless stimulates nitrite reduction, presumably through some regulatory mechanism. The reduction in CO_2 fixation rate after several minutes is due to the use of HEPES buffer instead of glutathione.

A transfer of the reconstituted chloroplast system from light to dark resulted in an immediate cessation of nitrite reduction (Fig. 3). The reduction was then restored to the initial rate upon

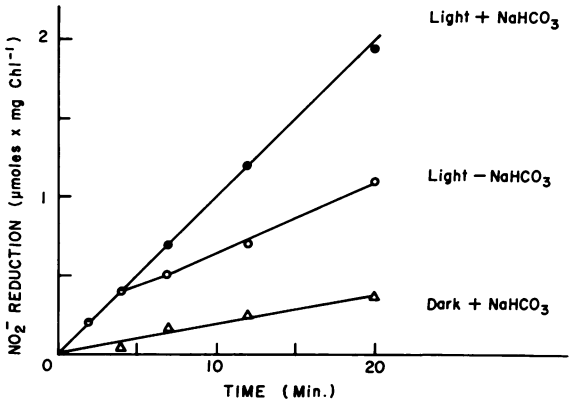

FIG. 1. Nitrite reduction by whole isolated spinach chloroplasts.

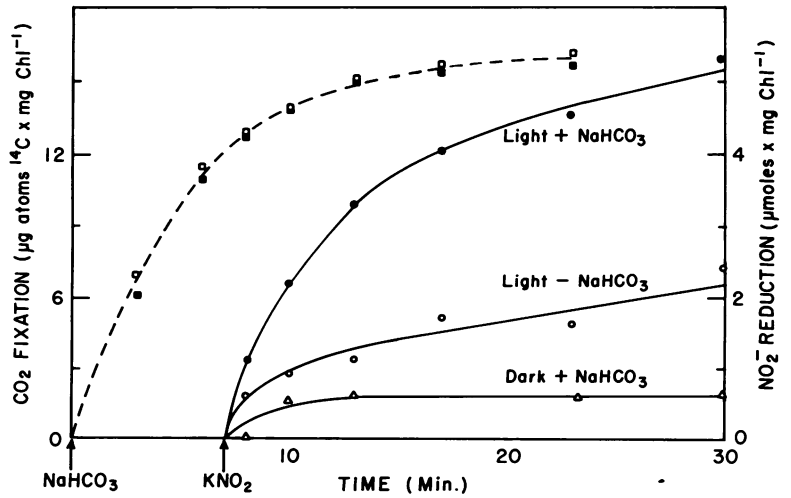

FIG. 2. Nitrate and CO_2 reduction by reconstituted spinach chloroplasts. After 7 min of preillumination, $NaHCO_3$ was added at time zero. KNO_2 was added at 7 min. (- - -); CO_2 fixation; (□): no nitrite addition; (■): nitrite addition after 7 min.

retransfer of the system back to light. The rise in nitrite in the media during the first 5 min in the dark could not be due to nitrate reductase activity since it was established in other experiments that there was no nitrate reductase activity present in these preparations in either light or dark. Incidentally, this finding suggests that the chloroplast preparations used were free of cytoplasmic contamination, since cytoplasm does contain nitrate reductase. Furthermore, as seen below, there does not appear to be oxidation of NH_4^+ to nitrite; thus, the apparent increase in nitrite does not come about from NH_4^+ oxidation. The remaining possibility is that there is an artifact in which reducing power formed in the light interferes with the development of color in the light. If so, this may be in part responsible for the apparent decrease in rate of nitrite reduction after several minutes in the light, as well as the apparent reappearance of nitrite in the dark. Medina and Nicholas (9) have reported that reduced pyridine nucleotides interfere with the color reaction. Various postassay treatments, such as adding zinc acetate and phenozine methosulfate as recommended by Scholl, *et al.* (16) to minimize this effect might have been employed but were not used by us in these studies, as the principal results could be seen in spite of this interference.

An attempt was made to study the effects of varying the times of addition of nitrite relative to bicarbonate (Table I). There is clear indication that the stimulation of nitrite reduction by bicarbonate required that the bicarbonate be present for several minutes in the light prior to the addition of nitrite. This stimulation by bicarbonate increased with time and was mostly remarkable during the latter phase of nitrite reduction, as bicarbonate sustained the reduction rate. When bicarbonate was added after the initiation of nitrite reduction, the rate of nitrite reduction was nearly unaffected during either the initial rapid phase or during the later slow phase of reduction. In other experiments (data not shown), bicarbonate added simultaneously with nitrite caused no initial stimulation of nitrite reaction, but did cause variable stimulation after several minutes. Also, note that although CO_2 fixation had virtually ceased 15 min after nitrite addition (Fig. 2), there remains a stimulation of the low rate of nitrite reduction, suggesting that prior CO_2 fixation is responsible for stimulation of nitrite reduction.

The decline in rate of nitrite reduction with time is apparently not due to build-up of NH_4^+ since in the presence of 1 mM NH_4^+ from the start nitrite reduction continues at a linear rate for a longer time than in its absence (Fig. 4). Possibly this increased rate (at longer times) in the presence of NH_4^+ is due to uncoupling of photosynthetic phosphorylation, leading to an improved supply of reduced ferredoxin for nitrite reduction. That this

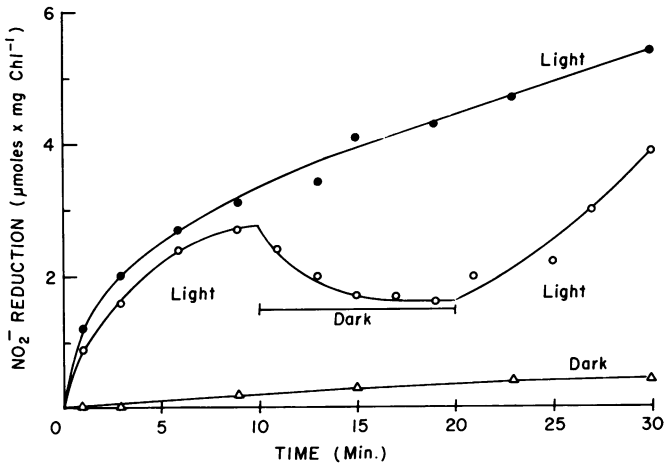

FIG. 3. Nitrite reduction by reconstituted spinach chloroplasts. Chloroplasts were either transferred from light to dark and back or maintained in constant conditions throughout the experiment.

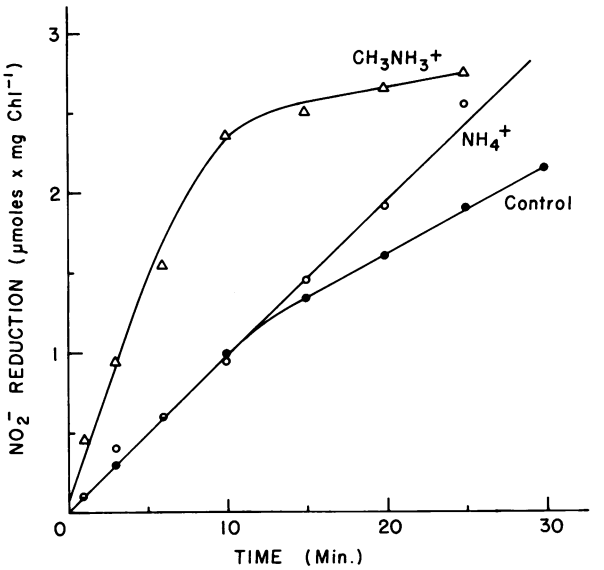

FIG. 4. Effect of NH_4^+ and methyl amine on nitrite reduction by reconstituted spinach chloroplasts.

Table I: Average rates of nitrite reduction by reconstituted spinach chloroplasts as affected by the timing of the addition of $NaHCO_3$.

Times in last three columns refer to periods over which rates of nitrite reduction were measured relative to time of nitrite addition.

Experiment	Bicarbonate	Nitrite Reduction Rate (μmole x mg Ch^{-1} x hr^{-1})		
		3-7 min	7-13 min	13-30 min
Bicarbonate added 7 min after nitrate addition	-	35.0	5.0	4.8
	+	37.5	6.5	4.5
		3-5 min	5-10 min	10-25 min
Bicarbonate added 7 min before nitrate addition	-	30	5.5	4.1
	+	40	9.0	9.2

might be so is indicated by the greatly increased rate of nitrite reduction in the presence of methyl amine, a more effective uncoupler.

The stimulation of nitrite reduction in the reconstituted system requires bicarbonate and added cofactors (ADP + $NADP^+$), but does not seem to require the presence of a primer, such as 1 mM PGA, in the medium (Table II, lines 1, 2, and 4), when one examines only the rate from 3 to 7 min after addition of nitrite (see, however, Table IV, described below). It is likely that neither ATP nor NADPH was directly involved in the stimulation of nitrite reduction since the presence of ADP + $NADP^+$ without bicarbonate did not increase the rate of nitrite reduction in the light (lines 3 and 4). That the stimulation of nitrite reductase cannot be due to a direct effect of bicarbonate can be concluded from the fact that bicarbonate did not restore nitrite reduction unless bicarbonate was being fixed and reduced photosynthetically due to presence of added ADP + $NADP^+$ in the illuminated reconstituted system (lines 4 and 5).

Examination of the labeled products of photosynthesis with $H^{14}CO_3^-$, when the bicarbonate was added simultaneously with the nitrite, showed no significant differences in photosynthetic cycle intermediates such as 3-phosphoglycerate, dihydroxyacetone phosphate, and sugar diphosphates and sugar monophosphates, at any time from zero to 40 min in the light (active photosynthesis occurs only during the first 10 to 15 min with HEPES buffer).

The incorporation of ^{14}C into amino acids (alanine + glutamic acid + glycine + serine) by the reconstituted chloroplast system in the absence of nitrite was only about 0.7% of the total labeled products. It was increased to 1.2% under conditions of nitrite reduction. The insignificant quantities of the administered $H^{14}CO_3^-$ incorporated into amino acids are presumably because of the lack of a system for converting 3-phosphoglycerate to alpha keto acids. Small amounts of these acids are present when the chloroplasts are isolated from the leaves, but their concentration in the isolated or reconstituted system would probably not be affected by the addition of HCO_3^-.

Nitrite reduction by illuminated, reconstituted chloroplast system without bicarbonate added, could be stimulated to about the same extent as with bicarbonate by the addition of fructose-6-P or dihydroxyacetone-P (Table III) and to a smaller extent by ribulose-1,5-P but not by fructose-1,6-P or ATP. Note that these metabolites were added earlier, and could have been substantially converted by the time of nitrite addition. Since 3-PGA is already used as a primer in the control of experiments described above (Table II, line 3), it might appear that it does not stimulate nitrite reduction, especially since when PGA was absent but HCO_3^- was added (Table II, line 2), stimulation was seen.

Interpretation of these results is complicated by the fact that one is looking at rates measured over a period of minutes, the rates are changing, the pool sizes of metabolites in the system are changing, and the effects, where present, of these metabolites on the rates of nitrite reduction may require some time to be manifested. When all conditions are the same except for the concentration of added PGA (Table IV), there is a short term effect of PGA concentration. With 1 mM PGA added as primer at the beginning of the experiment, there is a considerably higher rate of nitrite reduction during the first 3 min following nitrite addition than when the concentration of primer PGA is only .05 mM. This dependence of stimulation on PGA concentration disappears after 3 min of nitrite reduction (lines 1 and 2), in agreement with the results in Table II (lines 1 and 2). That the effect of PGA concentration is so transitory is due perhaps to change in PGA concentration with time. At this level of PGA also, there is an additional stimulatory effect of added bicarbonate. Furthermore, the maximum effect of added PGA on the rate of nitrite reduction during the first 3 min is seen only if the PGA is added prior to the addition of the nitrite (Table V).

DISCUSSION

Two factors appear to be required for the most active nitrite reduction in chloroplasts: a sufficient rate of ferredoxin photoreduction, and active CO_2 fixation. Nitrite reduction responded very quickly to light-dark transitions and was stimulated by uncouplers probably due to enhanced electron transport, although CO_2 in whole isolated spinach chloroplasts and reconstituted chloroplasts results in stimulation of nitrite reduction upon subsequent addition of nitrite. This activation is not due to bicarbonate *per se*, since omission of both primer and cofactors (ADP + $NADP^+$) in the presence of light and bicarbonate abolishes the stimulation (Table II). The stimulation was only evident when nitrite was added several minutes after bicarbonate, possibly allowing the formation of CO_2-fixation products. The same degree of stimulation can indeed be seen in the

Table III: The effect of the reductive pentose phosphate cycle intermediates and ATP on nitrite reduction.

No.	$NaHCO_3$	Compound Added	Nitrite Reduction Rate
		1 mM	(μmole x mg Ch^{-1} x hr^{-1})
1	+	-	11.8
2	-	-	7.0
3	-	dihydroxyacetone-P	13.8
4	-	fructose 1,6-diP	6.6
5	-	Fructose 6-P	12.0
6	-	ribulose 1,5-diP	9.6
7	-	ATP	7.2

Metabolites were added to the incubation mixture prior to addition of lamellae (see Materials and Methods), to give a final concentration of 1 mM for each added compound. Rates were measured between 3 and 7 min after addition of nitrite.

Table II: Nitrite reduction by a reconstituted chloroplast system. Rates were measured between 3 and 7 min after addition of nitrite.

	Reaction Mixture			Nitrate Reduction Rate
	Bicarbonate 7 mM	PGA 1.0 mM	NADP + ADP 0.1 mM 0.1 mM	μmole x mg Chl^{-1} x hr^{-1}
1. Light	+	+	+	28.8
2. Light	+	-	+	26.4
3. Light	-	+	+	10.5
4. Light	+	-	-	9.4
5. Light	-	-	-	10.3
6. Dark	+	+	+	0.8

Table IV: Nitrite reduction by a reconstituted chloroplast system. Rates of nitrite reduction were calculated from the time of nitrite addition.

	Reaction Mixture			Nitrate Reduction Rate	
	Bicarbonate	PGA	NADP + ADP	0 - 3 min	5 - 12 min
		mM		(μmole x mg Chl^{-1} x hr^{-1}	
1. Light	+	1	+	26.5	9.1
2 Light	+	.05	+	11.8	10.6
3. Light	-	.05	+	5.8	4.7
4. Dark	+	1	+	1.2	0.8

Table V: Nitrite reduction by a reconstituted chloroplast system. Rates of nitrite reduction were calculated from the time of nitrite addition. Reaction mixtures as described in Materials and Methods contained 7 mM $NaHCO_3$.

Time of PGA Addition (1 mM)	Rate of Nitrite Reduction	
	0 - 3 min	5- 12 min
1. Before initiation of experiment	25.1	10.2
2. Simultaneously with $NaHCO_3$	24.0	8.8
3. Simultaneously with KNO_2	15.5	14.3

absence of bicarbonate with the illuminated system when dihydroxyacetone-P, fructose-6-P, or to a lesser extent, ribulose-1, 5-diP (each 1 mM), are added to the reconstituted system. The fact that with PGA alone, further activation occurs with added HCO_3^- (Table IV, lines 2 and 3) is seen as indicating that the presence of both PGA and HCO_3^- in the light are required to provide the optimal levels of effectors. These effectors may be the sugar phosphates which gave stimulation (Table III), but this cannot be decided unambiguously at the present time, since rapid conversion of these substances to other active effectors is occurring during the course of the observation.

We concluded that *in vivo*, a combination of photosynthetic carbon reduction cycle intermediates is able to regulate to some extent the rate of reduction of nitrite to ammonia. Perhaps under physiological conditions (such as low light intensity or damaged photosynthetic apparatus) where CO_2 fixation is limited by the rate of photoelectron transport, it is a useful regulatory function to limit also the transfer of electrons to nitrite reduction. When photosynthetic activity is higher, however, the ammonia formed by nitrite reduction can be utilized by available carbon skeletons for synthesis of amino acids.

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