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Site of Action of Inhibitors of Carbon Dioxide Assimilation by Whole Lettuce Chloroplasts

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ABSTRACT

The sites of action of several compounds, reported to inhibit CO_2 fixation by chloroplast preparations were located by developing assays in lettuce chloroplasts to test their effect on partial reactions of the carbon cycle and on carbonic anhydrase. The results indicated that: D,L-glyceraldehyde and 5'-AMP inhibit phosphoribulose kinase or isomerase. 3-Phosphoglyceric acid and 6-phosphogluconate inhibit ribulose diphosphate carboxylase. Azide, Mg^{2+}, and nitrite inhibit the activity of carbonic anhydrase of lettuce chloroplasts and light-dependent CO_2 fixation by intact chloroplasts with similar sensitivities. None of these inhibited CO_2 fixation in ruptured chloroplasts. It is suggested that the inhibition by azide, nitrite, and magnesium ions of CO_2 fixation by intact chloroplasts is due to their inhibition of the activity of carbonic anhydrase.

Most of the compounds which inhibit CO_2 assimilation by isolated whole chloroplasts are relatively unspecific, since they show a multiplicity of effects involving both "light" and "dark" reactions. D,L-Glyceraldehyde came nearest to what would be expected of a purely "dark" inhibitor of CO_2 fixation. It apparently blocks the conversion of triose-phosphate into RuDP[2] without any effect on partial reactions of electron transport and photophosphorylation (18). The difficulty in locating the site of action of an inhibitor of CO_2 fixation by whole chloroplasts lies in the fact that the structural integrity of the chloroplast is a prerequisite for its ability to assimilate CO_2 via the complete carbon cycle (11). The study of partial reactions of the cycle, under conditions in which the structural integrity of the chloroplasts is not limiting, opens the possibility for locating sites of action of inhibitors or activators. Such partial reactions were studied previously (4, 5, 14, 18) by assaying in media containing intact chloroplasts rendered envelope-free by resuspension in hypotonic medium. These systems involved several phases of the carbon cycle: (*a*) from R5P to PGA, in the dark with R5P, ATP, and CO_2 as substrates, assaying PRI, PRK, and RuDPCase; (*b*) from RuDP to PGA in the dark with RuDP and CO_2 as substrates, assaying essentially RuDPcase; (*c*) from R5P to PGA, with intact chloroplasts which were preilluminated under conditions optimal for the activation of PRK, followed by rupturing of the chloroplasts, photophosphorylation of Ru5P and a CO_2 fixation period in the dark; (*d*) from R5P to RuDP, assaying PRI and PRK in intact chloroplasts with ^{32}P, using R5P as the phosphate acceptor.

In this paper, we located the site of action of a number of compounds reported to inhibit CO_2 fixation by chloroplast preparations utilizing these and other systems of partial reactions of the carbon cycle, and assaying their action on carbonic anhydrase.

MATERIALS AND METHODS

Preparation of Intact Chloroplasts. Lettuce leaves (*Lactuca sativa var. Romaine*) were obtained from a local store. Prior to chloroplast preparation the leaves were illuminated for 1 hr in the cold room. Intact chloroplasts were prepared as follows. Fifteen g of leaves were cut into small pieces and placed with 50 ml of blending solution (0.33 M sorbitol, 10 mM EDTA, 1 mM $MgCl_2$ 0.5 mM K_2HPO_4, 5 mM, $Na_4P_2O_7$, 10 mM Tricine, 2 mM Na-ascorbate, 0.2 mg/ml BSA, pH 8.3) in a VirTis homogenizer (Model 45 set at 65/110 v) and blended for 3 to 5 sec at 0 C. The suspension was filtered through four layers of gauze and centrifuged at 2000*g* for 50 sec. The pellet was resuspended in a small volume of the resuspension solution which had the same content as the grinding solution but with 0.1 mg/ml BSA, and used as the "intact chloroplasts" preparation. Chl was assayed as previously described (3).

Assays for CO_2 and Phosphate Incorporation. Reactions were run in small test tubes placed in a water bath at 25 C and either illuminated with 8000 ft-c of white light or placed in a black box at room temperature. The reactions were terminated by the addition of trichloroacetic acid to the 1-ml reaction mixtures to give a final concentration of 3%. The suspensions were centrifuged at 1000*g* for 10 min. A sample of 0.3 ml was analyzed for $^{14}CO_2$ incorporation into acid-stable products and 0.5-ml samples for the assay of ^{32}Pi esterification. $^{14}CO_2$ was removed from the samples by placing the 0.3-ml samples in a desiccator under aspirator vacuum for 1 hr (5). $^{14}CO_2$ incorporated was determined by addition to the sample of 2.7 ml of scintillation fluid (200 mg of POPOP; 4 g of PPO, 60 g of naphthalene, 175 ml of methanol, 40 ml of ethyleneglycol and dioxane to a total volume of 1 liter) and counting the radioactivity in a Packard 3320 scintillation counter. Total radioactivity (cpm) contained in the reaction mixture was obtained by placing 0.05 ml of the reaction mixture in 4.95 ml of 1 M unbuffered tris and withdrawing 0.3 ml from it for radioactivity determination. ^{32}Pi esterified was assayed by the isobutanol-benzene extraction procedure as described previously (3).

Light-dependent CO_2 Fixation. The reaction medium contained in a total volume of 1 ml: 0.33 M sorbitol, 0.5 mM EDTA (pH

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[2] Abbreviations: RuDP: D-ribulose 1,5-diphosphate; R5P: D-ribose 5-phosphate; PGA: 3-phosphoglyceric acid; PMS: phenazine methosulfate; FDP: D-fructose 1,6-diphosphate; PRI: phosphoriboisomerase; PRK: 5-phosphoribulokinase; RuDPCase: D-ribulose 1,5-diphosphate carboxylase; 6 PG: 6-phosphogluconate.

Table I. *Activities of Several Reactions Catalyzed by Chloroplasts*

Reaction	Additions		
	None	1 mM R5P	1 mM FDP
	μmoles mg Chl^{-1}·hr^{-1}		
Light CO_2 fixation[1]	36	45	43
Dark CO_2 fixation[1]	0.1	120	10
Post-illumination CO_2 fixation[1]	0.1	48	4
Two step dark CO_2 fixation[1]	0.1	54	6
Phosphoribulokinase[2]	5	76	7
Carbonic anhydrase[3]	1.2×10^4		

[1] CO_2 fixed.
[2] Inorganic phosphate esterified.
[3] CO_2 hydrated.

8.3), 1 mM $MgCl_2$, 1 mM $MgCl_2$, 0.5 mM K-phosphate (pH 8.3), 5 mM $Na_4P_2O_7$ (pH 8.3), 10 mM Tricine (pH 8.3), 2 mg/ml BSA, 2 mM Na-ascorbate, 5 mM $NaCHO_3$ (containing between 1 and 2 μCi of $NaH^{14}CO_3$), and intact chloroplasts (containing between 40 and 60 μg Chl). After 2 min of equilibration in the dark, the light was turned on for 15 min. The reaction was terminated by turning the light off and adding to a final concentration of 3%.

Postillumination CO_2 Fixation (5). Intact chloroplasts (containing between 40–60 μg of Chl) were osmotically ruptured in the reaction medium and illuminated for 15 min, leading to the accumulation of RuDP (kinase step). At this point, the light was turned off, radioactive bicarbonate and $MgCl_2$ were added, and postillumination CO_2 fixation was followed for 15 min (carboxylation step). The reaction was terminated by adding trichloroacetic acid to a final concentration of 3%. At the kinase step the reaction medium contained: 0.5 mM EDTA (pH 8.3), 2 mM $MgCl_2$, 10 mM K Phosphate (pH 8.3), 2 mg/ml BSA, 2 mM Na-ascorbate, 1 mM R5P (pH 8.3), 0.04 mM ATP (pH 8.3), 0.01 mM PMS. For the carboxylase step 16 mM $MgCl_2$ and 5 mM $NaHCO_3$ (containing between 1 and 2 μCi of $NaH^{14}CO_3$) were added.

Two-step Dark CO_2 Fixation. Intact chloroplasts (containing between 40 and 60 μg) were osmotically ruptured in the reaction mixture and were first incubated under conditions which led to accumulation of RuDP in the dark for 15 min (kinase step). At this point, radioactive bicarbonate was added and dark CO_2 fixation followed (carboxylation step). For the kinase step, the reaction mixture contained the same components as in the kinase step in the postillumination system except that PMS was excluded and 4 mM ATP and 20 mM $MgCl_2$ were included. For the carboxylation step 5 mM radioactive bicarbonate was added. The reaction was terminated after 15 min, as above.

Phosphoriboisomerase and Phosphoribulokinase. This activity was assayed essentially as described before (3) for spinach chloroplasts. Since no activation of PRK by preillumination was observed in lettuce chloroplast (see ref. 3), no preillumination period was employed. The reaction medium contained in a total volume of 1 ml: 0.5 mM EDTA (pH 8.3), 2 mM $MgCl_2$, 10 mM Tricine, (pH 8.3), 2 mg/ml BSA, 2 mM Na-ascorbate, 1 mM R5P (pH 8.3), 0.04 mM ATP, 0.01 mM PMS, 5 mM $NaHCO_3$, 2 mM K_2HPO_4, (containing 5–10 $\times 10^5$ cpm ^{32}Pi). The reaction was run for 15 min in the light and terminated by turning off the light and adding trichloroacetic acid as above.

Carbonic Anhydrase. The activity of this enzyme was measured by following (at 0 C) the initial change in pH, upon adding CO_2 saturated H_2O to a buffered solution, on a recorder connected to a pH meter. The reaction mixture contained in a total volume of 2 ml: 60 mM Tricine (pH 8.3), and chloroplasts and other components made up with H_2O to an additional volume of 0.9 ml. The reaction was started by the rapid addition from a syringe of 0.1 ml of H_2O saturated with CO_2, and the change in pH was followed as a function of time. The amount of chloroplasts added was adjusted so as to have a constant rate for at least 10 sec. The total pH change during the reaction did not exceed 0.1 pH unit. The scale was calibrated by the addition of known quantities of acid. The rate of the enzymic reaction was corrected for the slow nonenzymic rate and was expressed in moles H^+/mg Chl^{-1} hr^{-1}.

RESULTS

Properties of Reactions of Cycle. Table I lists typical specific activities obtained in reactions with intact or with osmotically ruptured lettuce chloroplasts under the optimal conditions described under methods. The rate of light-dependent CO_2 fixation by intact lettuce chloroplasts is similar to that obtained in our hands with spinach chloroplasts (5). The stimulation by the addition of sugar phosphates like R5P or FDP varied with different chloroplast preparations from 10 to 100%.

Dark CO_2 fixation with osmotically ruptured chloroplasts could be carried out only in the presence of a sugar phosphate, with R5P much superior to FDP. The dark CO_2 fixation reactions listed in Table I enabled us to assay partial reactions of the carbon cycle, either from FDP to PGA or from R5P to PGA when ATP for the phosphoribolukinase step was supplied from the outside. Similarly, the postillumination CO_2 fixation reaction is a partial reaction of the cycle from FDP to PGA or from R5P to PGA but here ATP was supplied by cyclic photophosphorylation

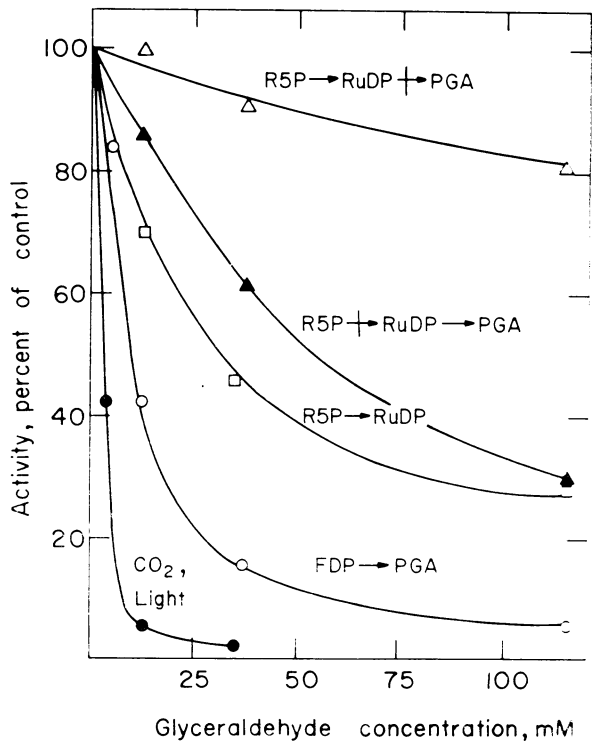

FIG. 1. Inhibition of CO_2 fixation reactions by D,L-glyceraldehyde. Light-dependent CO_2 fixation (CO_2, light) in the presence of 1 mM R5P: 100% = 34 μmoles CO_2 fixed/mg Chl·hr; Dark CO_2 fixation in the presence of 1 mM FDP (FDP → PGA): 100% = 2.3 μmoles CO_2 fixed/mg Chl·hr; Two-step dark CO_2 fixation: D,L-glyceraldehyde added before the carboxylase step (R5P → RuDP + PGA) or initially (R5P + RuDP → PGA) in the presence of 1 mM R5P + 4 mM ATP: 100% = 51 μmoles CO_2 fixed/mg Chl·hr; PRI + PRK R5P → RuDP): 100% 36 μmoles Pi esterified mg Chl·hr.

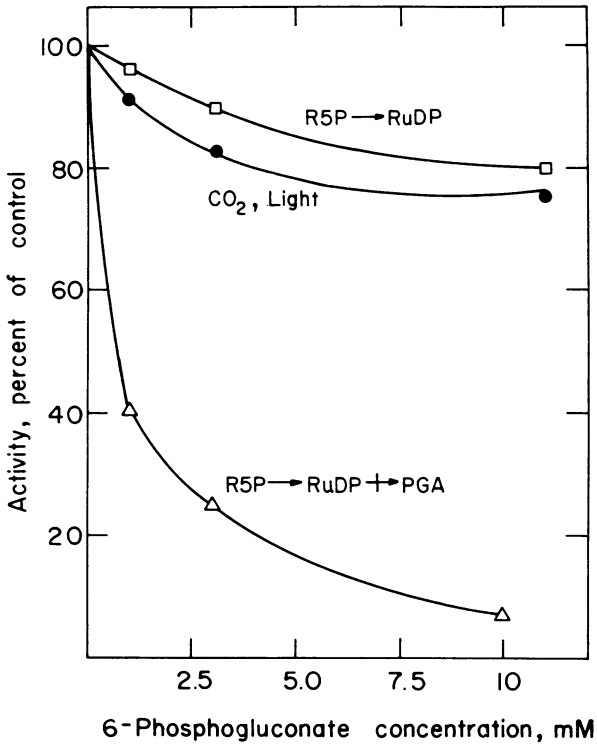

FIG. 2. Inhibition of CO_2 fixation reactions by 6-PG. Light-dependent CO_2 fixation in the presence of 1 mM R5P (CO_2, light): 100% = 18.5 μmoles CO_2 fixed/mg Chl·hr; Two-step dark CO_2 fixation: 6-PG added at the carboxylase step (R5P → RuDP ↛ PGA): 100% = 40 μmoles CO_2 fixed/mg Chl·hr; PRI + PRK activity in the presence of 1 mM R5P (R5P → RuDP): 100% = 52 μmoles Pi esterified mg Chl·hr.

(5). This reaction is dependent therefore on the presence of PMS, supplied at a low concentration in order to avoid inhibition of CO_2 fixation, and a very low ATP concentration, which is continuously regenerated by cyclic photophosphorylation and so leads to the accumulation of ribulose diP.

The two-step dark CO_2 fixation reaction was devised as a means to distinguish between the effects on PRI and PRK or ribulodiphosphate carboxylase activities of the carbon cycle. The phosphoribulokinase assay with ^{32}P enabled us to follow the kinase activity directly without the involvement of the carboxylation reaction, starting with different intermediates of the carbon cycle as substrates.

Utilizing these partial reactions of light-dependent CO_2 fixation, we tried to evaluate more closely the site of action of some compounds, which were reported as inhibitors of CO_2 fixation with intact chloroplasts. Four sites were tested: (*a*) between FDP and R5P; (*b*) between R5P and PGA; (*c*) the carboxylation reaction itself; (*d*) carbonic anhydrase which has been postulated to play an important role in the transfer of CO_2 from the outside solution into the intact chloroplasts (8) but is not required in the partial reactions with osmotically ruptured chloroplast preparations.

D,L-Glyceraldehyde. Stokes and Walker (18) reported that CO_2 fixation and associated CO_2-dependent O_2 evolution were almost completely inhibited by 10 mM D,L-glyceraldehyde. They suggested that D,L-glyceraldehyde blocks the conversion of triose-P into R5P. As shown in Figure 1, 10 mM D,L-glyceraldehyde inhibited CO_2 fixation by isolated intact lettuce chloroplasts. This inhibition was not affected by the addition of FDP or R5P.

CO_2 fixation in the dark with osmotically ruptured chloroplasts was severly inhibited by D,L-glyceraldehyde in the presence of FDP and less so in the presence of R5P. When tested in two-step dark CO_2 fixation, D,L-glyceraldehyde was essentially not inhibitory when added at the carboxylase step. These observations implicate at least two inhibitory sites: (*a*) a more sensitive site in the conversion of FDP to R5P, and (*b*) a less sensitive site in the conversion of R5P to RuDP. The latter site was checked by an independent measurement of the conversion of R5P to RuDP with ^{32}P which confirmed that this path is inhibited by D,L-glyceraldehyde (Fig. 1) to a degree comparable with that of dark CO_2 fixation.

6-Phosphogluconate. 6-PG was shown to be a potent inhibitor of RuDPCase (7, 19) and inhibited postillumination CO_2 fixation but not light-dependent CO_2 fixation (4; Fig. 2). In the dark systems 6-PG was a potent inhibitor of CO_2 fixation even when added at the carboxylase step. The same inhibition pattern was obtained in a postillumination CO_2 fixation system or in dark CO_2 fixation with FDP (not shown). Independent measurements of phosphoribulokinase with ^{32}P (Fig. 2) indicated that PRI and PRK were only slightly affected by 6-PG. The inhibition observed may be due to the inhibition of the carboxylase which uses up the product of PRK (RuDP). These data agree with previous data (7, 19) that the site of 6PG inhibition is at the carboxylation step. Light-induced CO_2 fixation in intact chloroplasts is not affected, most probably because the inhibitor does not enter the intact chloroplast (5).

3-Phosphoglyceric Acid. PGA is a noncompetitive inhibitor of purified spinach RuDPcase with respect to RuDP (K_i = 8.3 mM) and a competitive inhibitor (K_i = 9.5 mM) with respect to HCO_3^- (17). PGA at 10 mM almost completely inhibited light-dependent CO_2 fixation in intact spinach chloroplasts (1).

With lettuce chloroplasts, light-dependent CO_2 fixation was more than 90 % inhibtted with 10 mM 3-PGA (Fig. 3). When no

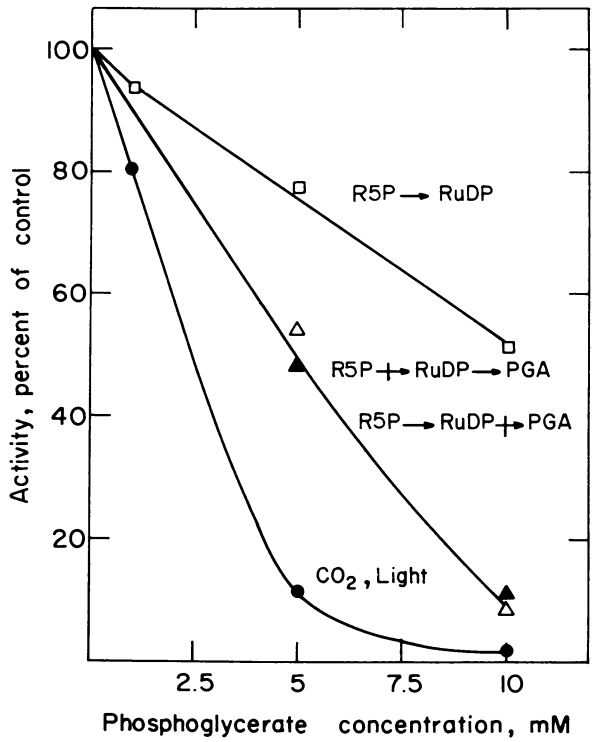

FIG. 3. Inhibition of CO_2 fixation reactions by P-glycerate. Two-step dark CO_2 fixation with P-glycerate added before the carboxylase step (R5P → RuDP ↛ PGA) or initially (R5P ↛ RuDP → PGA) in the presence of 1 mM R5P: 100% = 70 μmoles CO_2 fixed/mg Chl·hr; PRI + PRK activity in the presence of 1 mM R5P (R5P → RuDP): 100% = 12.7 μmoles Pi esterified/mg Chl·hr; light-dependent CO_2 fixation in the presence of 1 mM R5P (CO_2 light): 100% = 40 μmoles CO_2 fixed/mg Chl·hr.

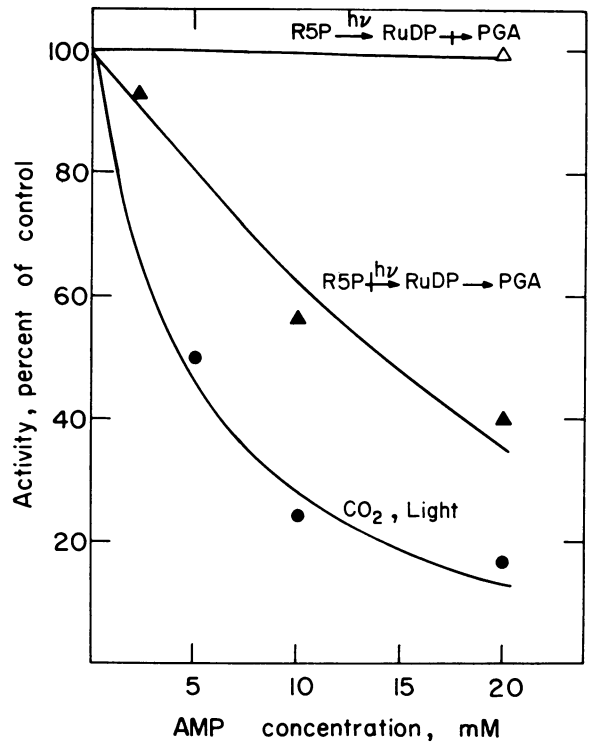

FIG. 4. Inhibition of CO_2 fixation reactions by 5'-AMP. Light-dependent CO_2 fixation in the presence of 1 mM R5P: 100% = 23 μmoles CO_2 fixed/mg Chl·hr; postillumination CO_2 fixation in the presence of 1 mM R5P; 5'-AMP added initially (R5P ⇢ RuDP → PGA) or at the carboxylase step (R5P → RuDP ⇢ PGA). 100% = 34 μmoles CO_2 fixed/mg Chl·hr.

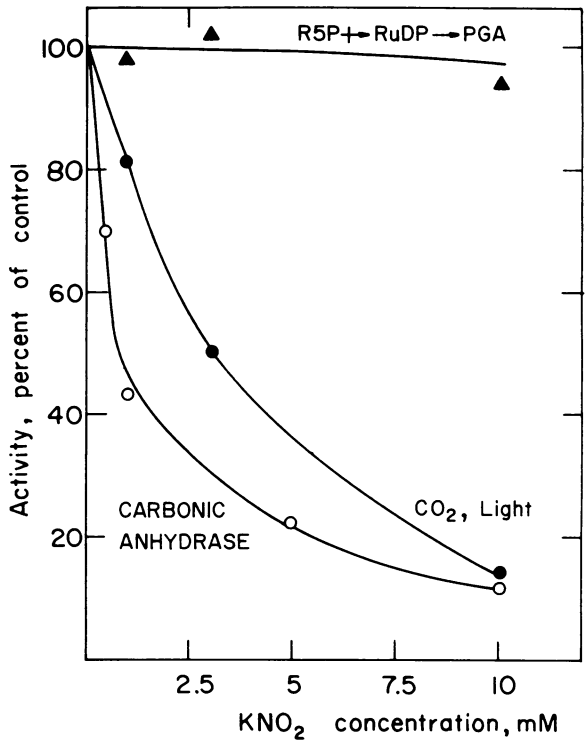

FIG. 6. Inhibition of CO_2 fixation reactions by nitrite. The control rates (100%) were: carbonic anhydrase: 12 mmoles H^+/mg Chl·hr; light-dependent CO_2 fixation in the presence of 1 mM R5P: 42 μmoles CO_2 fixed/mg Chl·hr; dark CO_2 fixation in the presence of 1 mM R5P: 78 μmoles CO_2 fixed/mg Chl·hr. Symbols as described under previous figures.

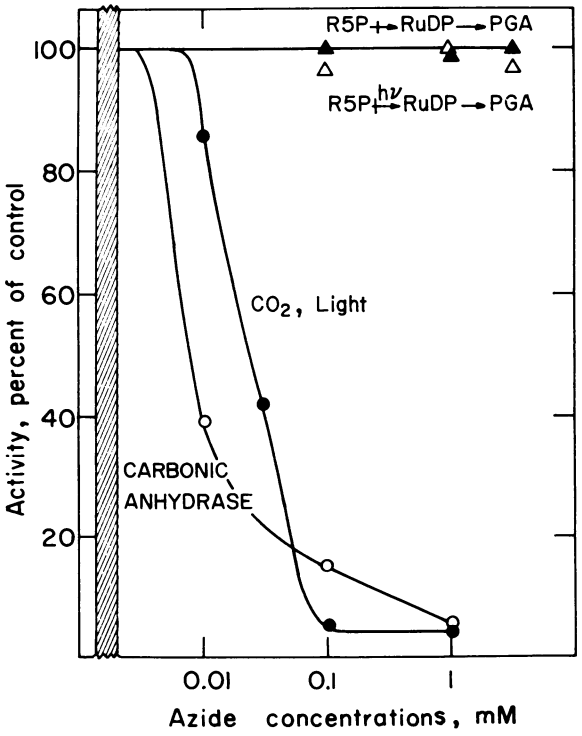

FIG. 5. Inhibition of CO_2 fixation reactions by azide. The control rates (100%) were: carbonic anhydrase activity: 13 mmoles H^+/mg Chl·hr; light-dependent CO_2 fixation: 19.5 μmoles CO_2 fixed/mg Chl·hr; two-step dark CO_2 fixation: 24 μmoles CO_2 fixed/mg Chl·hr; postillumination CO_2 fixation: 12.3 μmoles CO_2 fixed/mg Chl·hr. Symbols as described in previous figures.

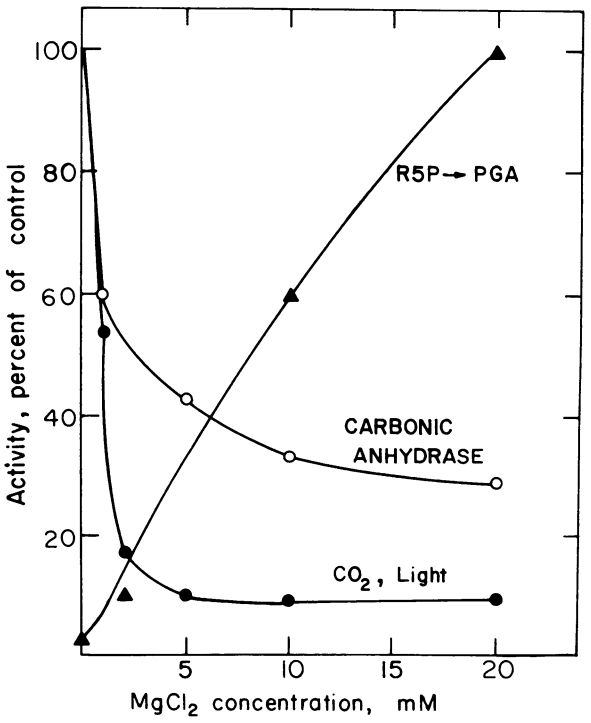

FIG. 7. Inhibition of CO_2 fixation reactions by magnesium: Control rates (100%) were: light-dependent CO_2 fixation in the presence of 1 mM R5P: 45 μmoles CO_2 fixed/mg Chl·hr; dark CO_2 fixation in the presence of 1 mM R5P: 42 μmoles CO_2 fixed/mg Chl·hr; carbonic anhydrase activity: 10 mmoles H^+/mg Chl·hr. Symbols as described under previous figures.

sugar phosphate was added to the system, a slight increase in the rate of CO_2 fixation was observed at concentrations below 1 mM PGA, followed by the same inhibitory effect at the higher PGA concentrations. In the dark CO_2 fixation reaction, PGA inhibited either when added at the kinase step or, to the same extent, when added at the carboxylase step. These data suggest that RuDP carboxylase is the site of inhibition by PGA. Independent measurements of PRI + PRK with ^{32}P indicate that in 10 mM 3-PGA the enzymes still maintain 50% of their capacity.

5'-AMP. Forsee and Kahn (10) reported that CO_2 fixation in isolated *Euglena* chloroplasts is strongly inhibited by 1 mM 5'-AMP. The site of inhibition was not determined. 5'-AMP was reported to inhibit PRI (2) but inhibition of CO_2 fixation was not observed (15). As shown in Figure 4, light-dependent CO_2 fixation with lettuce chloroplasts was inhibited 50% by 5 mM 5'-AMP in the presence of 1 mM R5P.

In the postillumination system, it is clearly seen that 5'-AMP is an inhibitor of PRI + PRK. When it was added at the carboxylase step, no inhibition occurred even at 20 mM 5'-AMP, but when added at the kinase step, CO_2 fixation was inhibited. Similar results were obtained in the two-step dark CO_2 fixation. It was not possible to test 5'-AMP in the direct PRI + PRK assay, since 5'-AMP via myokinase served to produce ADP which interfered with the assay employed.

Azide. Azide, at concentrations below 0.10 mM inhibited both light-dependent CO_2 fixation by whole lettuce chloroplasts and carbonic anhydrase activity of the same chloroplasts (Fig. 5). None of the CO_2 fixation reactions tested with ruptured lettuce chloroplasts were affected by sodium azide. As was previously shown, azide does not inhibit electron transport or photophosphorylation (4, 5).

Salts. Carbonic anhydrase is very sensitive to inhibition by a variety of salts. Thus, 1 to 10 mM concentrations of KNO_3, KNO_2, KCl, Na_2SO_4, $MgCl_2$ and $MgSO_4$ (but not sucrose) inhibit the activity of carbonic anhydrase by 50% (see also 6, 13). Of these only KNO_2 and $MgCl_2$ inhibit light-induced CO_2 fixation by intact chloroplasts (Figs. 6 and 7) but none had any inhibitory effect on reactions run with ruptured chloroplasts. As previously shown, high concentrations of Mg^{2+} were required for maximal dark CO_2 fixation (Fig. 7). Carbonic anhydrase was not inhibited by glyceraldehyde, PGA, 6-PG, or 5'-AMP.

DISCUSSION

By separating in time the activities of RuDPCase, PRI, and PRK in partial reaction with R5P as substrate, the sites of inhibition of four inhibitors of CO_2 fixation by lettuce chloroplasts were determined. D,L-Glyceraldehyde (Fig. 1) and 5'-AMP (Fig. 4) clearly inhibit PRI or PRK. Independent assays of PRI and PRK with ^{32}P confirmed the site of inhibition of glyceraldehyde. PGA (Fig. 3) and 6-PG (Fig. 2) inhibited RuDP carboxylase. However, these inhibitors of RuDP carboxylase inhibited also the independent assay of PRK with ^{32}P to a much lesser degree. This might be due to a "product inhibition" of PRK by RuDP when RuDP carboxylase is severely inhibited. When these inhibitors were studied in the partial reactions of CO_2 fixation with FDP as the substrate rather than R5P, the systems were more sensitive toward the inhibitors (Fig. 1). This extra inhibition may be due to the strongly limiting step between FDP and R5P in the path toward RuDP, as indicated by the much lower activities observed when FDP was the substrate in the dark experiments (Table I).

The apparent discrepancy between the requirement for high concentrations of Mg^{2+} ions for dark CO_2 fixation reactions with ruptured chloroplast preparations and the inhibitory effect of Mg^{2+} ions in whole chloroplast light-dependent CO_2 fixation reactions (5) may be resolved by the present data. Mg^{2+} ions inhibit the activity of carbonic anhydrase as they do inhibit light dependent CO_2 fixation. Carbonic anhydrase seems to be required only in whole chloroplasts, presumably to enhance the transport of CO_2 to the carboxylation site in the stroma (9, 12). Mg^{2+} ions, required by RuDP carboxylase, are available within the chloroplast. Carbonic anhydrase activity is not needed in ruptured chloroplast preparations since the carboxylation site is exposed to CO_2 in the reaction mixture and therefore neither azide nor magnesium nor nitrite are inhibitory. However, Mg^{2+} ions for RuDP carboxylase must be provided in the reaction mixture in the broken preparations. The fact that many salts inhibit carbonic anhydrase but not CO_2 fixation with whole chloroplasts may be taken as confirming previous data (16, 20) that carbonic anhydrase is located within the stroma of whole chloroplasts and is not available to react with externally added compounds which are impermeable through the double envelope.

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