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

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ABSTRACT

Ribulose 1,5-diphosphate carboxylase, when activated by preincubation with 1 mM bicarbonate and 10 mM $MgCl_2$ in the absence of ribulose 1,5-diphosphate, remains activated for 20 minutes or longer after reaction is initiated by addition of ribulose diphosphate. If as little as 50 μM 6-phosphogluconate is added during this preincubation period, 5 minutes before the start of the reaction, a further 188% activation is observed. However, addition of 6-phosphogluconate at the same time or later than addition of ribulose diphosphate, or at any time with 50 mM bicarbonate, gives inhibition of the enzyme activity. Possible relevance of these effects *in vivo* regulatory effects is discussed.

The carboxylation enzyme of the photosynthetic reductive pentose phosphate cycle, ribulose 1,5-diphosphate carboxylase, mediates the reaction of ribulose 1,5-diphosphate with CO_2 and water to give 2 molecules of 3-phosphoglycerate (22, 23). This reaction seems likely to be subject to metabolic regulation *in vivo*, since it is accompanied by a high negative free energy change under conditions of steady state photosynthesis (1, 3), and thus is a rate-limiting step. Other observations of metabolic level change *in vivo*, such as after the light-to-dark transition, also suggest that RuDPCase[2] changes in activity (16). When *Chlorella pyrenoidosa* have been photosynthesizing with $^{14}CO_2$, and either the light is turned off (16), vitamin K_5 is added (10), or octanoate is added (15), the levels of labeled metabolite change in a way, indicating a shift from operation of the reductive pentose phosphate cycle to oxidative pentose phosphate cycle. Among the manifestations of this shift are the inactivation of phosphoribulokinase (which converts ribulose 5-phosphate and ATP to RuDP and ADP) and the inactivation of RuDPCase. At the same time, labeled 6-phosphogluconate appears, indicating the onset of the operation of the oxidative pentose phosphate cycle.

Strong inhibition of isolated RuDPCase can be caused by levels of 6-PGluA (6, 21) much lower than the levels of other metabolites such as fructose 1,6-diphosphate, which can also inhibit this enzyme (5, 6). In our previous report of this inhibition of RuDPCase by 6-PGluA (6), the reaction was initiated by adding the enzyme to the otherwise complete reaction mixture. It had been reported that preincubation of the enzyme with bicarbonate and Mg^{2+} ions increased the activity of the enzyme when assayed for 5 min (11, 17). In the course of further studies of the effects of 6-PGluA on the activity of RuDPCase, we have found that preincubation of the enzyme with Mg^{2+} and bicarbonate produces a long lasting activation of the enzyme, and that inclusion of 6-PGluA in the preincubation mixture causes a large additional activation (rather than inhibition) of the enzyme. However, when the enzyme is activated by preincubation with Mg^{2+} and bicarbonate and the reaction is initiated with addition of RuDP, subsequent addition of 6-PGluA causes inhibition. These and other results in this study suggest a complex regulatory mechanism, involving inactive and active forms of the enzyme, which respond differently to 6-PGluA, with the response further modified by presence or absence of RuDP, concentration of bicarbonate and Mg^{2+}, and other factors.

MATERIALS AND METHODS

Materials. In addition to the materials used in a previous report (6), glucuronic acid 1-phosphate, the potassium salt, was purchased from Sigma Chemical Co. The sodium salt of 6-PGluA was used in the present studies.

Enzyme Isolation. The enzyme was isolated from spinach leaves. The isolation method was the same as the one previously described (6), except in the following respects: (a) the step of heating at 50 C was omitted, and (b) 20 mM phosphate buffer solution containing 0.1 mM EDTA at pH 7.4 to 7.5 was used instead of 0.05 M tris-HCl buffer solution with 10 mM $MgCl_2$, 2 mM glutathione, and 0.2 mM EDTA at pH 7.4. The isolated enzyme was stored in the precipitated form under 60% saturation of ammonium sulfate in 20 mM phosphate buffer, pH 7.4 to 7.5. The purified enzyme had a specific activity of about 1.2 units/mg protein. One unit is defined as 1 μmole of carbon incorporated into acid-stable compound per min at 25 C.

Assay Method. The enzyme activity was determined by measurement of the radioactive acid-stable compound formed when $^{14}CO_2$ is used as a substrate in the reaction mixture. Because different assay procedures have been found to give very different results, we shall describe detailed experimental methods used in assaying the activities. The assays were conducted in the 17- $\times$ 60-mm glass vials. Each vial was first flushed with N_2 gas and sealed with a rubber serum stopper. Tris-HCl buffer solution and solutions of RuDP, $NaH^{14}CO_3$, effectors, and en-

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[2] Abbreviations: RuDPCase: ribulose 1,5-diphosphate carboxylase; RUDP: ribulose 1,5-diphosphate; PGA: 3-phosphoglycerate; 6-PGluA: 6-phosphogluconate; GlcuA-1-P: glucuronic acid 1-phosphate.

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zyme were injected into the vials by microsyringes at different stages according to the assay method used. All the solutions, except NaH¹⁴CO₃, had been bubbled with N₂ gas in order to remove CO₂ and O₂ dissolved in the solution from the air.

The stored enzyme was first dissolved in phosphate buffer and then dialyzed against the same buffer with at least six changes. The dialyzed enzyme solution was centrifuged. The supernatant enzyme solution usually contained 50 mg/ml of protein. Before the experiment, this enzyme solution was diluted with tris-HCl buffer, 0.1 M, pH 7.8, which contained either 10 mM or no MgCl₂. (Tris-HCl buffer had been bubbled with N₂ gas previously, but not the enzyme solution.) Usually a 200-fold diluted enzyme solution was used for the assay. In all assays, unless otherwise indicated, the final concentration of each component was the following: tris-HCl, 0.1 M; pH 7.8; MgCl₂, 10 mM; RuDP, 0.5 mM; enzyme, 10 μg; NaH¹⁴CO₃, 50 mM (0.10 μc/μmole) or 1 mM (4.1 μc/μmole). The amounts of effector (inhibitor or activator), the preincubation or reaction time (see below) or both are indicated for each experiment. The final volume of the reaction mixture was 0.4 ml. All the preincubations and reactions were conducted at 25 C. At the end of the reaction, 0.1 ml of glacial acetic acid was added to stop the reaction. Then the vials were flushed with N₂ gas at room temperature to dryness. Water (0.5 ml) was added to dissolve the remaining material in the vial. Six milliliters of Aquasol were then added. After mixing, the vials in the vial holders (Isolab Inc., Akron, Ohio) were counted in the liquid scintillation spectrometer. With ¹⁴C-toluene as internal standard, the counting efficiency for carbon-14 with Aquasol and the small vial in the holder was about 75%.

The preincubation time is the length of time in which the enzyme was incubated with part(s) of the reaction ingredients

before the start of the reaction. The reaction time is the length of time in which the reaction had taken place.

Assay Method I. The reaction was started by adding the enzyme to the reaction mixture which contained all the required ingredients.

Assay Method II. As in I, except that the enzyme had been preincubated with MgCl₂ for at least 10 min.

Assay Method III. The enzyme had been preincubated with NaH¹⁴CO₃ for 10 min; then the reaction was started by adding the mixture of RuDP and MgCl₂.

Assay Method IV. The enzyme had been preincubated with MgCl₂ and NaH¹⁴CO₃ for a specific length of time before adding RuDP to start the reaction.

For the studies of activation or inhibition, the order and procedure for adding the effector will be indicated below.

Assay Method V. Method II was used; the effector was added in the reaction mixture before the addition of the enzyme.

Assay Method VI. Method IV was used; the enzyme had been preincubated with the effector along with MgCl₂ and NaH¹⁴CO₃ for a specific length of time before the addition of RuDP to start the reaction.

Assay Method VII. The enzyme had been preincubated with MgCl₂ and NaH¹⁴CO₃ for a specific length of time before starting the reaction by adding the mixture of RuDP and the effector.

Assay Method VIII. The enzyme reactions were carried out in several vials according to Method II (preincubation with MgCl₂ but not with NaH¹⁴CO₃); one reaction was stopped at 5 min. The effector was added to three other vials at the end of 5 min, and the reactions were allowed to proceed for another 1.5, 4.5, and 7.5 min. The enzyme activity was measured from the reaction rate between 5 and 12.5 min of reaction in each case.

Assay Method IX. As in VIII, except that Method IV (preincubation with both MgCl₂ and NaH¹⁴CO₃) was used instead of Method II. The enzyme activity was measured as above.

For the kinetic studies, the assays were conducted in separate vials for various preincubation or reaction times or both. In the study of 6-PGluA activation, 6-PGluA was added to the enzyme which had been preincubated with MgCl₂ and NaH¹⁴CO₃ so that the preincubation time with 6-PGluA in each vial was different, but the total preincubation time with MgCl₂ and NaH¹⁴CO₃ was kept the same.

The fact that inhibition and activation was induced by 6-PGluA and not a contaminant in a commercial preparation was checked by generating fresh 6-PGluA as described under Table III.

In all the inhibition and activation studies, a control test (H₂O) and a comparison test (GlcuA-1-P) were also carried out at the same time.

Protein concentration was determined by UV absorption at 280 nm. The factor, $A = 1.0$ for 0.61 mg enzyme/ml, was used for the calculation of protein concentration (15).

RESULTS

It was found that dissolved CO₂ in the buffer solution from air could activate the enzyme activity. Because the amount of CO₂ in the solution varies from time to time due to the storage condition, it is necessary to expel the dissolved CO₂ in the solution so that the activation effects due to preincubation with MgCl₂ and NaH¹⁴CO₃ can be accurately determined.

With 1 mM NaH¹⁴CO₃, the kinetic studies using Assay Methods I to IV showed that the enzyme gave by far the highest activity when it was preincubated with both MgCl₂ and NaH¹⁴CO₃ (Method IV, Fig. 1). The enzyme, when preincu-
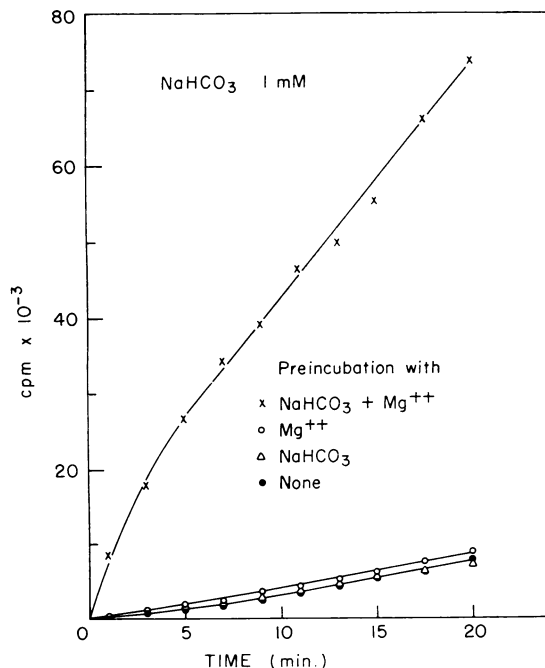

FIG. 1. Fixation of ¹⁴CO₂ via the carboxylation reaction with or without preincubation *versus* time of reaction. In the nonpreincubated system, the reaction was started by adding the enzyme to the reaction mixture which contained all the required ingredients (Method I). For the preincubated cases, the reactions were started by the additions of RuDP to the enzymes which had been preincubated with MgCl₂, NaH¹⁴CO₃ or both (Method II, III, and IV). Preincubation time: 10 min; NaH¹⁴CO₃: 1 mM.

bated with only $MgCl_2$ (Method II) or $NaH^{14}CO_3$ (Method III) or no preincubation at all (Method I), gave only one-tenth of the activity obtained under the above preincubation conditions. While the highest rate with the enzyme preincubated with both Mg^{2+} and $NaH^{14}CO_3$ was during the first 5 min, the rate remained about 6 times greater than the rate for the other assay conditions for the period from 5 to 20 min. The one which was preincubated with $MgCl_2$ had a slightly higher activity than the other two.

At a much higher concentration of $NaH^{14}CO_3$ (50 mM), the reaction rates from 9 to 20 min were almost the same (Fig. 2), irrespective of whether or not the enzyme was preincubated, although during the first 9 min the "preincubated" enzyme gave the higher activity.

The activities of enzymes which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ for various lengths of time indicated that the enzyme reached the maximal activity after about 2 min of preincubation at 25 C when the concentration of $NaH^{14}CO_3$ was low (1 mM) (Fig. 3). At high $NaH^{14}CO_3$ concentration (50 mM), 30 sec were enough to reach the maximum activity.

With 1 mM $NaH^{14}CO_3$, inhibition was observed when 6-PGluA was added to the reaction mixture 5 min after initiation of the reaction (Table I), whether the rate was high following preincubation with both Mg^{2+} and $NaH^{14}CO_3$ (Assay Method IX) or low following preincubation with only $MgCl_2$ (Assay Method VIII). Similar inhibition was seen upon addition of 6-PGluA to reaction mixtures containing 50 mM $NaH^{14}CO_3$ 5 min after reaction initiation following preincubation according to Method IX or Method VIII (Table II). The degree of inhibition in each case was calculated from the reaction rate. Addition of GlcuA-1-P gave no appreciable inhibition in any of these cases.

It was found that 6-PGluA could either activate or inhibit the enzyme activity, and the effect was completely dependent on the assay method (Tables I and II). At low $NaH^{14}CO_3$, 6-PGluA activated the enzyme if the enzyme was preincubated with 6-PGluA, as well as with $MgCl_2$ and $NaH^{14}CO_3$. A maximum of 188% activation was observed with 0.05 mM 6-PGluA following preincubation with 1 mM $NaH^{14}CO_3$ and Mg^{2+}. Even after 15 min reaction time, substantial activation (about 100%) of the enzyme was observed in the presence of 50 μM 6-PGluA. In all of the other assay methods with 1 mM $NaH^{14}CO_3$, 6-PGluA gave strong inhibition. At high $NaH^{14}CO_3$, inhibition was observed in every case, although different degrees of inhibition could be seen. In low $NaH^{14}CO_3$, every method, except Method VI ("preincubation"), gave almost the same degree of inhibition—about 60% inhibition by 0.5 mM 6-PGluA. GlcuA-1-P gave no significant effect. At high $NaH^{14}CO_3$, "no preincubation" Method V resulted in a stronger inhibition than the "preincubation" Method VI did (also see Fig. 6).

The enzyme reached its highest activity only after 3 to 4 min preincubation with 6-PGluA (0.05 mM) in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (Fig. 4). This may suggest that the binding of 6-PGluA to the enzyme is rather a slow process.

When the enzymes were preincubated with various concentrations of 6-PGluA along with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM), the maximal activation was obtained at 0.05 to 0.1 mM of 6-PGluA (Fig. 5). As the concentration was increased beyond 0.1 mM, the activation decreased or an inhibition effect appeared. At as low concentration as 5 μM, 6-PGluA gave 70 to 80% activation. However, with Method V ("no preincubation"), inhibition was obtained in every level of 6-PGluA.

GlcuA-1-P was used for comparison with 6-PGluA. Since both compounds have the same carboxyl and phosphate groups, differences in the charge effect and the ionic strength effect which might influence the enzyme activity are minimized. Pre-
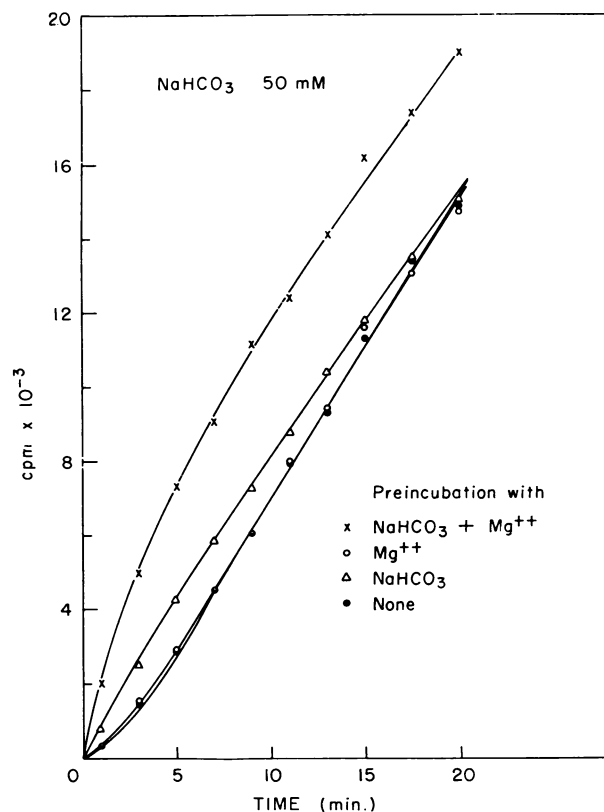

FIG. 2. Same as in Fig. 1, except $NaH^{14}CO_3$ was 50 mM.
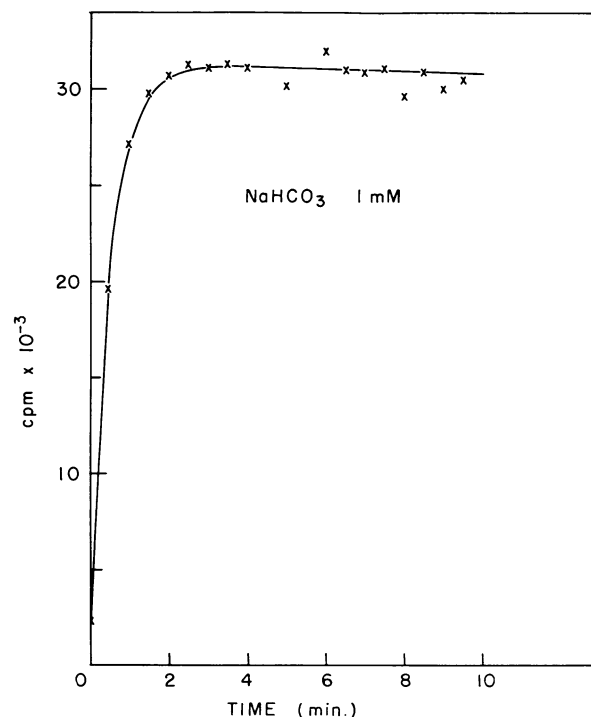

FIG. 3. Dependence of carboxylation reaction on time of preincubation of carboxylase with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM). Reaction time: 5 min.

incubation of the enzyme with 2 mM GlcuA-1-P, in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (Method VI), gave about 30% activation. The "activation" curve (Fig. 5) for enzyme preincubated with GlcuA-1-P was similar to curves for inorganic phos-

Table I. *Inhibition and Activation of RuDP Carboxylase Activity by 6-PGluA with 1 mM $NaH^{14}CO_3$*

Effector	% Activity of Control[1]				
	Assay Method				
	V[2]	VI[3]	VII[4]	VIII[5]	IX[6]
H_2O (control)[1]	100(2,430)[7]	100(26,505)	100(26,320)	100(2,843)	100(15,506)
6-PGluA, 0.5 mM	41	177	43	39	45
GlcuA-1-P, 0.5 mM	93	128	94	95	105
6-PGluA, 0.05 mM	89	288	87	85	84
GlcuA-1-P, 0.05 mM	103	105	104	105	102

[1] In each method, the enzyme activity was taken as 100%, when H_2O, instead of effector, was used in the assay mixture.

[2] Method V. The assay reactions were started by adding $MgCl_2$-preincubated enzymes to the reaction mixtures which contained the effector (or H_2O). The activities were the reaction rates between 0 and 5 min.

[3] Method VI. The assay reactions were started by adding RuDP to the enzymes which had been preincubated with the effector (or H_2O) along with $MgCl_2$ and $NaH^{14}CO_3$ for 5 min. The activities were the reaction rates between 0 and 5 min.

[4] Method VII. The assay reactions were started by adding the mixtures of RuDP and the effector (or H_2O) to the enzymes which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ for 5 min. The activities were the reaction rates between 0 and 5 min.

[5] Method VIII. The enzyme reactions were carried out in several vials according to Method II (preincubation with $MgCl_2$); one reaction was stopped at 5 min. The effector (or H_2O) was added to three other vials at the end of 5 min, and the reactions were allowed to proceed for another 1.5, 4.5, and 7.5 min. The activities per 5 min were measured from the reaction rates between 5 and 12.5 min in all the cases.

[6] Method IX. As in Method VIII, except that Method IV (preincubation with both $MgCl_2$ and $NaH^{14}CO_3$) was used instead of Method II.

I [7] The numbers in parentheses are the amounts of carbon incorporated in cpm/5 min.

Table II. *Inhibition and Activation of RuDP Carboxylase Activity by 6-PGluA with 50 mM $NaH^{14}CO_3$*

Effector	% Activity of Control[1]				
	Assay Method				
	V[2]	VI[2]	VII[2]	VIII[3]	IX[3]
H_2O (control)[1]	100(3791)[4]	100(5732)	100(5705)	100(3930)	100(4300)
6-PGluA, 0.5 mM	50	55	58	40	41
GlcuA-1-P, 0.5 mM	99	104	98	105	103
6-PGluA, 0.05 mM	87	89	85	83	85
GlcuA-1-P, 0.05 mM	105	99	103	98	102

[1] In each method, the enzyme activity was taken as 100%, when H_2O, instead of effector, was used in the reaction mixture.

[2] The activities were measured as those in Table I, except that the reaction rates were taken between 2.5 and 7.5 min; during this period, the rates were linear with time (Fig. 2).

[3] The activities of Methods VIII and IX were measured as those in Table I.

[4] The numbers in parentheses are the amounts of carbon incorporated in cpm/5 min.

phate. We were unable to confirm significant activation by 0.5 mM fructose 6-phosphate, reported by Buchanan and Schurmann (5).

At 50 mM $NaH^{14}CO_3$, no activation could be observed by either method. Nevertheless, Method V ("no preincubation") gave stronger inhibition than Method VI ("preincubation") (Fig. 6).

In order to check whether the above activation or inhibition effect is really induced by 6-PGluA, a control experiment was performed in which 6-PGluA was freshly generated using G-6-P, G-6-P dehydrogenase, and NADP⁺. From the results (Table III), it is clear that 6-PGluA, not impurities in the chemicals purchased, was the cause of the activation or inhibition.

DISCUSSION

After observing the activation of RuDPCase by preincubation of the enzyme with Mg^{2+} and bicarbonate, Pon *et al.* (17) offered three possible explanations which can now be examined in the light of the present data. There may be a requirement that in the reaction mechanism CO_2 entering the carboxylation reaction at the active site must be bound prior to the binding of RuDP. This order of binding may be required, but if so, cannot explain the persistent activating effect of preincubation. After one carboxylation in the presence of RuDP, there would no longer be a preincubation effect. A second possibility was that since radioactive PGA is measured, there might be an exchange or displacement of unlabeled bicarbonate by labeled bicarbonate. This also is ruled out in the present study by the careful exclusion of unlabeled bicarbonate and CO_2. The third possibility is that both Mg^{2+} and bicarbonate activate the enzyme, but that the activation bicarbonate bound to the enzyme is not the reacting species. In terms of data in the present study, this seems to be the most likely explanation of the preincubation effect.

It might be suggested, however, that the even higher reaction rate seen during the first 5 min of the reaction following pre-
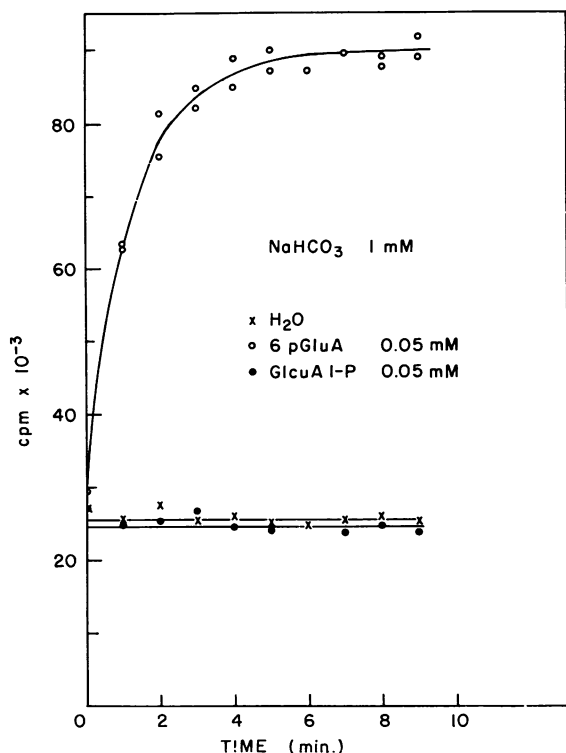
FIG. 4. Dependence of the carboxylation reaction on the time of preincubation of carboxylase with effectors (0.05 mM) in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (1 mM). The assays were conducted in separate vials. Effector or H_2O was added to the enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ so that when RuDP was added, the preincubation time with the effector in each vial was different, but the total preincubation time with $MgCl_2$ and $NaH^{14}CO_3$ was kept the same. Total preincubation time with $MgCl_2$ and $NaH^{14}CO_3$: 5 min; reaction time: 5 min.

FIG. 5. Effects on carboxylase activity caused by various concentrations of effectors with two different assay methods. $NaH^{14}CO_3$: 1 mM; reaction time: 5 min (between 0 and 5 min). A. Enzyme had been preincubated with effector in the presence of $MgCl_2$ and $NaH^{14}CO_3$ for 5 min (see Assay Method VI). B. Enzyme had been preincubated with $MgCl_2$ only (see Assay Method V).

FIG. 6. Same as in Fig. 5, except $NaH^{14}CO_3$ was 50 mM. Reaction time: 5 min (between 5 and 10 min).

incubation could, in fact, be the result of carboxylation of previously bound CO_2, the species which has been shown to be involved in carboxylation (7). In our preincubation experiments, $H^{14}CO_3^-$ was added to the buffer at least 30 min prior to initiation of the reaction, so that complete equilibration of CO_2 and HCO_3^- species was assured. To account for the increased rate seen during the first 5 min (compared to the subsequent period) for the preincubated enzyme, about 100 molecules of CO_2 would have to be bound to the enzyme at the start of the reaction period. A more likely explanation of the higher initial rate, decreasing over the first 5 min, would seem to be that binding of RuDP to the enzyme decreases its activity. The enzyme would be in the most active form at the start, and binding of RuDP until some steady state number of binding sites are occupied might take several minutes.

Since the higher rate of enzymic reaction persists for as long as 20 min, we suggest that preincubation with Mg^{2+} and HCO_3^- either modifies the enzyme conformation to give a more active form or prevents excessive binding of RuDP to the enzyme or both. Since the enzyme presented with RuDP without preincubation with HCO_3^- and Mg^{2+} is much less active and remains inactive for at least 20 min, it is suggested that RuDP binding by the nonpreincubated enzyme does convert the enzyme to (or maintain it in) a less active form from which it cannot change in low levels (1 mM) HCO_3^- as long as RuDP is present. If RuDP had not had any influence on the enzyme conformation and activity, the reaction rate should increase after several minutes (in the nonpreincubated case). It is proposed that the enzyme is in the active form as long as
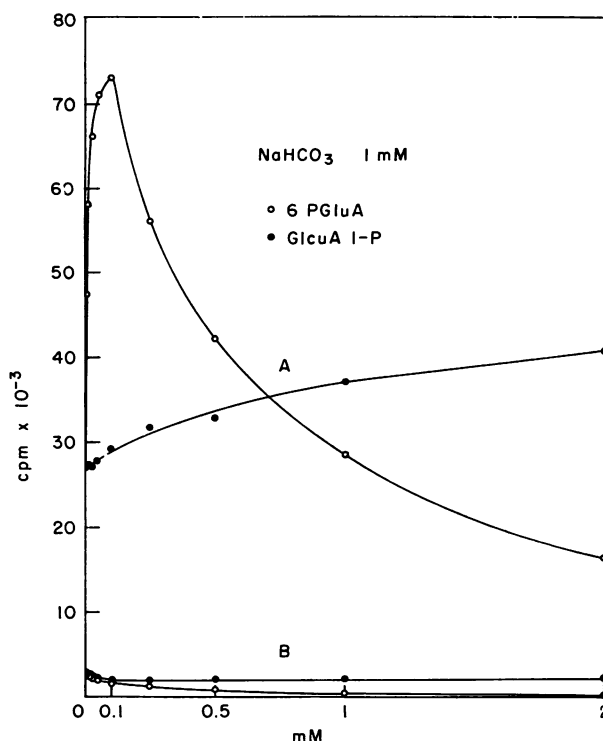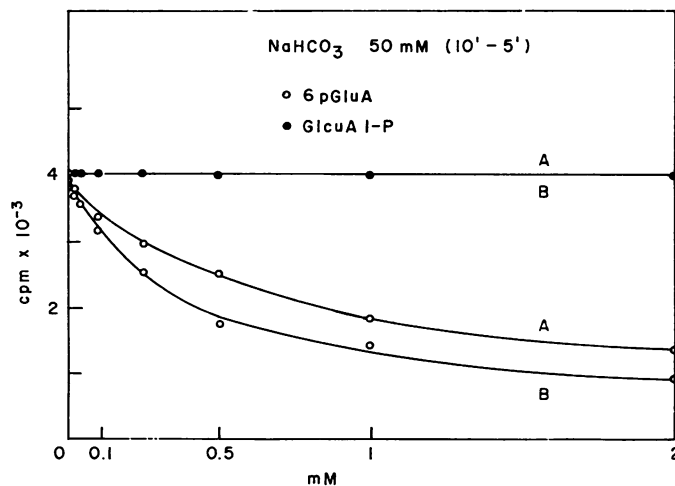
it is in an environment of Mg^{2+} and CO_2 and is never presented with RuDP in the absence of Mg^{2+} and CO_2. (However, as shown in Fig. 2, the effect caused by RuDP without preincubation with CO_2 and Mg^{2+} can be overcome by high levels of CO_2.)

It is known that RuDPCase contains a number of subunits and many binding sites for substrates (18–20, 25). It is suggested that the activity of the enzyme depends in some way on the number of CO_2 molecules and RuDP molecules bound to the enzyme, and that a high number of CO_2 molecules bound to the enzyme tends to reduce the number of RuDP molecules

Table III. *Control Experiment*

6-PGluA was first generated by the reaction of 5 mM of glucose 6-phosphate (G-6-P) with 5 mM of NADP in the presence of glucose 6-phosphate dehydrogenase (10 units) in 0.1 M tris-HCl buffer, pH 7.8. In each of the other three tubes, either G-6-P, NADP, or G-6-P dehydrogenase was omitted. All the tubes were incubated for 20 min at 25 C. After the reaction, part of the reaction product in each tube was diluted 10-fold with the same buffer. An aliquot (40 μl) from each of the original and diluted reaction products was added to the vials for assay of RuDP carboxylase activity, using either Method V or Method VI, so that the concentrations of the effectors were decreased 10-fold (*i.e.* the final concentration in the complete system was 0.5 or 0.05 mM).

Incubation System	Assay Method V[1] Activity		Assay Method VI[1,2] Activity	
	cpm	%	cpm	%
Original products				
All ingredients	871	38	50,974	183
Minus NADP	2,267	99	29,296	105
Minus G-6-P	2,173	95	34,003	122
Minus G-6-P dehydrogenase	2,239	98	30,487	109
Minus all ingredients (control[3])	2,291	100	27,861	100
10-fold dilution of above				
All ingredients	1,886	82	77,978	287
Minus NADP	2,234	98	28,602	103
Minus G-6-P	2,245	98	29,090	104
Minus G-6-P dehydrogenase	2,225	97	27,490	99

[1] Reaction time: 5 min; $NaH^{14}CO_3$: 1 mM.

[2] Preincubation time: 5 min.

[3] In each method, the enzyme activity was taken as 100% when only buffer solution (without G-6-P, G-6-P dehydrogenase, or $NADP^+$) was used in the assay.

bound to the enzyme, but not necessarily at the active catalytic sites. It is further suggested that RuDP binding at some sites tends to prevent CO_2 binding at activating sites, thus reducing the activity of the enzyme. Sufficiently high levels of bicarbonate provide enough CO_2 to overcome the effects of the RuDP binding at sites which inactivate the enzyme.

As previously reported, and as seen in these studies (Tables I and II), 6-PGluA inhibits the enzyme when added with or after RuDP, or under any condition with 50 mM HCO_3^-. Surprisingly, when the enzyme is preincubated with 1 mM $H^{14}CO_3^-$ and Mg^{2+}, and when 6-PGluA is added during the preincubation period, considerable additional activation of the enzyme can be seen. The maximal activation occurs with only 0.1 mM 6-PGluA. An activation by 75% is seen with only 5 μM 6-PGluA. Thus, the 6-PGluA is a very specific effector under these conditions. At 2 mM, a net inhibitory effect is observed. Activation (with preincubated RuDPCase) is not seen when the 6-PGluA is added simultaneously with the RuDP. Thus, it appears that the binding of RuDP is faster than the binding of 6-PGluA at those sites involved in the conversion of active to inactive enzyme. Furthermore, binding of RuDP prior to incubation of the enzyme with Mg^{2+}, CO_2 and 6-PGluA precludes not only the activation by Mg^{2+} and CO_2, but also the additional activation by 6-PGluA.

The additional activation (when the enzyme is preincubated with CO_2 plus Mg^{2+}) due to 6-PGluA may be due to the 6-PGluA binding in such a way as to prevent some binding of RuDP when it is subsequently added. The binding of 6-PGluA is a relatively slow process as indicated by the fact that the maximal stimulation of the enzyme by preincubation with HCO_3^-, Mg^{2+} and 6-PGluA requires about 5 min (Fig. 4). The

slow response of the enzyme to 6-PGluA may be an example of the hysteretic phenomenon proposed by Frieden (8). The preincubation effect of CO_2 alone required 2 min (Fig. 3), and the binding of RuDP which leads to lower activity must be assumed to be even faster, since simultaneous addition of RuDP, Mg^{2+}, and CO_2 gives the less active form of the enzyme.

The inhibition by 6-PGluA reported previously and seen under all conditions except preincubation with Mg^{2+} and low CO_2, may be due to competition of 6-PGluA with RuDP at the active catalytic sites. Kinetic analysis of the inhibition of the enzyme by 6-PGluA without preincubation suggests that the inhibition might be of the linear noncompetitive type, but, as we pointed out, simple kinetic treatment might not be strictly valid for RuDPCase, which has several subunits, two substrates, and multiple binding sites. Thus, we are now inclined to view the inhibition by 6-PGluA as due to competition with RuDP for the RuDP-catalytic binding sites, while the rather small displacement of the crossing point of the reciprocal plots (1/V vs. 1/[RuDP]) for different 6-PGluA concentrations from the zero value of 1/[RuDP] could be due to the effect of 6-PGluA in affecting the binding of RuDP at the allosteric RuDP binding sites.

What significance can be attached to the regulation of RuDPCase by levels of RuDP, CO_2, Mg^{2+}, and 6-PGluA? When the light is on, and physiological conditions are favorable for photosynthesis, we may assume that the level of Mg^{2+} is high, CO_2 is not too low, RuDP concentration is adequate, 6-PGluA is nearly absent, and RuDPCase is active.

During the light to dark transition, the level of RuDP though falling rapidly is enough to sustain the carboxylation reaction for about 2 min. If the level of Mg^{2+} free in the soluble part of the chloroplasts decreases greatly, as indicated by the study of Lin and Nobel (12, 13), this would of itself decrease the activity of the active form of RuDPCase, which has a pH dependence shift to the alkaline with reduced Mg^{2+} (4). The appearance of 6-PGluA during the 1st min of darkness would further inhibit the enzyme. These and perhaps other effects (9, 24) may account for the slow rate of the carboxylation reaction observed in isolated chloroplasts and in *Chlorella pyrenoidosa* after about 2 min darkness.

After a long period of darkness, the level of RuDP would have gone to zero. The level of CO_2 inside chloroplasts would be much higher in the dark, due to absence of photosynthetic uptake, and generation of CO_2 by the oxidative pentose phosphate cycle. When the light is turned on again, particularly if the intensity is low, low levels of 6-PGluA will still be present (even in bright light for the few minutes after a period of darkness [2]). When the light comes on, the Mg^{2+} level will also increase, so that the enzyme *in vivo* would be "preincubated" with CO_2, Mg^{2+}, and a low concentration of 6-PGluA, the conditions required for activation. Thus, RuDP can be actively carboxylated as soon as it is formed from ribulose 5-phosphate.

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