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The Glyceraldehyde 3-Phosphate and Glycerate 3-Phosphate Shuttle and Carbon Dioxide Assimilation in Intact Spinach Chloroplasts[1]

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ABSTRACT

The regulation of CO_2 assimilation by intact spinach (*Spinacia oleracea*) chloroplasts by exogenous NADP-linked nonreversible D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) was investigated. This dehydrogenase mediated a glyceraldehyde 3-phosphate/glycerate 3-phosphate shuttle for the indirect transfer of NADPH from chloroplast to the external medium. The rate of NADPH formation in the medium reflected glyceraldehyde 3-phosphate efflux from the chloroplast. Increasing enzyme concentrations stimulated NADP reduction and, in turn, CO_2 fixation. Pyrophosphate increased CO_2 fixation by apparently inhibiting glyceraldehyde 3-phosphate efflux. Increasing the glycerate 3-phosphate concentration above 0.1 mM stimulated glyceraldehyde 3-phosphate efflux but inhibited CO_2 fixation. Addition of up to 0.5 mM orthophosphate enhanced both glyceraldehyde 3-phosphate efflux and CO_2 fixation while each was inhibited by higher orthophosphate concentrations. The mechanism by which the extent of glyceraldehyde 3-phosphate efflux regulated the rate of CO_2 fixation in chloroplasts was discussed.

During photosynthesis, NADPH and ATP are produced and subsequently utilized to drive the photosynthetic carbon reduction cycle. Photosynthetically generated ATP and NADPH are not directly available for extra chloroplastic reactions due to the impermeability of the inner chloroplast membrane to these compounds (11, 13). However, a shuttle system involving G3P[4] and PGA, both of which move readily across the chloroplast envelope (3, 13), has been reported for the indirect transfer of NADH and ATP from chloroplast to cytoplasm (12, 22). This shuttle system requires the oxidation of G3P to PGA in the cytoplasm by two enzymes: NAD-linked reversible D-glyceraldehyde-3-P dehydrogenase and 3-P-glycerate kinase and is dependent on the concentrations of ATP, ADP, NADH, NAD, and Pi.

Recently, Kelly and Gibbs (16) proposed another shuttle system for the indirect transfer of photosynthetically reduced NADP from chloroplast to cytoplasm. According to this scheme, G3P which is formed from PGA in the chloroplast, moves into the surrounding milieu where it is reoxidized to PGA by NR-G3P-DH. Extra chloroplastic NADPH is generated in this oxidation; PGA returns to the chloroplast carbon reduction cycle, completing the shuttle.

Cytoplasmic NADPH formation thus reflects the rate of G3P efflux from the chloroplast. Stoichiometric studies indicate that 1 mole of PGA is formed and 1 mole of NADP is reduced for each mole of G3P oxidized. The irreversible G3P dehydrogenase is particularly suitable for this role, since the affinity for G3P and NADP is the lowest of all the triose-P dehydrogenases hitherto characterized in the plant cell (19) and the enzyme is located in the cytoplasm (17).

Kelly and Gibbs (16) observed a stimulatory effect of this shuttle system on light-dependent O_2 evolution in intact spinach chloroplasts. We have further investigated the kinetics of the shuttle along with its effect on CO_2 fixation in intact spinach chloroplasts. Additionally, we have studied the effect of several components on the shuttle system, including the concentrations of NR-G3P-DH, PGA, Pi, and PPi. Finally, the importance of the shuttle system in studying the known inhibition of CO_2 fixation by high concentrations of Pi and PGA, as well as the stimulation caused by PPi and low concentrations of PGA and Pi, are considered.

MATERIALS AND METHODS

Chloroplast Preparation. Intact chloroplasts were isolated from spinach leaves by a method similar to the procedure of Jensen and Bassham (14). Chopped leaves (10–15 g) were homogenized in a VirTis homogenizer (Model 45 set at 65 v) for 3 to 5 sec at 0 C in 50 ml of a solution, pH 6.8, containing 50 mM HEPES-NaOH, 330 mM sorbitol, 2 mM EDTA, 2 mM $MgCl_2$, 5 mM $Na_4P_2O_7$, and 5 mM sodium ascorbate. The homogenate was filtered through Miracloth and centrifuged at 2000*g* for 50 sec. The pellet was resuspended in a small volume of the solution used above, except that no ascorbate was added and the pH was set at 7.6. Chlorophyll was assayed by the method of Arnon (1).

Spinach (*Spinacia oleracea* L. var. Savoy, Winter Bloomsdale, and Bloomsdale Longstanding) was grown in sterile potting soil mixed with vermiculite. Leaves approaching

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[4] G3P: D-glyceraldehyde 3-phosphate; PGA: 3-phosphoglyceric acid; NR-G3P-DH: nonreversible D-glyceraldehyde-3-phosphate dehydrogenase; R5P: D-ribose 5-phosphate; FDP: D-fructose-1,6-diphosphate; DHAP: dihydroxyacetone phosphate; G6P: D-glucose 6-phosphate.

maturity were taken from plants 6 to 10 weeks of age. All experiments were carried out with individual preparations of the three spinach varieties. We recognize the high variability in the CO_2 fixation rates and believe them to be the result of changes in the environmental growth conditions.

Determination of G3P Efflux and CO_2 Fixation. G3P efflux and CO_2 fixation were evaluated in two ways. In one, $^{14}CO_2$ and NADP reduction (G3P efflux) were determined together in the same test tubes placed in a water bath illuminated with Sylvania flood lamps. Samples were removed at intervals for the determination of $^{14}CO_2$ assimilated, and NADPH was assayed enzymically with L-glutamic acid dehydrogenase. In the second procedure, they were measured simultaneously in a cuvette in an Eppendorf 1101m fluorimeter. NADP reduction was measured by recording the increase in fluorescence due to NADPH formation. The rates of NADP reduction obtained in the light were corrected for the rates of NADP reduction obtained under the same conditions in the dark. Thus the NADPH values represent the light-dependent efflux of G3P from the chloroplast. It was assumed in these calculations that the rate of G3P formation from an endogenous store such as a polyglucan was not affected by light.

Table I. *Effect of NR-G3P-DH on Rates of CO_2 Fixation, O_2 Evolution, and G3P Efflux (NADP Reduction) out of Chloroplast*

$^{14}CO_2$ fixation and NADP reduction were carried out in the same test tubes placed in a 25 C water bath illuminated for 10 min with two Sylvania flood bulbs so that the total light intensity was 2000 ft-c. The 1-ml reaction mixture set at pH 8.3 contained: 0.5 mM NADP, 50 mM HEPES-KOH, 330 mM sorbitol, 2 mM EDTA, 2 mM $MgCl_2$, 1 mM $Na_4P_2O_7$, and either 1 mM unlabeled $NaHCO_3$ or 5 mM $NaHCO_3$ containing 1.5×10^6 cpm. PGA, R5P, or FDP (final concentration 1 mM) was included in the reaction mixture as indicated. One enzyme unit of NR-G3P-DH was placed in the appropriate tubes. The concentration of Chl was 40 μg/ml. Parallel reactions were carried out in test tubes covered with aluminum foil. CO_2 fixation was determined as described under "Materials and Methods." NADPH was assayed enzymically (after removal of the chloroplasts by centrifugation) with L-glutamic acid dehydrogenase and followed in a spectrophotometer at 340 nm.

Oxygen evolution was determined in parallel reaction mixtures with the same composition and chloroplast preparation as for the $^{14}CO_2$ fixation and NADP reduction determinations. The reaction mixtures were contained in a 1-ml compartment connected to a Clark-type oxygen electrode which had been previously calibrated. Oxygen evolution was recorded during 10 min of photosynthesis with a light intensity of 2000 ft-c. Actinic light was provided from a 500-w projector lamp filtered through a saturated $CuSO_4$ solution.

Component Added	O_2 Evolution		CO_2 Fixation		NADP Reduction (Light)		NADP Reduction (Dark)
	−Enzyme	+Enzyme	−Enzyme	+Enzyme	−Enzyme	+Enzyme	+Enzyme
	μmoles/mg Chl·hr						
1 mM $NaHCO_3$	4	12			1	5	0.6
5 mM $NaHCO_3$	15	22	17	22	1	3	1
5 mM $NaHCO_3$, 1 mM PGA	13	16	13	16	3	8	1
5 mM $NaHCO_3$, 1 mM R5P	15	18	15	21	2	18	12
5 mM $NaHCO_3$, 1 mM FDP	14	18	14	19	3	21	22

In both experimental procedures, the photosynthetic rate was measured in terms of the fixation of $^{14}CO_2$ into acid-stable products. After following NADP reduction for the desired time period, 0.6 ml of the reaction mixture was transferred to a test tube containing 0.1 ml of 30% trichloroacetic acid. The suspension was centrifuged at maximum speed in a bench-top centrifuge for 5 to 10 min, and a 0.2-ml sample was analyzed for radioactivity. Excess $^{14}CO_2$ was removed from the samples by placing the aliquots in glass tubes kept in a desiccator under aspirator vacuum. $^{14}CO_2$ fixed was determined by the addition to the samples of 0.5 ml of H_2O and 3.5 ml of scintillation fluid (100 g of naphthalene, 5 g of PPO, and dioxane to a total volume of 1 liter) and counting the radioactivity in a Beckman LS-255 liquid scintillation counter using the $^3H + ^{14}C$ window. The total $^{14}CO_2$ radioactivity introduced into the reaction mixture was determined by placing 0.05 ml of the reaction mixture in 4.95 ml of 1 M unbuffered tris solution and withdrawing 0.1 ml for radioactivity determination.

The following chemicals and enzymes were obtained from Sigma Chemical Co.: ATP, NADP, NADPH, NADH, α-ketoglutarate, R5P, FDP, PGA, DHAP, L-glutamic acid dehydrogenase, triose-P isomerase. All chemicals were prepared as sodium salts at the recommended pH before use. $NaH^{14}CO_3$ was obtained from the Amersham-Searle Co.

Nonreversible-D-G3P dehydrogenase (EC 1.2.1.9) was purified 100-fold from 13-day dark-grown pumpkin (*Cucurbita pepo* L. var. Spookie) cotyledons and assayed according to Kelly and Gibbs (17). One enzyme unit is the amount of enzyme which catalyzes the reduction of 1 μmole NADP/hr at pH 7.6 and 25 C.

RESULTS

Effect of NR-G3P-DH on CO_2 Fixation, Oxygen Evolution, and G3P Efflux. NR-G3P-DH brought about an increase in CO_2 fixation, O_2 evolution, and exogenous NADP reduction in the presence of bicarbonate, PGA, R5P, or FDP (Table I). The effect of R5P or FDP on NADP reduction was difficult to evaluate because of the high rates observed in the dark. We, therefore, refrained from the use of these two sugar phosphates in future experiments.

Effect of $NaHCO_3$ Concentration on Shuttle System. In this experiment, the rates of CO_2 fixation and G3P efflux were measured as a function of increasing concentrations of bicarbonate. This resulted in a rise in CO_2 fixation concomitant with an increase in G3P efflux, as reflected by a rise in NADP reduction (Fig. 1). Between 0.5 and 5 mM $NaHCO_3$, the ratio of NADP/CO_2 remained fairly constant at about 0.4.

Kinetics of CO_2 Fixation and G3P Efflux in Presence of NR-G3P-DH. In order to study the effect of the shuttle system on the time course of CO_2 fixation, the rates of exogenous NADP reduction and CO_2 fixation were measured in the presence and in the absence of NR-G3P-DH with and without 0.1 mM PGA. The results presented in Figure 2A show a significant stimulatory effect of the enzyme on CO_2 fixation. These rates were higher with 0.1 mM PGA both in the presence and absence of enzyme. We also obtained a corresponding increase in G3P efflux (Fig. 2B), and once again these rates were greater in the presence of 0.1 mM PGA.

G3P Efflux in Light and in Dark. G3P efflux was measured with and without 0.1 mM PGA in the presence and in the absence of NR-G3P-DH (Table II). Each reaction was kept in the dark for the first 5 min, followed by 15 min of light, and finally 5 more min in the dark. We observed higher rates of G3P efflux with 0.1 mM PGA both with and without enzyme. The initial rates of G3P efflux during the first dark period were identical to those of the second dark period in the case of

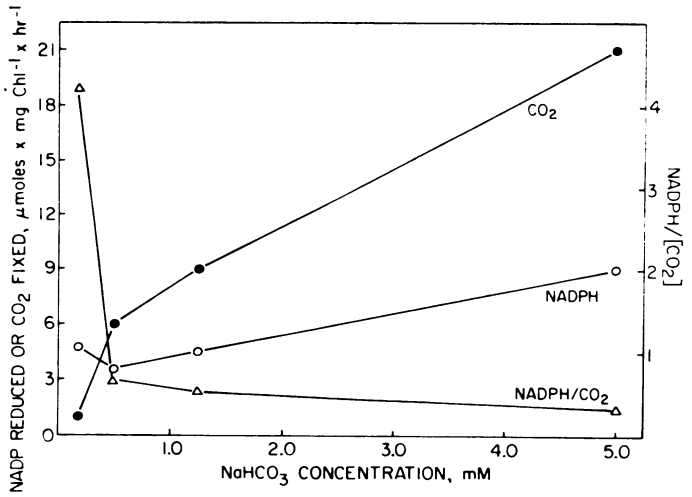

FIG. 1. Effect of $NaHCO_3$ concentration on the rate of CO_2 fixation and the efflux of G3P in the presence of NR-G3P-DH. CO_2 fixation and G3P reduction were determined as described in Table I. The reaction mixture of 1 ml contained the same components as listed in Table I but with 0.5 mM Pi in place of $Na_4P_2O_7$. The Chl concentration was 25 μg/ml. Rate of CO_2 fixation (●); rate of NADP reduction (○); ratio NADPH (G3P efflux)/CO_2 (△).

Table II. *Effect of NR-G3P-DH on Rate of G3P Efflux in Dark, in Light, and in Dark after a Light Period*

NADP reduction was followed fluorimetrically in a 2-ml reaction mixture as described in Figure 3 initially for 5 min in the dark. Then the actinic light source was turned on for 15 min followed by a 5-min dark period. The Chl concentration was 24 μg/ml and 2 units of NR-G3P-DH were added to the appropriate reaction mixture.

Substrates	Dark	Light			Dark
	0–5 min	5–10 min	10–15 min	15–20 min	20–25 min
	μmoles NADP reduced/mg Chl				
5 mM $NaHCO_3$	0.01	0.07	0.06	0.04	0.01
5 mM $NaHCO_3$, enzyme	0.03	0.11	0.18	0.12	0.03
5 mM $NaHCO_3$, 0.1 mM PGA	0.02	0.07	0.07	0.07	0.01
5 mM $NaHCO_3$, 0.1 mM PGA, enzyme	0.05	0.20	0.25	0.26	0.16

CO_2 minus enzyme and for CO_2 plus enzyme, where both dark period rates of NADP reduction were 0.01 and 0.03 μmole NADP/mg Chl, respectively. For CO_2 and PGA minus enzyme, the rate of G3P efflux during the first dark period was 0.02, whereas for the second dark period the rate was 0.01. In addition, in these three reactions the rate of G3P efflux was, on the average, seven times greater during the three 5-min light intervals than during either of the two dark periods. Even in the reaction containing CO_2, PGA, and enzyme, where G3P efflux during the second dark period was three times greater than during the first dark period (0.16 as compared to 0.05), the rate of G3P transport during the three 5-min light periods was, on the average, 50% greater than during this second dark period.

Based on these results, we designed an experiment to determine the substrate for this dark NADP reduction. There were two possibilities: (*a*) the action of NR-G3P-DH on G3P derived from the catabolism of starch released from ruptured chloroplasts or (*b*) the oxidation of G6P also derived from starch by glucose-6-P dehydrogenase with the generation of

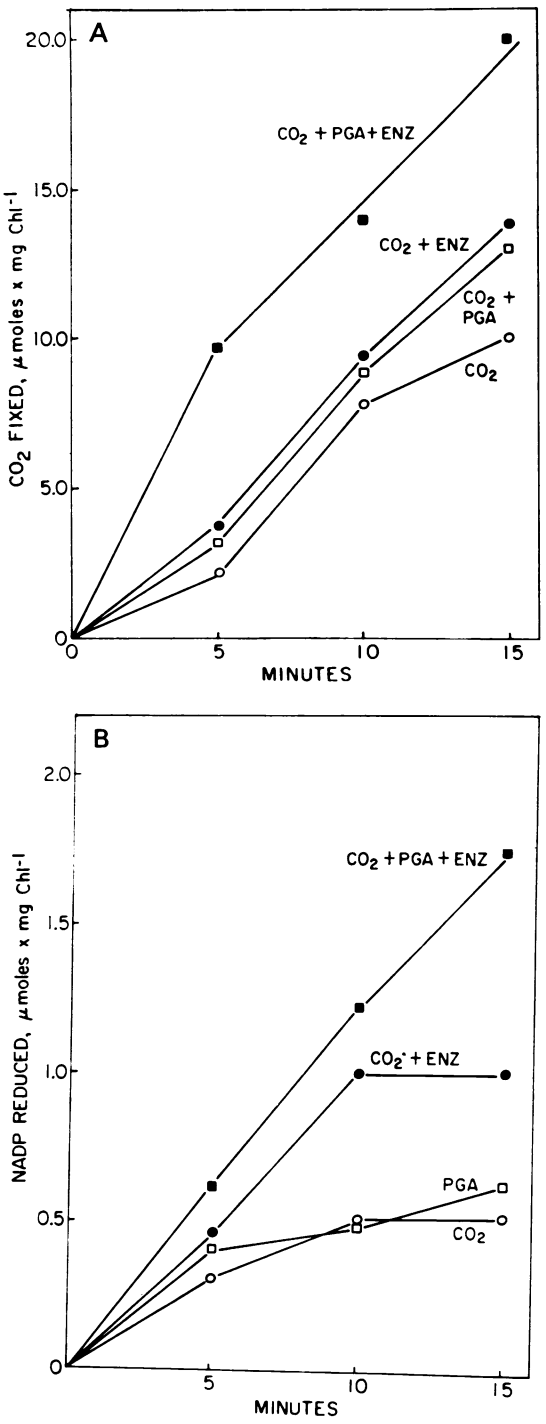

FIG. 2. Kinetics of CO_2 fixation (A) and of G3P efflux in the presence of NR-G3P-DH (B). CO_2 fixation and G3P reduction were determined as described in Table I. The reaction mixture of 1 ml contained the same components as listed in Table I but with the addition of 0.15 mM Pi in place of $Na_4P_2O_7$ and with the pH at 7.6 the Chl concentration was 40 μg/ml.

NADPH. In order to investigate (*a*), the intact chloroplast preparations were incubated with concentrations of ATP, up to 4 mM, because the nucleotide triphosphate would be required for the conversion of starch to G3P. Inhibition of the rate of NADP reduction by increasing levels of ATP (data not shown) indicated that the dark NADPH formation observed, probably did not result from polysaccharide breakdown to triose-P. On the other hand, the addition of G6P resulted in a stimulation of

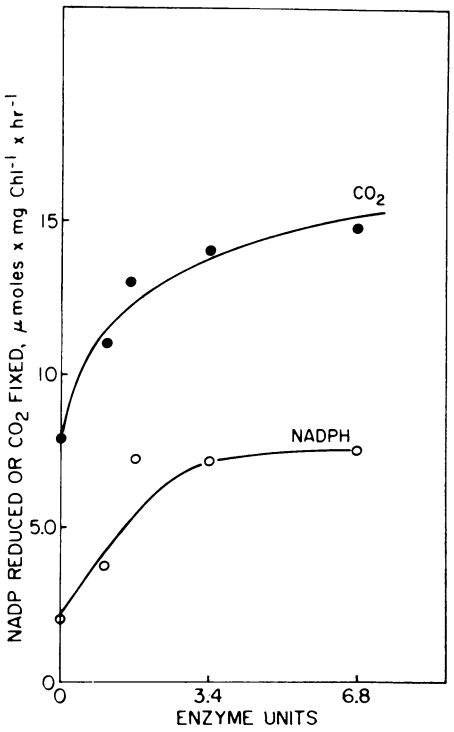

FIG. 3. Rate of CO_2 fixation and G3P efflux as a function of NR-G3P-DH concentration. CO_2 fixation (●), and G3P efflux (○) from the chloroplast were measured in the same cuvette in an Eppendorf 1101M fluorimeter modified for actinic light illumination of the cuvette and connected to a Beckman 25-cm recorder. The fluorescent exciting light was passed through an Eppendorf Hg 313 + 363 nm filter. The photomultiplier was protected by a Rohn and Haas Plexiglas No. 2424 filter. Actinic light was provided by a 500-w projector lamp filtered through a Baird-Atomic, Inc. No. 6400 interference filter. The incident light intensity was of 2.4 erg cm^{-2} sec^{-1}. Chloroplasts containing 18 mg of Chl were placed in a cuvette containing in a volume of 1 ml: 0.5 mM NADP, NR-G3P-DH, 50 mM HEPES-KOH, 330 mM sorbitol, 2 mM EDTA, 2 mM $MgCl_2$, 5 mM $NaH^{14}CO_3$, containing 1 to 5 × 10^6 cpm. This reaction mixture was set at pH 7.6. In this experiment, PPi was omitted from the chloroplast resuspension fluid. Experiments were carried out at room temperature with the actinic light source turned on after 2 min of equilibration in the dark. NADP reduction, during both the dark equilibration and light periods, was measured by recording the increase in fluorescence due to NADPH formation. NADPH fluorescence was standardized against a known amount of authentic NADPH oxidized enzymatically with L-glutamic acid dehydrogenase and followed at 340 nm. The rate of photosynthesis was measured as radioactivity in acid-stable products.

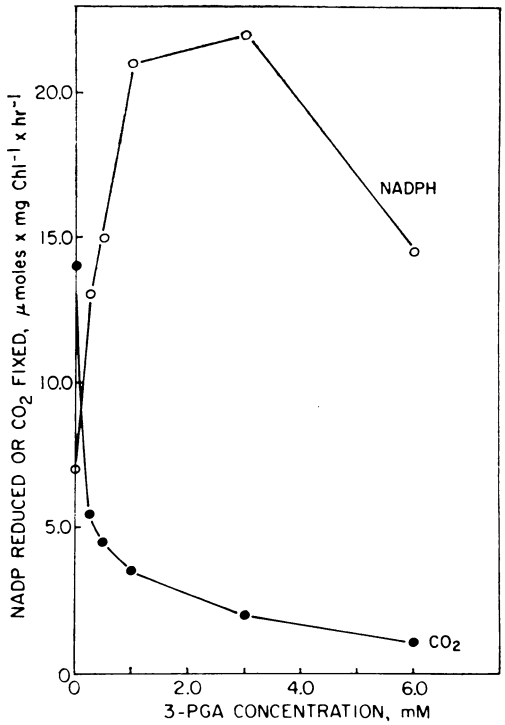

FIG. 4. Rate of CO_2 fixation and G3P efflux as a function of PGA concentration in the presence of NR-G3P-DH. CO_2 fixation (●) and NADP reduction (○) were determined simultaneously as described in the legend to Figure 3. The Chl concentration was 18 μg/ml and 3.4 enzyme units of NR-G3P-DH were placed in each reaction mixture. Pyrophosphate was deleted from the chloroplast resuspension solution.

NADP reduction (data not shown), suggesting that at least part of the dark NADP reduction was due to endogenous glucose-6-P dehydrogenase activity.

Effect of NR-G3P-DH Concentration on CO_2 Fixation and G3P Efflux. Figure 3 shows the result of increasing concentrations of NR-G3P-DH on CO_2 fixation and NADP reduction. Between 0 and 3.4 enzyme units, exogenous NADPH formation and CO_2 fixation increased from 2 to 7.1 and from 7.9 to 14 μmoles/mg Chl·hr., respectively, while at higher enzyme concentrations these rates leveled off.

Effect of PGA, Orthophosphate, and Pyrophosphate on CO_2 Fixation and G3P Efflux in Presence of NR-G3P-DH. Low concentrations of PGA (less than 0.1 mM) are known to stimulate the rate of CO_2 fixation in intact spinach chloroplasts, while a marked inhibition is observed at higher concentrations (2–4). As shown in Figure 4, the addition of increasing concentrations of PGA to the reaction mixture resulted in an inhibition of CO_2 fixation along with an increase in NADP reduction. Between 3 and 6 mM PGA, however, NADP reduction decreased, while CO_2 fixation continued to fall slightly.

Several studies have shown that concentrations of Pi, less than 0.5 mM, stimulate the rate of CO_2 fixation in intact chloroplasts, while concentrations above 0.5 mM cause an inhibition (5–8, 10). In the experiment depicted in Figure 5, CO_2 fixation and G3P efflux were measured as a function of Pi concentration. At concentrations less than 0.5 mM, we observed increases in both CO_2 fixation and G3P efflux. Conversely, at higher concentrations of Pi, G3P efflux declined, paralleling a decrease in CO_2 fixation.

Pyrophosphate is routinely added to intact chloroplasts because this compound stimulates the rate of CO_2 fixation (14, 18, 21). Increasing PPi concentrations led to a 48% decrease in G3P loss while doubling the rate of CO_2 fixation (Fig. 6). Studies in this laboratory demonstrated that PPi had no effect on NR-G3P-DH. Five mM PPi alleviated, to some extent, the inhibitory effects of increasing concentrations of PGA on CO_2 fixation (Fig. 7). An immediate inhibition of CO_2 fixation was observed in the presence of 5 mM PPi and increasing Pi concentrations (Fig. 8). This finding contrasts with that recorded in Figure 5, where CO_2 fixation was stimulated at low levels of Pi.

DISCUSSION

The observation of Kelly and Gibbs (16) that NR-G3P-DH doubled the rate of light-dependent O_2 evolution in intact spinach chloroplasts was confirmed along with the finding that NR-G3P-DH enhanced the rate of CO_2 fixation (Table I).

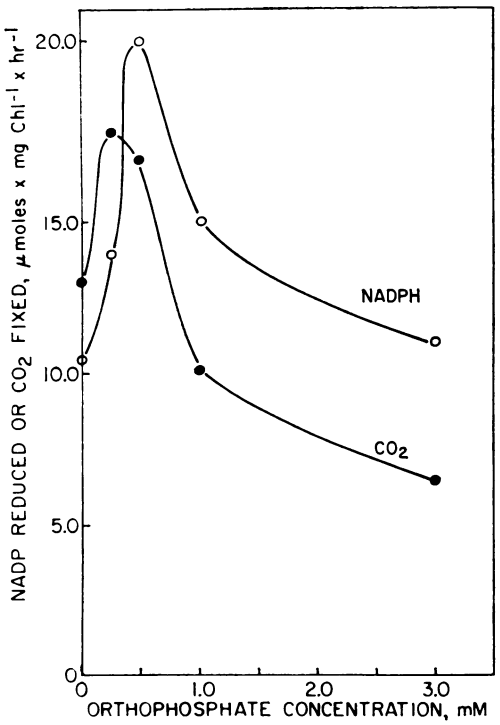

FIG. 5. Rate of CO_2 fixation and G3P efflux as a function of orthophosphate concentration in the presence of NR-G3P-DH. CO_2 fixation (●) and NADP reduction (○) were simultaneously determined as described in the legend to Figure 3. The Chl concentration was 20 μg/ml, and 3.4 enzyme units of NR-G3P-DH were placed in each reaction mixture. Pyrophosphate was deleted from the chloroplast resuspension solution.

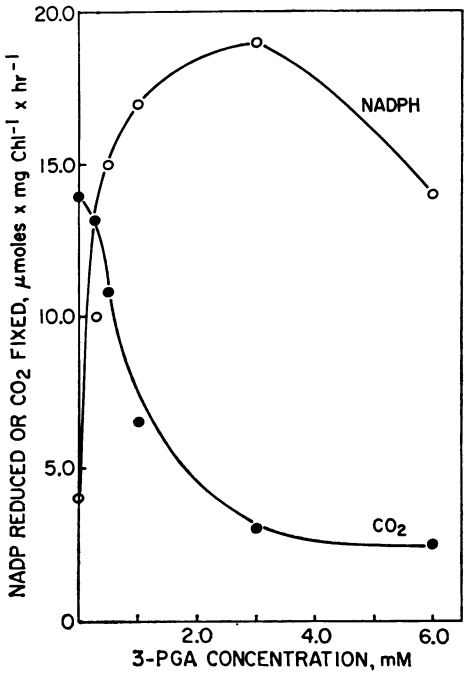

FIG. 7. Rate of CO_2 fixation and the leak of G3P as a function of PGA concentration in the presence of pyrophosphate and NR-G3P-DH. CO_2 fixation (●) and NADP reduction (○) were simultaneously determined as described in the legend to figure 3. The Chl concentration was 18 μg/ml, and 3.4 enzyme units of NR-G3P-DH were added to each reaction mixture including 5 mM PPi.

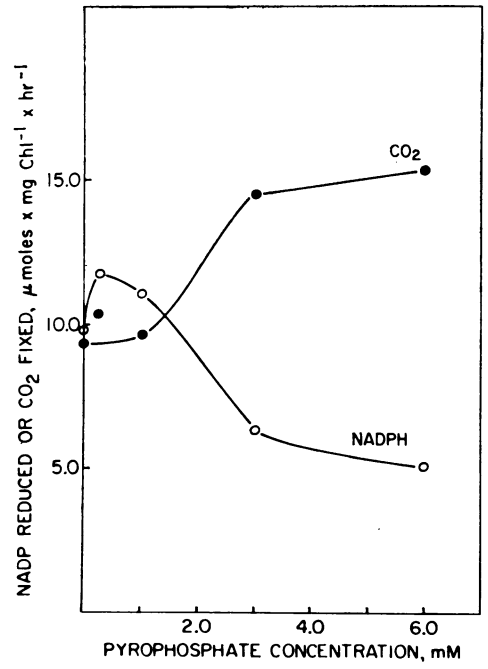

FIG. 6. Rate of CO_2 fixation and G3P leak as a function of pyrophosphate concentration in the presence of NR-G3P-DH. CO_2 fixation (●) and NADP reduction (○) were determined simultaneously as described in the legend to Figure 3. Pyrophosphate was deleted from the chloroplast resuspension solution but added as indicated to the reaction mixture. The Chl concentration was 20 μg/ml, and 3.4 enzyme units of NR-G3P-DH were added to each reaction mixture.

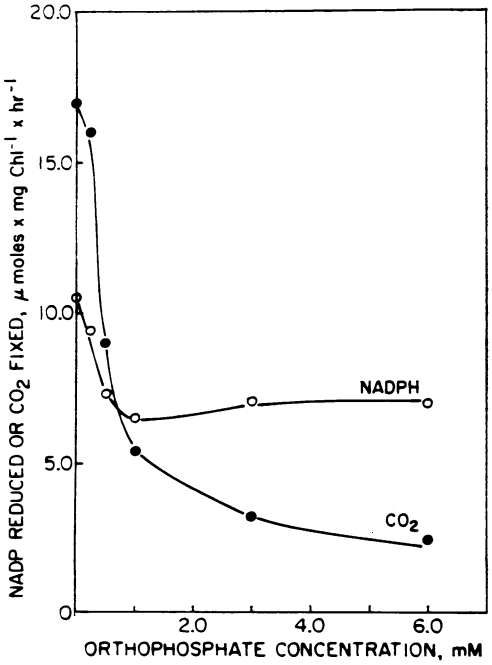

FIG. 8. Rate of CO_2 fixation and the leak of G3P as a function of orthophosphate concentration in the presence of pyrophosphate and NR-G3P-DH. CO_2 fixation (○) and NADP reduction (○) were simultaneously measured as described in the legend to Figure 3. The Chl concentration was 18 μg/ml, and 3.4 enzyme units of NR-G3P-DH were added to each reaction mixture including 5 mM PPi.

The high dark rates of NADP reduction observed in the presence of FDP and R5P and recorded in Table I probably resulted from the presence of enzymes of the carbon reduction cycle which transfers FDP and R5P to the level of G3P. This finding suggests that R5P and FDP, both known to overcome the initial induction period commonly observed in CO_2 fixation (23), were probably first broken down to triose-P before penetrating the chloroplast. This is consistent with the finding that the inner chloroplast membrane is impermeable to both FDP and R5P but permeable to triose-P (13).

Increased rates of CO_2 fixation which resulted from increasing bicarbonate concentrations (Fig. 1) and the addition of 3-PGA (Fig. 2), both in the presence of NR-G3P-DH, were observed along with a stimulation in G3P efflux (Figs. 1 and 2). These results suggest that the rate of G3P efflux was dependent on the rate of CO_2 fixation. In order to investigate this hypothesis further, G3P efflux and CO_2 fixation were measured in the light and in the dark (Table II). The finding that both dark period rates of G3P efflux were significantly less than the rates measured in the light suggest that G3P efflux is greater during light-dependent CO_2 fixation due to the increased flow of carbon in the photosynthetic carbon reduction cycle, thus making more G3P "available" for transport into the external medium. NADP reduction in the dark was probably due to the presence of glucose-6-P dehydrogenase in the chloroplast preparations.

We questioned whether NR-G3P-DH was increasing G3P transport from the chloroplast or if this efflux was independent of the level of NR-G3P-DH. The ability of higher concentrations of enzyme to cause an increase in G3P efflux (Fig. 3) indicated that NR-G3P-DH was actively involved in the transport of G3P from inside the chloroplast and, in so doing, enhanced CO_2 fixation. To explain the stimulatory effect of G3P efflux on CO_2 fixation we propose a "transport stimulation" mechanism exerted by the shuttle system. That is, the transport of G3P from the chloroplast into the cytoplasm affected the carbon reduction cycle by "pulling" more PGA toward the formation of G3P. This, in turn, would accelerate the flow of carbon substrate within the cycle, thus resulting in a faster regeneration of ribulose-1,5-diP acceptor and an increased rate of carboxylation. The over-all effect of the shuttle system is, therefore, to transfer NADPH, generated in the light-dependent conversion of PGA to G3P within the chloroplast, to the external medium resulting in an enhanced CO_2 fixation.

High concentrations (greater than 0.1 mM) of a 3-PGA are known to inhibit CO_2 fixation in intact spinach chloroplasts by a mechanism which remains unclear (2, 3, 6). In our study on the effect of exogenous PGA (Fig. 4), concentrations above 0.1 mM caused an inhibition of CO_2 fixation along with an increase in NADP reduction. We explain these results on the basis of an exchange of exogenous PGA with G3P in the chloroplast, since this would account for the observed rise in NADPH formation. Such a large exogenous PGA pool which continuously exchanged with G3P would result in a large efflux of G3P from the chloroplast, thus preventing the carbon reduction cycle from proceeding beyond the formation of G3P to any appreciable extent. Consequently, the regeneration of ribulose-1,5-diP acceptor is inhibited, thereby decreasing the rate of CO_2 fixation. It is suggested that this PGA/G3P exchange is a mechanism by which concentrations above 0.1 mM 3-PGA inhibit CO_2 fixation in spinach chloroplasts. This situation is in direct contrast to the shuttle system operating either in the absence of or with 0.1 mM PGA, with no large efflux of G3P and hence with no block in the carbon reduction cycle occurring subsequent to G3P formation. This latter situation resulted in enhancement of CO_2 fixation. Low concentrations (less than 0.1 mM) of exogenous PGA stimulated CO_2 fixation (Fig. 2), presumably because an initial shortage of intermediates of the photosynthetic carbon reduction cycle was eliminated (3, 4).

Low concentrations (0.5 mM) of Pi are required for maximum rates of CO_2 fixation in intact chloroplasts. On the other hand, concentrations above 0.5 mM of Pi significantly inhibit CO_2 fixation. Inorganic phosphate is known to penetrate the chloroplast envelope and, in so doing, has been shown to exchange, facilitated by a phosphate translocator, with PGA or triose-P (13). It has been proposed that this inhibition caused by Pi results from the exchange of Pi with either G3P or PGA within the chloroplast (18, 23). This large efflux of carbon substrate would, of course, inhibit CO_2 fixation. That this inhibition by Pi was completely reversed by the presence of triose-P and PGA (8) would seem to lend further support to this proposed mechanism of phosphate inhibition.

In confirmation of others, low concentrations of Pi (less than 0.5 mM) stimulated CO_2 fixation in our preparations (Fig. 5). The corresponding increase in G3P efflux may result from the greater availability of G3P at higher rates of CO_2 fixation as postulated earlier. Conversely, with concentrations of Pi above 0.5 mM, decreasing rates of G3P efflux were observed, paralleling the inhibition of CO_2 fixation. Clearly, the mode of Pi inhibition was not solely by an exchange with G3P. If this were the case, we would have seen a rise in exogenous NADPH formation along with the fall in CO_2 fixation. Thus Pi behaved differently than PGA which exchanged preferentially with G3P. Phosphate inhibition of CO_2 fixation could also result from its inhibitory action at some other site in the carbon reduction cycle. For example, Pi is a competitive inhibitor of ribulose-1,5-diP carboxylase (15).

Schwenn *et al.* (21) and Lilley *et al.* (18) reported that the PPi-mediated stimulation of CO_2 fixation in intact spinach chloroplasts was actually due to the Pi formed in the external hydrolysis of PPi by pyrophosphatase released from damaged chloroplasts. Pyrophosphate itself does not permeate the chloroplast envelope (13, 18). When the chloroplasts were carefully prepared so as to prevent the release of Pi or when Mg^{2+} ions required for the activation of this enzyme were deleted from the reaction mixture, PPi had no effect on CO_2 fixation (21). These observations suggested that PPi affected photosynthetic CO_2 fixation by a mechanism similar to Pi, although the former acted indirectly. The results in Figure 6 suggested that high concentrations of PPi stimulated CO_2 fixation by blocking the loss of G3P from the chloroplast in addition to its stimulatory effect via its hydrolysis to Pi. This was indicated by the decline in NADP reduction. We interpret our study to indicate that the effect of PPi was more complex than had been previously envisaged.

The immediate inhibition of CO_2 fixation observed at low Pi concentrations in the presence of 5 mM PPi (compare Fig. 8 with Fig. 6) was probably due to the Pi resulting from the additive effect of Pi and Pi derived from the hydrolysis of PPi by pyrophosphatase. Figure 8 also shows that the efflux of G3P was less than with increasing concentrations of Pi alone (Fig. 6). This was again probably due to the prevention of G3P efflux by PPi.

The effect of PPi was, therefore, opposite to that of PGA which readily exchanged with G3P. When the concentration of PGA was increased in the presence of 5 mM PPi, however,

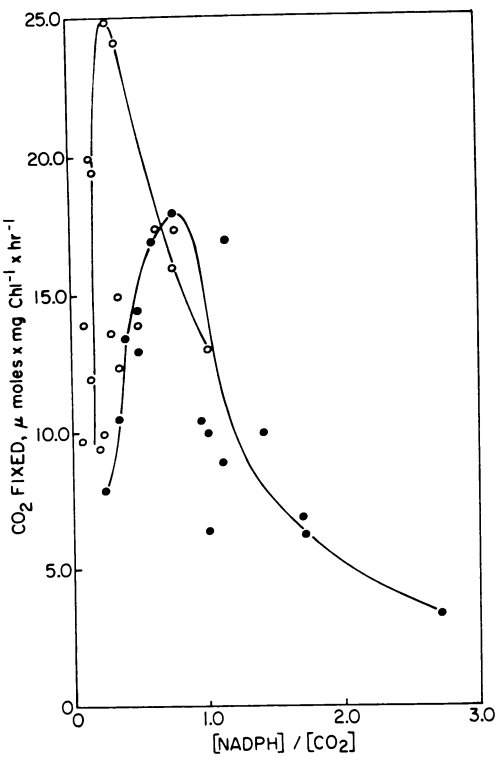

FIG. 9. Rate of CO_2 fixation as a function of the ratio between G3P efflux and CO_2 fixed (NADPH/CO_2). The data presented here were compiled from the various experiments discussed before and from experiments not included in this report. NADPH/CO_2 ratios from experiments with PPi present in the reaction mixture (○); NADPH/CO_2 ratios from experiments with Pi in the reaction mixture (●). The highest rates of photosynthesis were recorded when the PPi and Pi concentrations were 1 mM and 0.3 to 0.5 mM, respectively.

the PPi effect was overcome by PGA which promoted the efflux of endogenous G3P to the same extent as in the case where no PPi was present (compare Fig. 7 with Fig. 4).

Figure 9 illustrates the rate of CO_2 fixation as a function of NADPH formed (G3P transported) CO_2 fixed and summarizes much of our observations on the shuttle system. This figure represents experiments where Pi was present in the reaction mixture and experiments where PPi replaced Pi.

Both curves are bell-shaped and this aspect can be interpreted as follows. When the rate of G3P efflux was relatively low, CO_2 fixation increased due to the proposed "transport stimulation" mechanism. This interpretation implies that a high level of G3P brought about by a rapid reduction of PGA in contrast to a slow conversion of triose-P to fructose-P regulated CO_2 assimilation. Control was presumably at the equilibrium reaction catalyzed by glyceraldehyde-3-P dehydrogenase since the aldotriose has not been demonstrated to be effective at any other enzymic reaction comprising the Calvin cycle. On the other hand, the rate of G3P efflux, if sufficiently high, could result in an inhibition of CO_2 fixation due to an impairment in the regenerative phase of the Calvin cycle.

It can be seen in Figure 9 that the curve representing experiments where PPi was in the reaction mixture peaked at a NADP: CO_2 ratio of 0.33, while the Pi-containing reaction mixtures were characterized by a value of 0.78. The ratio of 0.33 is in accord with a stoichiometry observed earlier with spinach chloroplasts by Cockburn *et al.* (7) and implies that the predominant reactions in our preparations utilizing PPi as the source of Pi were:

$$3\ CO_2 + 3\ H_2O + Pi \rightarrow G3P + H_2O + 3\ O_2$$

A further implication is a regulated hydrolysis of PPi to Pi, since in the presence of Pi alone a ratio of 0.78 was observed. A ratio higher than 0.33 points to a source of NADP-reducing substances whose origins were not from the newly assimilated CO_2 and whose formation was subject to regulation by the levels of Pi and PPi. We have presented preliminary evidence that a portion of the NADPH could result from the oxidation of glucose-6-P catalyzed by a glucose-6-P dehydrogenase in our chloroplast preparations. However, the release of endogenous-oxidizable substrates was low and could not account entirely for a doubling of the NADPH:CO_2 ratio (Table II). This statement was predicated upon the assumption that light did not affect the breakdown of endogenous stores in the isolated organelles. There are a number of reports that light stimulates starch dissimilation in intact tissue (20) and this phenonomen requires evaluation in the spinach chloroplast.

The differential effect of Pi and PPi on the phosphate translocator may be the more critical factor. According to Heldt *et al.* (13), the transport mediated by the phosphate translocator is a strict counter exchange between triose-P, PGA, and Pi. On the other hand, PPi is an inhibitor of the translocator and impairs the exchange. An increase in Pi would result in a concomitant rise in the efflux of G3P derived from newly assimilated CO_2 or from endogenous sources while PPi, which probably binds to the translocator, prevents the exchange. The observed ratio may reflect the balance between G3P which is retained within the organelle due to PPi and G3P which is forced out of the plastid under other conditions where PPi is replaced by Pi or PGA. The possibility that endogenous stores and/or the translocator play a vital role in the regulation of photosynthesis in the isolated chloroplast is currently under study.

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