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Inhibition of Ribulose 1,5-Diphosphate Carboxylase by 6-Phosphogluconate[1]

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ABSTRACT

6-Phosphogluconate is a much more effective inhibitor of the photosynthetic carboxylation enzyme, ribulose-1,5-diphosphate carboxylase, than other sugar phosphates and sugar acids of the reductive and oxidative pentose phosphate cycles. The inhibition appears to be noncompetitive with ribulose 1,5-diphosphate. Since 6-phosphogluconate is unique to the oxidative cycle and inhibits at concentrations comparable to those found *in vivo*, it is proposed that its inhibition of the carboxylase may be a regulatory factor. If so, it would operate during darkness as a different control factor from those factors postulated to activate the carboxylase during photosynthesis.

In the carboxylation reaction of the photosynthetic reductive pentose phosphate cycle (3, 4) RuDP[2] reacts with CO_2 and water to give 2 molecules of PGA (18, 19). The specific activity of the isolated enzyme in early studies seemed too low for it to be able to catalyze CO_2 fixation at the rates observed for green cells *in vivo* (15).

From analysis of steady-state levels of RuDP in the dark following photosynthesis, Pedersen *et al.* (14) concluded that the mechanism for the carboxylation reaction *in vivo* becomes inactive after about 3 min of darkness, since the level of RuDP drops very slowly after this time, despite the fact that the free energy change for the carboxylation reaction is about 10 kcal negative (4, 6). Other studies showed that the carboxylation reaction is inactivated, even with the light on, by the addition of fatty acids (13) which are thought to interfere with ion pumping in the thylakoids. Inactivation also occurred with addition of vitamin K_5, thought to interfere with electron transport (11).

Jensen and Bassham (10) found that CO_2 fixation in isolated spinach chloroplasts ceases within about 2 min after the light is turned off, even though the level of RuDP does not drop more than 60% from its value in the light. If ATP was added to the chloroplasts, the level of RuDP was maintained as high in the dark as in the light, yet CO_2 fixation still stopped completely in the dark. Thus the dark inactivation of the carboxylation reaction apparently occurs in isolated chloroplasts as well as *in vivo*.

It has been proposed that the RuDPCase is activated in the light in chloroplasts by changes in the ionic content of the chloroplast and by the higher ratio of reduced to oxidized coenzymes (1, 2, 5). Both the changes in ionic content (especially Mg^{2+} and H^+) and the higher ratio of reduced to oxidized cofactors would be consequences of the photoelectron transport reactions occurring in the thylakoid membranes, and it was proposed that these changes, as reflected in the stroma region, provide a general regulatory mechanism whereby RuDPCase and other key regulated enzymes are activated in the light. Such a light-activated mechanism does not necessarily obviate the need for another mechanism to turn off more completely the carboxylation in the dark.

From studies of the metabolites in *Chlorella pyrenoidosa* in light and dark, it was found that the one metabolite which is most notably higher in concentration in the dark than in the light is 6-PGluA (5). This compound, which is considered by us to be an indicator of the operation of the oxidative pentose phosphate cycle, appears immediately in the dark and disappears within 1 min in the light. The appearance of 6-PGluA, whether in the dark *in vivo* or upon the addition of vitamin K_5 in the light to either *Chlorella pyrenoidosa* or isolated spinach chloroplasts (11), thus seems to be correlated with the inactivation of the carboxylation reaction (as well as with certain other regulated reactions). The present study shows that 6-PGluA is an effective inhibitor, at low levels, of the RuDPCase.

MATERIALS AND METHODS

Enzyme Purification. Carboxylase was purified from spinach (*Spinacia oleracea*) leaves. The purification procedures basically follow methods of others, with some modifications (12, 16, 17). Briefly, the purification procedures include homogenization in a Waring Blendor for 3 min, heating at 50 C for 20 min, Sephadex G-25 column chromatography, precipitation by 30 to 60% saturation with ammonium sulfate, DEAE-cellulose column chromatography with a linear gradient of NaCl (0–1 M), and Sephadex G-200 column chromatography. The purified enzyme had a specific activity of about 0.5 unit/mg protein (one unit is defined as 1 μmole of carbon incorporated into acid-stable compound per min under assay conditions).

Materials. RuDP, 6-PGluA, fructose-1,6-diP, and NADPH were purchased from Sigma Chemical Co. The acid forms of RuDP and of 6-PGluA were generated from solutions of the respective barium and tri-monocyclohexylammonium salts with Dowex-50. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Calbiochem, and NADP was purchased from Nutritional Biochemical Corp. The scin-

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[2] Abbreviations: RuDP: ribulose-1,5-diphosphate; PGA: 3-phosphoglycerate; RuDPCase: ribulose 1,5-diphosphate carboxylase; 6-PGluA: 6-phosphogluconate.

tillation fluid, "Aquasol," was purchased from New England Nuclear.

As another test, 6-PGluA was first generated by incubation of 5 mM glucose-6-P with 5 mM NADP and glucose-6-P dehydrogenase (10 units) for 20 min at pH 7.8 and 23 C. In control flasks, either glucose-6-P, NADP, or glucose-6-P dehydrogenase were omitted. An aliquot volume from each of these flasks was added to each of four separate flasks for assay of RuDPCase (described below) so that the concentrations of 6-PGluA, glucose-6-P, and NADP, were decreased 10-fold (*i.e.*, 6-PGluA concentration in the complete system was approximately 0.5 mM).

Enzyme Assay. In a final volume of 200 μl, the reaction mixture contained the following components and their concentrations: tris-HCl, 175 mM; $MgCl_2$, 10 mM; pH 7.8; RuDP; $NaHCO_3$; 6-PGluA; and enzyme in different amounts as shown in each figure. The reaction was started with the addition of enzyme solution to the reaction mixture contained in the serum cap sealed tube. After 5- or 10-min incubation in the water bath at 23 C, 100 μl of 6 N acetic acid was added to stop the reaction. An aliquot (250 μl) of this mixture was transferred to a scintillation counting vial and was dried in an oven at 90 C for 1 hr. Two hundred fifty μl of water was added to the vial, followed by 18 ml of Aquasol. The radioactivity was measured by scintillation counter with a counting efficiency around 90%.

Spinach chloroplasts were isolated and allowed to photosynthesize with $H^{14}CO_3^-$ as described previously (10). Rates of ^{14}C uptake into acid-stable compounds were determined, and analysis was made of these compounds by paper chromatography and radioautography (14). These rates and patterns were compared for chloroplast suspensions with and without additions of 6-PGluA to concentrations of 0.34 mM and 0.68 mM.

RESULTS

In the presence of 0.5 mM 6-PGluA, RuDPCase was inhibited 75%, as compared with 14% inhibition by 0.5 mM fructose-1,6-diP (Table I). Essentially no inhibition was seen with glucose-6-P or with gluconate. Other compounds tested which caused less than 10% inhibition when added at 0.5 mM concentration included fructose-l-P, fructose-6-P, glucose-l-P, glucose-6-P, galactose-l-P, and PGA.

When the RuDPCase-mediated reaction was carried out in a reaction mixture in which approximately 0.5 mM 6-PGluA was previously generated by reaction of glucose-6-P with NADP in the presence of glucose-6-P dehydrogenase (see "Materials and Methods"), 69% inhibition was observed. No inhibition was seen in controls in which either glucose-6-P, NADP, or glucose-6-P dehydrogenase were omitted. No inhibition was seen in a separate control where NADPH (0.5 mM) only was added.

Table I. *Inhibition of Ribulose 1,5-Diphosphate Carboxylase by 6-Phosphogluconate and Related Compounds*

The mixture contained protein, 10.0 μg; RuDP, 0.5 mM; $NaH^{14}CO_3$, 50 mM (0.26 μc/μmole); incubation time was 10 min.

Compound	^{14}C Total Fixed cpm	% of Control (H_2O)
Control	14,990	100
Fructose 1,6-diP, 1.0 mM	11,540	77
6-Phosphogluconate, 1.0 mM	2,265	15
Glucose-6-P, 1.0 mM	14,524	97
Gluconate, 1.0 mM	14,754	98
Fructose-1,6-diP, 0.5 mM	12,900	86
6-Phosphogluconate, 0.5 mM	3,763	25
Glucose-6-P, 0.5 mM	14,775	99
Gluconate, 0.5 mM	14,716	98

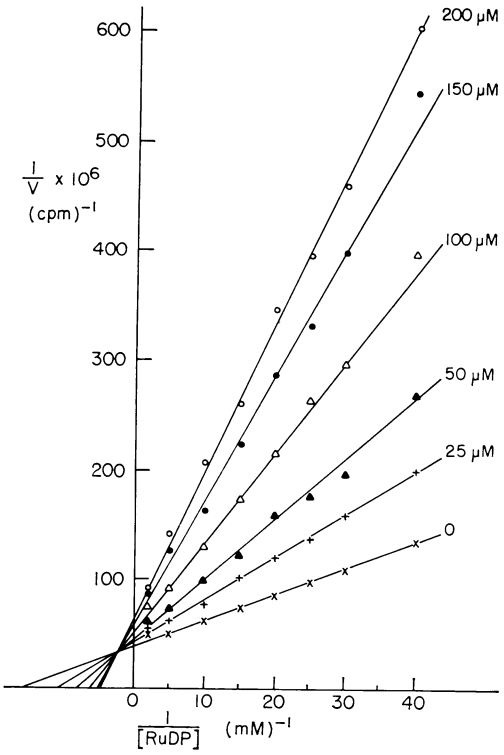

FIG. 1. Inhibition of RuDPCase by 6-PGluA. Concentrations of 6-PGluA are indicated in the figure. Protein, 5.0 μg; $NaH^{14}CO_3$, 50 mM (2.6 μc/μmole); incubation time, 10 min.

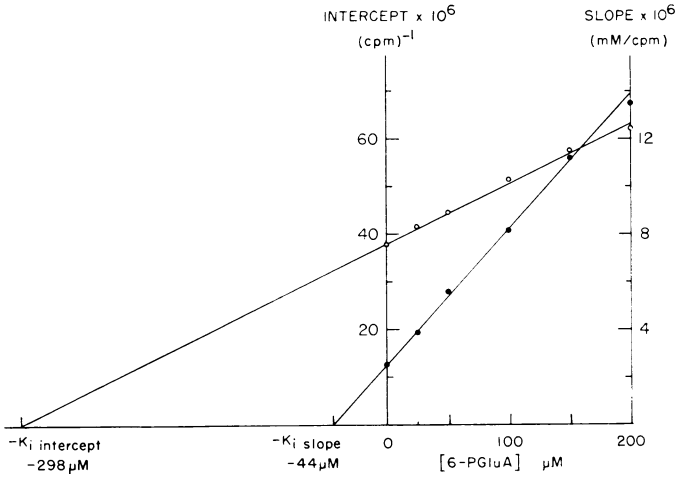

FIG. 2. Replots of intercepts and slopes of Figure 1 *versus* 6-PGluA concentration.

A plot of 1/v *versus* 1/[RuDP] at 50 mM $NaHCO_3$ and at several levels of 6-PGluA gives a noncompetitive inhibition pattern (Fig. 1). Replots of the vertical intercepts and slopes of Figure 1 *versus* [6-PGluA] suggest that this is a linear noncompetitive inhibition, with $K_{i\ intercept} = 298$ μM and $K_{i\ slope} = 44$ μM (Fig. 2), according to the nomenclature and theory of Cleland (8). It should be noted, however, the RuDPCase is a large protein with several subunits, two substrates, and several binding sites, so that simple kinetic treatment may not be

strictly valid. Also, 10 mM Mg^{2+} was present in the assays, and substrate, inhibition, and enzyme are affected to some extent by Mg^{2+}.

The vertical intercepts in Figure 1 are reciprocals of the maximum velocity of the enzyme with an excess of substrate in the presence of inhibition, so that $K_{i\ intercept}$ indicates the amount of inhibitor required to reduce the maximum velocity to one-half. Of more interest in the present context is $K_{i\ slope}$, which is indicative of the concentration of inhibitor necessary to reduce velocity to one-half as RuDP concentration approaches zero.

With a constant (saturating) concentration of RuDP of 0.5 mM, and varying $NaHCO_3$ concentration, plots of 1/v *versus* $1/[HCO_3^-]$ also show a noncompetitive inhibition pattern (Fig. 3). In this case, replots of the intercepts and slopes from Figure 3 give a result consistent with a hyperbolic noncompetitive inhibition (Fig. 4). Replots of the reciprocals of the differences between Figure 3 slopes with and without inhibitors ($1/slope_I - slope_0$), or of the reciprocals of the differences between Figure 3 vertical intercepts with and without inhibitors ($1/intercept_I - intercept_0$), in each case *versus* 1/[6-PGluA], give $K_{i\ intercept} = K_{i\ slope} = 415\ \mu M$ (Fig. 5) according to Cleland (8).

The rate of CO_2 fixation and the metabolic pattern of ^{14}C incorporation by isolated spinach chloroplasts were found to be unaffected by the addition of 6-PGluA to the suspending medium.

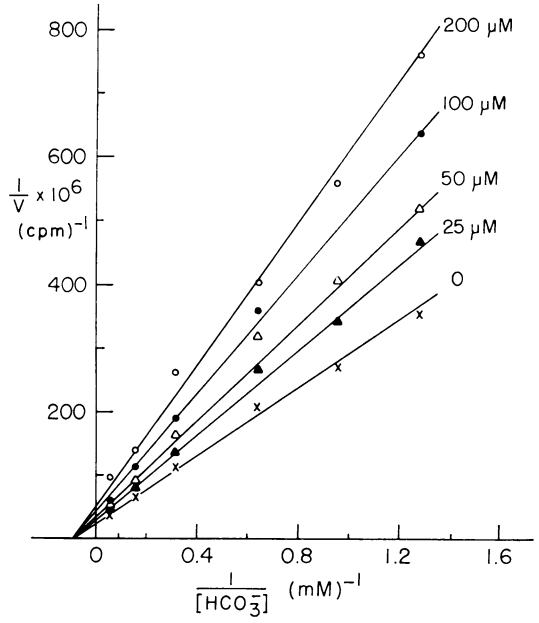

FIG. 3. Inhibition of RuDPCase by 6-PGluA. Concentrations of 6-PGluA are indicated in the figure. Protein, 5.0 μg; RuDP, 0.5 mM; incubation time, 5 min; $NaH^{14}CO_3$, 13.9 μc/μmole.

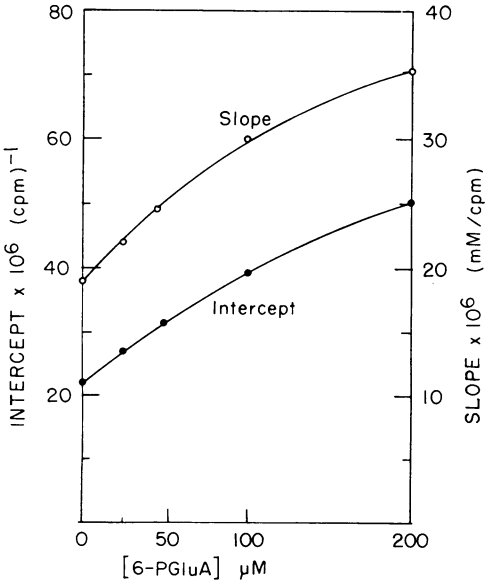

FIG. 4. Replots of intercepts and slopes of Figure 3 *versus* 6-PGluA concentration.

DISCUSSION

The inhibitory constants observed in this study may be compared with *in vivo* concentrations of metabolites estimated by using the saturating ^{14}C label of metabolites in *Chlorella pyrenoidosa* during steady-state photosynthesis in the light and during respiration in the dark to determine the gram atoms of carbon in each compound per cm^3 of packed algae cells used in making up the algal suspension (6). The arbitrary assumption was made that the metabolically active space containing the metabolites was one-fourth of the packed cell volume. This gave a RuDP concentration of 2.04 mM in the light, and a 6-PGluA concentration of 0.047 mM in the dark. The dark RuDP concentration was not reported, but the level of RuDP in the comparable study of Pedersen *et al.* (14) was 1.36 mM in the light and 0.20 mM in the dark, based on the same assumptions. Thus, the several values for K_i are slightly higher, but in the worst case within an order of magnitude of the estimated concentration of 6-PGluA in the dark. It should be noted that, in the dark, the oxidative pentose phosphate cycle is in operation in the chloroplasts (11), and CO_2 is being liberated but not consumed in the chloroplasts. Thus the K_i values obtained with 50 mM HCO_3^- (Figs. 1 and 2) are not necessarily unrealistic in terms of *in vivo* metabolism. However, the *in vivo*

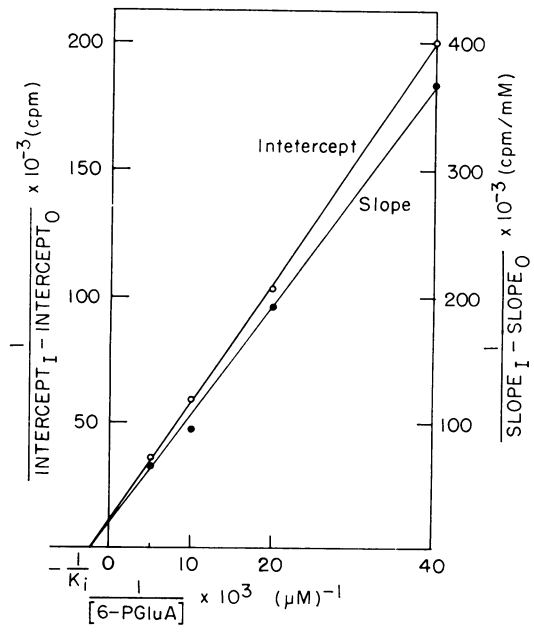

FIG. 5. Replots of $1/(intercept_I - intercept_0)$ and $1/(slope_I - slope_0)$ of Figure 3 *versus* 1/(6-PGluA concentration).

concentrations would include 6-PGluA in both cytoplasm and chloroplasts. In the report of the appearance of 6-PGluA in isolated chloroplasts, the amount of ^{14}C label found was smaller than in the *in vivo* experiments (11).

In any event, these comparisons between estimated K_i values and 6-PGluA concentration in the dark *in vivo* suggest some possibility that non-competitive inhibition of RuDPCase by 6-PGluA in the dark plays a role in the inactivation of carboxylation reaction required by the switch from the reductive pentose phosphate cycle during photosynthesis to the oxidative pentose phosphate cycle in the dark. The need for a light-dark switch in metabolism of chloroplasts has been discussed elsewhere (1, 2, 5). The enzymes characteristic of the oxidative pentose phosphate cycle have been found to be present in isolated spinach chloroplasts, although there were larger amounts of such enzymes in the cytoplasm (9).

Evidence for some of these changes can be seen upon either addition of vitamin K_5 to *Chlorella pyrenoidosa* in the light (11) or without additions when the light is turned off (14). It has been proposed that vitamin K_5 in its oxidized state diverts electrons from the reduction of ferredoxin and NADP, and that the resulting increased ratio of NADP/NADPH or of $ferredoxin_{ox}/ferredoxin_{red}$ activates glucose-6-phosphate dehydrogenase. Similar changes in activities occur when the light is turned off, without any additions.

Data in the present study suggest that the increased level of 6-PGluA may then further inactivate the RuDPCase, thus completely stopping the carboxylation reaction. The lack of inhibition of photosynthesizing spinach chloroplasts by 6-PGluA added to the medium is presumed to be due to lack of penetration of the limiting double membrane of the intact chloroplasts.

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