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BIOCHEMICAL AND PHYSICAL STUDIES OF RIBULOSE 1,5-DXPHOSPHATE CARBOXYLASE FROM SPINACH LEAVES

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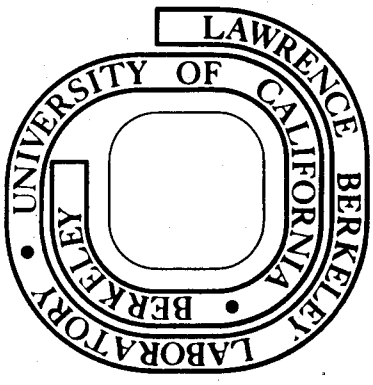

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DISCLAIMER

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CONTENTS

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Biochmical and Physical Studies of Ribulose 1,5-Diphosphate Carboxylase from Spinach Leaves

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August, 1974

ABSTRACT

Ribulose 1,5-diphosphate carboxylase, which catalyzes the CO_2 fixation in photosynthetic organisms, is a regulatory enzyme of the Calvin cycle. The enzyme isolated from spinach leaves has been studies by biochemical and physical methods.

When activated by preincubation with 1 mM bicarbonate and 10 mM $MgCl_2$ in the absence of ribulose 1,5-diphosphate, the enzyme remains activated for 20 min or longer after the reaction is initiated by addition of ribulose 1,5-diphosphate. Partially-preincubated (preincubated with either bicarbonate or $MgCl_2$) or non-preincubated enzyme has an activity of about one tenth of that of fully-preincubated enzyme. Ribulose 1,5-diphosphate is a strong inhibitor for the non-preincubated enzyme while bicarbonate and $MgCl_2$ are activators.

If as little as 0.05 mM 6-phosphogluconate is added during the preincubation period, 5 min 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 1,5-diphosphate, or at any time with 50 mM bicarbonate, gives inhibition

of the enzyme activity.

When the enzyme is activated by preincubation with bicarbonate and $MgCl_2$ in the absence of ribulose 1,5-diphosphate, it can be further activated about 170% with 0.5 mM NADPH present in the preincubation mixture. $NADP^+$, NADH, and NAD^+ are ineffective. The activation by NADPH is comparable to that seen with 0.05 to 0.1 mM 6-phosphogluconate in that these specific preincubation conditions are required, but the effects of NADPH and 6-phosphogluconate are not additive. Moreover, where higher concentrations of 6-phosphogluconate inhibit the enzyme, higher concentrations of NADPH give a greater activation, saturating at 1 mM and 200%.

Under the specific conditions of preincubation, fructose 1,6-diphosphate has an activation curve similar to that of 6-phosphogluconate, peaking at 0.1 mM and 70%. Above this level, activation decreases, and inhibition is seen at still higher concentrations. Other metabolites tested produced smaller or no effects on the enzyme activity assayed under these conditions.

When either NADPH or 6-phosphogluconate is present in the preincubation mixture, it becomes possible to determine the K_m for bicarbonate using a Lineweaver-Burk plot, and the K_m for bicarbonate under these conditions is 2.8 mM, corresponding to 0.3% CO_2 at pH 7.8 and 25 C.

The activation by bicarbonate and $MgCl_2$ during the preincubation period is a rather slow process; 2 min is required to reach maximum activation with 1 mM bicarbonate and 10 mM $MgCl_2$. The further activation by the presence of either 6-phosphogluconate or NADPH in the preincubation mixture needs about 5 min to reach a maximum.

The inhibitions observed at higher concentrations of either 6-phosphogluconate or fructose 1,6-diphosphate in the preincubation assay method and at any concentration in other assay methods are due to the competitive inhibitions at ribulose 1,5-diphosphate catalytic site(s). The K_i of 6-phosphogluconate is 20 μM and that of fructose 1,6-diphosphate is 190 μM. Among the metabolites of the oxidative and reductive pentose phosphate cycles, 6-Phosphogluconate is the strongest inhibitor discovered so far.

Since 6-phosphogluconate is unique to the oxidative pentose phosphate cycle and inhibits at concentrations comparable to those found in vivo, it is proposed that its inhibition on carboxylase may be a regulatory factor. The specific activation induced by NADPH, which is an important reducing compound produced during the light period, may also play the necessary activating role for the enzyme in vivo. The possible mechanisms by which ribulose 1,5-diphosphate carboxylase is regulated by these two metabolites, especially during light-dark-light transition are discussed.

ABBREVIATIONS

ATP: adenosine 5'-triphosphate

ADP: adenosine 5'-diphosphate

AMP: adenosine 5'-monophosphate

FDP: fructose 1,6-diphosphate

F6P: fructose 6-phosphate

G6P: glucose 6-phosphate

GlcuA 1-P: glucuronic acid 1-phosphate

NAD: nicotinamide adenine dinucleotide, oxidized form

NADH: nicotinamide adenine dinucleotide, reduced form

NADP: nicotinamide adenine dinucleotide phosphate, oxidized form

NADPH: nicotinamide adenine dinucleotide phosphate, reduced form

6PGluA: 6-phosphogluconate

PGA: 3-phosphogluconate

Ru5P: ribulose 5-phosphate

RuDP: ribulose 1,5-diphosphate

RuDPCase: ribulose 1,5-diphosphate carboxylase

tris: tris(hydroxymethyl) aminomethane

K_i: inhibition constant

K_d: dissociation constant

K_m: Michaelis-Menten constant

cpm: counts per minute

v: initial velocity of an enzymatic reaction

ESR: electron spin resonance

DTT: dithiothreitol

UV: ultraviolet (spectrum)

INTRODUCTION

Historically, the chemical reactions involved in plant photosynthesis are divided into two phases, the light and dark phase reactions; the former deals with the reactions which convert light quanta into chemical energy: ATP and NADPH. The chemical reactions which use this energy to synthesize carbohydrates and other compounds for physiological needs are called dark phase reactions, since these reactions only use the energy to carry out the syntheses and have nothing to do with the light directly. The primary reactions involved in the dark phase reactions are those in the carbon reduction cycle (Calvin cycle) (5). This cycle consists of a series of clclic reactions which convert CO_2 and H_2O into glucose at the expense of ATP and NADPH. The net reaction of the cycle is:

$$6\ CO_2 + 12\ H^+ + 18\ ATP + 12\ NADPH + 12\ H_2O$$
$$= \text{hexose} + 18\ P_i + 18\ ADP + 12\ NADP^+$$

Since the reductive pentose phosphate cycle was elucidated by Calvin and co-workers in 1950's, the regulations of the chemical reactions in this cycle have been studied intensively throughout the world. Photosynthetic carbon metabolism is controlled by many ways such as membrane transport, light-dark-light transition, ion flux, etc. (3). One category of such regulation is the enzymatic control of the reaction rates of some of the metabolic steps.

Bassham and co-workers have been studying the regulations of photosynthetic carbon metabolism either in green algae cells in vitro or in isolated chloroplasts from spinach leaves (4). Their studies demonstrated that some of the enzymes of the Calvin cycle are really

the regulatory enzymes which control the reaction rates of several metabolic processes. Fig. 1 indicates such regulation steps in the reductive and oxidative pentose phosphate cycles.

In can be shown that each reaction of the cycle can be carried out without light, in vitro, as long as the necessary reactants are present and the specific enzyme and co-factors exist to accelerate the reaction rate. However, as we know, chemical reactions in the living systems are not as simple as those in vitro; they are well under control so that the metabolic balance can be maintained. Although each reaction in the Calvin cycle can proceed in the dark in vitro, for some reasons indicated below it is reasonable to ask, "Can the Calvin cycle be operating in the dark in vivo? If not, What are the possible mechanisms which regulate this cycle?"

If the Calvin cycle functions in the dark, because the syntheses of sugars from CO_2 and H_2O definitely need ATP and NADPH, it is quite reasonable to ask where the energy and reductants come from while there is no energy supply from light. The limited amount of ATP or NADPH left over from the light period (from cyclic and non-cyclic photophosphorylations) must be used up in a very short time, if light is the only source of energy. However, we know that the degradations of sugars and lipids and oxidative phosphorylation in the mitochondria can liberate ATP and NADPH. Is it possible that ATP and NADPH so produced can be used to synthesize sugars? Logically there is no reason to believe that a living system would degrade a compound in order to synthesize the same compound. If this were true, there would be a large waste of energy according to the second law of thermodynamics. Therefore, it is thought that

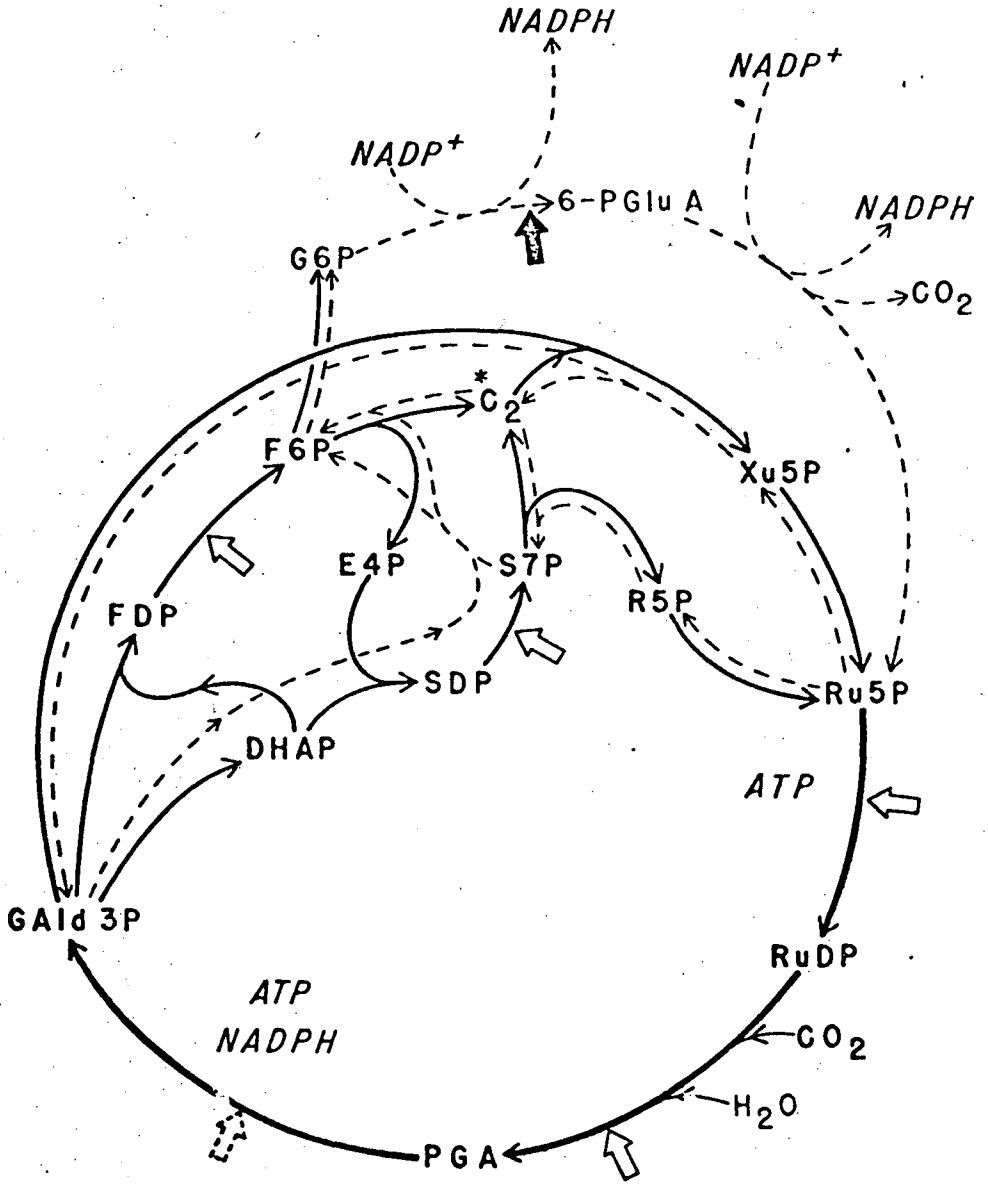

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Fig. 1. The Reductive Pentose Phosphate Cycle (Calvin Cycle) of Photosynthesis. Solid lines indicate reactions of reductive cycle, dashed lines reactions of oxidative cycle. Open arrows indicate positions of enzymes activated in light; dark blunt arrow indicates position of enzyme activated in dark.

the carbon reduction cycle does not (except for a short time) operate in the dark, in vivo. When Chlorella pyrenoidosa have been photosynthesizing with $^{14}CO_2$ and the light is turned off (32), the levels of labeled metabolite change in a way indicating a shift from the 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 Ru5P and ATP to RuDP and ADP) and the inactivation of RuDPCase. At the same time, labeled 6PGluA appears (6), indicating the onset of the operation of the oxidative pentose phosphate cycle. Therefore, it is seen that light-dark-light transition is one of the most important regulations of photosynthetic carbon metabolism in vivo.

In biological systems, it is a common phenomenon that a series of consecutive reactions is controlled by the enzyme which catalyzes the first reaction in the sequence. The enzyme, ribulose 1,5-diphosphate carboxylase (EC, 4.1.1.39), is the first enzyme in the Calvin cycle which catalyzes the following reaction (45, 46):

$$RuDP + CO_2 + H_2O \Longrightarrow 2\ PGA \qquad \Delta G = -9.8\ Kcal/mole$$

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 (7), 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 changes in activity.

From the analysis of steady-state levels of RuDP in the dark following photosynthesis, Pedersen et al. (32) concluded that the

mechanism for the carboxylation reaction in vivo becomes inactive after 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/mole negative (7). Other studies showed that the carboxylation reaction is inactivated, even with the light on by the addition of fatty acids (31) which are thought to interfere with the ion pumping in the thylakoids. Inactivation also occured with the addition of vitamin K_5, thought to interfere with electron transport (21).

Jensen and Bassham (18) found that CO_2 fixation in isolated chloroplasts ceased within about 2 min after the light was turned off. 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 RuDPCase is activated in the light in chloroplasts by changes in the ionic content of the chloroplasts, and by the higher ratio of reduced to oxidized coenzymes (3,4,6). 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 occuring 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.

The specific activity of the isolated RuDPCase in early studies seems too low for it to be able to catalyze CO_2 fixation at the rates observed for green cells *in vivo* (33). The low enzyme activity, especially at low CO_2 concentration, affords doubt as to its real significance in CO_2 fixation *in vivo*. One of the factors responsible for the low enzyme activity observed in an isolated enzyme system is the lack of some factor(s) which may actually activate the enzyme activity so that the reaction rate can be increased. The earlier studies of Pon et al. (34) indicated that if the enzyme is preincubated with both bicarbonate and $MgCl_2$ it will achieve maximum activity. This fact reveals the possibility that $MgCl_2$ and bicarbonate have an activating effect. One recent report of the activation of RuDPCase activity is the F6P effect discovered by Buchanan and Schurmann (10). The light activation factor discovered by Wildner and Criddle (48) also shows activation in the presence of light. These results clearly demonstrate the importance of the activators in the regulation of RuDPCase activity. As indicated above, the light-dark-light transition turns on and off some of the reactions in the oxidative and reductive pentose phosphate cycles. Some metabolite concentrations should change drastically during this transition. Also, it is known that ATP and NADPH are produced by cyclic and non-cyclic photophosphorylations when the light energy is available. These metabolites in the chloroplasts would be the most possible candidates for the control of RuDPCase activity, especially during the light-dark-light transition.

The study of an enzyme activity requires an accurate assay

method to measure its ability to catalyze the reaction. Since Pon et al. (34) found that the preincubated and non-preincubated enzyme gave significantly different activities, it is important to establish an accurate assay method. The discrepancies between the results of several reports might be due to the poorly described assay methods used by different authors. Because the carboxylation reaction needs three substrates (CO_2, RuDP and H_2O) and a divalent cation is necessary for enzyme activity, there are a variety of ways in which the enzyme activities can be measured. When an effector participates in the enzymatic reaction, the effector can be added before, after, or simultaneously with the substrate(s). The changes of enzyme activities due to the different orders of addition of the substrates, metal ion, and the effector might reveal the binding mechanisms of these small molecules on the enzyme.

As mentioned above, the carboxylation reaction has an absolute requirement for a divalent cation and it has been suggested that control of metal ion activation might play a role in the regulation of the enzyme activity in vivo (6). However, little is known about the stoichiometry of the metal ion-enzyme interaction, the nature of the groups on the protein involved in the interaction, or the mechanism of the activation. In fact, the only case where binding of metal ions to the enzyme has been studied involved inactive ternary complexes of the enzyme with a substrate analogue or complexes with cyanide (49). Putative evidence for the existence of an enzyme-CO_2-Mg^{+2} has been reported (1).

Studies of other investigators indicate that Mn^{+2} ion can partially activate enzyme activity as compared to Mg^{+2}. Mn^{+2}, not

Mg^{+2}, has the unpaired spin which is necessary to give an ESR signal. Free Mn^{+2} ion in solution has an intense isotropic ESR spectrum but the ESR signal of Mn^{+2} bound to small molecules and proteins is often broadened and undetectable providing a convenient means of measuring binding constant (12, 24, 25).

Because Mg^{+2} is the ion which activates the enzyme *in vivo*, it is assumed that the binding if Mg^{+2} is more effective than that of Mn^{+2}. Addition of Mg^{+2} to the Mn^{+2}-bound enzyme will liberate Mn^{+2} from the Mn^{+2}-enzyme complex, if both ions have the same binding site. The competitive bindings of these two ions should be indicated by the signal intensities.

RuDPCase was first discovered by Quayle et al. (35) in green algae and by Weissbach et al. (46) in spinach leaves in 1954. Later it was found that this enzyme is actually the same protein as Fraction I protein isolated by Wildman and Bonner in 1947 (47). Since the discovery of this enzyme almost 20 years ago, enzymes have been isolated from a large variety of sources, from higher plants to photosynthetic bacteria.

The enzyme isolated from spinach leaves has a molecular weight of about 560,000 and contains two kinds of subunits. The bigger subunit has a molecular weight of 54,000-60,000 and the small one of 12,000-16,000. The whole enzyme probably consists of 8 subunits of each kind. According to Wishnick et al. (50) this enzyme also has one copper atom per whole enzyme molecule. The number of binding sites of RuDP was determined to be 4 ± 1 to 8, depending on the ionic strength used in the measurement (49). Recently, eight binding sites for Mn^{+2} ion has been reported when a saturated

amount of bicarbonate was present (27). Several review articles about this enzyme from different sources have been published recently (20, 23, 40).

Some of the possible mechanisms which have been proposed to regulate RuDPCase activity are: the dependence of optimal pH of the enzyme on Mg^{+2} ion concentrations (8), the light activation factor (48), F6P activation and FDP deactivation (11) and heat activation (19). Although all the mechanisms just mentioned offer possible ways that the enzyme activity is regulated by metabolites or other factors, they are not necessarily the only means of regulation. It is my intention to study the problems mentioned previouly and hope that some other regulation mechanisms can be obtained from these studies and thus broadening our understanding in the control of photosynthetic carbon metabolism.

MATERIALS

Spinach leaves used for the isolation of RuDPCase were purchased from local markets. In the study of CO_2 fixation in isolated chloroplasts, fresh leaves were obtained from spinach grown in vermiculite.

The commercial available chemicals and enzymes were purchased from several companies as indicated below.

Sigma Chemical Co. (St. Louis, Mo.): Sodium salts of F6P, FDP, PGA, NAD, NADH, NADP, NADPH, 6PGluA and G6P; α-ketoglutarate; Glcu A 1-P, potassium salt; Dowex-50, H^+ form; Barium salts of Ru5P and RuDP; L-glutamate dehydrogenase; F6P kinase; Ru5P kinase; DTT.

Calbiochem (La Jolla, Calif.): ATP, sodium salt; G6P dehydrogenase; glutathione, reduced form.

The scintillation fluid, " Aquasol", was the product of New England Nuclear (Boston, Mass.).

Acid forms of RuDP and Ru5P were generated from solutions of barium salts of these compounds with Dowex-50, H^+ form.

EXPERIMENTAL

1. Purification of RuDPCase. In order to study the characteristics of this enzyme, it is necessary to establish a reproducible method of purification. Methods described by others were tried. The following purification steps employed in this study are adapted or modified from those studies (30, 37, 43).

Crude extract. Spinach leaves were washed in tap water and distilled water. The destemmed leaves were chopped and homogenized in the Waring Blender for 3 min in 2 vol of phosphate buffer (0.02 mM, pH 7.4-7.5). The homogenate was filtered through several layers of cheese cloth. The filtrate was then centrifuged in the Sorvall RC-2B centrifuge (rotor ss-34; 17,500 rpm, 30 min). The supernatant was termed "crude extract".

Sephadex G-25 column chromatography. The crude extract was loaded on the Sephadex G-25 column which had been equilibrated with phosphate buffer. The eluate fractions were assayed for carboxylase activities and the fractions with high activities were pooled together. The volume of this "G-25" fraction was about the same as that of "crude extract".

Ammonium sulfate fractionation. Ammonium sulfate was added slowly to the "G-25" fraction to 30% saturation. The solution was kept at 0 C for at least 5 hr. The precipitate was discarded after centrifugation at 10,000 g in the Sorvall centrifuge (rotor GSA, 8,000 rpm, 30 min). The supernatant was brought to 60% saturation with ammonium sulfate. After keeping at 0 C for at least 5 hr, the precipitate was collected by centrifugation at 10,000 g in the Sorvall centrifuge (rotoe GSA, 8,000 rpm, 30 min). The precipitate

was dissolved in phosphate buffer. This fraction was named "ammonium sulfate precipitate".

DEAE-cellulose column chromatography. A washed DEAE-cellulose column was equilibrated with phosphate buffer. The dialyzed "ammonium sulfate precipitate" solution was layered on the top of the resin. After it was absorbed, the resin was eluted with two volumes of phosphate buffer and then with a linear gradient of NaCl (0 - 1 M). The eluate fractions with high carboxylase activities were combined and precipitated with ammonium sulfate at 60% saturation. The protein collected by centrifugation was dissolved in phosphate buffer. It was named "DEAE" fraction.

Sephadex G-200 column Chromatography. Swelled Sephadex G-200 gel was equilibrated with phosphate buffer in a column. The dialyzed "DEAE" fraction was put onto the top of the gel and eluted with phosphate buffer. The fractions with high activities were pooled and precipitated with ammonium sulfate at 60% saturation. The protein collected by centrifugation was termed "G-200" fraction. This last prepared enzyme was used in the following studies.

2. Assay methods of enzyme activity.

A. Genaral principle and procedure. The enzyme activity was determined by measurement of the radioactive acid-stable compound formed when $^{14}CO_2$ was used as a substrate in the reaction mixture. Because different assay prodedures 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 17x60 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, $NaH^{14}CO_3$, effector(s), and enzyme were injected into vials by microsyringes at different stages according to the assay method used. All the solutions, except $NaH^{14}CO_3$, had been bubbled with N_2 gas in order to remove CO_2 and O_2 dissolved in the solutions from the air.

The "G-200" fraction 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 $MgCl_2$. Tris-HCl solution had been bubbled with N_2 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; $MgCl_2$, 10 mM; enzyme, 10 µg; RuDP, 0.5 mM. The amounts of $NaH^{14}CO_3$ (and its specific radioactivity), effector(s), the preincubation and/or reaction time (see below) 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 vials were flushed with N_2 gas at room temperature to dryness. 0.5 ml water was added to dissolve the remaining material in the vial. Six ml of "Aquasol" was then added. After mixing, the vials in the vial holders (IsoLab Inc., Akron, Ohio) were counted in the liquid scintillation spectrometer. With ^{14}C-toluene as internal standard, the counting efficiency for

carbon-14 with "Aquasol" and the small vial in the vial holder was about 75%.

The preincubation time is the length ot time in which the enzyme is 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 have taken place.

Protein concentration was determined by UV absorption at 280 nm. The factor, O.D. = 1.0 for 0.61 mg enzyme/ml, was used for the calculation of protein concrntration (30).

B. Assay methods with no effector.

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 pre-incubated with $MgCl_2$ for at least 10 min.

Assay Method III. The enzyme had been preincubated with $NaH^{14}CO_3$ for 10 min before the reaction was started by adding the mixture of RuDP and $MgCl_2$.

Assay Method IV. The enzyme had been preincubated with both $MgCl_2$ and $NaH^{14}CO_3$ for a specific length of time before adding RuDP to start the reaction.

C. Assay methods with one effector. For the studies of activation and 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 pre-incubated with the effector along with $MgCl_2$ and $NaH^{14}CO_3$ for a

specific length of time before the addition of RuDP to start the reaction.

Assay Method VII. The enzyme had been preincubated with both $MgCl_2$ and $NaH^{14}CO_3$ for a specific length of time before starting the reaction 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 $MgCl_2$ but not with $NaH^{14}CO_3$); one reaction was stopped at 5 min. The effector was added to other three 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 $MgCl_2$ and $NaH^{14}CO_3$) 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 and/or reaction time. In the kinetic study of the activation of one effector, the effector was added to the enzyme which had been preincubated with both $MgCl_2$ and $NaH^{14}CO_3$ so that 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.

D. Assay methods with two effectors.

Assay Method X. The enzyme was preincubated simultaneously with both effectors, in the presence of $MgCl_2$ and $NaH^{14}CO_3$, for a specific length of time before the addition of RuDP to initiate the reaction.

Assay Method XI. The enzyme was preincubated with one effector in the presence of $MgCl_2$ and $NaH^{14}CO_3$ for a specific length of time before the addtion of second effector. After the completion of the second preincubation, RuDP was added to initiate the reaction.

In kinetic studies of these assays with two effectors, the enzyme was first preincubated in separate vials with the first effector in the presence of $MgCl_2$ and $NaH^{14}CO_3$ for a specific length of time before the additions of second effector. After the completion of the second preincubations, with various lengths of time for each vial, RuDP was added to initiate the reactions.

E. Control experiments. In order to check that the above activating and inhibitory effects are really induced by effectors so indicated, several control experiments have been performed to varify their effects on enzyme activity.

a. 6PGluA: 6PGluA was freshly generated from G6P, NADP, and G6P dehydrogenase. In each of the other three reactions, one of the three ingredients was omitted. The reaction products mixture of each tube was used to test its effect on RuDPCase activity.

b. NADPH: The commercial NADPH, which gave the activating effect on RuDPCase activity, was treated with NH_4Cl, α-ketoglutarate, and L-glutamate dehydrogenase. In each of the other four tubes, one of the four ingredients was omitted. The reaction products mixture of each tube was used to test its effect on RuDPCase activity.

c. FDP: FDP was produced in the reaction of F6P, ATP, and F6P kinase. In each of the other three tubes, either F6P, ATP, or F6P kinase was omitted. The reaction products mixture in each tube was used to test its effect on RuDPCase activity.

RuDP: RuDP was first generated from Ru5P and ATP in the presence of Ru5P kinase. In each of the other three tubes, either ATP, Ru5P,or Ru5P kinase was omitted. The reaction products mixture in each tube was used to test its effect on RuDPCase activity.

3. CO_2 fixation in isolated chloroplasts. Spinach chloroplasts were isolated and allowed to photosynthesize with $NaH^{14}CO_3$ as described previously (18). Rates of ^{14}C uptake into acid-stable compounds were determined, and analysis was made of these compounds by paper chromatography and radioautography (32). These rates and patterns were compared for chloroplast suspensions with and without additions of 6PGluA to concentrations of 0.34 and 0.68 mM.

4. ESR study. Tris-HCl buffer solution (0.05 M, pH 7.8) was used throughout this study. X-band ESR spectra of either standard Mn^{+2} solution, the mixture of RuDPCase and Mn^{+2}, or the mixture of RuDPCase, Mn^{+2}, and Mg^{+2} was recorded on a Varian E-3 spectrometer. Same experiments were also performed with two other concentrations of tris-HCl buffer, 0.01 and 0.2 M.

RESULTS

1. Enzyme purity. The enzyme purified by the methods used in this study had a specific activity of about 1.2 unit/mg protein. One unit is defined as 1 μmole of carbon incorporated into acid-stable compound per min. The purity of the enzyme was checked with gel electrophoresis. The final "G-200" fraction contained only one protein as shown by gel electrophoresis (Fig. 2).

2. Enzyme activities of several assay methods with no effector. It was found that dissolved CO_2 in the buffer solution from air could activate the enzyme activity. Because the amount of CO_2 in the solution varies from time to time due to the storage condition, it is necessary to expel the dissolved CO_2 in the solution so that the activation effects due to preincubation with $MgCl_2$ and bicarbonate can be accurately determined.

With 1 mM $NaH^{14}CO_3$, the kinetic studies using Assay Methods I-IV showed that the enzyme gave by far the highest activity when it was preincubated with both $MgCl_2$ and $NaH^{14}CO_3$ (Method IV, Fig. 3). The enzyme when preincubated with only $MgCl_2$ (Method II), or $NaH^{14}CO_3$ (Method III), or no preincubation at all (Method I) gave only 1/10 of the activity obtained under the above preincubation conditions. While the highest rate with the enzyme preincubated with both $MgCl_2$ 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

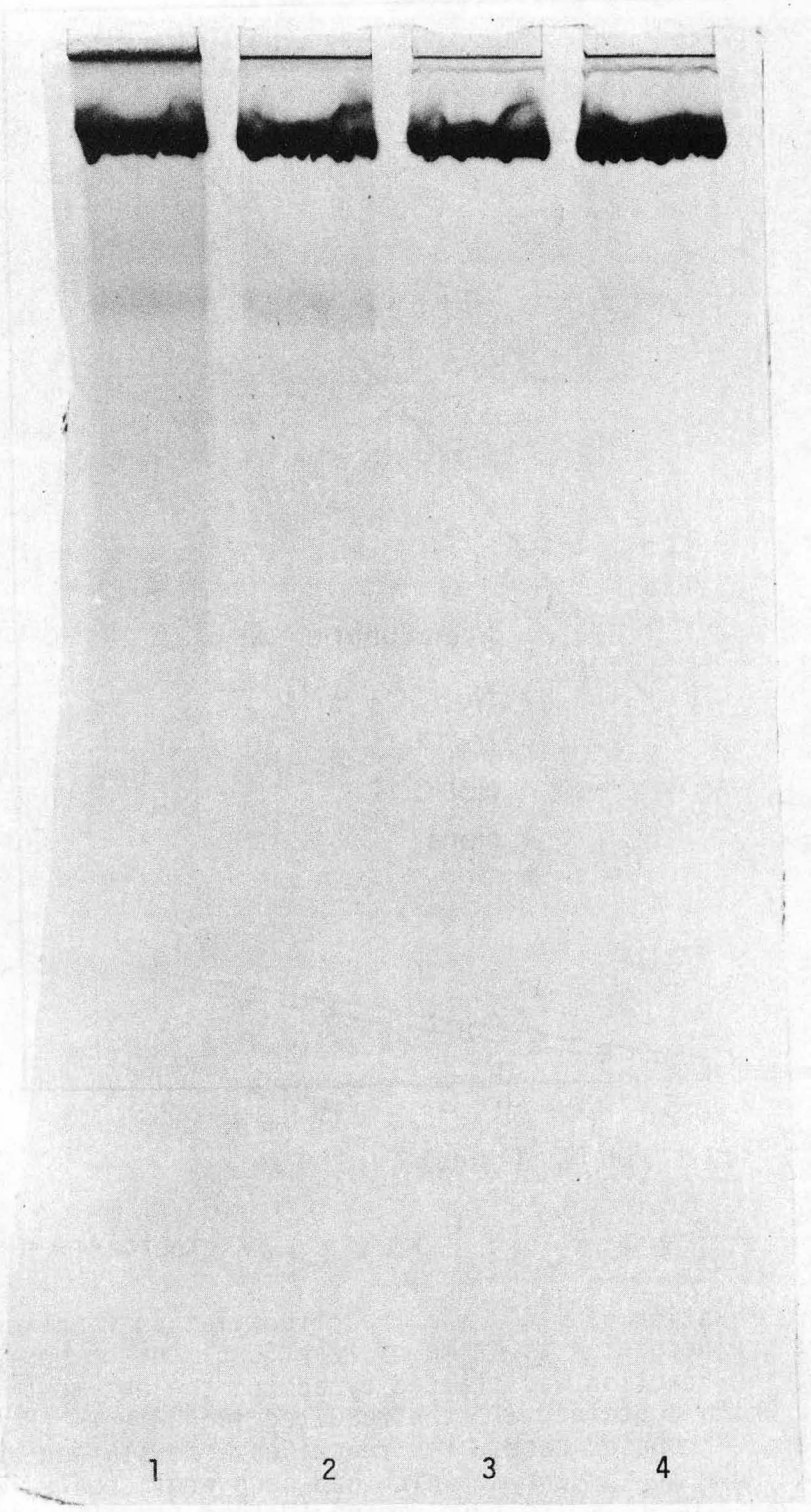

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Fig. 2. Gel electrophoresis of two fractions from the purification of RuDPCase. 1 and 2 are "DEAE" fractions. 3 and 4 are "G-200" fractions. Top thick bands are RuDPCase.

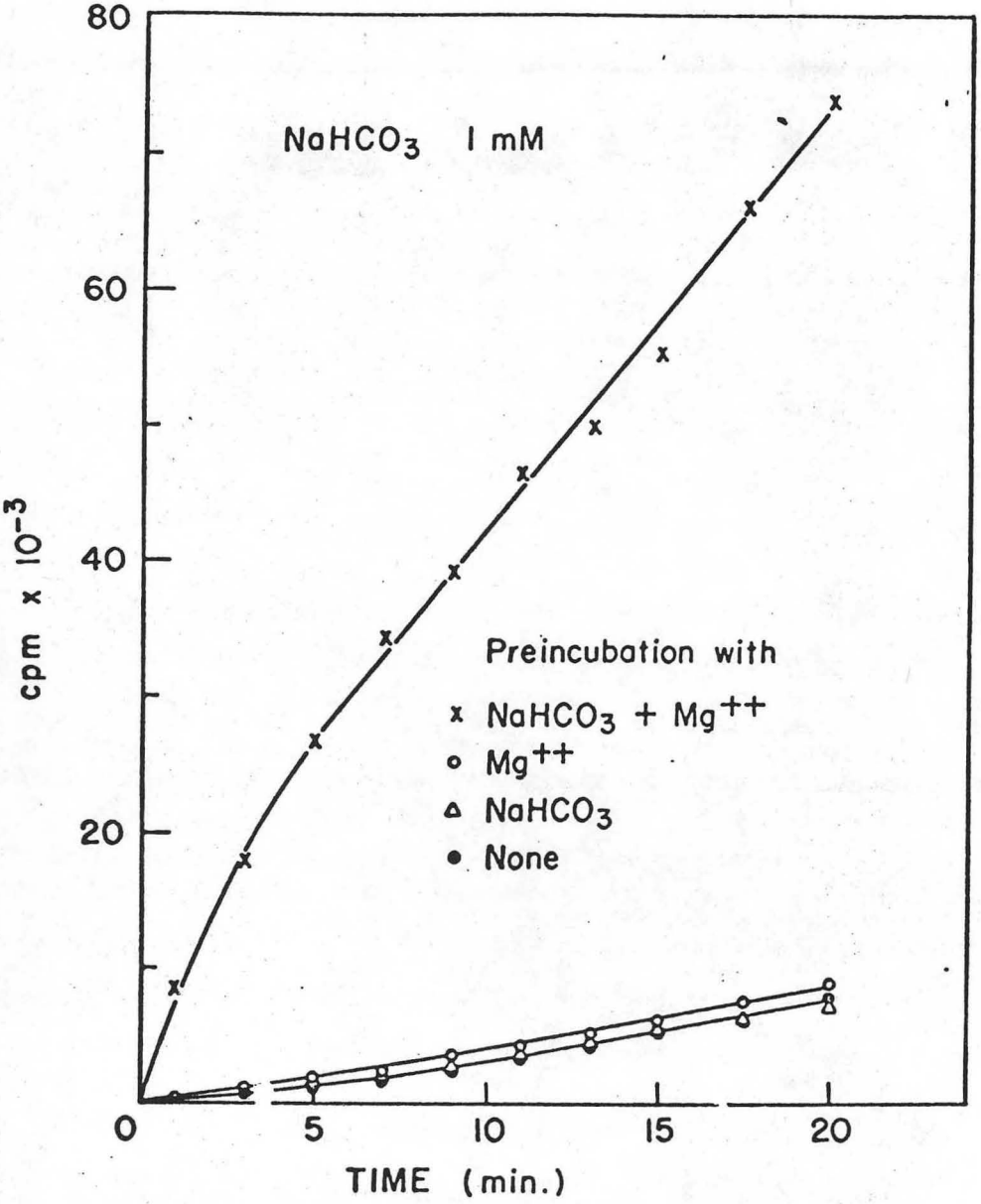

Fig. 3. Fixation of $^{14}CO_2$ via the carboxylation reaction with or without preincubation vs. time of reaction. In the non-preincubated system, the reaction was started by adding the enzyme to the reaction mixture which contained all the required ingredients (Method I). For the preincubated cases, the reactions were started by the additions of RuDP to the enzyme which had been preincubated with $MgCl_2$ and/or $NaH^{14}CO_3$ (1 mM, 4.1 μc/μmole) (Method II, III, and IV). preincubation time: 10 min.

rates from 9 to 20 min were almost the same (Fig. 4), 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 preincubation at 25 C when the concentration of $NaH^{14}CO_3$ was low (1 mM) (Fig. 5). At high $NaH^{14}CO_3$ concentration (50 mM), 30 sec was enough to reach the maximum activity.

3. Effects of 6PGluA on enzyme activity. With 1 mM $NaH^{14}CO_3$, inhibition was observed when 6PGluA was added to the reaction mixture 5 min after initiation of the reaction, whether the rate was high following preincubation with both Mg^{+2} and $NaH^{14}CO_3$ (Method IX, Fig. 6A) or low following preincubation with only $MgCl_2$ (Method VIII, Fig. 6B). Similar inhibition was seen upon addition of 6PGluA to reaction mixtures containing 50 mM $NaH^{14}CO_3$ 5 min after reaction initiation following preincubation according to Method IX (Fig. 7A) or Method VIII (Fig. 7B). The degree of inhibition in each case was calculated from the reaction rate. Addition of Glcu A 1-P gave no appreciable inhibition in any of these cases.

It was found that 6PGluA could either activate or inhibit the enzyme activity, and the effect was completely dependent on the assay method (Table I, II). At low $NaH^{14}CO_3$, 6PGluA activated the enzyme if the enzyme was preincubated with 6PGluA in the presence of $MgCl_2$ and $NaH^{14}CO_3$. A maximum of 188% activation was observed. Even after 15 min reaction time, substantial activation (about 2-fold) of the enzyme was observed in the presence of 0.05 mM 6PGluA.

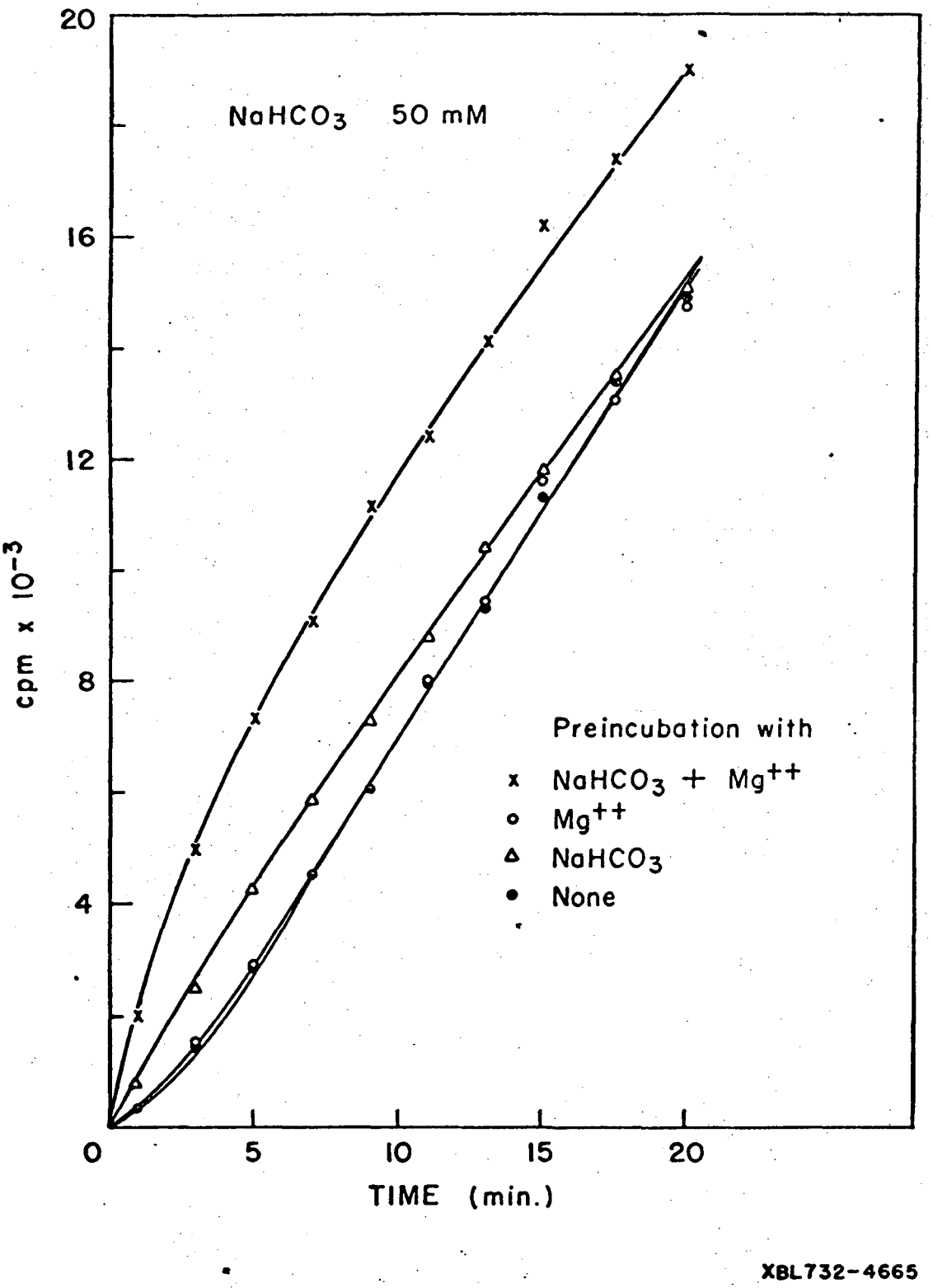

Fig. 4. Same as in Fig. 3 except $NaH^{14}CO_3$ was 50 mM (0.1 μc/μmole).

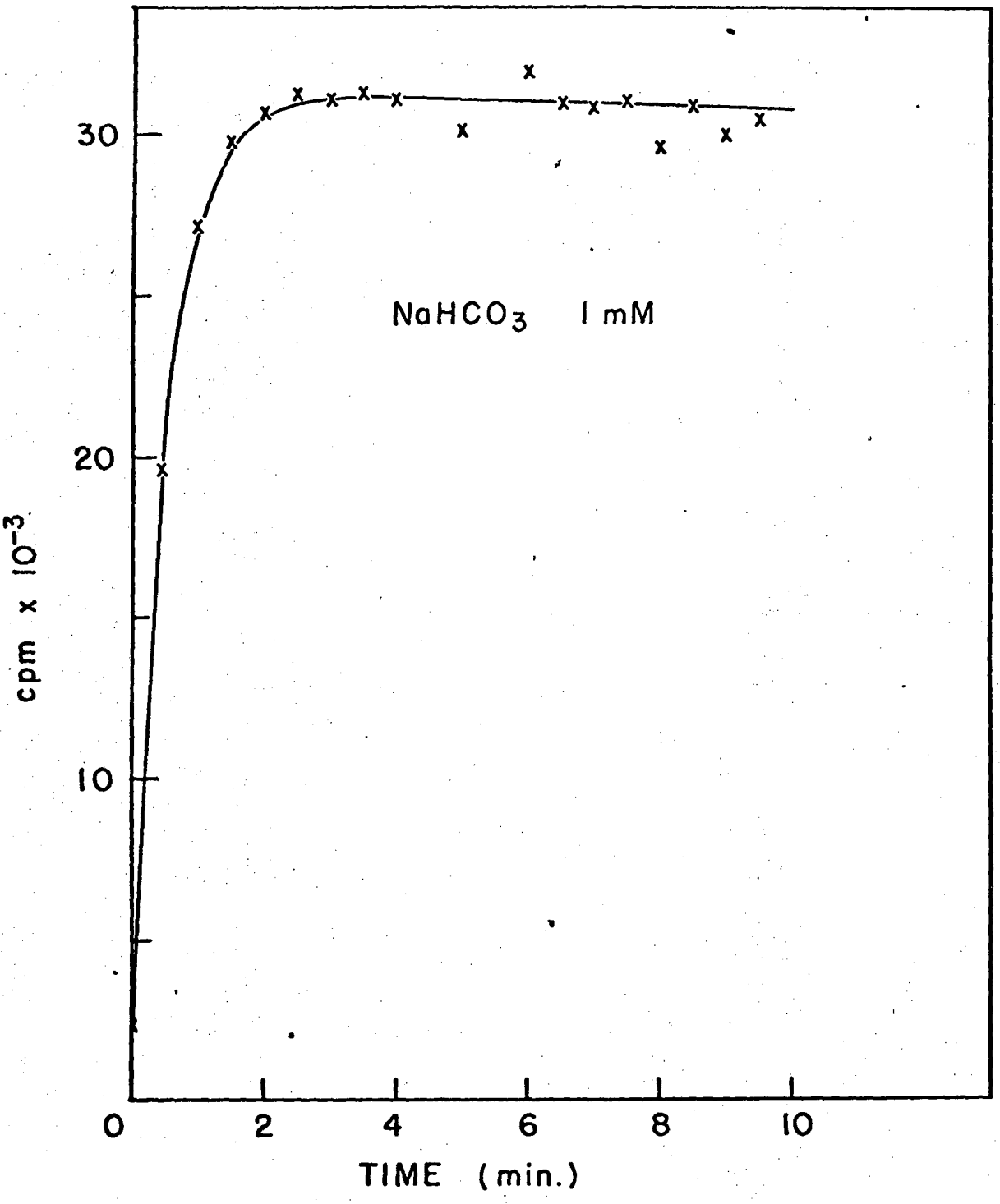

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Fig. 5. Dependence of carboxylation reaction on time of preincubation of carboxylase with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 4.1 μc/μmole). reaction time: 5 min.

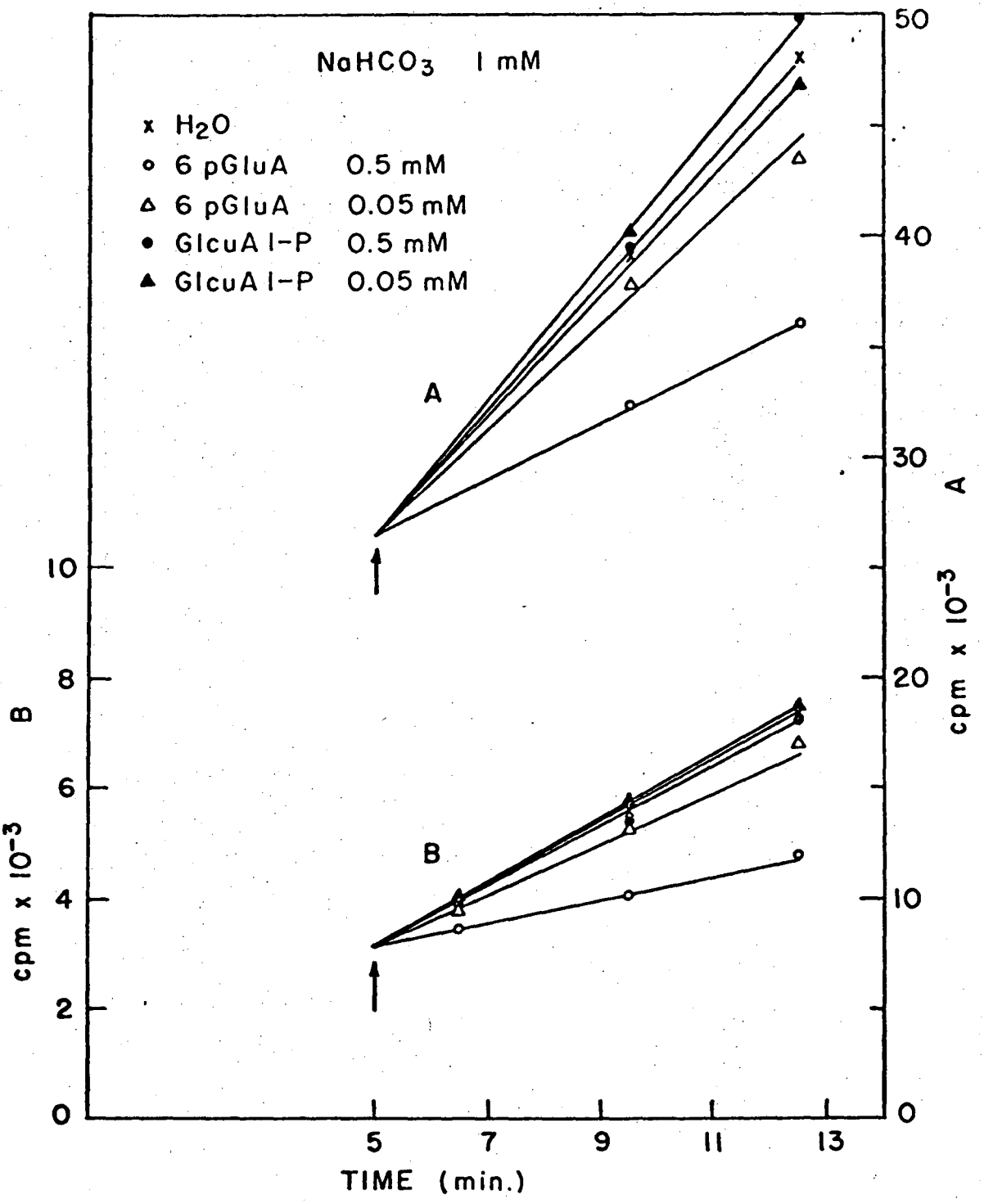

Fig. 6. The effect induced by adding effector into the reacting carboxylation reaction mixture. Arrows indicate the time of additions of effectors. A: Method IX. B: Method VIII. pre-incubation time: 5 min; $NaH^{14}CO_3$: 1 mM, 4.1 μc/μmole.

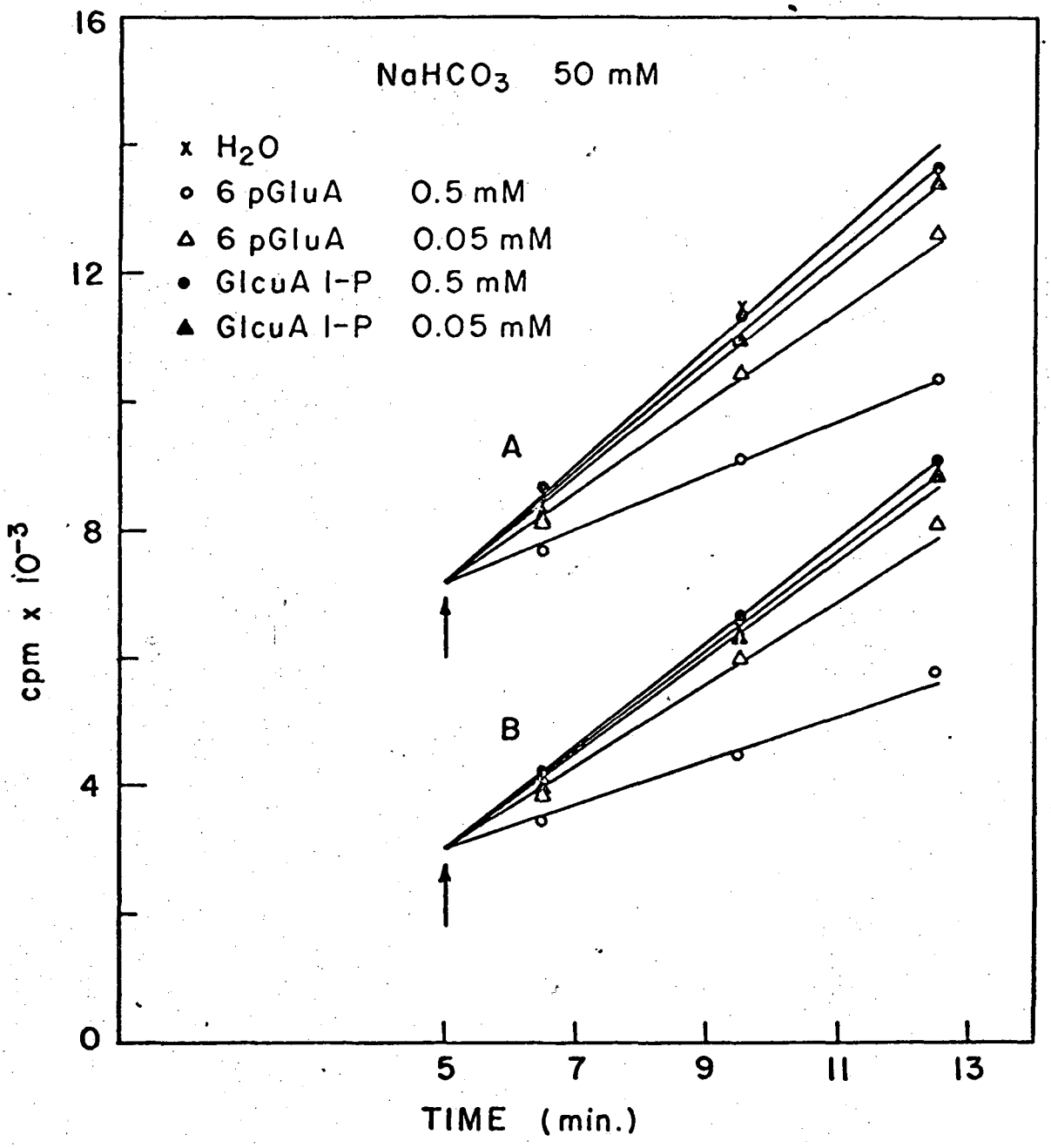

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Fig. 7. Same as in Fig. 4 except $NaH^{14}CO_3$ was 50 mM (0.1 μc/μmole).

Table I. Inhibition and Activation of RuDPCase activity by 6PGluA with 1 mM $NaH^{14}CO_3$

Effector	% Activity of Control* Assay Method V	VI	VII	VIII	IX
H_2O (control)*	100 (2430)+	100 (26505)+	100 (26320)+	100 (2843)+	100 (15506)+
6PGluA, 0.5 mM	41	177	43	39	45
GlcuA 1-P, 0.5 mM	93	128	94	95	105
6PGluA, 0.05 mM	89	288	87	85	84
GlcuA 1-P, 0.05 mM	103	105	104	105	102

* In each method, the enzyme activtiy was taken as 100% when H_2O, instead of effector, was used in the assay mixture.

+ The amounts of carbon incorporated in cpm/5 min of reaction.

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

Table I (continued)

Method VI: The assay reactions were started by adding RuDP to the enzyme 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.

Method VII: The assay reactions were started by adding the mixtures of RuDP and the effector (or H_2O) to enzyme 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.

Method VIII: The 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 other three 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 cases.

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

$NaH^{14}CO_3$: 4.1 μc/μmole.

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

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

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

\+ The amounts of carbon incoporated in cpm/5 min of reaction. $NaH^{14}CO_3$: 0.1 μc/μmole.

** 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 (see Fig. 4).

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

In all of the other assay methods with 1 mM $NaH^{14}CO_3$, 6PGluA 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 6PGluA. Glcu A 1-P gave no significant effect. At high $NaH^{14}CO_3$, Method V (no-preincubation) showed a stronger inhibition than the Method VI (preincubation) did (see Fig. 11).

The enzyme reached its highest activity only after 4-5 min preincubation with 6PGluA (0.05 mM) in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (Fig. 8). This may suggest that the binding of 6PGluA to the enzyme is a rather slow process.

When the enzyme was preincubated with various concentrations of 6PGluA along with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM), the maximum activation was obtained at 0.05-0.1 mM of 6PGluA. As the concentration was increased beyond 0.1 mM, the activation decreased or an inhibition effect appeared. At as low as 5 μM, 6PGluA gave 70-80% activation (Fig. 9). However, with Method V (no-preincubation), inhibition was obtained in every level of 6PGluA (Fig. 10).

GlcuA 1-P was used as a comparison with 6PGluA. 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. Preincubation 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. 9) for enzyme preincubated with GlcuA 1-P was similar to that of inorganic phosphate. We were unable to confirm significant

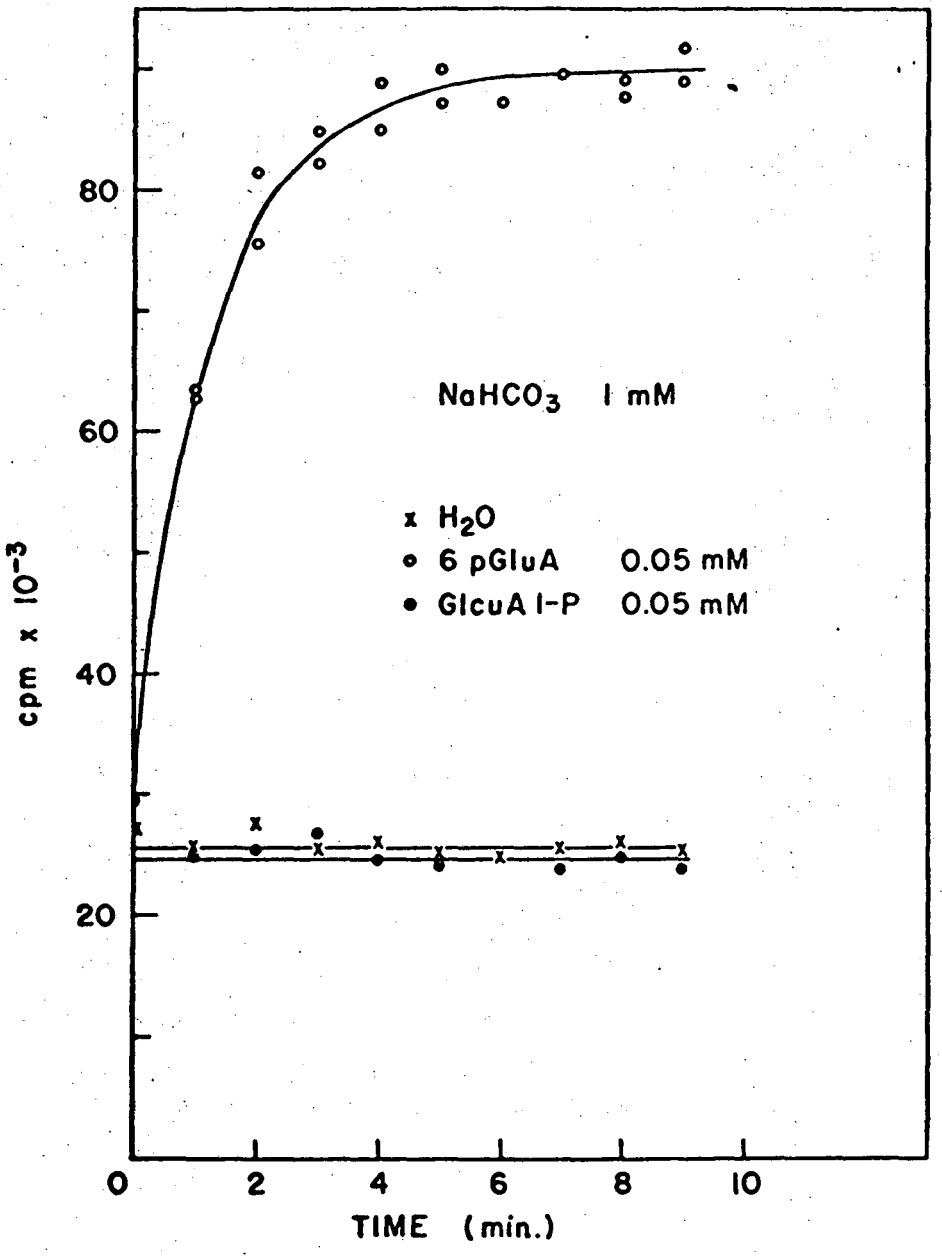

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Fig. 8. Dependence of the carboxylation reaction on the time of preincubation of carboxylase with the effector in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 4.1 μc/μmole). The assays were conducted in separate vials. Effector was added to the 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 $NaH^{14}CO_3$: 10 min; reaction time: 5 min.

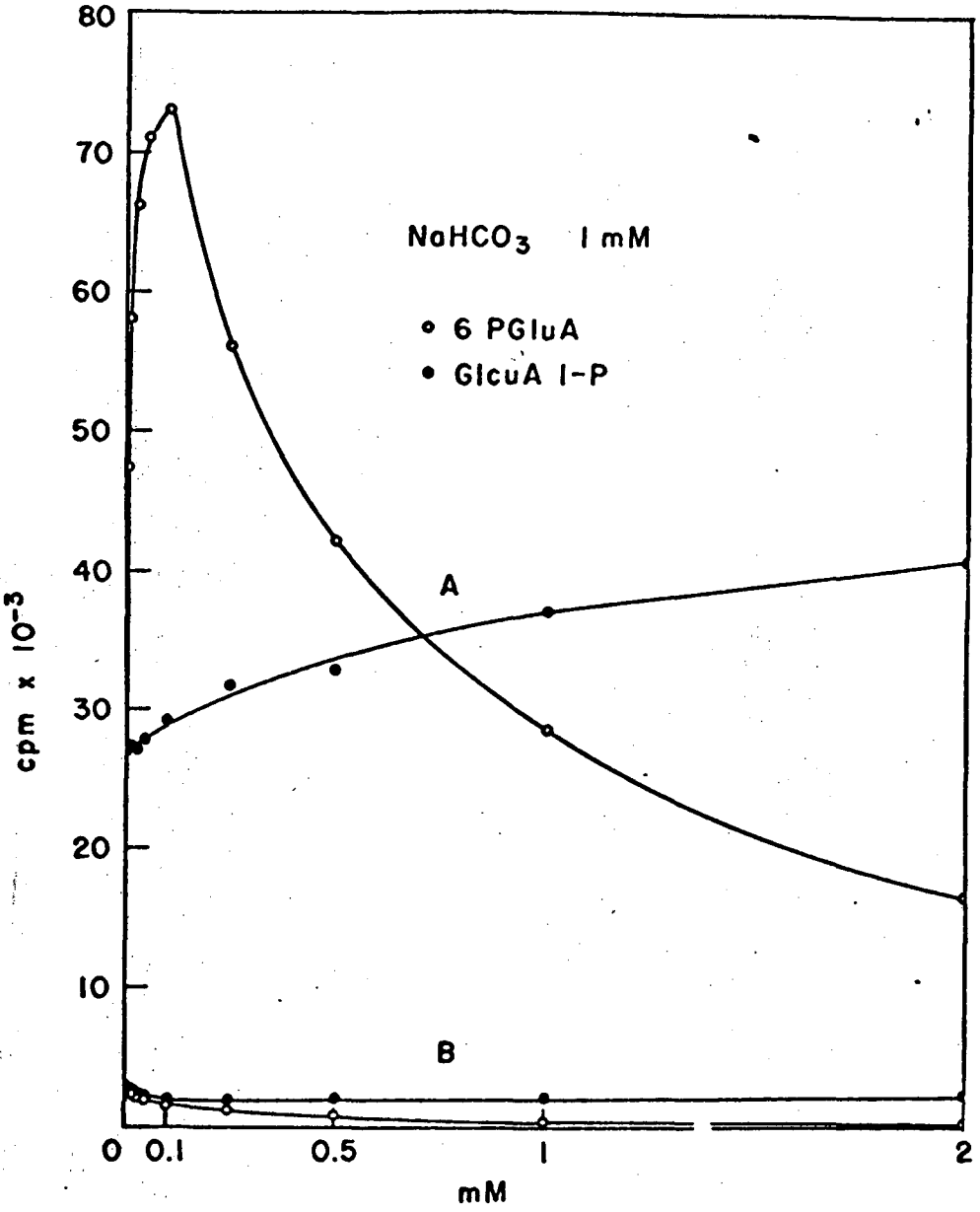

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Fig. 9. The effects on RuDPCase activity caused by various concentrations of effectors with two different assay methods. $NaH^{14}CO_3$: 1 mM, 4.1 μc/μmole; reaction time: 5 min (between 0 and 5 min). A: Enzyme had been preincubated with the effector in the presence of $MgCl_2$ and $NaH^{14}CO_3$ for 5 min (Method VI). B: Enzyme had been preincubated with $MgCl_2$ only (Method V).

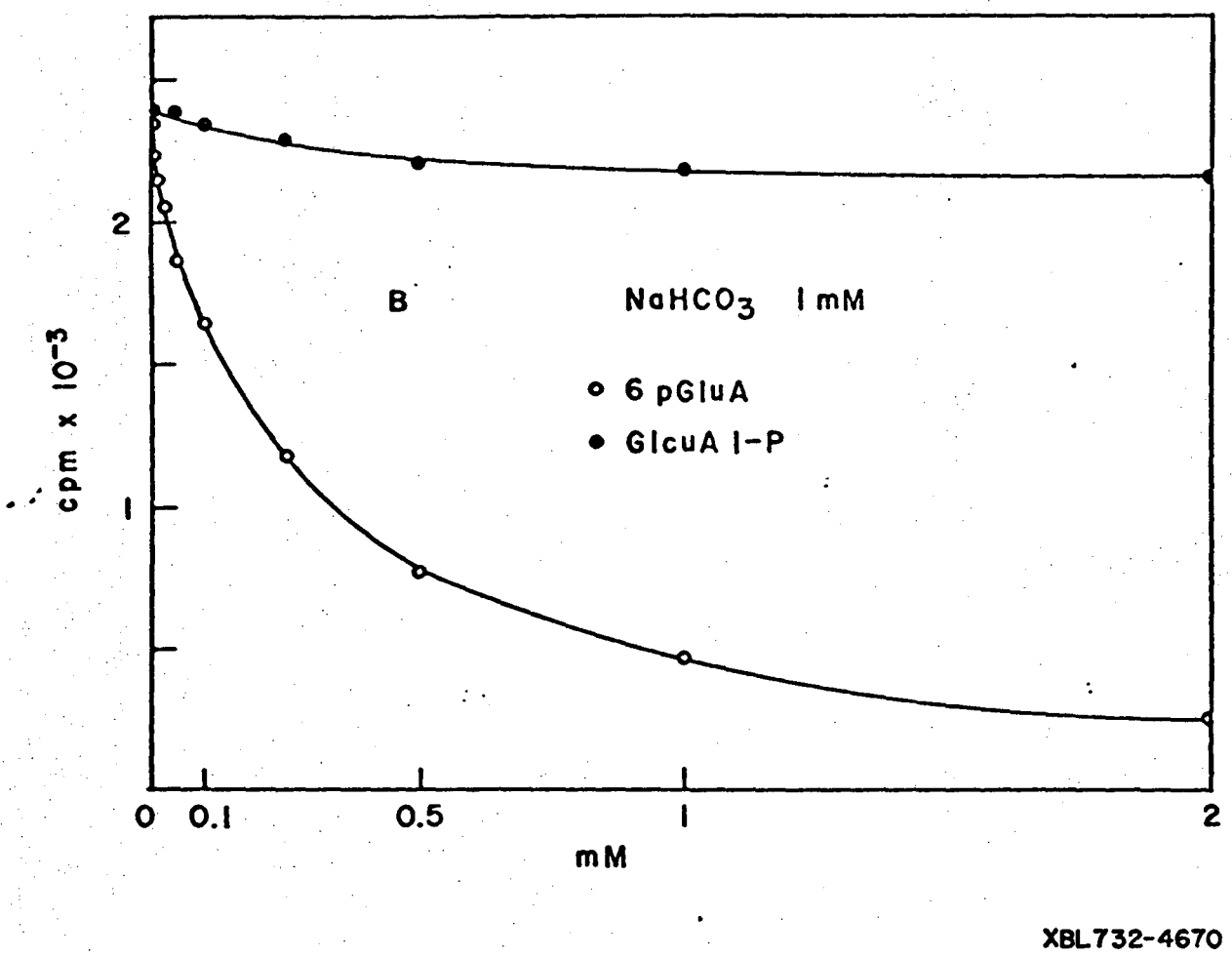

Fig. 10. Expanded curve of B from Fig. 9.

activation by 0.5 mM F6P, reported by Buchanan and Schurmann (10). At that level of F6P we saw less than 20% activation.

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) did (Fig. 11).

4. Effects of other chloroplast metabolites on enzyme activity. When RuDPCase is assayed following preincubation with 10 mM $MgCl_2$ and 1 mM $NaH^{14}CO_3$, together with various metabolites present in chloroplasts, many metabolites have little effect in either activating or inhibiting the enzyme (Fig. 12). Among those compounds that do affect the enzyme activity, two types of dependence of activity on effector concentration are seen. With 6PGluA, as previously described, a 180% activation is seen at 0.1 mM; above this concentration the activation decreases, with higher concentrations causing inhibition. The effect of FDP is similar, but the maximum activation at 0.1 mM is only about 70%.

A different kind of dependence of enzyme activity on effector concentration is seen with NADPH and with PGA. Activation of the enzyme continues to rise with effector concentration, saturating at about 1 mM at nearly 200% for NADPH but about only 50% for PGA. The concentration of NADPH needed to give 100% (about half of the maximum) activation is 0.2 mM. Although both PGA and NADPH cause activation with a similar type of dependence on effector concentration, these two activation effects appear to be rather specific, since little or no activation or inhibition of the enzyme is seen with $NADP^+$, NADH, NAD^+ (Fig. 12 and 14), or F6P (Fig. 12). Also

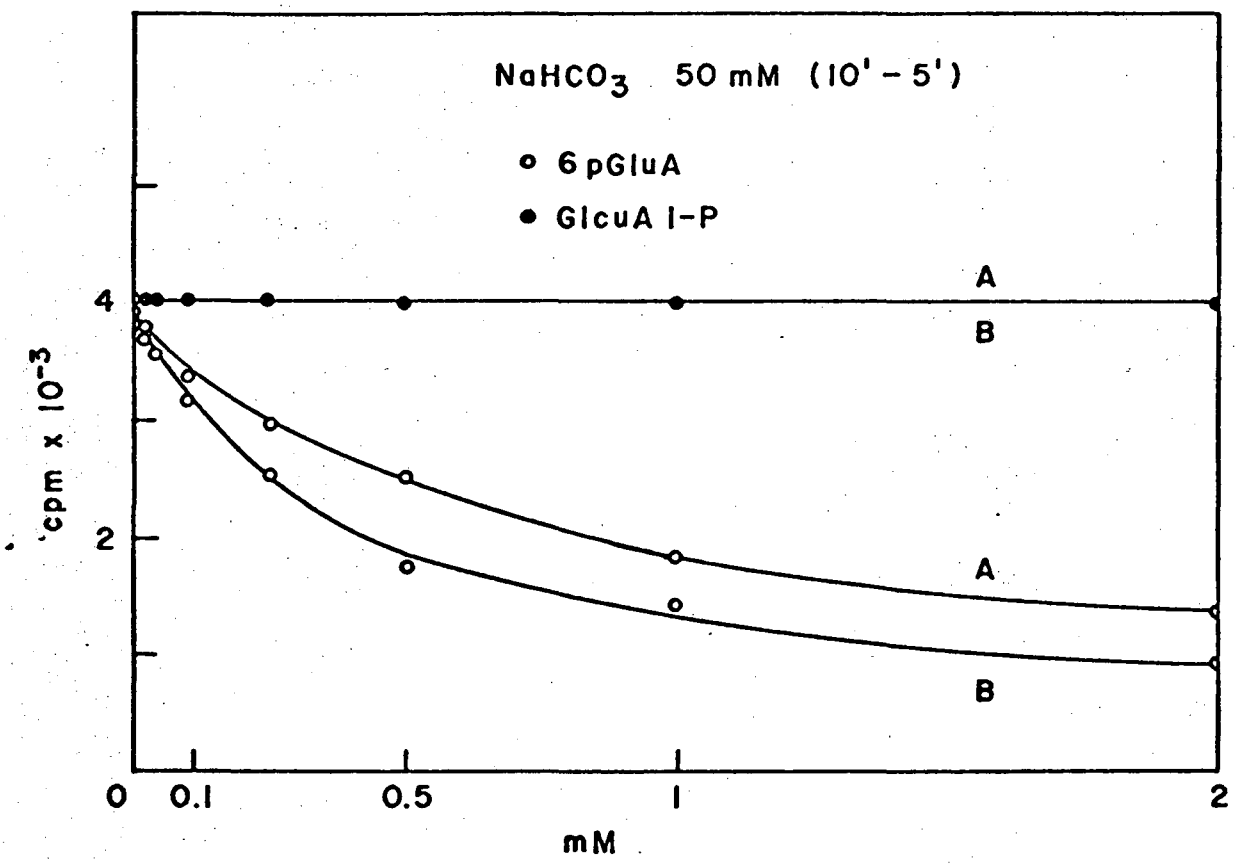

Fig. 11. Same as in Fig 9 except $NaH^{14}CO_3$ was 50 mM (0.1 μc/μmole). reaction time: 5 min (between 5 and 10 min).

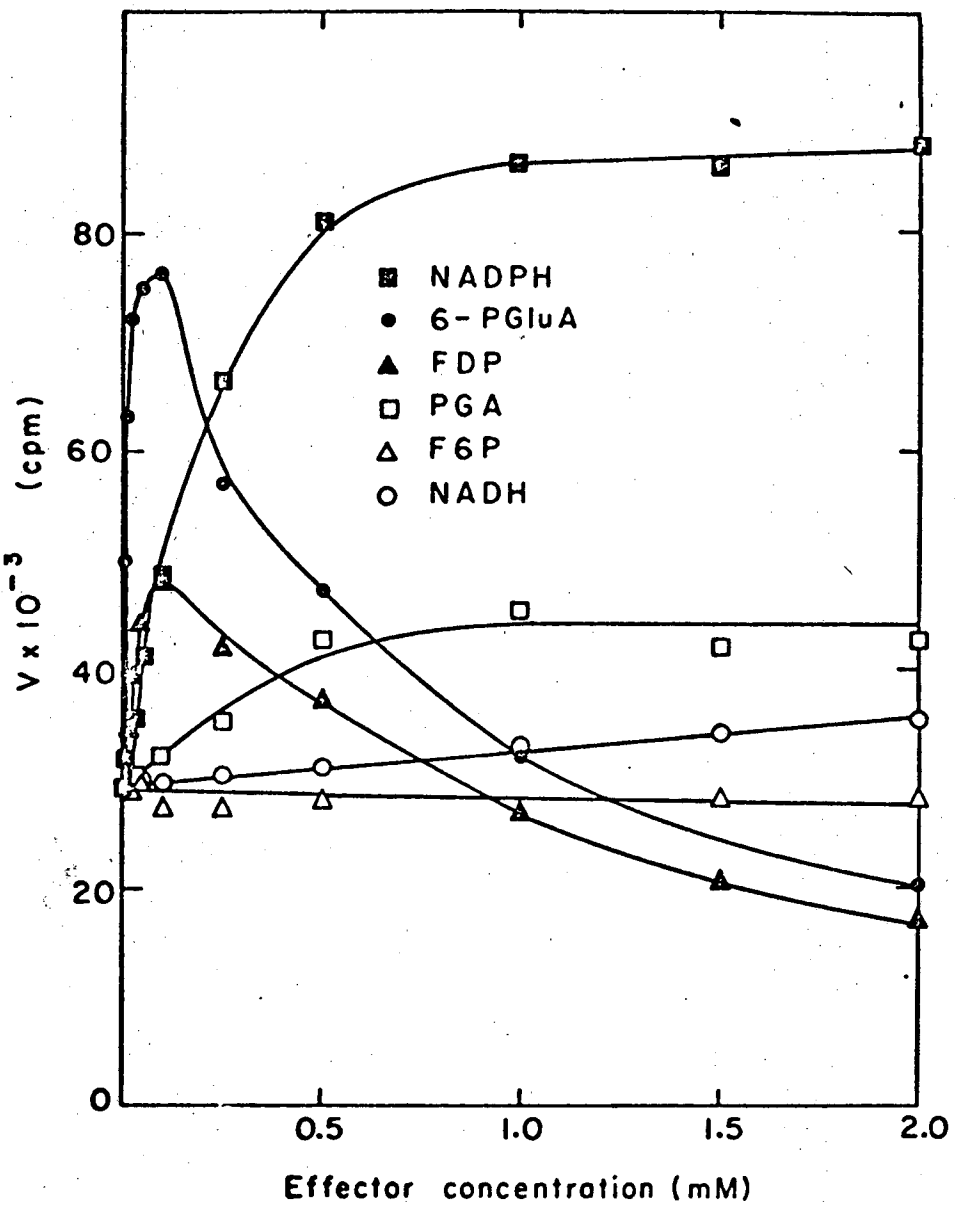

Fig. 12. The effects on RuDPCase activity caused by various concentrations of effectors with the preincubation assay method (Method VI). The enzyme had been preincubated with the effector in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 4.1 μc/μmole) for 5 min before the reaction was started by the addition of RuDP. reaction time: 5 min.

without effect were reduced glutathione, DTT, and ATP (not shown). Earlier studies showed GlcuA 1-P and G6P to be without effect.

The kinetic experiment indicated that NADPH, like 6PGluA, caused an activation effect only when it was added to the enzyme before RuDP was added. The reaction which was started by addition of the mixture of RuDP and NADPH to the preincubated enzyme did not show any activation (Fig. 13).

When the enzyme activities were measured by the preincubation method in the presence of effector (Method VI) at different concentrations of RuDP, it was found that the activations induced by 6PGluA or NADPH were reduced or inhibitions were observed at low RuDP concentrations. This effect was more significant in the case of 6PGluA.

If the enzyme was assayed by adding $MgCl_2$-preincubated enzyme to the reaction mixture which contained all the required ingredients for the reaction (Method V), FDP caused less inhibition than 6PGluA did. Other metabolites, including NADPH, gave no significant effect (Table III).

A kinetic study showed that the enzyme reached its highest activity only after 5-6 min preincubation with NADPH (0.5 mM) in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (Fig. 14).

5. Effects of 6PGluA and/or NADPH on $K_m(HCO_3^-)$. When the enzyme was preincubated with $MgCl_2$ and different concentrations of $NaH^{14}CO_3$, a plot of enzyme activity vs. $NaHCO_3$ concentration gave a curve not in accordance with Michaelis-Menten kinetics. It was not possible to obtain an accurate determination of the K_m by a double reciprocal (Lineweaver-Burke) plot (1/v vs. $1/(NaHCO_3)$)

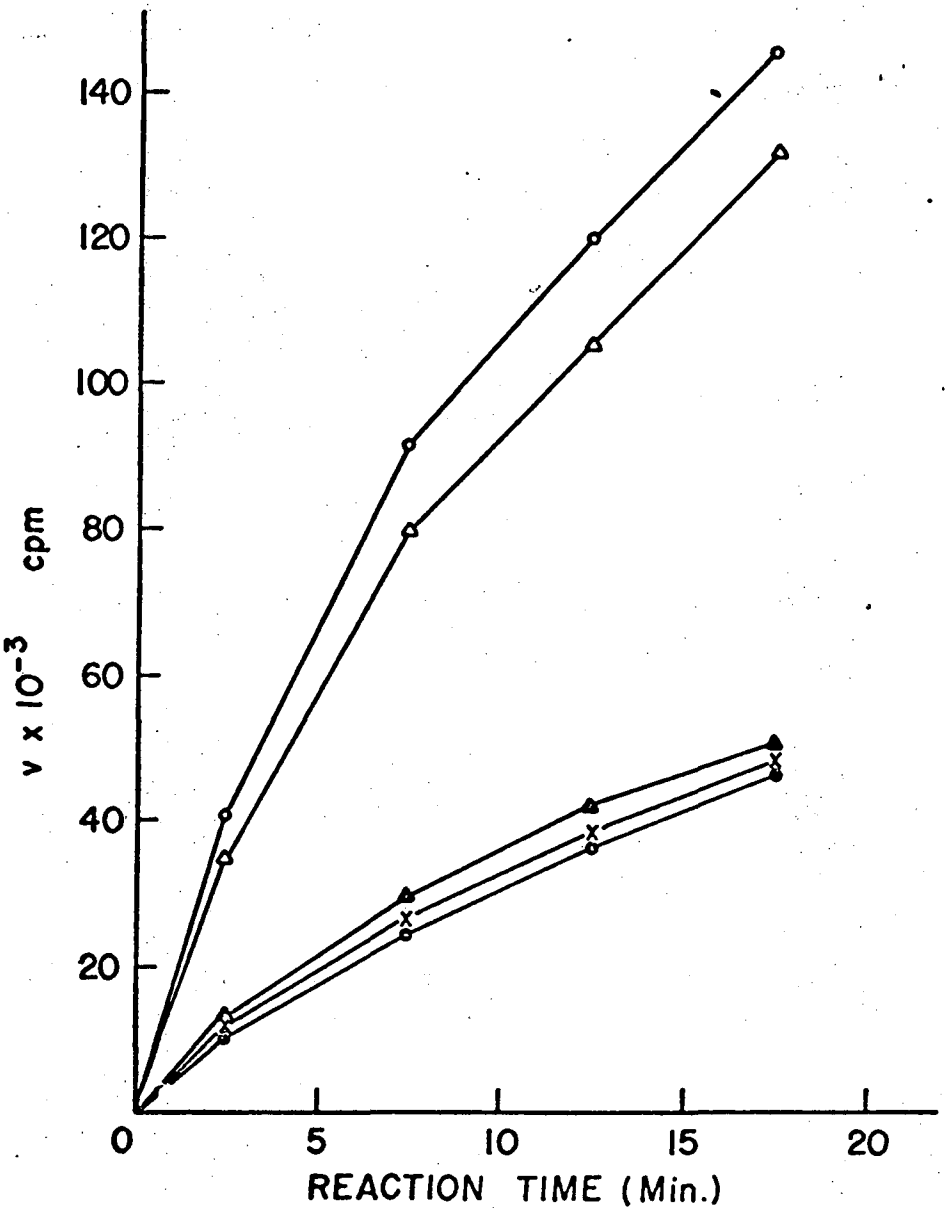

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Fig. 13. Fixation of $^{14}CO_2$ via the carboxylation reaction with different assay methods in the presence of effector vs. the time of reaction. The reactions were started by adding RuDP to the enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ in the presence of either 6PGluA (o——o) or NADPH (Δ——Δ). Other reactions were started by adding the mixture of RuDP and 6PGluA (●——●) or RuDP and NADPH (▲——▲) to the enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$. The control reaction is shown by x——x . preincubation time: 5 min; $NaH^{14}CO_3$: 1 mM, 1.08 μc/μmole; NADPH: 0.5 mM; 6PGluA: 0.05 mM; RuDPCase: 15 μg.

Table III. Inhibition of PuDPCase Activity by Several Chloroplast Metabolites.

The enzyme activities were assayed by Method V. The enzyme reactions were started by adding the enzyme which had been preincubated with $MgCl_2$ to the reaction mixtures containing all the required ingredients and the effector at different concentrations. $NaH^{14}CO_3$: 1 mM, 4.1 μc/μmole. reaction time: 5 min.

	Relative Activity*, %		
Effector added	0.5 mM	1.0 mM	2.0 mM
6PGluA	54	39	18
FDP	94	78	61
F6P	99	97	95
PGA	97	96	95
NAD(H)	99	98	95
NADP(H)	98	96	94

*The control rate was 4,400 cpm (100%) when H_2O, instead of effector, was added in the reaction mixture.

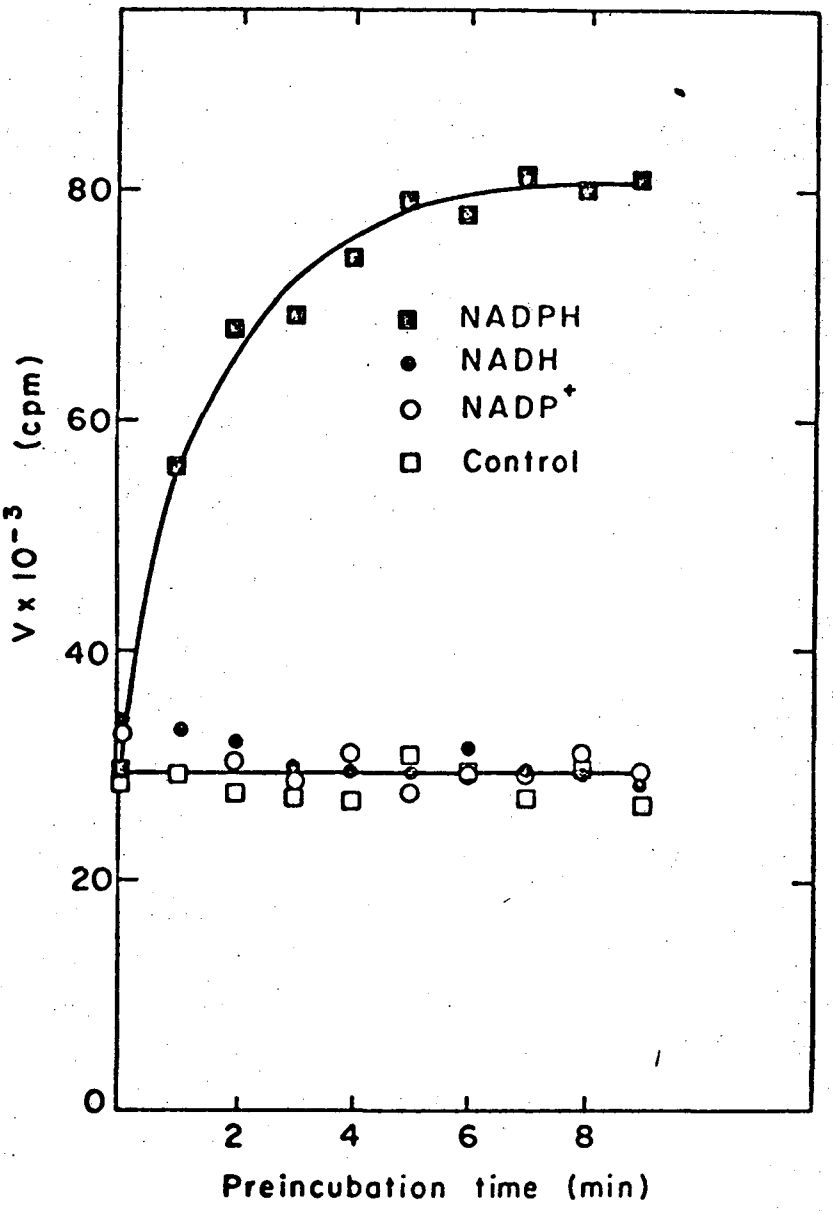

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Fig. 14. Dependence of the carboxylation reaction on the time of preincubation of RuDPCase with the effector (0.5 mM) in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 4.1 μc/μmole). The assays were conducted in separate vials. Effector or buffer solution 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 $NaH^{14}CO_3$: 10 min; reaction time: 5 min.

(Fig. 15). However, if the enzyme was preincubated with $MgCl_2$ and different concentrations of $NaH^{14}CO_3$ in the presence of 6PGluA (0.05 mM), NADPH (0.5 mM), or both, the K_m for HCO_3^- could be determined and was 2.8 mM. This corresponds to a K_m for CO_2 of about 0.3% at pH 7.8 and 25 C.

6. RuDP effects on preincubated and non-preincubated RuDPCase. When the enzyme was assayed by preincubation method (Method IV), the higher the RuDP concentration, the higher the reaction rate observed; however, in the non-preincubated system (Method I), the lower the RuDP concentration, the greater the rate obtained. In the latter system, the reaction rate at lower RuDP concentration increased gradually and more rapidly; at higher concentration the rate increased much slower (Fig. 16).

7. K_i's of 6PGluA and FDP. When the enzyme activity was assayed by adding the mixture of RuDP and 6PGluA (or FDP) to start the reaction, inhibition was observed at every concentration of the effector. A series of assays were performed with several concentrations of RuDP at different levels of effector. The plots of 1/v vs. 1/(RuDP) indicated that both 6PGluA and FDP induced competitive inhibition with regard to RuDP (Fig. 17 and 18). The K_i's have been calculated from the intercepts at the 1/(RuDP) axis. K_i for 6PGluA was 20 μM and that for FDP was 190 μM. It shows that 6PGluA ia a stronger inhibitor than FDP. The former can bind at the RuDP catalytic site about 10 times as strongly as FDP can. K_m(RuDP) determined in the same experiment was about 35 μM.

8. Combined effects of two effectors on RuDPCase activity. When the enzyme activity was assayed by Method VI (preincubation with

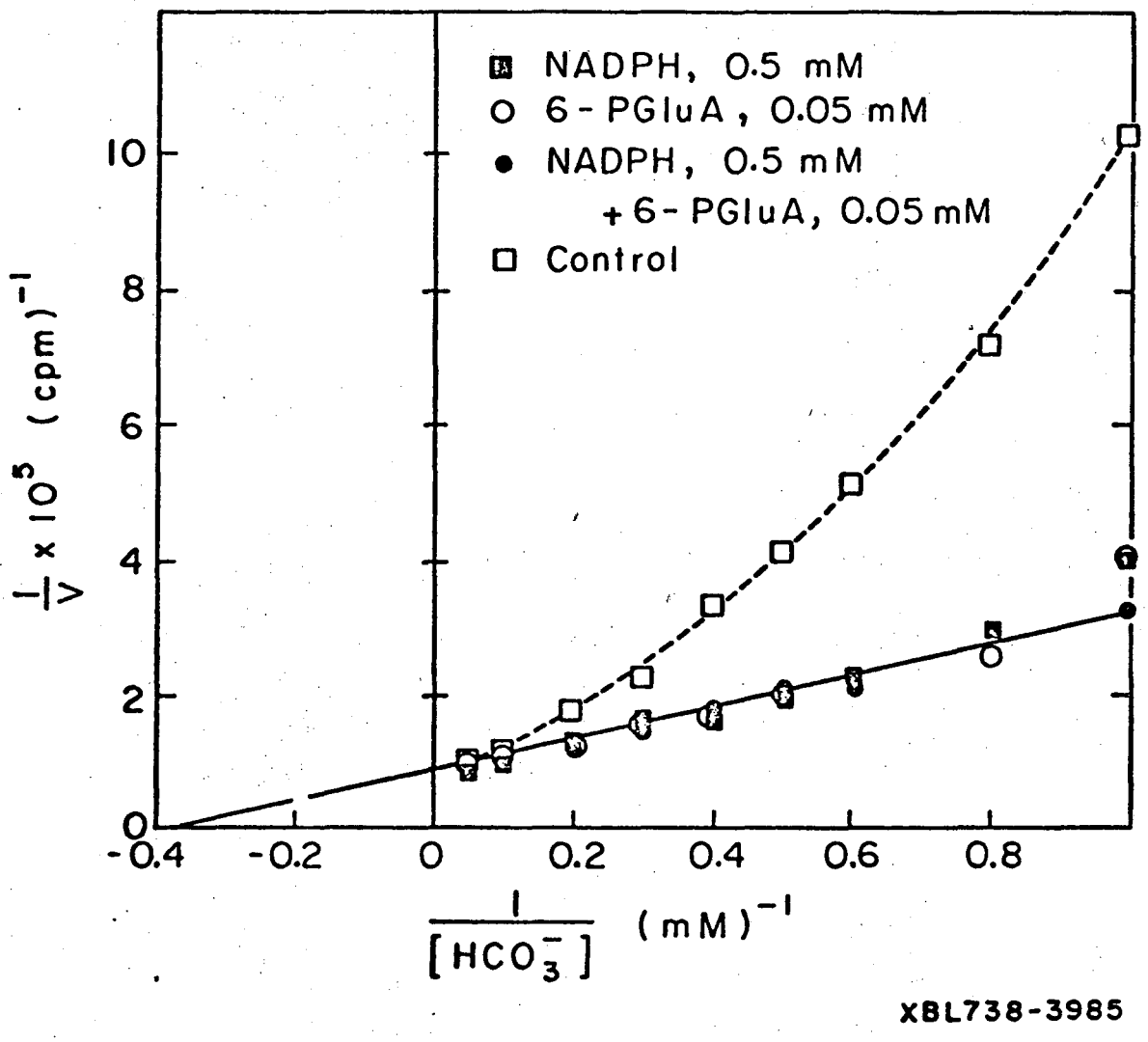

Fig. 15. Lineweaver-Burk plot for the effects of $NaH^{14}CO_3$ concentration on the RuDPCase activity in the presence of effector(s). In each case the enzyme had been preincubated with the effector in the presence of $MgCl_2$ and different concentrations of $NaH^{14}CO_3$ (1.68 μc/μmole) for 5 min before starting the reaction by the addition of R-DP. reaction time: 5 min.

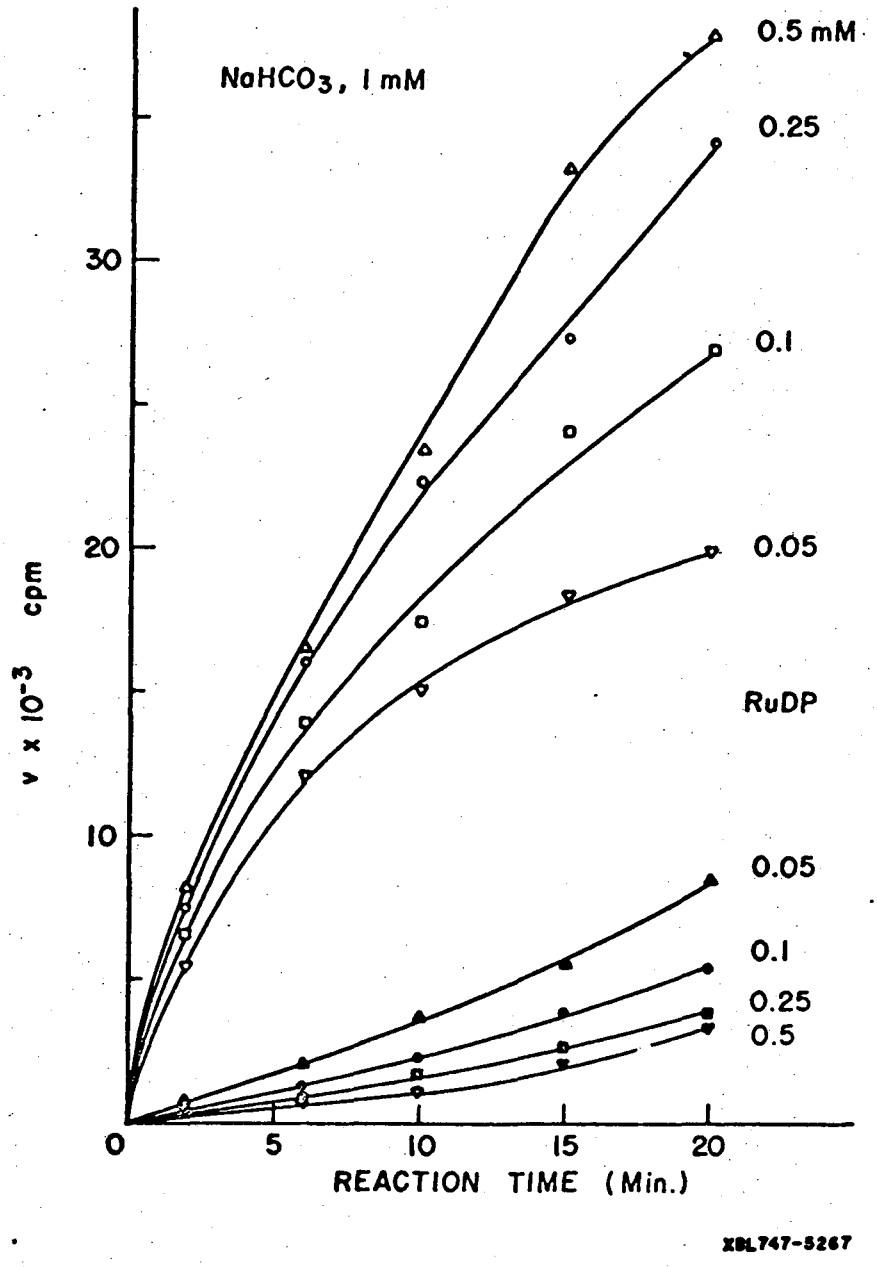

Fig. 16. The effects of RuDP on preincubated and non-preincubated enzyme. The carboxylation reactions were started by additions of different concentrations of RuDP to enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 2.0 μc/μmole) for 5 min (open markers). The reactions were initiated by adding the non-preincubated enzyme to the reaction mixtures which contained the same amounts of ingredients as in the above reactions (closed markers).

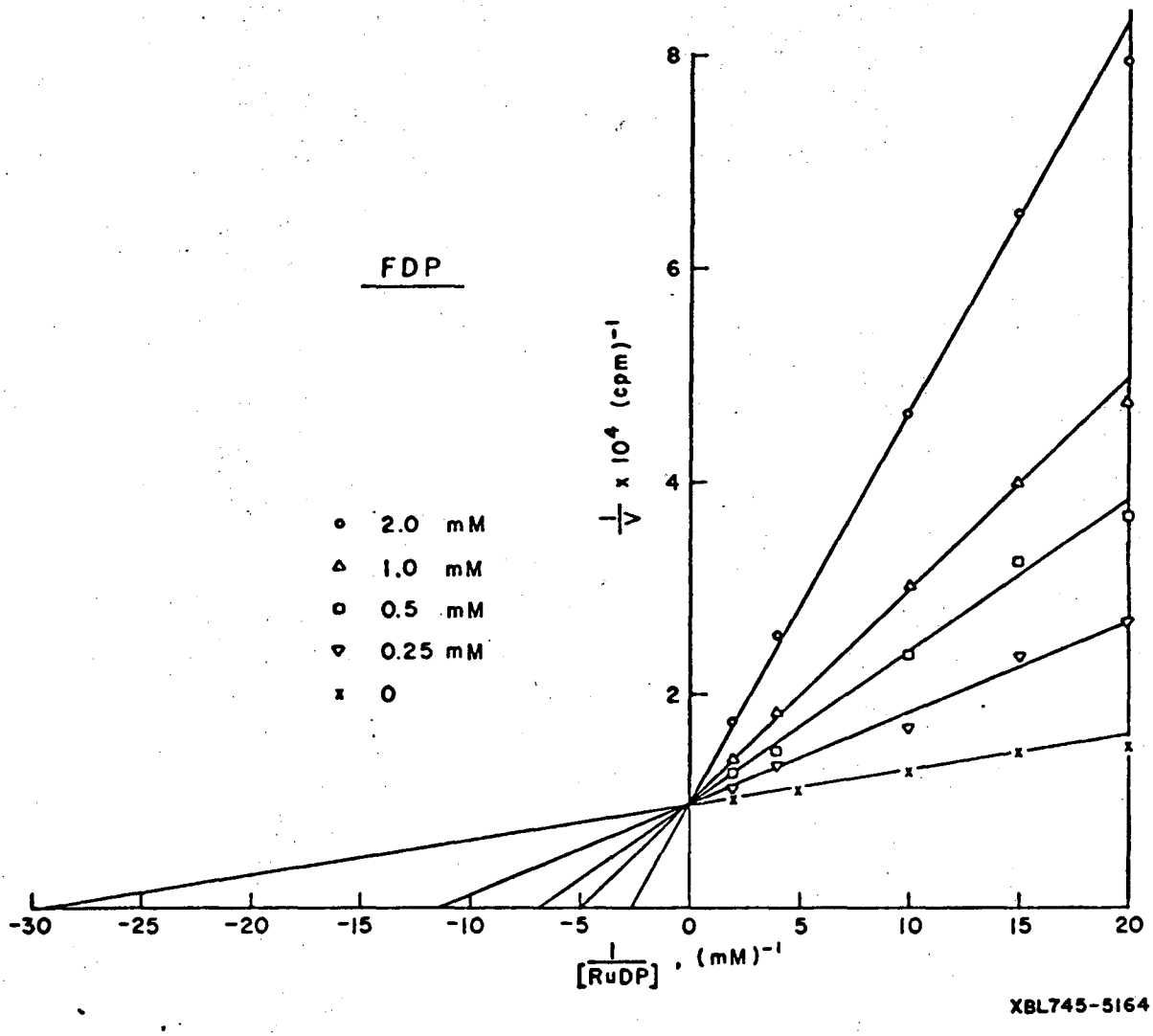

Fig. 17. Inhibition of RuDPCase by FDP. The reactions were started by additions of the mixtures of RuDP and FDP to the enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 1.4 μc/μmole). preincubation time: 5 min; reaction time: 5 min; concentrations of FDP are indicated in the figure.

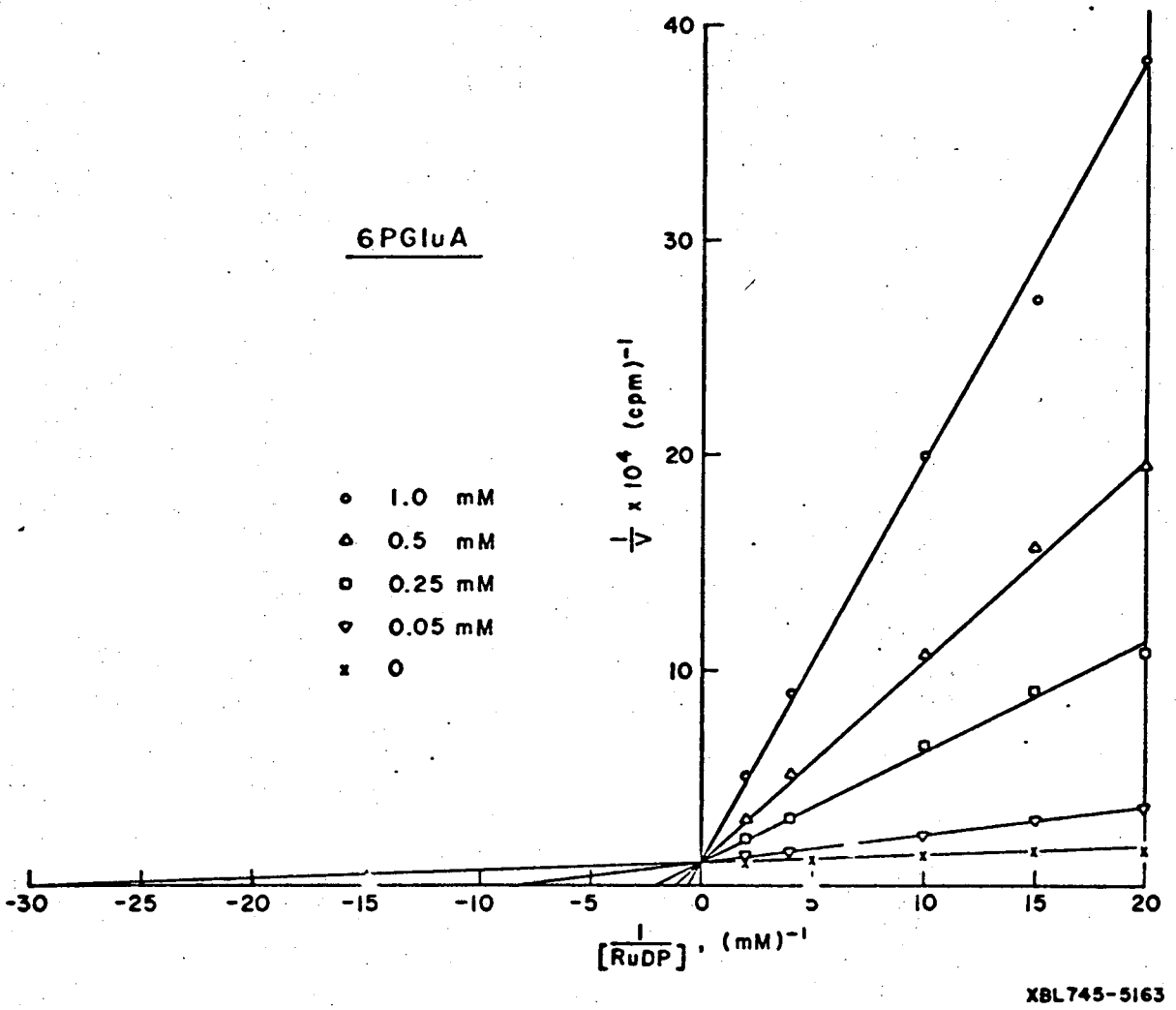

Fig. 18. Inhibition of RuDPCase by 6PGluA. The reactions were started by additions of the mixtures of RuDP and 6PGluA to the enzyme which had been preincubated with $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 1.4 μc/μmole). preincubation time: 5 min; reaction time: 5 min; concentrations of 6PGluA are indicated in the figure.

the effector along with $MgCl_2$ and $NaH^{14}CO_3$), both 6PGluA and NADPH showed activating effects as described before. With NADP or NADH in the above preincubation medium, no effect could be observed. Thus the activation induced by 6PGluA (0.05 mM) or NADPH (0.2 mM) is not reduced by either NADP or NADH (1 mM). However, if FDP was added in the preincubation medium (Method X), the activation induced by either 6PGluA or NADPH was partially or completely abolished, depending on the order of addition of the effectors. FDP, at 0.25 mM, induced about 50% activation and this activation was not reduced by another addition of FDP, at 0.75 mM, which was added with RuDP after the preincubation had been completed. The same total amount of FDP, i.e. 1 mM, had a deactivating effect if it was added in the preincubation medium. Table IV summarizes the combined effects of two effectors.

As shown in Fig. 19, the activation induced by 6PGluA during the first preincubation period can be gradually reduced by FDP in the second preincubation period. On the other hand, the deactivating effect induced by FDP (1 mM) can be gradually removed by 6PGluA during the second preincubation period. Both the activation with 6PGluA and deactivation with FDP are rather slow processes.

8. Control experiments.

a. 6PGluA: From results obtained (Table V), it is clear that 6PGluA, not impurities in the chemicals purchased, was the cause of activation and inhibition observed.

b. NADPH: The control experiment indicated that NADPH, which gave the above activation on RuDPCase activity, could not show any activating effect after it was oxidized in the reaction catalyzed by

Table IV. Combined effects of two effectors on RuDPCase activity.

The enzyme activity was assayed according to the following scheme:

$NaH^{14}CO_3$, $MgCl_2$ enzyme and first effector	5 min ------>	second effector	5 min -------->	reaction started with	relative activity, %
H_2O		--------		RuDP*	100**
6PGluA, 0.05 mM		--------		RuDP	275
NADPH, 0.5 mM		--------		RuDP	265
FDP, 1.0 mM		--------		RuDP	95
6PGluA, 0.05 mM + FDP, 1.0 mM		--------		RuDP	100
NADPH, 0.5 mM + FDP, 1.0 mM		--------		RuDP	104
H_2O		--------		RuDP + FDP, 1.0 mM	85
6PGluA, 0.05 mM		--------		RuDP + FDP, 1.0 mM	250
NADPH, 0.5 mM		--------		RuDP + FDP, 1.0 mM	240
6PGluA, 0.05 mM		FDP, 1.0 mM		RuDP	188
NADPH, 0.5 mM		FDP, 1.0 mM		RuDP	174

(to be continued)

Table IV (continued)

FDP, 1.0 mM	6PGluA, 0.05 mM	RuDP	101
FDP, 1.0 mM	NADPH, 0.5 mM	RuDP	104
FDP, 0.25 mM	--------	RuDP	155
FDP, 0.25 mM	--------	RuDP + FDP, 0.75 mM	145

* RuDP concentration was 0.5 mM for all assays.

** The control was 11,000 cpm/5 min of reaction.

$NaH^{14}CO_3$: 1 mM, 1.5 μc/μmole.

$MgCl_2$: 10 mM

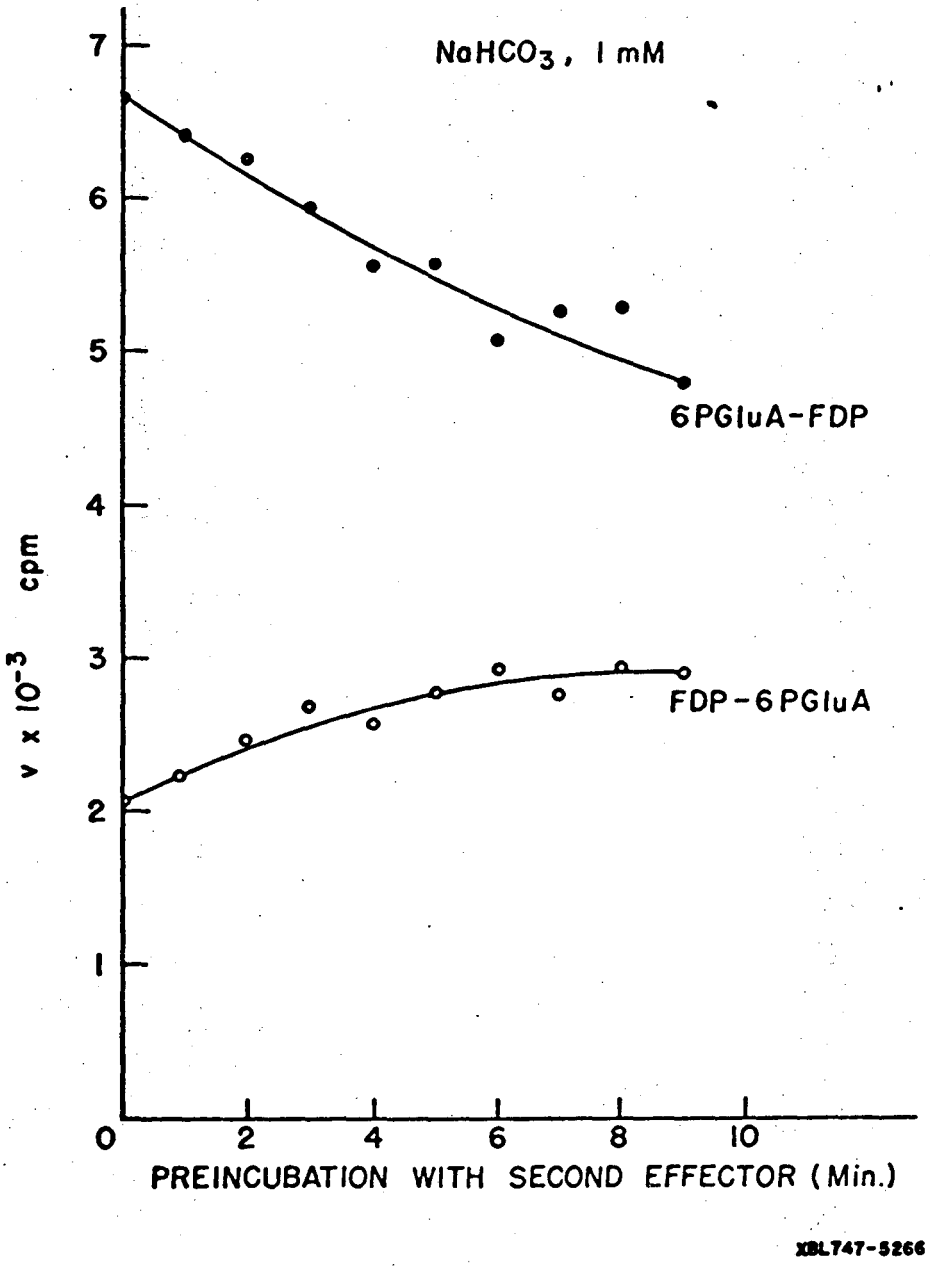

Fig. 19. The effects of 6PGluA activation and FDP deactivation. The enzyme was first preincubated in separate vials with 6PGluA in the presence of $MgCl_2$ and $NaH^{14}CO_3$ (1 mM, 0.5 μc/μmole) for 5 min before the additions of the second effector, FDP (1 mM). After the completion of the second preincubations with various lengths of time for each vial, RuDP was added to initiate the reactions (upper curve). The lower curve shows the result when FDP was the first effector and 6PGluA was the second effector. reaction time: 5 min.

Table V. Control Experiment of 6PGluA.

6PGluA was first generated by the reaction of 5 mM of G6P with 5 mM of NADP in the presence of G6P dehydrogenase (10 units) in 0.1 M tris-HCl buffer, pH 7.8. In each of the other three tubes, either G6P, NADP, or G6P dehydrogenase was omitted. All the tubes were incubated for 20 min at 25 C. After the reaction, part of the reaction products mixture in each tube was diluted 10-fold with the same buffer. An aliquot (40 µl) from each of the original and diluted reaction products mixtures was added to the vials for assay of RuDPCase 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*		Assay Method VI*,+	
	activity (cpm)	%	activity (cpm)	%
All ingredients	871	38	50974	183
minus NADP	2267	99	29296	105
minus G6P	2173	95	34003	122
minus G6P dehydrogenase	2239	98	30487	109
minus all ingredients (control)**	2291	100	27861	100

Table V (continued)

10-fold dilution of above incubation system:

All ingredients	1886	82	77978	287
minus NADP	2234	98	28602	103
minus G6P	2245	98	29090	104
minus G6P dehydrogenase	2225	97	27490	99

* reaction time: 5 min; $NaH^{14}CO_3$: 1 mM, 4.1 μc/μmole.

\+ preincubation time: 5 min

** In each method, the enzyme activity was taken as 100% when only buffer solution (without G6P, G6P dehydrogenase, or NADP) was used in the assay.

L-glutamic acid dehydrogenase in the presence of α-ketoglutarate and NH_4Cl. When either α-ketoglutarate or NH_4Cl was omitted in the reaction, the reaction mixture induced about the same activation on RuDPCase activity as the original NADPH.

c. FDP: FDP produced in the reaction of F6P, ATP, and F6P kinase could cause deactivating effect on RuDPCase. The reaction mixture which was lacking any of those three ingredients could not demonstrate any deactivating effect.

d. RuDP: RuDP generated from Ru5P and ATP in the presence of Ru5P kinase showed the inhibitory effect on RuDPCase activity by using Method I. The reaction mixture which was lacking any of those ingredients did not give any inhibitory effect.

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

11. The ESR study of the binding between RuDPCase and metal ion(s). When RuDPCase was added to solution of Mn^{+2} a quantitative reduction in the intensity of the Mn^{+2} ESR signal was observed. No changes in the ESR spectrum of the aquo-complex appeared, and no evidence for any new signal was obtained. A titration of the enzyme was made assuming that the heights of the hyperfine components in the first derivative ESR spectrum are proportional to the concentration of free Mn^{+2}. The results of three experiments, using different enzyme preparations and concentrations, are presented in the form of a Hughes-Klotz plot (17, 25) in Fig. 20. In the absence of competing divalent cations the data fit a single straight line

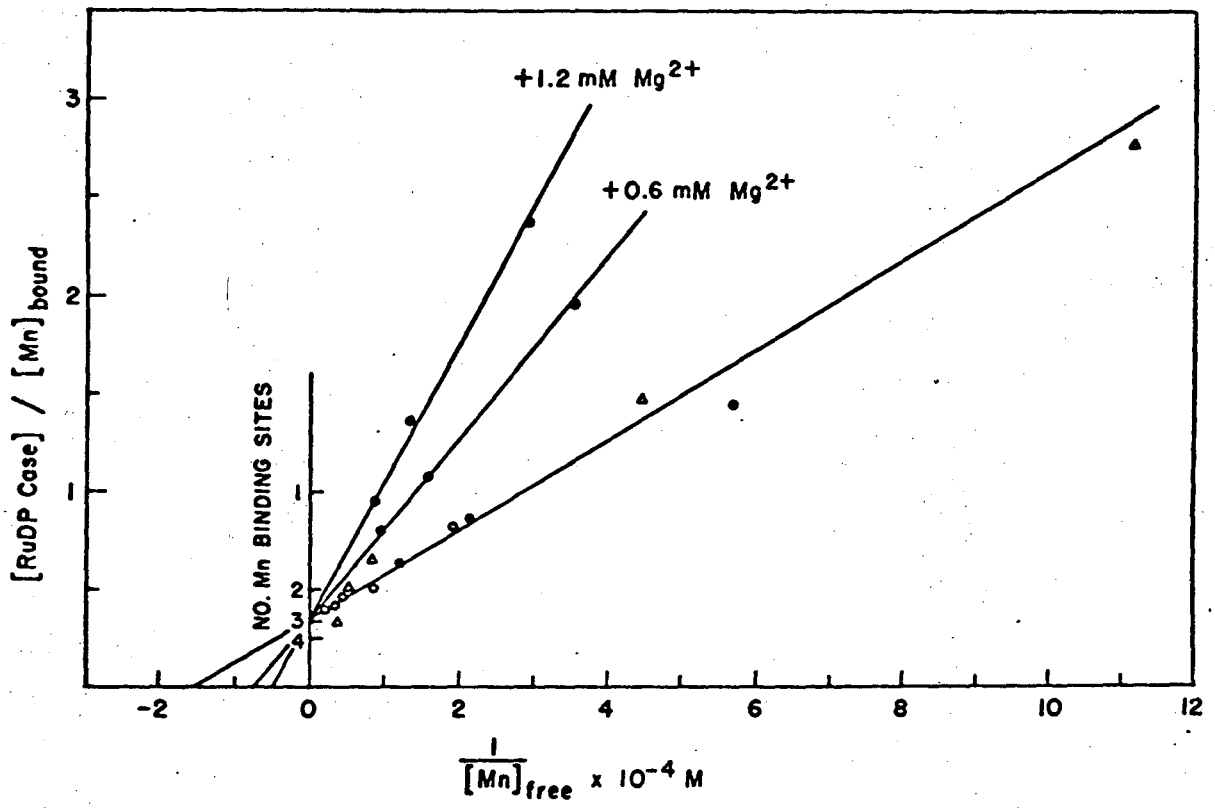

Fig. 20. Titration of RuDPCase with Mn^{+2}. Hughes-Klotz plot of the concentrations of free and bound Mn^{+2}, determined by ESR (see text). Experimental conditions: buffer, 0.05 M tris-HCl; pH 7.8: temperature, 22 C; sample volume, 0.15 ml; enzyme concentrations: Δ—Δ 55 mg/ml, o—o 33 mg/ml, ●—● 18 mg/ml.

reasonably well. The x and y intercepts are equal to the reciprocals of the dissociation constant and the number of binding sites, respectively. Within the Mn^{+2} concentration range used, 0.80 mM to 0.04 mM, K_d = 0.06 mM and n = 3. When Mg^{+2} was added to solutions of enzyme and Mn^{+2}, an increase in the free Mn^{+2} ESR signal was observed (Fig. 20). In the presence of 0.6 and 1.2 mM Mg^{+2} the K_d for Mn^{+2} increased to 0.12 mM and 0.18 mM, respectively, while n remained equal to 3. Assuming competitive binding of the two cations, K_d for Mg^{+2} can be determined (15, 26). The results of separate calculations at each Mn^{+2} and Mg^{+2} concentration are shown in Table VI. The average value for $K_d(Mg^{+2})$ = 0.54 mM.

Table VI. Dissociation Constants of Mn^{+2} and Mg^{+2} RuBPCase.

Experimental conditions are given in Fig. 20 and text. $K_d(Mn^{+2})$ was determined from best straight line fit to data; $K_d(Mg^{+2})$ was calculated for each data according to reference 15, assuming competition of Mg^{+2} and Mn^{+2}.

Mn^{+2} (mM)	Mg^{+2} (mM)	$K_d(Mn^{+2})$, mM	$K_d(Mg^{+2})$, mM
0.04 to 0.60	0	0.06	----
0.06	0.6	0.12	0.54
0.12	0.6	0.12	0.53
0.18	0.6	0.12	0.57
0.06	1.2	0.18	0.53
0.12	1.2	0.18	0.48
0.18	1.2	0.18	0.58
		average	0.54

DISCUSSION

After observing the activation of RuDPCase by preincubation of the enzyme with Mg^{+2} and bicarbonate, Pon et al. (34) offered three possible explanations which can now be examinated in the light of the present data. One possibility is that 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 of RuDP. This order of binding may be required, but if so, it can not 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 is that since radioactive PGA is measured, there might be an exchange or displacement of unlabelled bicarbonate by labelled bicarbonate. This is ruled out in the present study by the careful exclusion of unlabelled bicarbonate and CO_2. The third possibility is that both Mg^{+2} and bicarbonate activate the enzyme, but that the activating bicarbonate bound to the enzyme is not the reaction species. In terms of the 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 five minutes of the reaction following preincubation could in fact be the result of carboxylation of previously bound carbon dioxide, the species which has been shown to be involved in carboxylation (13). In our preincubation experiments, $H^{14}CO_3^-$ was added to the buffer either at least 30 min prior to initiation of the reaction or in the presence of carbonic anhydrase, so that complete equilibration of HCO_3^- and CO_2 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 the 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. When the enzyme is presented with RuDP without preincubation with Mg^{+2} and HCO_3^-, it is much less active and remains as such for at least 20 min. This suggests that RuDP binding by the non-preincubated enzyme converts the enzyme to (or maintains it in) a less active form from which it can not change in the presence of RuDP and low levels (1 mM) of $NaHCO_3$. If RuDP had not had any influence on the enzyme conformation and activity, the reaction rate should increase after several minutes (in the non-preincubated 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. 4, 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 (38, 39, 41, 49). 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. A high number of CO_2 molecules bound to the enzyme tends to reduce the number of RuDP molecules bound to the enzyme, but not necessarily at the catalytic sites. It is further suggested that RuDP binding at some sites tends to prevent CO_2 binding at the 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 mentioned, and as seen in this study (Table I & II), 6PGluA 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 $NaH^{14}CO_3$ and Mg^{+2}, and when 6PGluA is added during the preincubation period, considerable additional activation of the enzyme can be seen. The maximum activation occurs with only 0.1 mM 6PGluA. A 75% activation is seen with only 5 μM 6PGluA. Thus 6PGluA is a very specific effector under these conditions. At 2 mM, a net inhibitory effect is observed. Activation (with preincubated enzyme) is not seen when 6PGluA is added simultaneously with RuDP. Thus it appears that the binding of 6PGluA at those sites involved in the conversion of active to less active enzyme. Furthermore, binding of RuDP prior to incubation of the enzyme with Mg^{+2}, CO_2 and 6PGluA precludes not only the activation by Mg^{+2} and CO_2, but also the additional activation by 6PGluA.

The additional activation (when the enzyme is preincubated with CO_2 and Mg^{+2}) due to 6PGluA may be caused by 6PGluA binding in such

a way as to prevent some binding of RuDP when it is subsequently added. The binding of 6PGluA is a relatively slow process as indicated by the fact that the maximum stimulation of the enzyme by preincubation with HCO_3^-, Mg^{+2} and 6PGluA requires about 5 min (Fig. 8). The slow response of the enzyme to 6PGluA may be an example of the hysteretic phenomenon proposed by Frieden (14). The preincubation effect of CO_2 alone requires 2 min (Fig. 5). 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 results in the less active form of the enzyme.

The similar, though smaller, activating effects of FDP seen in the present study could be explained by a similar hypothesis.

The physical study of the binding between this enzyme and its substrates and/or metal ion by Trown and Rabin (44) indicates that the binding with RuDP is very fast and the binding with Mg^{+2} and bicarbonate is rather slow. Their results are consistent with the findings obtained in the biochemical assays of enzyme activities in present study.

The competitive inhibition of the enzyme activity by either 6PGluA or FDP demonstrates that either of these two metabolites can compete with RuDP at its catalytic site. The affinity of 6PGluA to this site is 10 times that of FDP. These facts thus support the view that at higher concentrations of either 6PGluA or FDP the observed inhibitory effects are due to competitive inhibition. This shows that 6PGluA is a much more effective inhibitor than FDP. Such inhibition is also seen with either 6PGluA or FDP with the less active form of the enzyme (Table IV) (42). Buchanan and Schurmann (11)

reported that with 1 mM Mg^{+2}, FDP at all concentrations studied is inhibitory. Bowes and Ogren (9) found that the enzyme from soybean was inhibited 46% by 1 mM FDP at 5 mM Mg^{+2}, while Avron and Gibbs (2) found no significant activation or inhibition by either F6P or FDP using freshly prepared broken spinach chloroplasts. From these reports and our experience, it seems clear that the effects of FDP on enzyme activity are very dependent on enzyme preparation and assay methods. Further detailed study of the action of this enzyme, preincubation with Mg^{+2} and CO_2, substrate and inhibitor levels, etc. seems to be needed before these differences and their significance can be understood.

The activation caused by the presence of NADPH and PGA during preincubation of the enzyme might be ascribed to mechanisms similar to those suggested for the effect of 6PGluA. Given the structural dissimilarity between NADPH and 6PGluA and the specificity of the NADPH effect (no effect by $NADP^+$, NADH, NAD^+), it may be more reasonable to expect that there is a different binding site for NADPH. In the case of PGA and NADPH, there is little or no inhibition of the enzyme in its less active form, and presumably no competitive inhibition of the enzyme in its active form either. Thus, higher concentrations of these effectors do not result in loss of the activation or in inhibition.

In earlier studies of RuDPCase, the $K_m(HCO_3^-)$ has been reported as 11 mM (46) and 20 mM (36). With the non-preincubated enzyme, at a very high Mg^{+2} concentration (45.5 mM) and a very low RuDP concentration (0.136 mM), the K_m was determined to be 2.5 mM (8). However, V_{max} was not reported and was presumably considerably lower

than V_{max} seen with the preincubated enzyme.

Buchanan and Schurmann (11) reported that in the presence of added F6P, the value of $K_m(HCO_3^-)$ for the spinach enzyme was 3.3 mM, while with 6PGluA it was 2.5 mM. We find a similar lowering of K_m in the presence of 6PGluA, NADPH or both, but saw no effect with F6P.

What significance can be attached to the regulation of RuDPCase by levels of RuDP, CO_2, Mg^{+2} and 6PGluA? 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, 6PGluA 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 free Mg^{+2} in the soluble part of the chloroplasts decreases greatly, as indicated by the study of Lin and Nobel (22, 29), 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} (8). The appearance of 6PGluA during the first minute of darkness would further inhibit the enzyme. These and perhaps other effects (18, 48) may account for the slow rate of the carboxylation reaction observed in isolated chloroplasts and in Chlorella pyrennoidosa after about 2 min of darkness.

The K_i for 6PGluA observed in this study may be compared with in vivo concentration estimated by Bassham et al. (7). In the dark, 6PGluA was measured to be 0.047 mM. Thus, the K_i of 6PGluA is lower than the estimated concentration of 6PGluA in the dark. It should

be noted that, in the dark, the oxidative pentose phosphate cycle is in operation in the chloroplasis (21), and CO_2 is being liberated but not consumed in the chloroplasts. Thus the K_i value obtained with 1 mM $NaHCO_3$ is not necessarily unrealistic in terms of in vivo metabolism. However, the in vivo concentration would include 6PGluA in both cytoplasm and chloroplasts. In the report of the appearance of 6PGluA in isolated chloroplasts, the amount of ^{14}C label found was smaller than in the in vivo experimrnts (21).

In any event, these comparisons between estimated K_i value and 6PGluA concentration in the dark in vivo suggest some possibility that competitive inhibition of RuDPCase by 6PGluA 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 (3, 4, 6). 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 (16).

Evidence for some of these changes can be seen upon either addition of vitamin K_5 to Chlorella pyrenoidosa in the light (21), or without additions when the light is turned off (32). 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 G6P dehygrogenase.

Data in the present study suggest that the increased level of

6PGluA may then further inactivate RuDPCase, thus completely stopping the carboxylation reaction. The lack of inhibition of photosynthesizing spinach chloroplasts by 6PGluA added to the medium is presumed to be due to lack of penetration of the limitimg double membrane of the intact chloroplasts.

The further activation of preincubated enzyme by NADPH seems likely to play some physiological role, given the specificity of this activation (lack of activation by $NADP^+$, NADH, and NAD^+). One possibility arises during the transition of the plant cells from dark to light. During the dark, the level of RuDP will have become very low or zero (7), but CO_2 will be present due to respiratory reactions. Moreover, the level of Mg^{+2} in the chloroplasts may drop to a low level in the dark, thus lowering the activity of RuDPCase (22, 29). When the light is turned on, or the sun rises, the level of Mg^{+2} ions in the chloroplasts is expected to increase (22, 29). During the first seconds of bright light, or longer in dim light, 6PGluA is present due to operation of the oxidative pentose phosphate cycle during darkness in the chloroplasts (6, 21). This combination of preincubation of the enzyme with CO_2, Mg^{+2}, and 6PGluA can cause maximum activation of RuDPCase, as suggested earlier. However, as the light brightens, or is on longer, 6PGluA will disappear (6) and NADPH will appear. Thus the role of keeping RuDPCase fully activated can be taken over by NADPH.

As illumination continues, sugar phosphates will be converted by phosphoribulokinase to RuDP until an effective substrate concentration is reached. The RuDP concentration <u>in vivo</u> was estimated to be 2 mM in chloroplasts (7), and is higher than 0.5 mM used in this

study. Even a concentration of 0.5 mM is sufficient to saturate the enzyme.

The smaller effects of FDP and of PGA are not easily explainable. Perhaps these effects are merely unavoidable consequences of structural similarity of FDP to RuDP and of PGA to 6PGluA. Activation of RuDPCase by FDP and PGA in vivo would seem to be adverse to the physiological needs for metabolic control, since an increase in either of these compounds may signal a decreased utilization of photosynthetic products for biosynthesis.

Based on the information we have from this study and from other workers, interaction mechanisms of the enzyme and its substrates and effectors can be proposed. Nishimura and Akazawa (28) recently demonstrated that RuDPCase has its catalytic sites on the large subunits and its regulatory sites on small ones. The ternary complex of the enzyme bound to metal ion and CO_2 has been shown (1). Therefore, it is reasonable to think that the binding sites of RuDP, CO_2, and Mg^{+2} are closely located on big subunits.

NADPH has a unique activating effect which none of the other reducing agents or related compounds, such as NADP and NAD(H), exhibit. Up to 2 mM, NADPH shows no inhibition at all regardless of how the enzyme activity is assayed. Hence, it shows that the enzyme must have a regulatory site for NADPH.

6PGluA can either activate or inhibit the enzyme and the inhibition is due to the competition with RuDP at the catalytic site. One possibility is that the activation by 6PGluA results from its binding at a regulated site. The activation caused by low concentrations of 6PGluA can persist for at least 20 min. This means that

the binding between the enzyme and 6PGluA is kept intact for at least 20 min after the addition of RuDP. The dissociation constant, K_d, between the enzyme andRuDP was determined to be less than 1 μM; the K_d of 6PGluA with the enzyme at RuDP catalytic site is about 20 μM. The binding of RuDP is much stronger than 6PGluA and RuDP/6PGluA ratio used in the assay of 6PGluA activation was 10 to 1. Thus, it is very likely that RuDP could replace 6PGluA during the 20 min reaction period following the 5 min preincubation if both RuDP and 6PGluA bind at the same catalytic site.

At lower concentrations, 6PGluA causes significant activation. The concentration at which 6PGluA causes half of the maximum (188%) activation is between 5-10 M, while the K_i of 6PGluA at the RuDP catalytic site is about 20 M. The activation effect induced by 6PGluA has been shown to be a slow process, which needs about 5 min to reach maximum activation. The Lineweaver-Burke plots in the 6PGluA inhibition study fit very well with the kinetic equation of competitive inhibition, which assumes that the binding between the enzyme and the inhibitor is very fast, i. e., equilibrium must be established in a very short time. Therefore, the binding of 6PGluA at the catalytic site is much faster than that at the regulatory site. It suggests that there are two different sites for 6PGluA; one is the regulatory (activation) site, the other is the RuDP catalytic site. The binding constants suggest that the binding at the former is stronger than that at the latter.

FDP, like 6PGluA, also can either activate or inhibit the enzyme activity. Because it has been shown that FDP can also compete with RuDP at its catalytic site and the structural similarity between FDP

and 6PGluA, it is proposed that FDP can also bind at the 6PGluA regulatory site to show an activation.

Since both 6PGluA and NADPH can activate the carboxylation reaction, it is interesting to see whether these two effectors bind the enzyme at the same site or not. At least two mechanisms can be proposed at this moment.

First, for the simple reason that 6PGluA and NADPH are not chemically related, it is hard to believe that both compounds bind the enzyme at the same site. If indeed these two sites are independent, the binding of one of them should not interfere with the binding of the other. Suppose that the 6PGluA binding site can be blocked by RuDP when it was added before or simultaneously as 6PGluA was added to the enzyme, then the same blockage should not take place in the case of NADPH because NADPH is not structurally similar to RuDP. The experiment in which the reaction was started by the addition of the mixture of RuDP and NADPH to the enzyme which had been preincubated with Mg^{+2} and $NaH^{14}CO_3$ indicated that the binding of NADPH was also blocked by RuDP. Therefore, the dilema of this mechanism is that two chemically different compounds can activate the enzyme in the same way (only Method VI gives activation) and the activation can be abolished in both cases even though only one of them (6PGluA) is structurally similar to the inhibitor (RuDP).

The second mechanism proposed is that there is only one common binding site on the enzyme. This mechanism would be consistent with the non-additive activation by 6PGluA and NADPH. The difficulty that arose in that two binding sites hypothesis can be dissolved by this one site mechanism as well. However, it is really difficult

to see that these two chemically unrelated compounds would bind the enzyme at the same site.

It is found that FDP, like RuDP, also can deactivate the activation induced by either 6PGluA or NADPH, if 1 mM of FDP is present in the preincubation medium. As mentioned above, it is reasonable to think that FDP can compete with 6PGluA at the regulatory site due to the structural similarity. Because of the high FDP/6PGluA ratio used in this study (1 mM/ 0.05 mM), it is likely for FDP to occupy the 6PGluA site and thus abolish the activation observed in the absence of FDP. The same mechanism is not likely to happen in the case of NADPH since FDP does not chemically resemble NADPH. The fact that FDP also eliminates the activation induced by NADPH suggests that a complicate mechanism is needed to explain all the results we have obtained.

As discussed previously, the model of either two independent sites or one single site for 6PGluA and NADPH is not consistent with the facts we observed and for the structural reasons. Therefore, the model or two dependent sites can be proposed. The binding of either RuDP or FDP at 6PGluA site might induce structural changes so that the binding of NADPH at the other site is blocked and therefore no activation can be observed.

A specific inhibitor of one of these two activators should have no effect on the activation induced by the other activator in the case of two independent binding sites. If such an inhibitor can be found, it will tell whether there are two independent sites or not. NADP or NADH was the likely candidate for the inhibitor of NADPH. The result shows that neither NADP nor NADH can reduce the activation

induced by NADPH or 6PGluA. At present it seems that there is no easy way to solve this binding problem.

As indicated before, the activation effect induced by every effector can be seen only in the specific preincubation condition. Other assay methods in which the activator is added simultaneously or after the addition of RuDP result in no activation at all (in the case of 6PGluA, inhibition effect is observed). These results and the facts that the activating effects produced by activators can persist for a long time strongly suggest that the binding of RuDP is very rapid and also can cause drastic structural changes so that the enzyme conformation is "locked" and can not be easily transformed. In the absence of any effector, it has been shown that RuDP can inhibit the binding of CO_2 or Mg^{+2}, which are the necessary factors which convert the enzyme to become active. Thus the binding of RuDP on the enzyme first can not only prevent the activator from binding at the regulatory site, but also delay the activating effect of CO_2 and Mg^{+2}. Therefore, it is believed that the RuDP binding causes structural changes on catalytic site as well as on regulatory site.

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