Plant Physiol. (1986) 80, 676–684
0032-0889/86/80/0676/09/$01.00/0

Carbon Dioxide and Nitrite Photoassimilatory Processes Do Not Intercompete for Reducing Equivalents in Spinach and Soybean Leaf Chloroplasts

Received for publication August 1, 1985 and in revised form November 20, 1985

J. MICHAEL ROBINSON
Plant Physiology Institute, Plant Photobiology Laboratory, United States Department of Agriculture/ Agricultural Research Service, Beltsville Agricultural Research Center-West, Beltsville, Maryland 20705

ABSTRACT

Previously, C Baysdorfer and JM Robinson (1985 Plant Physiol 77: 318–320) demonstrated that, in a reconstituted spinach chloroplast system, NADP photoreduction functioning at most maximal rate and reductant demand, was the successful competitor with NO_2^- photoreduction for reduced ferredoxin. This resulted in a repression of NO_2^- reduction until all NADP available had been almost totally reduced. Further experiments, employing isolated, intact spinach leaf plastids and soybean leaf mesophyll cells, were conducted to examine competition for reductant between CO_2 and NO_2^- photoassimilation, *in situ*. In isolated, intact plastid preparations, regardless of whether the demand for reductant by CO_2 photoassimilation was high (5 millimolar 'CO_2') with rates of CO_2 fixation in the range 40 to 90 micromoles CO_2 fixed per hour per milligram chlorophyll, low (0.5 millimolar 'CO_2') with rates in the range 5 to 8 micromoles CO_2 per hour per milligram chlorophyll, or zero (no 'CO_2'), NO_2^- photoreduction displayed equal rates in the range of 8 to 22 micromoles per hour per milligram chlorophyll. In the absence of 'CO_2', but in the presence of saturating white light, 3-phosphoglycerate photoreduction at rates of 82 to 127 micromoles per hour per milligram chlorophyll did not repress, and occasionally stimulated concomitant rates of NO_2^- reduction which ranged from 23.4 to 38.5. Conversely, in plastid preparations, NO_2^- at levels of 50 to 100 micromolar, stimulated plastid CO_2 fixation when 'CO_2' was saturating with respect to carboxylation. Further, levels of NO_2^- in the range 250 to 2500 micromolar, stimulated soybean leaf mesophyll cell net CO_2 fixation as much as 1.5-fold if 'CO_2' was saturating with respect to CO_2 fixation. It appeared likely that, in high light *in vivo*, CO_2 and NO_2^- photoassimilatory processes are not forced to intercompete for reduced ferredoxin in the intact chloroplast.

Higher plant chloroplasts simultaneously photoreduce and photoassimilate both CO_2 and NO_2^- into photosynthate employing reducing equivalents (NADPH, Fdrd[1]) and ATP derived from the photolysis of H_2O and transmitted by subsequent photosynthetic electron transport (2, 4, 32). Twelve mol of NADPH (24 mol of Fdrd) and 18 mol of ATP are required for the assimilation of 6 mol of CO_2 to 1 mol of glucose with the concomitant regeneration of 6 mol of RuBP in the PPRC (4) Reduced thioredoxin, resulting from the ferredoxin-thioredoxin reductase catalyzed transfer of electrons from Fdrd to oxidized thioredoxin, is employed for the reductive activation of PPRC enzymes such as FBP (C-1) phosphatase (4). However, the total magnitude of the stoichiometry for the Fdrd requirement for the sum of the plastid enzyme activations remains unknown (4).

Chloroplast reductive assimilation of 1 mol of NO_2^-, results first in formation of NH_4^+ (catalyzed by NiR), followed by the amidation of Glu to form Gln (catalyzed by GS), and finally followed by the transamination of the amide NH_4^+ of Gln to α-KG (catalyzed by GOGAT) to beget 2 mol of Glu (a net gain of 1 mol Glu) (2, 32). Since both NiR and GOGAT require Fdrd as reductant (2, 13), the assimilation of 1 mol of NO_2^- to gain an additional mol of Glu requires 8 mol of Fdrd and 1 mol of ATP (2). In C-3 plants, the GS and GOGAT system also appears to assimilate the NH_4^+ released by glycine decarboxylase during photorespiratory metabolism (32). Since rates of photorespiration are reputed to attain values as high as 50 μmol CO_2 and NH_4^+ released/h·mg Chl, then the magnitude of the total requirement for Fdrd for total NO_2^- plus NH_4^+ assimilation in the plastid also may be much higher than has been predicted (4, 32).

In leaves the magnitude of total CO_2 assimilation is 5 to 20 times higher than the magnitude of light dependent NO_2^- reduction reflecting that there are greater total amounts of enzyme molecules associated with the assimilation of carbon compared with those enzymes which catalyzed the assimilation NO_2^- (2). Based on demands for reductant, plastid electron transport activity supports more carbon than nitrogen assimilation. For example the enzyme, FNR, the tightly bound terminal enzyme in the photosynthetic electron transport chain (which is directly adjacent to the Fdox reducing site), delivers NADPH at rates of 300 to 400 μmol/h·mg Chl (minimal rates of 600–800 for Fdrd-Fdox recycling). These rates are more than sufficient to support total carbon assimilation (*e.g.* GAPase) (27). NiR, a stromal enzyme, usually exhibits rates in the range of 10 to 25 μmol NO_2^-/h·mg Chl, and the maximal required Fdrd-Fdox recycling rate to support total 'throughput' of NO_2^- for a net gain in Glu would be only 200 μmol/h·mg Chl.

A shortage of Fd (*i.e.* Fdrd), or a preferential allocation of Fdrd to FNR over NiR, could produce, in the plastid, a competition for Fdrd between CO_2 and NO_2^- assimilation. Indeed, there is evidence for competitiveness between photosynthetic carbon and nitrogen assimilation in intact plant leaves and cells as well as in isolated chloroplast systems. Rather recent studies,

[1] Abbreviations: Fdrd, Fd reduced; PPRC, pentose phosphate reductive cycle; CO_2, CO_2 gas dissolved in solution; HCO_3^-, bicarbonate; 'CO_2', CO_2 plus HCO_3^-; RuBP, ribulose-1,5-bisP; 3-PGA or PGA, 3-phosphoglycerate; FBP, fructose-1,6-bisP; GAP, glyceraldehyde-3-P; DHAP, dihydroxyacetone-P; triose-P, GAP plus DHAP; α-KG, α-ketoglutarate; Fd, total ferredoxin (oxidized plus reduced); Fdox, Fd oxidized; FNR, ferredoxin-NADP reductase; GAPase, NADP-glyceraldehyde-3-P dehyrogenase; NiR, nitrite reductase; GS, glutamine synthetase; GOGAT, glutamate synthase (glutamine oxoglutarate aminotransferase); TPS, total photosynthesis; N fed or inorganic N fed, NO_3^- plus NH_4^+ levels (mM) incorporated into modified Hoagland nutrient solutions.

which employed intact leaves of Bush Bean (20) or intact *Scenedesmus* cells (11), both indicated that the light-dependent assimilation of NO_2^- was repressed during concomitant net CO_2 photoassimilation. Also, in order to derive maximal NO_2^- photoreduction in isolated, intact, pea leaf chloroplasts, Anderson and Done (1) found it necessary to employ the inhibitor DL-glyceraldehyde in order to eliminate PPRC activity.

Baysdorfer and Robinson (3, 28) employed a reconstituted spinach leaf chloroplast system (purified stromal fraction, plastid lamellae, and saturating Fd, NADP, and NO_2^-), and found that the photoreduction of NADP at very high rates, completely repressed concurrent NO_2^- reduction until almost 90% of the supplied NADP had been reduced. At that point, NO_2^- reduction commenced at maximal rates. Since Fd had been supplied in amounts saturating both to FNR and NiR, the results suggested that there was a preferential allocation of Fdrd to FNR bound to the lamellae until nearly all the NADP had been converted to NADPH, whereupon Fdrd was released to support NiR catalysis in the stromal fraction (3, 28).

However, in contradiction to the foregoing examples (3, 11, 20), there are studies which suggest that plastid carbon and nitrogen photoassimilatory processes do not intercompete for reductant, *in vivo*. An important observation is that the concentration of Fd has been measured and found to be as high as 2 mM (25). At least in high light, there should be levels of Fdrd sufficient to be saturating with respect to all of the Fdrd requiring processes in the chloroplast (2, 4, 25). Further, Grant and Canvin (7) observed that in preparations of intact spinach leaf plastids, NO_2^- photoreduction rate was the same regardless of whether or not there was simultaneous CO_2 assimilation. Also, reports from several laboratories indicated that the net photoassimilation of CO_2, in isolated leaf mesophyll cells, was actually accelerated during the concomitant assimilation of NO_3^- (28) and NH_4^+ (19, 28, 33). Concurrently, inorganic nitrogen assimilation was in no way repressed, and often was stimulated (19, 28, 33). Furthermore, Plaut *et al.* (21), employing a reconstituted spinach plastid system (stromal and lamellae fractions), found that intermediates generated during CO_2 assimilation, *e.g.* DHAP and fructose-6-P, enhanced the rate of light dependent NO_2^- photoreduction, ostensibly through an activation of NiR.

A reality of whole plant growth is that plant leaf tissues from grain crops (*e.g.* soybean), often may derive as much as 40 to 100% of the newly acquired reduced nitrogen (amino acids) during light dependent (Fdrd and ATP dependent) assimilation (23, 24). These observations (23, 24) imply that competition between chloroplast carbon and nitrogen photoassimilation, were it to occur, would limit the magnitude of the gain in α-amino nitrogen. This could be counterproductive to plant growth and grain development, and the fact remains that increased levels of NO_3^- and NH_4^+, fed to intact plants in the range 9 to 36 mM, usually simultaneously will enhance both net CO_2 fixation and the assimilation of NO_3^- and NH_4^- (28).

The main purpose of the studies described in this report has been to positively define whether or not there is competition between CO_2 photoassimilation and NO_2^- photoreduction for plastid reductant (Fdrd). The approach has been one of examination, both in preparations of isolated intact spinach leaf plastids and soybean leaf mesophyll cells, of the process of NO_2^- photoreduction during points in time in which the demands for reductant by CO_2 or 3-PGA photoassimilation are either absent, minimal (*e.g.* rate-limiting CO_2), or maximal (*e.g.* saturating CO_2). I have observed, both in preparations of isolated intact spinach leaf plastids and isolated intact soybean leaf mesophyll cells, maintained in high (saturating) light, at 25°C, and with varying reductant demand by CO_2 assimilation from none to very maximal, that there was not visible mutual competition for reductant between CO_2 and NO_2^- reductive assimilation. In fact, isolated spinach plastid preparations fixing CO_2 in high light, occasionally displayed a stimulation of light dependent NO_2^- reduction, ostensibly caused by the presence of CO_2 photoassimilation. Additionally, in isolated spinach plastids and in soybean leaf mesophyll cells, NO_2^- reduction often appeared to significantly stimulate both total and net CO_2 photoassimilation. The implications of these mutual stimulations are discussed briefly.

MATERIALS AND METHODS

Plant Material. Leaf samples and isolates of intact chloroplasts were prepared from leaves of 20 to 30 d old, growth chamber propagated *Spinacia oleracea* cv Wisconsin Bloomsdale, or cv Wisconsin Dark Green. Trifoliolate samples, as well as preparations of isolated intact leaf mesophyll cells, were prepared from *Glycine max* (L.) Merr. cv Amsoy and cv Williams. Plants were propagated from emergence in growth chambers on a 12 h light-12 h dark cycle, 25°C constant, 65% RH, and 650 $\mu E/m^2 \cdot s$ incident energy upon the upper foliage. Each growth chamber was fitted with 24, 2.44 m, 210 W Sylvania VHO[2] or Westinghouse SHO fluorescent bulbs mixed with 10, 60 W incandescent bulbs.

Spinach and soybean plants were propagated in vermiculite held in 15.24 cm plastic pots, and each pot was surface irrigated twice daily with a modified Hoagland solution previously described (27). Incorporated in the nutrient solution were levels of inorganic N optimal for normal growth in the form of $Ca(NO_3)_2 \cdot 4\ H_2O$, 3.33 mM and NH_4NO_3, 1.67 mM; the ratio $NO_3^-:NH_4^+$ was 5:1 or 8.33 mM:1.67 mM. For comparative purposes, in some experiments, a second set of plants were irrigated with a growth superoptimal level of inorganic N in the form of $Ca(NO_3)_2 \cdot 4\ H_2O$, 5 mM; NH_4NO_3, 6 mM; KNO_3, 7 mM; and $Mg(NO_3)_2 \cdot 6H_2O$, 3.5 mM. In this latter case, the ratio of $NO_3^-:NH_4^+$ was also 5:1 or 30 mM NO_3^-:6 mM NH_4^+.

Net and TPS and Nitrite Levels in Intact Leaves. Soybean plant leaves were numbered acropetally, from the first leaves, and leaves numbered 3 and 4 were selected for measurements from each of 5 uniform test plants for gas exchange. Leaves 3 and 4 were from a second set of 5 test plants (identical to the first set of 5) were excised, pooled, placed in liquid N_2, and subsequently lyophilized. For spinach plants, the identical procedures were employed for testing except that two of the most mature leaves in the rosette configuration were selected for the samples; pools from 5 plants per set were employed as in the studies with soybean plants.

Foliar net phostosynthesis, as well as photo- and dark respiration were estimated, using the previously described methods (27), so that total photosynthesis could be calculated (27).

Leaves were sampled in the dark or light in the growth chamber, excised leaf blades were immediately inserted into liquid N_2 (in beakers) into pools of 5 leaves (15 leaflets) or 5 spinach leaves per sample. The leaf samples were homogenized and lyophilized according to previously described methods (27).

Tissue NO_2^- was extracted and quantitated from pools (50 mg per sample) of homogenized soybean or spinach leaf samples employing the methods described by Scholl *et al.* (29). To recover at least 70 to 73% of 70 nmol of standard $NaNO_2$ included with the 50 mg of lyophilized tissue, it was necessary to employ the heat step, as well as the addition of phenazine methosulfate and zinc acetate just subsequent to the heat step described by Scholl *et al.* (29). Prior to, and subsequent to lyophilization, leaf blades were weighed so that fresh and dry weight could be determined.

[2] Mention of a trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exculsion of other products or vendors that may also be suitable.

This facilitated the computation of the volume of H_2O in the tissue, and the expression of leaf NO_2^- as a μM concentration.

Spinach Leaf Chloroplast Isolation. Intact spinach leaf chloroplasts were prepared, plastid Chl was isolated and quantitated, and plastid intactness was estimated employing previously described methods (25, 26). In this report, Chl amounts reported in reaction mixtures (Fig. legends) will be that associated with only the intact plastids.

Soybean Leaf Mesophyll Cell Isolation. Mesophyll cells were isolated from trifoliolates, numbered acropetally, 3, 4, 5, and 6, from 25 to 30 d old soybean plants (cv Amsoy) grown in the pots irrigated with the optimal N nutrient media described above (10 mM N). Isolated intact mesophyll leaf cells were prepared mechanically employing methods described by Oliver *et al.* (18). Cells were isolated in the absence of inorganic N in the isolation and resuspension media which were otherwise identical with that employed by Oliver *et al.* (18). However, to increase the rapidity of cell isolation, rather than allowing the cells to settle out of the suspending and washing solutions (18), centrifugation (50 *g*/1.5 min) was employed, both for removal of cells from the initial homogenization medium and from the cell washing medium. This facilitated an isolation of cells requiring only 20 to 30 min just prior to measurement of cell CO_2 and NO_2^- photoreduction. Estimated by the Evans Blue cell exclusion technique (18), cell intactness ranged from 60 to 70%.

Chl was extracted from 0.1 ml of final cell resuspension added to 9.9 ml of 100% methanol, centrifuged at 1000*g*/3 min, and the resulting supernatant was spectrophotometrically analyzed at 650 and 665 nm. The relationships developed by MacKinney (14) were employed to compute Chl.

Simultaneous CO_2 Photofixation and NO_2^- Reduction in Plastids and Cells. In preparations of isolated, intact spinach leaf as well as isolated, intact soybean leaf mesophyll cells, simultaneous $^{14}CO_2$ photoassimilation and light dependent NO_2^- reduction (NO_2^- disappearance) was monitored employing modifications of the methods described by Miflin (16) and Reed and Canvin (24).

Isolated spinach leaf chloroplasts (from Wisconsin Bloomsdale) were incubated to 10 min dark followed by 30 min light (1000 $\mu E/m^2 \cdot s$) in reaction mixtures, containing, in 3.00 ml, 50 mM Tricine (pH 8.1); 330 mM sorbitol; 2 mM diK EDTA; 1 mM $MgCl_2$; 1 mM $MnCl_2$; 0.25 mM Pi; 1500 units catalase (Sigma-Thymol free); either 0.5 or 5.33 mM '$^{14}CO_2$' (10 μCi-^{14}C); and 0 to 1 mM $NaNO_2$. Plastids (those totally intact) were equivalent to 7 to 24 μg Chl. Mixtures were maintained at 25°C.

Intact soybean leaf mesophyll cells were incubated in the dark-light and temperature regimes identical to those used for the spinach leaf plastids, except that the cells were aerated, at 1 to 2 ml/min with streams of 20% O_2/80% N_2. Isolated mesophyll leaf cells were incubated in reaction mixtures containing in 3.5 ml, 50 mM Hepes (pH 7.8); 330 mM sorbitol; 2 mM DiK EDTA, 1 mM $CaCl_2$; 1 mM $MgCl_2$; 1 mM $MnCl_2$; 0.5 mM Pi; either 0, 0.55, or 5.5 mM '$^{14}CO_2$' (10 μCi-^{14}C); 0 to 0.5 mM $NaNO_2$; and cells (those totally intact) equivalent to 30 μg Chl.

Samples (0.5 ml) were withdrawn from the mixtures initially, and at 10 min intervals, and combined with 0.5 ml 1 M zinc acetate; reaction termination was instantaneous. Subsequently, consecutive time-course terminated samples were agitated to remix plastids and cells, 25 or 50 μl samples were combined with 5 μl of 12 N HCl in 12 ml scintillation vials, unincorporated $^{14}CO_2$ was allowed to escape, 5.0 ml scintillation cocktail was added to the vial, and the time-course of $^{14}CO_2$ incorporation into acid stable products was determined with scintillation spectrometry. Counting efficiency was estimated by including 0.01 μCi of standard [^{14}C]glycolate in designated time-course samples. Subsequent to $^{14}CO_2$ photofixation measurements, 0.7 ml of each zinc acetate time course terminate was centrifuged at approximately 12,000*g*/5 min in a Beckman Microfuge 12. Resulting supernatants were measured for NO_2^- employing the methods described by Miflin (16) with some modifications. An aliquot of the supernatants (known to contain approximately 15 nmol $NaNO_2$) were combined with pure H_2O to a volume of 0.5 ml, and to this mixture was added 0.25 ml 1% sulfanilimide (in 3 N HCl, w/v), and 0.25 ml, 0.02% *N*-(1-napthyl-)ethylene-diamine dihydrochloride (final volume of assay mixture, 1.0 ml). To ensure maximum color development, 10 min was allowed prior to reading the samples spectrophotometrically at 540 nm; readings were quantitated against standard curves in the range of 0 to 25 nmol NO_2^-. The components of the plastid or mesophyll cell preparation or reaction mixtures, *e.g.* sorbitol, EDTA, etc., did not influence the standard NO_2^- curves, relative to the curve for which standard NO_2^- amounts were prepared in pure H_2O.

Simultaneous 3-PGA and NO_2^- Photoreduction in Plastids. Since NO_2^- is oxidized to NO_3^- by strong acid, it was necessary to further modify procedures associated with time-course measurements of concomitant 3-PGA and NO_2^- photoreduction preparations of isolated, intact spinach leaf plastids. These measurements were made with plastids isolated from the variety, Wisconsin Dark Green.

Light dependent disappearance of 3-PGA was monitored according to the methods outlined by Latzko and Gibbs (12), but several modifications were employed. Except as noted, reaction mixtures and conditions for monitoring the simultaneous photoreduction of 3-PGA and NO_2^- (disappearance) were identical with those employed to determine isolated plastid CO_2 fixation. In all cases, 'CO_2' was rigorously excluded, and in its place, the mixtures contained, in 5 ml: 1 mM 3-PGA; 0.6 mM ADP; 0.25 mM Pi; 0.5 mM each $NaNO_2^-$, Glu, and α-KG; and 2500 units catalase. Plastid Chl reported represents that which is associated with intact plastids. At reaction initiation, and at subsequent time intervals, two consecutive 600 μl aliquots were removed (per time point). The first aliquot, for use in 3-PGA determination, was rapidly added to 60 μl of 61% $HClO_4$ to make a final concentration of 5.6% perchlorate. The second aliquot (600 μl), for NO_2^- determination, was rapidly added to 40 μl of 12 N NaOH to bring the mixtures to 0.75 M NaOH.

For determination of 3-PGA, 500 μl aliquot of perchlorate extract was added to 500 μl of glass distilled H_2O along with 100 μl of 1 M Mes (acid form), and the pH of this mixture was titrated, with 5 M KOH, to 6.8 to 7.2; the H^+ ion step was monitored with pH electrode. The resulting K-perchlorate sediment was removed by centrifugation at 1000*g*/10 min in polyethylene conical centrifuge tubes. The final volume of the clear reaction mixture extract was 3.0 ml, and 3-PGA was estimated in 200 or 500 μl aliquots of this mixture. The presence of NO_2^-, Glu, Gln, and/or α-KG did not influence the assays for 3-PGA which were determined enzymically (12).

Nitrite disappearance was determined in the NaOH treated samples. The samples were frozen and thawed, placed in 1.5 ml conical tubes, and centrifuged at 9000*g*/5 min on a Beckman model B Microfuge. One hundred μl of the resulting supernatant was combined with 0.9 ml pure H_2O, 1 ml of 1% sulfanilimide (in 3 N HCl), 1 ml of 0.02% naphthalenediamine dihydrochloride, the resulting mixture was vortexed and 30 min was allowed for complete color development. Subsequently, 2 ml of H_2O was supplied, the solution remixed, and the color was quantitated spectrophotometrically at 540 nm. Absorbance readings were compared to a comparably prepared standard curve which adhered to Beer's Law in the range 10 to 100 nmol $NaNO_2$. It was found that no other components of the reaction mixtures, *e.g.* 3-PGA, Glu, Gln, or α-KG, produced a color change with the sulfanilimide-naphthalenediamine reagent. Indeed, $NaNO_2$ standards could be prepared in H_2O only, in 50 mM Tricine (pH 8.1), or in the plastid reaction mixtures containing all metabolites and identical standard curves could be obtained. Additionally, there was no loss or gain of NO_2^- in dark held plastid reaction

mixtures, and the presence of plastids in the strong base did not influence the 540 nm absorbance maximum of the sulfanilimide-naphthalenediamine color complex.

RESULTS

Nitrite Levels *in Vivo* and Foliar CO_2 Photoassimilation. *Spinach and Soybean Leaves.* Because NO_2^-, at levels as low as 0.2 mM, often mediates inhibition of photosynthetic carbon metabolism in plastid isolates (6, 7, 15, 22), it was necessary to measure in leaves, those levels of NO_2^- which are physiologically normal. It was then possible to study the simultaneous photoreduction of NO_2^- and assimilation of CO_2 in plastid isolates employing NO_2^- concentrations which were physiologically realistic.

Soybean and spinach plants were fertilized with nutrient solutions containing either NO_3^- plus NH_4^+ fed in concentrations either optimal (8.3 mM NO_3^-) or superoptimal (30 mM NO_3^-) with respect to growth. This was done in order to observe whether or not plants would accumulate higher NO_2^- levels and, should that be the case, whether or not foliar CO_2 photoassimilatory rates would be unaffected, inhibited, or enhanced. Thirty-six mM total N fed proved to be just on the threshold of that amount which inhibited both soybean and spinach plant growth (data not shown).

In soybean (Fig. 1A), the average foliar level (complete trifoliates) of NO_2^- in the dark was approximately 185 μM, regardless of the concentration of the N fed to the whole plants, and during the illumination period, the level (presumed steady state) diminished to 125 to 135 μM, reflecting the photoreduction of NO_2^-. There was a slight increase, but never any decrease in TPS rate in the 36 compared with the 10 mM N fed plants.

The mature leaves of spinach plants fed the higher N level, displayed both in the light and in the dark, an approximately 2-fold higher NO_2^- concentration when compared with the foliage of the optimal N fed plants (Fig. 1B). For example, in 10 and 36 mM N fed plants, the foliar level of NO_2^- prior to illumination was, respectively, approximately 40 and 80 μM; during illumination levels diminished, respectively, to 35 and 65 μM. Reflections of light dependent NO_2^- reduction was most pronounced in the higher N level fed plants. However, regardless of the increase in NO_2^-, brought on by the increased N nutrition, TPS was the same in both treatments (Fig. 1B), and, as in the case of soybean, TPS was never inhibited in the highest N fed plants.

Lack of Repression of NO_2^- Reduction by Plastid and Leaf Mesophyll Cell Carbon Assimilation. *Properties of Isolated Plastid and Cell NO_2^- Photoreduction.* Light dependent disappearance of NO_2^- in intact spinach plastid isolates displayed maximal velocity in high white light (1000 μE/m·2s), 25°C, and with levels of NO_2^- in the range 100 to 200 μM, while ½ V_{max} or lower rates were observed with approximately 50 μM NO_2^- (Fig. 2B). Employing 100 to 200 μM NO_2^- in mixtures with plastid isolates prepared from the spinach leaves of the variety Wisconsin Dark Green, maximal velocities of NO_2^- reduction, varied from 6.1 to 15.2 μmol reduced/h·mg Chl (Figs. 2, 3). However, in plastid isolates from the variety Wisconsin Bloomsdale, maximal rates were routinely as high as 23.4 to 38.5 (100–500 μM NO_2^-) (Fig. 5). The reason for these varietal differences is not known.

Maximal velocities of NO_2^- reduction in soybean mesophyll cell preparation from leaf tissue pools, comprised of the third through the sixth trifoliolate leaflets (var. Amsoy and Williams) routinely displayed maximal light dependent rates of NO_2^- reduction of 5 to 22 μmol/h·mg Chl (250–2500 μM NO_2^-) (Fig. 4B and data not shown). On a routine basis, cell preparations from var Amsoy exhibited the highest rates of NO_2^- photoreduction, although isolates from var Williams occasionally displayed rates as high as 22.

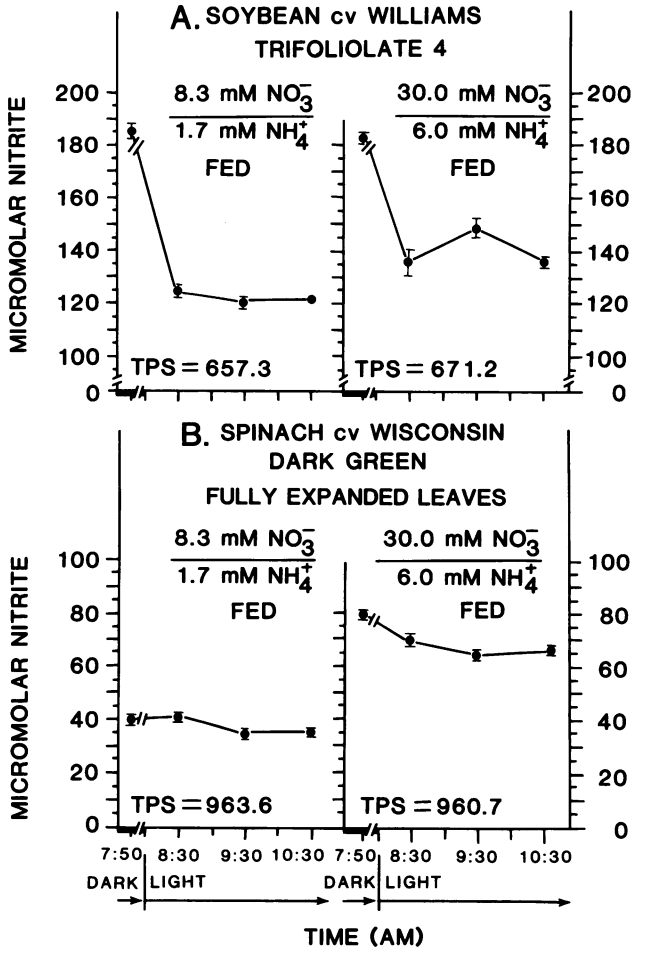

FIG. 1. Foliar levels of NO_2^- in dark held or illuminated leaves and net CO_2 photoassimilation rates in the same leaves of soybean (A) and spinach (B) plants growth in vermiculite pots irrigated with levels of NO_3^- plus NH_4^+ either just optimal (10 mM), or superoptimal (36 mM), with respect to plant growth. TPS rates are expressed as μmol CO_2 assimilated/h·dm².

In all cases the dark rate of NO_2^- reduction was quite minimal or nonexistent when compared with subsequent light dependent rate (Figs. 3–5). Certainly, in some cases it was clear that there was a rather low, but measurable linear dark NO_2^- reduction rate, ostensibly due to the starch degradative dependent, dark reduction of Fdox in the chloroplast (Figs. 3, 4; Ref. 10).

In the light, it was certainly clear that NO_2^- disappearance in plastid isolates was dependent upon the photolytic cleavage of H_2O. For example, in one experiment not shown, the rate of light dependent, NO_2^- dependent, O_2 evolution in plastid preparations held in the absence of exogenous CO_2, and with 0.4 mM $NaNO_2$, was 36.8 μmol/h·mg Chl while the concomitant disappearance of NO_2^- proceeded at a rate of 24.6. The ratio of O_2 photoevolved:NO_2^- reduced was 36.8:24.6 or 1.5:1.0 which reflected the transfer of 6 mol-equivalents of electrons from 3.0 mol H_2O to 1.0 mol NO_2^- (to form 1 mol NH_4^+).

Simultaneous CO_2 Photofixation and NO_2^- Reduction. The approach to the examination, in high light, of the possible competition for reductant between carbon and nitrogen photoassimilation in chloroplasts has been principally that of manipulating the demands by CO_2 photofixation and NO_2^- photoreduction for photolytically generated reductant (Fdrd). This was done by varying both the concentrations of 'CO_2' and NO_2^- in the plastid reaction mixtures in order to simultaneously establish

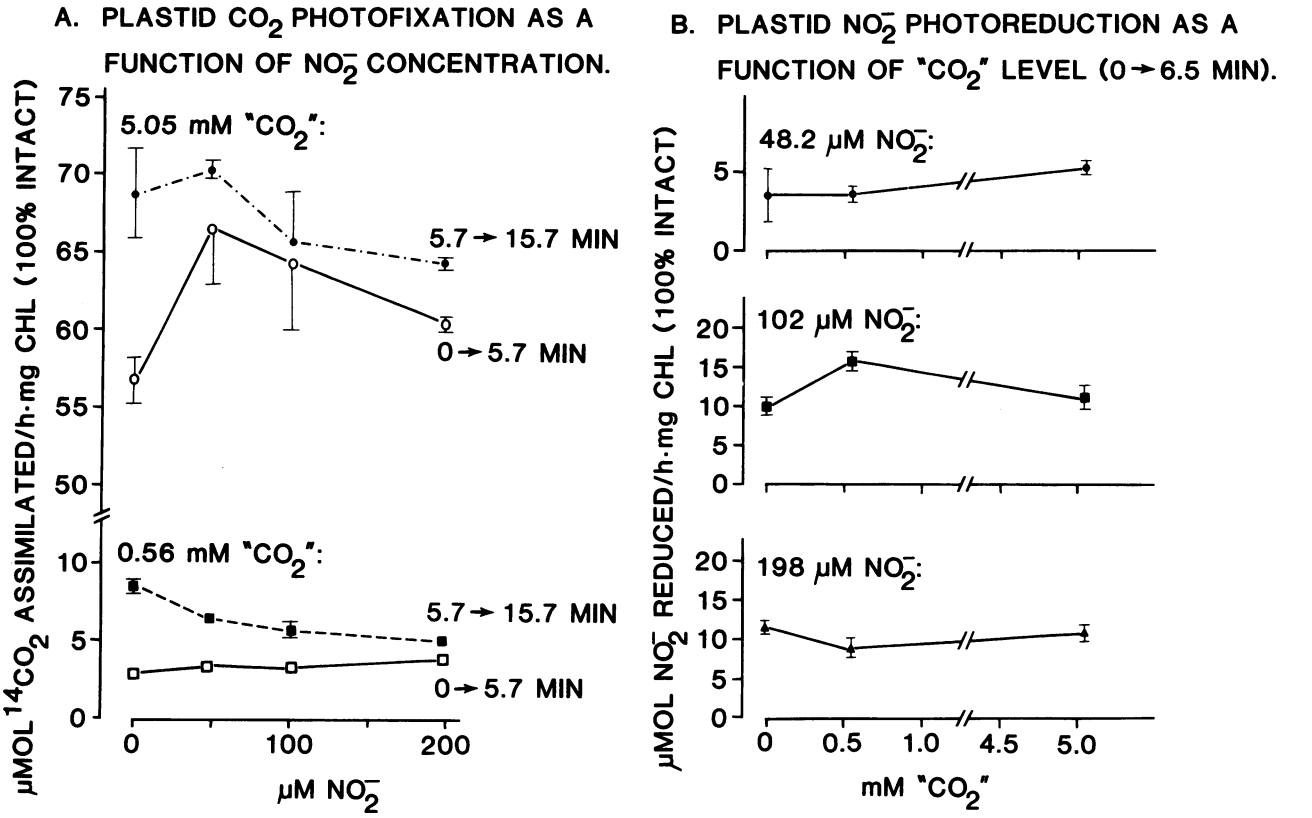

FIG. 2. Properties of plastid CO_2 and NO_2^- photoassimilation. A, Rates of CO_2 photoassimilation as a function of NO_2^- in the plastid reaction mixture at average time intervals of 0 to 5.7 min or 5.7 to 15.7 min with 'CO_2' either rate-limiting or saturating with respect to total CO_2 photofixation. B, Rates, in average time intervals (0–6.5 min) of NO_2^- photoreduction as a function of both NO_2^- level and 'CO_2' level (concomitant CO_2 and NO_2^- photoassimilation where both substrates are present). A and B represents one complete experiment, and all mixtures contained intact plastids equivalent to 24.1 μg Chl. All treatments were replicated, and the absence of standard deviation bars in A indicated ±0.0 μmol CO_2 fixed/h·mg Chl.

the rates of both processes. The experiment displayed in Figure 2 shows the results of simultaneously monitoring both CO_2 photofixation and NO_2^- reduction rate in isolated intact spinach leaf chloroplasts with conditions set where either (a) one or the other processes were absent, or (b) both processes were functioning at low rates, or (c) where one process was functioning at a low rate while the other process was functioning at a maximal rate, or (d) where both processes were functioning at maximal rates.

In the results in Figure 2A, the rates of CO_2 fixation were monitored at 'CO_2' levels both rate-limiting (0.5 mM) and saturating (5.0 mM). Additionally, at each 'CO_2' level, CO_2 fixation rate was measured as a function of NO_2^- reduction at levels of $NaNO_2$ either absent, rate-limiting (50 μM), or saturating (100–200 μM) with respect to light dependent NO_2^- disappearance (plastid NiR). Conversely, in Figure 2B, NO_2^- reduction rates were expressed as a function of either the absence, minimal, or maximal CO_2 photofixation ('CO_2' level), and rates of NO_2^- reduction were measured with NO_2^- levels either rate-limiting or saturating with respect to plastid NiR.

The results (Fig. 2, A and B) made it clear that regardless of whether or not CO_2 photofixation was absent, functioning at a minimal rate (rate-limiting 'CO_2') or functioning at a maximal rate (saturating 'CO_2'), concurrent NO_2^- photoreduction was not repressed, and in some cases it was actually slightly enhanced (Fig. 2B; Fig. 3, Experiment Two). For example, when maximal rates of CO_2 fixation were 65 to 70 μmol/h mg Chl (saturating 'CO_2'), then when initial NO_2^- concentrations were saturating with respect to plastid NO_2^- reduction, the rates of NO_2^- reduction concurrent with CO_2^- assimilation were, when compared to NO_2^- reduction in the absence of CO_2 photoassimilation, either unaffected or slightly enhanced (Fig. 2, A and B). Additionally, when NO_2^- reduction rate was limited by the amount of NO_2^- present, then when CO_2 fixation rate increased, by increasing 'CO_2' level, NO_2^- reduction rate was also slightly increased (Fig. 2B).

In a separate set of experiments displayed in Figure 3, and where NO_2^- concentration was initially approximately 200 μM, time-course kinetics for CO_2 fixation and concomitant NO_2^- reduction are shown for isolated intact spinach plastids. Nitrite is saturating with respect to NO_2^- reduction, and CO_2 is either rate-limiting or saturating with respect to CO_2 photofixation. Again, it was clear that, over time in the light, and regardless of reductant demand by carbon assimilation, there was no inhibition of NO_2^- photoreduction while there was concurrent carbon assimilation. Indeed, in some cases, as reflected in Figure 3, Experiment Two, NO_2^- reduction was stimulated during the first 20 min in the presence of CO_2 photofixation.

In soybean leaf cell isolates (Fig. 4B) it was also clear that, regardless of the magnitude of net CO_2 assimilation, light dependent NO_2^- reduction was not affected by the presence of concurrent carbon assimilation. The rate of NO_2^- photoreduction remained approximately 20 to 22 when the net CO_2 assimilation rate increased by a factor of more than 10-fold by increasing the 'CO_2' from rate-limiting to saturating.

3-PGA and NO_2^- Photoreduction in Plastid Isolates. To better examine the potential for competitiveness between NO_2^- reduction and plastid carbon photoassimilation, the major reductive

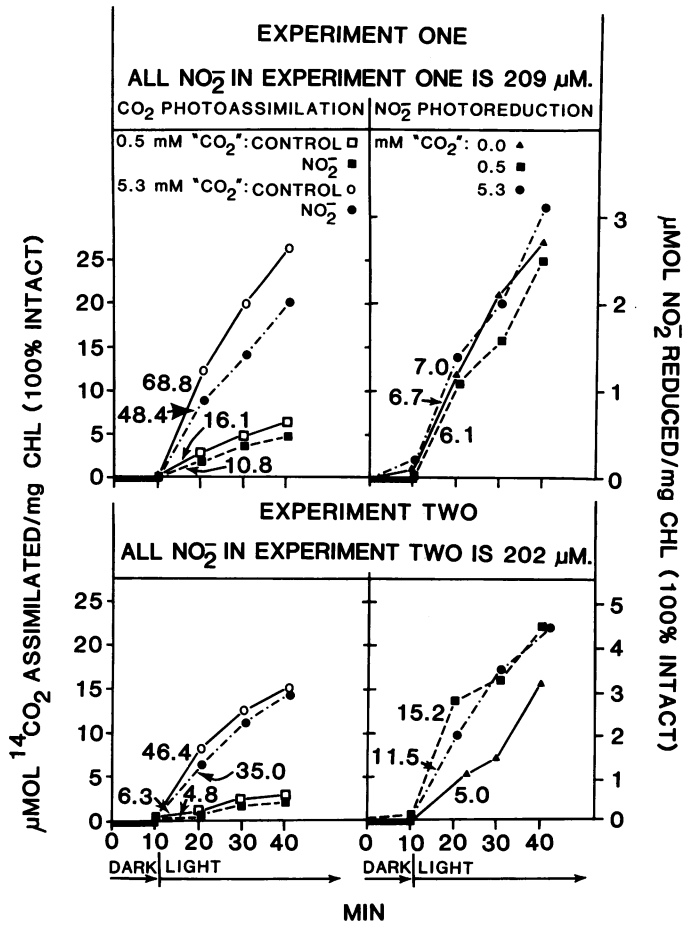

FIG. 3. Time course kinetics of CO_2 photoassimilation and (where applicable) concomitant NO_2^- photoreduction in preparations of intact plastids at levels of 'CO_2' rate-limiting or saturating with respect to CO_2 photoassimilation, and with saturating NO_2^-. Experiments One and Two represented two separate experimental periods, but within each experiment CO_2 photofixation and NO_2^- reduction were simultaneously monitored. In Experiments One and Two: intact plastids were present in the mixtures equivalent to, respectively, 46.5 μg Chl and 20.8 μg Chl. Standard deviations about the means for quantities of CO_2 fixed at each consecutive time point were in the range of ±0.2 to 0.7 μmol CO_2 fixed/mg Chl for treatments at 5.3 mM 'CO_2', and ±0.02 for treatments at 0.5 mM 'CO_2'. For NO_2^- disappearance, time-course point standard deviations ranged from ±0.03 to 0.12 μmol NO_2^-/mg Chl. Numerical values recorded on the time-course traces represent the rates in approximately the first 10 min of illumination, of total CO_2 photofixation or NO_2^- photoreduction, as μmol/h·mg Chl. For CO_2 photofixation rates, at both high and low 'CO_2', standard deviations about the means were, respectively, ±0.5 to 1.0 μmol/h·mg Chl, and for NO_2^- photoreduction rates, deviations were ±0.2 to 1.0 μmol/h·mg Chl.

step of the PPRC, *i.e.* 3-PGA reduction (3-PGA kinase and GAPase) was poised against NO_2^- reduction (NiR) as well as the two other steps in the plastid nitrogen assimilatory pathway (GS and GOGAT). Intact plastid preparations, when supplied with 3-PGA, in the absence of 'CO_2', and in the presence of ADP and Pi, displayed average rates of light dependent 3-PGA disappearance in the range of 120 to 127 μmol/h·mg Chl when NO_2^- was absent (Fig. 5). Although when supplied at 0.5 mM, NO_2^- mediated some mild inhibition of 3-PGA reduction (20–35%), plastid preparations were fed $NaNO_2^-$ at that level to ensure complete saturation of plastid NiR with NO_2^- (Fig. 5 and data not shown). Chloroplast NO_2^- photoreduction was monitored in the absence or presence of 3-PGA photoreduction, and it was clear that NO_2^- photoreduction either was unaffected or that the rate was slightly enhanced in the presence of ongoing 3-PGA photoreduction (Fig. 5 and data not shown).

Since it has been established that Glu and α-KG are rapidly transported across the chloroplast envelope into the plastid stromal compartment (8), and that these intermediates are subsequently metabolized in the assimilation of NH_4^+ in the stromal compartment via GS and GOGAT (2, 32), these intermediates were supplied along with NO_2^- to the plastids. However, neither Glu, nor Glu plus α-KG, enhanced nor inhibited NO_2^- reduction rates beyond those rates observed in the absence of those intermediates (data not shown). This same result occurred independent of the presence or absence of 3-PGA reduction (data not shown). For example, in one experiment the rate of plastid NO_2^- photoreduction with only NO_2^- present (0.5 mM) was 24.6 μmol/h·mg Chl. In the presence of Glu plus α-KG (each at 0.5 mM) the rate was 23.4. With 1 mM 3-PGA, NO_2^-, and Glu plus α-KG, the rate was 26.3; the concurrent rate of 3-PGA reduction was 102.1.

The Influence of NO_2^- upon CO_2 Assimilation. *Soybean Leaf Mesophyll Cell Isolates.* Nitrite stimulated soybean leaf cell net CO_2 fixation 1.3- to 1.5-fold when present in a concentration range from 0.25 to 2.5 mM (Fig. 4A and data not shown). The stimulation of CO_2 fixation was most predominant when the 'CO_2' level was saturating with respect to CO_2 assimilation (Fig. 4A; compare the stimulations of net CO_2 fixation by NO_2^- at high compared with low 'CO_2').

Spinach Leaf Plastid Isolates. In contrast to the influence of NO_2^- upon soybean mesophyll cell CO_2 assimilation, NO_2^- at levels as low as 200 μM may cause an inhibition of intact plastid isolate CO_2 photoassimilation (Figs. 2A, 3). I found, as others have (6, 7, 15, 22), that levels of 500 to 1000 μM NO_2^- mediated 50 to 70% inhibition of intact plastid isolated CO_2 photofixation, even when reaction mixture pH was maintained at 8.1 (data not shown). Since it was clear that levels of NO_2^- *in vivo* do not rise above 200 μM, then in experiments with plastid isolates, I only used NO_2^- at levels of 200 μM or lower (Figs. 1A, 2). However, in some instances even 200 μM NO_2^- was slightly inhibitory to plastid CO_2 fixation (Fig. 3), and the NO_2^- mediated inhibition of CO_2 fixation often was most severe when 'CO_2' level was rate-determining to carbon assimilation. For example, the results in Figure 2A show that in the period 5.7 to 15.7 min, 200 μM NO_2^- mediated 4% inhibition of CO_2 photofixation at high 'CO_2' compared with 40% inhibition at low 'CO_2'.

At lower, more physiological levels of NO_2^-, *e.g.* 50 to 100 μM NO_2^- was slightly stimulatory with respect to CO_2 assimilation and, similar to the situation with soybean leaf cells, this stimulation was most pronounced when the 'CO_2' level was saturating (*e.g.* 5.0 mM). For example, plastid isolates, incubated with high 'CO_2' and 50 μM NO_2^-, displayed 1.2-fold stimulation of CO_2 photoassimilation rate in the first 5 to 6 min of the light period. Although lower in magnitude, this stimulation still was apparent in the subsequent 5.7 to 15.7 min period of illumination (Fig. 2A).

DISCUSSION

Absence of Competition for Reductant. The results in Figures 2 to 5 clearly indicated that in high light, and regardless of whether CO_2 or NO_2^- was rate-determining or saturating, respectively, to plastid carboxylation and NO_2^- reduction, there was no apparent competition for Fdrd at sites where it was required. In contrast, Baysdorfer and Robinson (3, 28), employing a reconstituted spinach leaf chloroplast system (recombined stromal and lamellae fractions) illuminated with saturating white light, found that the terminal enzyme of photosynthetic electron transport (FNR) demanded Fdrd until almost 90% of the available NADP had reduced. At that point, Fdrd became available

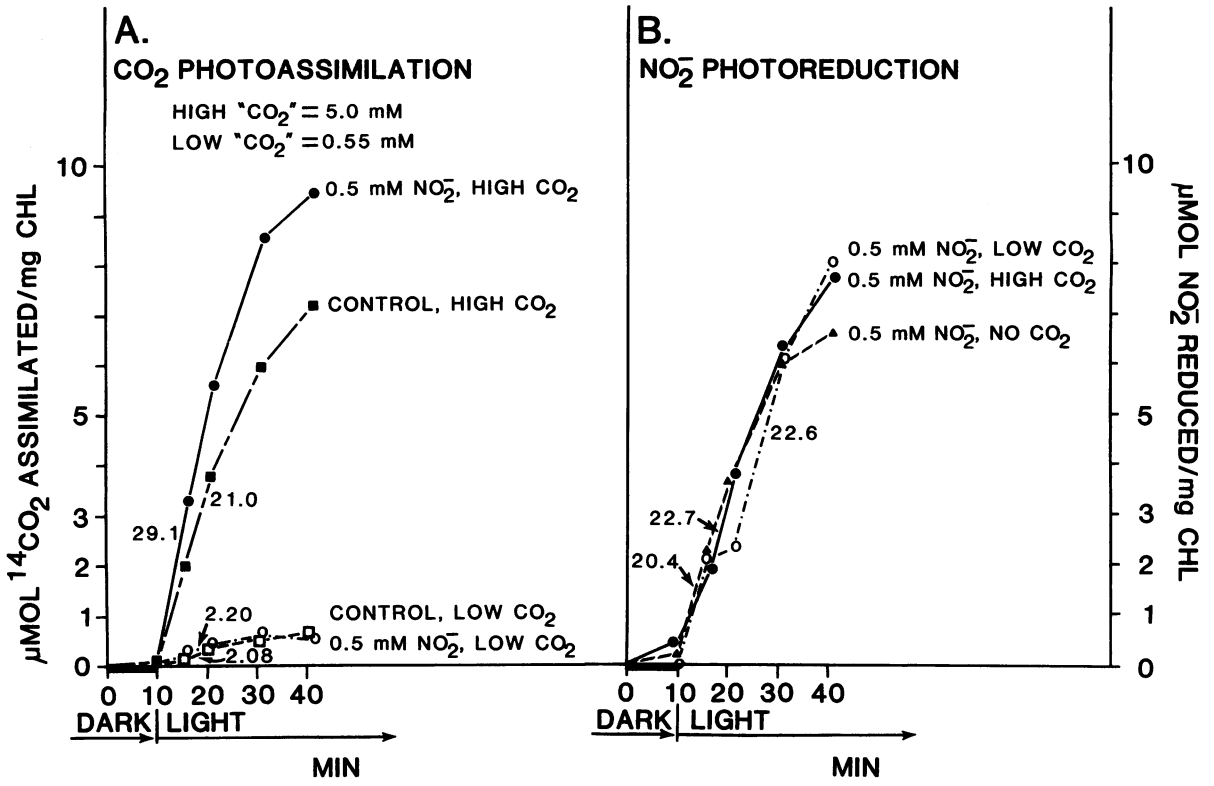

FIG. 4. Simultaneous CO_2 photoassimilation (A) and NO_2^- reduction (B) in isolated, intact soybean leaf mesophyll cells. Concentrations of 'CO_2' were either rate limiting or rate saturating with respect to light dependent CO_2 assimilation, and $NaNO_2$ (where employed) was saturating with respect to cell NiR. Light dependent NO_2^- disappearance was monitored in the absence as well as the presence of CO_2 photofixation as indicated. All treatments were replicated, and intact cells are present in each mixture equivalent to 29.7 μg Chl. Numerical values on the traces represent the most maximal rates of CO_2 fixation or NO_2^- reduction expressed as μmol/h·mg Chl.

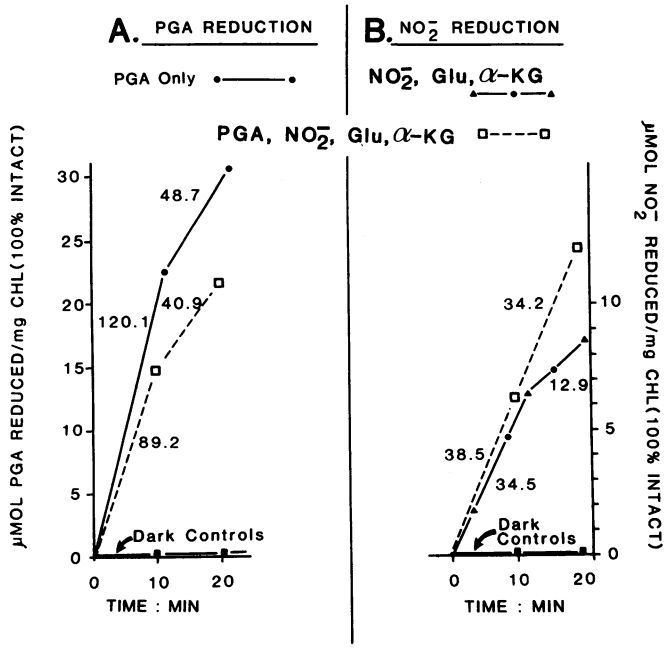

FIG. 5. Intact spinach plastid isolate time-course transients for 3-PGA (A) and NO_2^- (B) photoreduction, concomitantly monitored (where indicated) in the absence of 'CO_2'. Intact plastids are present in each mixture equivalent to 30.1 μg Chl.

to the NiR in the stromal fraction, and NO_2^- reduction was observed to proceed at maximal velocities.

It could be surmised that in the reconstituted chloroplast systems the affinity of FNR for Fdrd was much greater than that of NiR for this reductant. Indeed, the K_m of Fd (presumably Fdrd) for highly purified FNR has been estimated to be as low as 0.33 μM (30), while the K_m of NiR for that protein reductant was estimated to be 10 to as high as 70 μM (13). However, C Baysdorfer and JM Robinson (unpublished data) observed that the K_m of Fd for both FNR and NiR in the lamellae-stromal system was approximately 1 to 5 μM. Therefore, since we (3, 28) employed the Fd in our reconstituted plastid systems in saturating amounts with respect to both FNR, bound to the lamellae, and NiR, soluble in the stromal fraction, it seemed very unlikely that the results derived from the reconstituted system could be explained on the basis of there being a higher affinity of FNR than NiR for Fdrd. Why did the reconstituted plastid systems reflect a competition in high light between FNR and NiR for Fdrd when the intact systems did not?

Obviously, since in the preparations of intact plastids and isolated mesophyll cells, CO_2 and NO_2^- assimilation processes in no way appeared to be mutually exclusive (Figs. 2–5), it must be concluded that in both spinach and soybean leaf plastids there was sufficient Fdrd available *in vivo* (25). Furthermore, if the experiments with the reconstituted plastid systems (3, 28) are compared with the data presented in this report (Figs. 2–5), then I must conclude that there is a mechanism for allocation of Fdrd to NiR which was functional in intact plastids cells, but which was nonfunctional in the reconstituted plastid systems (3, 25). Presently, I must conclude that this mechanism could involve: (a) the binding of molecules of NiR to the Fdox reducing sites,

or (b) there may be electron transport systems which are specialized in delivering Fdrd to NiR, and in the reconstituted chloroplast system (3, 28), these electron transport chains were inactivated. In that reconstructed system (3, 28), NiR could only draw on Fdrd that was being alloted to FNR. When NADP was no longer available, Fdrd was released from the lamellae to NiR in the stromal fraction, and NO_2^- reduction could proceed. Currently these hypotheses are under investigation.

Artifacts Caused by NO_2^- Toxicity. It is unfortunate that NO_2^- may mediate inhibition of CO_2 fixation in intact plastid isolates, because this situation could lead to an erroneous conclusion. Since inhibition of chloroplast CO_2 assimilation was usually proportional to the level of NO_2^- (Fig. 2; Ref. 15), it could be concluded that NiR was the more successful competitor for Fdrd in the intact plastid. Certainly this could not be the case. It must be noted that plastid NO_2^- reduction was saturated with respect to NiR activity at NO_2^- levels of approximately 100 μM (Fig. 2B), and this level of the anion actually slightly stimulated plastid CO_2 assimilation (Fig. 2A). Also, a 200 μM, NO_2^- is usually more than saturating with respect to plastid NiR; that level of the anion is just on the threshold of slight inhibition with respect to CO_2 fixation (Figs. 2A, 3). Additionally, in the results displayed in Figure 3, 200 μM NO_2^- was visibly repressive to CO_2 fixation only in the initial 10 min of illumination; during the subsequent 20 min, sensitivity to the anion was only barely visible. I concluded that NO_2^- assimilation was not repressing carbon assimilation due to successful competition for Fdrd.

Several mechanisms of inhibition appear to be associated with NO_2^- toxicity at sites associated with plastid carbon assimilation. (a) Everson (6) observed that even at levels as low as 200 μM, NO_2^- mediated inhibition of carbonic anhydrase. In correlation with that observation, he demonstrated that the inhibition of CO_2 photofixation could be relieved by increasing the 'CO_2' level in the reaction mixtures. (b) Purczeld *et al.* (22) related the inhibition of plastid photosynthesis by NO_2^- to the prevention of the light dependent alkalization of the plastid stromal compartment mediated by an increased level of H^+ ions. This was caused by the dissociation of HNO_2 which had concentrated in the stromal compartment; the acid form had diffused from the reaction mixture across the plastid outer envelope. Prevention of stromal akalization resulted in loss of activation of FBP (C-1) phosphatase and other rate-determining PPRC enzymes. (c) Enzyme sulfhydryl groups may interact with nitrite to form nitrothiol groups (5) with the result that the active site on an enzyme such as GAPase, which is an -SH group, was blocked (17).

Because I often observed that increased 'CO_2' level almost completely removed NO_2^- mediated inhibition of CO_2 fixation (Fig. 2A), and because there often was observed a slight inhibition of PGA reduction (Fig. 5), I have to conclude that inhibition by NO_2^- was mixed, *i.e.* at several sites. Certainly carbonic anhydrase, PGA kinase, and GAPase appeared to be possible sites of inhibition in the isolated intact chloroplasts, and it is feasible that all of the above cited mechanisms of inhibition are involved. Of cource, to avoid the strongest inhibitions by NO_2^-, and at the same time to examine its assimilation, it was necessary to maintain the anion's concentration below 200 μM (Fig. 2A). In reality, NO_2^- is not toxic with respect to carbon metabolism in leaf tissues, probably because levels of that anion are present below 200 μM (Fig. 1) and, also, because that anion appears to be sequestered in a microbody with nitrate reductase (31). Within this microbody, NO_2^- may be transported across the plastid envelope and into the chloroplast stromal at some controlled rate (31) and, additionally, it may be transported to, and stored in, the cell vacuole. Klepper *et al.* (9) found that NO_2^- could be vacuum infiltrated into leaf tissues to the extent that the anion attained concentrations as high as 1 mM, apparently without detrimental effect upon carbon assimilation. Also, when isolates of soybean leaf mesophyll cells were incubated with $NaNO_2$ at concentrations in the range of 250 μM to 2.5 mM, they displayed enhanced, and not repressed, rates of net CO_2 fixation (Fig. 4A, data not shown); 5 mM NO_2^- caused no inhibition of photosynthesis in isolates of spinach leaf cells (34). Indeed, it also must be concluded that the leaf mesophyll cell is able to control NO_2^- concentrations so that toxicity by the anion is prevented.

Stimulation of Carbon Assimilation by Inorganic N Assimilation. When NO_2^- was present in the spinach plastid preparations at 50 to 100 M, CO_2 fixation rate was stimulated significantly, if 'CO_2' concentration was saturating with respect to carboxylation (Fig. 2A). Similarly, if 'CO_2' level was high, soybean leaf mesophyll cell net CO_2 assimilation was stimulated by NO_2^- at levels in the range of 250 to 2500 μM (Fig. 4A). The results of our previous study (28) also indicated that soybean mesophyll cell isolates displayed maximal stimulation of net CO_2 fixation rate by both NH_4^+ and NO_3^- when 'CO_2' level was high. Woo and Canvin (34) found no stimulation of net CO_2 fixation by NO_3^- in isolates of spinach mesophyll cells. However, this same consortium (33) using spinach cells, as well as Paul *et al.* (19) employing poppy leaf cells, also found that NH_4^+ mediated significant stimulations of net CO_2 fixation when 'CO_2' concentrations were saturating. Because in all leaf cell preparations that have been tested (19, 28, 33), NH_4^+ always mediated a stimulation of CO_2 fixation, I must conclude that there was a metabolic interaction of plastid NH_4^+ assimilation with plastid carbon assimilation that resulted in the stimulation of CO_2 fixation rate. Also, I conclude that since NO_3^- and NO_2^- (Figs. 2A, 4A) are converted to NH_4^+, then the mechanism of stimulation of CO_2 fixation mediated by those anions must be synonymous to that mechanism of stimulation related to NH_4^+ or its assimilation. What remains an enigma is that there was little or no stimulation of CO_2 assimilation by inorganic nitrogen assimilation unless the plastid system sensed that a maximal or near maximal plastid carboxylation activity was present (high 'CO_2') (Figs. 2A, 4A). The exact mechanism and control of this stimulation is under investigation.

However, the identity of the carbon assimilatory enzymes that are stimulated by the onset of inorganic nitrogen assimilation is becoming more certain. For example, the increase in photosynthetic rate in C-3 plant leaves, attributable to increased NO_3^- and NH_4^+, has been found to be correlated with an increase in the total specific activities of important rate-determining PPCR enzyme activities, *e.g.* RuBP carboxylase and FBP (C-1) phosphatase. Importantly, both the total and specific activities of anaplerotic pathway enzymes including pyruvate kinase and phosphoenolpyruvate carboxylase are increased in response to increasing NO_3^- and NH_4^+ (19, 28).

The resultant impact of inorganic nitrogen assimilation upon carbon metabolism in photosynthetic tissues is extremely important to the understanding of photosynthate partitioning controls. The work of Paul *et al.* (19) as well as studies done in this laboratory (28), have made it clear that in higher plant leaves, the onset of inorganic nitrogen assimilation, concomitant with photosynthic carbon assimilation, elicits a shift in the direction of the flow of carbon skeletons away from the synthesis of starch (28), and toward the syntheses of products in the anaplerotic pathway (*e.g.* pyruvate) (19, 28). The rather important final result is that there are significant increases in the magnitudes of the rates of syntheses of organic acids such as malate and citrate as well as those of amino acids such as Ala, Asp, and Gln (19). These increases in amino and organic acid syntheses were attributable, at least in part, to increases in the rate of net and total CO_2 assimilation (19, 28).

Influence of CO_2 Assimilation upon NO_2^- Reduction. Intermediates of nitrogen 'throughput', such as Glu and α-KG, did not serve to mediate any stimulation of plastid NiR activity

("Results"). Also, apparently GS and GOGAT in no way rate-limited the total activity expressed by plastid NiR ("Results").

On the other hand, in some experiments, plastid NO_2^- photoreduction was stimulated during CO_2 fixation or 3-PGA reduction (Figs. 2B; 3, Experiment Two; 5B). This may relate to whether or not plastid NiR is fully activated by one or more carbon flow intermediates. Miflin (16) found that NO_2^- reduction in isolates of intact spinach plastids was significantly stimulated by 100 μM levels of either phosphoenolpyruvate, pyruvate, or a combination of 3-PGA plus FBP. Furthermore, Plaut *et al.* (21) observed, using a reconstituted spinach leaf chloroplast system, that the stimulation of NO_2^- photoreduction, ostensibly caused by CO_2 assimilation, was caused by DHAP or fructose-6-P, generated during PPRC activity. In regard to the data presented in this report, I must conclude that if carbon metabolite effectors are required to fully activate NiR, then, in situations where CO_2 assimilation appeared to be required for full activity of NiR (Figs. 3, Experiment Two; 5B; ref. 21), a pool of the metabolite effector would have had to be synthesized in the early stages of the light period prior to NiR stimulation.

It is not clear why the stimulation of NO_2^- reduction by CO_2 fixation was variable in magnitude, and, in some cases, totally absent (*e.g.* Figs. 3, Experiment One; 4B). It is conceivable that there are situations in which the enzyme would be fully activated even in the darkened intact plastid isolates. This would result if there was available to NiR, prior to the light period, a sufficient pool of an activator compound, *e.g.* DHAP. Certainly the degradation of starch in the chloroplast has been shown to produce triose-P as well as Fdrd (10). Active starch degradation could result in a pool of the positive effector (*e.g.* DHAP and fructose-6-P) that would activate NiR even in the dark.

Acknowledgments—I thank Dr. Freeman W. ('Woody') Snyder for discussion and help with spinach and soybean plant nutrition and growth; Dr. Neal M. Barnett and Mr. Steve J. Corak for many valuable discussions and considerable help during the initial stages of this study; Drs. Chris Baysdorfer and Zvi Plaut for valuable discussions during manuscript preparation; Mr. David R. Lee for foliar gas exchange measurements; Mr. Walter Stracke, Jr. for many dedicated hours of excellent work on isolated soybean leaf cell photosynthesis measurements; Mr. Phillip Romas, Mr. Jeff Strank, and Mr. Jack Thompson for many hours of excellent technical assistance; and Ms. Alice Green for typing the manuscript.

LITERATURE CITED

1. ANDERSON JW, J DONE 1978 Light dependent assimilation of nitrite by isolated pea chloroplasts. Plant Physiol 61: 692–697
2. ANDERSON JW 1981 Light-energy-dependent processes other than CO_2 assimilation. *In* MD Hatch, NK Boardman, eds, The Biochemistry of Plants, Vol 8, Photosynthesis. Academic Press, New York, pp 473–500
3. BAYSDORFER C, JM ROBINSON 1985 Metabolic interactions between spinach leaf nitrite reductase and ferredoxin-NADP reductase: competition for reduced ferredoxin. Plant Physiol 77: 318–320
4. BUCHANAN BB 1980 Role of light in the regulation of chloroplast enzymes. Annu Rev Plant Physiol 31: 341–374
5. BYLER DM, DK GOSSER, H SUSSI 1983 Spectroscopic estimation of the extent of S-nitrothiol formation by nitrite action on sulfhydryl groups. J Agric Food Chem 31:523–527
6. EVERSON RG 1970 Carbonic anhydrase and CO_2 fixation in isolated chloroplasts. Phytochemistry 9: 25–32
7. GRANT BR, DT CANVIN 1970 The effect of nitrate and nitrite on oxygen evolution and carbon dioxide assimilation and the reduction of nitrate and nitrite by intact chloroplasts. Planta 95: 227–246
8. HEBER U, HW HELDT 1981 The chloroplast envelope: structure, function, and role in leaf metabolism. Annu Rev Plant Physiol 32: 139–168
9. KLEPPER L, D FLESHER, RH HAGEMAN 1971 Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves. Plant Physiol 48: 580–590
10. KOW YW, DL ERBES, M GIBBS 1982 Chloroplast respiration. A means of supplying oxidized pyridine nucleotide for dark chloroplastic metabolism. Plant Physiol 69: 442–447
11. LARSSON M, T OLSSON, C-M LARSSON 1985 Distribution of reducing power between photosynthetic carbon and nitrogen assimilation in *Scenedesmus*. Planta 164: 246–253
12. LATZKO E, M GIBBS 1974 Measurement of the intermediates of the photosynthetic carbon reduction cycle using enzymatic methods. Methods Enzymol 24: 261–268
13. LOSADA M, A PANEQUE 1971 Nitrite reductase from spinach. Methods Enzymol 23: 487–491
14. MACKINNEY G 1941 Absorption of light by chlorophyll solutions. J Biol Chem 140: 315–322
15. MAGALHAES AC, CA NEYRA, RH HAGEMAN 1974 Nitrite assimilation and amino nitrogen synthesis in isolated spinach chloroplasts. Plant Physiol 53: 411–415
16. MIFLIN BJ 1974 Nitrite reduction in leaves: studies on isolated chloroplasts. Planta 116: 187–196
17. O'LEARY V, M SOLBERG 1976 Effect of sodium nitrite inhibition on intracellular thiol groups and on activity of certain glycolytic enzymes in *Clostridium perfringes*. Appl Environ Microbiol 31: 208–212
18. OLIVER DJ, JH THORNE, R POINCELOT 1979 Rapid isolation of mesophyll cells from soybean. Plant Sci Lett 16: 149–155
19. PAUL JS, KL CORNWELL, JA BASSHAM 1978 Effects of ammonia on cell metabolism in photosynthesizing isolated mesophyll cells from *Papaver somniferum* L. Planta 142:49–54
20. PEIRSON D 1985 Effect of bicarbonate on *in vivo* nitrite utilization of bush bean leaves. Plant Physiol 77: S-8
21. PLAUT Z, K LENDZIAN, JA BASSHAM 1977 Nitrite reduction in reconstituted whole chloroplasts and spinach during carbon reduction. Plant Physiol 59: 184–188
22. PURCZELD P, CJ CHON, AR PORTIS JR, HW HELDT, U HEBER 1978 The mechanism of the control of carbon fixation by the pH in the chloroplast stroma. Studies with nitrite-mediated proton transfer across the envelope. Biochim Biophys Acta 501: 488–498
23. REED JA, DT CANVIN, JH SHERRARD, RH HAGEMAN 1983 Assimilation of ^{15}N nitrate and ^{15}N nitrite in leaves of five plant species under light and dark conditions. Plant Physiol 71: 291–294
24. REED JA, DT CANVIN 1983 Light and dark controls of nitrate reduction in wheat (*Triticum aestivum* L.) protoplasts. Plant Physiol 69: 508–513
25. ROBINSON JM 1977 The relationship of chloroplast envelope permeability to plastid carbon metabolism. PhD thesis. University of California, Davis, pp 142–145
26. ROBINSON JM, MG SMITH, M GIBBS 1980 Influence of hydrogen peroxide upon carbon dioxide photoassimilation in the spinach chloroplast. I. Hydrogen peroxide generated by broken chloroplasts in an "intact" chloroplast preparation is a causal agent of the Warburg effect. Plant Physiol 65: 755–759
27. ROBINSON JM 1984 Photosynthetic carbon metabolism in leaves and isolated chloroplasts from spinach plants grown under short and intermediate photosynthetic periods. Plant Physiol 75: 397–409
28. ROBINSON JM, C BAYSDORFER 1985 Interrelationships between carbon and nitrogen metabolism in mature soybean leaves and isolated leaf mesophyll cells. *In* RL Heath, J Preiss, eds, Regulation of Carbon Partitioning in Photosynthetic Tissues. American Society of Plant Physiologists, Rockville, MD, pp 333–357
29. SCHOLL RL, JE HARPER, RH HAGEMAN 1974 Improvement of nitrite color development in assays of nitrate reductase by phenazine methosulfate and zinc acetate. Plant Physiol 53: 824–828
30. SHIN M 1974 Ferredoxin-NADP reductase from spinach. Methods Enzymol 23: 440–447
31. VAUGHN KC, SO DUKE 1981 Histochemical localization of nitrate reductase. Histochemistry 72: 191–198
32. WALLSGROVE RM, AJ KEYS, P LEA, BJ MIFLIN 1983 photorespiration and nitrogen metabolism. Plant Cell Environ 6: 301–309
33. WOO KC, DT CANVIN 1980 Effect of ammonia on photosynthetic carbon fixation in isolated spinach leaf cells. Can J Bot 58: 505–510
34. WOO KC, DT CANVIN 1980 Effect of ammonia, nitrite, glutamate, and inhibitors of N metabolism on photosynthetic carbon metabolism in isolated spinach leaf cells. Can J Bot 58: 511–516